A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201904157
Angew. Chem. 10.1002/ange.201904157

Link to VoR: http://dx.doi.org/10.1002/anie.201904157
http://dx.doi.org/10.1002/ange.201904157

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10.1002/anie.201904157

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COMMUNICATION

Multi-function oxidase activity of CYP102A1 (P450BM3) in the oxidation of quinolines and tetrahydroquinolines

Yushu Li,[a] and Luet L. Wong*[a,b]

Abstract: Tetrahydroquinoline, quinoline and dihydroquinolinone are common core motifs in drug molecules. Screening of a 48-variant library of the cytochrome P450 enzyme CYP102A1 (P450BM3) followed by targeted mutagenesis based on mutation-selectivity correlations from initial hits, has enabled the hydroxylation of substituted tetrahydroquinolines, quinolines and 3,4-dihydro-2-quinolinones at most positions around the two rings in good to high yields at synthetically relevant scales (1.5 g/L/day). Other oxidase activities such as C–C bond desaturation, aromatisation and C–C bond formation were also observed. The enzyme variants, with mutations at the key active site residues S72, A82, F87, I263, E267, A328 and A330, provide direct and sustainable routes to oxy-functionalised derivatives of these building block molecules for synthesis and drug discovery.

Tetrahydroquinoline (THQ), quinoline and dihydroquinolinone are ubiquitous core motifs in natural products with important medicinal properties, e.g. alkaloids such as quinine, camptothecin and integriquinolone,[1] and in synthetic drugs with varied pharmacological activities including anti-viral, anti-microbial, and anti-tumour compounds. A variety of routes have been developed for the synthesis of compounds with these core motifs for use as building blocks for generating biologically active compounds. Strategies for accessing THQ derivatives include (a) reduction of the heterocyclic ring in the corresponding quinoline derivative, (b) construction of the heterocyclic ring by reactions that form one, two, and three C–C bonds, and (c) heterocyclic ring contraction and expansion.[2] Direct functionalisation of the heterocyclic core is a less common strategy due to the modest product selectivity and often more forcing conditions with transition-metal catalysts.

In contrast to chemical systems, enzymatic C–H bond activation could provide sustainable and more selective routes for direct functionalisation. CYP102A1 ($P450_{BM3}$) from *Bacillus megaterium*,[3] is a good candidate system on account of its catalytic self-sufficiency,[4] high-level expression in *E. coli* and the excellent scalability of its reactions.[5] The enzyme has been extensively engineered for C–H activation of unnatural substrates for applications in synthesis.[6] Rational design,[7] random mutagenesis,[7d, 8] site-saturation mutagenesis and combinatorial active-site saturation test (CAST)[9] have been applied with great success. Another approach is to screen a library of variants for initial hits followed by iterative site saturation mutagenesis.[10] Smaller libraries generated from 4–5 mutations at two active site residues have shown high product selectivity for some substrates.[11] We have adopted a similar approach based on CYP102A1 variants found to have increased activity for unnatural substrate oxidation as a result of the heme iron–axial water interaction being weakened by a number of mechanisms.[12] Further mutations were introduced at two to four active site residues (S72, A74, V78, F81, A82, F87, A184, I263, E267, A328, P329 and A330) in these base variants to afford a library of ~100 enzymes with diverse substrate pocket topologies. Screenings have shown this library to be capable of C–H bond oxy-functionalisation of different classes of organic compounds.[13] We report here the oxidation of THQs and quinolines by CYP102A1 variants with the aim to functionalise these building-block molecules selectively at as many positions as possible and thus provide synthetic handles to compounds with biological activity. In addition to C–H bond oxidation, we found that CYP102A1 displayed other oxidase activities including desaturation,[14] aromatisation,[15] and C–C bond formation reactions.

Screening scale reactions for an initial library of 48 variants [Tables S1 & S2 in Supporting Information (SI)] were carried out in 24-well plates at 25°C, shaking at 120 rpm. The 1 mL reactions in 200 mM phosphate buffer (pH 8.4) contained 1 μM CYP102A1 enzyme, 40 μM $NADP^+$ and 2 mM substrate. Glucose and glucose dehydrogenase were used to recycle the NADPH cofactor. Organics were extracted with ethyl acetate and analysed by gas chromatography. Products were isolated from preparative scale reactions (20–100 mg) by silica gel chromatography and characterised by NMR and MS data. The variant library oxidised **1** to a mixture of products (Fig. 1), including 6-hydroxy-THQ, **2**, the aromatisation product quinoline, **3**, 3,4-dihydro-2-quinolinone, **5**, and the dimers **4**, **10** and **11** (See Supporting Information). On prolonged reaction, the THQ derivative **2** was fully converted by most of the variants to the aromatised product, 6-quinolinol, **6**.

