Biosynthesis of Violacein: Origins of Hydrogen, Nitrogen and Oxygen Atoms in the 2-Pyrrolidone Nucleus[†]

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The biosynthetic origins of the hydrogen, nitrogen and oxygen atoms in the pyrrolidone ring of violacein were established by an analyses of the ^1H , ^{13}C NMR and MS spectra of its isotope-enriched metabolites. Feeding experiments of $[2\text{-}^2\text{H}]$ and $[3\text{-}^2\text{H}_2]$ tryptophans have revealed that the hydrogen in the pyrrolidone ring was derived from the methylene protons at the 3-position of tryptophan. The stereochemical fate of the prochiral hydrogens was determined to be in the retention of the *pro-S* hydrogen by these feeding experiments using $[3R\text{-}^2\text{H}]$ and $[3S\text{-}^2\text{H}]$ tryptophans. The incorporation experiment of $[\alpha\text{-}^{15}\text{N}]$ tryptophan demonstrated that the nitrogen atom in the ring originated from the α -amino group of tryptophan. The incorporation experiment of $^{18}\text{O}_2$ gas verified that all the oxygen atoms of violacein were derived from the molecular oxygen.

Violacein is a blue pigment produced by the *Bacterium* sp., *Chromobacterium violaceum*.¹⁾ Its structure consists of three units, *i.e.*, the 5-hydroxyindole, oxindole and 2-pyrrolidone nuclei. Among a number of natural pigments, the blue-colored pigments having a dimeric structure of two indole rings are rarely found.²⁾ As typical blue pigments, violacein from the microorganism and trichotomine³⁾ from the plant have been isolated. In 1960 Demoss and Evans demonstrated by using various ^{14}C -compounds that L-tryptophan is a biosynthetic precursor of violacein and is accompanied by a decarboxylation process.⁴⁾ However, no report of the biosynthetic investigations has appeared so far since their studies. Violacein contains a pyrrolidone nucleus, which is considered to be a derivative of the pyrrole ring. Except for the porphyrins, corrins, prodigiosin, pyrrolnitrin, *etc.*, the pyrrole derivatives are rare in nature.⁵⁾ The rare structure and ambiguous biosynthetic origin, as well as the formation mechanism, of the pyrrolidone ring have stimulated

us to investigate the biosynthesis of violacein.

In the previous paper,⁶⁾ we demonstrated that the carbon skeleton of the pyrrolidone ring is constructed by condensation of the side chains of two tryptophan molecules, *i.e.* by the bond formation of C3-C3 and C2-N-C2 between the two side chains. We also found an interesting phenomenon that the indole ring rearranges to the 2-position of the side chain. This rearrangement with an indole 1,2-shift has not previously been reported in tryptophan metabolisms. In order to investigate the mechanism for the rearrangement and condensation between the two side chains of tryptophan, the origins of constitutive atoms other than carbon in the ring must be established. In this paper, we describe the biosynthetic origins of the hydrogen, nitrogen and oxygen atoms in the pyrrolidone moiety.

We planned to elucidate the biosynthetic origins with the aid of stable isotopes (^2H , ^{15}N and ^{18}O). For detecting the labeled positions in the stable isotope-enriched metabolites, the complete assignments of the ^{13}C and ^1H NMR

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spectra of violacein and deoxyviolacein are indispensable. The assignments of the NMR spectra were obtained by such NMR techniques as ^1H - $\{^1\text{H}\}$ homonuclear decoupling experiments, C-H COSY NMR, as described in the previous paper.⁶⁾

Origin of the hydrogen atom

Taking into consideration the origin of the carbon skeleton in the pyrrolidone ring (Fig. 6), the following possibility could be anticipated regarding the biosynthetic origin of the hydrogen in the pyrrolidone ring: (i) from the

hydrogen at the 2-position of L-tryptophan, or (ii) from the hydrogen at the 3-position of the tryptophan, or (iii) from the water in the medium.

