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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.0c01890 • Publication Date (Web): 14 Apr 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

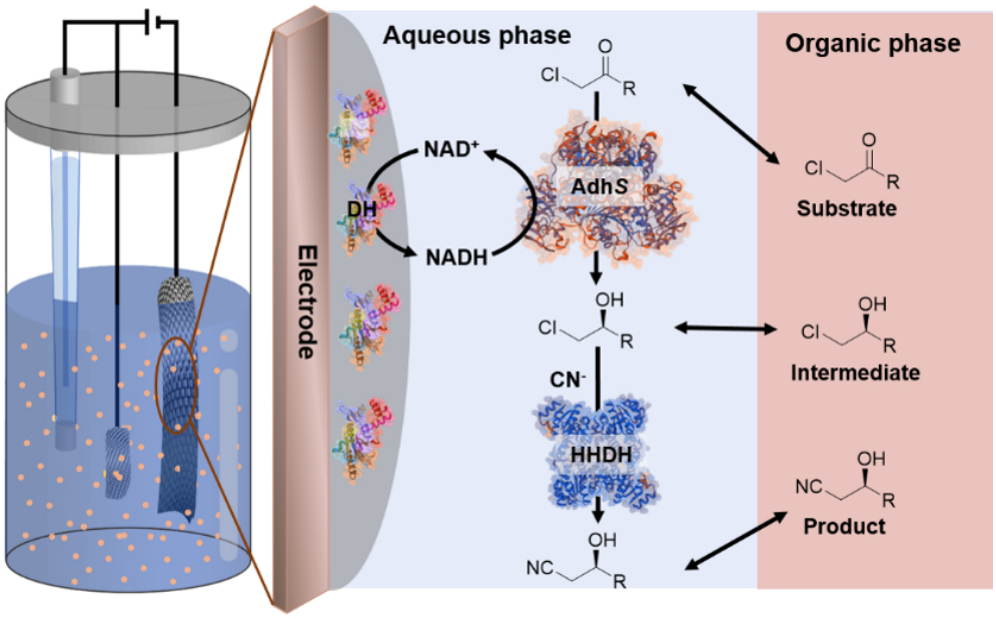

table of contents entry

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Biphasic bioelectrocatalytic synthesis of chiral β-hydroxy nitriles

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Abstract

Two obstacles limit the application of oxidoreductase-based asymmetric synthesis. One is the consumption of high stoichiometric amounts of reduced cofactor. The other is the low solubility of organic substrates, intermediates, and products in the aqueous phase. In order to address the two obstacles of oxidoreductase-based asymmetric synthesis, a biphasic bioelectrocatalytic system was constructed and applied. In this study, the preparation of chiral β-hydroxy nitriles catalyzed by alcohol dehydrogenase (AdhS) and halohydrin dehalogenase (HHDH) was investigated as a model bioelectrosynthesis, since they are high-value intermediates in statin synthesis. Diaphorase (DH) was immobilized by a cobaltocene-modified poly(allylamine) redox polymer on the electrode surface (DH/Cc-PAA bioelectrode) to achieve effective bioelectrocatalytic NADH regeneration. Since AdhS is a NAD-dependent dehydrogenase, the diaphorase modified biocathode was used to regenerate NADH to support the conversion from ethyl 4-chloroacetoacetate (COBE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE) catalyzed by AdhS. The addition of methyl tert-butyl ether (MTBE) as an organic phase not only increased the uploading of COBE, but also prevented the spontaneous hydrolysis of COBE, extended the lifetime of DH/Cc-PAA bioelectrode, and increased the Faradaic efficiency and the concentration of generated (*R*)-ethyl-4-cyano-3-hydroxybutyrate ((*R*)-CHCN). After 10 hours of reaction, the highest concentration of (*R*)-CHCN in the biphasic bioelectrocatalytic system was 25.5 mM with 81.2% enantiomeric excess (ee_p). The conversion ratio of COBE achieved 85%, which was 8.8 times higher than the single-phase system. Besides COBE, two other substrates with aromatic ring structures were also used in this biphasic bioelectrocatalytic system to prepare corresponding chiral β-hydroxy nitriles. The results indicate that the

biphasic bioelectrocatalytic system has the potential to produce a variety of β-hydroxy nitriles with different structures.

Keywords

Bioelectrocatalysis, asymmetric synthesis, biphasic system, cofactor regeneration, chiral β-hydroxy nitriles

INTRODUCTION

Asymmetric synthesis has a prominent position in the chemical industry as enantiomerically pure chemicals are key ingredients in high-value pharmaceutical and agrochemical synthesis.[1] Enzymatic asymmetric catalysis is an effective approach to produce enantiomerically pure chemicals due to the excellent enantioselectivity of enzymes, mild reaction conditions, few by-products, and avoidance of residual metals.[2-4] Enantioselective oxidoreductases, such as alcohol dehydrogenases,[5] amino acid dehydrogenases,[6] amine dehydrogenases,[4] glycerol dehydrogenases,[7] carbonyl reductases, and enoate reductases,[8-9] have been utilized in the preparation of chiral alcohols, amines, and amino acids via asymmetric synthesis and resolution. However, there are two obstacles that limit the real-world application of the oxidoreductase-based asymmetric synthesis. One is the consumption of high stoichiometric amounts of reduced cofactor.[10-11] Consequently, the adequate supply and effective regeneration of reduced cofactor is critical to ensure the smooth reaction. The other issue is the low solubility of organic substrates and products in aqueous phase, which limits the conversion ratio of substrates and the accumulation of products.

