Novel tryptophan metabolites, chromoazepinone A, B and C, produced by a blocked mutant of *Chromobacterium violaceum*, the biosynthetic implications and the biological activity of chromoazepinone A and B†

Takaaki Mizuoka,[a] Kazufumi Toume,[b] Masami Ishibashi[b] and Tsutomu Hoshino*[a]

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Chromobacterium violaceum produces tryptophan metabolites, purple pigments of violacein and deoxyviolacein. A blocked mutant was prepared with *N*-methyl-*N*′-nitrosoguanidine to gain insights into the biosynthetic mechanisms of the pigments. Five tryptophan metabolites were isolated: three novel compounds, named chromoazepinone A, B and C and two known compounds, chromopyrrolic acid and arcyriarubin A. The structure determinations of the three novel compounds are described. The biosynthetic pathways of these metabolites are proposed on the basis of the findings about violacein biosynthesis. Chromoazepinone A and B were found to have an interesting effect of inhibition of Wnt signal transcriptional activity, which is implicated in the formation of numerous tumors when aberrantly activated.

Introduction

Chromobacterium violaceum produces the purple pigments, violacein **1** and deoxyviolacein **2** (Fig. 1),[1,2] which exhibit many activities such as antibacterial, anticancer, antitryptanocidal activities *etc.*[3,4] Violacein has a bisindole nucleus, which is analogous to that of rebeccamycin **3**[5,6] and staurosporine **4**[7] (indolocarbazole skeleton), which exhibit potent inhibitory activities for protein kinase and topoisomerase-1, respectively. We have reported on the biosynthetic investigations of violacein since 1987.[8–18] Recent progress on the biosynthetic studies of **1** has been remarkable.[19] All the carbon, hydrogen and nitrogen atoms are exclusively derived from two molecules of L-tryptophan.[8,9] The most notable features are the 1,2-shift of the indole ring [8] and the incorporation of three molecules of oxygen gas into **1**.[9] We have now strong evidence that five open reading frames (VioA–E) are responsible for violacein biosynthesis.[20,21] The function of each enzyme has been identified. Scheme 1 shows the biosynthetic pathway.

VioA (L-amino acid oxidase, a flavoenzyme) oxidizes tryptophan into the imine of indole-3-pyruvic acid (**12** IPA imine).[18,21] Two molecules of **12** in turn undergo a condensation reaction by the action of VioB (heme protein) to afford an unstable dimeric structure with two tryptophan molecules **13**.[18,22] However, the structure has remained unclear. The unidentified dimeric structure is then subjected to a rearrangement reaction for one of the two indole rings, affording deoxyprotodeoxyviolaceinic acid **14** as an intermediate.[18] The 1,2-shift of the indole ring is catalyzed by VioE,[18,21–24] which is involved only in violacein biosynthesis, but not in the biosynthesis of **3** and **4**. VioD (a monooxygenase, falvin-dependent oxygenase) works to incorporate oxygen at the 5-position of the indole nucleus to generate protoviolaceinic acid **16**.[18,21,22] Intermediates **14** and **16** are further oxidized at the 16-position by the monooxygenase of VioC (flavin-dependent oxygenase) to give **15** and **17**.[18] Thereafter, non-enzymatic reactions proceed as shown in Scheme 1; the 1,4-addition of triplet oxygen to **15**, followed by decarboxylation and dehydration, lead to the final products **1** and **2**, as shown in Scheme 1.[18] Previously, we have isolated proviolacein **10**[14] and prodeoxyviolacein **11**,[14] which are produced by VioABDE and VioABE, respectively.[18] These compounds were produced from **14** and **16** by the oxidative decarboxylation reaction without participation of VioC;[18] **10** and **11** are formed in the same way as that depicted in non-enzymatic pathway of **15** and **17**. Thus, **10** and **11** are not the true biosynthetic intermediates.[18]

We have identified the biosynthetic intermediates or their related tryptophan metabolites by using the biosynthetic genes VioA–E.[18] In addition to the genetic studies, we have continued research on blocked mutants by employing the mutagenic agent of *N*-methyl-*N*′-nitrosoguanidine (NTG),[11,14,16] in order to obtain further insight into the biosynthetic mechanism of violacein **1**. This paper describes another blocked mutant named H-25, which produced five tryptophan metabolites, *i.e.*, three novel compounds **5**–**7** and two known compounds chromopyrrolic acid **8**[11] and arcyriarubin A **9**,[25] upon incubation of L-tryptophan with the washed whole cells of this mutant. In this paper, we describe the structures of the tryptophan metabolites, chromoazepinones A, B and C, which possess a novel bisindole scaffold, and discuss the biosynthetic pathways of these novel compounds in comparison with that of violacein **1**. Furthermore, we report that compounds **5**, **6**, and the methyl ester of **6** possessed an interesting effect of inhibiting Wnt signal transcriptional activity.

[a]*Department of Applied Biological Chemistry, Faculty of Agriculture, and Graduate School of Science and Technology, Niigata University, Nishi-ku, Ikarashi 2-8050, Niigata 950-2181, Japan. E-mail: hoshitsu@agr.niigata-u.ac.jp*
[b]*Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*

† Electronic supplementary information (ESI) available: Spectroscopic data (UV-visible, IR, EIMS and ^{1}H and ^{13}C NMR spectra) of chromoazepinone A, B and C, and arcyriarubin A. Detailed analyses of 2D NMR spectra are also given. See DOI: 10.1039/c003837g

