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Biocatalysis

BioLindlar Catalyst: Ene-Reductase-Promoted Selective Bioreduction of Cyanoalkynes to Give (*Z*)-Cyanoalkenes

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Abstract: The direct synthesis of alkenes from alkynes usually requires the use of transition-metal catalysts. Unfortunately, efficient biocatalytic alternatives for this transformation have yet to be discovered. Herein, the selective bioreduction of electron-deficient alkynes to alkenes catalysed by ene-reductases (EREDs) is described. Alkynes bearing ketone, aldehyde, ester, and nitrile moieties have been effectively reduced with excellent conversions and stereoselectivities, observing clear trends for the *E*/*Z* ratios depending on the nature of the electron-withdrawing group. In the case of cyanoalkynes, (*Z*)-alkenes were obtained as the major product, and the reaction scope was expanded to a wide variety of aromatic substrates (up to >99 % conversion, and *Z*/*E* stereoselectivities of up to >99/1). Other alkynes containing aldehyde, ketone, or ester functionalities also proved to be excellent substrates, and interestingly gave the corresponding (*E*)-alkenes. Preparative biotransformations were performed on a 0.4 mmol scale, producing the desired (*Z*)-cyanoalkenes with good to excellent isolated yields (63–97 %). This novel reactivity has been rationalised through molecular docking by predicting the binding poses of key molecules in the ERED-pu-0006 active site.

The selective reduction of triple bonds is an attractive synthetic strategy for the construction of valuable alkenes, key structural units in many natural products, agrochemicals, and pharmaceuticals.[1] However, efficient systems for the chemoselective reduction of alkynes stopping at the alkene stage require, in most cases, the use of expensive metal catalysts and/or a hazardous hydrogen atmosphere. In addition, poor stereoselectivities can result,[2] and all of these factors make them less attractive methods. Thus, the design of sustainable approaches to carry out this reduction process under mild conditions and with high stereoselectivities is highly sought after. In fact, the American Chemical Society Green Chemistry Institute's Pharmaceutical Round Table has recently stated ten key research areas which include, amongst others, the selective hydrogenation of unsaturated systems, highlighting the importance of developing new catalysts for the reduction of unsaturated compounds under mild conditions.[3] In this sense, biocatalysis is an excellent option for promoting transformations with the aforementioned features. However, the only enzymes capable of catalysing the reduction of multiple carbon-carbon bonds are the ene-reductases (EREDs), which, in nature, are restricted to the bioreduction of electron-deficient alkenes towards the corresponding alkanes in an asymmetric manner (Figure 1a).[4]

The most prominent class of EREDs is the flavin mononucleotide (FMN) dependent Old Yellow Enzymes (OYEs), which possess the capability for reducing multiple α,β-unsaturated substrates bearing different deactivating electron-withdrawing groups (EWG).[5] However, Rosche and co-workers unveiled, in 2007, the ability of OYE3 from *Saccharomyces cerevisiae* to reduce one electron-deficient alkyne (4-phenylbut-3-yn-2-one) into the corresponding alkene with excellent *E* stereoselectivity, although with a moderate conversion, and producing the alkane as a concomitant co-product (Figure 1b).[6]

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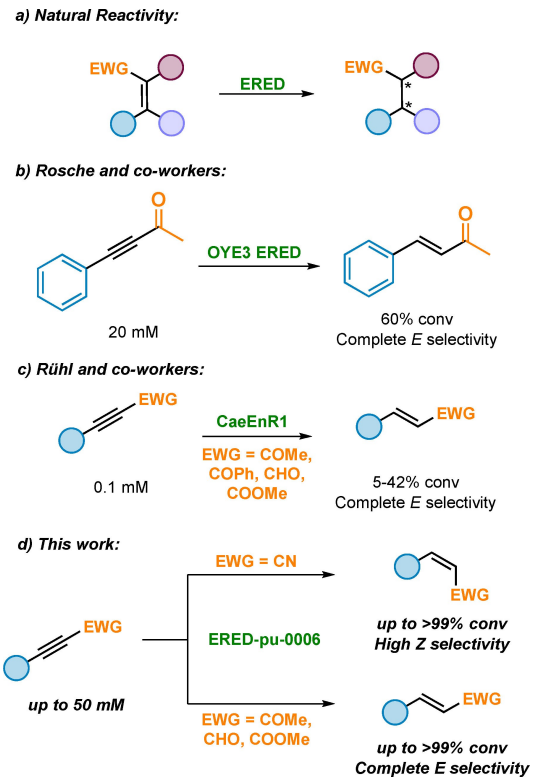


Figure 1. State-of-the-art in the ERED-catalysed partial reduction of alkynes, the source of inspiration for this work.