Currently, the cofactor regeneration methods can be summarized into four categories: 1) chemical,[12-13] 2) photochemical, [14-15] 3) microbial and enzymatic,[16-18] and 4) electrochemical methods, [19-22] Among these methods, electrochemical cofactor regeneration has the advantages of requiring no addition of a second reductant, avoiding the limitation caused by second substrate/product contamination and inhibition, easier downstream processing and higher catalytic yield. Moreover, the electrode can provide an interface at which the electrochemical potential is readily varied and can easily supply

electrons needed for the regeneration of all kinds of cofactors, including the nature's ubiquitous nicotinamide adenine dinucleotide (NAD/NADH),[20, 22-23] nicotinamide adenine dinucleotide phosphate (NADP/NADPH),[24] flavin mononucleotide (FMN/$FMNH_2$) and flavin adenine dinucleotide (FAD/$FADH_2$), as well as artificial mediators like cobaltocene $Cc(CO_2H)/Cc(CO_2H)_2$,[21, 25] methyl viologen,[26] or toluidine blue O (TBO).[27] The electrochemical cofactor regeneration method has great potential to be applied in the bioelectrosytheses of useful chemicals.[4, 27-30] For example, the electrochemical regeneration of methyl viologen has been used for N_2 and CO_2 fixation catalyzed by MoFe and VFe nitrogenase [21, 25-26] as well as the bioelectrochemical preparation of chiral amine intermediates and amino acid.[4,31] TBO has also been regenerated electrochemically to support decarbonylation of fatty aldehydes for alkanes synthesis.[27] In our previous research, a novel bioelectrode on which the diaphorase was immobilized in a cobaltocene-modified poly(allylamine) redox polymer was developed to realize the effective regeneration of NADH. The regenerated NADH can be used to support a variety of bioelectrosyntheses, including: CO_2 fixation and the production of methanol and propanol. The high efficiency and stability render the bioelectrode among the most promising means of NADH regeneration yet developed.[20]

In order to increase the solubility of hydrophobic substrates in the aqueous phase, the most common approach is the addition of co-solvents, such as DMSO, ethanol, or acetone.[5] However, the solubilizing effect of co-solvents is limited. Moreover, the excessive concentration of co-solvent may cause the denaturation of enzymes. In bioelectrocatalytic systems, the addition of co-solvent may also erode the polymers on electrode surfaces which further decreases the life-time of the modified electrodes. The addition of a second

phase, an organic phase in most cases, to form a biphasic system is an alternative approach to solve this problem. The added organic phase acts as a substrate reservoir and product sink. The organic substrate dissolved in the organic phase at high concentration can be continuously released into the aqueous phase and the generated product can be in situ extracted into the organic phase. This method can simultaneously increase the substrates loading and simplify the extraction process of the product. Meanwhile, the addition of the second phase can also significantly avoid the product or substrate inhibition.[32] Thus, multiphase catalytic processes have been increasingly exploited in pharmaceuticals and fine chemical industries to minimize financial efforts for catalyst recycling and product separation.[33] For organic electrochemical reactions, biphasic systems have been employed to perform some simple conversions. For instance, the biphasic system is usually employed for such a reaction in which the halide undergoes oxidation in the aqueous phase. The ensuing dihalogen or hypohalite can then effect the oxidation of nitroxyl at the aqueous/organic interface,[34] the bromination of cyclic and acyclic enes,[35] and the electro-oxidation of benzyl alcohol.[36-38] In these research examples, the bare electrodes were used to regenerate small inorganic electron mediators to support the redox reactions. For bioelectrocatalysis, only a few attempts of utilizing the biphasic system to produce chiral alcohols have been carried out.[39-40] In these studies, cofactor regeneration was decoupled from the substrate concentration to support the electroenzymatic reduction reaction. The main problems of these studies are the utilization of an expensive rhodium mediator to transfer electrons from electrode to $NAD(P)^+$ and the low conversion ratio of substrates. In comparison with the biphasic electrocatalytic system, the biphasic bioelectrocatalytic system is more complicated. The effect of the addition of organic phase on enzyme activity,

the stability of electrodes (especially the polymer or enzyme-modified electrode), and the cofactor regeneration efficiency are all challenging issues.

Chiral β-hydroxy nitriles are versatile building blocks for the synthesis of pharmaceutical compounds like β-blockers, cholesterol-lowering drugs,[41], and antidepressants.[1] For instance, ethyl *(R)*-4-cyano-3-hydroxybutyrate is an important intermediate to synthesize statins (HMG-CoA reductase inhibitors) which are the active ingredient of Lipitor. Lipitor is the first drug in the world with annual sales to exceed $10 billion.[41] As a result of their exceedingly high market value and the requirement for high chemical and optical purity, an efficient method for the production of chiral β-hydroxy nitriles is highly desired. Enzymatic synthesis of chiral β-hydroxy nitriles can be summarized into two types. The first type is kinetic resolution which involves lipase-catalyzed hydrolysis or esterification[42] and stereoselective ring-opening of racemic epoxide catalyzed by halohydrin dehalogenase.[43] The limitation of this method is the low yields of kinetic resolution (the maximum theoretical yield is only 50%). The second type is asymmetric synthesis which involves the asymmetric reduction of β-keto nitriles by oxidoreductase or bioreduction of chloroacetoacetate catalyzed by chiral alcohol dehydrogenase and halohydrin dehalogenase.[44-45] Chen et. al.[16] realized biosynthesis of chiral β-keto nitriles via the co-expression of enantioselective alcohol dehydrogenases, dehydrogenase for cofactor regeneration, and a halohydrin dehalogenase in *E. coli* cell. The enantioselective alcohol dehydrogenase was used to introduce the chiral center by reducing β-halo ketone to chiral β-halo alcohols.[5] Reduced cofactor was regenerated by co-expressed formate dehydrogenase or glucose dehydrogenase at the cost of ammonium formate or 2-propanol as a cosubstrate. The reduced chiral β-halo alcohols were finally converted to β-hydroxy

nitriles via the dehalogenation and cyanation substitute catalyzed by halohydrin dehalogenase. In comparison with kinetic resolution, the advantage of asymmetric synthesis is the 100% maximum theoretical yield, which makes asymmetric synthesis catalyzed by alcohol dehydrogneases and halohydrin dehalogenase a more promising method to prepare chiral β-hydroxy nitriles.

In this study, we constructed an efficient biphasic bioelectrocatalytic system which contains a diaphorase (DH, EC 1.6.99.3) from *Geobacillus stearothermophilus*, a (*S*)-specific alcohol dehydrogenase from *Lactobacillus kefir*[5] (LK-AdhS, EC 1.1.1.1) and a mutant halohydrin dehalogenase (HHDH, EC 4.5.1.-) from *Agrobacterium radiobacter* [46] to perform the synthesis of β-hydroxy nitriles. In this biphasic bioelectrocatalytic system, DH was immobilized by a low-potential redox polymer cobaltocene-modified poly-(allylamine) on the surface of the electrode (DH/Cc-PAA bioelectrode) to realize the effective regeneration of NADH to support the reductive reaction catalyzed by AdhS. In order to increase the substrate concentration, avoid the spontaneous hydrolysis of the substrate, and extend the life-time of the electrode, methyl tert-butyl ether (MTBE) was added as the second organic phase. The use of MTBE as the second organic phase decoupled the cofactor concentration from the substrate concentration. The organic phase can continuously supply fresh substrate while accumulating products to a higher concentration.

