Stereochemical Studies on the Formation of Melanin by Monophenol Monooxygenase

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The oxidation of tyrosine by monophenol monooxygenase (tyrosinase: EC 1.10.3.1) to melanin has been studied by a combination of ultraviolet, circular dichroism, and nuclear magnetic resonance techniques. It is demonstrated that the chiral intermediate (dopachrome) is generated stereoselectively in this enzymic reaction.

Keywords tyrosine; tyrosinase; dopa; melanin; dopachrome; chiral intermediate; stereoselective reaction

Melanin is the important black pigment that is found in dark skin, hair, eyes and insects. The study of melanin has a long history, but its structure is still unknown.[1] Synthetic melanin has been obtained by the action of monophenol monooxygenase (tyrosinase: EC 1.10.3.1) on 3,4-dihydroxyphenylalanine (dopa, **3**). 2*S*-Dopa (**3a**) is widely used in man for the treatment of Parkinson's disease,[2] and can be prepared from 2*S*-tyrosine (**1a**).[3,4]

Selective hydroxylation of aromatic substrates is a difficult task in preparative organic chemistry. Enzymatic hydroxylation can be used for fast, convenient and selective hydroxylation.[5] Arene oxides (**2**) are possible metabolic intermediates in the "National Institute of Health (NIH) shifts."

The enzymatic oxidation of tyrosine (**1**) to melanin (**9**) is thought to occur through the steps outlined in Fig. 1.[1,6,7] The initial steps are the hydroxylation of tyrosine (**1**) to dopa (**3**), oxidation of dopa (**3**) to dopaquinone (**4**), cyclization to leucodopachrome (**5**), oxidation to dopachrome (**6**) and finally a slow chemical decarboxylation to 5,6-dihydroxyindole (**7**). In this report we present the result of our investigation of these steps by a variety of physical methods.

Experimental Procedure

General Methods and Equipment Materials: All compounds were of the highest purity commercially available and were recrystallized prior to use. Monophenol monooxygenase (tyrosinase) was obtained from Sigma. Equipment: Ultraviolet (UV) spectra were measured on a JASCO UVIDEC-610C spectrometer. Circular dichroic (CD) spectra were taken on a JASCO J-500C equipped with a DP 500 data processor. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker WM 300-WB spectrometer. Chemical shifts are given δ units relative to tetramethylsilane (TMS) in a capillary as an external standard.

Results and Discussion

UV The dynamics of the enzymic transformations of 2*S*-tyrosine (**1a**) and 2*R*-tyrosine (**1b**), and 2*S*-dopa (**3a**) and 2*R*-dopa (**3b**) were monitored by UV spectrophotometry (Figs. 2 and 3). Completion of the reaction was