Case (i) indicates the involvement of the hydrogen 1,2-shift from the 2-position to the 3-position of the tryptophan side chain. Case (ii) means that the C-H bond at the 3-position of the tryptophan remains intact throughout the biosynthesis (note Fig. 6). To clarify the biosynthetic origin and the mechanism, washed whole cell systems were employed. Bacterial cells grown for 12 hr were washed with a saline solution and suspended in a buffered solution (pH 7.2) containing L-tryptophan. The incubation was then carried out, resulting in the gradual production of the pigments, the conversion rate of tryptophan into the pigment being in the range of 50~70%. A typical example is shown in Fig. 1. The maximum production of the pigments was attained by using an amount of washed cells corresponding to 100 mg or above of the dried cell weight, at a given concentration of L-tryptophan (2.5 mg) in the phosphate buffer (5 ml).

Two separate experiments, *i.e.*, incubation in deuterium oxide containing unlabeled L-tryptophan and one in water loaded with [2-

FIG. 1. Effect of the Amount of Washed Cells on Pigment Production under a Constant Concentration of L-Tryptophan (2.5 mg/5 ml of phosphate buffer).

FIG. 2. Synthetic Scheme for L-[3R-2H] and L-[3S-2H] Tryptophans.

^2H]tryptophan, were carried out. Violaceins prepared from the two experiments contained no deuterium atom, which was confirmed by the integration of H-13 in the ^1H NMR spectra. An incorporation experiment using $[3\text{-}^2\text{H}_2]\text{tryptophan}$ verified that the hydrogen atom of the pyrrolidone moiety came from the hydrogen at the 3-position of tryptophan. The amount of deuterium in the labeled violacein was estimated to be 35% from the integration of the ^1H NMR spectrum.

In the next step, the stereochemical course of the methylene protons at the prochiral center during biosynthesis was examined. Both $[3R\text{-}^2\text{H}]$ and $[3S\text{-}^2\text{H}]$ tryptophans were synthesized according to the method of Fig. 2. Similar feeding experiments using the whole cells were carried out. No incorporation of the deuterium atom was observed from feeding $[3R\text{-}^2\text{H}]$ tryptophan, while with $[3S\text{-}^2\text{H}]$ tryptophan, the deuterium atom was located at the 13-position of the pigment with a similar percentage of the isotope to the feeding experiment of $[3\text{-}^2\text{H}_2]\text{tryptophan}$. These experiments clarified that the hydrogen atom of the pyr-

rolidone nucleus originated from *pro-S* hydrogen at the 3-position of tryptophan, with stereospecific retention of the C3-H(S) bond of tryptophan throughout the biosynthetic pathway. In the case of deoxyviolacein, an identical incorporation and almost the same incorporation percentage as violacein were obtained.

In the course of these incorporation experiments, we found that the hydrogen at the 13-position of violacein pigments was readily exchanged by the proton of protic solvents by heating treatment; the hydrogen at the 13-position of violacein was substituted by a deuterium atom by approximately 100%, when a violacein solution in a mixed solvent of dimethylsulfoxide and deuterium oxide was heated at 120°C for 5 hr. The acidity of the hydrogen in the pyrrolidone ring was considered to be high; therefore, the isolation of the pigments was conducted by using only cold methanol and water, and without heating treatment, in order to trace the isotope of the deuterium compounds.

FIG. 3. (A) Proton-Noise Decoupled ^{13}C NMR Spectrum (50 MHz) and (B) ^1H NMR Spectrum (90 MHz) of the Labeled Violacein Obtained by Feeding $[\alpha\text{-}^{15}\text{N}]\text{tryptophan}$.