RESULT AND DISCUSSION

Design and construction of the biphasic bioelectrocatalytic system

As shown in **Figure 1**, the reaction cascade which converted ethyl 4-chloroacetoacetate (COBE) to (*R*)-ethyl-4-cyano-3-hydroxybutyrate ((*R*)-CHCN) was composed by AdhS and

HHDH. The initial substrate, COBE, was firstly reduced by AdhS to generate ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE) with the consumption of NADH. Then, CHBE was converted to ((*R*)-CHCN) through dehalogenation and cyanation substitute catalyzed by HHDH. As HHDH does not possess enantioselectivity, the optical purity was determined by the enantioselectivity of AdhS. In order to regenerate NADH to support the reaction catalyzed by AdhS, a DH/Cc-PAA bioelectrode was employed. DH was immobilized by a low-potential redox polymer, Cc-PAA, on the electrode surface. Herein, the electrode was the primary electron donor. The Cc-PAA polymer was able to meditate electrons from electrode to DH and further facilitate the NADH regeneration catalyzed by DH. The use of a porous carbon cloth electrode increased the conductivity and the specific surface area of the DH/Cc-PAA electrode, which further enhanced the NADH regeneration ability of the electrode. In order to increase the substrate concentration, the organic phase was added to form a biphasic bioelectrocatalytic system. The organic phase acted as a substrate reservoir and product sink which was able to continuously release COBE to the aqueous phase and extract produced CHCN to the organic phase. Moreover, COBE was reported to have an inhibitory effect on the activity of HHDH.[47] The use of the organic phase which decoupled the substrate from the aqueous phase effectively alleviated the inhibition of COBE towards HHDH.

Selection of organic phase

In order to select an appropriate organic phase for the biphasic bioelectrocatalytic system, four organic solvents including hexane, methyl tert-butyl ether (MTBE), ethyl acetate, and dichloromethane (DCM) were examined. As shown in **Figure 2a**, the addition of the organic phase had an obvious effect on the activity of AdhS and HHDH. Compared with

the Tris-HCl buffer single-phase reaction system, the addition of hexane, ethyl acetate, and DCM decreased the activity of AdhS and HHDH. In contrast, the addition of MTBE increased the activity of AdhS and HHDH slightly. On this basis, the partition number (the ratio of the concentration of target compound in the organic phase to that in aqueous phase) of COBE, CHBE, and CHCN in the Tris-HCl buffer/MTBE biphasic system was further measured (**Figure 2b**). The partition number of COBE was determined to be 9.1 suggesting that > 90% COBE remained in the MTBE phase. This was beneficial to relieve the inhibition of COBE to HHDH. For the intermediate, CHBE, the partition number was determined to be 47.1. The partition number of the final product, CHCN, was determined to be 24.1 suggesting that > 96% CHCN was extracted in the MTBE phase. Based on the above results, MTBE was used as the organic phase in the biphasic bioelectrocatalysis system.

Electrochemical capability of DH/Cc-PAA bioelectrode in biphasic bioelectrocatalytic system

After selecting MTBE as the organic phase, the ratio of aqueous phase to organic phase was further optimized. As shown in **Figure S5**, the DH/Cc-PAA electrode had the highest current response at the ratio of 5:1 and 5:2. Considering that more organic phase is good for increasing substrate loading, 5:2 was the optimal ratio that was used in this study.

Cyclic voltammetric (CV) analysis was used to evaluate the electrochemical capability of DH/Cc-PAA bioelectrode in the Tris-HCl/MTBE biphasic system. As shown in **Figure 3**, without the addition of NAD^+, the Cc/Cc^+ redox couple in both biphasic and single-phase systems was reduced at approximately −0.83 V vs Ag/AgCl. Upon addition of 20 mM NAD^+, current responses at -0.83 V vs. Ag/AgCl were observed in both biphasic and single-phase systems. This indicates that cobaltocene pendant of Cc-PAA mediated the

electron transfer from the electrode to DH. DH further transported the electrons to NAD^+ to realize the regeneration of NADH. More importantly, the addition of MTBE as an organic phase did not have a negative impact on the electrochemical capacity of DH/Cc-PAA bioelectrode. In order to minimize the overpotential applied while ensuring the quotient of the Co(II)/Co(III) couple shifted toward to the reduced Co(II) species, the operation potential of DH/Cc-PAA electrode for the regeneration of NADH was set at -0.85 V vs. Ag/AgCl.

Potentiostatic electrolysis analysis of the reaction process and NADH regeneration

The amperometric *i*-t analysis was used to analyze the reaction process of the biphasic system. As shown in **Figure 4a**, the entire reaction process was separated into two stages by two injections. Upon the 1st injection, 1 mM NAD^+ was added into the biphasic system. An obvious current response was observed, which indicated the reduction of NAD^+ catalyzed by DH/Cc-PAA bioelectrode. Upon the 2nd injection of 1 mg/mL AdhS, 0.3 mg/mL HHDH, 30 mM COBE and 50 mM NaCN into the biphasic system, a larger current response was observed. The added AdhS converted COBE to CHBE with the consumption of NADH, which further accelerated the electron transfer from the electrode to NAD^+. For the control electrode, the denatured DH was immobilized by Cc-PAA on the electrode surface. No catalytic current response was obtained after two times injection. The *in situ* detection of NADH was also performed to track the NADH concentration during the reaction process. The DH/Cc-PAA bioelectrode was able to regenerate NADH at a rate of 61 ± 0.21 μM/min/mg DH and the NADH accumulation and keep stable at approximately 600 μM within 15 min (**Figure 4b**). Upon the addition of 1 mg/mL AdhS and 30 mM COBE, NADH concentration rapidly decreased due to the consumption by NADH to