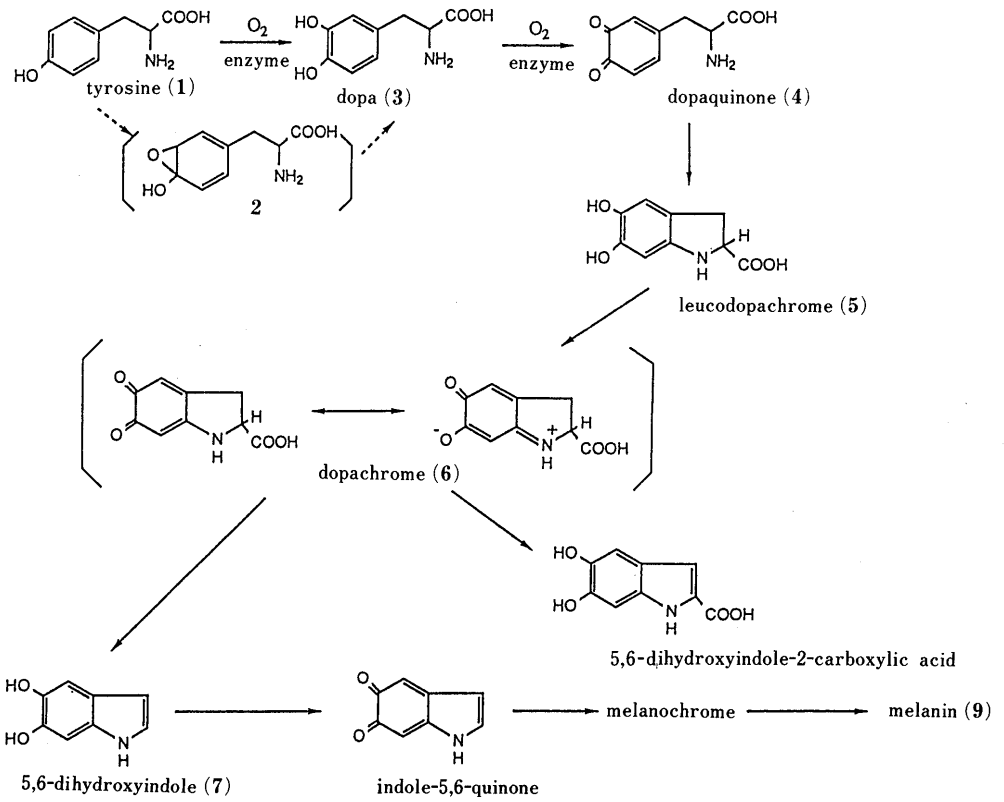

Fig. 1. Biosynthesis of Melanin (**9**)

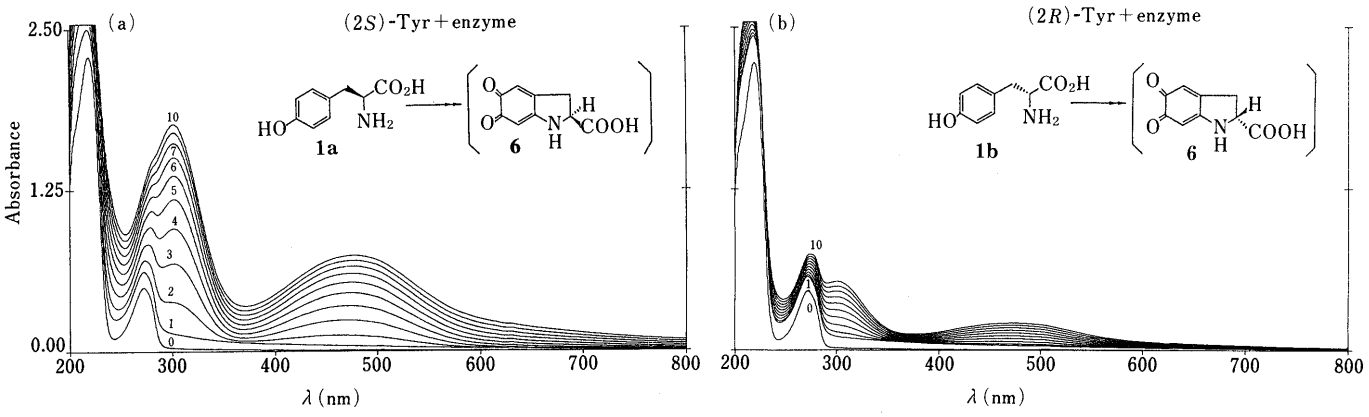

Fig. 2. Time Course of UV Spectra during the Reaction of Tyrosine (**1a**, **1b**) and Tyrosinase

(a) 0.1 ml of mushroom tyrosinase (233 units) in water was rapidly mixed at 23 °C with 1.0 ml of 2*S*-tyrosine (**1a**) (1 mM) in water, 1.0 ml of phosphate buffer (0.1 M; pH 6.6) and 0.9 ml of water. The enzymic reactions were monitored with a UV auto-spectrometer at 23 °C. No. 0, no enzyme; No. 1, after 3.5 min, then every 3.5 min (Nos. 2—10). (b) 2*R*-Tyrosine (**1b**) was used instead of 2*S*-tyrosine (**1a**).