Origin of the nitrogen atom

DL- $[\alpha\text{-}^{15}\text{N}]$ Tryptophan (10 mg) was fed to a 300 ml Erlenmeyer flask containing 100 ml of the production medium. Sixteen mg of violacein and 2 mg of deoxyviolacein were obtained from four flasks. The proton-noise decoupled ^{13}C NMR spectrum (50.1 MHz) of violacein in $\text{DMSO-}d_6$ is shown in Fig. 3. Around the carbon signals of C-11, C-12 and C-14, satellite peaks due to $^{13}\text{C}\text{-}^{15}\text{N}$ coupling ($^1J\text{ }^{13}\text{C}\text{-}^{15}\text{N}$ and $^2J\text{ }^{13}\text{C}\text{-}^{15}\text{N}$) were observed, indicating that the ^{15}N atom of the α -amino group was incorporated into the nitrogen atom of the pyrrolidone ring. Satellite peaks of C-13 and C-3 due to $^2J\text{ }^{13}\text{C}\text{-}^{15}\text{N}$ were not observed, due probably to their small coupling constants. This labeled position was also confirmed by the ^1H NMR spectrum in $\text{DMSO-}d_6$; satellite peaks arising from $^{15}\text{N}\text{-}^1\text{H}$ coupling ($J=95.5\text{ Hz}$) were found with the H-10 signal as a result of the high incorporation rate of the isotope (Fig. 3). Similar satellite signals due to the $^{15}\text{N}\text{-}^1\text{H}$ coupling were also observed at H-10 of deoxyviolacein, suggesting that an identical incorporation pattern to violacein occurred in the biosynthesis of deoxyviolacein. The content of the ^{15}N atom in the labeled violacein and deoxyviolacein was determined to be 29% from the EI-MS analyses. The high incorporation rate of the ^{15}N strongly suggests that the nitrogen atom of the pyrrolidone moiety originated from the α -amino group of tryptophan.

The possibility was investigated that the metabolism of a free ammonia molecule could be involved in the violacein biosynthesis. Incubation experiments using the washed cells were conducted with bufferized systems of the phosphate solution (pH 7.2, 5 ml) containing $^{15}\text{NH}_4\text{NO}_3$ (9.8 mg) and unlabeled L-tryptophan (2.5 mg). The molar ratio of $^{15}\text{NH}_4\text{NO}_3$ was 10 times that of the tryptophan. The ^1H NMR spectra of violacein and deoxyviolacein thus obtained showed a high-level enrichment of nitrogen-15 in the pyrrolidone ring; the proton signal of H-10 was relatively decreased with the stronger intensity of the satellite peaks. The atomic percentage of

^{15}N was estimated to be 52% from the analyses of EI-MS and the integration of the satellite peaks. One possible interpretation of the isotope incorporation may be that a transamination reaction between unlabeled tryptophan and $^{15}\text{NH}_4^+$ via indolylpyruvic acid occurred in the living cells, which was followed by the formation of $[\alpha\text{-}^{15}\text{N}]$ tryptophan and then by the incorporation of the labeled tryptophan into violacein molecules. To elucidate the incorporation mechanism of the free ammonia molecule, further experiments are now being undertaken.

Origin of the oxygen atom

In an open system under a normal atmosphere, the washed cell suspension in L-tryptophan solution produced blue pigments during the incubation. However, in a closed system where the normal atmosphere was replaced by nitrogen gas, no pigment production was observed. This observation suggests the necessity of oxygen gas for the pigment production. Indeed, in a closed flask containing oxygen gas, the cell suspension produced pigments. Therefore, the metabolism of molecular oxygen gas is involved in the biosynthetic pathway.

Violacein and deoxyviolacein possess three and two oxygen atoms, respectively. Their oxygen gas may be derived from water in the medium and/or from the atmosphere. To

FIG. 4. Effect of L-Tryptophan on Pigment Production under an Adequate Atmosphere.

FIG. 5. (A) EI-MS Spectra of Natural and ^{18}O -Incorporated Violaceins. (B) Proton-Noise Decoupled ^{13}C NMR Spectrum (125 MHz) of ^{18}O -Incorporated Violacein.

