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ORIGINAL ARTICLE

Unusual reactivity of β-hydroxy-serotonin, a forgotten serotonin derivative

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Abstract Serotonin (5-HT) is a well-known biogenic amine which regulates mood, sleep, and is involved in muscle contraction and blood coagulation. Based on an analogy to norepinephrine, a β-hydroxylated derivative of dopamine which has diverse physiological functions, beta-hydroxy-serotonin (β-OH-5-HT) originally encouraged interest as a potential pharmacological agent. Four decades ago, its organic synthesis was attempted. However, due to difficulties with the synthesis and the compound's instability, rigorous identification and characterization of β-OH-5-HT proved evasive. Here, we successfully synthesized β-OH-5-HT from 5-HT using a *Pseudomonas* enzyme, tryptophan side chain oxidase type I (TSOI), and we determined the structure by 2D-NMR and characterized β-OH-5-HT in detail. The CD spectra showed no optical activity, suggesting a racemic mixture. To separate DL-β-OH-5-HT, we synthesized L-Ala-5-HT and derivatized it into erythro- and threo-L-Ala-β-OH-5-HT with TSOI. Interestingly, both isolated fractions returned to a diastereoisomeric mixture within two hours at pH 5.0. Later, we found that, under acidic conditions, β-OH-5-HT readily reacted with nucleophiles like alcohols or thiols, yielding a variety of DL-β-substituted-5-HT. The unusual properties of β-OH-5-HT might be attributed to the unique nature of a β-hydroxyl group adjacent to an indole ring and amino group. The mechanism for the rapid racemization of β-OH-5-HT is discussed.

Keywords Serotonin · Serotonin derivatives · β-hydroxylation · Rapid racemization · Tryptophan · Enzymatic derivatization

Abbreviations

5-HT	5-Hydroxytryptamine or serotonin
β-OH-5-HT	β-Hydroxy-serotonin
TSO	Tryptophan side chain oxidase
FD/EI-MS	A field disorption/electron ionization mass spectrometry
2D-NMR	Two-dimensional nuclear magnetic resonance
β-OH-L-Trp	β-Hydroxy-L-tryptophan
β-OH-L-5-HTP	β-Hydroxy-L-5-hydroxytryptophan
5-HTP	5-Hydroxytryptophan
Z	Benzyloxycarbonyl
EDC-HCl	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
TMS	Trimethylsilane
β-keto-5-HT	β-Ketoserotonin
r.t.	Retention time

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an important biogenic amine ubiquitously distributed in animals

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(Essman 1977). It has a variety of pharmacological effects on the central nervous system through various 5-HT receptors (Hoyer et al. 2002). In particular, 5-HT is involved in the regulation of mood, appetite, sleep, muscle contraction, and some cognitive functions including memory and learning. In the other important biogenic aromatic amine (phenylethylamines) family, hydroxylation of the side chain at the β-position of phenylethylamines is a general metabolic reaction in animals and plants which results in products with different biological activities from the parent compounds. As shown in Fig. 1a, dopamine and tyramine are converted into norepinephrine and octopamine, respectively, by dopamine β-hydroxylase which oxidizes the side chain carbon adjacent to the aromatic ring. Epinephrine, which is further metabolized from norepinephrine, also contains a β-hydroxyl group in its chemical structure. Therefore, analogous to phenyletylamine metabolic pathways, it has been anticipated that β-hydroxylation of the side chain of 5-HT or other indolylalkylamines might result in new pharmacological agents.

In the past, the organic synthesis of β-hydroxy-serotonin (β-OH-5-HT) was attempted, but the task proved to be extremely difficult, and β-OH-5-HT had yet to be purified satisfactorily (Ames et al. 1956; Friedman and Vogel 1980; Plavsic et al. 1974). In this study, however, we became the first to succeed in obtaining highly pure β-OH-5-HT, and we did so via enzymatic derivatization of 5-HT. We used a unique enzyme called tryptophan side chain oxidase Type I (TSOI) from *Pseudomonas* (ATCC 29574) (Takai and Hayaishi 1987). TSO catalyzes the oxidation of the side chain of tryptophan, and it can be used for the modification of tryptophan derivatives or residues in peptides and proteins (Ito et al. 1981; Takai and Hayaishi 1987). There are two isozymes, TSOI and TSOII. Both enzymes are hemeproteins, but they show different substrate specificities (Ito et al. 1981) and only TSOI is active on free tryptophan and a variety of 3-substituted indole derivatives including 5-HT Fig. 1b (Takai and Hayaishi 1987). Further enzymatic conversion of the β-hydroxyl derivatives to the corresponding β-keto compound was also demonstrated at a much slower rate in previous literatures (Nakamaru et al. 1990; Takai and Hayaishi 1987).

Here, we provide a detailed report on the isolation and characterization of β-OH-5-HT by UV–visible absorption, CD spectra, fluorescence spectra, FD/EI-MS, and 2D-NMR analyses. Through this study, we found the unusually high reactivity of β-OH-5-HT, which has never been observed for β-hydroxy-L-tryptophan (β-OH-L-Trp), β-hydroxy-5-hydroxy-L-tryptophan (β-OH-L-5-HTP), or norepinephrine. The mechanism of the rapid racemization and the unusual reactivity of β-OH-5-HT are discussed as well as the potential medicinal implications of our work.

