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Regioselective hydroxylation pathway of tenatoprazole to produce human metabolites by *Bacillus megaterium* CYP102A1

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ABSTRACT

Tenatoprazole, a proton pump inhibitor drug candidate, is developed as an acid inhibitor to treat gastric acid hypersecretion disorders, such as gastric ulcer and reflux esophagitis. Tenatoprazole is known to be metabolized to three major metabolites—tenatoprazole sulfone, 5'-hydroxylated metabolite, and tenatoprazole sulfide—in human livers mainly by CYP2C19 and CYP3A4. In this study, an enzymatic strategy for the production of human metabolites of tenatoprazole was developed using bacterial P450 enzymes. A set of CYP102A1 mutants catalyzed the regioselective hydroxylation reactions of tenatoprazole. The major product of tenatoprazole by CYP102A1 is 5'-OH tenatoprazole, a major human metabolite produced by human CYP2C19 and CYP3A4. As another major metabolite of tenatoprazole, tenatoprazole sulfide is formed via a non-enzymatic conversion without a P450 system. In addition, 5'-OH tenatoprazole sulfide was found as a minor metabolite, which can be formed via a 5'-hydroxylation reaction of tenatoprazole sulfide by CYP102A1 and/or a non-enzymatic reduction of 5'-OH tenatoprazole to a sulfide form. Chemical synthesis of the 5'-OH tenatoprazole is not currently possible. In conclusion, an enzymatic synthesis of 5'-OH tenatoprazole, a major human metabolite of tenatoprazole, was developed by using mutants of CYP102A1 from *Bacillus megaterium* as a biocatalyst and tenatoprazole as a substrate.

1. Introduction

Tenatoprazole (5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl]-1*H*-imidazo[4,5-b]pyridine) is a proton pump inhibitor (PPI) drug candidate that is undergoing clinical testing as a potential treatment for reflux esophagitis and peptic ulcers [1]. Tenatoprazole has an imidazopyridine ring in place of the benzimidazole moiety found in most other PPIs, such as omeprazole (6-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methanesulfinyl]-1*H*-1,3-benzodiazole) and lansoprazole (2-[[3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl]methylsulfinyl]-1*H*-benzimidazole) (Fig. 1). Interestingly, it has a plasma half-life that is about seven times longer than that of other PPIs [2]. Tenatoprazole demonstrated better acid suppression than *S*-omeprazole in healthy subjects [3,4]. Furthermore, it was shown that higher doses of tenatoprazole produced greater acid suppression in a dose-dependent fashion [3]. As tenatoprazole is endowed with a markedly prolonged action duration, the clinical data have shown tenatoprazole to enable a degree of symptom relief and healing of gastric lesions that is superior to other PPIs. Thus, it can be effectively used in treating atypical and esophageal symptoms of gastroesophageal reflux, digestive bleeding, and dyspepsia [1].

Major metabolites of tenatoprazole in humans are 5'-OH tenatoprazole, tenatoprazole sulfone, and tenatoprazole sulfide [5]. CYP2C19 and CYP3A4 are known to oxidize tenatoprazole to 5'-OH tenatoprazole and tenatoprazole sulfone [6]. Omeprazole is also primarily oxidized in human livers by CYP2C19 and CYP3A4 to produce 5'-OH omeprazole and omeprazole sulfone, respectively [7]. Omeprazole sulfide is a major metabolite of omeprazole [8]. In addition, 5'-OH omeprazole sulfide was identified in human urine [9]. Previously known information regarding omeprazole metabolism suggests that the metabolism of tenatoprazole may be similar to the metabolic pathway of omeprazole [9,10].

The importance of drug metabolites in safety testing (MIST) during drug development was guided by the Food and Drug Administration in

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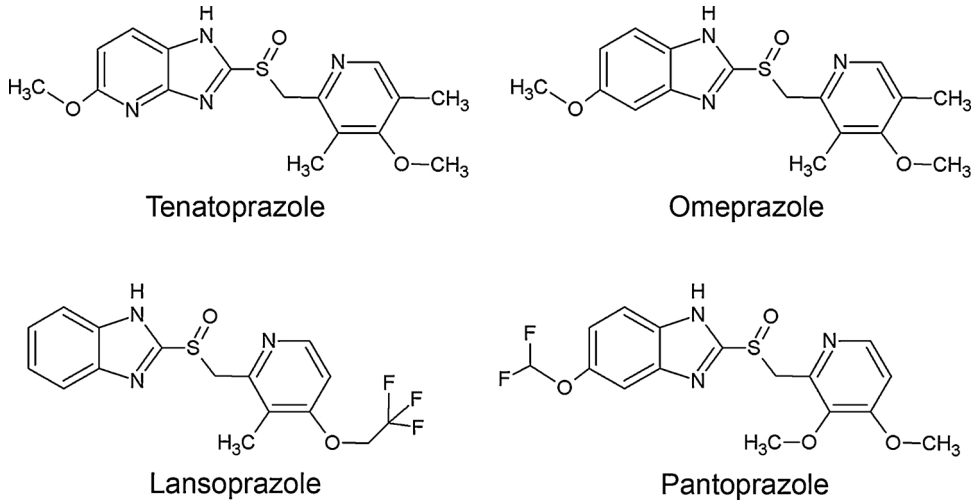

Fig. 1. Chemical structures of common proton pump inhibitors, including tenatoprazole, omeprazole, lansoprazole, and pantoprazole.

2008, and eventually, broader regulatory agreement was reached with the release of guidance from the International Conference on Harmonization (ICH M3(R2); 2009) [11]. During the last decade, major changes have occurred in experimental methods to identify and quantify metabolites, to evaluate the coverage of metabolites, and to determine the timing of critical clinical and nonclinical studies [12]. With the focus that MIST has brought to metabolites, there have been accompanying efforts to produce metabolites of interest on such a scale that they can be readily isolated, quantitated, and profiled. Standard organic synthesis procedures are frequently unable to produce the stereo- and regio-specific metabolites of drugs. Biocatalysts offer several advantages over chemical syntheses because they can often provide better chemo-, regio-, and stereo-selectivity under milder conditions. As cytochrome P450 (P450 or CYP) enzymes have poor catalytic efficiency relative to other enzyme systems, numerous efforts have been undertaken to engineer the catalytic improvements of P450 s. At present, most engineered P450 s could not achieve the high catalytic rates generally observed in bacterial P450 s, coupled with the broad substrate acceptance of human P450 s. However, researchers have suggested that the evolution of P450 s can be applied to produce drug metabolites for drug discovery and development [13].