make sure of their origins, an incorporation experiment using $^{18}\text{O}_2$ gas was conducted. Figure 4 shows that the addition of L-tryptophan to the medium caused an increase of pigment production. The maximum production was obtained by adding 30 to 50 mg of tryptophan and shake-culturing in a 500 ml-round flask with flat bottom containing 125 ml of the medium with a sufficient supply of the atmosphere. To produce a large amount of the pigments, tryptophan was added to the culture. The bacterium was grown for 10 hr in the round flask containing 30 mg of the unlabeled tryptophan in 125 ml of the medium, this giving little pigment production. A container of $^{18}\text{O}_2$ was subsequently connected with the flask, and cultivation was allowed to continue for a further 60 hr in a closed system under an atmosphere of $\text{N}_2 : ^{16}\text{O}_2 : ^{18}\text{O}_2$ (c 4 : 1 : 1). From two separate flasks, 23 mg of violacein and 3.5 mg of deoxyviolacein were isolated. Figure 5A shows the EI-MS of natural and ^{18}O -incorporated violaceins. The peak of M+6 can be clearly seen in the MS spectrum of ^{18}O -incorporated violacein, indicating that all of the oxygen atoms in the violacein molecule were derived from the molecular oxygen and not from the medium. In the case of deoxy-

violacein, the MS peak of M+4 was observed. These results were also confirmed by the proton-noise decoupled 125 MHz ^{13}C NMR spectrum (Fig. 5B). It is well known that the ^{13}C signal attached to the oxygen-18 is more upfield shifted with an observable magnitude ($\Delta\delta$ -0.01 to -0.05 ppm), compared with that connected to oxygen-16, and that the relative peak heights of the ^{16}O - and ^{18}O -derived signals are in the same ratio as the $^{16}\text{O} : ^{18}\text{O}$ composition.⁷⁾ The ^{18}O -induced isotope shifts were observed for C-11 and C-16 of violacein with a magnitude of $\Delta\delta$ -0.029 ppm. However, for the signal of C-6, an isotope shift was not found. Probably, the reason would be based on the very small magnitude of the ^{18}O -shift being hard to detect in the ^{13}C NMR measurement. The structural dependence of ^{18}O isotope shifts has been reported.^{8,9)} For phenols, the ^{18}O -isotope shift is of a very small magnitude. The relative peak heights of ^{16}O - and ^{18}O -derived signals for C-11 and C-16 were nearly equal, suggesting that the composition ratio of ^{16}O to ^{18}O was about 1 : 1. The isotopic shifts were also observed for C-11 and C-16 of deoxyviolacein with nearly equal peak heights for the ^{16}O - and ^{18}O -derived signals. The experiment using

FIG. 6. Structures of Violacein and Deoxyviolacein.

Incorporation patterns of various stable isotope compounds (^{13}C , ^{15}N , ^2H and ^{18}O).

$^{18}\text{O}_2$ gas proved that all the incorporation reaction of the oxygen atom into violacein was catalyzed by the oxygenase.

In order to determine the biosynthetic origin of the indole rings in the violacein molecule, a feeding experiment of L-[ring 4- ^2H]tryptophan was carried out. Five mg of deuterium-labeled tryptophan was administered to a 300 ml Erlenmeyer flask containing 100 ml of the medium. The fermentation of five flasks produced 17.2 mg of violacein and 1.5 mg of deoxyviolacein. The ^1H NMR spectrum (200 MHz) of deoxyviolacein showed that the deuterium atoms were located at both the 5- and 19-positions with the same content of 26% of D. On the other hand, in the molecule of violacein, the deuterium atom was only found at 19-position (27% of D), and hardly observed at the 5-position. This would probably be due to the elimination of the deuterium at the 5-position in the course of the hydroxylation reaction; hydrogen of the 6-position would be transferred to the 5-position *via* an NIH shift, resulting in the loss of the deuterium atom. This NIH shift has been reported in biological hydroxylation catalyzed by the monooxygenase, *e.g.* phenylalanine to tyrosine¹⁰⁾ and tryptophan to 5-hydroxytryptophan.¹¹⁾ The

epoxide has been proposed as the intermediate of the aromatic hydroxylation.¹²⁾ The elimination of deuterium provides additional evidence for the involvement of the molecular oxygen in the hydroxylation reaction at the 6-position of violacein.

