Accepted Article

Title: Chemo- and Enantioselective Photoenzymatic Ketone Reductions Using a Promiscuous Flavin-dependent Nitroreductase

Authors: Alejandro Prats Luján, Mohammad Faizan Bhat, Thangavelu Saravanan, and Gerrit J. Poelarends

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: ChemCatChem 2022, e202200043

Link to VoR: https://doi.org/10.1002/cctc.202200043
Chemo- and Enantioselective Photoenzymatic Ketone Reductions Using a Promiscuous Flavin-dependent Nitroreductase

Alejandro Prats Luján†, Mohammad Faizan Bhat†, Thangavelu Saravanan*, Gerrit J. Poelarends*

[*] A. Prats Luján, M. F. Bhat, Dr. T. Saravanan, Prof. Dr. G. J. Poelarends
Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen (The Netherlands).
E-mail: g.j.poelarends@rug.nl; Web: https://www.rug.nl/staff/g.j.poelarends/
Twitter: @GPoelarends

Dr. T. Saravanan
School of Chemistry, University of Hyderabad, P.O. Central University, Hyderabad 500046 (India).
E-mail: tsaravanan@uohyd.ac.in; Web: http://chemistry.uohyd.ac.in/~thsa/index.html

†A.P.L. and M.F.B. contributed equally to this work.
Abstract

Flavoenzymes are oxidoreductases that catalyze an extensive range of different types of reactions. An advanced and powerful approach to achieving transformations that are normally outside the realm of flavoenzymes is the synergistic combination of photocatalysis and biocatalysis. Here we report the identification of a promiscuous flavin-dependent nitroreductase, BaNTR1, that is able to promote enantioselective photobiocatalytic reductions of a broad range of structurally diverse ketones to yield the corresponding alcohols with high conversion (up to >99%) and outstanding enantiopurity (up to >99:1 e.r). Noteworthy, BaNTR1 was able to promote the photoenzymatic reduction of various α,β-unsaturated ketones to give the corresponding optically pure alcohols without reducing the C=C or C≡C bond, illustrating its remarkably high chemoselectivity. Our results highlight the usefulness of photocatalysis for expanding the catalytic repertoire of nitroreductases to include highly enantio- and chemoselective reductions of non-native ketone substrates to produce optically pure alcohols. This includes difficult to prepare allyl alcohols that are not accessible via photoenzymatic conversions using ene-reductases.
Introduction

Chirality plays an essential role in the development and synthesis of bioactive molecules. As a result, the synthesis of optically pure alcohols has attracted significant interest from academia and industry due to their broad applications.\textsuperscript{[1,2]} This is exemplified by the presence of chiral alcohols as structural elements in numerous pharmaceutically active compounds (Figure 1), such as Isoprenaline (\(\beta\)-adrenoreceptor agonist),\textsuperscript{[3]} Duloxetine (anti-depressant),\textsuperscript{[4]} and Epothilone A\textsuperscript{[5]} (anti-tumor), as well as in agrochemicals, fragrances and other biologically active molecules.

Figure 1. Chiral alcohols as structural elements in pharmaceuticals. A) Examples of benzylic and aromatic alcohols. B) Examples of allylic alcohols.

A common way to obtain enantioenriched alcohols is by the asymmetric reduction of ketones. Amongst the diverse chemical methodologies for chiral alcohol synthesis, the most powerful and widely used is the asymmetric hydrogenation of prochiral ketones with hydrogen gas in combination with a metal catalyst.\textsuperscript{[6,7]} The use of metal catalysts such as Ru, Ir, Rh or Ni allowed the synthesis of chiral alcohols in good yield and with high enantiopurity.\textsuperscript{[8–12]} However, these classic methodologies employ noxious metals and harsh reaction conditions. Another important issue is the difficult production of chiral allyl alcohols where the chemoselective reduction of the corresponding ketones remains a challenge due to the competition between the 1,2- and 1,4-reduction mechanisms.\textsuperscript{[10,13,14]} Because of this, there is a requirement for more selective and environmentally friendlier biocatalytic methodologies.
So far, carbonyl reductases or aldo-keto reductases are the most common type of enzymes used to perform ketone reductions.\cite{15-19} Fascinatingly, Hyster and co-workers recently reported that photoenzymatic catalysis allows radical-mediated ketone reduction in flavin-dependent ene-reductases, enabling the synthesis of several enantioenriched alcohols.\cite{20} Inspired by this pioneering work from the Hyster group, we decided to investigate different flavoenzymes in order to find a suitable candidate for the production of a wide variety of enantiopure alcohols, including difficult allyl alcohols that are not accessible with ene-reductases, via highly enantio- and chemoselective photoenzymatic ketone reductions. We focused our research on nitro- and azoreductases, which received much attention due to their broad applications in cancer pro-drug therapies, xenobiotic degradation and bulk chemical synthesis.\cite{21-24} These flavoenzymes have been shown to reduce aromatic nitro and azo compounds, as well as activated alkenes, but they are not known to perform carbonyl reductions.\cite{25-27}

