Organic Process Research & Development

Article

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Development of a Biocatalytic Process to Prepare (*S*)-*N*-Boc-3-hydroxypiperidine

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ABSTRACT: (*S*)-*N*-Boc-3-hydroxypiperidine (*S*-NBHP) is a useful synthon for the synthesis of pharmaceutical intermediates including ibrutinib, the API of the newly approved drug Imbruvica, for the treatment of lymphoma. To our knowledge, there are no published biotransformation methods scalable to prepare *S*-NBHP. We report here the development of an efficient process catalyzed by recombinant ketoreductase (KRED) to reduce *N*-Boc-piperidin-3-one to obtain optically pure *S*-NBHP. The process has been optimized and demonstrated to have commercial potential with 100 g/L of substrate concentration, product of >99% ee with under 5% of enzyme loading (w/w).

INTRODUCTION

A large number of natural and unnatural bioactive molecules have one or more piperidine rings.[1] There are many active pharmaceutical ingredients (API) which also contain this moiety.[2] The hydroxyl group on the C3-position would introduce a chiral carbon atom and may significantly affect the bioactivity of the molecule.[3] As a result, chiral hydroxypiperidines and their derivatives are important synthons used in the pharmaceutical industry.

Several methods have been reported for the synthesis of chiral piperidines including classic diastereomeric resolution,[4] asymmetric synthesis,[5] and asymmetric reduction, for example, of 4-oxo-piperidine-3-carboxylic acid esters followed by multi-step conversions (Scheme 1).[6] All these methods suffered from low yields or lengthy synthesis. Lacheretz et al. reported the bioreduction of cyclic 3-oxo-amines using the tissue of *Daucus carota*, where six piperidin-3-one derivatives could be reduced to *S*-alcohols with varying enantioselectivity.[7] Among the substrates, the reduction of *N*-Boc-piperidin-3-one showed the highest enantioselectivity, giving (*S*)-*N*-Boc-3-hydroxypiperidine (*S*-NBHP) with 95% optical purity. Unfortunately the reaction is not practical due to low substrate concentration (3 mM), high catalyst concentration (23%, m/v), moderate chiral purity (95% ee) and low yield (73%).

Ketoreductase (KRED)-mediated biotransformation has been applied more and more widely in pharmaceutical industry nowadays.[8] In general, there are two strategies to locate a KRED for practical application. On the one hand, the discovery of new natural KRED biocatalysts using activity screening and genome mining continues to enlarge the substrate spectrum.[9] On the other hand, enzyme engineering technologies including directed evolution and semirational and rational design can engineer KREDs with high efficiency and stability.[10] Directed evolution and rational design as the most important technique of protein engineering have emerged to be powerful tools for manipulating protein properties with numerous successful stories.[11] The availability of highly diversified KRED libraries makes it increasingly possible to discover biocatalysts to meet the metrics of industrial processes. Here we report such a work to screen and apply recombinant KREDs to catalyze the asymmetric preparation of *S*-NBHP on an industrial scale.

RESULTS AND DISCUSSION

The target enzymatic reduction of *N*-Boc-piperidin-3-one was proposed as in Scheme 2. We first screened our KRED library including 40 enzymes using 96-well plates. The KRED library was composed of natural enzymes from various sources and engineered enzymes, which had all been used to reduce a certain keto-aldo substrate efficiently in our laboratory.[8b,12a] All the KREDs were expressed in *Escherichia coli* and added into the screening in the form of lyophilized lysates without further purification.

From the results of the first-round screening shown in Table 1, there are 11 hits within the 40 enzymes which showed more than 90% conversion under the screening conditions, indicating good activity on the substrate, *N*-Boc-piperidin-3-one. We chose 10 enzymes with about 99% conversion to carry out the second-round screening in a 15-fold-scale system with the same ratio of enzyme and substrate, to verify the results and measure the product's optical purity. The results are shown in Table 2. It was found that the activity of KR-134 was not as high as the first-round screening, which might be a special case with high experimental deviation. The other nine enzymes showed good activity and a range of different enantioselectivity. Fortunately, the optical purity value of the *S*-NBHP catalyzed by KR-110 was as high as 99.3%. More importantly, KR-110 can use isopropanol (IPA) as the substrate to reduce NAD to NADH, which could avoid adding another enzyme to recycle the coenzyme and adjusting the reaction medium pH.[12]

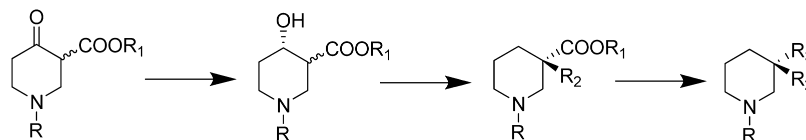
The conversion of the screening system was high because the substrate concentration was low (10 g/L). So the reaction conditions of the process were optimized with higher substrate