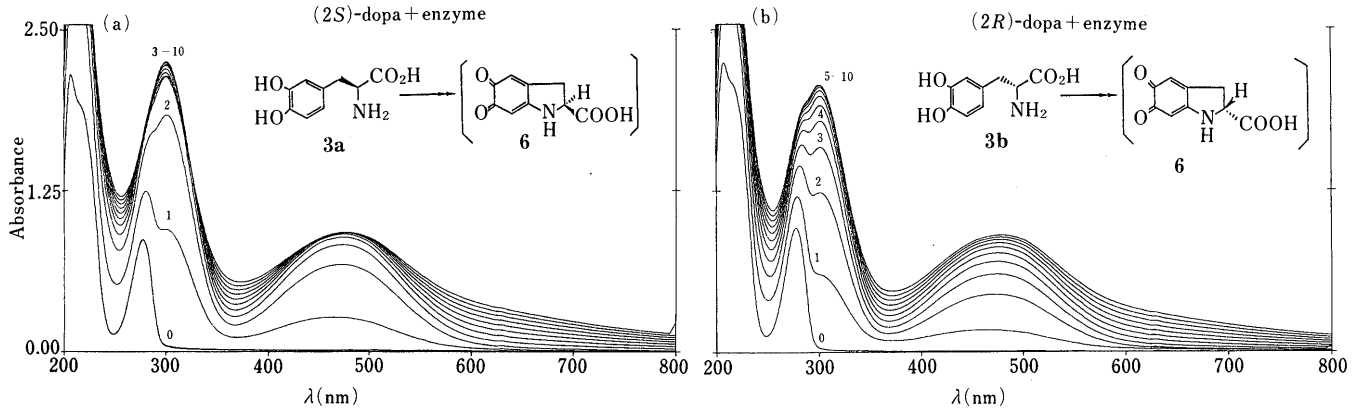

Fig. 3. Time Course of UV Spectra during the Reaction of Dopa (**3a**, **3b**) and Tyrosinase

(a) 2*S*-Dopa (**3a**) (1 mM; 1.0 ml) was used in place of 2*S*-tyrosine (**1a**). (b) 2*R*-Dopa (**3b**) was used instead of 2*S*-tyrosine (**1b**).

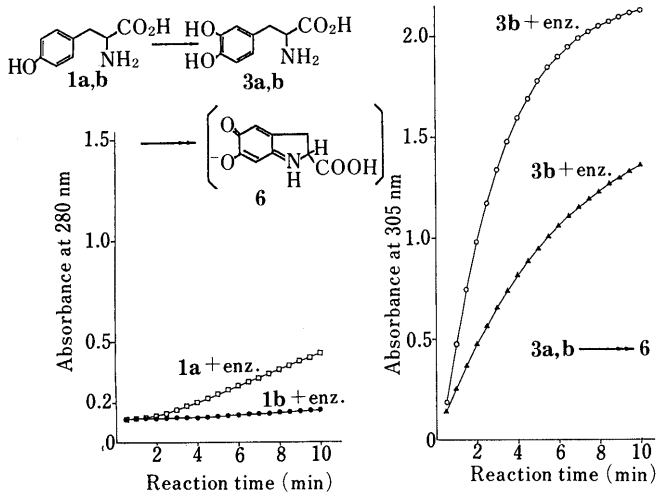

Fig. 4. Time Course of UV Absorbance

(a) 2*S*- or 2*R*-tyrosine (**1a** or **1b**)+enzyme. The absorbance at 280 nm was recorded every 30 s. The rate constant was calculated from the slope of the line between 3 min and 9 min.

(b) 2*S*- or 2*R*-dopa (**3a** or **3b**)+enzyme. The rate constant was calculated from the slope of the line between 0.5 min and 3.0 min.

indicated by the appearance of UV absorptions at 280, 305 and 475 nm (the solution turned red), which were confirmed to arise from dopachrome (**6**), by the study of Mason.[8] As these UV absorptions are similar to those of synthetic models of dopachrome (**6**), he concluded that the reaction intermediate (the red pigment) has the structure **6**.

Figure 2 shows the result of the enzymatic reaction on 2*S*-tyrosine (**1a**) and 2*R*-tyrosine (**1b**). It can be seen from the curves that the conversion of 2*S*-tyrosine (**1a**) to dopachrome (**6**) was much faster at 23 °C than the conversion of 2*R*-tyrosine (**1b**) (Fig. 4).[9]

Similarly, Fig. 3 shows that 2*S*-dopa (**3a**) is transformed to dopachrome (**6**) faster at 23 °C than 2*R*-dopa (**3b**).[10]

CD The enzymic reaction was shown to be stereospecific by means of time course measurement of CD spectra. Figures 5 and 6 demonstrate that the product of reaction of 2*S*-tyrosine (**1a**) and 2*S*-dopa (**3a**) shows (+)-Cotton effects at 490 and 310 nm, and a (−)-Cotton effect at 258 nm, where as the product of reaction of 2*R*-tyrosine (**1b**) or 2*R*-dopa (**3b**) show the reverse Cotton effects. Thus transformation of 2*S*-dopa (**3a**) produces dopachrome (**6a**) of opposite stereochemistry to that produced by reaction of 2*R*-tyrosine (**1b**) or 2*R*-dopa (**3b**).