reduce COBE by AdhS. The NADH concentration in the product solution was soon observed to recover which demonstrated the NADH regeneration ability of the DH/Cc-PAA bioelectrode (**Figure 4b**). The equilibrium concentration of NADH was at the same level as the K_m value of AdhS towards NADH (585 μM, **Figure S7**). That result means the amount of generated NADH by the DH/Cc-PAA bioelectrode can ensure the actual reaction rate of AdhS achieves approximately 50% of its maximum reaction velocity. The utilization of the DH/Cc-PAA bioelectrode to regenerate NADH and do CO_2 fixation in a single-phase system was first developed in our previous research.[20] In this current study, the DH/Cc-PAA bioelectrode was further used in the biphasic reaction system to realize the production of chiral β-hydroxy nitrile. The equilibrium concentration of regenerated NADH was also analyzed to evaluate how well the cofactor regeneration ability of the DH/Cc-PAA bioelectrode matches the reaction rate of oxidoreductase. For traditional biocatalytic cofactor regeneration, the most commenly used enzymes are glucose dehydrogenase (GDH) and formate dehydrogenase (FDH). These two enzymes catalyze the oxidation of glucose and formate, respectively, to realize the regeneration of reduced cofactor. As the reaction proceeds, the declining concentration of glucose or formate causes a decrease in the rate of cofactor regeneration. In order to keep a high regeneration rate of cofactor, substrates need to be added into the reaction system which is subsequently not fully consumed, contaminating the product solution and increasing the cost of downstream separation and purification.[48-49] In this study, DH utilizes the electrons from the electrode to realize the electrochemical regeneration of the cofactor without the consumption of any other substrate. Moreover, the regeneration rate of the cofactor can be kept stable without being affected by the variation of substrate concentration. Based on the advantage discussed

above, the electrochemical regeneration of cofactor with the use of DH has the potential to replace the traditional biocatalytic cofactor regeneration method.

Bioelectrosynthetic production of CHBE

The reaction was performed in a sealed reactor. As the specific activity of AdhS is significantly lower than that of HHDH, the pH value of the bioelectrosythetic system was set to be the optimum pH of AdhS (pH = 8, **Figure S4**) to ensure AdhS has the highest specific activity. Under this pH value, although the specific activity of HHDH is approximately one-fourth of its highest specific activity, it was still much higher than that of AdhS. Tris-HCl buffer (100mM, pH = 8) was used as the aqueous phase and MTBE was used as the organic phase. The volume ratio of aqueous phase to organic phase was set at 5:2 (v/v). As CHBE was the precursor of CHCN, the production of CHBE was firstly measured. As shown in **Figure S6**, 1 mg/mL (0.12 U/mL) AdhS is an optimized concentration. At this concentration, the conversion ratio of 30 mM COBE achieved approaching 100%. Lower concentration of AdhS caused low conversion ratio of COBE. As shown in **Figure 5a**, the final concentration of the produced CHBE was 29 ± 3.2 mM in the biphasic bioelectrocatalytic system after 8 hours reaction. Almost 100% conversion ratio of COBE was achieved. During the reaction, the Faradaic efficiency exhibited a steady and slow decline without obvious fluctuation. After 10 hours of reaction, the Faradaic efficiency of the biphasic system continuously decreased from 34.8% to 28.5%. Compared with the biphasic bioelectrocatalytic system, the final concentration of CHBE of the single-phase system was 8 mM after 6 hours of reaction. The conversion ratio of COBE was only 26%. For the faradaic efficiency of the single-phase bioelectrocatalytic system, it exhibited a continuous downward trend during the entire reaction process. The

highest Faradaic efficiency was achieved after 30 min reaction, which was 27%. After 10 hours of reaction, the Faradaic efficiency was only 5%. The low conversion ratio of COBE of the single-phase system was due to the spontaneous hydrolysis of COBE in basic aqueous conditions and the dissolution of Cc-PAA polymer in the existence of DMSO as co-solvent. As shown in **Figure S8**, the concentration of COBE in the single-phase system decreased continuously due to the spontaneous hydrolysis of COBE at the basic aqueous conditions. In contrast to the single-phase system, the concentration of COBE in the biphasic system could be kept stable for 10 hours. This result indicated that the addition of MTBE as an organic phase decoupled COBE from the basic conditions and further effectively prevented spontaneous hydrolysis. For the DH/Cc-PAA bioelectrode, it was also more stable in the biphasic bioelectrocatalytic system (**Figure S9)**. The current response of DH/Cc-PAA electrode in the single-phase system decreased rapidly which indicated the life-time of the DH/Cc-PAA electrode was short and rapidly lost the capability of NADH regeneration. In contrast to the single-phase bioelectrocatalytic system, the DH/Cc-PAA bioelectrode was more stable in the biphasic system. The low stability of DH/Cc-PAA bioelectrode in the single-phase system was due to that the Cc-PAA polymer was dissolved by DMSO as DMSO was used as the solvent in the synthesis of Cc-PAA (see Supporting information). The use of the biphasic system avoids the addition of DMSO as co-solvent and further extends the life-time of DH/Cc-PAA bioelectrode.

Bioelectrosynthetic production of chiral β-hydroxy nitriles

In order to ensure the reaction catalyzed by HHDH is not the limiting step, excessive HHDH was added into the reaction system. In the biphasic system, in the presence of approximately 3 mM COBE (30 mM substrate, partition number = 10), the specific activity

of HHDH is 1.07 U/mL. 0.3 mg/mL (0.3 U/mL) HHDH was added into the reaction system to ensure the amount of HHDH is in excess. As shown in **Figure 6**, the concentration of generated CHCN increased during the time course. For the single-phase bioelectrocatalytic system, the highest concentration of CHCN was 2.9 mM after 10 hours reaction. For the biphasic bioelectrocatalytic system, the highest concentration of CHCN achieved 25.5 ± 2.2 mM, which was 8.8 times higher than that of the single-phase system. The conversion ratio of the initial substrate, COBE, achieved 85%. The addition of MTBE as the organic phase significantly improved the conversion from COBE to CHCN due to the increased the uploading of COBE without spontaneous hydrolysis of COBE and the extended lifetime of the DH/Cc-PAA bioelectrode. As shown in **Figure S3**, the initial substrate, COBE, could inhibit the activity of HHDH. In the biphasic system, the concentration of COBE in the aqueous phase was approximately 3 mM as the partition number of COBE is 10. At this concentration, the specific activity of HHDH decreases from 2.88 U/mg to 1.07 U/mg. For the single-phase system, the presence of 30 mM COBE reduced the specific activity of HHDH to 0.07 U/mg. The organic phase acts as the substrate reservoir stores most COBE and largely relieves the inhibition effect of COBE on the specific activity of HHDH. Separating the reaction catalyzed by AdhS and HHDH is another option to eliminate the inhibition effect of COBE on HHDH activity. In contrast, the one-pot reaction is a more promising method as the simple reaction process without additional separation steps. The inhibition effect of COBE on the activity of HHDH can be relieved, even eliminated, via the modification of HHDH. Protein engineering (rational design and directed evolution) methods can be employed to enhance the tolerance of HHDH to COBE. The HHDH used in this study is a modified one (F326V/W249F).[47] The tolerance to COBE

