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Biosynthesis of Violacein: Intact Incorporation of the Tryptophan Molecule on the Oxindole Side, with Intramolecular Rearrangement of the Indole Ring on the 5-Hydroxyindole Side*

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Feeding experiments with a mixture of [2-^{13}C]- and [indole-3-^{13}C]tryptophans, of [3-^{13}C]- and [indole-3-^{13}C]tryptophans (1:1 molar ratio) and of others have proved that the 1,2-shift of the indole ring occurred *via* an intramolecular process for formation of the left part (5-hydroxyindole side) of the violacein skeleton and demonstrated that the C–C bond from C2 of the indole ring to C2 of the side chain was completely retained for formation of the right part (oxindole side) during the entire biosynthetic process. Due to the involvement of transaminase, it has remained unresolved whether indolylpyruvic acid is the biosynthetic intermediate and/or from where the nitrogen atom of the pyrrolidone ring originates. An incorporation experiment with a mixture of [2-^{13}C]- and [α-^{15}N]tryptophans (1:1 molar ratio) verified that the nitrogen atom in the central ring was exclusively derived from the right-side tryptophan. Thus, all the carbon and nitrogen atoms in the right part of the violacein skeleton were constructed by intact incorporation of the tryptophan molecule, with decarboxylation probably occurring at a later biosynthetic stage.

Key words: violacein; *Chromobacterium violaceum*; tryptophan; biosynthesis; stable isotope

Violacein **1** is a blue pigment produced by the bacterium species, *Chromobacterium violaceum*.[1-3] Its deoxy-analogue, deoxyviolacein **2**, is a minor product. Violacein consists of three structural units: 5-hydroxyindole (the left side), oxindole (the right side) and 2-pyrrolidone nuclei (the central part) (Fig. 1). All the carbon skeleton is biosynthesized from two molecules of L-tryptophan **3** accompanying the decarboxylation process.[2,3] We have previously reported, by using ^{13}C-labeled tryptophan **3** and oxygen-18 gas, that rearrangement of the indole ring occurred[3] and that the oxygen atoms in violacein skeleton were derived from molecular oxygen.[4] Rearrangement of the aromatic ring of natural products during biosynthetic processes is rarely found; phenyl ring rearrangement has been found for the interconversion between flavanone and isoflavone in plants,[5] while an indole shift has been found for the synthesis of violacein produced by a prokaryotic bacterium sp.[3] The incorporation of all oxygen atoms into **1** is catalyzed by oxygenase, and hydroxylation at the 6-position of **1** is mediated by a monooxygenase, a copper-containing enzyme.[6] The addition of the copper-chelating agent, sodium *N'*,*N'*-diethyldithiocarbamate, accumulated proviolacein and prodeoxyviolacein,[7] whose structure is analogous to that of **1**, only differing in the absence of an oxygen atom at the 16-position. The structure of proviolacein suggests that oxygenation at the 16-position may be the final biosynthetic step.[7] Based on the finding that oxygenase-mediating reactions are responsible for biosynthesis, we have recently succeeded in constructing a cell-free system which allows efficient synthesis of the pigment from a tryptophan precursor; NADPH cofactor is the most important factor among several elements.[8] No supplementation of NADPH to a cell-free extract leads to the accumulation of a green pigment, named deoxychromoviridans,[9] which has a tetraindole skeleton, but no oxygen atom is involved in the molecule. This finding is further suggests the requirement of the NADPH cofactor for oxygen incorporation reactions. During the biosynthetic studies, we have succeeded in isolating the gene cluster for the biosynthesis (*ca.* 9 kb) encoding all the enzymes from the tryptophan precursor and with a high expression in *E. coli* (unpublished result by this laboratory, the DNA sequence will be reported in due course).[10] The cell-free system from the *E. coli* JM 109 transformant allowed the production of **1** in

* The nucleotide sequence data for the biosynthetic gene cluster of violacein is available under accession number AB032799.

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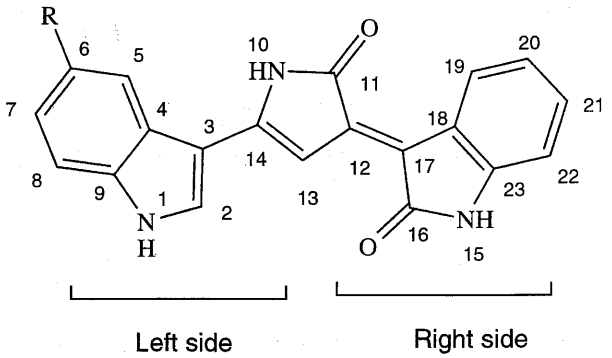
Abbreviations: INADEQUATE, incredible natural abundance double quantum transfer experiment; p.n.d. ^{13}C-NMR, proton-noise decoupled ^{13}C-NMR



1: R=OH, violacein (1a)
2: R=H, deoxyviolacein

Fig. 1. Structures of Violacein **1** and Deoxyviolacein **2**.
The left side denotes 5-hydroxyindole and half of the 2-pyrrolidone ring, while the right side represents oxindole and half of the central ring.

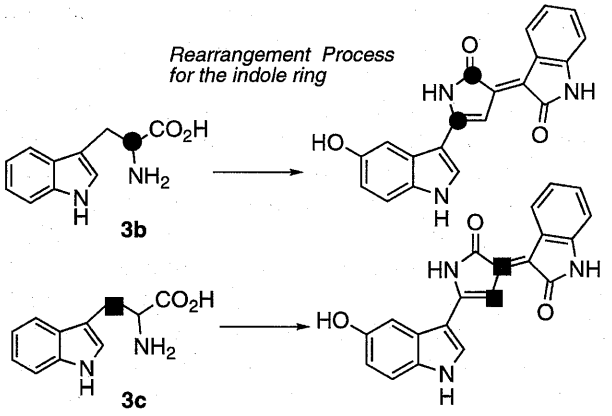


Fig. 2. Labeling Patterns of Violacein Produced by the Independent Incubation of [2-^{13}C]-**3b** and [3-^{13}C]-L-Tryptophans **3c**.
This result has been reported in the previous paper.[3]

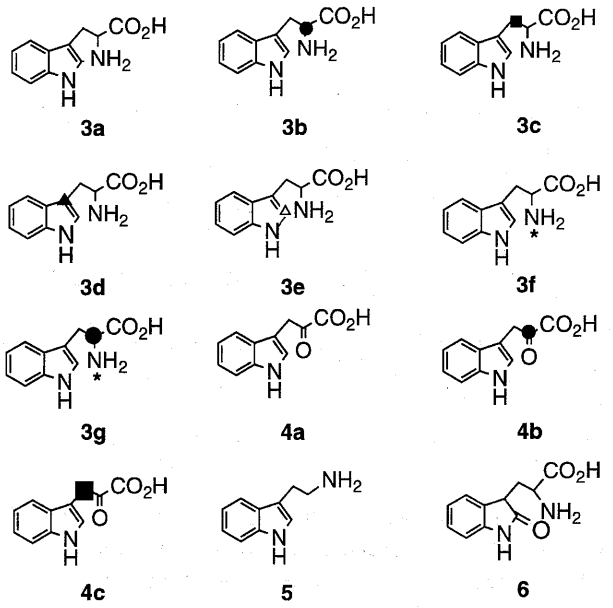


Fig. 3. Tryptophans and Analogs Used and Discussed in the Present Studies.
The C-13 and N-15 labeled positions are marked with the symbols ●, ■, ▲, △, ★.

a larger amount, usually by 30–40 fold, compared to that from the parent strain *C. violaceum*.