It was not until 2021 when Rühl and co-workers expanded the scope of this approach using the ERED CaeEnR1, isolated from a filamentous fungus of the phylum *Basidiomycota*.[7] In this report, the enzyme efficiently reduced a series of alkenes bearing electron-withdrawing groups such as esters, ketones, and aldehydes substituted at the β-position, with different moieties that could be either aromatic or aliphatic. However, when the bioreduction of alkynes was studied, the conversions turned out to be low (up to 42 %), even when the substrate concentration was just 0.1 mM using two equivalents of the nicotinamide adenine dinucleotide phosphate (NADPH) cofactor (Figure 1c). Although the concept described was very interesting, the limitations of the methodology as a synthetically useful approach were evident, opening up the opportunity for the development of new strategies which could overcome all these issues.

Herein, a novel reduction of cyanoalkynes was explored, employing a nicotinamide-dependent cyclohexenone reductase (NCR) from *Zymomonas mobilis*[8] and ERED-commercial preparations for the selective production of (*Z*)-cyanoalkenes. The reactivity of differently activated alkynes was also studied, to establish the potential of using these catalysts for the ERED-catalysed selective bioreduction of triple bonds towards α,β-unsaturated compounds (Figure 1d).

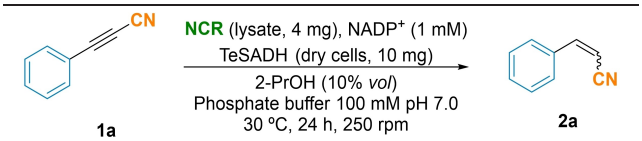
Taking into account that the nitrile group has been neglected in several ERED-catalysed bioreductions of electron-deficient alkenes, and at the same time that it is considered to be a "borderline activating group",[9] we selected 3-phenylpropiolonitrile (**1a**) as model substrate. This was subjected to standard reaction conditions using the NCR enzyme as a cell lysate[6,8] based on previous work (Table 1).

Our first attempt involved carrying out the reaction at a 5 mM substrate concentration to test the feasibility of the proposed transformation using 2-propanol (2-PrOH) and the alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH)[10] for cofactor recycling purposes. Pleasingly, the reaction proceeded well with an excellent conversion of 96 % but, surprisingly, the only product detected was cyanoalkene **2a** with a *Z*/*E* ratio of 91/9 towards the *Z* stereoisomer (Table 1, entry 1). Following the previously established *trans*-ERED mechanism,[5] it was envisaged that (*E*)-alkenes would have been formed, and this unexpected result is discussed further below. The substrate concentration was then raised (5–40 mM) to develop a more synthetically useful semi-reduction protocol. An increase in the concentration of **1a** up to 20 mM afforded even better results, reaching full conversion to the (*Z*)-alkene with almost complete stereoselectivity (Table 1, entries 2 and 3). However, when the substrate concentration was raised to 40 mM, the conversion dropped to 50 % (Table 1, entry 4). Further attempts to improve the catalytic performance by increasing the temperature up to 40 °C failed, leading to a complete loss of the NCR activity (Table 1, entry 5).

In order to further increase the substrate concentration of the proposed transformation and show an improvement in the production of stereo-defined alkenes, we decided to perform a screening of commercial enzymes (Codexis, Inc., for enzyme sequences, see Section VI in the Supporting Information), to see if any of them could carry out the proposed transformation at higher substrate loadings (i.e., 40 mM) and temperature (40 °C). The results obtained are shown in Table 2.