has been significantly enhanced. It can be expected that the inhibition effect of COBE on the activity of HHDH could be completely eliminated via constant iterative mutation. The enantiomeric excess of generated CHCN (ee_p, **Table 1**) was 81.2%. Besides COBE, another two substrates with aromatic rings, 2-chloroacetophenone and 1-chloro-3-phenylacetone, were used as substrates to produce corresponding aromatic chiral β-hydroxy nitriles ((*R*)-3-hydroxy-3-phenylpropanenitrile and (*S*)-3-hydroxy-4-phenylbutanenitrile). As shown in **Table 1**, after 10 hours reaction, the concentration of generated (*R*)-3-hydroxy-3-phenylpropanenitrile was 4.8±0.97 mM with 96.8 % ee_p values. The concentration of generated (*S*)-3-hydroxy-4-phenylbutanenitrile was 7.8± 0.90 mM with 94.6% ee_p values.

By using the traditional biocatalysis method, the chiral β-hydroxy nitrile has been successfully produced. In Ma and coworkers' research,[50] they reported a highly efficient two steps process using ketoreductase (KRED), glucose dehydrogenase and halohydrin dehalogenase to synthesize ethyl (*R*)-4-cyano-3-hydroxybutyrate with COBE as substrate. In the first step, with 160 g/L (~ 1M) of COBE and 0.9 mg/mL KRED and 1.64M glucose as a sacrificial reagent for NADPH regeneration, 96% conversion ratio of COBE and >99.5% ee_p of (*S*)-CHBE were achieved after 8 hours of reaction. In the second step, with1.2 g/L HHDH, approximately 780mM (*R*)-CHCN was obtained with >99.5% ee_p after 5 hours reaction. Due to the higher activity and higher enantioselectivity of KRED compared with AdhS, the space-time yield and optical purity of produced (*R*)-CHCN was higher than that of this study. However, this process is a separate one. It requires an extra CHBE separation step before the second reaction, which complicates the operation. Moreover, KRED is a $NADP^+$ dependent reductase. the higher price and lower stability of $NADP^+$ limit the

application of this enzyme.[51-52] In this study, the one-pot way of reaction simplified the operation. The biphasic way of reaction allowed for the extraction of the product. In Chen and coworkers' study, AdhS, HHDH, and formate dehydrogenase were co-expressed in one *E.coli* cell. The recombinant *E.coli* whole cells were used to perform the conversion from COBE to (*R*)-CHCN with 200 mM ammonium formate as a sacrificial reagent for NADH regeneration.[16] After 24 hours of reaction, 10 mM COBE was converted to (*R*)-CHCN with 60.2% ee_p. A common problem of the traditional biocatalytic method is the addition of a high concentration of sacrificial reagent for cofactor regeneration. In this study, inexpensive and/or renewable electricity can be used as an electron source for the cofactor regeneration. In addition, the Cc-PAA polymer and DH were immobilized on electrodes, not in the electrolyte, which was conducive to the improvement of mass transfer and the product separation after the reaction.

CONCLUSION

In this study, a biphasic bioelectrocatalytic system was constructed and applied to synthesis chiral β-hydroxy nitriles. On the basis of previous research, the DH/Cc-PAA bioelectrode was first used in a biphasic reaction system to realize regeneration of NADH the production of chiral β-hydroxy nitrile. The equilibrium concentration of regenerated NADH was further analyzed to evaluate how well the cofactor regeneration ability of the DH/Cc-PAA bioelectrode matches the reaction rate of oxidoreductase. In comparison with the traditional biocatalytic cofactor regeneration method, the bioelectrocatalytic NADH regeneration with the utilization of the DH/Cc-PAA bioelectrode does not require the addition of cofactor regeneration enzymes and the sacrificial substrates. The addition of MTBE as an organic phase did not disrupt the electron transfer between electrode and NADH. More importantly,

the organic phase increased the concentration of initial substrate, prevented the spontaneous hydrolysis of COBE, avoided the dissolution of the Cc-PAA polymer in the existence of DMSO, extended the life-time of the DH/Cc-PAA bioelectrode, relieved the inhibition effect of COBE on HHDH specific acitivty, and finally significantly increased the conversion ratio of COBE, the Faradaic efficiency of the conversion process, and the concentration of generated (*R*)-CHCN. In addition, the addition of the organic phase also caused the in situ extraction of the generated chiral β-hydroxy nitrile, which simplified the purification process of the product. Beside COBE, two other substrates with aromatic rings were used in this biphasic bioelectrocatalytic system as well to prepare the corresponding chiral β-hydroxy nitriles. All the generated three products had high optical purity, which indicated the biphasic bioelectrocatalytic system has the potential to produce a variety of chiral β-hydroxy nitriles.

ASSOCIATED CONTENT

Supporting Information. This Supporting Information is available free of charge via the Internet at http://pubs.acs.org. Material and methods, SDS-PAGE gel of enzymes used in this study, ^{1}H-NMR spectrum of synthesized Cc-PAA, The inhibition effect of COBE on the specific activity of HHDH, pH optimization of AdhS and HHDH, The optimization of volume ratio of aqueous phase to organic phase (MTBE), The optimization of AdhS concentration, Apparent Michaelis-Menten kinetics of AdhS corresponding to different concentration of NADH, The spontaneous hydrolysis of COBE following time course, representative CV of DH/Cc-PAA bioelectrode in one-phasic and biphasic system, the GC-Mass analysis of the generation of CHCN in two phasic reaction, ^{1}H-NMR spectrum of CHCN produced by biphasic bioelectrocatalytic system, the GC-Mass analysis of the

generation of 3-hydroxy-3-phenylpropanenitrile in two phasic reaction, ^{1}H-NMR spectrum of 3-hydroxy-3-phenylpropanenitrile produced by biphasic bioelectrocatalytic system, the GC-Mass analysis of the generation of 3-hydroxy-4-phenylbutanenitrile in two phasic reaction, ^{1}H-NMR spectrum of 3-hydroxy-4-phenylbutanenitrile produced by biphasic bioelectrocatalytic system, and the SFC analysis of the enantiomeric excess of the generated (*R*)-CHCN, (*R*)-3-hydroxy-3-phenylpropanenitrile and (*S*)-3-hydroxy-4-phenylbutanenitrile (PDF).