In conjunction with the previous report,⁶⁾ we could determine the biosynthetic origins of all the constitutive atoms of violacein molecules, the results being summarized in Fig. 6. All of the carbons, nitrogens and hydrogens were derived exclusively from L-tryptophan, and all of the oxygen atoms originated from the molecular oxygen. Figure 6 also shows that various metabolic reactions of tryptophan occurred during the biosynthesis: rearrangement of the indole ring, decarboxylation, stereospecific elimination of *pro-R* hydrogen at the prochiral center, oxygenation and hydroxylation with an NIH shift (monooxygenase), and involvement of nitrogen atom of the α -amino group in constructing the pyrrolidone ring. The following reactions must also be noted: the C-C bond formation between C12 and C13, and the introduction of double bonds of C13-C14 and C12-C17. The series of biosynthetic reactions should be arranged in a row, although at the present time, the reaction

sequence remains uncertain. It may help to elucidate the pathway by determining the sequence for oxygenation and decarboxylation, and various biosynthetic intermediates presumed from these reactions were fed to the washed whole cell systems and to the growing cells. In the course of these feeding experiments, we have found some interesting phenomena. Trials are also in progress to isolate the biosynthetic intermediates among the metabolites of *Chromobacterium violaceum*, and these findings will be reported in the subsequent papers.

EXPERIMENTAL

Instruments. NMR spectra were recorded on a JEOL FX-90Q, FX-200 or GX-500 spectrometer. Mass spectra were recorded on a Hitachi M60 at 20 or 70 eV (direct inlet system).

Isotopes. The isotopes of D_2O and $^{18}O_2$ that were used throughout the investigations were purchased from MSD, Canada. Their purities were 99.8 atom% for D_2O and 97.8% for $^{18}O_2$. The nitrogen isotope of $^{15}NH_4NO_3$ (99.5 atom%) was obtained from Shōkō Tsūshō, Tokyo.

Culture conditions and measurements of the pigments. *Chromobacterium violaceum* JCM 1249 was used. The culture medium (nutrient broth) consisted of meat extract (3 g), polypeptone (5 g), NaCl (5 g) and water (1 l), the pH being adjusted to 7.0. The seed culture (1 ml) grown for 20 hr was inoculated into 100 ml of the medium in a 300 ml Erlenmeyer flask and then cultured at 25°C for 48 hr on a rotary shaker (180 rpm). The production of the pigments reached a maximum after about 24 to 36 hr of cultivation. The cells were harvested by centrifugation at 10,000 rpm for 10 min. The pigments were localized within the cells, and were extracted with DMSO and then centrifuged to remove the decolorized cells. The amount of pigments was determined by measuring the absorbance at 570 nm.

Pigment production by a washed cell system. Ten ml of seed culture grown in the medium for 20 hr was inoculated into 1 l of the medium in a 2 l Erlenmeyer flask containing 12 ml of 10% (v/v) aq. polyoxyethylene (20) sorbitan monooleate, and then cultured under the same culture conditions previously described. The bacterial cells were harvested by centrifugation (9000 rpm) after cultivating for 12 hr. The supernatant was colored with blue and the precipitated cells were almost decolorized. The use of a detergent was effective for excreting the pigments into the medium from the cells; however, after longer culture, the pigments were accumulated in the cells. The harvested cells

were washed with a saline solution. The washed whole cells were suspended in a 1/20 volume of the original culture with a solution containing the labeled or unlabeled tryptophans. The washed cells collected from 100 ml of the culture broth, corresponding to about 100 mg of the dry weight, were suspended in a solution (pH 7.2) consisting of L-tryptophan (2.5 mg), K_2HPO_4 (5 mg), $MgSO_4 \cdot 7H_2O$ (1 mg) and H_2O (5 ml). Incubation at 25°C for 48 hr on a rotary shaker (180 rpm) caused gradual production of the pigments.

^{18}O Incorporation experiment. After growth for 10 hr in the open system of a 500 ml-round flask with flat bottom, the fermentation was continued in a closed system under an atmosphere of $N_2:^{16}O_2:^{18}O_2$ (c 4:1:1) for 60 hr at 25°C on a rotary shaker (180 rpm). The administration of unlabeled tryptophan (30 mg) into the nutrient broth medium in the flask caused a sufficiently large amount of the pigments for measuring the ^{13}C NMR spectra.