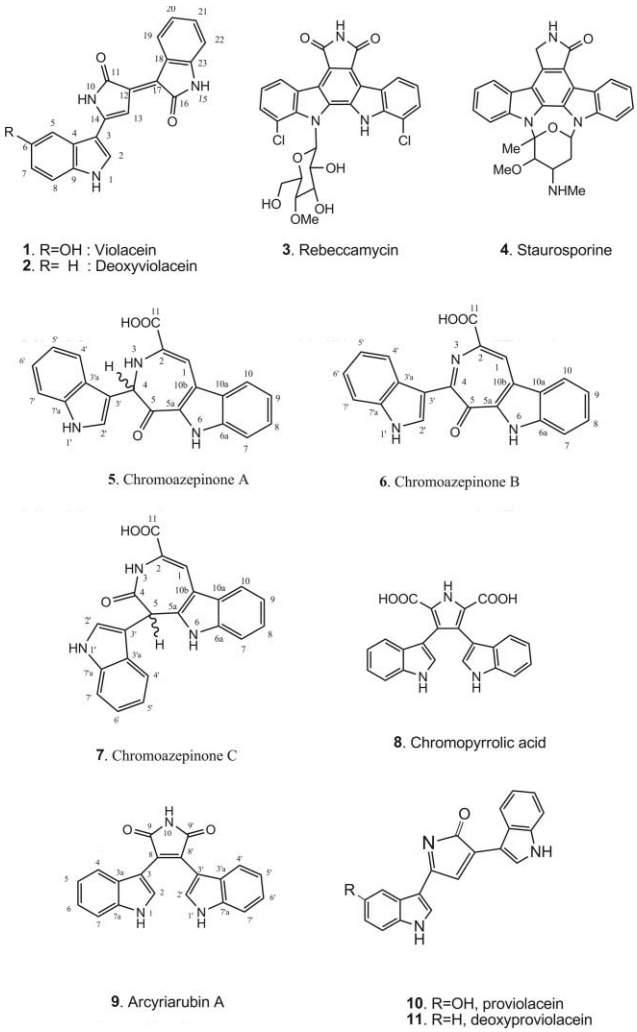
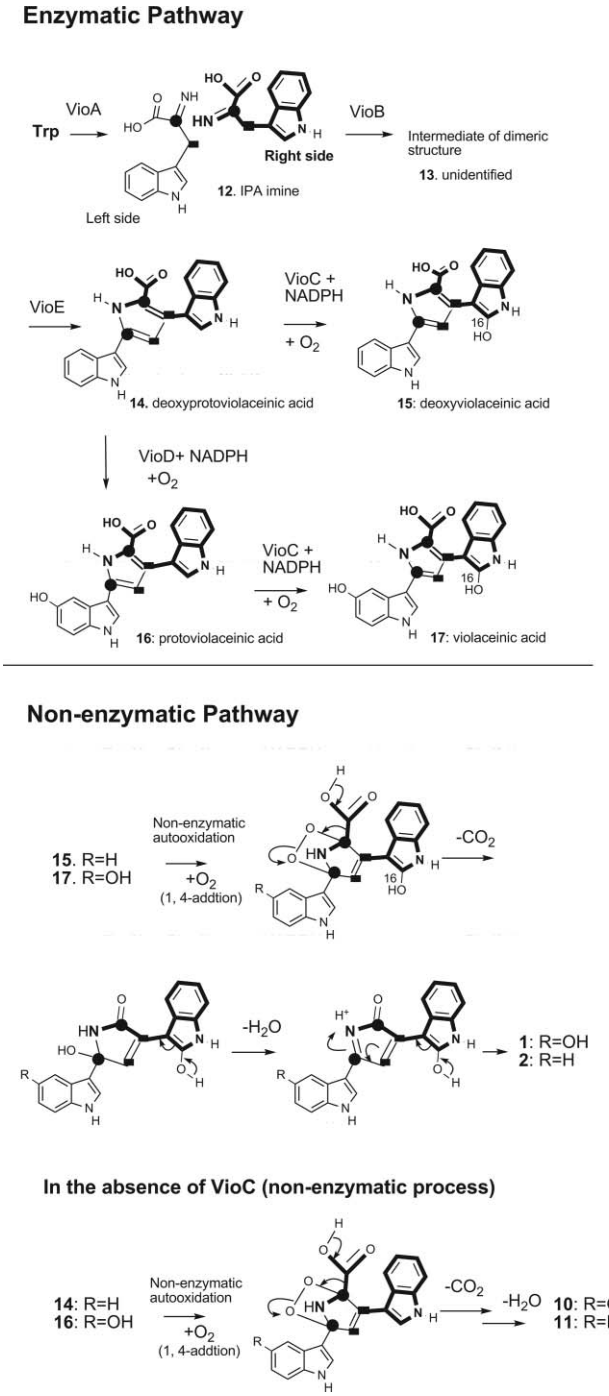


Fig. 1 Tryptophan metabolites discussed in the text.

Results and discussion

Isolation and structures of tryptophan metabolites 5–7

The grown cells of H-25 (90 L) were collected by centrifugation and washed with saline solution. The washed cells thus obtained were suspended in ammonium buffer solution (0.2 M, pH 8.5, 4.5 L) containing 4 g of L-tryptophan and then incubated for 24 h at 25 °C on a rotary shaker. The pigments accumulated in the orange-colored cells were extracted with MeOH (3 L). To the methanolic extract was added 3 L of water. The solution was concentrated to evaporate MeOH, and the pH then was adjusted to 3.0 with 2 N HCl after which 3 L of EtOAc was added to extract the pigments. The EtOAc solution was diluted with aqueous 2 N NaOH until the pH was 10.0 in order to transfer the pigments into the aqueous layer. Next, this aqueous layer was acidified to pH 3.5 and the pigments were extracted with 1 L of EtOAc. The EtOAc fraction was concentrated into a small volume, which was loaded on a Sephadex LH-20 column, and then eluted with MeOH. The three yellow or orange colored-pigments (**5**–**7**) were separated with the following elution order: compound **7**, **5** and **6**. The repeated column chromatography of the partially purified **5** over Sephadex LH-20 led to the isolation of **5** in a pure state. Pure



Scheme 1 The biosynthetic pathway from tryptophan to violacein **1** and deoxyviolacein **2**, which consists of two distinct pathways; the first one is the enzymatic processes, which proceed by the actions of five enzymes (VioA–E), and the second one is non-enzymatic reactions (autooxidation). In the absence of VioC, autooxidation products, **10** and **11**, are formed from **14** and **15**, respectively, leading to the conclusion that **10** and **11** are not the biosynthetic intermediates for **1** and **2**.