Experimental procedures

Materials

DL-5-Hydroxytryptophan ethyl ester (DL-5-HTP-OEt), leucine aminopeptidase (type III-CP), aminopeptidase M were purchased from Sigma (St. Louis, MO). Ninhydrin spray, Z-Ala, EDC-HCl, Palladium-black, and *p*-aminophenol were obtained from Wako Pure Chemicals (Osaka, Japan). AG1-X8 was from Bio-Rad (Hercules, CA). TMS, DMSO-d6 (99.9 %), acetone-d6 (99.5 %), D_2O (99.9 %) were from Aldrich. Amberlite XAD-2 was from Rohm & Hass (Philadelphia, PA). Silica gel thin layer chromatoplate (Kieselgel 60-F254) was from Merck (Germany). All other chemicals were of reagent grade and obtained from commercial sources.

Enzyme purification

Pseudomonas (ATCC 29574) was grown aerobically at 25 °C in nutrient broth and harvested in the late stationary phase and then stored frozen at −80 °C. TSOI was purified

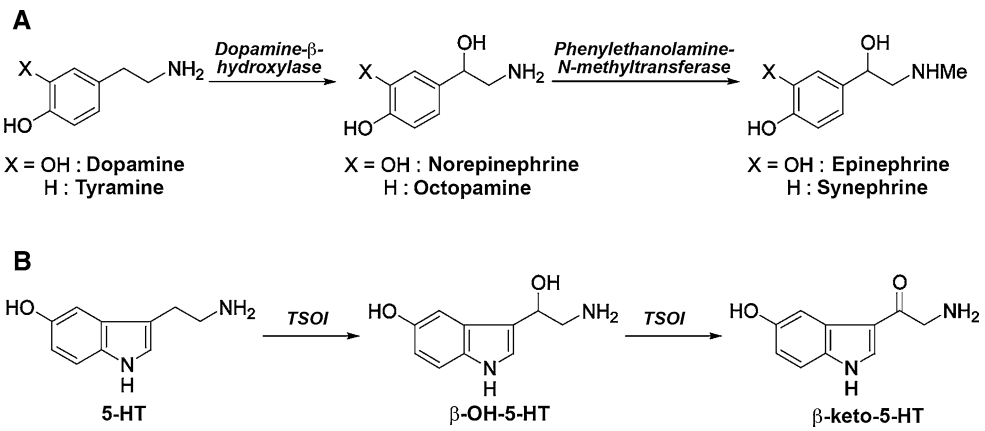

Fig. 1 Parallel metabolic pathways. **a** β-Hydroxylated phenylethylamines. **b** The TSOI-catalyzed conversion of 5-HT to β-OH-5-HT and β-keto-5-HT

essentially as described previously (Takai and Hayaishi 1987) with moderate modifications.

Analytical HPLC

HPLC was performed on a Toso CCDE pump with two UV detectors, Toso UV-8 model 2 (at 280 nm) and Hitachi 655A (at 315 nm) at 25 °C, or a photodiode array (SPD-M6A, Shimadzu). A reverse phase, 5C18 column (Wakosil or Cosmosil ODS, 4.6 × 150 mm) was used for analyses. The isocratic solvent systems used were: (a) methyl alcohol: 50-mM ammonium acetate at pH 5.0 (3:7, v/v) for Trp and 5-HTP derivatives; (b) methyl alcohol: 50-mM ammonium acetate at pH 5.0 (5:95, v/v) for 5-HT derivatives. We employed acetonitrile (a 15-min linear gradient from 0 to 40 %) in 50-mM sodium phosphate pH 3.0 containing 4-mM sodium octyl sulfate when isolating methanol or other adducts of β-OH-5-HT. For chiral separation, CROWNPAK CR(+) (4 × 150 mm, Daicel, Japan) was used with 0.1 % trifluoroacetic acid pH 2 at 11 °C at a flow rate of 1 ml/min.

Preparation of erythro-L-threo-β-OH-L-Trp, β-OH-5-HT, and β-OH-5-HTP

To avoid TSO-catalyzed deamination and decarboxylation of free tryptophan, L-Trp-Leu and DL-5-HTP-OC_2H_5 were used as substrates. To obtain erythro-L-threo-β-OH-Trp, L-Trp-Leu (400 mg) was dissolved in 50 ml of 50-mM ammonium acetate, pH 5.0 at 50 °C and bubbled with pure O_2 gas for 5 min and then incubated at 25 °C with 7.5 units of TSOI. After 2–3 h, the substrate was completely consumed. The reaction was then stopped by heat treatment (at 100 °C for 5 min). The denatured enzyme was removed by centrifuge at 4 °C for 10 min or ultra-filtration (PM-10) under nitrogen pressure. The supernatant was applied to a column (1.5 × 11.3 cm, 20 ml) of Amberlite XAD-2 that was equilibrated with H_2O at 4 °C. After the column was washed with 200 ml of H_2O, the elution was performed with 15 % aqueous methyl alcohol. The eluate was concentrated to 15 ml on a rotary evaporator at 60 °C and then adjusted to pH 8.0 by 5-mM Tris–HCl and treated by leucine aminopeptidase (100 μg) in the presence of 5-mM $MgCl_2$ at 37 °C for 1–2 h. After the 1–2 h, threo diastereoisomer of β-OH-Trp-Leu was completely digested, but a significant amount of erythro diastereoisomer still remained unhydrolyzed. The reaction mixture was freed from the previously added enzyme by filtration and applied to an Amberlite XAD-2 column at 4 °C. By monitoring free leucine with the electroconductivity meter and TLC, the main products corresponding to erythro- and threo-β-OH-L-Trp were collected and lyophilized to a white powder, 4.5 and 27 mg, respectively. For preparation of β-OH-5-HT, a similar strategy was used as described above except that we used 5-HT hydrochloride (200 mg) as a substrate and Bio-Rex 70 (H^+-from, 10 × 150 mm, 11.7 ml) for the isolation. Fractions eluted with 100-mM ammonium acetate at pH 5.0 that contained β-OH-5-HT were combined, concentrated on a rotary evaporator at room temperature and desalted through a Sephadex G-50. After lyophilization, 86 mg of β-OH-5-HT acetate salt was obtained (43 % yield). If necessary, a counter ion, acetate, was exchanged with a Cl^- by AG1-X8. For preparation of β-OH-5-HTP, DL-5-HTP-OEt (75 mg) was used as a substrate and incubated with TSO under air because 5-HTP is very unstable under pure O_2 conditions. Diastereoisomers of β-OH-L-5-HTP were separated on a semi-preparative YMC Pack D-ODS-5 (20 × 250 mm) with a methyl alcohol linear gradient (5–30 %) HPLC system. The modified ester forms were hydrolyzed with the 0.1-M NaOH. 2.5 and 2.9 mg of erythro- and threo-β-OH-L-5-HTP were obtained, respectively.