In the previous paper,[3,4] we have inferred a 1,2-shift of the indole ring *via* an intramolecular process, based upon the results obtained from independent feeding experiments with each labeled tryptophan **3b** or **3c** (Fig. 2, and compounds shown in Fig. 3). However, another possible interpretation for this rearrangement mechanism remains that fragmentation and exchange reactions may occur between each side chain and each indole ring to biosynthesize **1** *via intermolecular* rearrangement (Fig. 4–1), which leads to the same labeling pattern as that of intramolecular shift. If one assumes that such an enzyme as tryptophanase is involved, the exchange reaction might be presumed between the side chain of the left side and the indole ring of the right side, and *vice versa*; tryptophanase cleaves the C–C bond between the indole ring and the side chain to yield indole, pyruvic acid and ammonia. To determine which process, *i.e.*, either intra- or intermolecular shift, is responsible for the biosynthesis, we planned to use two types of ^{13}C-labeled **3**, having different labeled positions; one is labeling of side chain **3b** or **3c**, while alternative labeling is that of indole ring **3d** or **3e** (Fig. 4). When a mixture of **3b** and **3d** or of **3c** and **3e** has been administered, the incorporation pattern shown in Fig. 4–2 could be presumed, which allows the discrimination between the intra- and intermolecular mechanisms. As an enzymic source, the cell-free extract from the *E. coli* clone was used throughout the present studies, due to its high activity. These mixed feeding experiments (1:1 molar ratio), have established that the indole shift took place *via* an *intramolecular* and not intermolecular process. Due to the involvement of transaminase, it has remained inconclusive whether indolylpyruvic acid **4** is the biosynthetic intermediate or not.[4] To date, this unresolved point has interfered with progress in biosynthetic studies on **1**. A feeding experiment on ^{13}C-**3b** and ^{15}N-labeled **3f** has permitted the proposal that the nitrogen atom (N10) in the central pyrrolidone ring is exclusively derived from the *right side tryptophan*, and not from the left side one. This finding means that **4** is not a biosynthetic intermediate for the right side. All the C–C and C–N bonds containing α-amino nitrogen from the indole nucleus are kept intact for the right side tryptophan during the entire biosynthetic processes. The reactions of decarboxylation and oxygenation at the 16-position would proceed at a later reaction stage, probably after the condensation reaction of two molecules of

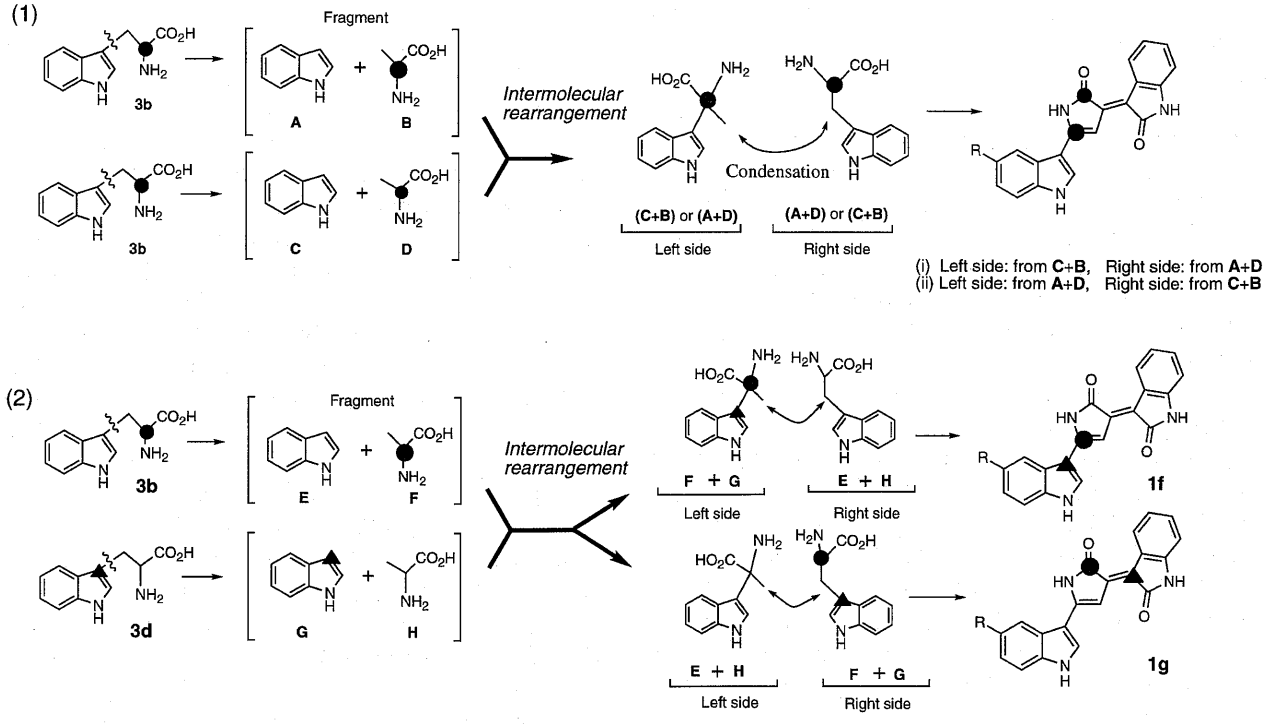


Fig. 4. Presumed Labeling Patterns in the Case of Fragmentation Between the Indole Ring and the Side Chain Being Followed by an Exchange Reaction Between the Two Different Parts of the Side Chain and the Indole Ring.

Cases 1 and 2 show two examples of violacein being produced as a result of intermolecular rearrangement of the indole ring. It is noted that the incorporation pattern of **3b** into violacein (●) shown in case 1 is identical to that of Fig. 2.[3] Therefore, only the usage of side chain-labeled tryptophan **3b** or **3c** does not lead to the inference of which process of intra- or intermolecular rearrangement would be responsible for biosynthesis. As shown in 2, it is possible to differentiate, however, between the two different mechanisms of intra- and intermolecular processes by using indole ring-labeled tryptophan **3d**; labeled violacein (**1f** and **1g**, see Fig. 6), which is marked with ● and ▲, must be produced *via* an intermolecular shift, but **1f** and **1g** are not formed *via* the intramolecular process (also see Fig. 6). The fragments of **B**, **D**, **F** and **H** may be pyruvic acid, if tryptophanase were responsible for the biosynthesis.

3, because tryptamine **5** and oxindolylalanine **6** were not incorporated into **1**. We describe here the new finding that the right side tryptophan is directly incorporated without any modification during the biosynthesis.

Materials and Methods

Instruments. ^{1}H- and ^{13}C-NMR spectra were measured with a Bruker DPX 400 instrument, EIMS spectra with a JEOL SX 100 mass spectrometer, and UV spectra with a JASCO Ubest-30 spectrophotometer.