In particular, a large set of CYP102A1 mutants are known to have the ability to produce human metabolites of a number of marketed drugs and steroids [14–16]. They can produce human metabolites of a large set of clinical drugs, such as pantoprazole, lansoprazole, rabeprazole, simvastatin, lovastatin, fluvastatin, atorvastatin, diclofenac, tamoxifen, propafenone, astemizole, ibuprofen, tolbutamide, and mefenamic acid and other nonsteroidal anti-inflammatory drugs [15,17–21]. They also can oxidize various non-natural substrates, such as natural products and environmental chemicals [16,22,23]. Therefore, CYP102A1 can be considered a prototype P450 for the versatile biocatalysts used in drug discovery and synthesis [15,24]. Recently, the 5'-hydroxylation activity of CYP102A1 mutants on omeprazole [17–19] and omeprazole sulfide [25] with high selectivity and productivity has been reported. These results suggest that engineered CYP102A1 can be used as a biocatalyst to produce the 5'-OH metabolites of omeprazole [17–19] and omeprazole sulfide [25] on a scale used for safety testing.

The aim of this study was to find an efficient enzymatic strategy for the synthesis of human major metabolites of tenatoprazole using bacterial P450 enzymes. Here, we found that CYP102A1 mutants can catalyze the regioselective hydroxylation of tenatoprazole and tenatoprazole sulfide at the C-5' position with a high regioselectivity. To our knowledge, this is the first report to produce major human metabolites of tenatoprazole using bacterial P450 s.

2. Materials and methods

2.1. Materials

Tenatoprazole (racemic mixture) was purchased from Abcam Biochemicals (Cambridge, UK). Nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced $NADP^+$ (NADPH), omeprazole (racemic mixture), glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Tenatoprazole sulfide was obtained from 4Chem Laboratory (Suwon, Gyeonggi-do, Korea). Other chemicals were of the highest grade commercially available.

2.2. CYP102A1 mutants for screening highly active hydroxylases toward tenatoprazole

From our previous work to produce human metabolites of drug molecules and natural products using CYP102A1 mutants, a large set of CYP102A1 mutant collections has been made [15,25–31]. On the other hand, the wild-type (WT) CYP102A1 did not show apparent activities toward the non-natural substrates. We selected 50 mutants from the mutant collection to screen highly active mutants toward tenatoprazole (Supplementary Table S1). These mutants were constructed using site-directed mutagenesis of the active site residues [19,27,28–31] and error-prone PCR [15,25,26] of the heme domain (1–430 amino acid residues) of the chimera M16V2. The triple mutant M10 (R47 L/F87 V/L188Q) was chosen, as this mutant showed a high activity of omeprazole 5'-hydroxylation [19]. Mutants M10 and M16 have mutations in the substrate channel and active site [27]. Each mutant bears amino acid substitution(s) relative to WT CYP102A1, as summarized in Supplementary Table S1. The M16V2 chimeric fusion protein was obtained by the reductase domain swapping of the M16 mutant at key catalytic residues (R47 L/F81I/F87 V/E143 G/L188Q/E267 V) of the heme domain with that of a natural variant (CYP102A1.2) of CYP102A1 from *Bacillus megaterium* [32]. A DNA library of the chimeric mutants was obtained by error-prone PCR of the chimera M16V2 heme domain (1–430 amino acid residues). Chimeric mutants were selected based on their ability to show 4-nitrophenol hydroxylase activity. The selected chimeric mutants showed high activity toward several typical human P450 drug substrates, including chlorzoxazone, simvastatin, lovastatin, fluvastatin, atorvastatin, diclofenac, tamoxifen, propafenone, and astemizole [15].

The selected mutants were expressed in the *Escherichia coli* and partially purified as lysate [15]. The plasmids of WT CYP102A1 (pCWBM3) and its mutants were transformed into *E. coli* strain DH5αF-

IQ (ThermoFisher Scientific, Massachusetts, USA), according to the manufacturer's instructions. The first culture was inoculated from a single colony into 5 ml of Luria-Bertani medium, supplemented with 100 μg/ml ampicillin and grown at 37 °C. This culture was used to inoculate 250 ml of Terrific Broth medium, supplemented with 100 μg/ml ampicillin. The cells were grown at 37 °C, with shaking at 250 rpm, to an OD_{600} ~ 0.8, at which time gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (final concentration, 0.50 mM) with δ-aminolevulinic acid (final concentration, 1.0 mM). Following induction, the cultures were allowed to grow for another 36 h at 30 °C. Cells were harvested using centrifugation (15 min, 5000 *g*, 4 °C). The cell pellet was resuspended in TES buffer [100 mM Tris-HCl (pH 7.6), 500 mM sucrose, 0.5 mM EDTA] and lysed via sonication (Sonicator, Heat Systems – Ultrasonic, Inc.). The lysate was centrifuged at 100,000 *g* (90 min, 4 °C) to collect the soluble cytosolic fraction, and it was used for the activity assay. The soluble fraction (so-called "lysate") was dialyzed against 50 mM potassium phosphate buffer (pH 7.4) and stored at -80 °C. Enzymes were used within 1 month of purification. CYP102A1 concentrations were determined from the CO-difference spectra using $\varepsilon = 91\ mM^{-1}\ cm^{-1}$. For all of the WT and mutated enzymes, a typical culture yielded 200 to 700 nM of P450 protein.

2.3. Hydroxylation of tenatoprazole by CYP102A1

As tenatoprazole has a similar chemical structure to omeprazole, a high-performance liquid chromatography (HPLC) method was based on an analytical method for omeprazole and its metabolites, as previously described [19,25]. Racemic mixture of tenatoprazole was used in this study because each chiral tenatoprazole is not currently available commercially. The reaction mixture included 0.20 μM CYP102A1 and 0.10 mM tenatoprazole in 0.25 ml of potassium phosphate buffer (0.10 M, pH 7.4). The reaction was started by the addition of an NADPH regeneration system (10 mM glucose-6-phosphate, 0.50 mM $NADP^+$, and 1.0 IU yeast glucose-6-phosphate dehydrogenase/ml) at 37 °C. After the indicated time, it was stopped by adding cold ethyl acetate (0.50 ml). Omeprazole was added as an internal standard to this solution at a final concentration of 20 μM [5]. The reaction mixtures were then vortexed for 2 min. After centrifuging at 3000 rpm for 10 min, the upper layer was removed for drying under a stream of nitrogen gas. After the residues were dissolved in 180 μl of mobile phase, 30 μl samples were injected onto a Gemini C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA) with an acetonitrile/5 mM potassium phosphate buffer (pH 7.3) (25:75, v/v) as the mobile phase. Product formation was analyzed by HPLC at A_{302}.

Calibration standards of tenatoprazole and omeprazole were constructed from a blank sample (a reaction mixture without substrate and internal standard) and twelve samples (a reaction mixture with tenatoprazole and omeprazole) covering 1–500 μM. The peak area ratio of tenatoprazole to the internal standard (omeprazole) was linear with respect to the analyte concentration over the range of 1–500 μM. When two volume ethyl acetate was used to extract tenatoprazole and internal standard in buffer solution, the extraction efficiencies of tenatoprazole and internal standard were 70% and 74%, respectively, at the concentrations used in the assay. Quantitation of the metabolites was done by comparing the peak areas of each metabolite to the mean peak areas of the internal standard (20 μM).