Herein we report the identification of a promiscuous nitroreductase, BaNTR1, that is capable of promoting highly enantioselective photoenzymatic reductions of a wide variety of non-native ketone substrates to yield the corresponding alcohols with high conversion (up to >99%) and outstanding enantiopurity (up to >99:1 e.r.). BaNTR1 also shows remarkably high chemoselectivity, promoting the photoenzymatic reduction of various \(\alpha,\beta\)-unsaturated ketones to give the corresponding optically pure alcohols without reducing the \(C=\) or \(C=\) bond. This photobiocatalytic strategy underscores the potential of combining two different catalytic systems for the generation of non-natural reactivities in enzymes. Furthermore, it offers an alternative synthetic choice to prepare optically pure alcohols, including difficult to prepare allyl alcohols, starting from structurally diverse prochiral ketones.

**Results and Discussion**

We started our investigations by selecting a panel of flavin-dependent nitroreductases and azoreductases composed of the well characterized enzymes AzoR, NfsA and NfsB from *Escherichia*
coli,[26,28,29] EcNR from *Enterobacter cloacae*,[30] and NRSal from *Salmonella typhimurium*. In addition, we also included enzymes that have not been studied in-depth, and therefore their catalytic potential is yet to be established, such as AzoR1 from *Pseudomonas aeruginosa*,[32] BaNTR1 from *Bacillus amyloliquefaciens*[^33] and YdgI from *Bacillus subtilis*.[^34] The genes coding for these enzymes were synthesized, cloned and expressed in *E. coli* BL21(DE3). Notably, YdgI production was considerably lower compared to the rest of the enzymes, yet enough to continue with the analysis.

After the successful purification of these eight flavoenzymes (Fig. S1), they were examined for their ability to promote the photoenzymatic reduction of two representative ketone substrates, acetophenone (1a) and trans-4-phenyl-3-buten-2-one (1p), to give the corresponding alcohols 2a and 2p (Table 1). Guided by a previous study of Hyster and coworkers,[20] we used Ru(bpy)₃Cl₂ as photocatalyst and irradiation with blue light. Remarkably, BaNTR1 was the only enzyme capable of efficiently reducing ketone 1a into the corresponding alcohol 2a, with the other enzymes showing low or no activity (Table 1). Moreover, BaNTR1 was also able to promote the conversion of the α,β-unsaturated ketone 1p to the corresponding allyl alcohol 2p without reducing the C=C bond, thus demonstrating excellent chemoselectivity. In contrast, the other seven flavoenzymes showed exclusively ene-reductase activity, reducing the C=C bond to give 3p without achieving keto reduction (Table 1).

Based on these initial results, we selected nitroreductase BaNTR1 as the best candidate from the panel of flavoenzymes to promote enantio- and chemoselective photoenzymatic ketone reductions. Analysis of different photocatalysts and irradiation with blue or white light indicated that the combination of [Ru(bpy)₃]Cl₂ with blue light was indeed optimal to perform the photoenzymatic reaction (Table 2). Furthermore, we have chosen MOPS buffer for these reactions, as the morpholine molecule has been reported to stabilize flavoenzymes in the presence of light irradiation and reactive oxygen species.[35,36,37]
Table 1. Flavoenzyme screening for selective photoenzymatic reduction of 1a and 1p.

