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Asymmetric azidohydroxylation of styrene derivatives mediated by a biomimetic styrene monooxygenase enzymatic cascade

Artificial nicotinamide coenzyme biomimetics provide an alternative to the native NAD(P)H to promote biocatalysis, and can act as an electron donor to reduce flavins in free solution. In this work, a flavin was reduced to fuel a one-pot enzymatic cascade for the asymmetric azidohydroxylation of styrenes. First the asymmetric epoxidation of styrenes was catalysed by a flavin-dependent monooxygenase with the coenzyme biomimetic. Then regioselective epoxide ring opening with azide, tuned with a halohydrin dehalogenase, allowed access to chiral 1,2-azido alcohols isomers with up to two stereocenters.

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Asymmetric azidohydroxylation of styrene derivatives mediated by a biomimetic styrene monooxygenase enzymatic cascade†

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Enantioenriched azido alcohols are precursors for valuable chiral aziridines and 1,2-amino alcohols, however their chiral substituted analogues are difficult to access. We established a cascade for the asymmetric azidohydroxylation of styrene derivatives leading to chiral substituted 1,2-azido alcohols *via* enzymatic asymmetric epoxidation, followed by regioselective azidolysis, affording the azido alcohols with up to two contiguous stereogenic centers. A newly isolated two-component flavoprotein styrene monooxygenase StyA proved to be highly selective for epoxidation with a nicotinamide coenzyme biomimetic as a practical reductant. Coupled with azide as a nucleophile for regioselective ring opening, this chemo-enzymatic cascade produced highly enantioenriched aromatic α-azido alcohols with up to >99% conversion. A bi-enzymatic counterpart with halohydrin dehalogenase-catalyzed azidolysis afforded the alternative β-azido alcohol isomers with up to 94% diastereomeric excess. We anticipate our biocatalytic cascade to be a starting point for more practical production of these chiral compounds with two-component flavoprotein monooxygenases.

Chiral epoxides are highly sought after to access optically active molecules *via* ring opening by nucleophiles such as azide, which affords 1,2-azido alcohols.[1] These compounds are intermediates for chiral aziridines, diamines,[2] and 1,2-amino alcohols found in bioactive compounds, chiral auxiliaries for asymmetric synthesis,[3] and give access to click chemistry.[4] Besides azidolysis,[5] the synthesis of 1,2-azido alcohols traditionally involves conversion of β-diols *via* sulfites,[6] or carbonyl reduction of α-azido ketones.[7,8] The challenge is to allow for a wide array of substituents and reach enantiopurity. Enzymatic aminohydroxylation of styrenes was recently described with an engineered cytochrome c,[9] and through enzymatic cascades using epoxide hydrolases, ADHs and transaminases.[10–14] On the other hand, enzymatic azidohydroxylation of aromatic alkenes remains to be investigated and applied *in vitro*.[7,15]

We envisioned to produce chiral 1,2-azido alcohols from aromatic alkenes *via* enzymatic epoxidation with a styrene monooxygenase (SMO).[16] SMO can activate and incorporate oxygen in alkenes to produce chiral epoxides with high stereo- and regioselectivity.[17–19] SMOs are two-component flavoprotein monooxygenases: the reductase component StyB (EC 1.5.1.36) uses nicotinamide adenine dinucleotide (NADH) to reduce flavin adenine dinucleotide (FAD), which freely diffuses to the oxygenase component StyA (EC 1.14.14.11) and reduces molecular oxygen, forming a hydroperoxyflavin that oxidizes styrene to its chiral epoxide (Fig. 1A).[20,21] StyA enzymes accept a range of aromatic[17,19] and aliphatic[22,23] alkene substrates, and can be easily produced recombinantly in *E. coli*.