To our delight, several enzymes could carry out the envisioned transformation, showing the capability of the nitrile group to sufficiently promote the selective reduction of the triple bond. ERED-pu-0006[11] (Table 2,

Table 1: NCR-catalysed bioreduction of cyanoalkyne **1a**.

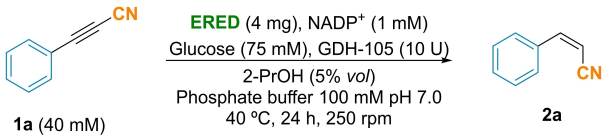


1a **2a**

Entry	**1a** (mM)	c (%)[a]	*Z*/*E*[a]
1	5	96	91/9
2	10	>99	97/3
3	20	>99	98/2
4	40	50	97/3
5[b]	40	<1	–
6[c]	5	<1	–

[a] Conversion and selectivity values determined by GC analysis, observing exclusively **2a** as reaction product. [b] Reaction carried out at 40 °C. [c] Biotransformation in the absence of ERED.

Table 2: ERED-catalysed bioreduction of cyanoalkyne **1a**.



1a (40 mM) **2a**

Entry	ERED	c (%)[a]	*Z*/*E*[a]
1	pu-0001	2	–
2	pu-0002	46	99/1
3	pu-0003	<1	–
4	pu-0004	21	98/2
5	pu-0005	27	98/2
6	pu-0006	99	98/2
7	pu-0007	55	98/2
8	pu-0006[b]	<1	–
9	OYE3	14	99/1

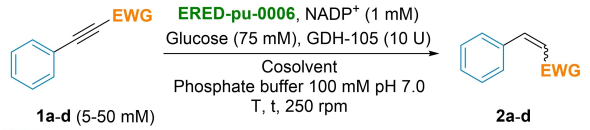
[a] Determined by GC analysis. [b] In the absence of glucose dehydrogenase (GDH).

entry 6), led to complete conversion to the desired cyanoalkene **2a**, even when high concentrations of the substrate were used (40 mM). This loading is remarkable when compared with other ERED-catalysed processes,[12] the NCR-catalysed reactions described in Table 1, and in particular previous examples describing alkyne bioreductions.[6,7] Since this enzyme presents a high similarity with OYE3 from *Saccharomyces cerevisiae* (91 % sequence similarity, see Section IV in the SI), we performed the bioreduction with substrate **1a** (40 mM) using OYE3 as a cell lysate, obtaining (*Z*)-**2a** with high selectivity, although in lower yields (Table 2, entry 9). The reaction was also completely selective toward the formation of the alkene and no alkane production was observed. Furthermore, almost total *Z* selectivity was achieved in every case, as noted before. A very interesting fact is that, when both cyanoalkenes (*Z*)-**2a** and (*E*)-**2a** (commercially available cinnamonitrile), were subjected to the same reaction conditions, no bioreduction took place, leaving the alkenes completely unaltered after 24 h. This outcome is in accordance with the limited literature published on this matter,[9,13] implying the preference of EREDs to reduce cyanoalkynes rather than cyanoalkenes. This effect may also be related to a specific substrate orientation in the active site, as the alkyne moiety has a linear geometry, which is likely to result in a different accommodation in the active centre of the enzyme compared to the (*Z*)-cyanoalkene. Next, we decided to synthesise (*E*)-3-phenylbut-2-enenitrile (**3**), and subject it to the same reaction conditions (see Supporting Information for further details), since this more electron-rich β,β-disubstituted alkene has proven to be a good substrate for EREDs.[13] However, in our case no conversion was observed in the transformation catalysed by ERED-pu-0006.

To test the performance of ERED-pu-0006 with other similar substrates, and compare its catalytic ability with previous reports,[6,7] we also optimised the reaction conditions (see Supporting Information for further details) and applied this enzyme to a variety of alkynes bearing different electron-withdrawing groups (Table 3).

