Ene Reductase Enzymes for the Aromatisation of Tetralones and Cyclohexenones to Naphthols and Phenols

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Abstract: Ene reductases (EREDs) have great potential as oxidation biocatalysts, as demonstrated by their efficient conversion of a number of tetralones to the corresponding naphthols. Of 96 enzymes tested, 57 were able to produce 2-naphthol in this way. Further tests with substituted tetralones revealed typically high conversions up to >99%. The reactions were performed under mild conditions in aqueous buffer with only co-solvent, biocatalyst and oxidation substrate required for conversion. Production of a methoxy-substituted naphthol was also successfully performed on a gram scale, with 91% yield. This methodology provides a new avenue to produce substituted naphthols as valuable building blocks, with the possibility to extend the approach to the production of phenols also being demonstrated.

Keywords: aromatisation; biocatalysis; ene reductases; enzyme catalysis; naphthols; oxidation; phenols

The application of biocatalysis in organic synthesis continues to grow year on year for a number of reasons including the high chemoselectivity, regioselectivity and enantioselectivity that enzyme catalysis offers to the organic chemist. In addition, the application of enzymatic processes translates into hard cost savings for the fine chemical and pharmaceutical industry, by increasing net yield and purity and reducing waste for enantiopure products.

The synthetic potential of flavin-dependent ene reductases (EREDs) in the asymmetric bioreduction of activated olefins has been well studied to date. They accept a wide range of activated alkene substrates including α,β-unsaturated ketones, aldehydes and nitriles, as well as carboxylic acids and their esters and nitroalkanes, as discussed in detail by several reviewers. Examples of synthetically relevant chiral ERED products include (R)-levodione,[4] (S)-citronellal,[5] Roche ester derivatives[6] and γ-butyrolactones (when coupled with alcohol dehydrogenase).[7] ERED enzymes are also highly amenable to scale-up as demonstrated by the one-pot, three-enzyme system used to produce a chiral intermediate on a 70-g scale within our laboratories.[8]

Besides alkene reduction, EREDs also exhibit a flavin-catalysed, disproportionation activity. During this reaction the flavin mononucleotide (FMN) prosthetic group is reduced by a suitable substrate (typically a conjugated enone), rather than NAD(P)H. Subsequent reduction of an activated alkene or of molecular oxygen (generating $\text{H}_2\text{O}_2$) by the FMN group completes the reaction cycle.[9]

Recently, Faber and colleagues[9a] demonstrated how a range of cyclohexenones and cyclic ketones could be used, as an alternative to NAD(P)H, to drive the asymmetric reduction of a number of industrially relevant, activated alkenes. This approach resulted in two products: the reduction product from the main target substrate and the oxidation product from the sacrificial co-substrate. Removal of [2H] from the co-substrate was typically followed by tautomerisation to give the aromatic (phenolic) compound, as illustrated in Scheme 1.

In the present work, the potential of EREDs as ‘aromatases’ in their own right is investigated, focusing on the production of naphthols. Naphthols are valuable building blocks for a wide variety of synthetic drugs and natural products. Several well-known pharmaceuticals including Propranolol, Naproxen and...
Agomelatine are naphthol derived, highlighting the importance of this moiety in commercial drugs (Figure 1). A proposed route to the anti-Ebola compound BCX4430 uses (S)-1-(α-aminobenzyl)-2-naphthol as a key chiral auxiliary in the synthesis.

Substituted naphthols provide a route to biologically active naphthopyrans, similar to the natural product mollugin. The methoxylated naphthyl compound guieranone A (isolated from the leaves of Guiera senegalensis), has been shown to have potent antifungal activity. Furthermore, synthetic approaches to oxidative coupling of naphthols have attempted to mimic the natural biosynthesis of compounds such as nigerone, hypocrellin, calphostin D, phleichrome, and cercosporin.