Preparation of L-Ala-5-HT

5-Hydroxytryptamine hydrochloride (1.00 g, 4.70 mmol) and equimolar Z-Alanine were conjugated with EDC-HCl in the presence of triethylamine (Sheehan et al. 1965). The obtained crude product was hydrogenated with Pd black to give 642 mg of L-Ala-5-HT (2.59 mmol, 55.2 % overall yield) (Bergmann and Zervas 1932).

Thin layer chromatography

TLC was carried out on precoated silica gel plates (Stahl 1969). The solvent systems used were: (a) *n*-butyl alcohol:acetic acid:water (4:1:5, v/v); (b) *n*-butyl alcohol:acetic acid:water (65:13:22); (c) *n*-butyl alcohol:acetic acid:water (12:3:5). Products were located on the plate either by irradiation with an ultraviolet lamp (253.7 and 365.0 nm) or by spraying with ninhydrin reagent for amines, Ehrlich's reagent for indole compounds, or 0.1-M *o*-phenylenediamine in 2-M KOH for ketoaldehyde.

Other methods

Spectrophotometric measurements were performed on a Shimadzu MPS 2000. Fluorescence spectra were recorded on a Hitachi F-4010 in a quartz cuvette of a 4-mm light path. Melting points were determined with a Yanagimoto micro-melting pointing apparatus using 1-naphtol (95–97 °C), anthranilic acid (146–147 °C), and *p*-aminophenol (183–185 °C) as standards. NMR spectra were obtained at 400 MHz in Fourier transform mode on a JEOL JNM GX-400. Field desorption mass spectrometry was performed on a JEOL Model. CD measurements were carried out using a J-720 Spectropolarimeter (JASCO, Japan), at 25 °C in

1-cm path-length quartz cuvettes. NMR spectrometry and FD/EI-MS were performed in the Meijiseika Pharmaceutical Institute by courtesy of Dr. Hiroshi Tanno. Any variations from the procedures and details are described in the figure legends.

Beta-hydroxy-5-hydroxytryptamine (β-OH-5-HT) acetate

White powder (99.9 % purity by HPLC). Highly hygroscopic, but stable for a month at 4 °C or several months at 80 °C. M.p. (dec.) was 190–200° C. U.V. spectrum: λ_{max} 272, inflection at 295 nm; $\varepsilon_{mM} = 5.6$ at pH 5. FDMS: m/z 192. ^{1}H NMR (400 MH_Z, DMSO-d6): 10.7 ppm (indole NH, H-1), 6.6, 6.9 and 7.2 ppm (4H, indole CH protons, H-6, H-4, H-2/H-7, respectively), 4.8 ppm (1H, β-CH), and 2.9 ppm (2H, α-CH). The large chemical shift of the β-CH methylene proton (1H, β-CH) was observed (from 3 ppm to 4.8 ppm), in comparison with the ^{1}H NMR data of 5-HT.

Results

Synthesis and isolation of β-OH-5-HT

β-OH-5-HT has previously appeared in the literature but without a detailed description and characterization due to the compound's instability (Plavsic et al. 1974). In comparison to the difficulties of the chemical synthesis, the enzymatic conversion of 5-HT to β-OH-5-HT with TSOI was much simpler and easily achieved. As shown in Fig. 1b, 5-HT underwent two consecutive dehydrogenations by TSOI. The first dehydrogenation followed by hydration gave β-OH-5-HT. This conversion was complete in less than 16 min (Fig. 2a, upper trace). Upon prolonged incubation, β-OH-5-HT then underwent a further dehydrogenation to give β-keto-L-Trp. The second dehydrogenation step was approximately 100 times slower than the first step (Fig. 2a, lower trace). When we prepared a larger amount of β-OH-5-HT, O_2 bubbling was needed to facilitate the reaction. The isolated, pure (more than 99.9 % assessed by HPLC), white powder of β-OH-5-HT was highly hygroscopic, but it was stable for a month at 4 °C and stable for several months at −80 °C. The melting point was around 190–200 °C with decomposition, which was lower than the reported value of 215 °C (Plavsic et al. 1974). The ultraviolet absorption spectrum of β-OH-5-HT showed a small blue shift from 275 to 272 nm and a small increase of the inflection at 295 nm to over 300 nm in comparison with 5-HT (Fig. 2b). These characteristics were similar to those of β-OH-Trp in comparison with tryptophan (Ito et al. 1981). For β-keto-5-HT, the difference from 5-HT is more distinct with three increased peaks at 251, 273, and 305 nm. This has been recognized as a typical feature in the spectra of