Enzymatic recycling of the required NADH generally involves a dehydrogenase and a co-substrate (Fig. 1A), typically added in large excess. Non-enzymatic approaches have been investigated by shortcutting NADH/StyB using various photo- or electrochemical methods to reduce FAD.[24–29] In particular, 1-benzyl-1,4-dihydronicotinamide (BNAH) was shown to be effective and simple to use as a reductant with StyA[16,30] and other two-component flavoprotein monooxygenases such as halogenases,[31] a bacterial luciferase,[32] and an FMN-dependent type II Baeyer–Villiger monooxygenase.[33] BNAH thus circumvents the use of two enzymes (StyB, dehydrogenase) and NADH, for using oxygenase StyA in biocatalytic reactions (Fig. 1B).

Previous enzymatic cascades involving two-component SMOs have led to the synthesis of amino acids and other

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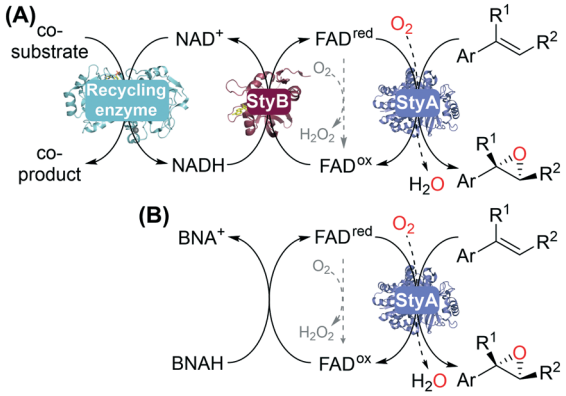


Fig. 1 SMO-Catalyzed asymmetric epoxidation of aromatic alkenes: (A) two-component flavoprotein monooxygenase StyA and reductase StyB with the required recycled NADH cofactor; (B) oxygenase StyA with 1-benzyl-1,4-dihydronicotinamide (BNAH) as reductant for direct FAD reduction.

valuable chiral compounds.[10,34–37] Herein we establish the proof of principle for a one-pot biocatalytic cascade *via* StyA-catalyzed epoxidation, mediated by a nicotinamide coenzyme biomimetic (NCB), followed by either chemical or enzymatic azidolysis with halohydrin dehalogenases (HHDHs), towards the desired product isomer (Fig. 2). Our linear artificial cascade combines two (bio)chemical steps in one pot without isolation of intermediates,[38] and allows for substituents on the α- or β-carbon to produce tertiary alcohols, an advantage over other cascades limited from aromatic haloketones.[8,39,40] The regioselective ring opening depends on the nucleophile and epoxide, among other parameters.[41]

To establish the first step of the cascade, we explored three available StyA enzymes, recombinantly produced in *E. coli* and purified, for the selective asymmetric oxidation of aromatic alkenes: StyA from *Pseudomonas* sp. VLB120,[42] StyA1 from *Rhodococcus opacus* 1CP,[43] and *Sf*StyA from *Sphingopyxis fribergensis* Kp5.2.

Compared with using reductase StyB, NADH and its recycling system (Fig. 1A), the oxygenase StyA had previously displayed similar catalytic activity using BNAH as hydride donor to reduce FAD (Fig. 1B).[16] Initially, we screened a panel of aromatic alkenes **1a–s** -mostly styrene derivatives- with the

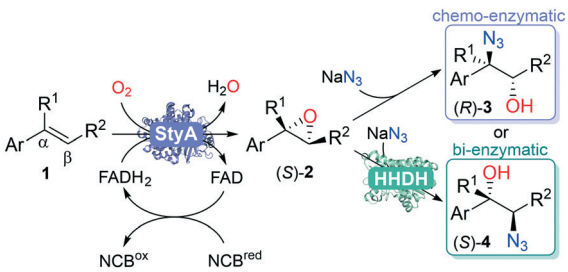


Fig. 2 Linear one-pot chemo- or bi-enzymatic cascade from aromatic alkenes **1** towards chiral 1,2-azido alcohols. First step: StyA, FAD, NCB to enantiopure epoxides **2**; second step: with NaN_3 to α-azido alcohol products **3**, or with NaN_3 and HHDH to β-azido alcohol products **4**.

recently isolated *Sf*StyA (see ESI†) to establish its substrate scope (Table 1). The aromatic alkene substrates explored bore different substitution patterns: a methyl substituent at the α- or β-position (**1b–d**, **1q–r**), a ring substituent in *para*-, *meta*- and *ortho*-positions (halogen atoms and methyl groups **1e–o**), a non-conjugated alkene (allylbenzene **1p**) and a heterocyclic ring (2-vinylpyridine **1s**).

