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Highly selective and controllable synthesis of arylhydroxylamines by the reduction of nitroarenes with an electron-withdrawing group using a new nitroreductase *Ba*NTR1†

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A new bacterial nitroreductase has been identified and used as a biocatalyst for the controllable reduction of a variety of nitroarenes with an electron-withdrawing group to the corresponding *N*-aryl-hydroxylamines under mild reaction conditions with excellent selectivity (>99%). This method therefore represents a green and efficient method for the synthesis of arylhydroxylamines.

N-Arylhyroxylamines are important intermediates in synthetic chemistry, where they have been used for the synthesis of fine chemicals[1] and a variety of useful biologically active compounds,[2] and a wide range of methods have been developed for the preparation of arylhydroxylamines, including stoichiometric reduction (by zinc, tin or sulfide),[3] electrochemical reduction,[4] and catalytic hydrogen transfer.[5] More recently, considerable interest has been focused on the direct synthesis of arylhydroxylamines *via* the selective catalytic hydrogenation of nitroarenes,[6] and we previously reported the development of biocatalytic methods for the preparation of arylhydroxylamines.[7] Although these processes proceeded smoothly to give the hydroxylamines, they were sometimes difficult to control, with the products themselves being further reduced to give the corresponding amines. The development of a method for the controllable synthesis of arylhydroxylamines therefore remains a considerable challenge.

Nitroreductases (NTRs) are flavin-containing enzymes that use NADH or NADPH as an electron source to reduce nitroarenes to the corresponding hydroxylamines and amines,[8] and are therefore regarded as ideal catalysts for the synthesis of hydroxylamines and amines. Hydroxylamines, however, are usually formed as intermediates during the reduction of nitroarenes with NTRs, and they can be difficult to separate from the reaction mixture because they possess similar polarities to the corresponding amines.[9] With this in mind, we became interested in exploring the use of novel and highly substrate-selective NTRs for the controllable synthesis of arylhydroxylamines from nitroarenes.

To begin with, several putative NTRs (ESI,† Table S1) were selected from GenBank, and cloned and overexpressed in *E. coli* BL21 (DE3). The activities of the resulting enzymes were assayed using 4-cyanonitrobenzene as an indicative substrate, and the results revealed that the NTR from *Bacillus amyloliquefaciens* (named as *Ba*NTR1 hereafter) exhibited the highest activity (ESI,† Fig. S1). Furthermore, the reduction of 4-cyanonitrobenzene using *Ba*NTR1 as a biocatalyst afforded the corresponding hydroxylamine, which was detected by HPLC and confirmed by ^{1}H NMR spectroscopy (ESI†). Further investigations were conducted to establish the optimal reaction conditions. As shown in Table 1, the pH of the reaction mixture played an important role in the bioconversion of the substrates. At pH 7.0, the reaction gave the hydroxylamine with >99% selectivity and 100% conversion after only 1 h (Table 1, entry 2). When the pH was decreased to 6.0 or increased to 8.0, lower levels of substrate conversion were observed after 1 h (Table 1, entries 1 and 3). Pleasingly, a reduction in the concentration of the externally supplemented cofactor ($NADP^+$) to 0.3 mM at pH 7.0 was well tolerated, with the substrate being completely converted within 1 h (Table 1, entry 4). A further

Table 1 Reduction of 4-cyanonitrobenzene under different conditions using the nitroreductase *Ba*NTR1[a]

Entry	pH	$NADP^+$ (mM)	Conv.[b] (%)	Sel.[c] (%)	Yield[d] (%)
1	6.0	0.5	62.7	>99	50
2	7.0	0.5	100	>99	83
3	8.0	0.5	98.7	>99	80
4	7.0	0.3	100	>99	82
5	7.0	0.1	81.2	>99	65

[a] Reduction conditions: substrate (100 mM), *Ba*NTR1 (10 mg, crude enzyme powder (lyophilized cell free extract)), glucose dehydrogenase (*Bm*GDH, 10 mg, crude enzyme powder (lyophilized cell free extract)), glucose (400 mM), and 0.1 M sodium phosphate buffer (10 mL) at 30 °C for 1 h. [b] The conversion was determined using HPLC. [c] The selectivity is defined as the molar ratio of hydroxylamine to amine. [d] Isolated yield.