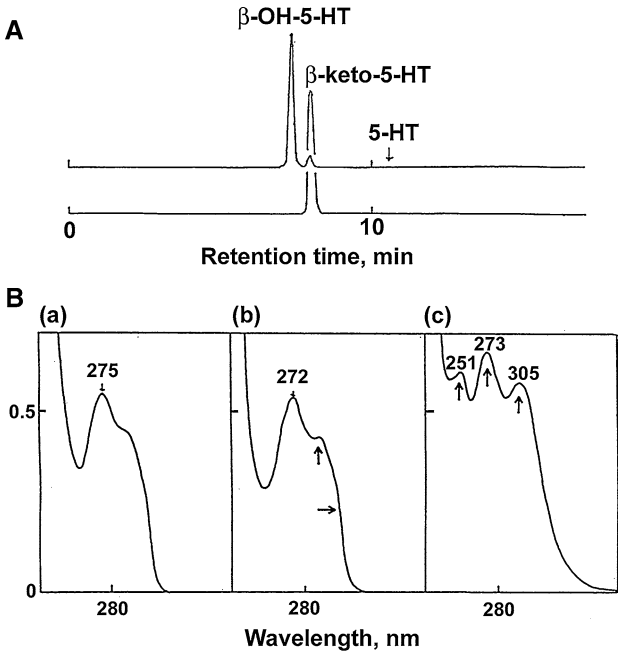

Fig. 2 **a** Elution profile on HPLC analysis of the reaction of 5-HT by TSOI. The reaction was performed at 25 °C in 0.1 ml of H2O containing 1-mM 5-HT and 0.05 units of TSOI. After 16 min (*upper*) and 48 h (*lower*) of incubation, aliquots (5 μl) were subjected to HPLC with solvent B at a flow rate of 0.5 ml/min. The elution was monitored at A280 nm (arbitrary units). **b** Ultraviolet absorption spectra of 5-HT (**a**), β-OH-5-HT (**b**), and β-keto-5-HT (**c**) at 0.1-mM in 50-mM ammonium acetate at pH 5.0. *Arrows* indicate the direction of the changes in absorbance from 5-HT

keto-indoles such as indolealdehyde, indoleacetoaldehyde, indoleglycolaldehyde, or β-keto-tryptophan (246, 264, and 314 nm) (Ito et al. 1981). During the overall spectral changes from 5-HT to β-keto-5-HT in the reaction with TSOI, the isosbestic points were clearly present (data not shown), indicating that the resulting product was formed without detectable accumulation of an intermediate. These data were in line with the HPLC analyses (Fig. 2a).

Identification of β-OH-5-HT

Field adsorption mass spectrometry of the β-OH-5-HT showed a molecular ion peak at m/z 192, corresponding to the total mass of 5-HT ($M_r = 176$) plus an oxygen atom ($M_r = 16$) which is the molecular formula of β-OH-5-HT (data not shown). The fragmentation pattern on electron ionization mass spectrometry (EI/MS) of the TMS derivative showed a base peak at m/z 306 $(TMS\text{-}O\text{-}indole\text{-}NH\text{-}CH\text{-}OTMS)^+$ and another peak at m/z 378 $(TMS\text{-}O\text{-}indole\text{-}N\text{-}TMS\text{-}CH\text{-}OTMS)^+$, suggesting that the site of the hydrogen group was at the β-carbon atom of the side chain of 5-HT (Fig. 3a). Although we could not attain a molecular ion peak at m/z 480 (M^+, 4TMS), it has

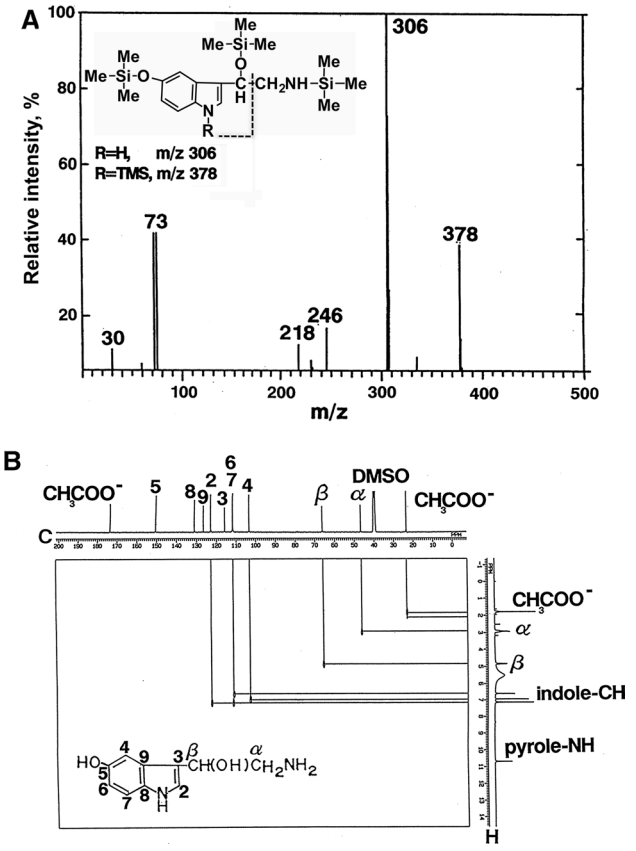

Fig. 3 **a** Mass spectrum of the TMS derivative of β-OH-5-HT transformed from 5-HT by TSOI. **b** CH-COSY NMR spectrum of β-OH-5-HT. The spectrum was recorded in dimethyl sulfoxide-d6 at room temperature