Mechanistically, **2** is formed via the NIH shift pathway whereby the initially formed 5,6-arene oxide undergoes ring-opening to form the C5 carbocation preferentially because of stabilisation by the nitrogen lone pair (Scheme 1). Aromatisation of **1** and **2** is likely to be initiated by hydroxylation at C2, the activated α-position to the amine nitrogen. The carbinolamine **A** loses OH^- to give the iminium species which is in equilibrium with the enamine tautomer, the C4–H bond of which is highly activated to hydrogen atom abstraction by Compound I to generate the stabilised allylic-benzylic radical. Aromatisation could occur by H-atom abstraction from the enamine NH or spontaneous elimination of water from the enamine alcohol formed by radical rebound. The enamine can also undergo nucleophilic attack on its iminium tautomer to form the dimeric iminium species **B**; under the reaction conditions, the iminium/enamine half of the dimer is

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10.1002/anie.201904157

aromatised to give **4**. This 2,3′-THQ-quinoline dimer is hydroxylated at C4 of the THQ moiety to give **10** which is aromatised to the 2,3′-quinoline-quinoline dimer **11**, most likely via an unobserved enamine-alcohol intermediate.

When **5** was screened with the library, we observed hydroxylation at the α-position to give **12**, desaturation of the Cα–Cβ bond to form **13**, benzylic oxidation to give **14**, and formation of the 6-phenol **15** and 8-phenol **16** (Fig. 1). The greater product diversity for **5** compared to **1** suggested that the NH group in THQ might be involved in restricting substrate binding within the active site. Therefore, the *N*-Boc and *N*-acyl derivatives of THQ (**17** and **20**) were tested with the library. We only observed the C4 hydroxylation products **18** and **21**. On prolonged incubation, the ketones **19** and **22** from further oxidation of these benzylic alcohols were obtained in quantitative yield (Fig. 1, Tables 1, S11 & S12). Quinoline (**3**) was also a substrate for the variants, being oxidised to 3-quinolinol, **7**, and a stable 5,6-arene oxide, **8**. Acid-catalysed arene oxide ring-opening of **8** gave 5-quniolinol, **9**.

Within the screening library, 29 of 48 variants gave >50% conversion of **1** while the WT showed negligible activity. Of the products, the variant RP/H171L/I263G (RP = R47L/Y51F/I401P) gave the highest proportion of the 6-phenol, **2** (54%, TON = 1080, Fig. 1, Tables 1 & S5), which was then fully converted to the aromatised product **6** at longer reaction times. Unexpectedly, this variant, and some others (Tables S5 & S6), did not show any further oxidation of **2** until **1** had been fully consumed. When purified **2** was used as substrate these variants fully converted it to **6** with 100% selectivity (TON = 2,000). The variant

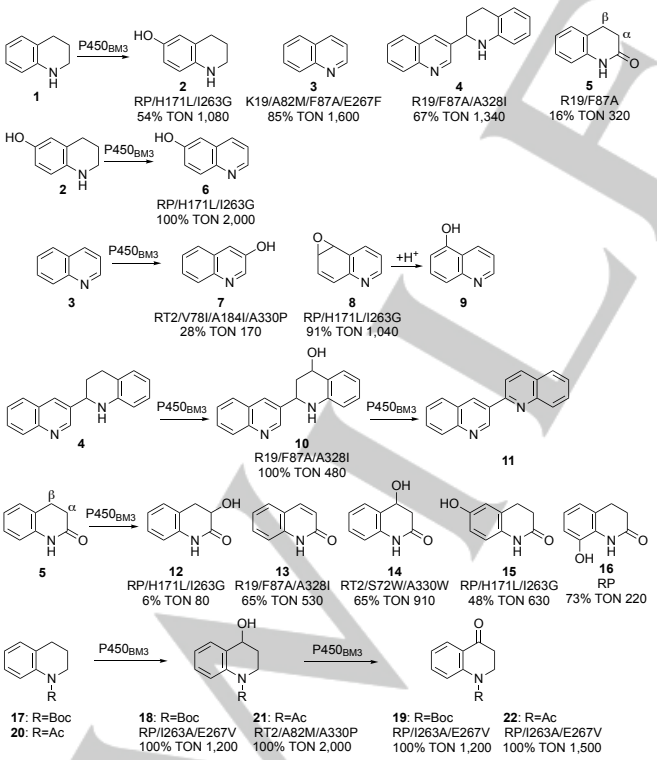
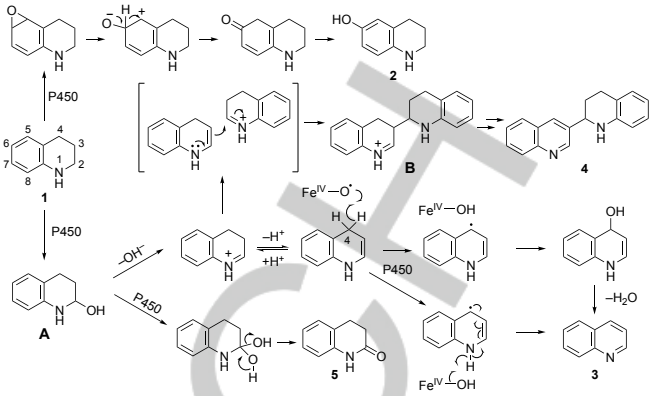