NMR spectra. All the ^{1}H-NMR and ^{13}C-NMR spectra were recorded in DMSO-d_6. Chemical shifts are given relative to the signal of DMSO-d_6 (δ_C, 39.5 ppm and δ_H, 2.49 ppm). Five hundred μg of the labelled violacein were usually used for the ^{13}C-NMR measurements. In the INADEQUATE experiment, the ^{13}C–^{13}C coupling constants (cnst3) and d4 = $1/(4J_{CC})$ were estimated from the p.n.d. ^{13}C-NMR spectra and adjusted to obtain good correlation of the cross peaks; J_{CC} = 8.0 Hz for the violacein, which was prepared from a mixture of **3b** and **3d**, and J_{CC} = 4.0 and 55.0 Hz for the labelled violacein from a mixture of **3c** and **3d**. The running times were usually 48–72 h.

Synthesis of the labelled L*-tryptophans.* L-[2-^{13}C]-**3b**, L-[3-^{13}C]-**3c**, L-[indole-3-^{13}C]-**3d** and L-[indole-2-^{13}C]tryptophans **3e** were synthesized according to the literature[11] and subjected to optical resolution with L-amino acylase from Sigma to give the L-forms. As starting materials, diethyl [2-^{13}C]-acetamidomalonate (99 atom%), [^{13}C]formaldehyde (20% aq. solution, 99 atom%), [3-^{13}C]indole (99 atom%) and [2-^{13}C]indole (99 atom%) were used, these being purchased from Isotech. L-[α-^{15}N]Tryptophan **3f** was prepared by reductive amination of indolylpyruvic acid by using $^{15}NH_4NO_3$ (99.2 atom%) and $NaCNBH_3$,[12] and then the L-form was obtained by optical resolution. $^{15}NH_4NO_3$ was purchased from Shokotsusho Co. The isotopic contents of all the labeled tryptophans thus prepared were higher than 97% from their MS analyses.

Preparation of oxindolylalanine **6**. **6** was prepared from tryptophan according to the literature.[13]

Preparation of the cell-free system and isolation

method for violaceim. E. coli JM 109 harboring pVBG04,[10] which encodes all the enzymes responsible for the biosynthesis of **1**, was grown on a reciprocal shaker at 30°C for 12 h in an LB medium (1 l) composed of polypeptone (1%), yeast extract (0.5%), NaCl (0.5%), and Tween 80 (0.002%) at pH 7.2. The *E. coli* cells grown under these culture conditions were free of endogenous **1**. To cell pellets collected by centrifugation, 50 ml of an ammonium buffer (0.2 M, pH 8.5) containing 200 μl of 0.1% (w/v) Triton X-100 and 50 μl of 10 mM 2-mercaptoethanol was added, and the mixture sonicated at 4°C for 15 min, before being centrifuged at 10,000 × *g* for 15 min to remove the cell debris. The crude cell-free extract thus prepared was employed as the enzymic source. To 5 ml of the cell-free extract, which had been adjusted to pH 8.5, 1 mg of L-tryptophan (1 mM) and 9 mg of NADPH (2 mM) were added. The mixture was incubated at 25°C for 24 h on a rotary shaker at 200 rpm, and then lyophilized. The powdered residue was dissolved in MeOH. Separation of **1** and **2** was achieved by column chromatography with an adsorbent of Sephadex LH 20 and subsequent elution with MeOH. The conversion ratio from **3** was usually 32–35% for **1** and 0.23% for **2**. The pigment amounts were determined by measuring the absorbance at 570 nm (ε 28,000) for **1** and at 560 nm (ε 26,000) for **2**. In the case of mixed feeding experiments on **3b** and **3f**, a phosphate buffer solution (0.2 M, pH 7.5) was used to prepare the cell-free extract.

*Calculation of the ^{15}N and ^{13}C atom% values of labeled violacein prepared by incorporation experiments with a mixture of [2-^{13}C]-**3b** and [α-^{15}N] tryptophans* **3f**. The p.n.d. ^{13}C-NMR spectrum showed two pairs of satellite peaks ($^2J_{C14\text{-}C11}$ = 7.7 Hz and $^1J_{C14\text{-}N10}$ = 12.2 Hz) around the central signal at C14 (δ_C 147.68) (Fig. 7B). These satellite peaks show the presence of two species **1c** and **1o**, while the appearance of the central signal indicates the presence of **1p** in labeled **1** (Fig. 7B). The signal at C11 (δ_C 171.75) had the satellite peak ($^2J_{C14\text{-}C11}$ = 7.7 Hz) and central signal, exhibiting the involvement of **1c** and **1m**, respectively. The satellite signal assignable to **1c** appeared at both C11 and C14. The peak heights of **1o** and **1c** were 1.1 and 3.1, respectively, after assigning the peak height of **1p** as 1.0. The satellite peak height of **1c** at C11 should be the same as that of the satellite peak at C14, which enabled us to estimate the peak height of **1m** to be 1.9 from the ratio between satellite peak **1c** (3.1) and central signal **1m**. The presence of **1a** was confirmed from the EIMS spectrum; the peak intensity of m/z 343 (M^+ for **1a**) was 14.6%. The other peak intensities were assigned as follows: 44.4% for m/z $M^+ + 1$ and 41.0% for m/z $M^+ + 2$. The peak of m/z $M^+ + 2$ is representative of the two species of **1c** and **1o**. Therefore, the contents of **1c** and **1o** were estimated to be 30.2% and 10.8%, respectively, from the ratio between **1o** and **1c** in the ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum (Fig. 7A) shows the central signal accompanying the satellite peaks ($^1J_{H10\text{-}N10}$ = 95.5 Hz) at H10 (δ_H 10.72). The ^{15}N-content was estimated to be 27% by integrating each signal. Taking into account the content of **1o** (10.8%), **1n** having the ^{15}N-atom must have been present in a content of 16.2%. The peak of m/z $M^+ + 1$ denotes the sum (44.4% from MS) of the three species **1m** + **1n** + **1p**. Thus, the sum of **1m** + **1p** was 28.2%. As already mentioned (from the ^{13}C-NMR spectrum), the ratio of **1m** to **1p** was 1.9: 1.0, leading to the contents of 18.5% and 9.7%. Therefore, the contents of species **1m**, **1n**, **1o**, **1c**, **1a** and **1p** were determined to be 18.5%, 16.2%, 10.8%, 30.2%, 14.6% and 9.7%, as shown in Fig. 8. These values were estimated without considering the content of isotopes such as ^{2}H, ^{17}O, ^{18}O and ^{15}N.

Results and Discussion

The amount of **1** produced by the *E. coli* transformant was significantly larger than that by *C. violaceum*. We have previously reported that a cell-free extract from the parent strain, which had been fortified with NADPH (2 mM), produced several pigments other than **1** and **2** from precursor L-tryptophan **3a**.[8,9,14-17] The conversion ratios from **3a** with a cell-free system of the parent strain were as follows: 0.8–1.0% for **1**, 0.11% for **2**, 0.13% for prodeoxyviolacein, 0.15% for proviolacein, 4.1% for deoxychromoviridans and 3.0% for chromoviridans. On the other hand, with the cell-free extract of the *E. coli* clone, which had been supplemented with NADPH (2 mM), a large quantity of **1** was produced in a yield of 32–35% together with a trace amount of **2**, but the amount of proviolacein or deoxychromoviridans was little.