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NOTE

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was mainly supported by the National Science Foundation Center for Synthetic Organic Electrosynthesis for funding (CHE-1740656).

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Figure captions

Figure 1. The schematic representation of the biphasic bioelectrocatalytic system for the preparation of (*R*)-CHCN.

Figure 2. The selection of organic phase. A) The activities of AdhS and HHDH in different biphasic systems; B) phase partition number (the ratio of the concentration of target compound in the organic phase to that in aqueous phase) of COBE, CHBE and CHCN in Tris-HCl buffer/MTBE biphasic system.

Figure 3. Representative cyclic voltammograms (CV) of DH/Cc-PAA bioelectrode in biphasic (orange) and single-phase (blue) system with (solid) and without (dash) 20 mM NAD^+. CVs were conducted at a scan rate of 2 $mV \cdot s^{-1}$ in Tris-HCl buffer (100 mM, pH = 8) under anaerobic condition. For the biphasic bioelectrocatalytic system, MTBE was used as the organic phase. The volume ratio of the aqueous phase and the organic phase was 5:2 (v/v).

Figure 4. (a) Amperometric *i*-t analysis of the biphasic bioelectrocatalytic system. The biphasic bioelectrocatalytic system with DH/Cc-PAA or denatured DH/Cc-PAA bioelectrode was sequentially supplemented with 1 mM NAD^+ (1^s injection), 1 mg/mL AdhS, 0.3 mg/mL HHDH, 30 mM COBE and 50 mM NaCN (2^{nd} injection) at 40 ^oC. The experiments were performed in sealed vials with string. The headspace was filled with argon. The volume ratio of the aqueous phase and the organic phase was 5:2 (v/v). -0.85 V vs Ag/AgCl was applied; (b) Monitoring the NADH concentration during the time course. After 18 min of reaction, 1 mM NAD^+, 1 mg/mL AdhS, 0.3 mg/mL HHDH, 30 mM COBE, and 50 mM NaCN were added simultaneously. The volume ratio of the aqueous phase and the organic phase was 5:2 (v/v). -0.85 V vs Ag/AgCl was applied.

Figure 5. (a) The concentration of generated CHBE in the biphasic and single-phase bioelectrocatalytic system. (b) Faradaic efficiency following a 10 h time course. For the single-phase bioelectrocatalytic system, the reactions contain DH/Cc-PAA bioelectrode, Tris-HCl buffer (100 mM, pH = 8), 1 mM NAD^+, 1 mg/mL AdhS, 30 mM COBE, and 5% DMSO as co-solvent. For the biphasic bioelectrocatalytic system, the reactions contain DH/Cc-PAA bioelectrode, Tris-HCl buffer (100 mM, pH = 8), 1 mM NAD^+, 1 mg/mL AdhS, 30 mM COBE, and MTBE as organic phase. The volume ratio of the aqueous phase and the organic phase was 5:2 (v/v).

Figure 6. The concentration of generated CHCN in the biphasic and single-phase bioelectrocatalytic system following a 10 h time course. For the single-phase bioelectrocatalytic system, the reactions contain DH/Cc-PAA bioelectrode, Tris-HCl buffer (100 mM, pH = 8), 1 mM NAD^+, 1 mg/mL AdhS, 0.3 mg/mL HHDH, 30 mM COBE, 50 mM NaCN and 5% DMSO as co-solvent. For the biphasic bioelectrocatalytic system, the reactions contain DH/Cc-PAA bioelectrode, Tris-HCl buffer (100 mM, pH = 8), 1 mM NAD^+, 1 mg/mL AdhS, 0.3 mg/mL HHDH, 50 mM NaCN, 30 mM COBE, and MTBE as organic phase. The volume ratio of the aqueous phase and the organic phase was 5:2 (v/v).

Figure 1.

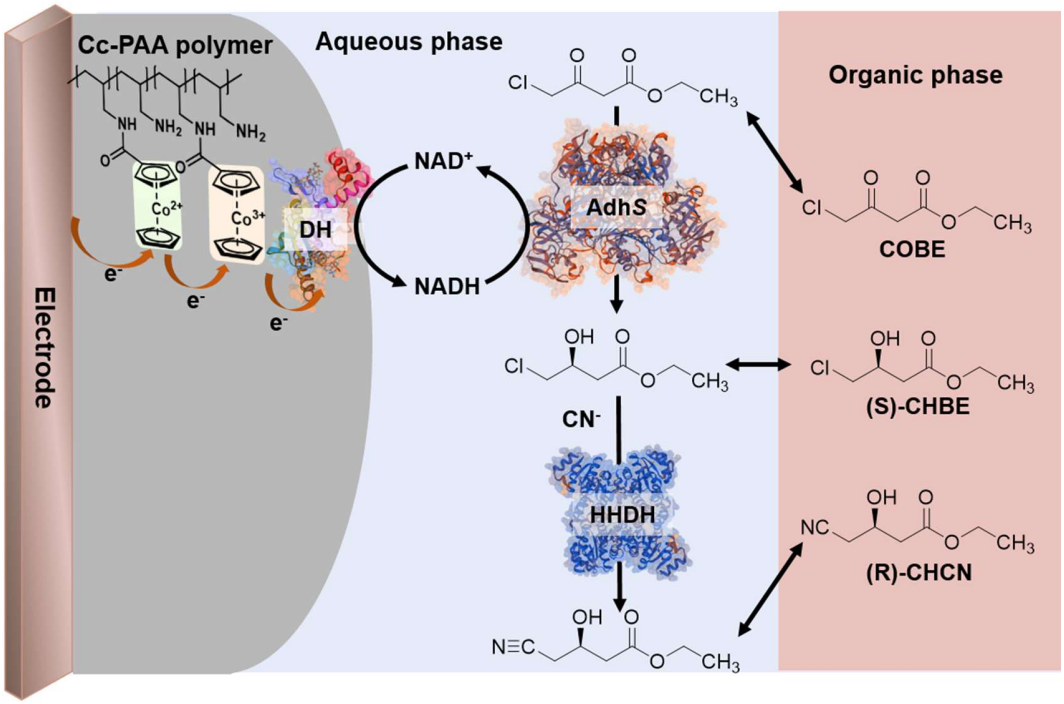

Cc-PAA polymer
Aqueous phase
Organic phase
Electrode
DH
NAD+
NADH
AdhS
COBE
CN-
(S)-CHBE
HHDH
(R)-CHCN
e-
e-
e-

Figure 2.

