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Regioselective preparation of 5-hydroxypropranolol and 4′-hydroxydiclofenac with a fungal peroxygenase

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ABSTRACT

An extracellular peroxygenase of *Agrocybe aegerita* catalyzed the H_2O_2-dependent hydroxylation of the multi-function beta-adrenergic blocker propranolol (1-naphthalen-1-yloxy-3-(propan-2-ylamino)-propan-2-ol) and the non-steroidal anti-inflammatory drug diclofenac (2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid) to give the human drug metabolites 5-hdroxypropranolol (5-OHP) and 4′-hydroxydiclofenac (4′-OHD). The reactions proceeded regioselectively with high isomeric purity and gave the desired 5-OHPand 4′-OHD in yields up to 20% and 65% respectively. ^{18}O-labeling experiments showed that the phenolic hydroxyl groups in 5-OHP and 4′-OHD originated from H_2O_2 which establishes that the reaction is mechanistically a peroxygenation. Our results raise the possibility that fungal peroxygenases may be useful for versatile, cost-effective, and scalable syntheses of drug metabolites.

Selective hydroxylations of aromatic compounds are among the most challenging reactions in synthetic chemistry and have gained steadily increasing attention during the last decade because hydroxylated aromatics are important precursors and products in the pharmaceutical industry.[1] For example, 5-hydroxypropranolol (5-OHP), a human metabolite of the beta blocker propranolol (1-naphthalen-1-yloxy-3-(propan-2-ylamino)propan-2-ol), is of pharmacological interest as it is frequently used in metabolic studies and has been demonstrated to be equipotent to propranolol as a ß-receptor antagonist.[2] Another important human drug metabolite is 4′-hydroxydiclofenac (4′-OHD), a major metabolite of the anti-inflammatory drug diclofenac (2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid) in humans.[3]

Although a four-step chemical synthesis of 5-OHP from 1,5-naphthalenediol is available, a low overall yield (<5%)[4] and problems with the removal of byproducts have prevented the cost-effective use of this approach. The chemical synthesis of 4′-OHD has likewise proved difficult.[3] A simpler approach would be to use an enzyme to hydroxylate these drugs in one step. One possibility would be the use of monooxygenases such as cytochrome P450s (cytP450s). This approach has been shown to work for 4′-OHD synthesis,[3,5,6] but is currently restricted to whole-cell biotransformations because cytP450s are poorly stable and catalytically slow. Moreover, their intracellular location makes them difficult to produce in quantity.[7,8] Another approach, the use of laboratory-evolved. engineered cytP450s for H_2O_2-dependent hydroxylation of propranolol to 5-OHP via the so-called ‘peroxide shunt’. has been demonstrated but needs further optimization.[9]

We used a recently discovered heme-thiolate enzyme from the basidiomycete *Agrocybe aegerita* to hydroxylate propranolol and diclofenac. This stable, secreted peroxygenase (AaP) oxidizes a wide range of aromatic substrates and appears to be a versatile oxidoreductase for biotechnological applications.[10-15] We treated each of the compounds (0.5 mM) with purified AaP (0.4 µm) and four equivalents of H_2O_2 (2 mM) in the presence of ascorbic acid (4 mM). The last ingredient was added to prevent polymerization

Abbreviations: AaP, *Agrocybe aegerita* peroxygenase; cytP450, cytochrome P450; 4′–OHD, 4′–hydroxydiclofenac; 5-OHD, 5-hydroxydiclofenac; 4-OHP, 4-hydroxypropranolol: 5-OHP, 5–hydroxypropranolol.

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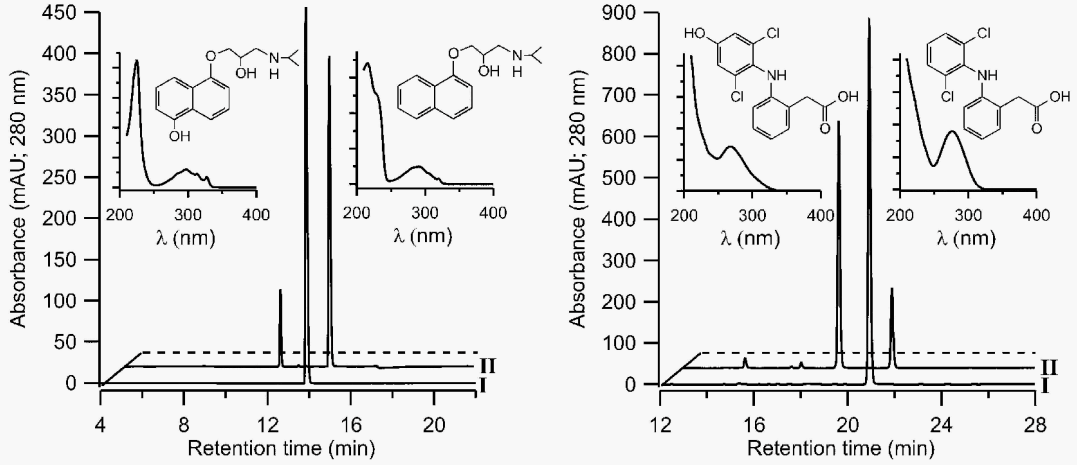