Reaction mixtures were extracted after one hour for conversion comparison. All alkenes except 2-vinylpyridine **1s** were converted to over 3.3 mM epoxide product within one hour (Table 1, >66% conversion, turnover frequency (TOF) >1100 h^{-1}). Full conversion was achieved when the reaction was left to completion (Fig. 3A) and no diol was detected (see ESI†). The formation of a ketone side product was observed in two cases, with 1,2-dihydronaphthalene **1q** giving 5% 2-tetralone (Table 1 entry 17), and indene **1r** leading to 54% 2-indanone (entry 18). The enantiomeric excess (ee) obtained for oxide products bearing different substituents were mostly excellent (94 to >99% ee, entry 1, 3–5, 8–9, 11–15, 17–19), very good (89% ee for α-methylstyrene oxide **2b** entry 2) and moderate for the non-conjugated alkene in allylbenzene **1p**, affording epoxide **2p** with 68% ee (entry 16).

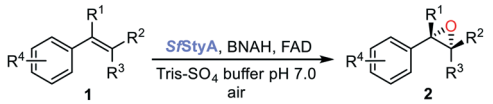
This substrate scope shows substituent-position effects on *Sf*StyA, observed with other characterized StyA enzymes.[44] The position of the substituent affected the ee, especially in the case of bromostyrene: the *para*-substituted product **2e** has an ee of 96% (*S*), compared with 81% and 70% ee (*S*) obtained for *meta*- **2f** and *ortho*-substituted **2g**, respectively (Table 1 entry 5–7). This effect was less pronounced with chlorostyrenes **2h–j** (83 to 95% ee, entry 8–10) and even less with fluorostyrenes **2k–m** (94 to >99% ee, entry 11–13). Methylstyrenes **2n–o** (95 to 99% ee, entry 14 and 15) showed a similar pattern as chlorostyrenes (entry 8 and 9).

The high conversions and enantioselectivity obtained with *Sf*StyA led to its selection for further reactions, as StyA1 is less stable and gives lower TOFs,[16] and StyA affords slightly lower *ee* for desired chiral epoxides *in vitro* (**2a** 98.0% ee, **2c** 97.6% ee,[24] compared with >99% ee with *Sf*StyA in both cases). We were especially interested in substrates leading to products with two chiral centers: (*S*)-α-methylstyrene oxide **2b** (Table 1, entry 2), (1*S*,2*S*)-1-phenylpropylene oxide **2c** and (1*S*,2*R*)-1-phenylpropylene oxide **2d** (entry 3 and 4) were obtained with excellent ee.

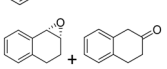
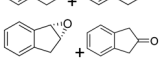
Next, we determined the time course (Fig. 3A) and various reaction parameters for the *Sf*StyA-catalyzed epoxidation of styrene to demonstrate the practical use of the *Sf*StyA/BNAH system with this oxygenase component: enzyme concentration (Fig. 3B), FAD concentration (Fig. 3C), the number of BNAH equivalents (Fig. 3D), and hydride donor (Fig. 3E), which influenced product formation.