Good to excellent conversions were achieved for 4-phenylbut-3-yn-2-one (**1b**, Table 3, entry 2), 3-phenylpropiolaldehyde (**1c**, Table 3, entry 4), and methyl phenylpropiolate (**1d**, Table 3, entry 7). These substrates were accepted, however, as previous reports have shown,[6,7] the α,β-unsaturated products were obtained with total *E* selectivity, the opposite trend to when the nitrile group was present.[14] Moreover, once the alkenes were formed, a subsequent bioreduction towards the corresponding alkanes was observed, giving by-products, in cases as the only products of the reaction. A fine-tuning of the reaction conditions (controlling temperature and time, see Section VIII in the SI), allowed us to stop the reaction in

Table 3: ERED-catalysed bioreduction of alkynes **1a–d** with distinct EWGs.[a]



1a-d (5-50 mM)
EWG: CN (**a**), COMe (**b**)
CHO (**c**), CO_2Me (**d**)

2a-d
$Ph(CH_2)_2COMe$ (**4b**)
$Ph(CH_2)_2CHO$ (**4c**)
$Ph(CH_2)_3OH$ (**5c**)
$PhCH{=}CHCH_2OH$ (**6**)

Entry	Substrate	EWG	[1] (mM)	Cosolvent (% *vol*)	ERED (mg)	T (°C)	t (h)	Product[b]	*Z*/*E*[b]
1	**1a**	CN	50	EtOH (5)	4	40	24	**2a** (>99)	98/2
2	**1b**	COMe	40	2-PrOH (7.5)	2	15	1	**2b** (79)[c]	<1/99
3	**1b**	COMe	40	2-PrOH (5)	4	40	1.5	**4b** (>99)	–
4	**1c**	CHO	10	2-PrOH (5)	2	15	1.5	**2c** (65)[d]	<1/99
5	**1c**	CHO	20	2-PrOH (5)	4	10	2	**4c** (>99)	–
6	**1c**	CHO	10	2-PrOH (5)	4	15	16	**5c** (95)[e]	–
7	**1d**	CO_2Me	5	2-PrOH (5)	4	40	24	**2d** (96)	<1/99

[a] See Supporting Information for a detailed optimisation study towards the formation of each product: **2a** in Table S2; **2b** and 4-phenylbutan-2-one (**4b**) in Table S3; **2c**, 3-phenylpropanal (**4c**) and 3-phenylpropan-1-ol (**5c**) in Table S4; **2d** in Table S5. [b] Product percentages and selectivities determined by GC analysis. [c] **4b** (12%) was obtained as a concomitant product. [d] **4c** (16%) and **6** (2%) were obtained as concomitant products. [e] **6** (5%) was obtained as a concomitant product.

the alkene stage, or drive the reaction completely towards the corresponding alkanes, namely 4-phenylbutan-2-one (**4b**, Table 3, entry 3), 3-phenylpropanal (**4c**, Table 3, entry 5) and 3-phenylpropan-1-ol (**5c**, Table 3, entry 6) with excellent conversions. An experiment eliminating GDH-105 from the reaction medium but employing stoichiometric NADPH proved that the reduction of the carbonyl group taking place in entry 6 was due to this cofactor recycling enzyme rather than the ERED, which was unable to reduce the C=O scaffold. Sadly, more electron-rich alkynes such as 3-phenylpropiolic acid (**1e**), 3-phenylpropiolamide (**1f**), and 1-chloro-2-phenylacetylene (**1g**) failed to undergo the proposed transformation. Thus, this study provides initial insights into the reactivity of this enzyme defining some advantages and limitations that it possesses to perform the desired partial reduction of alkynes.

Once the reactivity of ERED-pu-0006 against different EWGs was studied, we focused on the scope with other cyanoalkynes due to the excellent results observed with **1a** (Figure 2). (*Z*)-Cyanoalkenes are valuable compounds and few methodologies for their stereoselective synthesis have been described. They usually require multiple prefunctionalisation steps,[15] the use of toxic or dangerous reagents,[16] and/or they struggle to achieve good stereoselectivities and/or conversions.[17] Furthermore, it is challenging to follow traditional hydrogenation methodologies, as the nitrile group can be readily reduced to the corresponding amine,[18] and only very specific conditions and reagents might promote this transformation in a chemoselective manner.[19]