NMR NMR spectroscopy is a powerful technique for investigating the course of enzymic transformations. In this study we applied ^{13}C-NMR spectroscopy to investigate the early stages of the tyrosinase reaction. The ^{13}C-NMR of (2*RS*)-[2-^{13}C]tyrosine in D_2O consists of a single resonance at δ 56.9. The results of incubation with the enzyme at

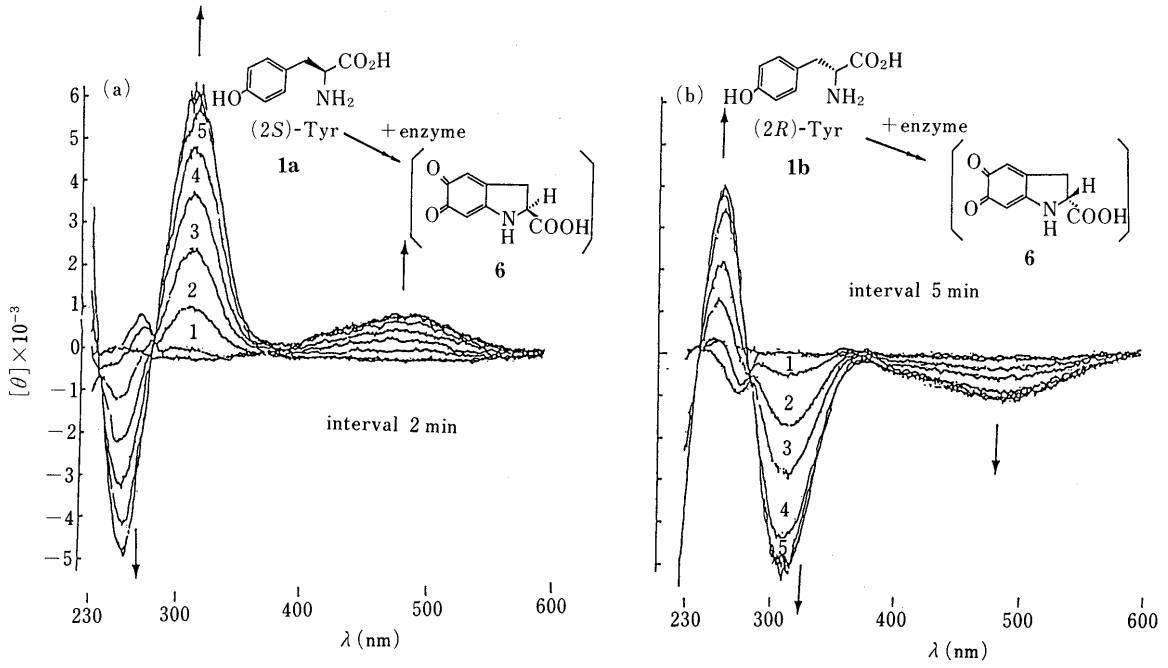

Fig. 5. Time Course of CD Spectra

(a) The enzymatic reaction mixture contained 0.1 ml of tyrosinase (233 units), 1.0 ml of 2*S*-tyrosine (**1a**) (1 mM) in 1 ml of phosphate buffer (0.1 M; pH 6.6) at 23 °C. Slit, 2 nm, TC, 1 s; scan, 200 nm/min (600—400 nm) or 100 nm/min (400—230 nm); cell length, 1 cm. One scan cycle took 2 min.
(b) 2*R*-Tyrosine (**1b**) was used in place of 2*S*-tyrosine (**1a**). One scan cycle took 5 min.