Higher enzyme concentration at 50 μM FAD led to higher product formation (Fig. 3B). FAD concentration was explored to ensure optimal product formation (Fig. 3C). FAD concentrations of up to 100 μM led to increased oxide formation, which translates to FAD reduction as the rate-limiting step. A plateau was then reached, hence the limiting

Table 1 Biocatalytic asymmetric epoxidation of styrene derivatives with the oxygenase *Sf*StyA[a]



a $R^1 = R^2 = R^3 = R^4 = H$
b $R^1 = CH_3$, $R^2 = R^3 = R^4 = H$
c $R^1 = H$, $R^2 = CH_3$, $R^3 = R^4 = H$
d $R^1 = R^2 = H$, $R^3 = CH_3$, $R^4 = H$
e $R^1 = R^2 = R^3 = H$, $R^4 = p$-Br
f $R^1 = R^2 = R^3 = H$, $R^4 = m$-Br
g $R^1 = R^2 = R^3 = H$, $R^4 = o$-Br
h $R^1 = R^2 = R^3 = H$, $R^4 = p$-Cl
i $R^1 = R^2 = R^3 = H$, $R^4 = m$-Cl
j $R^1 = R^2 = R^3 = H$, $R^4 = o$-Cl
k $R^1 = R^2 = R^3 = H$, $R^4 = p$-F
l $R^1 = R^2 = R^3 = H$, $R^4 = m$-F
m $R^1 = R^2 = R^3 = H$, $R^4 = o$-F
n $R^1 = R^2 = R^3 = H$, $R^4 = p$-Me
o $R^1 = R^2 = R^3 = H$, $R^4 = m$-Me
p allylbenzene
q 1,2-dihydronaphthalene
r indene
s 2-vinylpyridine

Entry	Product **2**		[**2**] (mM)	TOF[b] (h^{-1})	ee[c] (%)
1	**2a**		3.9	1300	>99 (*S*)
2	**2b**		4.7	1565	89 (*S*)
3	**2c**		4.7	1565	>99 (1*S*,2*S*)
4	**2d**		4.7	1565	>99 (1*S*,2*R*)
5	**2e**		4.3	1435	96 (*S*)
6	**2f**		4.1	1365	81 (*S*)
7	**2g**		3.3	1100	70 (*S*)
8	**2h**		4.7	1565	97 (*S*)
9	**2i**		4.7	1565	>99 (*S*)
10	**2j**		4.3	1435	83 (*S*)
11	**2k**		4.5	1500	>99 (*S*)
12	**2l**		4.3	1435	95 (*S*)
13	**2m**		4.7	1565	94 (*S*)
14	**2n**		4.2	1400	95 (*S*)
15	**2o**		4.8	1600	99 (*S*)
16	**2p**		3.8	1265	68 (*S*)
17	**2q**		4.0 + 0.2	1400	>99 (1*S*,2*R*)
18	**2r**		1.9 + 2.2	1365	>99 (1*S*,2*R*)
19	**2s**		2.5	835	>99 (*S*)

[a] Reaction conditions: [BNAH] = 15 mM, buffer (50 mM tris-SO_4 pH 7.0), catalase = 650 U mL^{-1}, [FAD] = 50 μM, [*Sf*StyA] = 3 μM, [alkene **1**] = 5 mM, 0.2% v/v dimethyl sulfoxide (DMSO), final volume 1 mL in a 2 mL plastic tube shaken on a thermomixer at 900 rpm and 30 °C, for 1 h. [b] Turnover frequency (TOF) = [product]/[enzyme] per hour. [c] Enantiomeric excess of epoxides determined by chiral GC-FID (see ESI†). Average of duplicates, standard deviation <10%.