To this end, thorough optimisation of the reaction conditions for each derivative was performed to achieve the highest possible performance (see Section VIII in the SI). This also allowed us to study the tolerance of the enzyme towards different substitutions on the aromatic ring, leading to high to excellent yields and excellent isomeric ratios in most cases. Thus, a weakly electron donating group in the 4-methyl derivative **1h**, weakly electron-withdrawing groups such as the chlorinated compound **1i** and the fluorinated derivatives **1j–l**, and strongly electron donating and withdrawing scaffolds were tested, employing the 4-methoxy **1m** and the 4-nitro **1n** derivatives, respectively, which underwent the proposed transformation without difficulties. Also, the heteroaromatic cyanoalkyne containing a thiophene ring (**1o**) was subjected to the reaction conditions, affording the desired (*Z*)-cyanoalkene **2o** with very good conversion and diastereoselectivity. On the other hand, when aliphatic substrates such as oct-1-yne **1p** and 3-cyclopropylpropiolonitrile **1q** were employed, no reaction was observed.

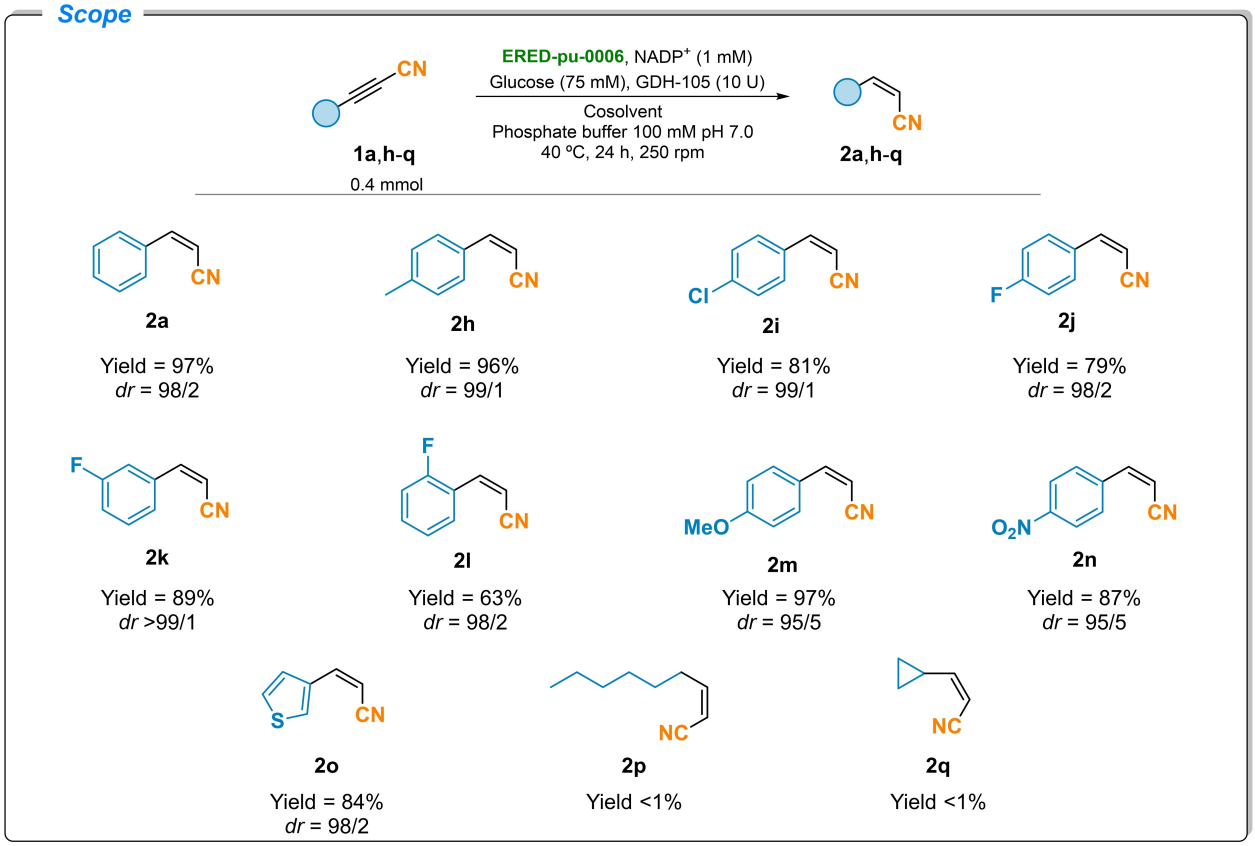


Figure 2. Scope of the ERED-pu-0006-catalysed bioreduction of cyanoalkynes **1a**,**h–q** at 0.4 mmol scale, showing isolated yields of all the compounds described. For substrates **1a**,**l–o**, the cosolvent employed was ethanol (EtOH, 5% *vol*). For **1h–k**,**p**,**q**, 2-PrOH (5% *vol*) was employed. For reaction details, see SI, Section IX.

These results showed the low affinity of the enzyme for non-aromatic substrates.

Once the scope of the reaction was established, we studied the viability of carrying out the procedure at a larger scale (Section IX in the SI), thus testing the feasibility of applying this synthetic strategy routinely in the laboratory. Thus, after adjusting the conditions for each substrate, transformations at 0.4 mmol could be achieved, with no inhibition observed, giving the desired (*Z*)-alkenes in high to excellent yields with excellent isomeric ratios in all cases (Figure 2).

In order to explain the observed experimental results, molecular docking studies were carried out by taking the crystal structure from OYE3 (Protein Data Bank, PDB ID: 5V4P) due its 91 % sequence similarity with ERED-pu-0006 and creating a model. Docking of the ligand **1a** was performed to explain the reactivity and stereoselectivities observed (see Section XI in the Supporting Information for further details). Alkynes bearing a nitrile group (**1a**, **h**–**o**) would be expected to be reduced to the corresponding alkenes (**2a**, **h**–**o**) in a *trans* manner, yielding (*E*)-alkenes, due to the established mechanism of ene-reductases.