Incorporation patterns by feeding a mixture of side-chain **3b** *or* **3c** *and indole ring-labeled tryptophan* **3d** *or* **3e** *in an equivalent amount*

Complete assignment of the ^{13}C-NMR signals of violacein have been reported in the previous paper.[3] Fig. 5A shows the p.n.d. ^{13}C-NMR spectrum of the labeled violacein, which had been prepared by incubating a mixture of [2-^{13}C]-**3b** and [indole-3-^{13}C] tryptophans **3d** (1:1 molar ratio). The EIMS spectrum showed m/z 345 as a base peak, but little ion at m/z 343 of **1a**, indicating that the violacein thus produced had two ^{13}C atoms in the molecule with little dilution of the C-13 atom% during the biosynthesis. The ^{13}C-NMR spectrum showed that four carbons were labeled at C3 (δ_C 105.78), C11 (δ_C 171.75), C14 (δ_C 147.68) and C17 (δ_C 118.77) as a result of the high incorporation rate (96% for m/z $M^+ + 2$ from EIMS), and also showed complex

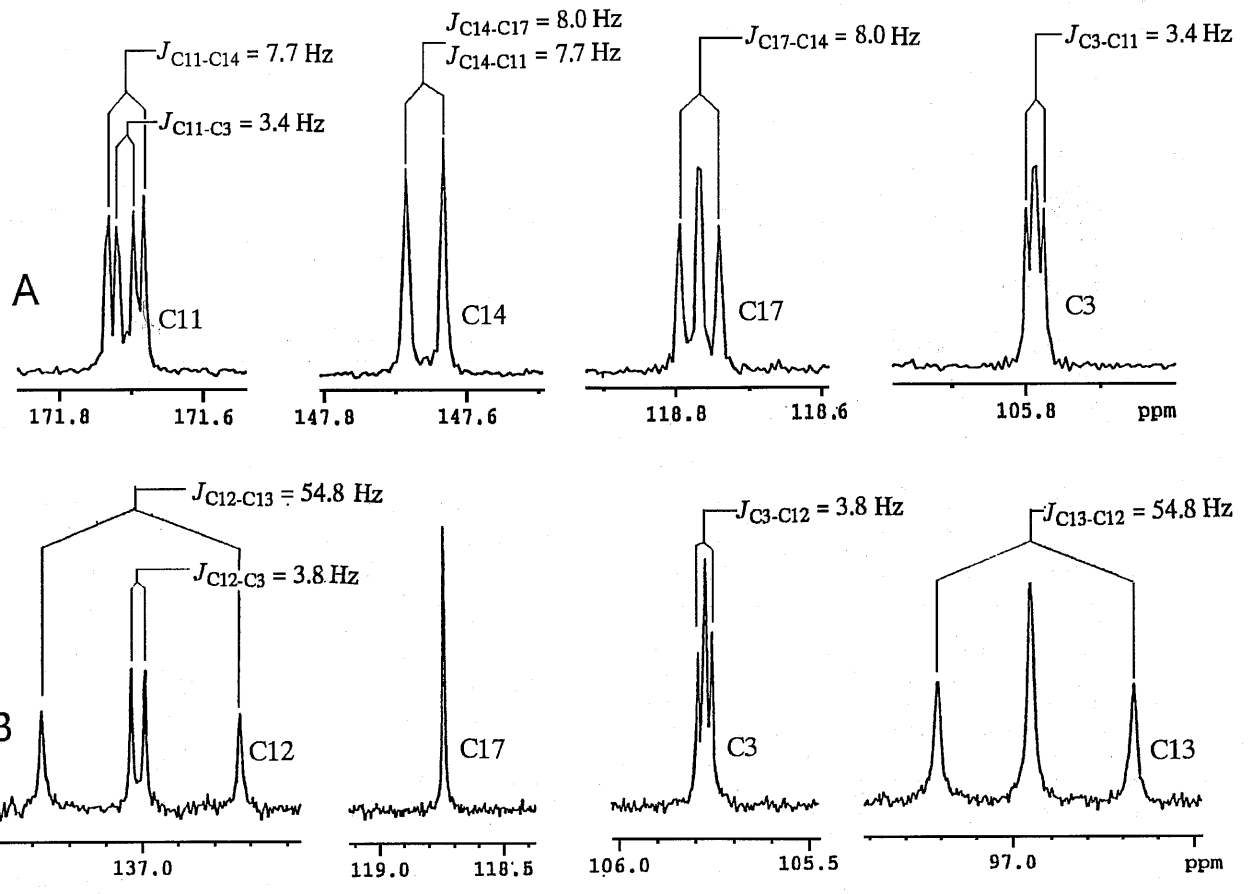


Fig. 5. p.n.d. ^{13}C-NMR Spectra of the Labeled Violaceins in DMSO-d_6.
A, violacein obtained by the feeding experiment on a mixture of [2-^{13}C]-**3b** and [indole-3-^{13}C]tryptophans **3d** (1:1 molar ratio). **B**, violacein similarly prepared from [3-^{13}C]-**3c** and [indole-3-^{13}C]tryptophans **3d** (1:1 molar ratio). The coupling constants of $^{2}J_{C14-C11}$ and $^{3}J_{C14-C17}$ were nearly equal (7.7 and 8.0 Hz, respectively), so these couplings were not separated at C14 (Fig. 4A).

splitting patterns (Fig. 5A) due to the involvement of different ^{13}C–^{13}C coupling constants. To elucidate the coupling network, the INADEQUATE experiment (data not shown) was carried out. Three cross peaks were found in the 2D NMR spectrum: C11/C14, C11/C3 and C14/C17. Thus, the intricate coupling patterns in the ^{13}C-NMR spectrum (Fig. 5A) were resolved as follows: C11 had two pairs of satellite peaks ($^{2}J_{C11-C14}$=7.7 and $^{3}J_{C3-C11}$=3.4 Hz); C14 showed two satellite peaks ($^{3}J_{C14-C17}$=8.0 and $^{2}J_{C11-C14}$=7.7 Hz), but lacked a central signal; with respect to C17 and C3, the central peaks appeared accompanying two pairs of satellite peaks having $J_{C14-C17}$=8.0 Hz and J_{C3-C11}=3.4 Hz, respectively (Fig. 5A). The central peaks at C3 and C17 would have appeared due to the lack of ^{13}C–^{13}C coupling ($^{4}J_{C3-C17}$=0 Hz for **1b** in Figs. 5 and 6). Detailed analyses of the coupling patterns and of the isotopic distribution by the signal intensities allow us to propose that four species **1b**, **1c**, **1d** and **1e** were included in the molar ratio of 1:1:1:1 (Fig. 6). This result indicates that **3b** and **3d** were equivalently metabolized to synthesize violacein. This incorporation experiment further shed light on the mechanism by which the central pyrrolidone ring was constructed between the side chains of two tryptophan molecules. If migration occurred *via* an intermolecular process as shown in Fig. 4, two extra species of **1f** and **1g** (Fig. 6) could have been produced in the labeled violacein. However, the NMR analyses unequivocally verify that no detectable amount of species **1f** and **1g** could be found, because neither the coupling of $^{1}J_{C3-C14}$ nor that of $^{2}J_{C11-C17}$ were detected (Fig. 5A). This finding strongly suggests that rearrangement of the indole ring on the left side **3** could never occur in an intermolecular manner, *i.e.*, according to such a mechanism as that shown in Fig. 4.