6 and **7** were obtained by a SiO_2 column chromatography using a mixture of $CHCl_3$ and MeOH (100 : 2–100 : 7). It was found that metabolite **5** was easily converted into **6** on SiO_2 TLC plate after evaporating the developing solvent. This suggests that **5** undergoes facile oxidation to give **6**. Therefore, we avoided using SiO_2 column

chromatography for the purification. The isolation yields of **5**, **6** and **7** were 2.6 mg, 8.0 mg and 2.4 mg, respectively.

Compound **5** had a carboxyl group; a reaction with CH_2N_2 gave the methyl ester [O-*CH*$_3$, δ_H 3.75 (3H, s) and δ_C 52.40 (q); C=O, δ_C 165.4 (s)]. In the ^{1}H- and ^{13}C NMR spectra (ESI, Figs. S2 and S3) of the methyl ester (DMSO-d_6), 17 protons and 22 carbons were found, respectively. The EIMS spectrum of the methyl ester (ESI, Fig. S1) showed an ion (M^+) at *m/z* 371, and the molecular formula $C_{22}H_{17}O_3N_3$ was established by HREIMS (found, *m/z* 371.1272; calcd, *m/z* 371.1270). Two indole rings were confirmed by detailed NMR analyses including 2D NMR spectra (^{1}H–^{1}H COSY, NOESY, HSQC and HMBC). Clear HMBC correlations were observed for H-1 (δ_H 7.09, s)/C-11 (δ_C 165.4, s, -*C*O$_2$Me), H-1/C-5a (δ_C 133.6, s), and H-1/C-10a (δ_C 125.4, s). A strong NOE was observed between H-1 and H-10 (δ_H 7.90, d, $J = 7.9$ Hz), indicating that an indole acrylic acid moiety is involved in **5**. Two protons that do not belong to indole rings were found in the ^{1}H NMR spectrum (DMSO-d_6): δ_H 5.46 (H-4, d, $J = 6.1$ Hz) and δ_H 7.07 (H-3, d, $J = 6.1$ Hz), these protons being spin-coupled to each other (a definitive cross peak in the ^{1}H–^{1}H COSY spectrum). In the HSQC spectrum, H-4 had a correlation with δ_C 56.97 (d), which is assignable to sp^3 carbon, but not to sp^2 carbon. No correlation was observed for H-3 in the HSQC spectrum. Furthermore, taking into account the molecular formula, H-3 was assigned to N*H*. Thus, the presence of an α,β-dehydrotryptophan moiety was confirmed. In addition, HMBC cross-peaks of H-4 to C-2 (δ_C 130.8, s), C-5 (δ_C 182.5, s), C-5a (δ_C 133.6, s), C-2′ (δ_C 123.6, d), C-3′ (δ_C 108.8, s) and C-3′a (δ_C 125.9, s) were observed (ESI, Fig. S6). A strong HMBC cross peak was observed between H-3 and C-1 (δ_C 102.8, d). These NMR data clearly revealed the involvement of an NH–CH–C=O partial structure in **5**, and this partial structure is connected as shown in Fig. 1, leading to the proposal for the whole structure of **5**. We propose the name chromoazepinone A for **5**. The absolute configuration at C-4 has remained unsolved $[\alpha]_D^{20} = +56.0$ (*c* 0.16, EtOH).

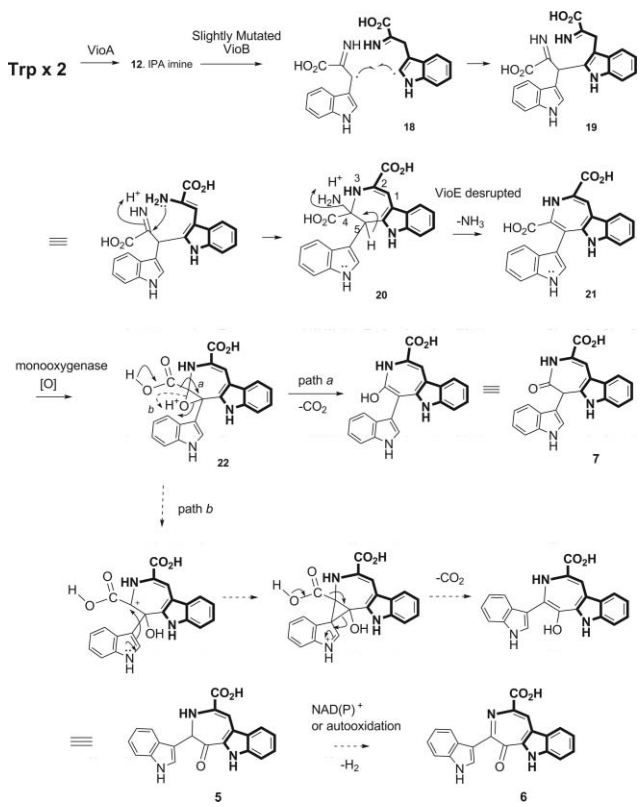
The UV-visible spectrum of compound **6** was dependent on the acidity (ESI, Fig. S10). In MeOH, the color was yellow (λ_{max} 405 nm) at neutral and basic solution, but red (λ_{max} 550 nm) in an acidic medium. The pK_a was determined to be 4.9, suggesting that **6** also has a carboxyl group. The IR spectrum (ESI, Fig. S11) showed a strong absorption (1680 cm^{-1}) for α,β-conjugated carbonyl group. In the ^{1}H- and ^{13}C NMR spectra (ESI, Figs. S8 and S9), 12 protons and 21 carbons were found, respectively. EI- (ESI, Fig. S7) and CIMS spectra showed peaks of *m/z* 355 and 356 as the highest ions, respectively, indicating that the molecular ion of **6** is *m/z* 355. In the MS spectra, the prominent ion of *m/z* 311 ($M^+ - 44$) for EI or 312 ($M^+ + H - 44$) for CI were observed, further indicating the involvement of a carboxyl group in **6**. The HREIMS exhibited an ion at *m/z* 355.0930, *cf.* *m/z* 355.0957: calcd. for $C_{21}H_{13}N_3O_3$. The presence of a carboxyl group was established by the preparation of the monomethyl ester with CH_2N_2; [O-*CH*$_3$, δ_H 4.04 (3H, s) and δ_C 52.59 (q); C=O, δ_C 167.3 (s)] (see ESI, Figs. S14 and S15); HREIMS, found *m/z* 369.1111 (M^+) (*m/z* 369.1113 calcd for $C_{22}H_{15}N_3O_3$). Detailed NMR analyses of **6** including DEPT 135 and 2D NMR (COSY 90, NOESY, HSQC and HMBC) revealed the presence of two indole rings. Clear HMBC correlations were observed for H-1 (δ_H 8.73, s)/C-2 (δ_C 134.8, s), H-1/C-11 (δ_C 168.5, s, -COOH), H-1/C-5a (δ_C 140.0, s), and H-1/C-10a (δ_C 125.2, s). A strong NOE was observed between H-1 and H-10 (δ_H 8.39, d, $J = 7.6$ Hz). These HMBC and NOE results (ESI, Fig. S12) unambiguously demonstrated the position of H-1 of **6** as shown in Fig. 1. The following two carbons remained to be assigned except for two indole nuclei: δ_C 153.4 (s) and 170.6 (s). No information for further inferring the structure of **6** was available from the HMBC data. As described previously, **5** was converted into **6** on a TLC plate, probably due to its high susceptibility to oxidation reaction. The EI-MS, the UV-visible spectra as well as the R_f value of the product prepared by this TLC treatment of **5** were identical to those of **6**. On the other hand, the reductive product of the methyl ester of **6** with $NaCNBH_3$ afforded the same R_f value and EIMS spectrum as the methyl ester of **5**. These results strongly indicate that the structure of **6**, named chromoazepinone B, is established as shown in Fig. 1. The protonation on the nitrogen atom in the acidic medium would have led to a longer wave-length absorption due to outcome of the extended resonance structure (ESI, Fig. S16).