[20] Modelling of the alkyne **1a** into the active centre of the enzyme showed that the nearest tyrosine residue for protonation at the α-position (Y376) was located in the same face as the flavin cofactor, thus affording the *cis* alkene (Figure 3). In the docked poses, the canonical catalytic tyrosine residue (Y197) was distant from the α-position of the alkyne. However, non-canonical tyrosine residues have been reported providing unexpected reactivities in EREDs,[21] making Y376 a plausible non-canonical protonating residue in ERED-pu-0006. The π-stacking interaction generated between the aromatic ring and the triple bond of the substrate and the flavin cofactor could account for this accommodation, also fixed by the hydrogen bond established between the hydroxyl group of Y376 and the nitrile moiety.[11]

Also, 3-cyclopropylpropiolonitrile **1q** was docked to rationalise why this enzyme was not able to reduce an alkyne bearing an aliphatic substituent. In the docking poses shown in Figure S6, it can be observed that on changing a phenyl to a cyclopropyl group, a different substrate orientation and interactions were obtained. Notably, the substrate was placed in a reversed conformation compared to aromatic nitrile **1a**, with the carbon of the triple bond at the α-position closer to the flavin cofactor instead of the more electrophilic β-position, leading to a non-productive conformation. We speculate that the lack of the aromatic ring system in **1q** may result in loss of any reaction in ERED-pu-0006. Moreover, a comparison between the quaternary structures of OYE3 (PDB ID: 5V4V) and NCR (PDB ID: 4A3U) revealed a high similarity with only 0.600 Å of deviation which can explain the reactivity observed between both enzymes. However, a Basic Local Alignment Search Tool (BLAST) sequence alignment carried out for both proteins gave only a 33 % of sequence similarity. When focused on the active centre (residues at <5 Å from FMN), a higher percentage of similarity was attained (59 %), including the key tyrosine catalytic residues (Y177 for NCR and Y197 for OYE3). Interestingly, when comparing OYE3 and ERED-pu-0006 active sites, tyrosines Y197 and Y397 were conserved in the final mutant.