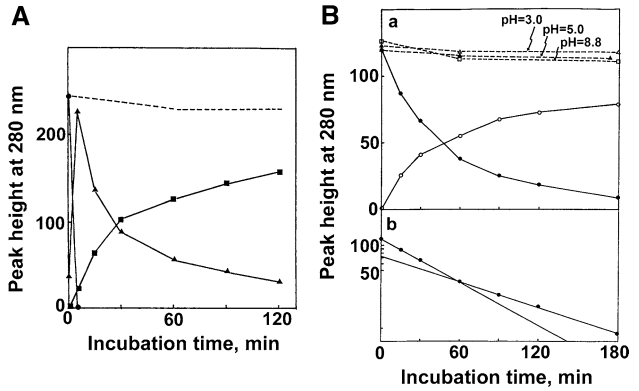

Fig. 4 **a** Time course of TSOI-catalyzed conversion of *threo*-β-OH-L-Trp (*filled circle–filled circle*, r.t. = 5.8 min) to the β-keto-L-Trp (*filled triangle–filled triangle*, r.t. = 10.8 min) through the intermediate X (*filled triangle–filled triangle*, r.t. = 7.8 min). **b** Time course of TSOI-catalyzed conversion of β-OH-5-HT (*filled circle–filled circle*, r.t. = 4.4 min) to β-keto-5-HT (*open circle–open circle*, r.t. = 9.0 min) (**a**) and the re-plot of the decrease of β-OH-5-HT in semi-log scale (**b**). The reaction was carried out at 25 °C in 50-mM ammonium acetate at pH 5.0 with 0.05 units and 5 units of TSOI in **a** and **b**, respectively, or without TSO (*dotted lines*) as control

been known that the splitting between α- and β-carbon of 3-alkylindole compounds was common (Powers 1968). The ^{1}H-NMR spectrum of β-OH-5-HT in DMSO-d6 or acetone-d6 showed that the shapes of the aromatic signals were almost identical to those of authentic 5-HT, and all indole protons in β-OH-5-HT were readily assigned (Fig. 3b). Compared to the chemical shifts and the coupling constant in the NMR spectra of 5-HT and erythro-L-threo-β-OH-L-Trp, the –CHOH–CH_2- structure in β-OH-5-HT was clearly established. One of the β-CH_2 protons of 5-HT was lost. Together with the decoupling experiments for the indole proton at C2 position by irradiation, all of the evidence here proved that hydroxylation has taken place on the side chain carbon adjacent to the indole ring. We also obtained ^{13}C-NMR spectrum in DMSO-d6 and assigned all the signals according to ref. (Silverstein and Bassler 1967) (Fig. 3b). The CH-COSY analysis showed that our assignments were consistent (Fig. 3b). Based on all of those results, the chemical structure of β-OH-5-HT was confirmed as *DL-β-OH-5-HT*. According to the reaction mechanisms of TSO, the β-OH-5-HT obtained here was expected to be a DL-racemic mixture because the β-addition of water to the reaction intermediate is a spontaneous, nonenzymatic process (Zavala et al. 1983). To clarify this matter, we first tried to distinguish DL-enantiomers of β-OH-5-HT using an enzyme reaction involved in the chiral environment. Generally, threo form was preferred to erythro form in the TSO reaction by 100 times for further oxidation to β-keto form. As in the case of β-OH-L-Trp, threo-β-OH-L-Trp readily reacted with TSOI and disappeared within 5 min, and a transient accumulation of the unidentified intermediate (r.t. = 7.8 min) occurred in a mirror image (Fig. 4a). Then, β-keto-L-Trp gradually formed as the intermediate decreased. In contrast, the conversion of β-OH-5-HT–β-keto-5-HT was much slower, and no intermediate appeared. When β-OH-5-HT was reacted with a large amount (100 times more than standard condition) of TSOI, β-keto-5-HT immediately formed Fig. 4b(a). When the decrease of β-OH-5-HT was re-plotted in semi-log scale, two different rates were clearly observed Fig. 4b(b), indicating that β-OH-5-HT was a DL-racemic mixture. As expected, the CD spectrum of β-OH-5-HT showed no optical activity (Fig. 5, upper). Although it is known that the 180–260 nm of the CD spectrum is normally predominantly associated with the amide backbone of a peptide and provides information about its secondary structure, tryptophan residues also contribute to CD spectra in the 225–250 nm region. In fact, the CD spectrum of threo-β-OH-L-Trp was readily distinguished from its erythro diastereoisomer (Fig. 5, lower).

Chiral separation of DL-β-OH-5-HT

Since any attempts on separation of DL-enantiomers of β-OH-5-HT by HPLC with chiral solvent system or by chiral crystallization failed, we tried to isolate D- and L-β-OH-5-HT by diastereoisomeric methods. For this purpose, L-Ala-5-HT was synthesized from Z-L-Ala and 5-HT and then modified into L-Ala-β-OH-5-HT with TSOI (Fig. 6a). The resulting diastereoisomers, Peaks 1 and 2, were separated by HPLC (Fig. 6b). The ultraviolet absorption spectra of those diastereoisomers were the same except for a small difference at ~245 nm (Fig. 6c) which was also seen in the spectra of erythro-L-threo-β-OH-L-Trp previously reported (Ito et al. 1981). The isolated Peak 1 and Peak 2 fractions were immediately adjusted to pH 7 and subjected to aminopeptidase M treatment at 25 °C (Fig. 7). Surprisingly, the individual isolated epimer fraction of L-Ala-β-OH-5-HT gradually became a diastereoisomeric mixture at pH 5.0 on the HPLC by rechromatography (data not shown) or, as seen in Fig. 7, at 0 time of the aminopeptidase treatment. Peak 1 seemed to be a preferred substrate for the peptidase, but the overall rates of digestion for Peaks 1 and 2 to give D- or L- enantiomer of β-OH-5-HT were not very different (Fig. 7). In addition, we found that each isomer of β-OH-5-HT gave another new peak on HPLC besides its original peak when it was eluted with the HPLC solvent at pH 3.0 containing methanol (data not shown). With further experiments, that newly emerged peak was identified as β-methoxy-5-HT, wherein the hydroxyl group at the β-position was replaced with a methoxy group from methanol in the solvent (data not shown). This tendency became more intense under the conditions of lower pH and higher ionic strength (Fig. 8a). Moreover, we found that β-OH-5-HT dissolved in 0.1-M HCl readily reacted with nucleophiles such as alcohols or thiols to give a variety of β-substituted-5-HT entities (Fig. 8b). Among the series of nucleophiles, pyrrole and ethanethiol were apparently the most effective in giving rise to a complete conversion of β-OH-5-HT into β-substituted-5-HT (Fig. 8b). This phenomenon was not observed with the β-OH- L-Trp and β-OH- L-5-HTP, or norepinephrine (data not shown).