Compound **7** also had a carboxyl group. Reaction with CH_2N_2 afforded its methyl ester [δ_H 3.74 (3H, s) and δ_C 52.37 (q) for O*CH*$_3$, δ_C 163.8 (s) for C=O]. The HREIMS exhibited *m/z* 371.1275 (M^+) and the molecular formula was determined to be $C_{22}H_{17}O_3N_3$ (calcd. *m/z* 371.1270), which was identical to that of **5**. The NMR analyses similar to those of **5** verified that two indole rings and an indole acrylic acid moiety are involved in **7**. The functional group, –NH–CO–CH–, differing from that of **5**, was confirmed by the following NMR evidence. H-3 (δ_H 9.11, s) had no cross peak of HSQC (^{1}H–^{13}C correlation), indicating that H-3 is a N*H* function. Furthermore, no ^{1}H–^{1}H COSY cross peak was found between H-3 and H-5 (δ_H 5.42, s), indicating that unlike compound **5**, H-3 was not adjacent to H-5. Strong HMBC correlations (ESI, Fig. S23) were observed for H-3/C-1 (δ_C 115.5, d), H-3/C-4 (δ_C 166.3, s) and H-3/C-5 (δ_C45.51, d), indicating that an α,β-dehydrotryptophan moiety is also involved in **7**. The chemical shift of C-4 indicated the involvement of an amide group. Definitive HMBC cross peaks of H-5 (ESI, Fig. S23) were found for C-4, C-10b (δ_C 107.9, s), C-2′ (δ_C 122.8, d), C-3′ (δ_C 106.9, s), C-3′a (δ_C 125.7, s). Thus, the positions of H-5 and the alternative indole ring were assigned as shown in Fig. 1. The position of H-5 was further supported by a strong NOE between H-5 and H-4′ (δ_H 7.55, d, $J = 8.0$ Hz). The whole structure of **7**, named chromoazepinone C, was deduced as shown in Fig. 1. The absolute configuration at C-5 of **5** has remained uncertain: $[\alpha]_D^{20} = +26.6$ (*c* 0.15, EtOH).

We isolated other tryptophan metabolites in relatively small amounts from the MeOH extracts of the incubated cells, which are known as chromopyrrolic acid **8** (0.6 mg) and arcyriarubin A **9** (1.0 mg). These compounds were isolated by SiO_2 column chromatography ($CHCl_3$–MeOH–AcOH = 98 : 2 : 2 for **8** or $CHCl_3$–MeOH = 95 : 5 for **9**). The ^{1}H- and ^{13}C NMR data for **8** were completely identical to those of chromopyrrolic acid previously isolated by us.[11] In the ^{13}C NMR spectrum of **9** (ESI, Fig. S26), 10 carbon signals were found, but HREIMS showed that 20 carbons were involved in **9** (*m/z* 327.1020 (M^+); calcd.: *m/z* 327.1008 for $C_{20}H_{13}N_3O_2$). Thus, **9** is a symmetrical molecule. The indole ring was confirmed by the HMBC spectrum measured in DMSO-d_6: H-1 (δ_H 11.65, s) were apparently correlated with C-2 (δ_C 129.7, d) and C-3 (δ_C 106.2, s). The amide function (ν_{max} 1700 cm^{-1}) was found at δ_H 10.89 (H-10, s) and δ_C 173.6 (C-9, s). Apparent HMBC cross peaks were observed for H-10/C-9 and H-10/C-8 (δ_C 128.3, s) (see ESI, Fig. S29). Thus, **9** was determined to be arcyriarubin A.