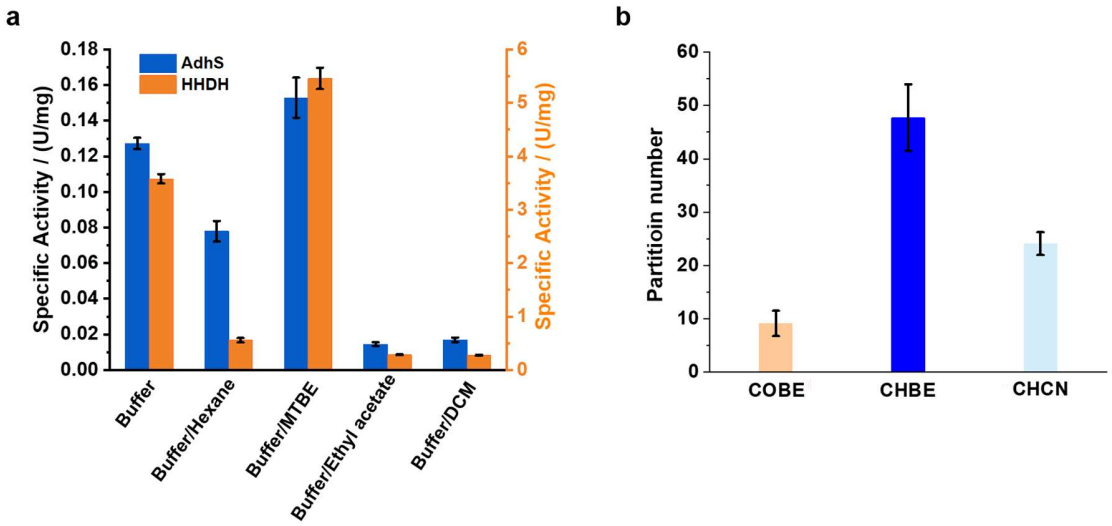

a
AdhS
HHDH
Specific Activity / (U/mg)
Specific Activity / (U/mg)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
6
5
4
3
2
1
0
Buffer
Buffer/Hexane
Buffer/MTBE
Buffer/Ethyl acetate
Buffer/DCM
b
Partitioin number
60
50
40
30
20
10
0
COBE
CHBE
CHCN

Figure 3.

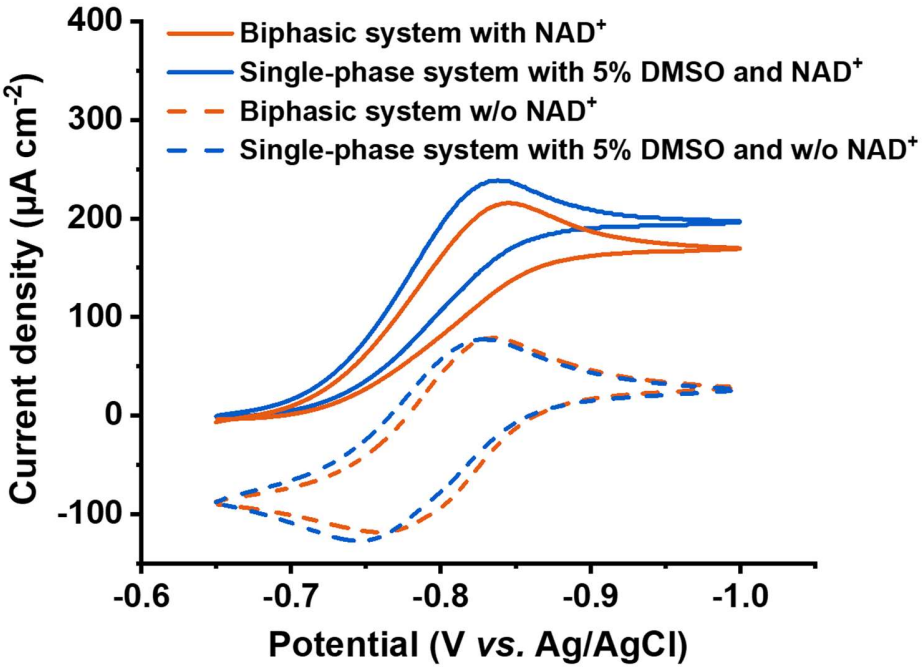

Biphasic system with NAD+
Single-phase system with 5% DMSO and NAD+
Biphasic system w/o NAD+
Single-phase system with 5% DMSO and w/o NAD+
Current density (μA cm-2)
400
300
200
100
0
-100
-0.6
-0.7
-0.8
-0.9
-1.0
Potential (V vs. Ag/AgCl)

Figure 4.