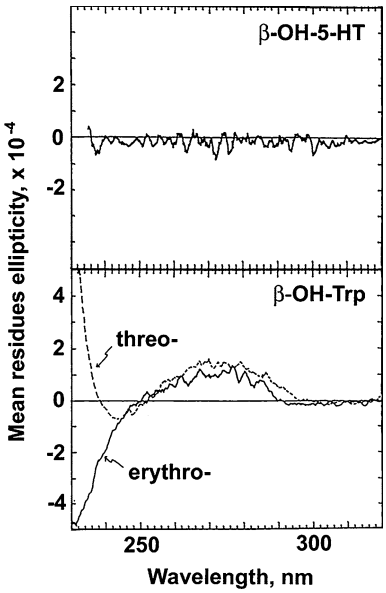

Fig. 5 CD spectra of β-OH-5-HT (*upper*) and β-threo-L-erythro-OH-L-Trp (*lower*) at room temperature. Each solution was at 0.2 mM in 50-mM ammonium acetate, pH 5.0

Isolation and DL-separation of the β-adducts of 5-HT

To identify the chirality of β-substituted-5-HT, the adduct with ethanethiol was prepared on a larger scale (Fig. 9a).

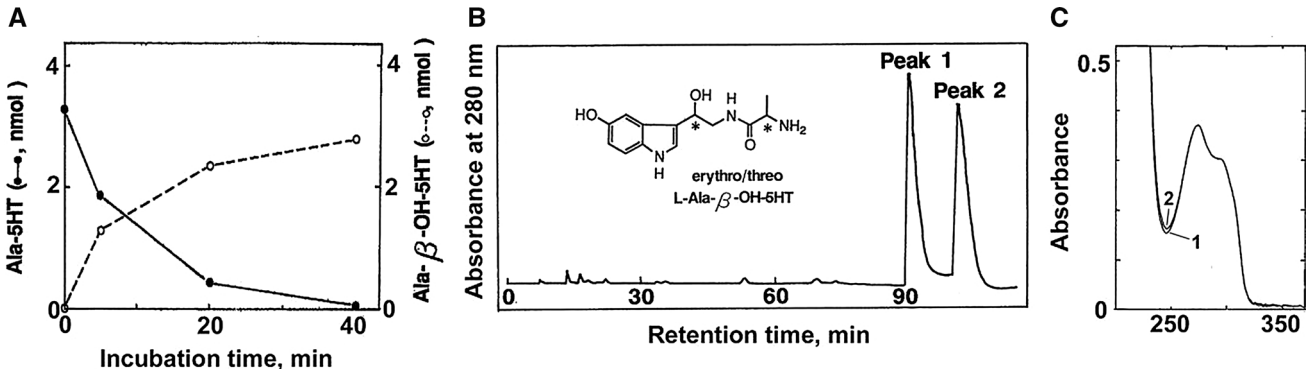

Fig. 6 β-Hydroxylation of Ala-5-HT by TSO. **a** Time course of TSOI-catalyzed conversion of Ala-5-HT (*filled circle–filled circle*) to Ala-β-OH-5-HT (*open circle–open circle*), the sum of threo and erythro diastereoisomers. Ala-5-HT at 0.2 mM was incubated at 25 °C in 50-mM potassium phosphate at pH 3.0 with 0.056 units of TSOI. **b** Elution pattern of semi-preparative HPLC analysis of threo and erythro diastereoisomers of L-Ala-β-OH-5-HT, Peaks 1 and 2. The reaction mixture was subjected to HPLC (YMC Pack D-ODS-5, 20 x 250 mm) with 50-mM ammonium acetate pH 5.0 at a flow rate of 8 ml/min at 25 °C. **c** Ultraviolet absorption spectra of the isolated Peak 1 and Peak 2 fractions (1 and 2) from (**b**)

Using a chiral column, we successfully detected the isolated D- and L-enantiomer peaks of β-ethanethiol-5-HT on HPLC (Fig. 9b). Together with Figs. 7, 8, this result strongly suggested that β-OH-5-HT in the acidic aqueous solution was under equilibrium of the DL-conversion at room temperature. Therefore, β-OH-5-HT could be readily trapped by a variety of nucleophiles depending on their nucleophilic strength with their respective intermediate(s) leading the DL-conversion.

Discussion

In the present study, we enzymatically synthesized β-OH-5-HT with TSOI in only one step and rigorously determined its chemical structure. We found unusual chemical properties of β-OH-5-HT. About 40 years ago, the organic synthesis of β-OH-indoleamines was of great interest. Their interest stemmed from the fact that these compounds could be regarded as analogs of epinephrine and ephedrine but containing an indole ring instead of a benzene ring. Due to their similarity to the other compounds, β-OH-indoleamines were suspected of having potential pharmacological relevance. However, besides Iskric's group in 1974 (Plavsic et al. 1974), nobody has succeeded in synthesizing and obtaining β-OH-5-HT or β-OH-tryptamine that is satisfactorily purified to be able to assess its unique properties. Furthermore, Iskric's group only reported on moderate pharmacological effects of β-OH-5-HT (Ferle-Vidovic et al. 1983; Plavsic et al. 1976). Beyond that limited report, there was no literature available on β-OH-5-HT or β-OH-tryptamine. We, however, had great success in synthesizing β-OH-5-HT via enzymatic derivatization with TSOI.