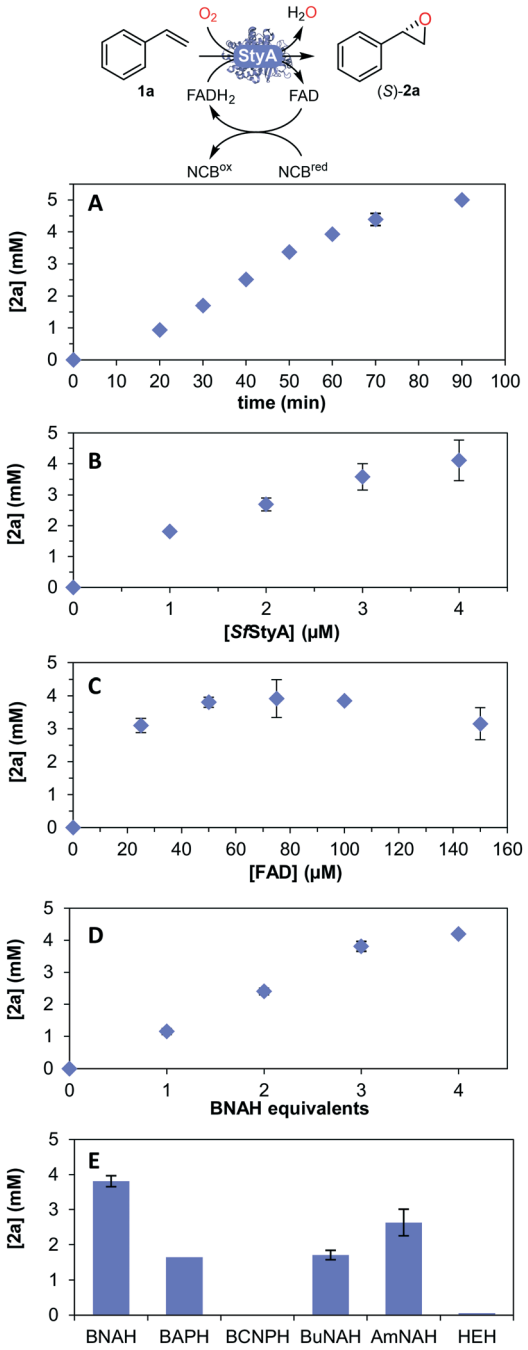


Fig. 3 Parameters for the *Sf*StyA-catalyzed asymmetric oxidation of styrene to its oxide **2a**: (A) time course; (B) [*Sf*StyA] = 0 to 4 μM; (C) [FAD] = 0 to 200 μM; (D) BNAH equivalents (1 equiv. = 5 mM); (E) type of NCBs hydride donor (see Fig. 4 for structures). General reaction conditions unless otherwise specified: [BNAH] = 15 mM, buffer (50 mM tris-SO_4 pH 7.0), catalase = 650 U mL^{-1}, [FAD] = 50 μM, [*Sf*StyA] = 3 μM, [styrene] = 5 mM, 0.2% v/v DMSO, final volume 1 mL in a 2 mL plastic tube shaken on a thermomixer at 900 rpm and 30 °C, for 1 h. Average of duplicates.

step most likely became the *Sf*StyA-catalyzed epoxidation. Less product was observed with >100 μM FAD, as previously reported with other StyA enzymes.[16,24,45] The lower product formation can be ascribed to FAD competing with $FADH_2$ for the flavin-binding site, although the affinity for $FADH_2$ is approximately 8000-fold higher.[18,43,45,46] Additionally, FAD and $FADH_2$ can disproportionate to give FAD radicals, reacting with molecular oxygen to form hydrogen peroxide,[47] leading to a futile loss of electrons.

Concerning the source of electrons, several NADH regeneration systems have been established with formate (FDH) or glucose (GDH) dehydrogenases (Fig. 1A). For our cascade design however, sodium azide can be an inhibitor for FDH, whereas high excess of glucose is typically used for GDH (see ESI†), and we aimed to achieve a more efficient and practical system. As we previously showed that the use of BNAH allows for cost-effective direct FAD reduction with up to 85% efficiency,[16] we explored other NCBs. Flavin reduction by NCBs such as BNAH is known to occur through direct hydride transfer in solution.[48,49] Different substituents were previously shown to affect the overall NCB redox potential and lead to different FAD reduction rates.[31] Therefore, we screened six NCBs bearing substituents varying from amide, acetyl, carboxylic acid to nitrile, with either a benzyl, butyl or alkyl amide chain on the nitrogen, and the classical Hantzsch ester (HEH, Fig. 3E and 4), as a hydride donor for the reduction of FAD without interfering with the epoxidation.[50] Within one-hour reactions, BNAH displayed the best overall conversion (3.9 mM), followed by AmNAH (2.6 mM). BCNPH and HEH proved to be the worst reductants. No product was observed in the absence of FAD, or enzyme, or BNAH.