The time course analysis of tenatoprazole hydroxylation using the mutant enzymes to measure yield of metabolites and percentage of each metabolite was determined by using 0.20 μM CYP102A1 with 0.10 mM tenatoprazole in 0.25 ml of potassium phosphate buffer (0.10 M, pH 7.4). An NADPH regeneration system was used to start the reaction. The samples were incubated at 37 °C for 2, 5, 10, 30, 60, and 120 min. Product formation was analyzed using HPLC, as described above.

To analyze the steady-state kinetics (k_{cat} and K_M) of each CYP102A1 mutant, the reaction mixture contained 0.20 μM mutant enzyme, an NADPH-generating system, and tenatoprazole (10–500 μM). After it was incubated at 37 °C for 5 min, the formation rate of products was determined by using HPLC as described above. The kinetic parameters (k_{cat} and K_M) were calculated using a Michaelis-Menten nonlinear regression analysis with GraphPad Prism (GraphPad Software, San Diego, CA).

To determine the total turnover number (TTN) for the four CYP102A1mutants, 1 mM of tenatoprazole was used. The reaction was initiated by the addition of the NADPH-generating system and incubated for 30 min, 1 h, and 2 h at 37 °C. The formation rate of 5'-OH Tenatoprazole, 5'-OH tenatoprazole sulfide, and an unidentified monohydroxylated product was determined by using HPLC as described above.

2.4. Liquid chromatography-mass spectrometry analysis

To identify the major metabolites of tenatoprazole produced by CYP102A1, a liquid chromatography-mass spectrometry (LC–MS) analysis was performed, and the LC profile and fragmentation patterns of the authentic compounds (i.e., tenatoprazole and tenatoprazole sulfide) were compared on a Shimadzu LCMS-2010 EV system (Shimadzu, Kyoto, Japan) with LC–MS solution software. The hydroxylation reaction of tenatoprazole by CYP102A1 was performed as described above. The separation was performed on a Shim-pack VP-ODS column (2.0 × 250 mm; Shimadzu) with a mobile phase of acetonitrile/water (50:50, v/v) at a flow rate of 0.10 ml/min. The mass spectra were recorded using electrospray ionization in the positive mode to identify the metabolites. The nebulization gas flow was set to 1.5 l/min. The interface, curve desolvation line, and heat block temperatures were 250 °C, 230 °C, and 200 °C, respectively.

Furthermore, to get more detailed information about the metabolites, the incubation mixtures were analyzed using a 6530 Quadrupole Time-of-Flight (QTOF) mass spectrometer (Agilent, Santa Clara, CA, USA) coupled with a 1290 Infinity ultra-performance liquid chromatography (UPLC) system (Agilent). Separation was performed using a HypersilGold C18 column (100 × 2.1 mm, 1.9 μm; Thermo, Milford, MA, USA); the gradient mobile phase was 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), delivered at a flow rate of 0.4 ml/min. The initial composition of mobile phase B was 10%; this increased to 30% over 9 min, decreased to 10% for 3 min over 0.2 min, and finally re-equilibrated to the initial conditions over 0.8 min. Thus, the total run time was 10 min. The temperatures of the column and autosampler were kept at 35 °C and 4 °C, respectively, and the injection volumes were 1 μl for all samples tested here. The electrospray ionization procedure was performed in the positive ion mode. The sheath gas flow rate was 8 l/min. The drying gas flow rate was 12 l/min at 350 °C, and the nebulizer temperature was 350 °C. The capillary voltage was 4500 V in the positive mode and that of the fragmentor was 175 V. All data were acquired over a scan range from 50 *m/z* to 1000 *m/z* in the centroid mode, and a reference compound ($C_{18}H_{18}O_6N_3P_3F_{24}$; $[M+H]^+ = 922.0098$) was used to correct all masses. Tandem mass spectrometry (MS/MS) data were acquired in the profile mode (via autoanalysis) for structural characterization; the collision energy was 30 eV.

2.5. Identification of major metabolites of tenatoprazole by NMR spectroscopy

To identify two hydroxylated metabolites, they were separated using HPLC and collected. The reaction mixture contained 0.4 μM of CYP102A1 M371 and 1 mM of tenatoprazole in 30 ml of potassium phosphate buffer (0.10 M, pH 7.4). An NADPH regeneration system was used to start the reaction. The sample was incubated at 37 °C for 2 h. Product formation was separated by using analytical HPLC as described above. Isolated yields of 5'-OH tenatoprazole and 5'-OH tenatoprazole sulfide were 8.2% and 1.3%, respectively. NMR experiments were performed at 25 °C on a Varian VNMRS 600 MHz NMR spectrometer equipped with a carbon-enhanced cryogenic probe. DMSO-d_6 was used