Isolation of the pigments. The isolation method was described in the previous paper.⁶⁾ In the case of the experiments to elucidate the origin of the hydrogen of the pyrrolidone moiety, a purification method without any heating process was necessary.

Preparation of DL-[2- 2H]tryptophan. DL-[2- 2H]Tryptophan was synthesized according to the method of Hochreiter *et al.*¹³⁾ The deuterium atom was incorporated at the 2-position of *N*-acetyl DL-tryptophan by a racemization process in a mixed solvent of AcOD–Ac $_2$ O at 100°C for 30 min. The acetyltryptophan was prepared by adding Ac $_2$ O into an aq. NaOH solution of DL-tryptophan. The D-labeled acetyltryptophan was hydrolyzed with 3 M HCl for 3 hr at 110°C. The solution was adjusted to pH 6 and allowed to stand overnight in a refrigerator. The precipitated labeled tryptophan was filtered off and recrystallized from aq. EtOH. The deuterium atom was estimated to be 85% from an EI-MS analysis of the tryptophan methyl ester, which was prepared with saturated HCl in MeOH at 60–70°C for a few hours.

Synthesis of L-[3- 2H_2]tryptophan. Formaldehyde- d_2 was prepared according to the method in ref. 14. The synthetic method for tryptophan via gramine¹⁵⁾ was applied to the synthesis of the labeled amino acid. Optical resolution was carried out as follows: To an aq. solution of pure DL-*N*-acetyltryptophan (200 mg), that had been adjusted to pH 7, were added 10 mg of acylase I from *Aspergillus* species (Sigma) and a trace of $CoCl_2$. The solution was incubated at 37°C for 48 hr and concentrated to a small volume. L-Tryptophan separated out as precipitates and was filtered. The pure labeled tryptophan was obtained by recrystallization from aq. EtOH (60 mg yield, 89% D_2).

Synthesis of L-[3R-²H] and L-[3S-²H]tryptophans. Synthetic program is shown in Fig. 2.

(i) *Indole-3-glyoxylic acid* (**1**) was prepared according to the method in ref. 16.

(ii) *Indole-3-carbaldehyde-d₁* (**2**).¹⁷⁾ A mixture of indole-3-glyoxylic acid (1 g), aniline (0.5 g) and D₂O (6 ml) was heated at 110°C to give a clear yellowish pink solution, which was then lyophilized. The deuterium anilium salt of indole-3-glyoxylate was subjected to pyrolysis in anisole (4.5 ml) under reflux. The solution was diluted with water, and aniline and anisole were removed by steam-distillation before being cooled. The deuterium aldehyde thus precipitated was filtered off and recrystallized from hot water (0.74 g yield, 87.5% D).

(iii) *Condensation of 3-indolecarbaldehyde-d₁ with pyrrolidine* (**3**).¹⁸⁾ A mixture of the anhyd. deuterium aldehyde (0.73 g) and pyrrolidine (0.43 g) in benzene (10 ml) was refluxed for 2 hr using a Dean-Stark apparatus to exclude the liberated water. During the reaction, the product separated out, and was filtered and then washed with benzene (0.84 g yield).

(iv) *α,β -dehydrotryptophan derivative-d₁* (**4**).¹⁹⁾ A solution of **3** and ethyl *N*-acetylglucinate in a mixed solvent of DMF and EtOH (9:1) was heated at 110°C for 8 hr. The resulting solution was diluted with EtOAc and washed with 1% HCl. The organic layer was washed with water, dried over anhyd. MgSO₄ and concentrated *in vacuo* to yield a brown residue, which was subjected to column chromatography over SiO₂ (Fuji Davison BW-820 MH; CHCl₃:MeOH = 98:2). The fraction containing **4** was recrystallized from MeOH. From 2.5 g of **2**, 0.8 g of pure **4** was obtained. UV $\lambda_{\max}$ (MeOH): 347 nm, the characteristic absorption of 3-vinylindole derivatives. EI-MS *m/z*: 273 (M⁺). NMR δ (DMSO-*d*₆): 1.25 (3H, t, *J* = 7 Hz, O-CH₂-Me), 2.08 (3H, s, COCH₃), 4.21 (2H, q, *J* = 7 Hz, O-CH₂-Me), 7.0~8.0 (4H, m, C-4 to C-7H, aromatic region of indole), 7.83 (1H, br. d, *J* = 3 Hz, C-2H), 9.3 (1H, br. s, amide NH), 11.7 (1H, br. s, indole NH). The chemical shift of the acetyl proton is diagnostic in the determination of an *E*- or *Z*-configuration of the double bond.¹⁹⁾ A peak of δ 1.85 ppm, ascribable to the *E*-isomer, was not observed, indicating that the product thus isolated was all of the *Z*-isomer.