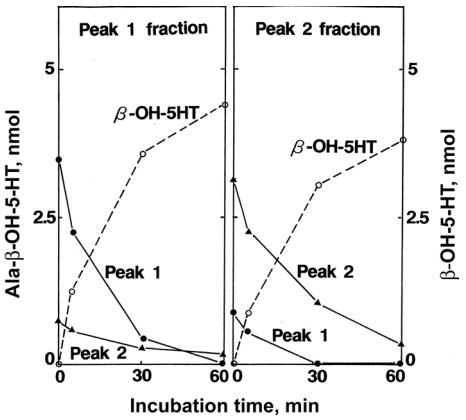

Fig. 7 Aminopeptidase M-catalyzed formation of β-OH-5-HT from Ala-β-OH-5-HT. The isolated Peak 1 and Peak 2 fractions were adjusted to pH 7 by trimethylamine and separately treated with aminopeptidase M (140 mU) in the presence of 1-mM $MgCl_2$ at 25 °C

Unique properties of β-OH-5-HT

After successfully synthesizing β-OH-5-HT, we were able to assess their respective structures and came across results that could be deemed as surprising. It is unusual and thus far unheard of that a simple compound like β-OH-5-HT in the acidic solution could be under equilibrium of the DL-racemization at room temperature. For free amino acids, the rate-determining step in the racemization reaction is the abstraction of the α-proton by a basic species such as OH^-, which produces a tertiary carbanion intermediate (Bada 1984; Steinberg et al. 1984). Such a conclusion suggests that the rate of racemization is controlled by the stability of the carbanion intermediate which is dependent upon the degree of protonation of the α-carboxyl and α-amino groups as well as the electron-withdrawing capacity of the R-group of the amino acid. Therefore, the rates of racemization of free amino acids may change as a function of pH and the ionization of functional groups in the R-group of

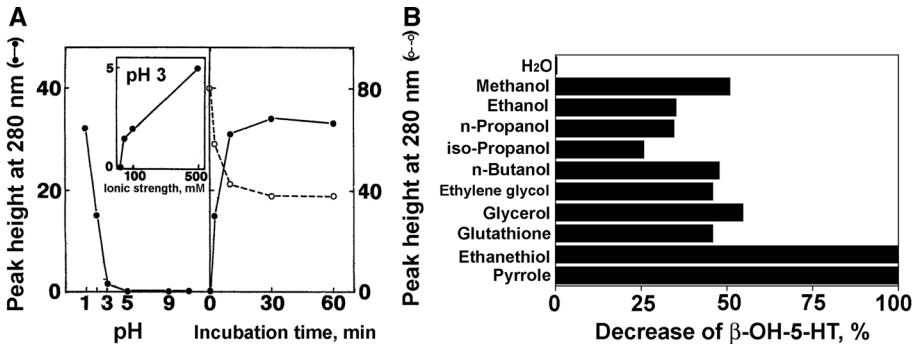

Fig. 8 **a** Nonenzymatic formation of the methanol adduct from β-OH-5-HT. Effect of pH and ionic strength on the methanol adduct formation in the presence of 30 % methanol (*left*) and the time course of the adduct formation at pH 1.0 (*right*) are displayed. **b** Relative reactivity of nucleophiles with β-OH-5-HT. 1-mM β-OH-5-HT in 0.1-M HCl was incubated with various nucleophiles (30 % solution, except for GSH at 0.5 M, and ethanethiol and pyrrole at 5 %) at 25 °C for 30 min

the amino acid. In fact, at pH 7.6, the β-OH derivative of serine is a more effective electron-withdrawing functional group than serine itself under similar conditions (Bada 1984). Generally, the rate of racemization for free amino acids is so slow that D-enantiomers cannot be readily detected under mild conditions (Manning 1970; Yamada et al. 1983). In contrast, as we have shown above, the rate of racemization of β-OH-5-HT is, surprisingly, extremely rapid even though racemization of β-OH-5-HT occurs at the β-carbon as opposed to the α-carbon. This leads us to believe that a different mechanism must be involved.

Hypothesized mechanism

When β-OH-5-HT is dissolved in the acidic solution, it seems that it immediately undergoes dehydration and rehydration by the general acid catalysis mechanism postulated in Fig. 10. Seemingly, the α-ammonium group next to β-hydroxyl group facilitates the dehydration process in the mild condition by a "proximity effect (Dugas 1996)." Usually, the resultant product of dehydration would be the dehydro product—in this case, that is β-dehydro-5-HT—but the dehydro product was never detected in our experiments. Instead, we successfully trapped the carbocation intermediate with a variety of nucleophiles which provides a stable β-substituted-5-HT that can be readily separated into D and L isomers on HPLC as shown in Fig. 9b. It strongly suggests that the unstable carbocation intermediate of β-OH-5-HT is immediately stabilized by electron disposition from indole nitrogen, yielding the indolenine intermediate (Fig. 10). Evidence suggests that it is very likely that the p*K*a value of α-hydrogen is an important key in this mechanism. In fact, β-OH-L-Trp dissolved in the acidic solution was not reactive with nucleophiles, but rather, it was slowly dehydrated to β-dehydro-Trp. This contrast can be explained by the differences of p*K*a of α-hydrogen between β-OH-5-HT (>35) and β-OH-L-Trp (<35) that are assumed from the studies on the p*K*a values of the α-protons of amino acids (Nibeilliu and Malthouse 2004; Richard and Amyes 2001). Since high racemization rates were found for N-terminal amino acids, 100 times faster than the C-terminal ones (Bada 1984), it is more likely that β-OH-5-HT racemizes, behaving as an N-terminal residue in a peptide, than that it facilitates dehydration to give a dehydro product. In the case of norepinephrine, which is the equivalent counterpart of β-OH-5-HT possessing benzene ring instead of indole (Fig. 1), very slow racemization rates at the β-carbon in aqueous solution at 25 °C have been reported (Hellberg 1955; Smithuis 1969), although it still seems to be faster than serine, a β-OH-α-amino acid. Since β-dehydration of norepinephrine produces a thermodynamically unstable species, para-quinone, the possibility