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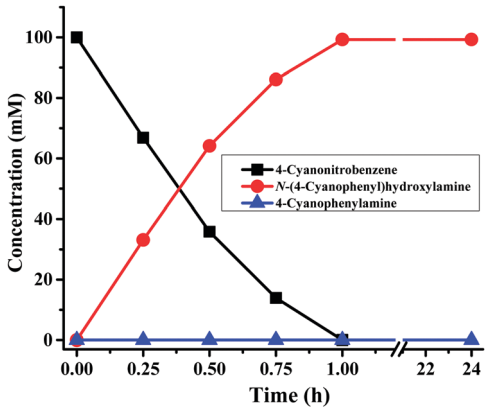


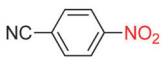
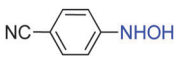
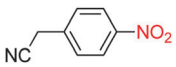
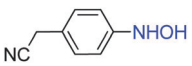
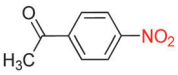
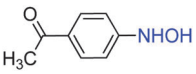
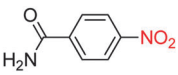
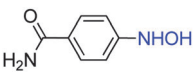
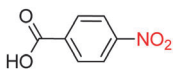
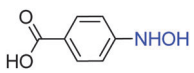
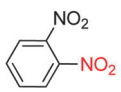
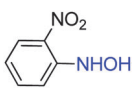
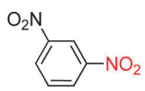
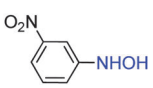
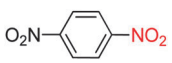
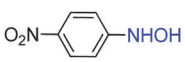
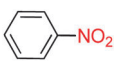
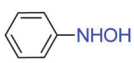
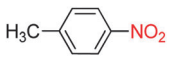
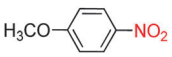
Fig. 1 The reductive process of 4-cyanonitrobenzene catalyzed by *Ba*NTR1. The bio-reduction was performed in 0.1 M sodium phosphate buffer (pH 7.0, 10 mL) at 30 °C with the substrate (100 mM), *Ba*NTR1 (10 mg), *Bm*GDH (10 mg), glucose (400 mM) and $NADP^+$ (0.3 mM).

reduction in the concentration of the cofactor to 0.1 mM, however, resulted in a lower level of conversion (Table 1, entry 5).

Under the optimal reaction conditions (Table 1, entry 4), the 4-cyanonitrobenzene was rapidly reduced to the corresponding hydroxylamine using a small quantity of the biocatalyst. It is noteworthy that the hydroxylamine product was not further reduced to the amine during an extended reaction period (1–24 h), and that the selectivity remained >99% after 24 h (Fig. 1). These results indicated that *Ba*NTR1 was extremely efficient and substrate specific for the controllable reduction of nitroarenes, and that this catalyst did not require strict control of the reaction time or the amount of catalyst added to the mixture to obtain high purity hydroxylamine.

To investigate the substrate scope of the *Ba*NTR1 catalyzed reduction, the optimal conditions were applied to a series of nitroarenes, and the results are shown in Table 2. Pleasingly, nitroarenes bearing a variety of chemically reducible functional groups, including nitrile, carbonyl, amide, and carboxyl (Table 2, entries 2–5) were well tolerated under the optimal conditions and gave the corresponding arylhydroxylamines in >99% selectivity and 100% conversion. Furthermore, the optimal conditions were successfully applied to substrates bearing two nitro groups, with only one nitro-group being

Table 2 Controllable reduction of a variety of nitroarenes using the nitroreductase *Ba*NTR1[a]

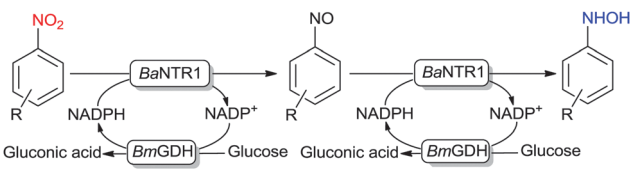
Entry	Substrate	Product	Time/h	Conv.[d] (%)	Sel.[e] (%)	Yield[f] (%)
1			1.0	100	>99	82
2			5.0	100	>99	49
3			3.0	100	>99	65
4			5.0	100	>99	58
5			2.5	100	>99	51
6[b]			4.0	90.5	>99	35
7[b]			1.0	100	98.6	42
8			1.0	100	>99	57
9[c]			1.0	100	98.3	60
10[c]		nd[g]	5.0	99.0	—	—
11[c]		nd[g]	10.0	96.7	—	—