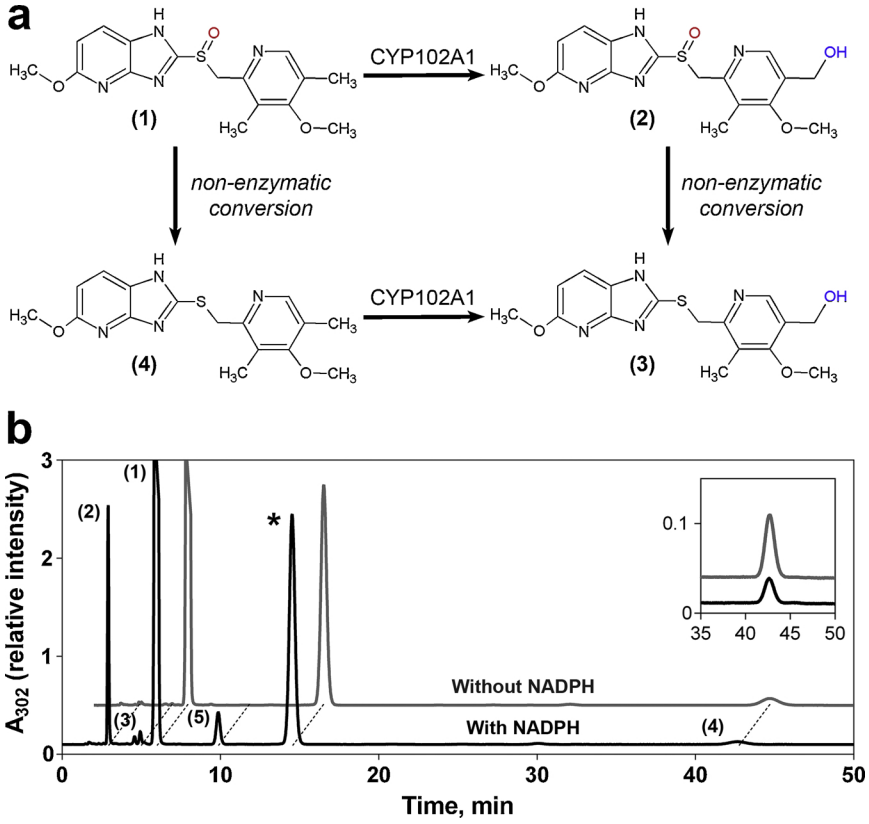

Fig. 2. (a) The metabolic pathway of tenatoprazole via the CYP102A1 mutant to produce human metabolites. It starts from tenatoprazole (**1**). There are two metabolites of tenatoprazole: 5'-OH tenatoprazole (**2**), produced by the CYP102A1 mutant, and tenatoprazole sulfide (**4**), which is formed non-enzymatically. The last product in the pathway is 5'-OH tenatoprazole sulfide (**3**), which comes from tenatoprazole sulfide via CYP102A1 and/or 5'-OH tenatoprazole via non-enzymatic conversion. The structures were created using ACD/Chemsketch (https://www.acdlabs.com). **(b)** HPLC chromatograms of the tenatoprazole metabolites produced by mutant M371 with (and without) NADPH. The reaction mixture containing P450 (0.20 μM) and tenatoprazole (0.10 mM) was incubated at 37 °C for 10 min. (1) Tenatoprazole: t_R = 6.82 min; (2) 5'-OH tenatoprazole: t_R = 3.05 min; (3) 5'-OH tenatoprazole sulfide: t_R = 4.90 min; (4) Tenatoprazole sulfide: t_R = 43.9 min; (*) internal standard omeprazole (20 μM): t_R = 14.5 min. The inset of panel (**b**) shows the peak of tenatoprazole sulfide using magnification.

as a solvent, and chemical shifts for proton NMR spectra were measured in parts per million (ppm) relative to tetramethylsilane. All of the NMR experiments were carried out with standard pulse sequences in VNMRJ (v. 3.2) library and processed with the same software.

2.6. NADPH oxidation

The reaction was performed in a spectrophotometric cuvette maintained at 37 °C. The reaction mixture contained 0.10 μM of CYP102A1 and 0.50 mM of tenatoprazole in 1 ml of potassium phosphate buffer (0.10 M, pH 7.4). The reactions were initiated by adding 10 μl of 10 mM NADPH (final concentration, 100 μM), and the decrease in A_{340} was monitored for 1 min. The rates of NADPH oxidation were calculated using ε_{340} = 6.22 M^{-1} cm^{-1} for NADPH.

2.7. Spectral binding titration

All spectrophotometric measurements were recorded with a Shimadzu 1601PC Spectrophotometer at 23 °C. The binding affinity of ligands to the CYP102A1 enzymes was determined by titrating 1.5 μM of the enzyme with the ligand in a total volume of 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4). The final CH_3CN concentration was < 2% (v/v). The reference cuvette, containing an equal concentration of the enzyme in the buffer, was titrated with an equal volume of the vehicle solvent. UV–vis spectra (350–500 nm) were recorded after each addition, and the absorbance differences were plotted against the added ligand concentrations. Spectral dissociation constants (K_D indicates a spectrally estimated dissociation constant) were estimated as previously described [27,33,34] using GraphPad Prism software (GraphPad software, San Diego, CA). Because of the high affinities of tenatoprazole (K_D within 5-fold of the P450 concentration), a nonlinear regression analysis using a quadratic equation was applied to determine the K_D for tenatoprazole. With tenatoprazole sulfide, the best fit was obtained using the Hill equation, as steady-state binding titration data were sigmoidal [33,34].

3. Results and discussion

3.1. Hydroxylation of tenatoprazole by CYP102A1

We used the WT enzyme and 50 mutants with substituted amino acid residue(s) in the substrate channel and active site to determine whether CYP102A1 can hydroxylate tenatoprazole. Tenatoprazole hydroxylation activity was examined at a fixed substrate concentration of 100 μM for 30-min incubation. A major hydroxylation product is 5'-OH tenatoprazole, and a minor product is 5'-OH tenatoprazole sulfide (Fig. 2). Tenatoprazole sulfide is another major product that is formed non-enzymatically, even in the absence of P450 and NADPH (Fig. 2b). Among all enzymes tested here, only 26 mutants (changed amino acid sequences are shown at Supplementary Table S1) showed apparent activities of hydroxylation (Fig. 3). In the case of the WT, any measurable activity was not observed (lower than 0.01 min^{-1}). Most of the active mutants showed two major hydroxylated products and tenatoprazole sulfide. On the other hand, mutant M10, chimera M16V2 (which the reductase domain of mutant M16 was replaced with the reductase domain of a natural variant V2 of CYP102A1), and two chimeric mutants of M16V2 (M413 and M416) [15] only showed one major hydroxylation product (5'-OH tenatoprazole). Although the activity of most mutants is less than 1 min^{-1}, two chimeric mutants (M371 and M387) showed the highest turnover numbers of 5'-OH tenatoprazole (3.43 and 1.72 min^{-1}, respectively) and 5'-OH tenatoprazole sulfide (0.33 and 0.29, respectively) among all the tested mutants. The identities of the metabolites and substrate were confirmed using LC–MS (Supplementary Fig. S1-S2) and NMR analyses (Supplementary Fig. S3-S7).

Spectral assignments of tenatoprazole and its metabolites, 5'-OH tenatoprazole (M1) and 5'-OH tenatoprazole sulfide (M2), were carried out with ^1H NMR, COSY, and 1-dimensional selective NOE experiments (Supplementary Fig. S3-S7). Chemical shift assignments for tenatoprazole (M0) in DMSO-d_6 was reported [35]. However, we found some discrepancies in the assignments for the H-6 and H-7 protons of

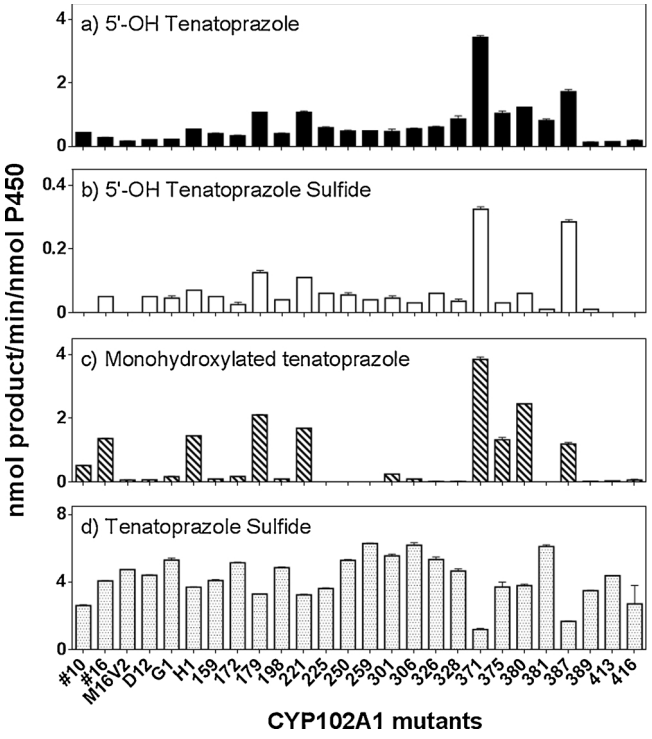

Fig. 3. Formation rate of the tenatoprazole metabolites catalyzed by CYP102A1 mutants. The formation rates of the products were determined using HPLC, as described in Materials and Methods. The values are presented as the means ± SD of triplicate measurements. (**a**) 5'-OH tenatoprazole; (**b**) 5'-OH tenatoprazole sulfide; (**c**) uncharacterized monohydroxylated metabolite; (**d**) tenatoprazole sulfide.