Towards the ERED-catalysed production of naphthols, a screen of 96 enzymes from Almac’s selectA-Zyme collection was initially undertaken with 2-tetralone 1a and 4-phenyl-3-buten-2-one 9a as model substrates (Scheme 2). Compound 9a was an inexpensive, readily available starting material and found to be well accepted by the ERED panel in previous work (unpublished data). Of the enzymes tested here, 57 produced detectable levels of 2-naphthol 1b, and 3 of these hits were taken forward in further tests. EREDs 61, 69 and 82 all gave >67% of the naphthol and varied in their levels of conversion of substrate 9a, giving 19%, 48% and 70%, respectively (Table 1).

As the reactions were conducted in air there was no expectation that the oxidation and reduction product conversions would match, and indeed this was the case. Atmospheric oxygen provides an alternative route for FMN oxidation as shown in Scheme 1, and can therefore uncouple naphthol production from reduction of 9a. As the primary aim was to promote naphthol production, the fate of the compound 9a was of less concern in any case.

In subsequent experiments a range of substituted tetralones was used (Scheme 2). Small-scale reactions were carried out with a number of 2-tetralones 1a–7a and with 1-tetralone 8a, either with or without 9a as a target substrate for reduction. The results, summarised in Table 1, showed that tetralones were very well accepted in general, with moderate to excellent conversions (up to >99%).

For the 2-tetralone series, the position of the aromatic substituent did appear to influence the tetralone acceptance. Specifically, position 6 was sensitive to substitutions, with drops in conversions most noticeable for the 6-methoxy compound 3a, although the 6-chloro (4a) and 6-bromo (5a) compounds were more readily accommodated, with up to 91% and >99% conversions respectively (ERED-61). Methoxy derivatives 2a, 6a and 7a performed similarly to the unsubstituted compound 2-tetralone 1a. The 7-methoxy compound 6a in particular gave the best conversions overall, with consistently over 90% and up to >99% of the corresponding naphthol produced.

The lowest conversions were evident with 1-tetralone 8a (although 44% conversion was still achieved), indicating that the position of the ketone moiety is important. This is consistent with the fact that substrate binding and orientation in EREDs is promoted by hydrogen bonding of the activating group with conserved histidine/histidine or histidine/asparagine residue pairs.

In light of the above results it would be interesting to test EREDs 61, 69 and 82 with more highly substi-
tuted tetralones, perhaps affording access to natural products such as guieranone A. Screening of the additional 54 EREDs shown to produce 2-naphthol, may also reveal even better activities with novel tetralone substrates.

For a given ERED, the ratio of the conversion of the reduction target 9a to that of the tetralone varied depending on the tetralone substrate used. This ratio should be constant if it is assumed that, following flavin reduction by the tetralone, the likelihood of 9a being reduced by the flavin is always the same. As the ratio is not consistent, this may indicate that different tetralones vary in their ability to promote FMNH$_2$ oxidation by O$_2$, or in their propensity to occupy the enzyme active site to the exclusion of 9a.

The requirement for the reduction substrate 9a was also of interest. Alongside the reactions that included 9a, reactions were also run with the tetralone as the sole substrate in each case. The results (also given in Table 1) show that the absence of 9a generally does not affect the ability of the enzyme to oxidise the tetralones. Indeed, conversions were often improved in many cases. In a separate experiment, ERED-69 was incubated in sealed vials under nitrogen, with substrate 6a, in the presence and absence of reduction substrate 9a (see the Supporting Information, S4). Under these conditions <1% conversion to the naphthol was seen without the reduction target whereas, in the presence of 9a, the conversion was 92%, in line with 94% conversion to the saturated ketone 9b (see Scheme 1). This result supports the idea that, in air, the dominant pathway for re-oxidation of the flavin, following reduction by the tetralone, is by oxygen (giving H$_2$O$_2$). In the absence of oxygen, 9a serves as an efficient reduction target to drive the oxidation of the tetralone.

In choosing a reaction scheme to move forward, it seemed preferable to omit the additional substrate 9a.
thus avoiding the need for sealed reactions under inert atmosphere and simplifying subsequent attempts at scale-up and product isolation.