A similar incorporation experiment was carried out by using a mixture of [3-^{13}C]-**3c** and [indole-3-^{13}C]tryptophan **3d** in a molar ratio of 1:1. The four carbon signals of C3 (δ_C 105.78), C12 (δ_C 137.22), C13 (δ_C 96.85) and C17 (δ_C 118.77) were labeled in the p.n.d. ^{13}C-NMR spectrum (Fig. 5B). The central signals at C3 and C13 were accompanied by satellite peaks of J_{CC}=3.8 Hz and J_{CC}=54.8 Hz, respectively. The INADEQUATE experiments showed the correlation between C12 and C13 and between C3

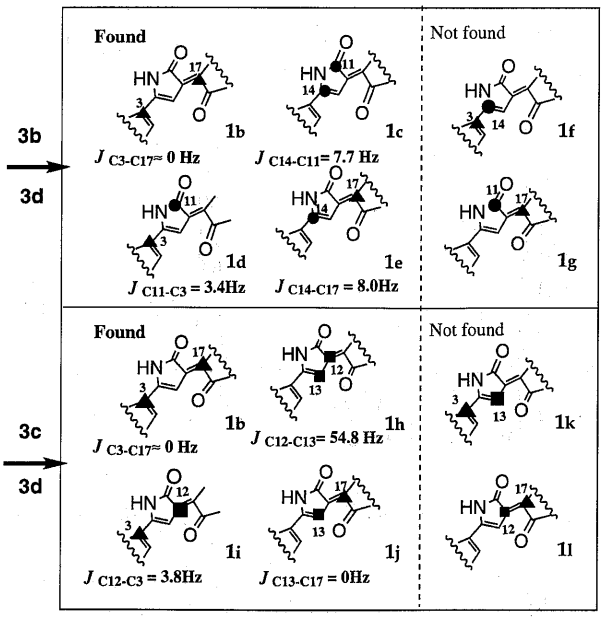


Fig. 6. Incorporation Patterns Obtained by Feeding a Mixture of the Side Chain-labeled and Indole ring-labeled Tryptophans.

Found (left side): incorporation results obtained by the feeding experiments on the two types of tryptophans (1:1 molar ratio) labeled at different positions. Not Found (right side): incorporation patterns presumed according to such a mechanism as that shown in Fig. 4 (2).

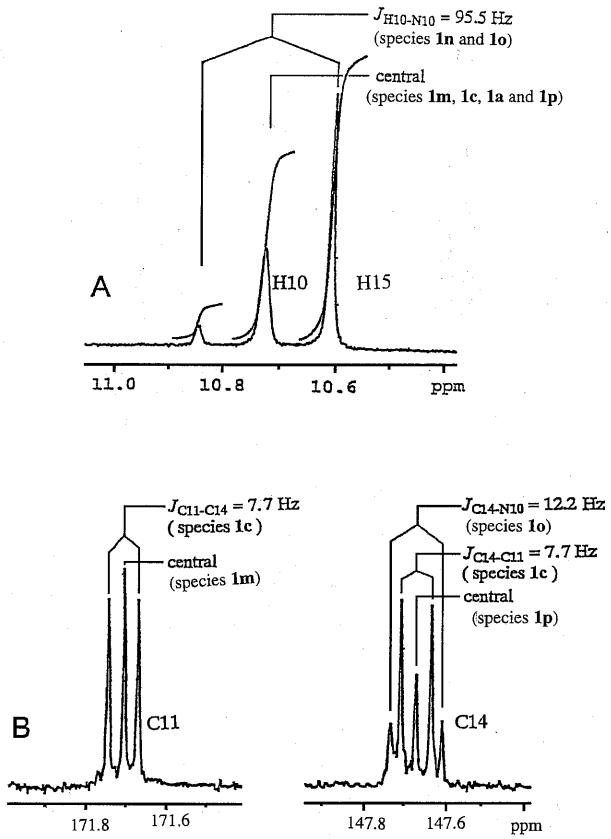


Fig. 7. ^{1}H- (**A**) and ^{13}C-NMR Spectra (**B**) of Labeled Violacein in DMSO-d_6, which Had Been Prepared from a Mixture of [α-^{15}N]-**3f** and [2-^{13}C]Tryptophans **3b** (1:1 molar ratio).

The product species inferred from the NMR spectra are shown. The satellite signal of H10 in a higher field due to the coupling of $J_{H10-N10}$ overlaps the signal of H15 at δ 10.61 ppm.

and C12, thus revealing the presence of **1h** and **1i**, respectively (Fig. 6). The central peaks at C3 and C13 would have appeared due to $^{4}J_{C3-C17}=0$ Hz for **1b** and $^{2}J_{C13-C17}=0$ Hz for **1j**. For C12, two pairs of satellite peaks due to $J_{CC}=54.8$ and 3.8 Hz were found without any observation of the central signal. For C17, only the central peak was observed. The ^{1}H-NMR spectrum also showed that C13 was labeled ($^{1}J_{C13-H13}=179$ Hz), and the ^{13}C content was determined to be *ca.* 48% (theoretically 50%). The peak heights of the satellite peaks to the central signals at each ^{13}C-labeled position in the ^{13}C-NMR spectrum show that the four species of **1b**, **1h**, **1i** and **1j** were involved in the labeled violacein in a molar ratio of 1:1:1:1 (Fig. 6). Species **1k** and **1l** are products which can be presumed from the biosynthetic mechanism shown in Fig. 4, but **1k** and **1l** were not found, because the coupling of neither $^{2}J_{C3-C13}$ nor $^{1}J_{C12-C17}$ could be detected (Fig. 5B). The reason is not clear why species **1j** exhibited only the central signal, *i.e.* $^{2}J_{C13-C17}=0$ Hz, despite the two-bond ^{13}C–^{13}C coupling usually being observed.

The mixed feeding experiments on **3c**+**3e** and **3b**+**3e** (1:1 ratio) also verified that biosynthesis occurred *via* an intramolecular mechanism, because the species presumed according to Fig. 4 were never found. The incorporation experiment on **3b**+**3c** revealed $^{2}J_{C11-C13}=5.4$ Hz and $^{2}J_{C12-C14}=2.1$ Hz, these values not having been reported before. The feeding result for **3d**+**3e** clarified the four labeling positions at C2, C3, C16 and C17.

All the ^{13}C-NMR spectra of ^{13}C-labeled **1**, which were prepared by using a mixture of tryptophans labeled at indole ring **3d** or **3e** and at side chains **3b** or **3c** (1:1 molar ratio), revealed the following mechanisms (Fig. 9): (1) Three C–C bonds (side chain C2 from indole C2) of the right side tryptophan were retained and incorporated into C16–C17–C12–C11 of the violacein skeleton (the right part). In other words, all the carbons of the right side tryptophan were directly incorporated into the right part of violacein, except for the carboxyl carbon. (2) On the other hand, for the left part of violacein, the bonds of both C13–C14 and C2–C3 (violacein numbering) were retained, but the C–C bond between the indole nucleus and the side chain of the left side tryptophan was cleaved to undergo an *intramolecular* 1,2-shift of the indole ring, probably in a concerted manner (Fig. 9). It can be concluded that the exchange reactions between the side chain and indole ring, which are described in Fig. 4, did not take place. These incorporation experiments not only reveal the biosynthetic mechanism, but also give information

of the previously unknown ^{13}C–^{13}C coupling constants (Fig. 6).