This procedure gives access to many different interesting (*Z*)-building blocks. For example, following the protocol reported by Ye et al., the synthesis of (*Z*)-allylic amines can be achieved (Figure 4a).[22] Also, *erythro*-epoxides can be prepared using a dioxirane as reported by Annese et al. (Figure 4b),[23] which could also be further transformed into interesting molecules such as cyanohydrins with total control of the selectivity.[24] Additionally, *syn*-iodinated cyanohydrins can be synthesised following the procedure published by Moorthy et al. (Figure 4c).[25] In order to exemplify synthetic possibilities using the methodology developed, the hydration of benzonitriles

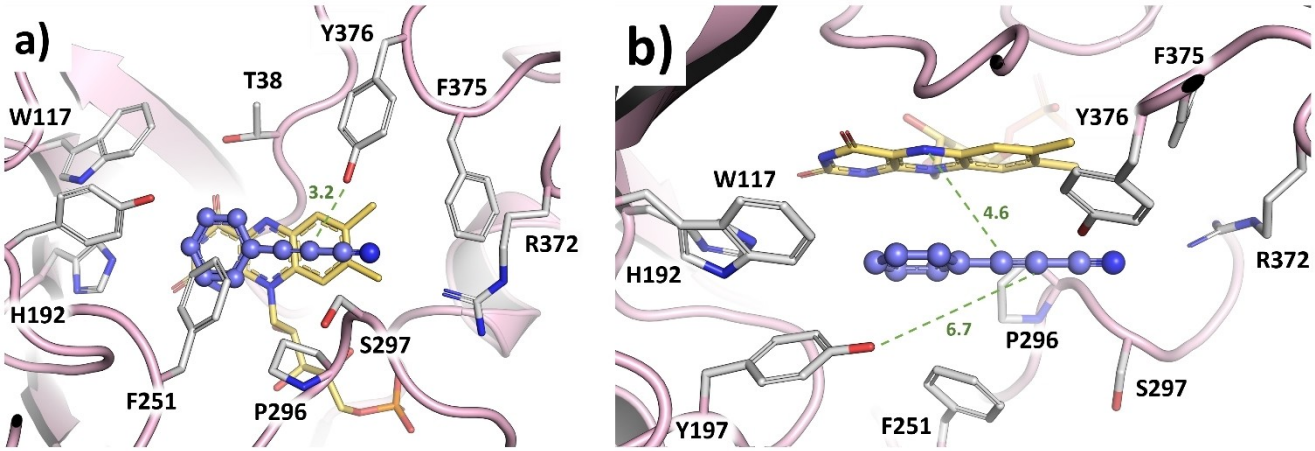


Figure 3. Docking images of substrate **1a** with enzyme ERED-pu-0006. Image on the left a) corresponds to the top view and image on the right b) to the side view. The cofactor, substrate and sidechains are shown in yellow, blue and grey, respectively. Distances in angstroms are represented with dotted green lines. See Supporting Information (Section XI) for further details.

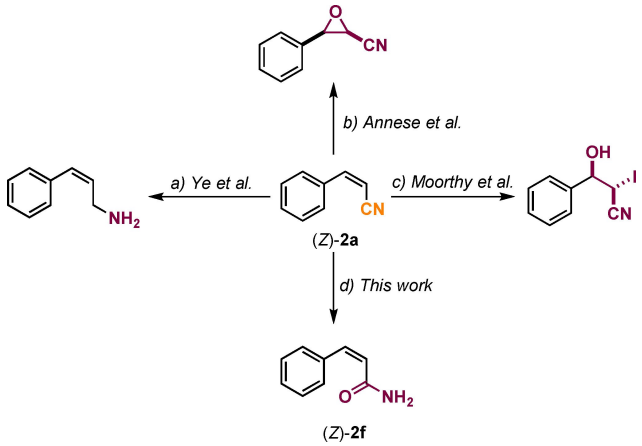
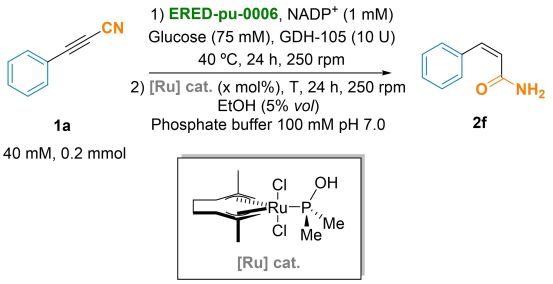