[a] Reduction conditions: substrate (100 mM), *Ba*NTR1 (100 mg, crude enzyme powder (lyophilized cell free extract)), *Bm*GDH (100 mg, crude enzyme powder (lyophilized cell free extract)), glucose (400 mM), $NADP^+$ (0.3 mM), and 0.1 M sodium phosphate buffer (pH 7.0, 100 mL) at 30 °C. [b] The *Ba*NTR1 charge was 200 mg. [c] The *Ba*NTR1 charge was 400 mg. [d] The conversion was determined using HPLC. [e] The selectivity is defined as the molar ratio of hydroxylamine to amine. [f] Isolated yield. [g] nd: the hydroxylamine was not detected.

reduced to the corresponding hydroxylamine (Table 2, entries 6–8). This is possible because the formed product resulted in the change of electronic density of another nitro-group and therefore another nitro-group could not be further reduced by *Ba*NTR1. Similar results have been reported in the previous literature.[7b,9e] The best result was observed at the *para*-position, with excellent (>99%) selectivity and 100% conversion being observed within 1 h (Table 2, entry 8). In the case of the *ortho*-nitro group, the reduction process occurred at a slower rate because of steric hindrance, with a lower conversion of 90.5% being achieved from a larger catalyst charge after 4 h, although the selectivity was still high (>99%) (Table 2, entry 6). For the *meta*-position dinitrobenzene, however, the *Ba*NTR1 charge had to be increased 2-fold to allow the conversion to reach 100% in 1 h, and trace amounts of side products and amine (1.4%) were detected in the reaction mixture (Table 2, entry 7).

We also investigated the reduction of nitrobenzenes bearing electron-donating groups, including *p*-nitrotoluene and *p*-nitroanisole. The results revealed that these substrates did not perform as well as those bearing electron-withdrawing groups. In the absence of any other substituent, the reduction of nitrobenzene required the catalyst charge to be increased 4-fold to give a conversion of 100% and a selectivity of 98.3% in 1 h (Table 2, entry 9). Under the same conditions, *p*-nitrotoluene and *p*-nitroanisole provided conversion levels of 99% and 96.7%, respectively (Table 2, entries 10 and 11), although large amounts of corresponding azoxybenzene as well as trace amounts of the amine were detected in the reaction mixture, suggesting that the hydroxylamine products of these reactions were further reduced to the amines and that the remaining hydroxylamines were rapidly and spontaneously condensed to azoxybenzene (ESI†). This suggestion would explain why the corresponding hydroxylamine was not detected after evaporation.

Based on our observations, we have proposed a mechanism for the reaction, as shown in Scheme 1. In contrast to other reported NTRs that are capable of further reducing arylhydroxylamines to arylamines, it was assumed that *Ba*NTR1 continuously reduces nitroarenes to arylhydroxylamines in two steps *via* the transfer of four electrons. The controllable reduction of nitroarenes bearing an electron-withdrawing group was very efficient with almost 100% conversion and >99% selectivity, whereas the controlled reduction of nitroarenes bearing an electron-donating group was more challenging because the formation of the corresponding amines was also observed. These results showed that nitroarenes bearing a strong electron-donating group required longer reaction times and produced more unidentified side products. In a similar manner to previous reports, azoxybenzene formation was observed to be a spontaneous condensation process.[10] In nitroarenes bearing an electron-withdrawing group, the N–O bond of arylhydroxyamine was strengthened and relatively stable,[11] with only trace amounts of the side product azoxybenzene being formed in the crude product. However, in nitroarenes bearing no electron-withdrawing or electron-donating group, the corresponding hydroxylamine was more unstable, and formed azoxybenzene rapidly.



Scheme 1 Proposed bioreaction pathways for the controllable reduction of nitroarene compounds to the corresponding *N*-aryl hydroxylamines by the nitroreductase *Ba*NTR1 coupled with an NADPH regeneration system (*Bm*GDH).

In summary, we have developed a controllable reduction method for the synthesis of arylhydroxylamines using a highly selective nitroreductase as a biocatalyst. In the reduction of nitroarenes bearing an electron-withdrawing group, this process allowed for the formation of high purity arylhydroxylamines in excellent yield and therefore represents a green, simple and highly efficient process.

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