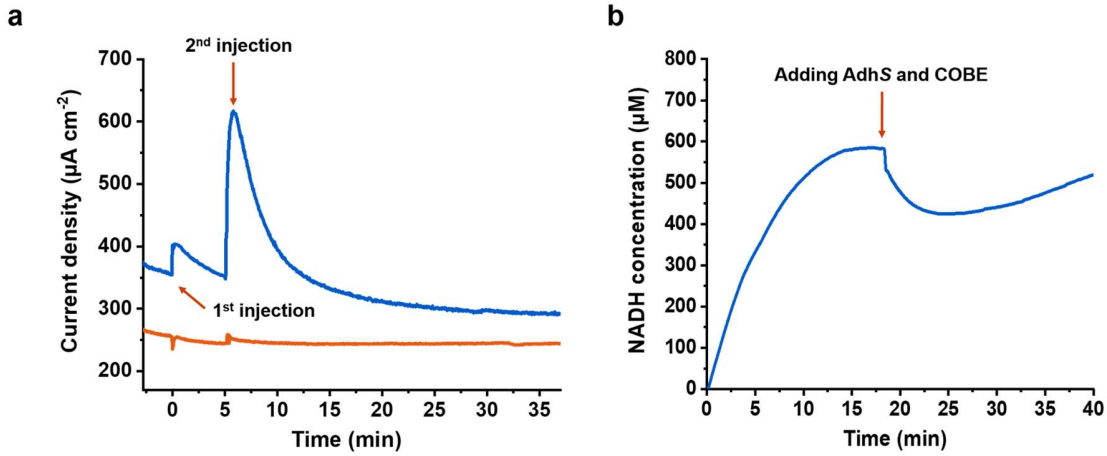

a
2nd injection
1st injection
Current density (μA cm-2)
Time (min)
b
Adding AdhS and COBE
NADH concentration (μM)
Time (min)

Figure 5.

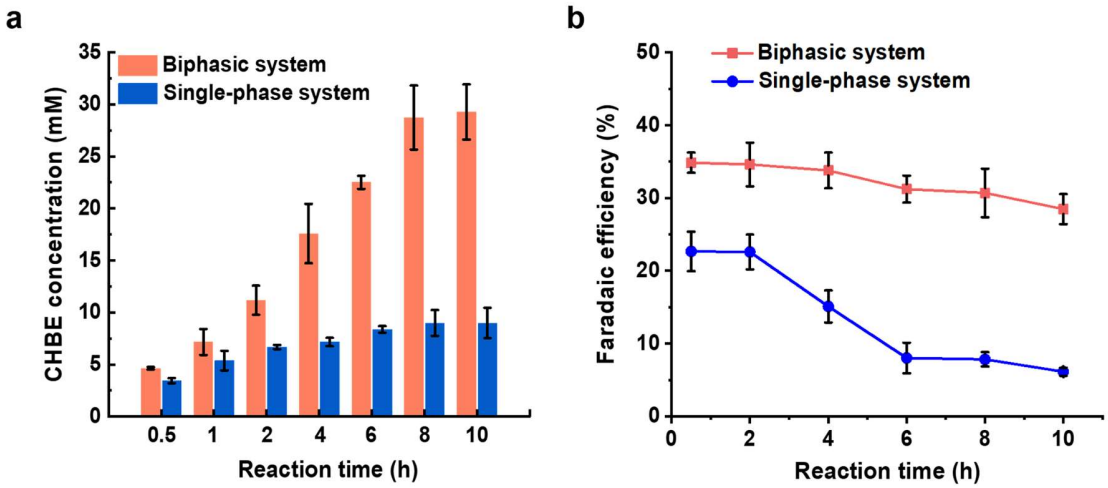

a
Biphasic system
Single-phase system
CHBE concentration (mM)
Reaction time (h)
b
Biphasic system
Single-phase system
Faradaic efficiency (%)
Reaction time (h)

Figure 6.

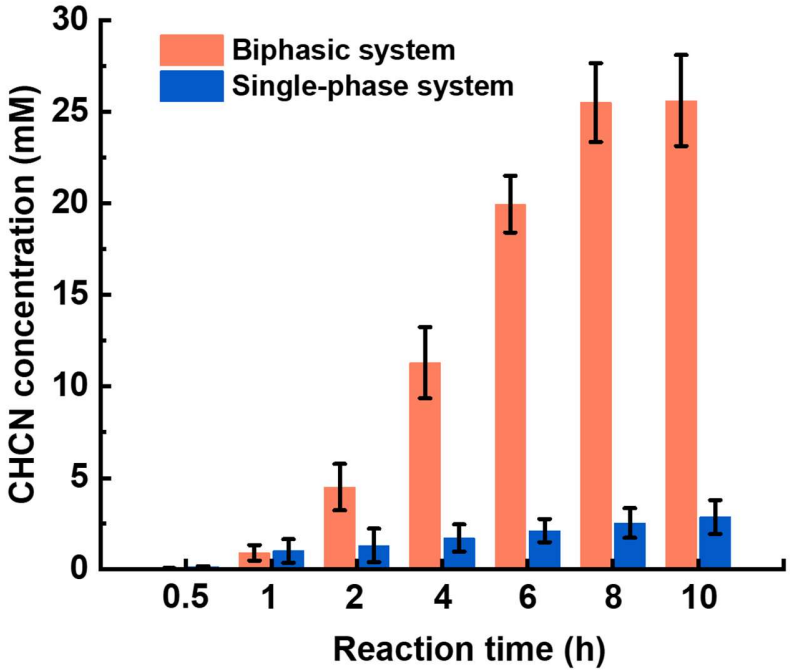
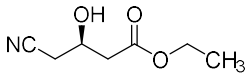
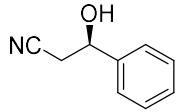
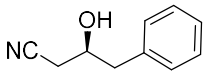

Biphasic system
Single-phase system
CHCN concentration (mM)
30
25
20
15
10
5
0
0.5
1
2
4
6
8
10
Reaction time (h)

Table 1. The preparation of chiral β-hydroxy nitriles with different structure by the single-phase and biphasic bioelectrocatalytic system[a]

Products	Single-phase system			Biphasic system		
	Product concentration (mM)	ee_p[b]	Yield rate ($mmol \cdot L^{-1} \cdot h^{-1}$)	Product concentration (mM)	ee_p[b]	Yield rate ($mmol \cdot L^{-1} \cdot h^{-1}$)
 **(*R*)-CHCN**	2.9 ± 0.62	ND[c]	0.29	25 ± 2.2	81.2%	2.5
 **(*R*)-3-hydroxy-3-phenylpropanenitrile**	0.65 ± 0.11	ND[c]	0.065	4.8 ± 0.97	96.8%	0.48
 **(*S*)-3-hydroxy-4-phenylbutanenitrile**	1.9 ± 0.31	ND[c]	0.19	7.8 ± 0.90	94.6%	0.78

[a] All values correspond to the mean (n=3). Errors correspond to one standard deviation.

[b] In [%]. Determined by SFC.

[c] No detection.

Table of content