<table>
<thead>
<tr>
<th>Flavoenzyme</th>
<th>2a</th>
<th>2p</th>
<th>3p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AzoR</td>
<td>N.C</td>
<td>N.C</td>
<td>74</td>
</tr>
<tr>
<td>AzoR1</td>
<td>N.C</td>
<td>N.C</td>
<td>20</td>
</tr>
<tr>
<td>BaNTR1</td>
<td>&gt;99</td>
<td>&gt;99</td>
<td>N.C</td>
</tr>
<tr>
<td>EcNR</td>
<td>4</td>
<td>N.C</td>
<td>&gt;99</td>
</tr>
<tr>
<td>NfsA</td>
<td>N.C</td>
<td>N.C</td>
<td>77</td>
</tr>
<tr>
<td>NfsB</td>
<td>N.C</td>
<td>N.C</td>
<td>&gt;99</td>
</tr>
<tr>
<td>NRSal</td>
<td>N.C</td>
<td>N.C</td>
<td>&gt;99</td>
</tr>
<tr>
<td>YdgI</td>
<td>N.C</td>
<td>N.C</td>
<td>24</td>
</tr>
</tbody>
</table>

Reaction conditions: 0.1 M MOPS buffer pH = 7 (1 mL), [Ru(bpy)$_3$]Cl$_2$ (0.15 mM), substrate (5 mM, 10% DMSO), NAD$^+$ (0.5 mM), glucose (100 mM), bmGDH (2 µM) and flavoenzyme (10 µM). Conversion determined by GC-MS (reaction time = 16 h). [N.C] no conversion.

Having established optimal reaction conditions, we performed a series of control reactions in order to determine the importance of the different reaction components (Table S1). In the case of acetophenone (1a), the absence of nitroreductase or any component from the cofactor recycling system, such as NAD$^+$, bmGDH or glucose, proved that all these components were required for the reduction of 1a to 2a, as there was no conversion of the starting material observed. Performing the reaction under aerobic conditions (in the presence of O$_2$), in the dark (reaction covered from LED light) or without photocatalyst ([Ru(bpy)$_3$]Cl$_2$) also resulted in no product formation. Regarding the α,β-unsaturated ketone 1p, most of the control reactions also resulted in no product formation. However, in the case of the control reaction without BaNTR1, and the reaction without enzymes but including NADH, we could observe some minor reduction of the C=C double bond. This ene-reduction is likely due to the photoexcitation of the reduced nicotinamide cofactor (NADH) under blue light, as previously reported.$^{[38,39]}$
Table 2. Effect of photocatalyst, buffer, and irradiation with blue or white light on the photoenzymatic reduction of ketones 1a and 1p to give alcohols 2a and 2p, respectively.

<table>
<thead>
<tr>
<th>Photocatalyst</th>
<th>LED Light</th>
<th>Buffer</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rose Bengal</td>
<td>White</td>
<td>MOPS</td>
<td>98</td>
</tr>
<tr>
<td>Rose Bengal</td>
<td>White</td>
<td>NaPi</td>
<td>90</td>
</tr>
<tr>
<td>[Ru(bpy)_3]Cl_2</td>
<td>White</td>
<td>MOPS</td>
<td>95</td>
</tr>
<tr>
<td>[Ru(bpy)_3]Cl_2</td>
<td>White</td>
<td>NaPi</td>
<td>94</td>
</tr>
<tr>
<td>FMN</td>
<td>Blue</td>
<td>MOPS</td>
<td>N.C</td>
</tr>
<tr>
<td>FMN</td>
<td>Blue</td>
<td>NaPi</td>
<td>33[a]</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Blue</td>
<td>MOPS</td>
<td>71</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>Blue</td>
<td>NaPi</td>
<td>62</td>
</tr>
<tr>
<td>Eosin B</td>
<td>Blue</td>
<td>MOPS</td>
<td>97</td>
</tr>
<tr>
<td>Eosin B</td>
<td>Blue</td>
<td>NaPi</td>
<td>93</td>
</tr>
<tr>
<td>[Ru(bpy)_3]Cl_2</td>
<td>Blue</td>
<td>MOPS</td>
<td>&gt;99</td>
</tr>
<tr>
<td>[Ru(bpy)_3]Cl_2</td>
<td>Blue</td>
<td>NaPi</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

Reaction conditions: 0.1 M buffer pH = 7 (1 mL), photocatalyst (0.15 mM), substrate (5 mM, 10% DMSO), NAD⁺ (0.5 mM), glucose (100 mM), bmGDH (2 µM) and BaNTR1 (10 µM). Conversion determined by GC-MS (reaction time = 16 h). [N.C] No conversion. [a] Reduction of the C=C bond, no alcohol product was observed. Note that the unselective reduction of activated C=C bonds by unbound FMN has been described before.[40]