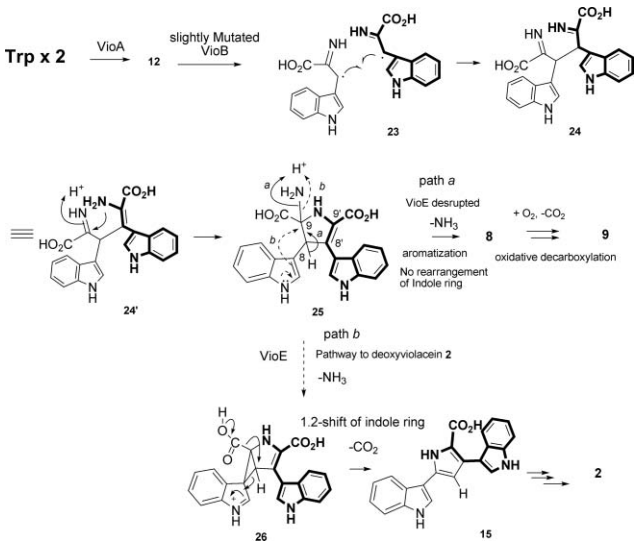
Proposed biosynthetic pathways from L-tryptophan

It is apparent that the tryptophan metabolites, chromoazepinone derivatives **5**–**7**, were produced by a pathway analogous to violacein biosynthesis (Scheme 2). Tryptophan oxidase (VioA) could give **12**, two molecules of which could be subjected to a condensation reaction to afford the dimeric structure **19**. **19** would be produced by the mutated VioB. Native VioB catalyzes the coupling reaction between the benzylic β-carbon atoms of two indolepyruvate imine **12** for the formation of violacein **1** (Scheme 1). However, in the biosyntheses of **5**–**7** the coupling reaction would have occurred between the Cβ-position of **12** and the C2-position of the indole ring to produce **19**, indicating that VioB would have been mutated but still active and catalyzed the coupling reaction shown in **18**. Intermediate **19** underwent a cyclization reaction to afford **20**, followed by elimination of an ammonia molecule, leading to the production of **21**. Thereafter, a monooxygenase could work to form the epoxide ring of **22**; this monooxygenase may be the native VioC or the mutated VioC, which may have accepted **21** with its broad substrate specificity. Otherwise, another monooxygenase involved in *Chromobacterium violaceum* may have worked to form the epoxide **22**. The epoxide opening reaction due to proton attack could give rise to two different pathways. Path *a* could lead to the production of **7** *via* a decarboxylation reaction. Path *b* afforded **5** *via* indole rearrangement in conjunction with a decarboxylation process. Compound **5** could be subjected to a dehydrogenation reaction with the aid of NAD(P)$^+$ cofactor to enzymatically produce **6**. Otherwise, **5** may have undergone in part an autooxidation during the incubation process under a vigorous aeration condition; the resonance structure of **6**, which is more extended than that of **5**, may have led to the formation of **6**. No hydroxy derivative on the indole rings of **5**–**7** was found in the incubation mixture. Thus VioD underwent the mutagenesis and its activity was completely lost. The ratio of path *b* to *a* was *ca.* 5 : 1, because the production amount of (**5** + **6**) was *ca.* five-fold larger than that of **7**.



Scheme 2 Proposed biosynthetic pathways of tryptophan to **5**–**7**. Indole shift proceeds *via* an epoxide ring cleavage.

Scheme 3 shows the proposed biosynthetic pathway for **8** and **9**. The coupling reaction between the two benzylic β-carbon atoms of **12** gave **23**, which underwent a cyclization followed by the elimination of an ammonia molecule and led to the production of **8**. The oxidative decarboxylation reaction of **8** could afford **9**. The coupling mode for the production of **23** is different from that of **19**, suggesting that the altered VioB, which was slightly modified and still in the active form, could lead to the two different coupling reactions (compare **23** with **18**).



Scheme 3 Proposed biosynthetic pathway for **8**, **9** and **2**. No indole shift is responsible for production of **8** and **9**, whereas in the formation of **2**, indole rearrangement is involved. A mechanism for how the 1,2-shift of the indole ring occurs is proposed.

No detectable amount of **10** and **11**, which are produced with an indole shift, was found in this incubation mixture. Furthermore, metabolites **8** and **9**, for which an indole shift is not involved in their biosynthetic processes, were isolated. These findings suggest that VioE had been disrupted in this mutant, thus we propose the biosynthetic pathways for **5**–**9** as shown in Schemes 2 and 3. It is noticeable that H-5 of **20** and H-8 of **25** are specifically removed as an outcome of the favorable aromatization or extension of conjugated double bonds, resulting in no indole rearrangement. In contrast, the native VioE catalyzes the formation of cyclopropane ring **26** *via* **25** (path *b* in Scheme 3) against the favorable aromatization *via* the deprotonation reaction of H-8 (path *a* in Scheme 3), leading to the genuine intermediate **15**. This mechanism for the indole shift by VioE is analogous to that recently suggested by Balibar and Walsh, who have also proposed the cyclopropane ring intermediate.[22] This cyclopropyl intermediate is most favorable for the decarboxylation reaction in conjunction with the indole shift, as depicted in **26** (see also the bottom of Scheme 2). We suggest that the proper orientation of the indole ring in the VioE enzyme