Figure 1. HPLC elution profiles showing products formed by AaP (2 U ml^{-1}. 0.4 μM) after conversion of propranolol (500 μM, left) and diclofenac (500 μM, right) in the presence of ascorbic acid (4 mM). Controls without enzyme (**I**). Complete reactions (**II**). The reactions (pH 7) were started by addition of H_2O_2 (2 mM). Insets show UV/visible absorption spectra of the reactant (right) and major product (left) in each chromatogram.[18]

of 5-OHP or 4′-OHD, an undesirable side reaction attributable to the general peroxidase activity of AaP.[16] The mixtures were stirred at room temperature for 3 min and stopped with 0.1 m1 of 50% wt/vol trichloroacetic acid, after which products were identified and quantified against authentic standards by HPLC.[17]

The reactions proceeded rapidly and regioselectively, converting about 20% of racemic propranolol to 5-OHP and about 65% of diclofenac to 4′-OHD (Figs. 1 and 2). For propranolol oxidation, the formation of byproducts previously reported for the cytP450-catalyzed reaction[8] was insignificant: 4-hydroxypropranolol and 1-naphthol occurred only in trace quantities, and *N*-desisopropylpropranolol was not found. The enantiomeric excess of *S*-5-OHP during AaP-catalyzed hydroxylation of propranolol was less than 2% (data not shown), that is, the reaction was not enantioselective. For diclofenac oxidation, the human drug metabolite 5-OHD[3] was not formed, but traces of several other unidentified byproducts were detected. Control reactions without AaP or with heat-inactivated enzyme gave no conversion of propranolol or diclofenac.

The failure of the reactions to proceed to completion was probably not a consequence of enzyme inactivation. because reactions conducted with more AaP did not give significantly higher yields (data not shown). It appears more likely that the phenolic products 5-OHP and 4′-OHD prevented further oxidation of the parent compounds because they also are AaP substrates. Under our reaction conditions, these phenols probably consumed some of the H_2O_2 by undergoing continuous, competitive AaP-catalyzed oxidation to the 5-OHP and 4′-OHD phenoxy radicals, which in turn were continuously re-reduced to 5-OHP and 4′-OHD by the excess ascorbate we included. This conclusion is supported by our observation that propranolol was rapidly polymerized when the reactions were conducted in the absence of ascorbate (data not shown).

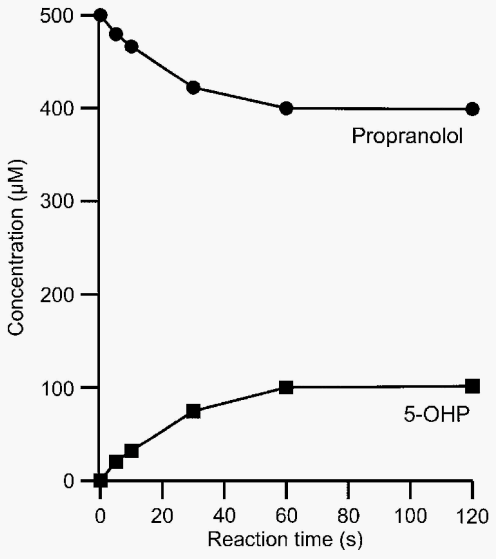

Figure 2. Time course of AaP-catalyzed 5-hydroxylation of propranolol (reaction conditions are the same as in Figure 1, standard deviation <5%).

When we conducted the AaP-catalyzed oxidation of propranolol with $H_2{}^{18}O_2$ in place of H_2O_2, mass spectral analysis[18] of the resulting 5-OHP (Fig. 3) showed that the principal $[M-H]^-$ ion had shifted from the natural abundance *m/z* of 274 to *m/z* 276 for propranolol. Similarly, the analogous experiment with diclofenac showed that the principal ion of 4′-OHD shifted from an *m/z* of 310 to an *m/z* of 312 (data not shown). Experiments using $H_2{}^{18}O$ gave no detectable ^{18}O incorporation (data not shown), as expected because phenolic oxygens are not readily exchangeable with water under our reaction conditions.[19] An additional experiment with natural abundance H_2O_2 in an N_2-purged reaction mixture showed that propranolol production was not inhibited by depletion of O_2 (data not shown), and therefore O_2 did not contribute significantly as an electron acceptor. These results show that the new phenolic oxygens in 5-OHP and 4′-OHD originated from H_2O_2.

