Selective biochlorination of hydroxyquinolines by a flavin-dependent halogenase

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ABSTRACT

Rdc2 is a flavin-dependent halogenase from *Pochonia chlamydosporia*. Through the introduction of a His$_6$-tag to both the N- and C-termini, the isolation yield of Rdc2 from *Escherichia coli* using Ni-NTA affinity chromatography was increased by three-fold. In vitro reaction of Rdc2 and a flavin reductase (Fre) with seven different hydroxyquinolines revealed that 3-hydroxyquinoline (**3**), 5-hydroxyquinoline (**5**), 6-hydroxyquinoline (**6**), and 7-hydroxyquinoline (**7**) can be specifically halogenated. These products were prepared by incubating the corresponding substrates with IPTG-induced *E. coli* BL21(DE3)/Rdc2. They were respectively characterized as 3-hydroxy-4-chloroquinoline (**3a**), 5-hydroxy-6-chloroquinoline (**5a**), 5-chloro-6-hydroxyquinoline (**6a**), and 7-hydroxy-8-chloroquinoline (**7a**) by NMR and MS analyses. This work represents the first enzymatic preparation of chlorohydroxyquinolines and provides a 'green' method to synthesize this group of medicinally important compounds.

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Introduction

Quinolines are common building blocks of numerous bioactive molecules, such as camptothecin (anticancer),[1] chloroquine (antimalarial),[2] quinine (antimalarial),[3] quinidine (antiarrhythmic),[4] and montelukast (anti-asthma) shown in Figure 1.[5] Hydroxyquinolines are used as versatile chelating agents and precursors to many pharmaceuticals and pesticides.[6] For example, the derivatives of 8-hydroxyquinoline can be used as multi-target compounds for the treatment of Alzheimer's disease.[7] Hydroxyquinolines can be chemically synthesized from quinoline (**1**, Fig. 2).

Halogenated compounds represent about 20% of pharmaceuticals on the market and a quarter of those in the development pipeline. Chlorohydroxyquinolines are known to possess important medicinal properties.[8] For example, it has been reported that 5-chloro-8-hydroxyquinoline has significant antimycoplasma activity.[9] However, preparation of halogenated molecules relies on chemical methods. Chemical halogenation typically requires harsh reaction conditions including high temperatures and toxic reagents (such as chlorine gas, hydrochloric acid, and hydrogen peroxide). This is often accompanied by poor selectivity, which results in unspecific halogenation and undesired byproducts, creating difficulties in the purification process.[10] For example, a previous research showed that chlorination of quinoline at 160–190 °C

yielded a mixture of five chlorinated products, including 3,4-dichloroquinoline, 3,4,6-trichloroquinoline, 3,4,8-trichloroquinoline, 3,4,6,8-tetrachloroquinoline, and 3,4,6,7,8-pentachloroquinoline.[11]