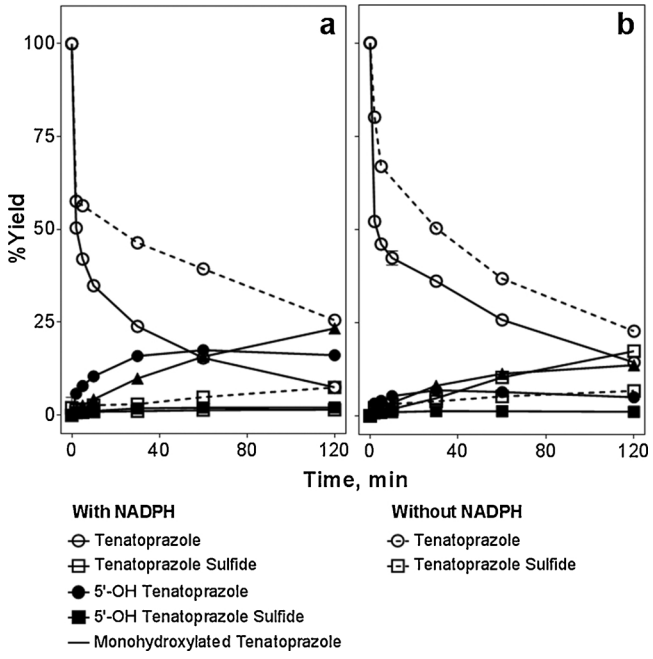

Fig. 4. Time course profile of tenatoprazole and its metabolites using mutants M371 (**a**) and M387 (**b**). Yields of each compound at the indicated reaction time were determined by using 0.20 μM CYP102A1 with 0.10 mM tenatoprazole in 0.25 ml of potassium phosphate buffer (0.10 M, pH 7.4). The samples were incubated at 37 °C for 2, 5, 10, 30, 60, and 120 min. Control experiments (dotted lines) in the absence of NADPH were done at the same experimental conditions. The values are presented as the means ± SD of triplicate measurements.

tenatoprazole after running a series of 1-dimensional selective NOE experiments (Supplementary Fig. S6). Correct ^{1}H NMR chemical shifts of TEN (M0) and its metabolites (M1 and M2) are listed in Supplementary Table S2. Important NOE results for the identification of tenatoprazole and its metabolites are shown in Supplementary Fig. S6.

Another metabolite (at t_R = ~10 min) was observed at the HPLC trace of all tested samples. As this metabolite is shown only in the presence of NADPH, it is also a metabolite of the CYP102A1-catalyzed reaction. UPLC-ESI-QTOFMS analysis has been done to obtain more information about metabolites, including another major unidentified metabolite (so-called "metabolite **5**," seen in Supplementary Fig. S2). The MS spectra analyses show that the metabolites of tenatoprazole include 5'-OH tenatoprazole, 5'-OH tenatoprazole sulfide, tenatoprazole sulfide, two monohydroxylated products, and tenatoprazole sulfone. The tenatoprazole sulfone was also detected, although the sulfone was not detected in the HPLC trace. We suggest that the unidentified metabolite (metabolite **5)** is a monohydroxylated product at the imidazopyridine ring of tenatoprazole (Supplementary Fig. S2 b, c, and h). Although some mutants show high turnover numbers (up to 3.9 min^{-1}), which were calculated based on the area of internal standard, for the formation of this monohydroxylated product, the product showed a very low detection level in the mass spectrometer. The extent of this metabolite's formation was variable among the mutant CYP102A1 lines, similar to the other metabolites described herein (Fig. 3c). However, the chemical structure of this metabolite was not identified in this study.

3.2. Time course analysis of hydroxylated metabolites of tenatoprazole by CYP102A1 mutants

Mutants M371 and M387, which exhibited high product formation rates, were selected for time course study and additional kinetic analyses. Time course measurement with tenatoprazole was performed at 100 μM. For mutant #371 (Fig. 4a), the substrate tenatoprazole gradually decreased during the reaction time, and 7.5% of tenatoprazole remained after a 2 h reaction. At the same time, the major product, 5'-OH tenatoprazole, reached the maximal yield (18%) after a 60 min reaction. Tenatoprazole sulfide and 5'-OH tenatoprazole sulfide were raised slightly (1.4% and 2.0% yield, respectively, after a 60 min incubation). However, the time course analysis of M387 (Fig. 4b) showed a distinct result when compared to that of M371. In the case of M387, tenatoprazole also gradually decreased to 14% yield after a 2 h incubation, while the rate of tenatoprazole sulfide formation was much faster than that of variant M371. Consequently, the highest percentage yield of 5'-OH tenatoprazole by M387 for a 30 min reaction was 6.9%, which is only one-third of the tenatoprazole sulfide formation by M371. Although tenatoprazole sulfide gradually increased with incubation time, reaching to 17% at 2 h of incubation, 5'-OH tenatoprazole sulfide only increased to 1.1%. The ratio of products as percentage after the 2 h reaction at neutral pH was shown at Supplementary Table S3.

We examined whether tenatoprazole sulfide formation, a reduction reaction of sulfoxide to sulfide, is catalyzed by a P450 system (P450 heme or reductase domain) or transformed non-enzymatically. When the concentrations of tenatoprazole and its sulfide were measured with the reaction mixtures of both mutants without NADPH, the tenatoprazole concentration rapidly decreased to 35% and 42% in 10 min for the mutants M371 and M387, respectively (Fig. 4). After that, its concentrations gradually decreased with incubation time. After 2 h of incubation, only 26% and 23% of tenatoprazole remained for the mutants M371 and M387, respectively. However, at the same time, the tenatoprazole sulfide concentration increased to a small extent during the incubation time. After 2 h of incubation, only 7.5% and 6.7% of tenatoprazole sulfide were formed for the mutants M371 and M387, respectively (Fig. 4 and Supplementary Table S3).

To investigate whether the non-enzymatic reduction of tenatoprazole to the sulfide requires the bacterial lysate, we only measured the

Table 1
The steady-state kinetics of hydroxylated product formation from tenatoprazole using CYP102A1 mutants.