Since cytP450s and AaP are both heme-thiolate enzymes, the catalytic cycle of AaP probably resembles the ‘peroxide shunt’ that is responsible for the hydroxylation of propranolol by some engineered cytP450s.[9] However, AaP appears to be the better choice as a biocatalyst because it is easier to produce, is more efficient, is more stable to H_2O_2, and in the case of propranolol exhibits higher regioselectivity (Table 1). On the other hand, although AaP regioselectively hydroxylates other precursors as well,[14] it has some limitations. For example. we have observed that, although AaP efficiently hydroxylates *N*-phenylacetamide to acetoaminophen (yields up to 80%, unpublished results), it very poorly adds the second 3-hydroxyl needed to produce the human drug metabolite 3-hydroxyacetaminophen. We are currently exploring the possibility that AaP may catalyze useful one-step monooxygenations of other pharmaceutically relevant aromatics.

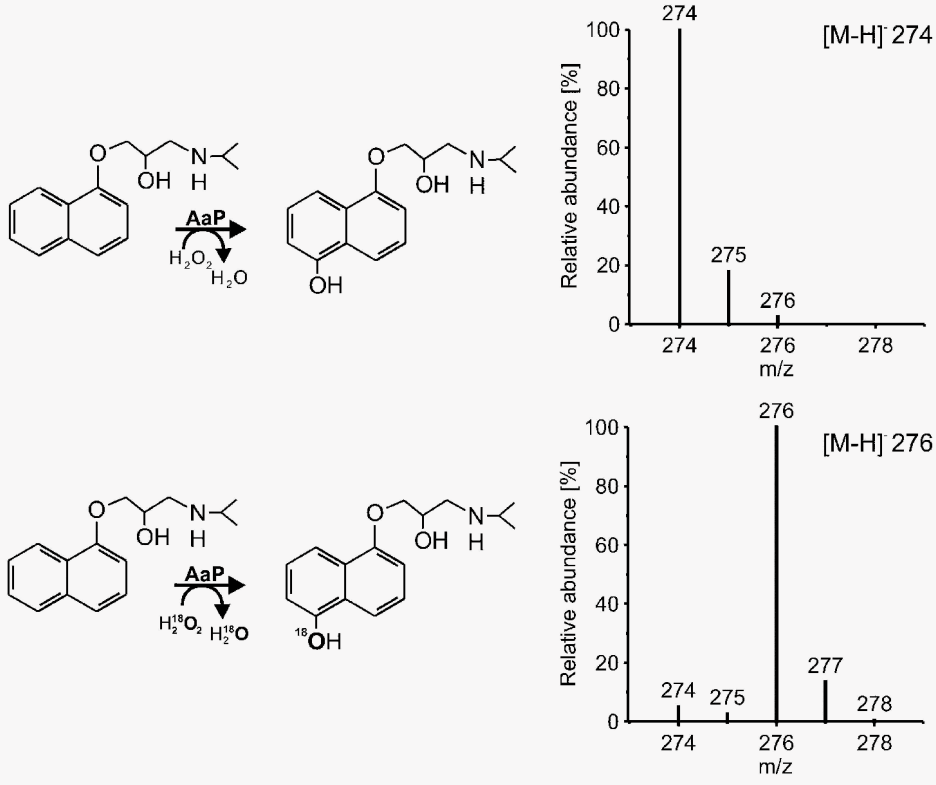

Figure 3. Mass spectra showing molecular ions of 5-OHP obtained from the oxidation of propranolol with AaP (1 U m^{-1}, 0.2 μM) in the presence of natural abundance H_2O_2 (top) or $H2^{18}O_2$ (bottom).[19]

Table 1
Conversion of propranolol (5 mM) to 5-OHP by AaP and cytP450.[c]

Enzyme in reaction (μM)	Conversion of propranolol to 5-OHP (%)	Reaction time (min)	Products formed
AaP[a] (0.6)	13.6	2	1
CytP450[b] (5.0)	0.5	180	4

[a] H_2O_2 concentration was 5 mM.
[b] H_2O_2 concentration was 1 mM. Data are for mutant D6H10 in Ref. 9.
[c] Conversion of diclofenac to 4′-OHD under these reaction conditions was 30%.

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17. HPLC was done on a Phenomenex Synergi Fusion RP-80A reverse phase column (4.6 by 150 mm, 4 μm particle size). The column was eluted at 40 °C and a flow rate of 1 m1 min^{-1} with a mixture of aqueous phosphoric acid solution (15 mM, pH 3) and acetonitrile, 95:5, for 5 min, followed by a 25-min linear gradient to 100% acetonitrile. Elution times of propranolol, 5-OHP, 4-OHP, 1-naphthol, N-desisopropylpropranolol. diclofenac. 5-OHD and 4′-OHD were checked against authentic standards.
18. Liquid chromatography/mass spectroscopic (LC/MS) analyses were performed using a reversed phase Synergi Gemini C6-Phenyl 110A column (4.6 by 150 mm, 5 μm particle size). The isocratic mobile phase consisted of 5% vol/vol acetonitrile and 95% aqueous 0.1% vol/vol ammonium formate that had been adjusted to pH 10 beforehand with NaOH. The column was operated at 40 °C and 1 ml min^{-1} for 5 min. Electrospray ionization was performed in the negative ionization mode.
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