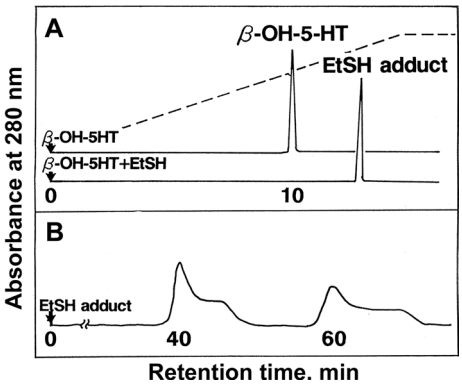

Fig. 9 Isolation **a** and optical resolution **b** of ethanethiol adduct. **a** shows a 15-min linear gradient to 40 % acetonitrile. The reaction mixture and the analytical conditions used were as described in the legend to Fig. 8 and "Experimental procedures," respectively

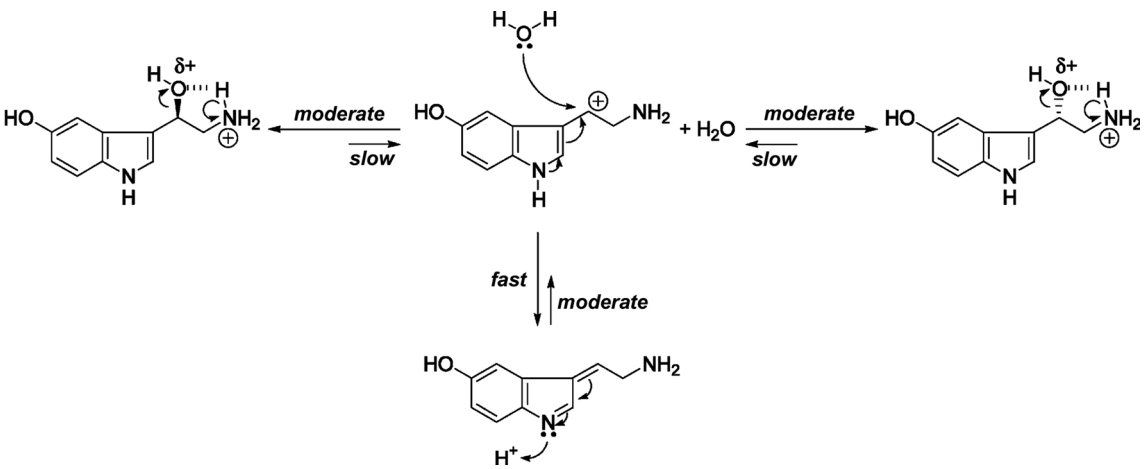

Fig. 10 The mechanism of DL-racemization of β-OH-5-HT at room temperature

of attaining this species at room temperature is almost zero. From the several lines of experimental evidence described here, we hypothesize that β-OH-5-HT in the acidic aqueous solution is under equilibrium of DL-conversion at room temperature (Fig. 10). These unusual properties of β-OH-5-HT may be attributed to the unique nature of the hydroxyl group at β-position adjacent to its indole ring and amino group.

Although the reaction mechanism of TSO has been extensively examined, it should be noted that the mechanism for racemization and nucleophilic substitution of β-OH-5-HT that we postulated here is in a sharp contrast with the oxazoline-mediated reaction mechanism described previously (Zavala et al. 1983). The isolated oxazoline intermediate produced from *N*-acetyl-L-tryptophanamide after the abstraction of two electrons by TSO can be converted to β-substituted products in the presence of various nucleophiles only at a higher pH (more than a neutral) or in low ionic strength (less than 20 mM) which is quite the opposite of the conditions required for the conversion of β-OH-5-HT. The readiness of nucleophilic substitution of β-OH-5-HT could be also explained by the previous observation of Iskric's group that on acidification of an aqueous solution, the spot belonging to β-OH-tryptamine or β-OH-5-HT disappeared and a new spot corresponding to their dimers appeared on TLC (Plavsic et al. 1974). This observation also supports the idea that β-OH-tryptamine or β-OH-5-HT can easily produce its carbocation intermediate in the acidic solution.

With the enzymatic synthesis and characteristics of β-OH-5-HT established, we are now able to apply this β-modification method to other bioactive indoleamines such as melatonin, *N*-acetyl-5-HT, and tryptamine. It will be interesting to see if β-OH-5-HT, β-keto-5-HT, and the related compounds will show some biological or pharmacological effects when they are injected to neuronal cells or mammalian brain. Among selective pharmacological agents for seven classes of 5-HT receptor subtypes, most of the 5-HT_3 receptor antagonists harbor β-keto-indole moiety, which shares a similar chemical structure with cocaine (Hibert et al. 1990). Various indole derivatives are also known to have antibacterial, antiviral, anticancer, or anti-inflammatory properties (Sharma et al. 2010). The synthesis of β-modified-indole compounds by TSOI described here might help understand the mechanism of their pharmacological effects and generate a new class of therapeutic agents in the future.

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Conflict of interest The authors declare no competing financial interest.

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