Having demonstrated that the nitroreductase was required to perform the ketone reduction, we explored the substrate scope with a range of structurally diverse aromatic and α,β-unsaturated ketones (Figure 2). We were pleased to find that BaNTR1 has a broad substrate range, accepting various substituted acetophenones (1a-1k) as non-native substrates to give the corresponding alcohols 2a-2k with high conversions (up to >99%) and excellent enantiopurity (up to >99:1 e.r.). While ketones 1l, 1n and 1o are comparatively poor substrates, giving the corresponding alcohols (2l, 2n, 2o) with 28-63% conversion, the bulky ketone 1m (2-acetonaphthone) was well accepted to give nearly enantiopure alcohol 2m with >99% conversion. Most interestingly, BaNTR1 was able to promote the conversion (59-99%) of the α,β-unsaturated ketones 1p-1s to the highly enantioenriched alcohols 2p-2s without reducing the C=C or C≡C bond, illustrating not only the excellent enantioselectivity of this enzyme but also its remarkably high chemoselectivity.
Figure 2. Photoenzymatic synthesis of chiral alcohols. Reaction conditions: 0.1 M MOPS pH = 7 (1 mL), [Ru(bpy)$_3$]Cl$_2$ (0.15 mM), substrate (5 mM, 10% DMSO), NAD$^+$ (0.5 mM), glucose (100 mM), bmGDH (2 µM) and BaNTR1 (10 µM). The conversion was determined by GC-MS, and the enantiomeric ratio (e.r) by chiral HPLC using chemically synthesized racemic standards. [a] The absolute configuration was determined by chiral HPLC using a commercially available authentic standard with defined (S) configuration. [b] The absolute configuration was determined using chiral HPLC analysis, comparing the retention pattern of racemic standard and enzymatic product with previously published chiral HPLC data. [c] The absolute configuration was tentatively assigned.
S (or R for products 2j and 2k) configuration on the basis of analogy and according to the chiral HPLC data. [d] Chiral HPLC separation could not be achieved.

To further demonstrate the synthetic usefulness of this photoenzymatic system, we performed semi-preparative scale experiments with substrates 4-cyannoacetophenone (1f) and 2-acetonaphthone (1m) (Fig. S40). The desired alcohol products 2f and 2m were obtained in high isolated yield (95% for 2f and 80% for 2m) and with excellent optical purity (e.r. = 99:1).

Conclusion
In conclusion, we discovered a promiscuous flavin-dependent nitroreductase, BaNTR1, that is able to promote enantio- and chemoselective photoenzymatic reductions of a broad range of ketones to yield the corresponding alcohols with high conversions (up to >99%) and outstanding enantiopurity (up to >99:1 e.r.). As postulated by Hyster and coworkers,[20] the catalytic mechanism of photoenzymatic ketone reduction by a flavoenzyme likely involves a single electron transfer from the excited photocatalyst to the enzyme-bound ketone, forming a ketyl radical intermediate. This generated ketyl radical is then probably quenched by a hydrogen atom transfer from the reduced flavin (hydroquinone), formed upon initial reduction with NADH, to generate the corresponding enantioenriched alcohol. This photoenzymatic system expands the toolbox of biocatalysts that can be used for asymmetric ketone reductions, a feature mainly accomplished by ketone-reductases (KREDs). We have initiated structural and mechanistic studies of BaNTR1 with the aim to unravel its precise catalytic mechanism of photoenzymatic ketone reduction, and to provide a structural basis for its high chemo- and enantioselectivity.

Current work in our group also focuses on elucidating alternative substrates and new catalytic activities for BaNTR1 and the other seven flavoenzymes to further enlarge the catalytic repertoire of this group of cofactor-dependent enzymes for the synthesis of valuable building blocks. The initial results show that BaNTR1 mainly functions as nitroreductase, while the other tested flavoenzymes possess both nitroreductase and ene-reductase activity. These findings are consistent with the
observed chemoselectivity of BaNTR1 towards α,β-unsaturated ketones, promoting their photoenzymatic reduction to give exclusively the corresponding allyl alcohols. These preliminary results will be reported in due course.

Acknowledgement

We acknowledge financial support from The Netherlands Organization of Scientific Research (VICI grant 724.016.002) and from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 754425.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: asymmetric synthesis · photobiocatalysis · nitroreductase · ketone reduction · chemoselectivity

References


The enantioselective photoenzymatic reduction of a wide variety of ketones to the corresponding alcohols using the promiscuous nitroreductase BaNTR1 is reported. This flavoenzyme not only shows outstanding enantioselectivity but also remarkably high chemoselectivity, promoting the photoenzymatic reduction of various α,β-unsaturated ketones to give the desired enantioenriched alcohols without reducing the C=C or C≡C bond.