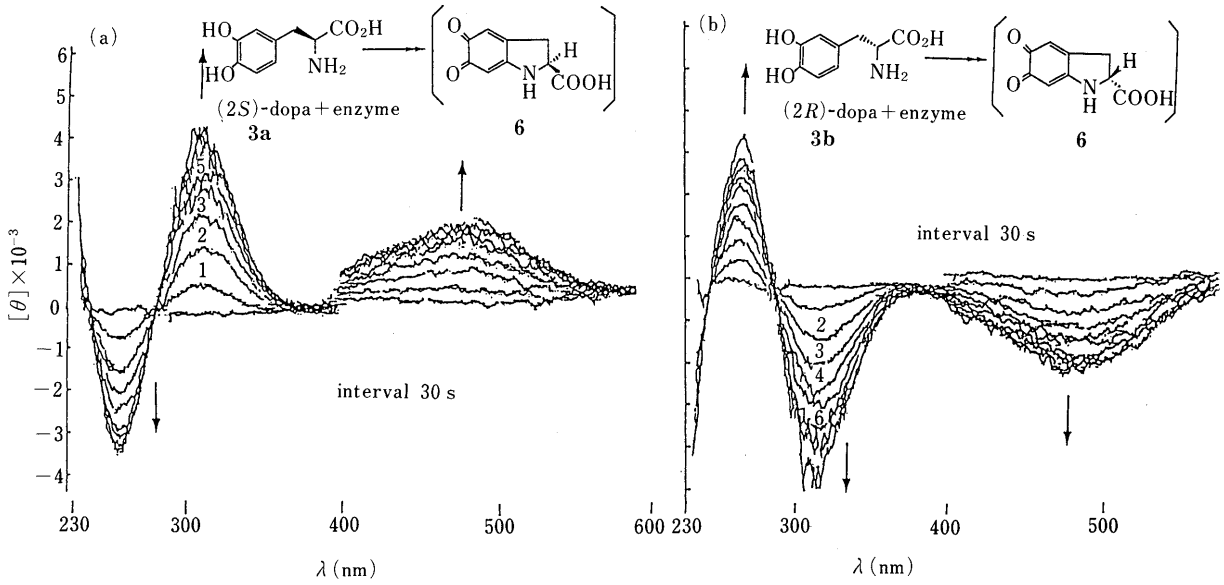

Fig. 6. Time Course of CD Spectra

(a) 2*S*-Dopa (**3a**) (1 mM; 1.0 ml) was used instead of 2*S*-tyrosine (**1a**). TC, 0.25 s (400—230 nm) or 0.5 s (600—400 nm); scan 500 nm/min. One scan cycle took 30 s (400—230 nm) or 36.4 s (600—400 nm).
(b) 2*R*-Dopa (**3b**) (1 mM; 1.0 ml) was used instead of **3a**.

5 °C are shown in Fig. 7. It can be seen that there is a build up of a species with a chemical shift of δ 65.2. As C-2 of dopa (**3**) shows the same chemical shift ($=\delta$ 56.9)[11] as that of tyrosine, this signal at δ 65.2 should originate from the reaction intermediate. Eventually this signal disappears and is replaced by a signal at δ 120.0. The chemical shift of the latter signal is similar to that of C-2 of indole ($=\delta$ 124.1),[11] so it can be assigned to C-2 of 5,6-dihydroxyindole (**7**). The resonance at δ 65.2 must belong to the same species (**6**) that was observed in the UV and CD experiments, by analogy with the ^{13}C spectrum of proline ($=\delta$ 61.6).[11]

Thus, by a combination of physico-chemical techniques, we have demonstrated that tyrosine (**1**) is converted rapidly by tyrosinase to dopachrome (**6**), that the rate of conversion is faster in 2*S*- than in 2*R*-amino acid and that in the conversions are stereospecific.

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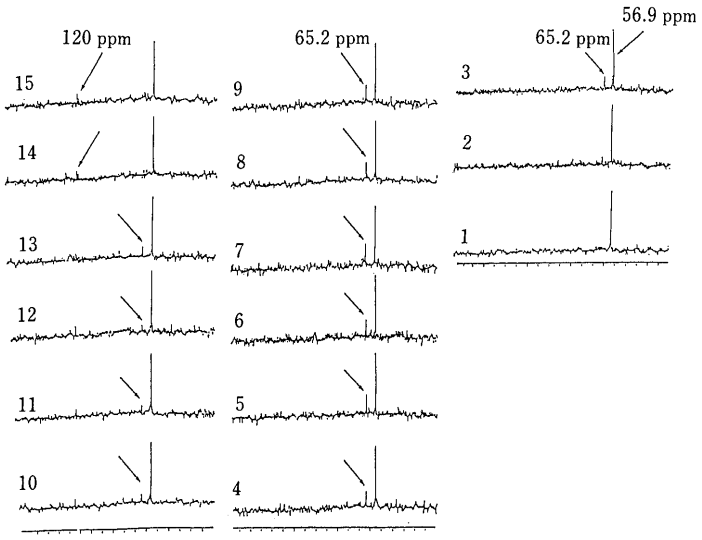

Fig. 7. Time Course of ^{13}C-NMR Spectral Changes

^{13}C enriched tyrosine (1 mg) was incubated with 1 mg of mushroom tyrosinase solution in 0.5 ml of D_2O. ^{13}C-NMR spectra were recorded at 75 MHz at 5 C. **1**: at 560 s, then every 112 min.