Special Issue: Biocatalysis 14

Received: January 20, 2014
Published: April 28, 2014

 © 2014 American Chemical Society dx.doi.org/10.1021/op500022y | *Org. Process Res. Dev.* 2014, 18, 827−830

Scheme 1



Scheme 2

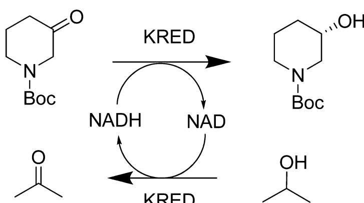


Table 1. Results of first-round screening

Name	Conv. (%)	Name	Conv. (%)	Name	Conv. (%)	Name	Conv. (%)
KR-101	56.6	KR-111	69.6	KR-121	76.8	KR-131	99.9
KR-102	80.6	KR-112	77.1	KR-122	32.0	KR-132	98.8
KR-103	61.5	KR-113	90.5	KR-123	24.4	KR-133	99.9
KR-104	63.1	KR-114	45.9	KR-124	20.6	KR-134	99.9
KR-105	-	KR-115	-	KR-125	50.1	KR-135	99.9
KR-106	-	KR-116	13.6	KR-126	66.5	KR-136	99.9
KR-107	27.0	KR-117	36.5	KR-127	62.1	KR-137	99.9
KR-108	38.6	KR-118	40.5	KR-128	32.6	KR-138	46.4
KR-109	66.6	KR-119	35.9	KR-129	66.4	KR-139	99.5
KR-110	99.9	KR-120	55.7	KR-130	99.5	KR-140	95.6

Table 2. Results of second-round screening

Name	Conv. (%)	eep (%) (Configure)
KR-110	99.9	>99 (*S*)
KR-130	99.9	98 (*R*)
KR-131	99.9	94 (*R*)
KR-132	99.9	95 (*R*)
KR-133	99.9	98 (*S*)
KR-134	53.9	84 (*S*)
KR-135	99.9	98 (*R*)
KR-136	99.9	98 (*R*)
KR-137	99.9	97 (*R*)
KR-139	99.9	75 (*R*)

concentration (100 g/L), testing different temperatures, cosolvents, and other process parameters. The results of reaction temperature optimization are shown in Figure 1. It was

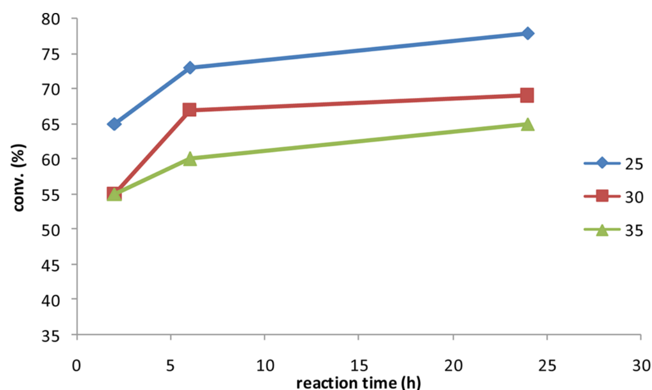


Figure 1. Optimization of the reaction temperature.

found that the reaction temperature optimum was 25 °C, and lower temperatures may be not economical for process control. Most KREDs showed the best activities at 30−40 °C, but higher temperature reduced the reaction rate. In this case, it was indicated that the KR-110 might be sensitive to thermodeactivation. After optimizing the reaction temperature, gram-scale preparation using lower catalyst loading (5% of KR-110 and 0.5% of NAD, w/w) was tested; however, the conversion only reached 91.6% after 24 h.

The KRED-mediated reduction could usually be improved in several aspects, such as substrate dispersity, acetone inhibition, and substrate/product inhibition; thus, further optimization was performed. It was reported that toluene was used as a cosolvent in previous studies.[13] Considering the poor solubility of the substrate, toluene was tested with other common cosolvents (MeOH, EtOH, CH_3CN, DMSO, and toluene) in the reaction system. However, after adding various cosolvents, the highest reaction conversion decreased to 85.8% (data not shown). Thus, it could be concluded that organic solvent (besides IPA) in the system would harm the enzyme reaction. Another beneficial process manipulation in the KRED system using IPA as a cosubstrate is the removal of the acetone from the reactor by either air/nitrogen bubbling, or running the reaction under reduced pressure.[14] When air was bubbled into the medium (0.1 VVM), the final conversion of 90.6% showed it made no improvement. This meant the accumulated acetone in the reaction mixture did not prevent the complete conversion of the substrate. This conclusion could also be made by adding a certain amount of acetone into the reaction mixture initially and comparing with the reaction without acetone. Finally we turned to test the substrate inhibition and added a 50 g/L substrate concentration with the same enzyme/substrate ratio. The reaction conversion reached to 98.6%, and then we added the substrate in two 50 g/L batches after 4 h, with the result of a 97.7% conversion (Figure 2). This showed that substrate inhibition might be a restrictive factor of the reaction, but its detailed mechanism is not clear.