Figure 4. Different derivatives that can be synthesised starting from (*Z*)-**2a**.

towards the corresponding arylamides was explored through ruthenium catalysis.[26] The aim was to achieve the synthesis of (*Z*)-cinnamamide (**2f**) in a one-pot chemoenzymatic approach[27] (Figure 4d), since the direct bioreduction of 3-phenylpropiolamide (**1f**) could not be achieved. The results obtained are shown in Table 4 (for experimental details, see Section X in the SI).

Initially, a concurrent cascade was explored in which the ruthenium catalyst was placed in the same reaction along with the enzyme. However, only a maximum of 40 % conversion towards (*Z*)-**2f** could be reached (Table 4, entries 1–3). On the other hand, the reaction between the Ru catalyst and **1a** occurred simultaneously, as large amounts of 3-phenylpropiolamide (**1f**) could be detected when increasing the metal loading (Table 4, entry 3). As previously indicated, this amide cannot be reduced by ERED-pu-0006, stopping the reaction at this point. Alternatively, a one-pot sequential approach was followed, which did not afford satisfactory conversions (Table 4, entry 4). However, when the temperature and the metal loading were raised to 60 °C and 6 mol %, respectively, the desired amide **2f** could be isolated with a 68 % yield (Table 4, entry 5). Attempts to increase the temperature further led to poor results (Table 4, entry 6).

Table 4: ERED–Ru-catalysed synthesis of (*Z*)-cinnamamide **2f** from **1a**.



Entry	T (°C)	ERED (mg)	[Ru] (mol %)	**2f** (%)[a]	*Z*/*E*[b]
1[c]	40	16	3	<1	–
2[c]	40	32	3	40	98/2
3[c]	40	32	6	<1[d]	–
4	40	32	3	28	98/2
5	60	32	6	75 (68)[e]	98/2
6	80	32	6	20	98/2

[a] Conversion values determined by GC analyses, observing only **2f** as a reaction product. [b] Determined by ^{1}H NMR of the crude mixtures. [c] Reaction carried out via a concurrent cascade. [d] 3-Phenylpropiolamide (**1f**) was obtained. [e] Isolated yield of amide **2f** in parentheses.

In conclusion, a biocatalytic methodology for the stereoselective partial reduction of alkynes has been developed using first NCR and then the ERED-pu-0006 enzyme. A series of electron-withdrawing groups attached to the triple bond have been explored with ERED-pu-0006, including nitrile, ester, ketone, aldehyde, carboxylic acid, amide, and halogen moieties, demonstrating the ability of this ERED to effectively reduce some of these derivatives in a very selective manner. In the particular case of cyanoalkynes, an unprecedented bioLindlar activity was found providing the corresponding (*Z*)-alkenes with a wide substrate scope, obtaining in all cases high to excellent yields and isomeric ratios (>90 %). When the reactions were reproduced at a synthetic scale (0.4 mmol), a similar efficiency was accomplished for all derivatives. On the other hand, other EWGs such as ketone, aldehyde, or ester moieties provided selectively the corresponding (*E*)-α,β-unsaturated or saturated compounds, depending on the reaction conditions. Regarding mechanistic considerations, docking experiments have been carried out, allowing us to propose a mechanism in which the π-stacking interactions between the FMN coenzyme and the substrate **1a** could explain its orientation in the active site as well as the *Z* stereoselectivity observed due to a protonation by a non-canonical tyrosine.

Finally, as an example of the synthetic potential that the products obtained offer, a one-pot chemoenzymatic process from cyanoalkyne **1a** has been proposed and optimised, consisting of an ERED bioreduction followed by ruthenium-catalysed hydration of the nitrile group of the (*Z*)-cyanoalkene intermediate, affording (*Z*)-cinnamamide in a sequential manner in a high yield.

Supporting Information

The authors have cited additional references within the Supporting Information.[8b,15,17b,28–32]

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: alkynes · biocatalysis · cyanoalkenes · ene-reductases · semireduction

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