CYP102A1	5'-OH tenatoprazole[a]			5'-OH tenatoprazole sulfide[b]			Monohydroxylated metabolite[c]		
	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M
M371	16 ± 1	210 ± 20	0.076	1.3 ± 0.1	230 ± 10	0.0057	17 ± 1	550 ± 20	0.031
M387	5.3 ± 0.6	98 ± 28	0.054	0.84 ± 0.10	180 ± 50	0.0047	3.2 ± 0.3	70 ± 20	0.046

The steady-state kinetics (k_{cat} and K_M) of [a]5'-OH tenatoprazole and [b]5'-OH tenatoprazole sulfide formation using CYP102A1 mutants were determined in a final volume of 0.25 ml of potassium phosphate buffer (0.10 M, pH 7.4). The reaction mixture contained 0.20 μM enzyme, an NADPH regeneration system, and tenatoprazole (10–500 μM). The samples were incubated for 5 min at 37 °C. Data represent mean values ± SD from triplicate measurements.

formation of tenatoprazole sulfide from tenatoprazole (100 μM) in a buffer solution (100 mM potassium phosphate, pH 7.4) (Supplementary Fig. S8). While the tenatoprazole gradually decreased during the incubation time, the tenatoprazole sulfide concentration increased to a small extent. After 10 and 30 min of incubation, 8% and 20% of tenatoprazole were broken down, respectively, although only 1% and 2% of tenatoprazole sulfide were formed. After 2 h of incubation, 45% of tenatoprazole remained and 6% of tenatoprazole sulfide was formed. After 8 h and 12 h, 96% and 99% of tenatoprazole were decomposed, respectively. After more than 8 h of incubation, most of tenatoprazole was decomposed, and only 20% of tenatoprazole sulfide remained. This result indicates that tenatoprazole is intrinsically unstable, even in a neutral buffer solution.

After 2 h of incubation of tenatoprazole with P450 containing lysate and NADPH, about 50% of tenatoprazole was transformed to other forms of metabolites; 5'-OH tenatoprazole, 5'-OH tenatoprazole sulfide, a monohydroxylated tenatoprazole, and tenatoprazole sulfide (Supplementary Table S3). The other 50% of tenatoprazole seemed to convert to other metabolites. However, incubation of tenatoprazole with P450 containing lysate without NADPH accelerates the decomposition of tenatoprazole. After 2 h of incubation, ~30-34% tenatoprazole remained, and only ~7% tenatoprazole sulfide was formed.

The formation of the sulfide occurred with or without NADPH; however, the rate of sulfide formation for mutant M387 was greater with NADPH (Fig. 4b). Conversely, for mutant M371, the rate of sulfide formation was much lower with NADPH than without NADPH (Fig. 4a). The sulfide formation in mutant M371 seems to be actively suppressed.

We found that the formation of tenatoprazole sulfide is mainly non-enzymatic and does not need NADPH (Fig. 4). Therefore, some portion of tenatoprazole will be converted to a sulfide form at a rapid pace when tenatoprazole is administered. This result suggests a possibility that tenatoprazole sulfide can be used as a substrate for P450-catalyzed reaction. There is another possibility: 5'-OH tenatoprazole sulfide is non-enzymatically formed from 5'-OH tenatoprazole. When considered together, the non-enzymatic decomposition of tenatoprazole to tenatoprazole sulfide at a neutral pH should be considered for the study metabolism of tenatoprazole during the drug development process.

On the other hand, omeprazole seems to be relatively stable at a neutral pH because omeprazole was reported to be stable at pH 7.5 for 24 h [36]. However, omeprazole is known to be unstable under acidic conditions, in which it easily degraded [8,37,38]. Omeprazole in aqueous solutions between pH 2.2 and pH 4.7 degraded completely in less than 4 h [38]. As a control experiment, the stability of omeprazole at pH 7.4 and pH 5.0 was compared (Supplementary Fig. S9). As omeprazole is stable at pH 7.4, it remains 99% and 95% after incubation for 2 h and 5 h, respectively. On the other hand, 93% and 99% of omeprazole degraded after 2 h and 5 h of incubation at pH 5.0, respectively. During the degradation of omeprazole at pH 5.0, 32% and 38% of omeprazole sulfide was formed for 2 h and 5 h, respectively. Several other degradation product peaks in addition to the peak of omeprazole sulfide were also observed during incubation under this acidic condition. This result is consistent with previous reports regarding the stability of omeprazole under acidic conditions.

3.3. Kinetic parameters of 5'-OH tenatoprazole and 5'-OH tenatoprazole sulfide formation by CYP102A1 mutants

Table 1 and Supplementary Fig. S10 show the steady-state kinetics of hydroxylated product formation from tenatoprazole by mutants M371 and M387. In the case of 5'-OH tenatoprazole formation, mutant M371 had higher k_{cat} value than that of mutant M387 (~ 3-fold). By contrast, the value of K_M by mutant M387 (98 μM) was 2.1-fold lower than that of mutant M371 (210 μM). The k_{cat} of mutant M371 for 5'-OH tenatoprazole sulfide formation was 1.3 min^{-1}, which is 1.5-fold of that by mutant M387. Data show that 5'-OH tenatoprazole formation exhibited significantly higher k_{cat} value of each enzyme than that of 5'-OH tenatoprazole sulfide formation by 6 ~ 12-fold. In addition, K_M values of M371 for 5'-OH tenatoprazole and 5'-OH tenatoprazole sulfide formation were higher than those of M387. Catalytic efficiencies of 5'-OH tenatoprazole formation by two mutants were much higher than those of 5'-OH tenatoprazole sulfide formation by 11 ~ 13-fold (Table 1). However, a direct comparison of the kinetic parameters to tenatoprazole and its sulfide form is not possible because the exact concentration of tenatoprazole sulfide cannot be determined in this experiment. This result indicates that 5'-OH tenatoprazole formation is the major reaction when tenatoprazole is used as a substrate.

When the kinetic parameters of an unidentified monohydroxylated product formation by CYP102A1 mutants were determined, the k_{cat} values of two mutants were comparable to those of 5'-OH tenatoprazole formation. The K_M value of M371 for unidentified monohydroxylated product formation was 2.6-fold higher than that of 5'-OH tenatoprazole formation. However, direct comparison of the kinetic parameters between 5'-OH tenatoprazole formation and unidentified monohydroxylated product formation is not possible because the exact concentration of the unidentified monohydroxylated product cannot be determined in this experiment. The results indicate that the unidentified monohydroxylated product is also a major metabolite, which is produced from tenatoprazole catalyzed by P450.

When the TTNs (nmol product/nmol P450) of M371 for the formation of 5'-OH tenatoprazole and the unidentified monohydroxylated product were determined with 1 mM tenatoprazole during the incubation of 30 min, 1 h, and 2 h, the overall range was 200–380 (Supplementary Fig. S11). While the formation of 5'-OH tenatoprazole decreased after 30 min of incubation, that of the unidentified metabolite formation increased with incubation time. This indicates that 5'-OH tenatoprazole can be converted to other metabolites. However, the TTNs of 5'-OH tenatoprazole sulfide formation was only 20–24. The TTNs of M387 were much lower than those of M371 for the formation of three metabolites. The TTNs for the 5'-OH tenatoprazole formation (120 ~ 382) by CYP102A1 mutants are much lower than those for the 5'-OH omeprazole (950 ~ 1200) [19].

When the rates of product formation and NADPH oxidation were compared, M387 (1.8%) showed lower coupling efficiency than M371 (3.3%) toward 0.5 mM tenatoprazole (Table 2). The coupling efficiency of the tenatoprazole oxidation is much lower than other non-natural substrates, such as omeprazole sulfide (16–82%) [25], testosterone (14–84%) [39], and norethisterone (14–67%) [39].