Figure 1. Products from the CYP102A1-catalysed oxidation of 1,2,3,4-tetrahydroquinoline (THQ, **1**), showing the most selective variant in the screening library, the proportion of each product and the turnover number (TON).



Scheme 1. The proposed mechanistic pathways for the oxidation of 1,2,3,4-tetrahydroquinoline (THQ, **1**) by CYP102A1.

K19/A82M/F87A/E267F gave 85% of the aromatisation product **3** (TON = 1,600, 94% conversion, K19 = H171L/Q307H/N319Y), whereas R19/F87A/A328I converted **1** to 67% of **4** (TON = 1,340, 100% conversion). The variant R19/F87A gave the highest percentage (16%, TON = 320) of 3,4-dihydro-2-quinolinone, **5**. For quinoline (**3**), RT2/V78I/A184I/A330P gave 28% of 3-quinolinol, **7** (TON = 170), and RP/H171L/I263G gave 91% of the 5,6-oxide, **8** (57% conversion, TON = 1,040). With **5**, the 3-alcohol **12** was a minor product, the RP/H171L/I263G giving 6% (TON = 80, Table S10); the 6-phenol **15** was the major product for this variant (48%, TON = 630). The RP variant gave the highest proportion of 8-phenol **16** (73%, TON = 220). The R19/F87A/A328I formed 65% (TON = 530) of the aromatised product 2-quinolinol, **13**, and RT2/S72W/A330W gave 65% of the C4-hydroxylation product **14** (TON = 910).

We then sought to improve product selectivity by comparing the results for different mutation combinations. The data for K19, K19/F87A, K19/A328I and R19/F87A/A328I indicated that the native residue F87 disfavoured C2 oxidation and gave more C5,C6 oxidation, likewise for the bulky substitution A328I. The F87A and F87V mutations promoted C2 oxidation, leading to aromatisation and formation of the lactam **5** and dimers. The series RP, RP/H171L and RP/H171L/I263G revealed that the I263G mutation promoted C5,C6 oxidation. Therefore, the I263G and I263A mutations were combined with A328V, A328L, A328I and A328F. These four A328 mutations were also combined with F87A in the R19/FA variant. From this new set of variants (Table S3), the R19/I263G showed 100% conversion of **1** to 80% of **2** (TON = 1,600) compared to 54% for RP/H171L/I263G (Tables 1 & S6). This was improved to 87% with the R19/I263G/A328L (TON = 1,740, 100% conversion). The change from A328I to A328L led to increased formation of the THQ-quinoline dimer **4** for the R19/F87A/A328L variant (78%, up from 67%). The new variants also showed increased selectivity for the oxidation of 3,4-dihydro-2-quinolinone **5**, with R19/I263G giving an albeit low 12% of the 3-alcohol **12** (TON = 170), and the R19/I263G/A328L forming 100% of the 6-phenol **15** (95% conversion).

Next, we explored the effect of substituents on THQ oxidation by both the initial library and 2nd-generation variants with 2-methyl-tetrahydroquinoline (2MTHQ, **23**) and 8-methyl-tetrahydroquinoline (8MTHQ, **31**). Formation of the 6-hydroxy