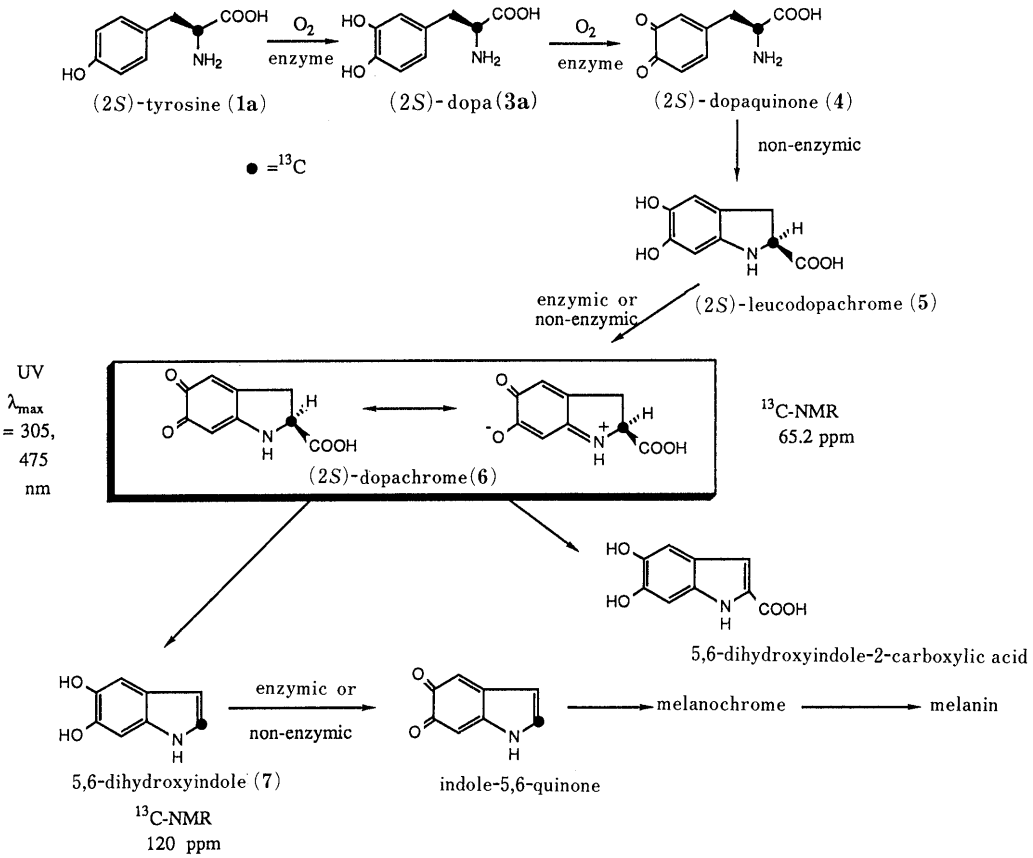

Fig. 8. Proposed Biosynthetic Pathway to Melanin (**9**)

References and Notes

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9) The rate constants are 4.0×10^{-2} min^{-1} (for the reaction of **1a**, and 6.8×10^{-3} min^{-1} (for that of **1b**). See Fig. 4a and its legend. As the rate-determining step is the conversion of **1** into **3** in the formation of dopachrome (**6**), the change of absorbance at 280 nm (λ_{max} of **3**) was monitored.
10) The rate constants are 4.3×10^{-1} min^{-1} (for the reaction of **3a**) and 1.9×10^{-1} min^{-1} (for that of **3b**). They were calculated from the changes of the absorbance at 305 nm (λ_{max} of **6**). See Fig. 4b and its legend.
11) E. Breitmaier and W. Voelter, "Carbon-13 NMR Spectroscopy," Third Edition,VCH, Weinheim, 1987.