Table 2
Catalytic properties of tenatoprazole oxidation using CYP102A1 mutants.

CYP102A1	Product[a]	NADPH oxidation (min^{-1})[b]	Product formation (min^{-1})[c]	Coupling efficiency (%)[d]	TTN[e] (reaction time)
#371		389 ± 21		3.3	
	5'-OH tenatoprazole		8.02 ± 0.24	2.06	383 ± 4 (0.5 h)
	5'-OH tenatoprazole sulfide		0.64 ± 0.03	0.16	24 ± 1 (0.5 h)
	Monohydroxylated metabolite		4.39 ± 0.05	1.13	313 ± 12 (2 h)
#387		445 ± 24		1.8	
	5'-OH tenatoprazole		4.37 ± 0.35	0.98	120 ± 3 (0.5 h)
	5'-OH tenatoprazole sulfide		0.54 ± 0.02	0.12	8.4 ± 0.2 (0.5 h)
	Monohydroxylated metabolite		2.97 ± 0.10	0.67	77 ± 8 (2 h)

[a] As shown, 5'-OH tenatoprazole, 5'-OH tenatoprazole sulfide, and monohydroxylated metabolite are considered to be the major metabolites when using CYP102A1 mutants.
[b] The NADPH consumption rates were measured over 1 min at 340 nm as nmol NADPH/min/nmol P450.
[c] The product formation rates were determined as nmol-indicated product/min/nmol P450 using tenatoprazole (0.2 mM) in the Materials and Methods section.
[d] The corresponding coupling efficiency is defined as the rate of product formation divided by the rate of NADPH consumption.
[e] The TTNs for the hydroxylation of omeprazole sulfide were determined as described in the Materials and Methods section. Data represent the mean values ± SD from triplicate measurements.

3.4. Binding titration of tenatoprazole and tenatoprazole sulfide toward CYP102A1 mutants

The mutants M371 and M387 contain a mixture of low- and high-spin heme iron (Supplementary Fig. S12). The addition of tenatoprazole and tenatoprazole sulfide to a solution with the mutants M371 and M387 produced difference spectra of reverse Type I and Type I, respectively (Fig. 5 and Supplementary Fig. S12). The binding affinity of the mutants toward the tenatoprazole and tenatoprazole sulfide substrates was determined from the titration curves. The mutants have a higher affinity for tenatoprazole (K_d = 0.89 ~ 1.0 μM) than tenatoprazole sulfide (K_d = 105 ~ 124 μM). The titration plots obtained for tenatoprazole sulfide binding to the mutants displayed apparent sigmoidicity. A fit to the Hill equation resulted in coefficients (n) of 1.7–1.9, suggesting a high degree of binding cooperativity. When the values of K_d and K_M of tenatoprazole to the mutants were compared, the values of K_d were much lower than those of K_M. Even though the tenatoprazole can tightly bind to the active site at a very low concentration of tenatoprazole, the oxidation reactions to make products do not seem to proceed easily. On the other hand, the addition of tenatoprazole or tenatoprazole sulfide to a WT solution did not cause any apparent spectral changes.

When considered together, the formation of 5'-OH tenatoprazole sulfide when tenatoprazole was used as a substrate suggests the possibility of two metabolic pathways of tenatoprazole. After 5'-OH tenatoprazole is produced by CYP102A1, it can be non-enzymatically transformed to 5'-OH tenatoprazole sulfide. On the other hand, tenatoprazole sulfide, a major metabolite of tenatoprazole, can be used as a

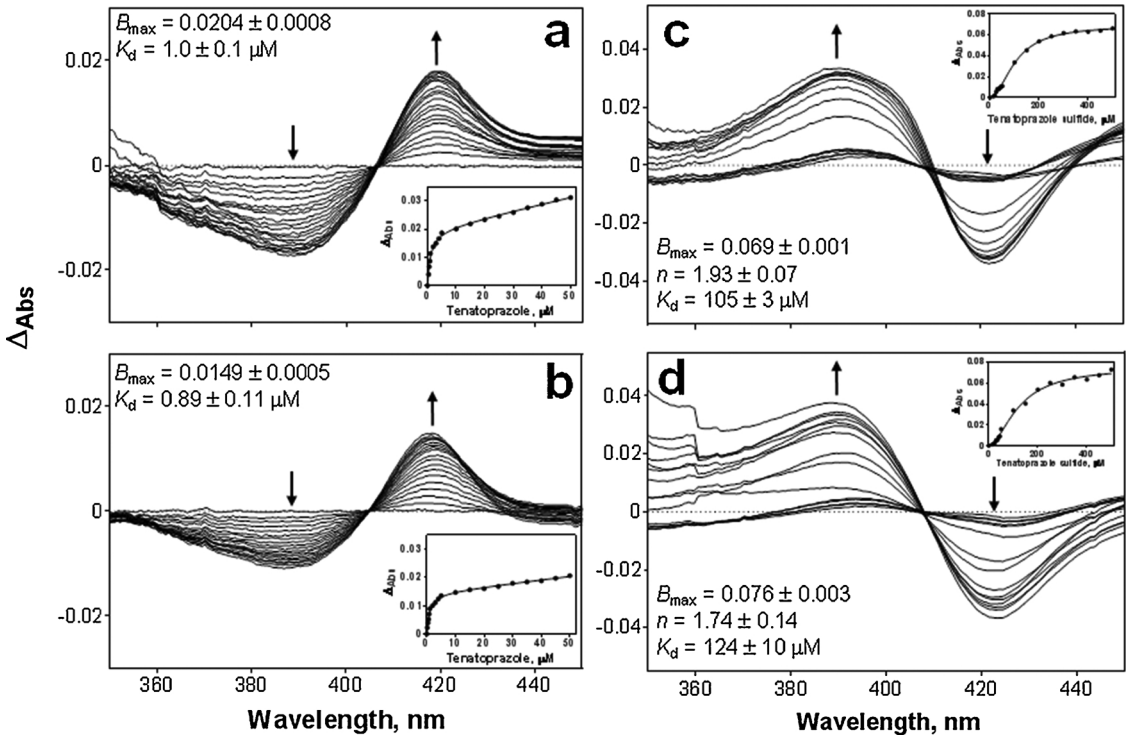

Fig. 5. Spectral binding titration of CYP102A1 with tenatoprazole and tenatoprazole sulfide. CYP102A1 (1.5 μM) was titrated as a function of ligand concentration in 100 mM potassium phosphate (pH 7.4), as described in Materials and Methods: mutants #371 (**a** and **c**); #387 (**b** and **d**) with tenatoprazole (**a** and **b**) and tenatoprazole (**c** and **d**). The inset of each panel shows a plot of induced Soret absorbance change ($\Delta A_{390-420}$) *versus* the relevant concentration of ligands for mutants M371 and M387. Dissociation constants (K_d) for the ligand binding and maximal changes of absorption (B_{max}) were shown. Spectral changes of the heme iron from the unbound to bound substrate were marked with arrows.

substrate for a hydroxylation pathway after non-enzymatic transformation to tenatoprazole sulfide from tenatoprazole (Fig. 2a).