10.1002/anie.201904157

derivatives was unaffected (Fig. 2). Gratifyingly, as with THQ, the 2nd-generation variant R19/I263G/A328L was also the most selective for both, with 100% selectivity at 93% conversion for **24** (TON = 1,860, Table 1) and 84% for **32** (TON = 1,680). On prolonged reaction both **24** and **32** were aromatised with total conversion to the corresponding methylquinolinols **26** and **36**. As observed with **2**, there was no further oxidation of **24** and **32** with some variants until **23** and **31** had been fully consumed. F87-based variants showed the highest aromatisation activity, at 66% for the formation of 2-methylquinoline (**25**, TON = 420) and 84% for 8-methylquinoline (**33**, TON = 1,010). It appears that aromatisation of THQs to the quinoline analogues is a general activity of CYP102A1 variants, which offers an unexpected route for interconversion between these two classes of compounds by hydrogenation/aromatisation. The K19/A82M/F87A variant gave the most (77%, TON = 1,540) of the dimer **34**. No dimer was observed with **23**, presumably because of steric hindrance by the 2-methyl substituent to nucleophilic attack by the enamine on the iminium species. F87A and F87V-based variants gave higher proportions of the dihydro-2-quinolinone **35** from 8MTHQ (GVQ/A264G 27%, TON = 280).

The methyl substituent had greater effect on quinoline oxidation. The 3-quinolinol derivatives were formed with moderate selectivity: 56% (TON = 290) of 2-methyl-3-quinolinol, **27**, by the RP variant, and 38% (TON = 280) of 8-methyl-3-quinolinol, **37**, by R19/I263A/A328L. The 5,6-oxide **29** from the oxidation of **25** was stable under turnover conditions and could be isolated; the R19/I263G variant gave **29** with 81% selectivity (TON = 1,150). Treatment of **29** with acid gave a mixture of 2-methyl-5-qunolinol (**30**, 62%) and 2-methyl-6-quinolinol (**26**, 38%). Here, stabilisation of the C5 carbocation from arene oxide ring opening by the 2-methyl substituent altered the selectivity compared to

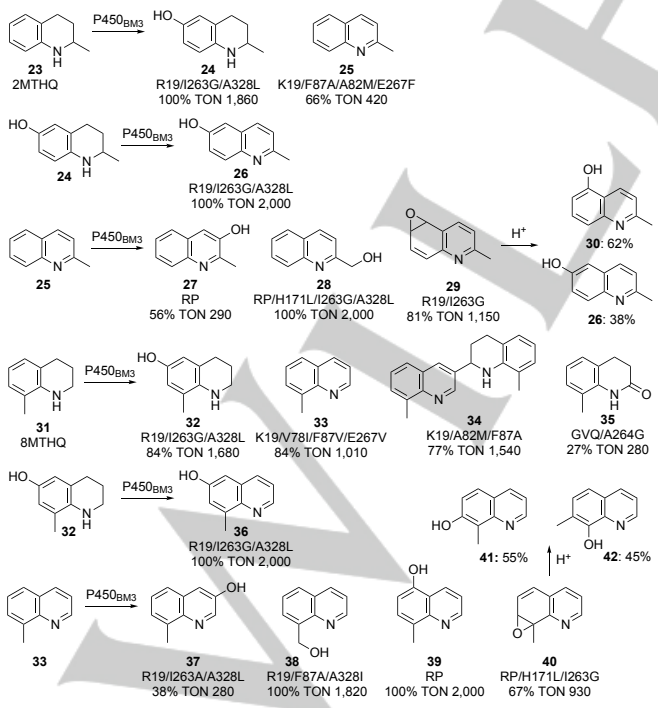


Figure 2. The reaction scheme of $P450_{BM3}$ oxidation of 2MTHQ and 8MTHQ.

Table 1. Activity and selectivity for the oxidation of tetrahydroquinolines and quinolines by CYP102A1 variants. * denotes a second-generation variant.

Product	CYP102A1 Variant	Selectivity	TON
2	R19/I263G/A328L*	87%	1,740
3	K19/A82M/F87A/E267F	85%	1,600
4	R19/F87A/A328L*	78%	1,510
5	R19/F87A/I263G*	20%	400
6	R19/I263G/A328L*	100%	2,000
7	RT2/V78I/A184I/A330P	28%	170
8	RP/H171L/I263G	91%	1,040
10	R19/F87A/A328L*	100%	1,150
12	R19/I263G*	12%	170
13	R19/F87A/A328I	65%	530
14	RT2/S72W/A330W	65%	910
	A74G/F87L/L188Q/I263G/A328G*	80%	380
15	R19/I263G/A328L*	100%	1,900
16	RT2/S72H/A330W*	51%	730
18	RP/I263A/E267V	100%	1,200
19	RP/I263A/E267V	100%	1,200
21	RT2/A82M/A330P	100%	2,000
22	RP/I263A/E267V	100%	1,500
24	R19/I263G/A328L*	100%	1,860
25	K19/A82M/F87A/E267F	66%	420
26	R19/I263G/A328L*	100%	2,000
27	RP	56%	290
28	RP/H171L/I263G/A328L*	100%	2,000
29	R19/I263G*	81%	1,150
32	R19/I263G/A328L*	84%	1,680
33	K19/V78I/F87V/E267V*	84%	1,010
34	K19/A82M/F87A	77%	1,540
35	GVQ/A264G*	27%	280
36	R19/I263G/A328L*	100%	2,000
37	R19/I263A/A328L*	38%	280
38	R19/F87A/A328I	100%	1,820
39	RP	100%	2,000
40	RP/H171L/I263G	67%	930

