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-1*H*-indol-3-yl)-3-(1*H*-indol-3-yl)-1*H*-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).[1b] The most remarkable features are the 1,2-shift of the indole ring[1a] and the incorporation of three molecules of O_2 into **1**.[1b] 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] and by us (named pVBG04)[1j] (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**,

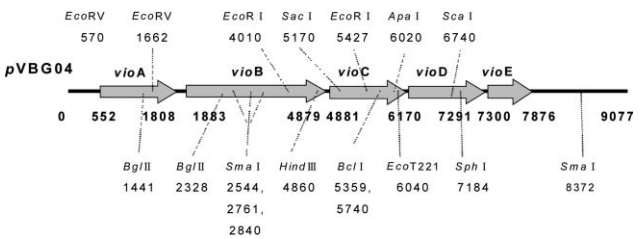

Fig. 1 Restriction map of the biosynthetic gene cluster of **1**, which was cloned from *Chromobacterium violaceum* (JCM1249) by us (accession number AB032799).

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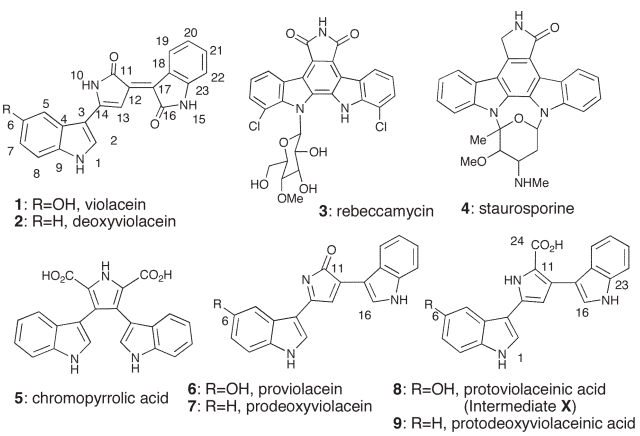
† Electronic supplementary information (ESI) available: Construction of VioABCDE, stability of **8**, NMR spectra of **8** and **9**, the assignments of the NMR data, EI- and FABMS, homologous alignment, FAD of VioA, C and D, *etc*. See DOI: 10.1039/b705358d

VioA is assumed to be a L-tryptophan oxidase to give the imine of indole-3-pyruvic acid (IPAimine), 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 acid **5**[1d] with aid of StaO and RebO.[6,7] However, **5** is not the biosynthetic intermediate of **1**,[1d] thus the role of VioB has remained uncertain. No homologous protein can be assigned to VioE. Based on the finding that proviolacein **6**[1g] and prodeoxyviolacein **7**[1g] are produced by incubating Trp with VioABDE and VioABE, respectively,[4,5] 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.[1g] 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 (NADPH is essential to the production of **1**[1h]). Furthermore, we describe the important finding that **X** is very labile to O_2, resulting in the generation of a shunt product **6**.



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

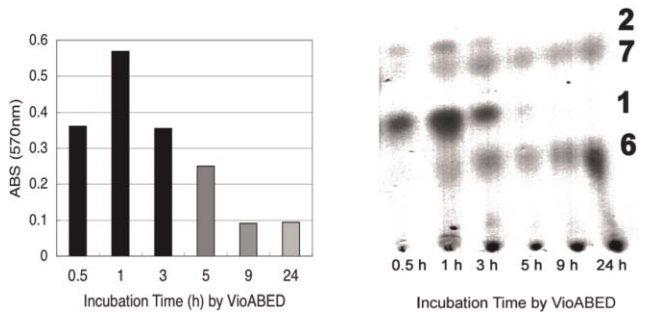


Fig. 2 (Left) Production amount of **1**, monitored at 570 nm in MeOH, against different incubation times (0.5–24 h) with VioABDE + NADPH. EtOAc was added to each of the mixtures incubated for different times. The EtOAc-extract, which was quickly evaporated into a small volume, was added to VioC and 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) SiO_2-TLC of the samples described left, which developed twice with $CHCl_3$: 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 SiO_2-TLC analysis revealed that a decreased production of **1** gave the higher production of **6**, suggesting that **6** is produced from **X**.

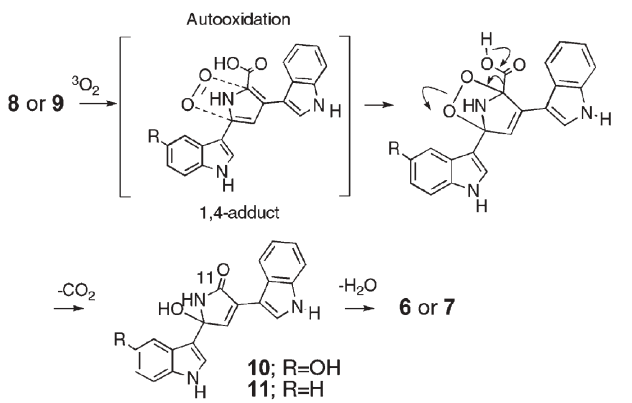
Next, we tested the stability of **X**† by estimating the amount of **1** produced. Intermediate **X** dissolved in the EtOAc gradually decomposed at room temperature, but the decomposition was suppressed at a low temperature of −20 °C. However, when the solvent was evaporated to dryness, the production of **1** significantly decreased, suggesting that a rapid decomposition occurred by the exposure to O_2. The TLC analysis clarified that the decomposed product was mainly **6** (EIMS, *m*/*z* 327, M^+). The bubbling of O_2 into the EtOAc-extract more significantly decreased the production of **1**, further proving that **X** is labile to O_2.†