After process optimization, we scaled up the reaction to gram scale. In a 5-g scale-up reaction, the conversion reached 99.8% in 24 h, and the yield was 97.6% with 93% chemical purity.

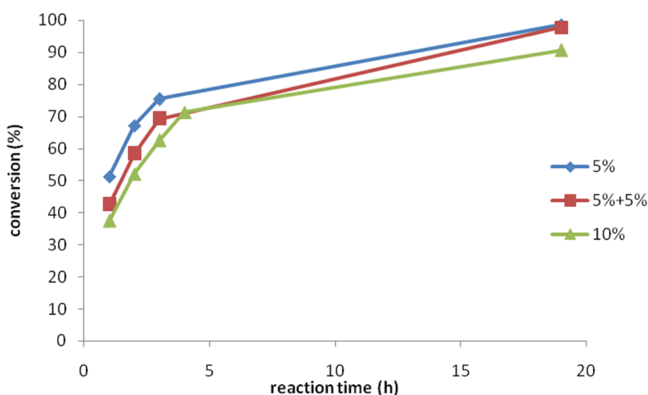


Figure 2. Conversion curves of substrate-adding process.

KRED-catalyzed biosynthesis reactions are regarded as the most promising applications nowadays. A benchmark (100 g/L of substrate concentration, 99% of product optical purity and 5% (w/w) of enzyme/substrate loading) was proposed by S. Luetz et al. to select those practical and scalable biocatalysis processes out of the numerous published articles.[15] In the preparation of *S*-NBHP, our work provided a practical solution for the first time to produce chiral *S*-NBHP using green biotransformation technology. Another standard of KRED process is even higher, proposed by J. Liang et al.[10b] So the enzyme loading and the substrate concentration of the process might be more competitive by further improvement.

■ EXPERIMENTAL SECTION

Chemicals. *N*-Boc-piperidin-3-one was provided by our cooperative partner with a purity of 99% and single impurity <0.3%. All other reagents were of analytical grade and supplied by commercial sources.

Enzyme Screening. The KREDs and glucose dehydrogenase (GDH) were expressed according to published papers[8b,12a] and prepared in the form of lyophilized powder from *E. coli* cell lysate without purification. The first round screening was performed on 96-well plates. Each well contained 10 mg KRED powder, 10 mg GDH powder, 10 mg *N*-Boc-piperidin-3-one, 1 mg NAD, 1 mg NADP, 10 mg glucose, and 1 mL PBS buffer (0.1 M, pH 7.0). After incubation, the plate was stirred at 30 °C for 24 h. The second round screening was performed with 100 mg KRED powder, 100 mg *N*-Boc-piperidin-3-one, 10 mg NAD, 10 mg NADP, and 1 mL IPA in 14 mL PBS buffer (0.1 M, pH 7.0) for 24 h. The reaction medium was sampled and detected by HPLC and extracted by ethyl acetate to detect the chiral purity of the product.

Process Optimization. The process optimization medium was composed with 7.5 mg KR-110 powder, 140 mg *N*-Boc-piperidin-3-one, 0.75 mg NAD, 100 μL IPA and 1.5 mL PBS buffer (0.1 M, pH 7.0). In temperature optimization, 25, 30, 35, and 40 °C were set as the reaction temperature. In cosolvent optimization, 5% (v/v) of cosolvent was added into the reaction system. In the bubbling reaction, air was bubbled with 0.1 VVM rate.

Gram-Scale Preparation. The reaction medium was composed with 250 mg KR-110 powder, 5 g *N*-Boc-piperidin-3-one, 25 mg NAD, 3 mL IPA, and 50 mL PBS buffer (0.1 M, pH 7.0). The reaction was conducted at 25 °C. The substrate was added equally in two batches after 4 h. After 24 h, the medium was filtered through Celite. The filtrate was extracted with equal volumes of ethyl acetate for three times. The filter cake was washed with 10 mL ethyl acetate for three times. The organic layers were combined, evaporated and dried, obtaining 4.25 g yellow liquid (yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44−1.46 (m, 11H, $-(CH_3)_3$, $-CH_2$), 1.73−1.88 (d, 2H, $-CH_2$), 3.04−3.13 (m, 2H, $-CH_2$), 3.54 (s, 1H, −CHOH), 3.73−3.77 (m, 2H, $-CH_2$). MS(ESI): 202 $(M + H)^+$.

Analysis. The HPLC analysis was performed using a Zorbax-C18 (4.6 × 150 mm, 5 μm, Agilent, Shanghai) column with a mobile phase of 40% acetonitrile and 60% water (v/v). The chiral HPLC was performed using a CHIRALPAK IC column (4.6 × 150 mm, 5 μm, DAICEL, Shanghai), with a mobile phase of 5% IPA and 95% *n*-hexane (v/v). The substrate and product were detected at 210 nm, and the retention time was 4.5 min (product) 7.6 min (substrate) and 14.7 min(product at chiral HPLC).

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Notes

The authors declare no competing financial interest.

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