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 [2-13C]- and [indole-3-13C]tryptophans, of [3-13C]- and [indole-3-13C]tryptophans (1:1 molar ratio) and of others have proved that the 1,2-$\delta$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 transaminate, 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 [2-13C]- and [$\alpha$-15N]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 13C-labeled tryptophan 3 and oxygen-18 gas, that rearrangement of the indole ring occurred3) 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 violacine 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 monoxygenase, a copper-containing enzyme.6) The addition of the copper-chelating agent, sodium N',N'-diethyldithiocarbamate, accumulated proviolacine and prodeoxyviolacine,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 proviolacine 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

* 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. 13C-NMR, proton-noise decoupled 13C-NMR
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.

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

Fig. 2. Labeling Patterns of Violacein Produced by the Independent Incubation of [2-¹³C]-3b and [3-¹⁵N]-L-Tryptophans 3c.
This result has been reported in the previous paper.

In the previous paper, 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 3e (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 ¹³C-labeled 3, having different labeled positions; one is labeling of side chain 3b or 3e, while alternative labeling is that of indole ring 3d or 3e (Fig. 4). When a mixture of 3b and 3d or of 3e 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 enzymatic 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. To date, this unresolved point has interfered with progress in biosynthetic studies on 1. A feeding experiment on ¹³C-3b and ¹⁵N-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

![Diagram of Structures](image-url)
Biosynthesis of Violacein

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 (I f and I g, see Fig. 6), which is marked with ● and A, must be produced via an intermolecular shift, but If and Ig 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. 1H- and 13C-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 1H-NMR and 13C-NMR spectra were recorded in DMSO-d6. Chemical shifts are given relative to the signal of DMSO-d6 (δc, 39.5 ppm and δh, 2.49 ppm). Five hundred µg of the labelled violacein were usually used for the 13C-NMR measurements. In the INADEQUATE experiment, the 13C-13C coupling constants (cnst3) and d4 = 1/(4JCC) were estimated from the p.n.d. 13C-NMR spectra and adjusted to obtain good correlation of the cross peaks; JCC = 8.0 Hz for the violacein, which was prepared from a mixture of 3b and 3d, and JCC = 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-[2-13C]-3b, L-[3-13C]-3c, L-[indole-3-13C]3d and L-[indole-2-13C]-tryptophans 3e were synthesized according to the literature13 and subjected to optical resolution with L-amino acylase from Sigma to give the L-forms. As starting materials, diethyl [2-13C]-acetamidomalonate (99 atom%), [13C]formaldehyde (20% aq. solution, 99 atom%), [3-13C]indole (99 atom%) and [2-13C]indole (99 atom%) were used, these being purchased from Isotech. L-[α-15N]Tryptophan 3f was prepared by reductive amination of indolylypruvic acid by using 15NH4NO3 (99.2 atom%) and NaCNBH3, and then the L-form was obtained by optical resolution. 15NH4NO3 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 violacein. *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%) 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 [L-\(^{15}N\)]-tryptophan 3f.** The p.n.d. \(^{13}C\)-NMR spectrum showed two pairs of satellite peaks (\(\Delta J_{14-17} = 7.7\) Hz and \(\Delta J_{13-14} = 12.2\) Hz) around the central signal at C14 (δc 147.68) (Fig. 7B). These satellite peaks show the presence of two species 1e and 1o, while the appearance of the central signal indicates the presence of 1p in labeled 1 (Fig. 7B). The signal at C11 (δc 171.75) had the satellite peak (\(\Delta J_{14-17} = 7.7\) Hz) and central signal, exhibiting the involvement of 1e and 1m, respectively. The satellite signal assignable to 1c appeared at both C11 and C14. The peak heights of 10 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 (\(\Delta J_{14-17} = 95.5\) Hz) at H10 (δh 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 \(^2H\), \(^17O\), \(^18O\) and \(^15N\).

**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 \(^13C\) 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 (δc 105.78), C11 (δc 171.75), C14 (δc 147.68) and C17 (δc 118.77) as a result of the high incorporation rate (96% for \(m/z\) M+ + 2 from EIMS), and also showed complex