Origin of the nitrogen atom in the pyrrolidone nucleus

In the previous paper,[17] we have reported that, by feeding [3-^{13}C]indolylpyruvic acid **4c** with growing cells, both C12 and C13 of **1** was labeled, *i.e.*, leading to the production of **1h** in Fig. 6. At that time, we assumed that the incorporation of **4c** into **1** would probably have occurred due to an active transamination reaction to yield **3c** from **4c**, because the transamination reaction is ubiquitous in living organisms. To examine the nitrogen source in the pyrrolidone ring, L-[α-^{15}N]tryptophan **3f** was incubated with a cell-free extract in a phosphate buffer solution instead of an ammonium buffer, because the ammonium buffer component is likely to be incorporated into the tryptophan precursor *via* a transamination reaction.[4] The ^{1}H-NMR spectrum of violacein, which had been produced by feeding **3f**, showed the satellite peak ($^1J_{N10\text{-}H10}$=95.5Hz) at H10 (δ_H 10.72). The ^{15}N content of the pyrrolidone moiety was 56% from an integration of the satellite and the central peaks. However, the ^{15}N content was up to 76% when **3f** was incubated in the presence of the transaminase inhibitor, aminooxyacetate (10 mM),[18] further supporting the transamination reaction being active in the cell-free system. The fact that the ^{15}N atom was incorporated also suggests that the nitrogen atom of the pyrrolidone ring came from an α-amino group, but the question has remained unanswered which side of the two tryptophans (either left or right) serves the nitrogen atom for central ring formation.

To clarify this unresolved point, a mixture of **3b** and **3f** (1:1) was incubated to prepare violacein labeled with both the ^{15}N- and ^{13}C-isotopes. Integration of the central and satellite peaks at H10 in the ^{1}H-NMR spectrum determined the ^{15}N-content to be 27% (Fig. 7A), this value being half that from a single feeding of **3f**, suggesting that **3b** and **3f** were

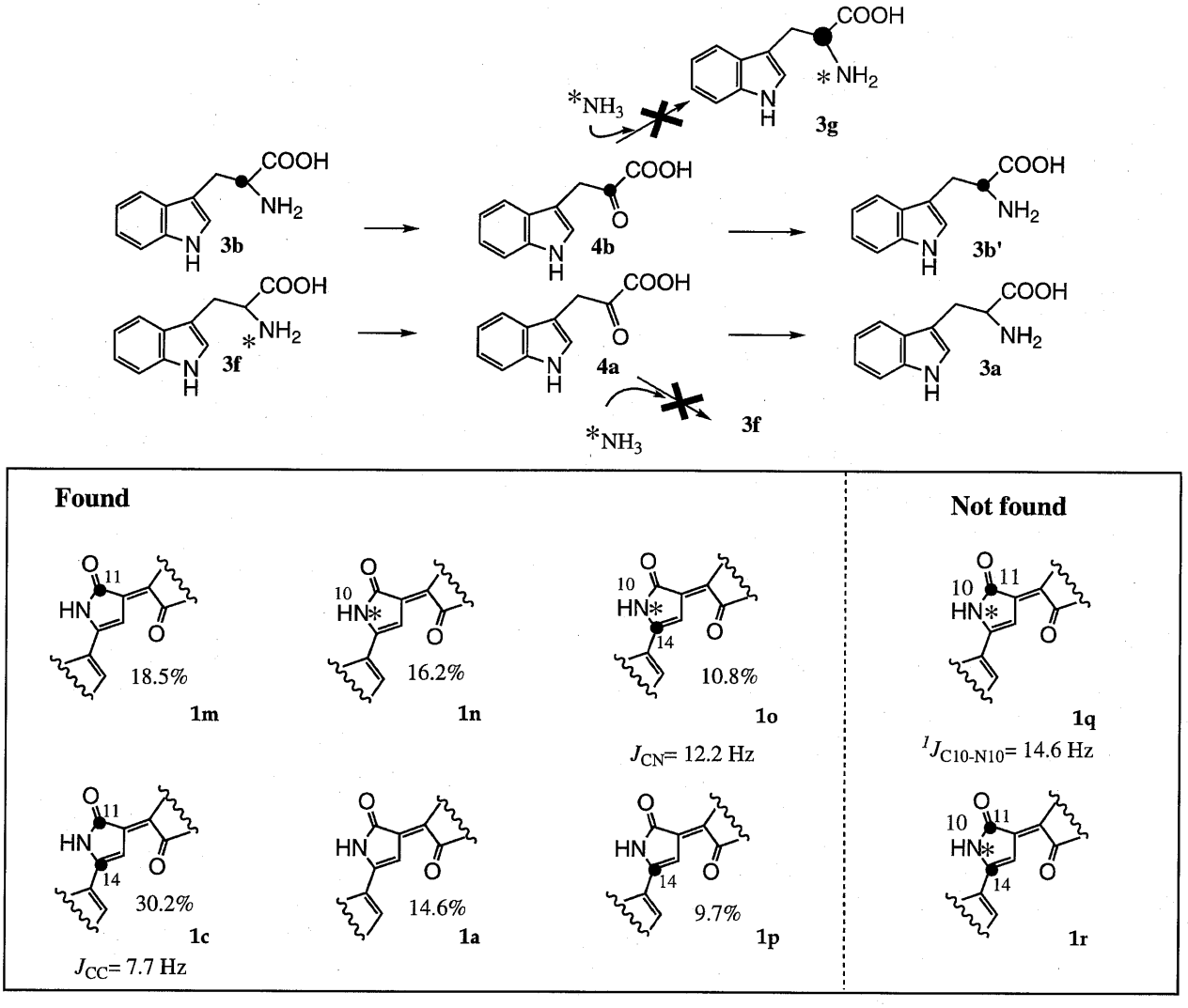


Fig. 8. Incorporation Patterns Obtained by Feeding a Mixture of [α-^{15}N]-**3f** and [2-^{13}C]Tryptophans **3b** (1:1 molar ratio) and the Substrate Species Involved in This Reaction System.

equivalently metabolized to biosynthesize **1**. The ^{13}C-NMR spectrum (Fig. 7B) shows one satellite (due to $^{2}J_{C11\text{-}C14}=7.7$Hz) along with the central peak at the position of C11 (δ_C 171.75). Two pairs of satellite peaks, due to $^{2}J_{C11\text{-}C14}=7.7$ Hz and $^{1}J_{C14\text{-}N10}=12.2$ Hz, were observed at C14 (δ_C 147.68) in addition to the central signal. The ^{13}C–^{13}C coupling between C11 and C14 was further confirmed by an INADEQUATE experiment. By analyzing the peak intensities of the splitting patterns, the isotopic distribution was determined as follows (Fig. 8): 18.5% for **1m**, 16.2% for **1n**, 10.8% for **1o**, 30.2% for **1c**, 14.6% for **1a** and 9.7% for **1p**. The presence of **1a** and **1n** was confirmed by a combination of EIMS and NMR analyses. The contents of these product species were estimated by the calculation method that had been described in the Materials and Methods section. Two species **1q** and **1r** were never detected in the ^{13}C-NMR spectrum (Fig. 7B), because the coupling of $^{1}J_{C11\text{-}N10}$ was absent at both C11 and C14.