With this in mind, the best performing substrate (7-methoxy compound 6a) and the best performing enzyme (ERED-69) were selected for preparation of the corresponding naphthol on a gram scale. In order to increase the substrate loading, a number of co-solvents were screened at the 500-μL scale. In initial reactions, up to 17% (v/v) DMSO, tBME, toluene or THF was tested, with up to 42 mM (for DMSO) or 500 mM substrate (Table 2, entries 1–13). Toluene and THF were the least suitable solvents, giving at best 47% and 3% conversion, respectively, at 31 mM substrate (Table 2, entries 12 and 13).

Further tests focused on the better co-solvents, tBME and DMSO, at 60 mM substrate concentration. The reaction buffer was adjusted to pH 7.5 (as normal) or pH 9.0. Improved conversions were observed at pH 9.0, with values of 60% for 20% (v/v) DMSO co-solvent, 70% for 30% (v/v) DMSO and 83% with 17% (v/v) tBME (Table 2, entries 15, 17 and 19, respectively).

The effect of adding catalase was also tested at this stage, based on the assertion that any H₂O₂ produced might be removed and thus help to drive catalysis and protect against oxidative damage. In practice the addition of catalase did not improve conversions under the conditions tested (see the Supporting Information, S5). Finally, at 60 mM substrate the effects of reaction duration and temperature were examined, although no substantial difference in conversions was seen beyond 16 h reaction time or by varying the temperature to 25°C or 37°C from the original 30°C (see the Supporting Information, S5).

With the optimised conditions in hand, the reaction was scaled to 190 mL. (158 mL buffer, 32 mL tBME) with 2 g of substrate (60 mM or 10.6 g L⁻¹) and 3.8 g of freeze-dried ERED-69 enzyme powder. Following extraction, 1.82 g of product was isolated, giving 91% yield, with product confirmed by ¹H NMR (Supporting Information, S7).

Extending the above approach, the results in Table 3 show how EREDs from the selectAZyme panel can also convert substituted cyclohexenones to the corresponding phenols (note: as with the tetralones, omission of 9a did not seem to have a detrimental effect on conversion – see the Supporting Information, S6). Starting with 3-methyl-2-cyclohexenone 10a, up to 96% conversion to meta-cresol 10b was observed with selected EREDs. Compound 10a can also serve as a target for reduction and the saturated product 10c was observed at low levels (typically <1%). Cyclohexenones with alternative substitution patterns were less efficient for phenol production. A methyl group at the α-carbon greatly reduced the conversions of 11a and 13a, with reduction to the saturated ketone favoured. A comparison of 11a with 13a and Table 3. Phenol production from enones 10a–14a using selected EREDs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Conv. [%] 10b–14b</th>
<th>Conv. [%] 10c–14c[a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>ERED-66</td>
<td>96</td>
<td>&lt;1</td>
</tr>
<tr>
<td>11a</td>
<td>ERED-61</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>12a</td>
<td>ERED-61</td>
<td>18</td>
<td>n.d.</td>
</tr>
<tr>
<td>13a</td>
<td>ERED-71</td>
<td>10</td>
<td>n.d.</td>
</tr>
<tr>
<td>14a</td>
<td>ERED-71</td>
<td>&lt;1</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

[a] Conversions determined by GC-FID peak area; n.d.= not detected.

![Table 2. Optimisation conditions towards the scale-up of 7-methoxy-2-naphthol 6b.](image)
10a with 14a shows that additional ring substituents curtail the aromatisation reaction. Proposed explanations for this include altered (unproductive) substrate binding modes and fewer accessible hydrogens. That being the case, molecular modelling studies and targeted mutagenesis should afford access to productive binding modes that allow the efficient aromatisation of these and other cyclohexenones, while mitigating the competing reductive reaction.