unsubstituted quinoline. The 5,6-oxide of **33** was not observed, with the RP variant forming 8-methyl-5-quinolinol (**39**) with 100% selectivity (TON = 2,000). The 8-methyl group likely played a role in this outcome by stabilising a C6 carbocation upon ring opening of the 5,6-arene oxide. Interestingly, the RP/H171L//I263G variant oxidised **33** to the 7,8-oxide **40** with 67% selectivity (TON = 930). On treatment with acid, **40** gave 8-methyl-7-quinolinol, **41** (55%), via an NIH shift after arene oxide ring opening to form the C8 carbocation but also the regioisomer 7-methyl-8-quinolinol, **42** (45%), from an NIH shift of the methyl group in a C7-cation intermediate. Benzylic oxidation of both methylquinolines **25** and **33** was observed across the variant library with up to 100% selectivity for the benzylic alcohols **28** (RP/H171L/I263G/A328L, TON = 2,000) and **38** (R19/F87A/A328I, TON = 1,820).

With the 2nd-generation variants included, less than 80 CYP102A1 variants in total were screened and yet a good fraction of the possible oxidation sites in the tested substrates, including aromatisation of THQs to quinolines, were accessible with good to high selectivity and TON (>1,000). Combinations of mutations

at F87, A328 and I263 were particularly effective, with mutations at S72, A82, E267 and A330 also playing a role. Interestingly, the variant library catalysed the benzylic (C4) oxidation of the *N*-Ac and *N*-Boc derivatives of THQ to the alcohol and then the ketone, and near-total selectivity for benzylic oxidation of methyl-quinolines was observed. Therefore, the combinations of mutations in this collection of variants demonstrated good control over the regioselectivity of attack on the two rings as well as the partitioning between reaction pathways after initial H-atom abstraction. The exceptions were the absence of C3, C7,C8 and benzylic methyl oxidation of THQs and the low yields of the 3-phenols from the quinolines. Further enzyme engineering is required to achieve oxidation at these positions. The biotransformation of THQ was scalable. For example, the oxidation of **1** by RP/H171L/I263G at 100 mL scale in shake flasks, without active aeration, agitation or pH control, afforded 100% conversion of >20 mM of **1** with a TON >10,000 by sequential addition of 4 mM aliquots of substrate over 2 days (1.5 g/L/day), with a total product yield of 160 mg (60%).

In summary, CYP102A1 shows multi-function oxidase activity in the oxidation of quinolines, tetrahydroquinolines and 3,4-dihydro-2-quinolinone. Substituents on both rings of these core motifs were well tolerated by the CYP102A1 variants, and there were good indications of the residues and mutations to be targeted for the selective oxy-functionalisation of these important building block compounds at specific positions.

Experimental Section

Experimental details are shown in Supporting Information.

Acknowledgements

YL is supported by a Vice Chancellor's Scholarship from Oxford University.

Keywords: P450 • Protein engineering • C–H activation • Nitrogen heterocyclic compounds • alkaloids

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10.1002/anie.201904157

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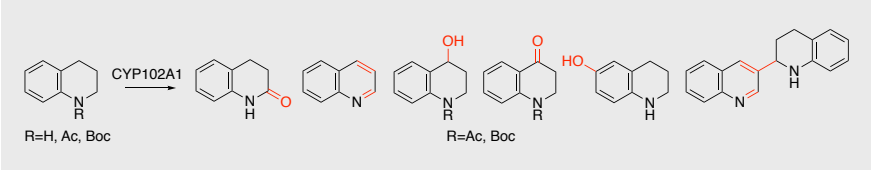

*Yushu Li and Luet L. Wong***

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Multi-function oxidase activity of CYP102A1 in the oxidation of quinolines and tetrahydroquinolines

A library of CYP102A1 (P450BM3) variants show desaturation, aromatisation and C–C bond formation in addition to the normal C–H bond oxidation activity in the oxidation of tetrahydroquinolines, quinolines and dihydroquinolinones with high selectivity and turnover numbers.