With this feeding system, the following six substrate species are presumed for the biosynthesis of violacein: **3b**, **4b**, **3b′**, **3f**, **4a** and **3a** (Fig. 8). Once nitrogen-15 has been released from **3f** to give **4a**, the formation of ^{15}N-labeled **3f** (the reverse reaction) would be little due to the large pool size of $^{14}NH_3$ in the cell-free system. Thus, the reverse reaction of **4a** or **4b** into **3f** or **3g**, respectively, would be impossible. **3a** would be formed from **4a** due to a transamination reaction. **3g** may be produced by the incorporation reaction of $^{15}NH_3$ into **4b**. **3g** is indispensable for the formation of **1r**, because **1r** can be produced from (**3b** or **3b′**+**3g**), (**4b**+**3g**) or (**3g**+**3g**), but **1r** was never detected in the ^{13}C-NMR spectrum (Fig. 7B). This finding strongly suggests that the reaction of **4b**→**3g** never occurs and also suggests that the assumption for the large pool size is valid; that is, the reaction of **4a**→**3f** also never occurs. There is the possibility that a condensation reaction between **4a** and **4a** occurs, this being followed by the incorporation of a nitrogen atom at a later stage, leading to the formation of **1**, but this idea is not supported. If so, there would be little incorporation of nitrogen-15 due to the involvement of

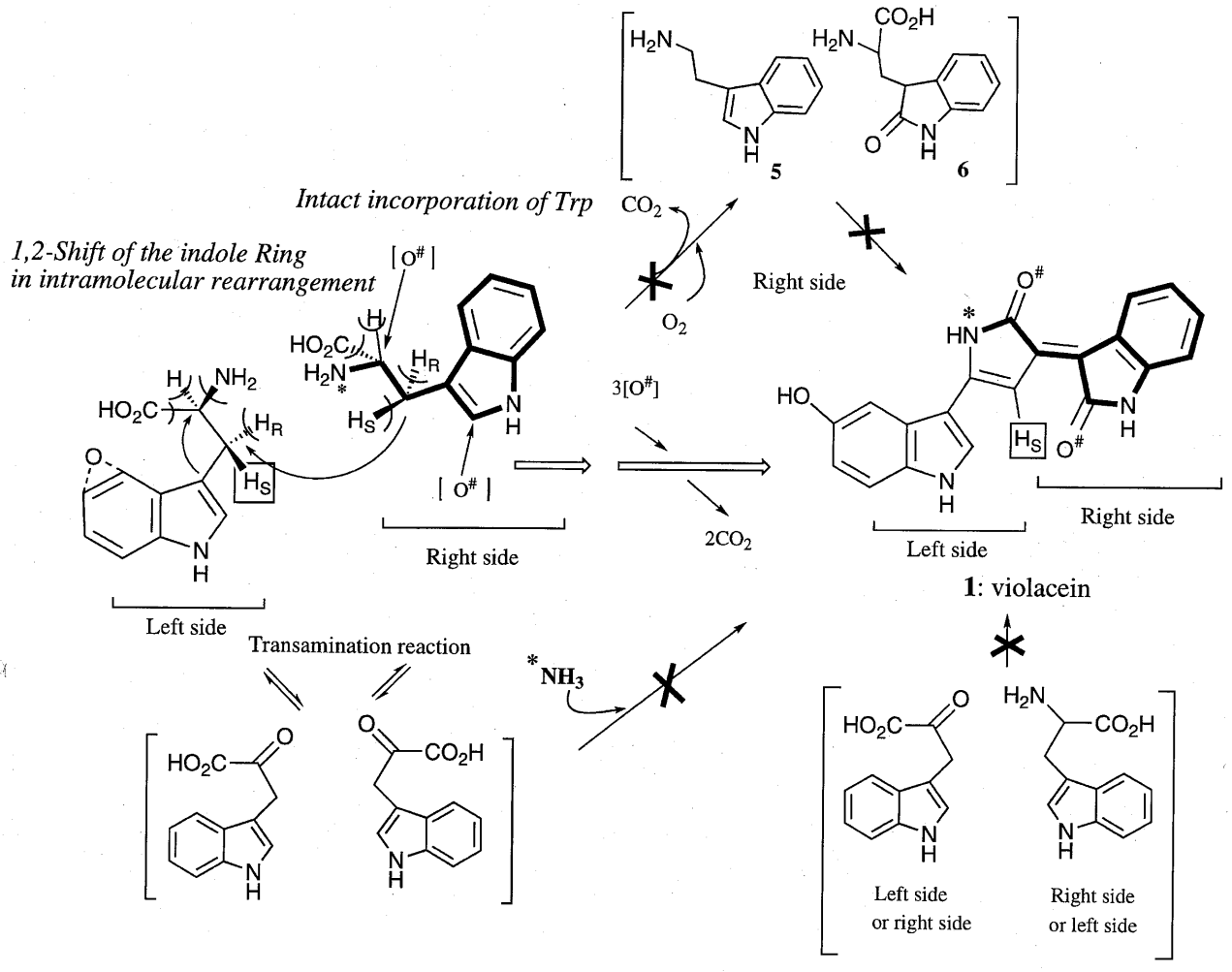


Fig. 9. Summarized Scheme for Violacein Biosynthesis.
The tryptophan molecule is directly incorporated into the right side, and decarboxylation probably takes place after condensation between each side chain. Intramolecular rearrangement of the indole ring occurs for the left side during the biosynthesis.

a large pool size of the nitrogen-14 source in the cell-free system; in contrast, the quantity of ^{15}N-labeled violacein (**1n** and **1o**) was significantly large (27%), as shown in Figs. 7A and 8.

Two molecules of tryptophan, or two indolic intermediates produced by tryptophan metabolism, are essential to the formation of the violacein skeleton. Due to the presence of the active transamination reaction in the cell-free system, the following four possibilities (A-D) are presumed for detecting all the product species shown in Fig. 8: (A) **3** for the left side and **4** for the right side, but the nitrogen of the central ring is from the left side **3**; (B) **3** for the right side and **4** for the left side, but the nitrogen is from the right side **3**; (C) **3** and **3** for both sides, but the nitrogen is from the left side **3**; (D) **3** and **3** for both sides, but the nitrogen is from the right side **3**. Table 1 shows all the possible combinations of the two substrate species for the biosynthesis of **1** and the presumed ratios of the product species. As shown in Table 1, **1q** must have been produced from the possibilities A and C, while **1o** must have been produced from B and D. **1q** could never be detected from the ^{13}C-NMR spectrum, but **1o** was found in a high amount (Fig. 7B). This important finding leads to the exclusion of possibilities A and C, and definitively demonstrates that the nitrogen atom in the pyrrolidone moiety was exclusively from the α-amino group of the right side **3**, and not from that of the left side **3**. To give a deeper insight into possibilities B and D, the ratios for all the product species found by this feeding experiment were compared with those by the presumed ones. If one assumes that the six substrate species would be present in an equivalent amount and that the biosynthesis of violacein proceeds only through one pathway, that is, a divergent pathway is not involved, the following ratios could be presumed for **1m**, **1n**, **1o**, **1c**, **1a** and **1p** (Table 1): case B, 2:1:1:2:1:1, respectively; case D, 2:1.5:1:3:1.5:1, respectively. The experimental ratios for **1m**, **1n**, **1o**, **1c**, **1a**, and **1p** were 1.9:1.7:1.1:3.1:1.5:1.0, respectively. The experimental result is closest to case D, suggesting that case D is most probable; thus, indolylpyruvic acid **4** may be unlikely as the intermediate for the left side.