Methylphenols find applications in the synthesis of drugs such as gemfibrozil[15] and α-tocopherol (vitamin E).[16] and also in the production of plastics and resins.[17] Other alkylphenols such as thymol, carvacrol and eugenol are important flavour and fragrance compounds, besides having anti-microbial properties.[18] Substituted phenols are therefore of great synthetic value and improving this new ERED-based route via enzyme engineering is an ongoing area of research in our laboratories.

In summary, an efficient enzymatic method for the production of substituted naphthols from the corresponding tetralones was presented. This approach takes advantage of the natural ability of ene reductases of the Old Yellow Enzyme family to work ‘in reverse’. This ability was shown to be widespread among the selectAZyme panel of EREDs, with 60% of the panel giving 2-naphthol when presented with 2-tetralone as a substrate. Selected EREDs from the panel proved highly effective in the production of a set of substituted naphthols, with moderate to excellent conversions of up to >99%. A representative reaction was performed on a 2-g scale with 91% isolated yield, demonstrating the robustness of these enzyme catalysts towards solvents and elevated substrate loadings. The selectAZyme EREDs are therefore an invaluable tool for synthetic organic chemists seeking clean, safe and efficient ways to make substituted naphthols, for incorporation into new and existing synthetic APIs and natural products. In addition, the methodology can also easily be applied to the production of phenols, an area of ongoing active research within Almac.

**Experimental Section**

General experimental approaches are described below. For more specific details see the Supporting Information, including section S1.

**Chemicals and Enzymes**

Chemicals were purchased from Sigma Aldrich UK. All enzymes were obtained from Almac as freeze dried cell free extracts of *Escherichia coli*.

**Analytical Methods**

For quantitative conversion calculations GC-FID analysis was performed using either a Perkin-Elmer AUTOSYSTEM XL Gas Chromatograph fitted with a Zebron ZB-5 column (30 m, 0.25 mm I.D., 0.25 µm film thickness) or a Finnigan Trace GC fitted with an Agilent HP5-MS column (30 m, 0.25 mm I.D., 0.25 µm film thickness).

For tetralone substrates with no commercially available naphthol product standard (5-methoxy-2-naphthol, 6-chloro-2-naphthol, 6-bromo-2-naphthol and 8-methoxy-2-naphthol), GC-MS analysis was carried out to allow assignment of product peaks. For this purpose, a Thermo Finnigan Trace GC with PolarisQ mass spectrometer was used, and fitted with a Zebron ZB-5 column (30 m, 0.25 mm I.D., 0.25 µm film thickness).

Following product isolation from the scaled up 7-methoxy-2-naphthol reaction, the naphthol product was dissolved in CDCl₃ and analysed by ¹H NMR using a Bruker 500 MHz Ultrashield system.

**Conditions for the Enzymatic Oxidation/Reduction of Tetralones and Enones**

For small-scale screening, enzymatic reactions were carried out in 2-mL 96-well deep-well plates containing 10 mg per well of lyophilised cell free extract for each ERED in the selectAZyme panel. To each well, 500 µL of 100 mM Tris buffer, pH 7.5, were added. Substrates were dissolved in DMSO to 80 mM and 12.5 µL added to the reaction mix. Where the reduction substrate 9a was included, 12.5 µL of an 80 mM DMSO stock were also added or alternatively 12.5 µL of DMSO when 9a was omitted. Plates were covered with a SealPlate film (Sigma Aldrich) and left to shake overnight (16–18 h) at 30°C and 1400 rpm in an Aosheng MB 100–4 A Thermo Shaker.

During optimisation of the ERED-69 reaction with 7-methoxy-2-tetralone, test reactions were carried out as above, except that 2 mL microfuge tubes were used (laid horizontally during shaking), and reaction parameters varied as described (see the Supporting Information, S5).

For GC analysis, an equal volume of ethyl acetate was added to each reaction and samples moved into 2 mL microfuge tubes. Tubes were shaken vigorously by hand for 1 min, vortexed briefly and then centrifuged at 16,000×g for 5 min. The organic layer was pipetted off to fresh tubes and dried over MgSO₄.

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References