(v) *Racemates of the N-acetyltryptophan-d₁* (**5** and **6**). A solution of **4** (0.5 g) in EtOH (38 ml) was allowed to hydrogenate with H₂ gas and 5% Pd-C at ambient pressure and temperature for 6 hr. After removing the charcoal by filtration, the solution was concentrated *in vacuo*. The residue of the ethyl ester was hydrolyzed in aq. 1N NaOH under reflux for 1.5 hr and then acidified to collect the racemates (**5** and **6**). Yield, 0.42 g. NMR δ (0.03N NaOD, ext. TMS): 2.33 (3H, s, COCH₃), 3.78 (β -H, d, *J* = 4.6 Hz), 4.97 (α -H, d, *J* = 4.6 Hz), 7.5~8.3 (5H, m, aromatic region). This clean AX spin system between α -H and β -H suggests that the hydrogenation reaction occurred with high stereoselectivity of the *cis* addition of H₂. The conformation analysis of the race-

mates of 3-deuterio *N*-acetylphenylalanine has already been discussed in detail.²⁰⁾

(vi) *L-[3R-²H] and L-[3S-²H]tryptophans* (**7** and **8**). To an aq. solution of the racemates (**5** and **6**), that had been adjusted to pH 7, was added 12 mg of aminoacylase, and the mixture then incubated in the same manner as with the preparation of L-[3-²H₂]tryptophan. From the solution of the racemates, only L-[3R-²H]tryptophan was precipitated (120 mg yield from the recrystallization with aq. EtOH); the remaining liquid was enriched with D-[3S-²H]-*N*-acetyltryptophan. The mother liquor was diluted with water and passed through a column of Dowex 50W (H⁺ form) to remove the trace of L-[3R-²H]tryptophan. The column was washed with water. The fractions that were negative to a ninhydrin test were concentrated *in vacuo* and acidified with 5N HCl to precipitate the D-*N*-acetyl amino acid. Epimerization at the 2-position took place by the method described previously. The solution was acidified to collect the DL-[3S-²H]-*N*-acetyltryptophan, and pure acetyltryptophan was obtained by recrystallization from aq. EtOH. Optical resolution was carried out by treating with aminoacylase. Recrystallization from aq. EtOH gave the pure L-[3S-²H]-tryptophan (40 mg yield). In the region of δ 3.2 to 3.8 ppm of the ¹H NMR (0.1N NaOD, ext. TMS), only the doublet of δ 3.60 ppm (*J* = 4.8 Hz) for the L-[3R-²H]tryptophan and only the doublet δ 3.44 ppm (*J* = 7.1 Hz) for the L-[3S-²H]amino acid were observed. The coupling constants are diagnostic in determining the stereochemistry of the 3-position of the 3-deuterio-phenylalanine.²¹⁾ The deuterium atom was retained in 87% for the L-[3R-²H] and 86% for the L-[3S-²H]amino acids from the MS analysis.

*Preparation of [α -¹⁵N]tryptophan.*²²⁾ This was synthesized by the reductive amination of 3-indolylpyruvic acid using ¹⁵NH₄NO₃ and NaCNBH₃.²²⁾ The atom of ¹⁵N was 96% from the MS analysis.

*Preparation of [ring 4-²H]tryptophan.*²³⁾ The regioselective incorporation of the deuterium atom into C-4 of the indole ring was carried out by a photochemical reaction of L-tryptophan in D₂O²³⁾ (75% D from the MS analysis).

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