Once the biocatalytic asymmetric oxidation process was established, we proceeded to investigate the use of the chiral epoxides in a chemo-enzymatic cascade. Azide can cleave epoxides under mild conditions. With aliphatic epoxides, ring opening occurs through a bimolecular reaction, an S_N2-type backside attack of the azide on the least substituted carbon of the protonated epoxide, forming a *trans*-1,2-azido alcohol as product. The rate of chemical epoxide ring opening is pH dependent, the more acidic, the faster the reaction, and also

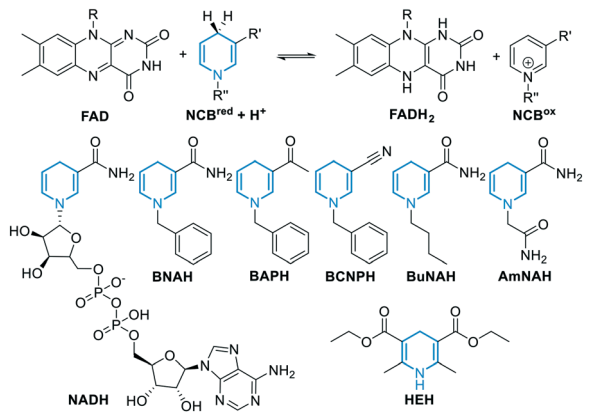
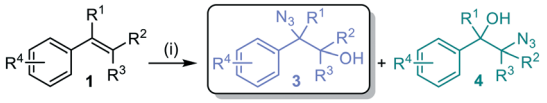
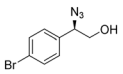
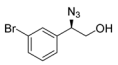
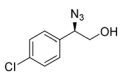
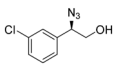
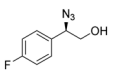
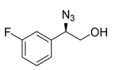
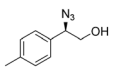
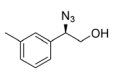


Fig. 4 Top: schematic FAD reduction by NCBs. Bottom: chemical structures of NADH and NCBs (1-benzyl-3-acetyl-1,4-dihydropyridine BAPH, 1-benzyl-3-cyano-1,4-dihydropyridine BCNPH, 1-butyl-1,4-dihydronicotinamide BuNAH, 1-carbamoyl-1,4-dihydronicotinamide AmNAH, Hantzsch ester HEH).

higher temperatures increase the reaction rate (see ESI†). Previous studies have shown that azidolysis of aryl-substituted epoxides show different regioselectivity, affording α-azido alcohols as the major products.[51,52] The nucleophilic attack occurs at the α-carbon of the aromatic substituted epoxide, with complete inversion of configuration (see ESI†).[41,53] The regioselectivity was explained by stabilization of positive charge formed through delocalization,[53] but recent

Table 2 One-pot chemo-enzymatic cascade from styrene derivatives to α-azido alcohols[a]



Entry	Major product		Conv.[b] (%)	**3 : 4** (%)
1	**3a**		>99	98 : 2
2	**3b**		98[c]	97 : 3[d]
3	**3c**		>99	100 : 0
4	**3d**		27	68 : 32
5	**3e**		99	96 : 4
6	**3f**		99	96 : 4
7	**3g**		82	98 : 2
8	**3h**		96	97 : 3
9	**3i**		92	93 : 7
10	**3j**		90	95 : 5
11	**3k**		98	98 : 2
12	**3l**		97	91 : 9
13	**3m**		98	94 : 6
14	**3n**		98	98 : 2
15	**3o**		99	98 : 2
16	**3q**		>99	94 : 6
17	**3r**		79	96 : 4
18	**3s**		62	53 : 47

[a] Reaction conditions (i): [BNAH] = 15 mM, buffer (50 mM tris-SO_4 pH 7.0), catalase 650 U mL^{-1}, [FAD] = 50 μM, sodium azide (7 equiv.), [*Sf*StyA] = 3 μM, [alkene] = 5 mM, 0.2% v/v DMSO, final volume 1 mL in a 2 mL plastic tube shaken on a thermomixer at 900 rpm and 30 °C, for 24 h. [b] Determined by chiral GC-FID. Average of duplicates, standard deviation <10%. [c] 5% diol product observed. [d] Assignment of isomers ratio not determined.