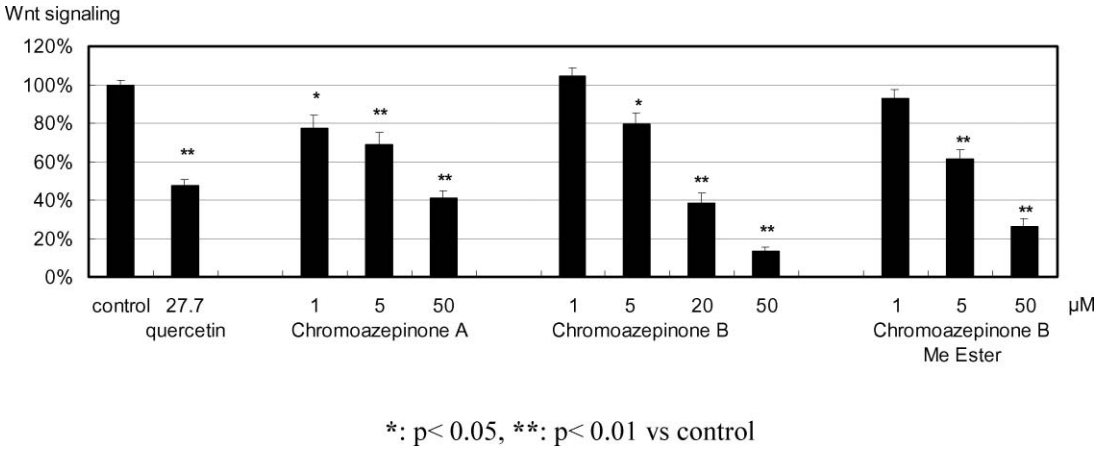


Fig. 2 Wnt signal inhibitory activity of chromoazepinones A, B, and methyl ester of chromoazepinones B. Quercetin was used as a standard positive compound.

cavity may facilitate the indole shift, with the left indole ring of **25** being arranged at the proximal position to C-9 of **25**. This proper indole orientation may be enforced by a π–π interaction with Phe50 and by the interaction of the NH of the left indole ring with Glu66 through hydrogen bonding.[24] The biosynthetic intermediate for **1** and **2** produced by VioB is short-lived, thus its successful isolation has not been reported,[22–24] but **24** (**24′**) or **25** may be proposed as a plausible intermediate.

Bioactivity of chromoazepinones A (5), B (6), and methyl ester of 6

Chromoazepinones A (**5**), B (**6**), and the methyl ester of **6**, which were available in substantive amounts, were tested for their effect on TCF/β-catenin transcriptional activity in the Wnt signaling pathway by using STF/293 cells.[26] As a result (Fig. 2), it was revealed that these compounds exhibited inhibitory activity of TCF/β-catenin transcription with IC_{50} values of 23.9, 13.8, and 10.3 μM, respectively, with almost no cytotoxicity at these concentrations against STF/293 cells. Wnt signaling is implicated in numerous aspects of development, cell biology, and physiology. Meanwhile, Wnt target genes have recently been found to be highly expressed in various cancers, some of which are likely to contribute to cancer formation.[27] Small-molecule inhibitors of the Wnt signaling pathway are therefore desired because it is expected that they would suppress cancer cell growth. Therefore, chromoazepinones and their analogues may be expected as a candidate for small-molecule Wnt signaling inhibitors.

In conclusion, we succeeded in generating novel tryptophan metabolites, chromoazepinone A–C, through chemical mutagenesis. This could have occurred due to the complex involvements of a cluster consisting of the five genes (VioA–E) and non-enzymatic processes in the biosynthetic pathway for violacein. One or more biosynthetic gene mutations with a slight modification would have given rise to some degree of substrate flexibility, leading to recognition of different intermediates. In addition, combination of the slight modification and deactivation of one or more gene(s) involved in the gene cluster would have resulted in creating the unnatural tryptophan metabolites, chromoazepinone A–C, which possess interesting biological activities. Further mutagenesis experiments may pave the way for generations of new alkaloids derived from the tryptophan molecule.

Experimental

Instruments

UV spectra were measured on a JASCO Ubest-30 spectrophotometer. IR spectra with a JASCO IR-700 spectrophotometer. NMR spectra were recorded in DMSO-d_6 on a Varian Gemini 200, a JEOL alpha 400, a Bruker DPX 400 or a Varian Unity 500 spectrometer, the chemical shifts being relative to the solvent peak δ_H 2.49 and δ_C 39.50 as the internal reference for ^1H and ^{13}C NMR spectra, respectively. MS spectra of (EI) were obtained with a JEOL SX 100 mass spectrometer (isobutane gas for CI). Specific rotation values were measured with a Horiba SEPA-300 polarimeter.

Culture of H-25 mutant and isolation of metabolites 5–9

Following a previous paper,[9,11] the blocked mutant H-25 was prepared with mutagenic agent NTG. The culture medium (nutrient broth) consisted of meat extract (3 g), polypeptone (5 g), NaCl (5 g) and water (1 L), with the pH adjusted to 7.2. A seed culture (1 mL) grown for 20 h was inoculated into 100 mL of the medium in a 300 mL Erlenmyer flask and cultured at 25 °C for 24 h on a rotary shaker (180 rpm). The cells were harvested by centrifugation at 9000 rpm for 10 min, followed by washing with a saline solution. The washed whole cells were suspended in a solution containing L-tryptophan (5 mg) in 5 mL of 0.2M ammonium buffer (pH 8.5), and then incubated at 25 °C for 48 h on a rotary shaker, which caused the gradual production of orange pigments. To isolate sufficient amounts of the pigments for structural determination, 90 L cultivation was conducted and 4.0 g of tryptophan was incubated at 25 °C for 24 h on a rotary shaker (180 rpm). The pigments of the orange-colored cells were extracted with MeOH (3 L). To the methanolic extract was added 3 L of water. The solution was concentrated to evaporate MeOH, and the pH was then adjusted to 3.0 after which 3 L of EtOAc was added to extract the pigments. To the EtOAc solution was added an aqueous NaOH (2 N) until the pH was adjusted to 10.0. This transferred the pigments into the aqueous layer, which was acidified to pH 3.5 with 2 N HCl before 1 L of EtOAc was added to it to reextract the pigments. The EtOAc fraction

was dried over Na_2SO_4, and concentrated into a small volume, which was loaded on a Sephadex LH-20 column, and eluted with MeOH. The three yellow or orange colored-pigments (**5**–**7**) were separated in the following elution order: compound **7**, **5** and **6**. The repeated column chromatography of the partially purified **5** over Sephadex LH-20 led to the isolation of **5** in a pure state. Pure **6** and **7** were obtained by SiO_2 column chromatography using a mixture of $CHCl_3$ and MeOH (100 : 2–100 : 7). It was found that metabolite **5** was easily converted into **6** on a SiO_2 TLC plate after evaporating the developing solvent, suggesting that **5** undergoes facile oxidation to give **6**. Therefore, the usage of SiO_2 column chromatography for purification was avoided. The isolation yields of **5**, **6** and **7** were 2.6 mg, 8.0 mg and 2.4 mg, respectively. Compounds **8** and **9** were also detected in the MeOH extracts from the orange-colored cells. The purification was carried out with SiO_2 column chromatography as described in the text.