Biological halogenation is considered as a better approach for its high selectivity. Flavin-dependent halogenases are major players in the introduction of halogen in the biosynthesis of halogenated natural products such as radicicol.[12] Most reported flavin-dependent halogenases are prokaryotic tryptophan halogenases. The use of these enzymes in the preparation of halogenated molecules was limited due to their strict substrate specificity. A recent work reported that a tryptophan 6-halogenase (Th-Fal) from a thermophilic halotolerant *Streptomyces violaceusniger* can halogenate several tryptophan derivatives and aromatic substrates, including methyltryptophan, 5-hydroxytryptophan, kynurenine, anthranilic acid, and anthranilamide.[13] However, there is still a lack of a highly flexible halogenase that can be used for a broad range of substrates. Rdc2 is the first flavin-dependent halogenase identified from fungi. This enzyme is involved in the biosynthesis of radicicol, specifically chlorinating monocillins (a group of resorcylic acids) at C-6. Rdc2 was found to have flexible substrate specificity and can work on similar macrolactones and other structurally different compounds such as oxytetracycline and curcumin.[14] A homology model of Rdc2 was built, which indicates that the space at the active site of this enzyme is large enough to accommodate different substrates. Two hydroxyisoquinolines were converted by Rdc2 to two new halogenated derivatives.[15] However, specific enzymatic preparation of chlorohydroxyquinolines has not been
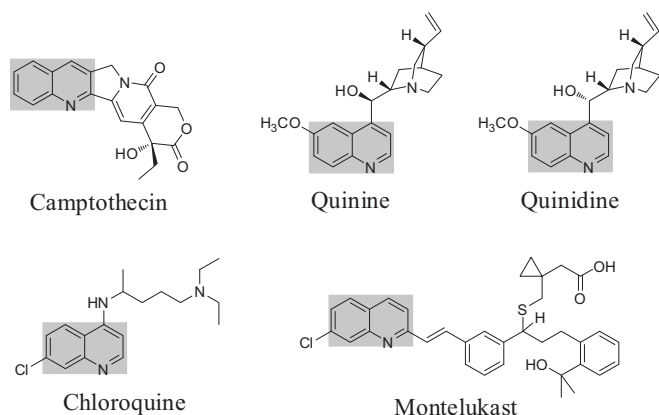

Figure 1. Quinoline-containing bioactive molecules. The quinoline moiety is shaded.

previously reported. In this work, we systematically tested the halogenation capability of Rdc2 using seven monohydroxylated quinolines (2–8, Fig. 2), including 2-hydroxyquinoline (2), 3-hydroxyquinoline (3), 4-hydroxyquinoline (4), 5-hydroxyquinoline (5), 6-hydroxyquinoline (6), 7-hydroxyquinoline (7), and 8-hydroxyquinoline (8).

Results and discussion

The reaction of Rdc2 requires a partner enzyme, flavin reductase (Fre), which reduces FAD to yield FADH$_2$. We previously constructed pZJ54 (pET28a-rdc2) and pZJ62 (pET28a-fre) to purify N-His$_6$-tagged enzymes for in vitro reactions.[14] In order to get a higher Rdc2 yield, we designed a construct with a His$_6$-tag at both N- and C-terminal ends. To this end, the intron-free rdc2 gene was amplified from the previously constructed pZJ54 by PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs) using a pair of specific primers, 5′-aaCATATGTCGGTACCCAAGTCTTG-3′ (the NdeI site is underlined) and 5′-aaAAGCTTAACTTTGTTGAGGC-CAA-3′ (the HindIII site is underlined). The amplified gene was ligated into the cloning vector pJET1.2 to yield pFC55. The Rdc2 gene was then excised from pFC55 with NdeI and HindIII and ligated into pET28a between the same sites to generate pFC56.

The plasmid pFC56 was introduced into E. coli BL21(DE3) for protein expression. The resulting strain was grown at 37 °C in Luria–Bertani (LB) medium supplemented with 50 μg mL^{-1} kanamycin. Protein expression was induced at OD$_{600}$ 0.4–0.6 with 200 μM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The double
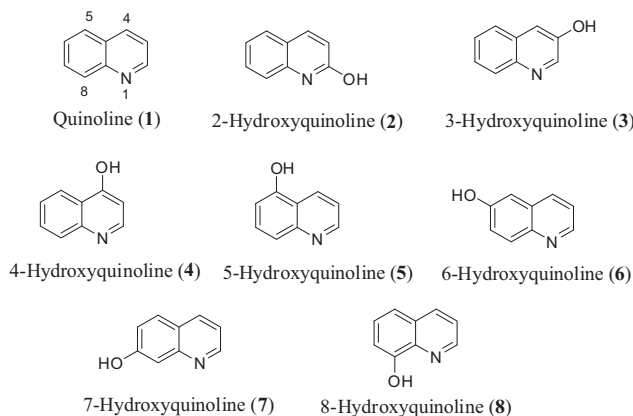