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A. Z. M. R. Momen and T. Hoshino
splitting patterns (Fig. 5A) due to the involvement of different $^{13}$C-$^{12}$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 ($^2J_{c11-c14}=7.7$ Hz and $^1J_{c3-c11}=3.4$ Hz); C14 showed two satellite peaks ($^2J_{c14-c17}=8.0$ Hz and $^1J_{c11-c14}=7.7$ Hz), but lacked a central signal; with respect to C17 and C3, the central peaks appeared accompanying two pairs of satellite peaks having $^1J_{c17-c14}=8.0$ Hz and $^1J_{c3-c11}=3.4$ Hz, respectively (Fig. 5A). The central peaks at C3 and C17 would have appeared due to the lack of $^{13}$C-$^{13}$C coupling ($^1J_{c3-c17}=0$ 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 $^1J_{c3-c14}$ nor that of $^2J_{c14-c17}$ 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 ($\delta_c=105.78$), C12 ($\delta_c=137.22$), C13 ($\delta_c=96.85$) and C17 ($\delta_c=118.77$) 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 $^1J_{cc}=3.8$ Hz and $^1J_{cc}=54.8$ Hz, respectively. The INADEQUATE experiments showed the correlation between C12 and C13 and between C3
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 \( J_{C3-C17} = 0 \) Hz for 1b and \( J_{C13-C17} = 0 \) Hz for 1j. For C12, two pairs of satellite peaks due to \( J_{C12-C13} = 54.8 \) and 3.8 Hz were found without any observation of the central signal. For C17, only the central peak was observed. The \( ^1H \)-NMR spectrum also showed that C13 was labeled \( (J_{C13-H13} = 179 \text{ Hz}) \), 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 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 \( J_{C3-C13} \) nor \( J_{C13-C17} \) could be detected (Fig. 5B). The reason is not clear why species 1j exhibited only the central signal, i.e. \( J_{C13-C17} = 0 \) 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 on 3b+3e revealed \( J_{C13-C17} = 5.4 \text{ Hz} \) and \( J_{C12-C14} = 2.1 \text{ Hz} \), 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
Biosynthesis of Violacein

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,\textsuperscript{17} we have reported that, by feeding $[3-^{13}$C]$\beta$-dolylpyruvic 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-[\alpha-^{15}$N]\warm 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.\textsuperscript{4*} The $^1$H-NMR spectrum of violacein, which had been produced by feeding 3f, showed the satellite peak ($J_{1h0-1h10} = 95.5$Hz) at H10 ($\delta_{1h}$ 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),\textsuperscript{10} 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 \alpha-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

![Chemical structure](image)

**Found**

- $1m$ (18.5%)
- $1n$ (16.2%)
- $1o$ (10.8%)
- $1p$ (9.7%)
- $1c$ (30.2%)
- $1a$ (14.6%)

$J_{CC} = 7.7$ Hz

**Not found**

- $1q$
- $1r$

$J_{C10-N10} = 14.6$ Hz

\textbf{Fig. 8.} Incorporation Patterns Obtained by Feeding a Mixture of [\alpha-$^{15}$N]-3f and [L-$^{13}$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 $^{3}$J$_{C_{11}-C_{14}}$ = 7.7 Hz) along with the central peak at the position of C11 ($\delta_{C}$ 171.75). Two pairs of satellite peaks, due to $^{3}$J$_{C_{11}-C_{14}}$ = 7.7 Hz and $^{1}$J$_{C_{14}-N_{10}}$ = 12.2 Hz, were observed at C14 ($\delta_{C}$ 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$_{C_{11}-N_{10}}$ 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
Table 1. Presumed Product Species Obtained by the Feeding Experiments on [2-15C]-3b and [α-15N] Tryptophan 3f and Presumed Ratio of the Product Species

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* 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 set ratio of product species 1m, 1n, 1o, 1c, 1a, 1p and 1q: Case A, 1/1:1/1:1/1:1/1:1; Case B, 2:1:1:1:1:1:1; Case C, 1:1/1:1/1:1/1:1; Case D, 2:1:1:1/1:1:1:1; the experimental ratio for 1m, 1n, 1o, 1c, 1a, 1p and 1q was 1/1:1/1:1/1:1/1:1.5:0.5, 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 violacin 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 tryptophane moieties was derived from the C2-N bond of the right side tryptophan. In the previous paper, we have described that the C2-CN bond of the two molecules of 3 were cleaved due to the active transamination reaction, but this is erroneous. We now have definitive evidence that the C2-CN 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 tryptophan skeleton. To examine whether the tryptophan molecule is directly incorporated into the right side of the violacein skeleton, a mixture of 3b and tryptophane 5 was incubated. If 5 were to be incorporated, the right side tryptophan would first undergo a decarboxylation process, and 5 would then be used for constructing the pyrrolidone ring. However, no incorporation was detected in the EIMS and 13C-NMR spectra. This result suggests that the decarboxylation reaction occurred at a later biosynthetic stage. Oxindolyllalanne 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. 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-3,13C, indole-3,13C] were to be used, the 13C-NMR spectrum would be more complicated than that obtained by using a mixture of single-labeled 3 and 4, because four carbon atoms are labeled. If a double-labeled [α-15N,2-13C]tryptophan is used for determining the nitrogen source, we cannot get any information on which side tryptophan is used for determining the nitrogen source, we cannot get any information on which side tryptophan is used for determining the nitrogen source. The left side tryptophane is 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 C-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 carboxyl 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.

References

9) Ruhul Momen, A. Z. M., Mizuoka, T., and Hoshino,
Biosynthesis of Violacein