Spectroscopic data of 5–7 and 9

Metabolite 5, chromoazepinone A: (*Z*)-4-(1*H*-indol-3-yl)-5-oxo-3,4,5,6-tetrahydro-azepino [4,5-*b*]indole-2-carboxylic acid. M.p. > 230 °C. $[\alpha]_D^{20} = +56.0$ ($c = 0.16$, EtOH); UV (MeOH) λ_{max} (log ε) 272 (4.30), 289 (4.31), 331 (3.89, shoulder), 428 (3.70) nm.

Me ester of 5. UV (MeOH) λ_{max} (log ε) 270 (4.14), 289 (4.23), 425 (3.66) nm; IR (KBr) ν_{max} 1690, 1640, 1518, 1260, 740 cm^{-1}; HREIMS *m/z* M^+ 371.1272 (calcd for $C_{22}H_{17}O_3N_3$, 371.1270). ^{1}H-NMR (400 MHz) in DMSO d_6: δ_H (ppm) 11.99 (H-6, br s), 10.75 (H-1′, brs), 7.90 (H-10, d, $J = 7.9$ Hz), 7.81 (H-4′, d, $J = 8.0$ Hz), 7.47 (H-7, d, $J = 7.9$ Hz), 7.37 (H-8, t, $J = 7.9$ Hz), 7.28 (H-7′, d, $J = 8.0$ Hz), 7.14 (H-9, t, $J = 7.9$ Hz), 7.09 (H-1, s), 7.07 (H-3, d, $J = 6.1$ Hz), 7.06 (H-6′, t, $J = 8.0$ Hz), 6.99 (H-5′, t, $J = 8.0$ Hz), 6.46 (H-2′, d, $J = 1.5$ Hz), 5.46 (H-4, d, $J = 6.1$ Hz), 3.75 (H-12, 3H, s, OCH_3); ^{13}C NMR (100 MHz) in DMSO-d_6: δ_C (ppm) 182.5 (C-5, s), 165.4 (C-11, s), 137.5 (C-6a, s), 136.0 (C-7′a, s), 133.6 (C-5a, s), 130.8 (C-2, s), 126.4 (C-8, d), 125.9 (C-3′a, s), 125.4 (C-10a, s), 123.6 (C-2′, d), 121.4 (C-6′, d), 120.6 (C-10, d), 120.4 (C-10b, s), 120.2 (C-9, d), 118.8 (2C, C-4′and C-5′, d), 112.5 (C-7, d), 111.5 (C-7′, d), 108.8 (C-3′, s), 102.8 (C-1, d), 56.97 (C-4, d), 52.40 (C-12, q). HREIMS *m/z* M^+ 371.1272 (calcd for $C_{22}H_{17}O_3N_3$, 371.1270).

Metabolite 6, chromoazepinone B: (1*Z*,3*Z*)-4-(1*H*-indol-3-yl)-5-oxo-5,6-dihydro-azepino[4,5-*b*]indole-2-carboxylic acid. M.p. > 230 °C. UV (MeOH): pH 2.0, λ_{max} (log ε) 282 (4.18), 322 (4.20), 422 (4.15), 550 (3.95) nm; pH 7 & 12, λ_{max} (log ε) 282 (4.26), 322 (4.32), 405 (4.21) nm; HREIMS *m/z* M^+ 355.0930 (calcd for $C_{21}H_{13}O_3N_3$, 355.0957). ^{1}H-NMR (400 MHz) in DMSO-d_6: δ_H (ppm) 13.17 (H-6, very brs), 11.80 (H-1′, s), 9.38 (H-4′, d, $J = 7.9$ Hz), 9.00 (H-2′, s), 8.73 (H-1, s), 8.39 (H-10, d, $J = 7.6$ Hz), 7.71 (H-7, d, $J = 7.6$ Hz), 7.62 (H-8, t, $J = 7.6$ Hz), 7.48 (H-7′, d, $J = 7.9$ Hz), 7.40 (H-9, t, $J = 7.6$ Hz), 7.22 (H-6′, t, $J = 7.9$ Hz), 7.17 (H-5′, t, $J = 7.9$ Hz); ^{13}C NMR (100 MHz) in DMSO-d_6:δ_C (ppm) 170.6 (C-5, s), 168.5 (C-11, s), 153.4 (C-4, s), 140.0 (C-5a, s), 139.0 (C-6a, s), 136.3 (C-7′a, s), 134.8 (C-2, s), 133.1 (C-2′, d), 128.6 (C-8, d), 126.8 (C-3′a, s), 125.2 (C-10a), 124.1 (C-4′, d), 122.6 (C-6′, d), 122.4 (C-10b), 122.2 (C-9, d), 121.9 (C-10, d), 121.1 (C-5′, d), 119.0 (C-1, d), 115.4 (C-3′, s), 113.2 (C-7, d), 111.6 (C-7′, d).