A mixture of **3b** and **3f** was incubated in the presence of a transaminase inhibitor (15 mM aminooxyacetate). The ^{15}N-content was increased to 46%, but was 27% without the inhibitor. The contents of the product species were as follows: 19.1% for **1m**, 25.7% for **1n**, 20.3% for **1o**, 22.7% for **1c**, 6.1% for **1a**, and 6.1% for **1p**. The lower amounts of **1a** and **1p** suggest that the transamination reaction was inhibited, leading to the increased amounts of **3b** and **3f**. Therefore, the ratio for **1m**:**1n**:**1o**:**1c** would be *ca.* 1:1:1:1. This ratio means that violacein is produced only from **3b** and **3f**. The exogenous addition of indolylpyruvic acid to the cell-free extract inhibited to some extent the biosynthesis of **1**; concomitant incubation of **4a** (1 mM) with **3a** (1 mM) gave a lower violacein production (12% inhibition). If **4** were to be the intermediate, no inhibition could

Table 1. Presumed Product Species Obtained by the Feeding Experiments on [2-^{13}C]-**3b** and [α-^{15}N] Tryptophan **3f** and Presumed Ratio of the Product Species

Substrates for forming violacein	Product species and probability (parentheses)		Substrates for forming violacein	Product species and probability (parentheses)	
	Case A*	Case B*		Case C*	Case D*
3b+**4b**	**1c** (1)	**1c** (1)	**3b**+**3b′**	**1c** (1)	**1c** (1)
3b′+**4b**	**1c** (1)	**1c** (1)	**3b**+**3b**	**1c** (1)	**1c** (1)
3b+**4a**	**1p** (1)	**1m** (1)	**3b′**+**3b′**	**1c** (1)	**1c** (1)
3b′+**4a**	**1p** (1)	**1m** (1)	**3b**+**3a**	**1m** (0.5)	**1m** (0.5)
				1p (0.5)	**1p** (0.5)
3f+**4a**	**1n** (1)	**1n** (1)	**3b′**+**3a**	**1m** (0.5)	**1m** (0.5)
				1p (0.5)	**1p** (0.5)
3f+**4b**	**1q**** (1)	**1o**** (1)	**3b**+**3f**	**1q**** (0.5)	**1o**** (0.5)
				1p (0.5)	**1m** (0.5)
3a+**4b**	**1m** (1)	**1p** (1)	**3f**+**3b′**	**1q**** (0.5)	**1m** (0.5)
				1p (0.5)	**1o**** (0.5)
3a+**4a**	**1a** (1)	**1a** (1)	**3f**+**3f**	**1n** (1)	**1n** (1)
			3f+**3a**	**1n** (0.5)	**1n** (0.5)
				1a (0.5)	**1a** (0.5)
			3a+**3a**	**1a** (1)	**1a** (1)

* Case A: **3** for the left side and **4** for the right side, but the nitrogen of the central ring is from left side **3**; Case B: **3** for the right side and **4** for the left side, but the nitrogen is from right side **3**; Case C: **3** and **3** for both sides, but the nitrogen is from left side **3**; Case D: **3** and **3** for both sides, but the nitrogen is from right side **3**. Presumed net ratio of product species **1m**, **1n**, **1o**, **1c**, **1a**, **1p** and **1q**: case A, 1:1:0:2:1:2 and 1; case B, 2:1:1:2:1:1 and 0; case C, 1:1:5:0:3:1.5:2 and 1; case D, 2:1.5:1:3:1.5:1 and 0; the experimental ratio for **1m**, **1n**, **1o**, **1c**, **1a**, **1p** and **1q** was 1.9:1.7:1.1:3.1:1.5:1.0:0, respectively. The presence of **1a** and **1p** suggests that transaminase is active. The presumed ratios are based upon the assumption that two molecules of the substrate species, which are responsible for the construction of the violacein skeleton, are present in equal amounts and are equivalently metabolized to biosynthesize **1**.

** It is noteworthy that **1q** must be found in cases A and C, while **1o** must be produced in cases B and D.

be assumed. This inhibition might suggest that the structure of the true intermediate for the left side is analogous to that of indolylpyruvic acid.

The feeding experiment on **3b** and **3f** (1:1 molar ratio) has established that the nitrogen atom of the pyrrolidone moiety originated from the α-amino group of the *right side tryptophan*. In the previous paper,[4] we have described that the C2-αN bonds of two molecules of **3** were cleaved due to the active transamination reaction, but this is erroneous. We now have definitive evidence that the C2-αN bond for the right side tryptophan is retained during the biosynthesis. In contrast to the right side tryptophan, the nitrogen atom of the left side tryptophan must be eliminated to form the pyrrolidone nucleus. To examine whether the tryptophan molecule is directly incorporated into the right side of the violacein skeleton, a mixture of **3b** and tryptamine **5** was incubated. If **5** were to be incorporated, the right side tryptophan would first undergoes a decarboxylation process, and **5** would then be used for constructing the pyrrolidone ring. However, no incorporation was detected in the EIMS and ^{13}C-NMR spectra. This result suggests that the decarboxylation reaction occurred at a later biosynthetic stage. Oxindolylalanine **6** was also not incorporated into **1**. We have previously reported the structure of proviolacein, which suggest that oxygenation at the 16-position is the final step.[7] No incorporation of **6** is in agreement with the isolation of proviolacein. These findings strongly suggest that the tryptophan molecule is directly incorporated into the right side of violacein without any modification during the metabolism (Fig. 9).

In the present investigation, we fed a mixture of single-labeled tryptophans in an equivalent amount. To inspect the C–C bond retention, double-labeled compounds can also be used for biosynthetic investigations. However, if double-labeled tryptophan such as [side chain-3-^{13}C, indole-3-^{13}C] were to be used, the ^{13}C-NMR spectrum would be more complicated than that obtained by using a mixture of single-labeled **3c** and **3d**, because four carbon atoms are labeled. If a double-labeled [α-^{15}N,2-^{13}C]tryptophan is used for determining the nitrogen source, we cannot get any information on which side tryptophan (either left or right) works as the nitrogen donor for central ring formation.

As shown in Fig. 9, the feeding experiments on the differently positioned ^{13}C-labeled tryptophans (side chain and indole ring, 1:1) and that of [α-^{15}N]- and [2-^{13}C]tryptophans (1:1) gives new insight into the biosynthetic mechanism for violacein: (1) The 1,2-shift of the indole ring, which occurs at the left side of tryptophan, proceeds in an *intramolecular* and concerted manner. (2) The skeletal carbons of the tryptophan molecule are directly incorporated into the right side part of violacein, but the carboxyl carbon is removed in a later reaction step, probably after the condensation between each side chain. (3) The nitrogen atom exclusively originates from the right side tryptophan. From the results of 2 and 3, it can be inferred that the right side of the violacein skeleton is constructed as the result of intact incorporation containing the α-amino group of the tryptophan molecule. Indolylpyruvic acid seems to be an attractive intermediate for the left side, because the high reactivity of the nucleophilic attack by the amino group on the carbonyl group can be presumed. However, it is unlikely that indolylpyruvic acid is the intermediate, as already described. An intermediate for the left side should have a functional group to both facilitate the nucleophilic attack by the amino group of the right side tryptophan and to assist the intramolecular rearrangement of the indole ring, probably in a concerted fashion, leading to the formation of the central pyrrolidone ring. Further studies are in progress to give greater insight into the cyclization mechanism between the side chains of the two tryptophan molecules.

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