When the hydroxylation activities of mutant M10 toward C-5' of tenatoprazole (this work) and omeprazole [19] were compared, this mutant showed much higher activity of omeprazole (52 min^{-1}) than that of tenatoprazole (0.44 min^{-1}). Mutant M10 is a well-known, highly active mutant toward several human substrates and has mutations in the substrate channel and active site (R47 L/F87 V/L188Q) [40]. However, the highest active mutant, M371 (k_{cat}, 16 min^{-1}), toward tenatoprazole has additional mutations in the substrate channel and active site (D23 G, F81I, F107 L, E143 G, and E267 V) and outside the active site and substrate channel (D136 G). Although M371 shows the highest activities for producing three major metabolites among tested mutants, M389, M413, and M416 show very low activities for producing the metabolites. M16, H1, M179, and M221 show a regioselective preference for the unidentified monohydroxylated metabolite, producing 5'-OH tenatoprazole (Fig. 3). Among the additional mutation sites for M16, M371, and M387 mutants, D23 is located at the entrance of the substrate access channel. F107 is located at the active site under the heme. However, F11, Q110, R190, and D136 are located outside the substrate access channel and active site. The mutations at the M371 and M387 appear to have different effects on the conformation of the active site upon binding to the substrates (Supplementary Fig. S13). It is well-known that the catalytic rate in enzymes (e.g., dihydrofolate reductase) is significantly affected by mutations far from the site of chemical activity in the enzyme [41]. Note that mutations at a small number of residues can significantly impact the structural organization of CYP102A1 and can induce major alterations in substrate selectivity [18]. When considered together, it can be suggested that combinatorial approaches, including random mutagenesis, rational design, and target-directed mutagenesis based on the crystal structure of CYP102A1 with substrates, would be necessary to make desired enzymes with high activity and regioselectivity.

When considered together, CYP102A1 mutants show different preferences regarding the substrates for the additional hydroxylation at the imidazopyridine ring in addition to the hydroxylation at the C-5' position of the pyridine ring, although tenatoprazole has a similar chemical structure to that of omeprazole. As tenatoprazole has a very similar chemical structure to omeprazole, differences in electronic distribution between the imidazopyridine ring of tenatoprazole and the benzimidazole moiety of omeprazole might cause the hydroxylation of tenatoprazole at the imidazopyridine ring. Our results in this study suggest the possibility of the different metabolic pathways between tenatoprazole and omeprazole.

Because the chemical synthesis of 5'-OH tenatoprazole is not currently possible, it should be obtained with human P450 enzymes or liver microsomes. In this study, engineered bacterial CYP102A1 enzymes showed the enhanced catalytic activity of tenatoprazole 5'-hydroxylation, and they increased the product yield of 5'-OH tenatoprazole. It is now possible to study the effects of 5'-OH tenatoprazole and 5'-OH tenatoprazole sulfide on gastric acid hypersecretion disorders in *in vitro*, *in vivo*, and animal model systems because scalable production of the metabolites is possible by using bacterial P450. Specifically, the effects of the 5'-OH metabolites from racemic, *S*-, and *R*-tenatoprazole on several diseases caused by gastric acid hypersecretion are of particular interest. The effects of these metabolites on gastric acid hypersecretion disorders may suggest that the metabolites could be used as "drug leads" to avoid interindividual variations in drug-metabolizing enzymes and drug–drug interactions. Furthermore, the metabolites can be subjected to further structural modifications to obtain improved clinical candidates during the lead optimization phase of the drug discovery process.

Although mutant M371 show a high k_{cat} value (16 min^{-1}) and a high yield (41%) among tested mutants, this P450 enzyme shows relatively low levels of activity when compared to a set of industrial biocatalysts [42]. Directed evolution and other protein engineering methods can be used to improve upon this low level of activity and regio-selectivity to produce the metabolites on a scale to meet the MIST issue [13].

In recent years, P450-catalyzed reactions have been of special interest for selective C–H oxygenation to produce fine chemicals and pharmaceuticals [42–44]. For industrial fine chemical production, biotransformations have been considered to require a minimum space-time yield (0.1 g l^{-1} h^{-1}) and a minimum final product concentration (1 g l^{-1}). For industrial pharmaceutical production, an efficient time-to-market strategy is more crucial than the production costs, with estimated minimum process requirements for volumetric productivity (0.001 g l^{-1} h^{-1}) and product concentration (0.1 g l^{-1}) [42,45]. Efficient and time-saving production seems to be important in fulfilling the minimal requirements for industrial pharmaceutical production. At present, several approaches using engineered CYP102A1 as biocatalysts seem to achieve the industrially relevant product concentrations. The Urlacher's group reported that the productivity of (+)-nootkatone was up to 360 mg l^{-1} and had a space-time yield of 18 mg l^{-1} h^{-1} for synthesis of the (+)-nootkatone from (+)-valencene using a CYP102A1 variant and an alcohol dehydrogenase (ADH) in one pot [46]. A combination of engineered CYP102A1, ADH, and engineered cyclohexanone monooxygenase could produce final concentrations of ~3 g l^{-1} enantholactone (2-oxocanone) from cycloheptane [47]. Another report described the L-Tyrosine derivatives up to 5.2 g l^{-1} were being obtained using an engineering CYP102A1 variant and tyrosine phenol lyase using substituted benzenes, pyruvate, and NH_3 as starting materials [48].

4. Conclusion

In this study, we found that the major hydroxylation product of tenatoprazole by CYP102A1 is 5'-OH tenatoprazole, a major human metabolite produced by human CYP2C19 and CYP3A4. Tenatoprazole sulfide, another major metabolite, is formed via a non-enzymatic conversion without P450 system. Because tenatoprazole sulfide can be used as a substrate for a regioselective hydroxylation reaction at C-5' by CYP102A1, 5'-OH tenatoprazole sulfide can be produced as a minor metabolite via CYP102A1 reaction. On the other hand, 5'-OH tenatoprazole sulfide can also be formed non-enzymatically from 5'-OH tenatoprazole. Here, we report the enzymatic one-step production of 5'-OH tenatoprazole from tenatoprazole, which was developed using the CYP102A1 mutant as a biocatalyst. The tenatoprazole metabolites can be used for other studies during the drug-development process, such as drug efficacy, safety, metabolism, and synthesis of derivatives. An enzymatic regioselective C—H hydroxylation strategy should be useful for the production of hydroxylated metabolites of other PPI drugs with similar chemical structures.

Contributors

CHY conceived the project, and procured grants, supervised the project, and reviewed the data. TKL, GSC, HHJ, THHN, TTMD, YJL, KDP, YS, and DHK, along with CHY, designed the experiments, and executed all the experiments and analyzed the data. TKL and CHY wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest. All authors have approved the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2019.09.014.

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