Me ester of 6. UV (MeOH); acidic: λ_{max} (log ε) 283 (4.00), 326 (4.06), 416 (4.06), 432 (4.08), 536 (3.86), 553 (3.86) nm; neutral: 283 (4.05), 318 (4.19), 416 (4.13), 420 (4.13) nm; basic: 283 (4.04), 333 (4.26), 429 (4.14), 507 (3.86, shoulder) nm. HREIMS *m/z* M^+ 369.1111 (calcd for $C_{22}H_{15}N_3O_3$ 369.1113). ^{1}H-NMR (500 MHz) in DMSO-d_6: δ_H (ppm) 12.97 (H-6, brs), 11.81 (H-1′, s), 9.36 (H-4′, m), 9.02 (H-2′, d, $J = 2.5$ Hz), 8.73 (H-1, s), 8.39 (H-10, d, $J = 8.0$ Hz), 7.73 (H-7, d, $J = 8.0$ Hz), 7.65 (H-8, t, $J = 8.0$ Hz), 7.51 (H-7′, m), 7.43 (H-9, t, $J = 8.0$ Hz), 7.24 (2H, H-5′ & H-6′, m), 4.04 (H-12, 3H, s); ^{13}C NMR (50 MHz) in DMSO-d_6: δ_C (ppm) 170.5 (C-5, s), 167.3 (C-11, s), 153.5 (C-4, s), 140.0 (C-5a, s), 138.9 (C-6a, s), 136.2 (C-7′a, s), 133.7 (C-2, s), 133.1 (C-2′, d), 128.6 (C-8, d), 126.7 (C-3′a, s), 125.1 (C-10a, s), 123.9 (C-4′, d), 122.5 (C-6′, d), 122.2 (C-9, d), 122.1 (C-10b, s), 121.8 (C-10, d), 121.1 (C-5′, d), 119.3 (C-1, d), 115.2 (C-3′, s), 113.7 (C-7, d), 111.6 (C-7′, d), 52.59 (C-12, q)

Metabolite 7, chromoazepinone C: (*Z*)-5-(1*H*-indol-3-yl)-4-oxo-3,4,5,6-tetrahydro-azepino[4,5-*b*]indole-2-carboxylic acid. $[\alpha]_D^{20} = +26.6$ ($c = 0.15$, EtOH).

Me ester of compound 7. M.p. > 230 °C. UV (MeOH) λ_{max} (log ε) 280 (4.04), 285 (3.99), 347 (3.93) nm; IR (KBr) ν_{max} 1702, 1640, 1620, 1258, 740 cm^{-1}; HREIMS *m/z* M^+ 371.1275 (calcd for $C_{22}H_{17}O_3N_3$, 371.1270). ^{1}H-NMR (400 MHz) in DMSO-d_6: δ_H (ppm) 11.86 (H-6, brs), 10.89 (H-1′, brs), 9.11 (H-3, s), 7.75 (H-10, d, $J = 8.0$ Hz), 7.67 (H-1, s), 7.55 (H-4′, d, $J = 8.0$ Hz), 7.47 (H-7, d, $J = 8.0$ Hz), 7.31 (H-7′, d, $J = 8.0$ Hz), 7.21 (H-8, t, $J = 8.0$ Hz), 7.15 (H-9, t, $J = 8.0$ Hz), 7.06 (H-6′, t, $J = 8.0$ Hz), 6.97 (H-5′, t, $J = 8.0$ Hz), 6.67 (H-2′, d, $J = 1.9$ Hz), 5.42 (H-5, d), 3.74 (H-12, 3H, s); ^{13}C NMR (50 MHz) in DMSO-d_6: δ_C (ppm) 166.3 (C-4, s), 163.8 (C-11, s), 136.3 (2C, C-5a & C-6a, s), 136.2 (C-7′a, s), 125.7 (C-3′a, s), 122.8 (C-2′, d), 121.4 (C-6′, d), 115.5 (C-1, d), 122.4 (C-8, d), 119.6 (C-2, s), 118.7 (C-5′, d), 118.2 (C-4′, d), 112.0 (C-7, d), 111.5 (C-7′, d), 106.9 (C-3′, s), 52.37 (C-12, q), 45.51 (C-5, d), the assignments of C-5a, C-6a and C-7′a being interchangeable due to the very close values.

Metabolite 9: arcyriarubin A[25]. UV (MeOH) λ_{max} (log ε) 276.5 (4.05), 369.0 (3.71), 458.0 (3.85) nm; IR (KBr) ν_{max} 3390, 1760, 1700, 1530, 1340, 740 cm^{-1}; HREIMS *m/z* M^+ 327.1020 (calcd for $C_{20}H_{13}N_3O_2$, 327.1008). ^{1}H-NMR (400 MHz) in DMSO-d_6: δ_H (ppm) 11.65 (H-1 & H-1′, 2H, s), 10.89 (H-10, 1H, s), 7.73 (H-2 & H-2′, 2H, d, $J = 2.5$ Hz), 7.36 (H-7 & H-7′, 2H, d, $J = 8.0$ Hz), 6.99 (H-6 & H-6′, 2H, t, $J = 8.0$ Hz), 6.80 (H-4 & H-4′, 2H, d, $J = 8.0$ Hz), 6.64 (H-5 & H-5′, 2H, t, $J = 8.0$ Hz); ^{13}C NMR (50 MHz) in DMSO-d_6: δ_C (ppm) 173.6 (2C, C-9 & C-9′, s), 136.57 (2C, C-7a & C-7′a, s), 129.7 (2C, C-2 & C-2′, d), 128.3 (2C, C-8 & C-8′, s), 126.0 (2C, C-3a & C-3′a, s), 122.2 (2C, C-6 & C-6′, d), 121.5 (2C, C-4 & C-4′, d), 119.9 (2C, C-5 & C-5′, d), 112.3 (2C, C-7 & C-7′, d), 106.2 (2C, C-3 & C-3′, s).

Luciferase assay (TCF/β-catenin transcriptional activity)[26]

STF/293 cells (3×10^4, generously provided from Prof. Jeremy Nathans, John Hopkins Medical School) were split into 96-well plates and 24 h later cells were treated with 15 mM LiCl and testing samples (DMSO solution). After incubation for 24 h, cells were lysed with CCLR (cell culture lysis reagent; 20 μL/well, Promega) and luciferase activities were measured with a Luciferase Assay System (Promega). We checked this system worked reasonably by

using quercetin as a standard positive compound. Assays were performed in triplicate.

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