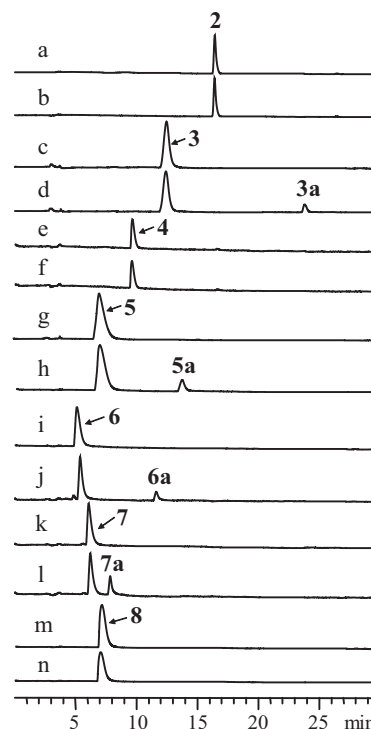Figure 2. Structures of quinoline (1) and hydroxyquinolines (2–8).

Figure 3. HPLC analysis (at 250 nm) of the in vitro reactions of Rdc2 with hydroxyquinolines (at 30 °C for 2 h) in the presence of Fre, NADH, FAD, O$_2$ and NaCl. (a) Inactivated Rdc2 + 2; (b) Rdc2 + 2; (c) inactivated Rdc2 + 3; (d) Rdc2 + 3; (e) inactivated Rdc2 + 4; (f) Rdc2 + 4; (g) inactivated Rdc2 + 5; (h) Rdc2 + 5; (i) inactivated Rdc2 + 6; (j) Rdc2 + 6; (k) inactivated Rdc2 + 7; (l) Rdc2 + 7; (m) inactivated Rdc2 + 8; (n) Rdc2 + 8.

His$_6$-tagged Rdc2 was purified by Ni-NTA column at a yield of 18.5 mg L^{-1}, which represents a three-fold increase compared to the N-terminal His$_6$-tagged Rdc2.[14] The plasmid pZJ62 was introduced into E. coli BL21-CodonPlus (DE3)-RIL for the purification of Fre, as previously reported.[14] Both enzymes were stored in 50% glycerol at −20 °C.

We then conducted in vitro enzymatic assays with Rdc2, Fre, NADH, FAD, NaCl, O$_2$, and seven different hydroxyquinolines 2–8 as substrates. The reactions were incubated at 30 °C for 2 h and the negative controls were maintained under the same conditions with inactivated Rdc2. As shown in Figure 3, HPLC analysis revealed that 3, 5, 6, and 7 can be converted to less polar products 3a, 5a, 6a, and 7a by Rdc2, respectively. By contrast, no products were synthesized from 2, 4, and 8. A comparison of the UV spectra of 3a, 5a, 6a, and 7a with the corresponding substrates showed a bathochromic shift (Fig. S1), indicating that these products are chlorinated derivatives of 3, 5, 6, and 7. ESI-MS of all these products showed the [M+H]$^+$ quasimolecular peaks at m/z 182 and 180, with a ratio of 3:1 (Fig. S2), representing a characteristic isotope pattern of monochlorinated compounds. All the hydroxyquinolines are isomers with a molecular weight of 145, while the molecular weight of 3a, 5a, 6a, and 7a is 179, which is 34 mass units larger than the substrates, indicating that they are indeed the chlorinated products.

To obtain sufficient amounts of chlorinated products for NMR spectroscopic analysis, we used a whole-cell biotransformation approach. A total of 15 mg of 3, 5, 6, and 7 were separately fed into 500 mL of IPTG-induced fermentation broth of E. coli BL21(DE3)/ pFC56, from which 4 mg of 3a, 3.2 mg of 5a, 4.5 mg of 6a, and 4.3 mg of 7a were isolated in pure form, respectively.

The purified products were dissolved in methanol-d$_4$ and the NMR spectra were acquired on a Bruker AvanceIII HD Ascend-500 NMR instrument. To determine the chlorination position for