The EtOAc-extract from the mixture incubated for 1 h was subjected to a column chromatography over SiO_2 by eluting with EtOAc, followed by a reversed phase HPLC (C_{18}) with MeOH : H_2O = 45 : 55. The isolation was quickly accomplished in less than 4 h. Argon gas was bubbled into the **X**-containing fraction, which was then stored at −80 °C. Intermediate **X** was extracted again with EtOAc, which was quickly dried over anhydrous Na_2SO_4, followed by evaporation to a small volume (the solvent was never dried to shield **X** from aerial O_2), to which acetone-d_6 was added and evaporated to a small volume by passing a stream of argon gas. The substitution with acetone-d_6 was repeated several times to remove the residual EtOAc. The 1D and 2D NMR spectra were then measured at −10 °C. No change of the ^{1}H NMR signals was found during the NMR measurements for 36 h, but gradual alteration was observed when measured at 25 °C. The ^{13}C signal at δ_c 162.9 (s) was clearly observed in addition to the bisindole core carbons, but the signal of H-11 was missing, suggesting that the carboxyl group is substituted at the 11-position; the reaction of **X** with CH_2N_2 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-1*H*-indol-3-yl)-3-(1*H*-indol-3-yl)-1*H*-pyrrole-2-carboxylic acid, named protoviolaceinic acid **8**. The methyl ester of **8** underwent no decomposition by the exposure to O_2. 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 O_2 into 6-postion of **9** to give **8**.

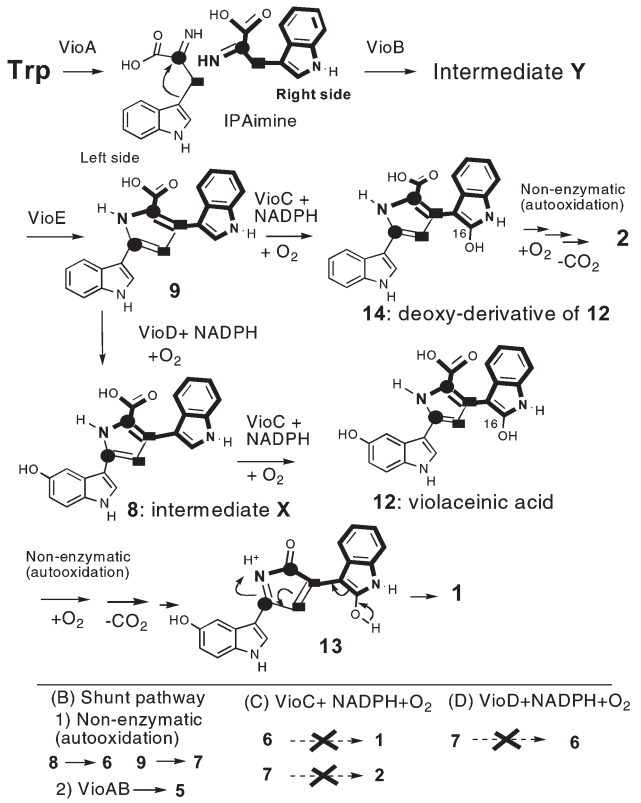
Pyrrole derivatives such as dimethylpyrroles are sensitive to aerial condition and undergo autooxidation.[8] Intermediates **8** and **9** could be allowed to react with triplet oxygen to form 1,4-adduct (Scheme 1), followed by decarboxylation to afford **10** and **11**, respectively. Dehydration from **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 O_2 into 11-position of **6** and **7** proceeded in a no-enzymatic fashion.

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.[4,5]

VioA has a highly homologous alignment to L-amino acid oxidase.† We detected H_2O_2 with quinoneimine dye and phenol 4-aminoantipyrine peroxidase,[9] and identified IPA as a hydroxylamine adduct. Our previous experiments[1j] 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 IPAimine are employed



Scheme 1 Autooxidation mechanism of **8** and **9** into shunt products **6** and **7**.



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**.[1j]

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.[10]† The electronic spectrum of VioC purified with Ni-NTA affinity column showed λ_{max} 448 nm and the supernatant, prepared by heating VioC at 100 °C for 10 min, had λ_{max} 370 and 450 nm, which are characteristic of flavo-proteins. HPLC (C_{18}) analyses showed that the cofactor was FAD, but not FMN.† VioA and VioD also had FAD.† 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 O_2, 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

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11 In the pyrrole autooxidation reaction, two indole rings act as electron donors, while O_2 works as electron acceptor.[8] 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 O_2 attack on **12**, leading to the failure of isolation of **12**. Furthermore, VioC requires O_2 for the catalysis, thus we cannot remove O_2 from the incubation system in order to trap **12**.

12 Walsh *et al.*[5] 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.

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