studies revealed that electrostatic interaction between the phenyl ring and the incoming nucleophile play a dominant role.[54]

We chose sodium azide salt for its high solubility in water and determined that the amount needed for fast azidolysis was seven equivalents with respect to the substrate (see ESI†). The reactivity and regioselectivity of the azidolysis in water is controlled by a neutral pH value, 30 °C temperature and low buffer concentration, thus avoiding diol formation stemming from the competition of hydroxide or water with the azide ion (see ESI†).[52] Under these conditions, we achieved full conversion and highly regioselective ring opening of styrene oxide: the desired α-azido alcohol (2-azido-2-phenyl-1-ethanol **3a**) was formed as the main product with only a trace amount of regioisomer **4a**, determined by gas chromatography with flame ionization detection (GC-FID, ratio of 98 : 2 **3a** : **4a**). The linearly increasing product formation with higher azide concentrations can be explained by the S_N2 type mechanism.

We then carried out the full azidohydroxylation cascade with the established *Sf*StyA-catalyzed epoxidation of alkenes **1a–o**, **q–s**, affording the corresponding (2*R*)-α-azido alcohols **3** with good to excellent conversions and regioselectivity (Table 2). The *ee* from the *Sf*StyA reaction was retained in all cases.

The difference in rate and degree of regioselectivity between azidolysis of *trans*- and *cis*-β-methylstyrene oxide (**2c** *versus* **2d**) could be explained by the orientation of the phenyl ring with respect to the epoxide (see ESI†). A further advantage of this chemo-enzymatic cascade, besides the ability to carry out the reactions in one pot *via* chiral epoxides, is the high reactivity of the epoxide intermediates: without the need to isolate the epoxide, the aldehyde and ketone previously observed with the simple biocatalytic epoxidation (Table 1, entries 17–18) do not have time to form. Therefore, we obtained full conversion with 1,2-dihydronaphthalene **1q** and 79% conversion with indene **1r** (Table 2, entry 16 and 17).

Encouraged by the enantioenriched 2-azido-2-phenyl-1-ethanol derivatives obtained, several reactions were carried out on a scale of 15 mg of substrate, increasing the substrate concentration to 10 mM. The corresponding azido alcohols obtained were simply extracted with ethyl acetate without further purification and identified and characterized by NMR spectroscopy (Fig. 5, ESI†). A higher scale reaction was performed with *trans*-β-methylstyrene **1c** (56 mg, 10 mM) as substrate. Using *Sf*StyA, BNAH and FAD, a 92% crude yield was obtained with >99% ee of (1*R*,2*S*)-1-azido-1-phenylpropan-2-ol **3c**. ^{1}H NMR showed a high purity product after extraction with ethyl acetate (see ESI†).

It should be noted that sodium azide may interfere with the steady-state kinetics of substrate epoxidation catalysed by the SMO.[55–57] Our one-pot cascade may be more efficient as a two-step approach, adding the sodium azide in the second step.

To access the β-azido alcohol isomers, several halohydrin dehalogenases (HHDHs, EC 3.8.1.2, Enzymicals screening kit,

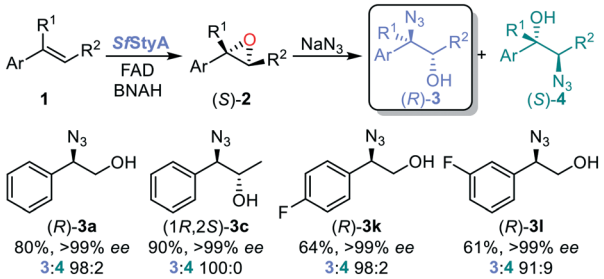


Fig. 5 One-pot chemo-enzymatic cascade from styrenes to chiral α-azido alcohols, 15 mg scale, % isolated yield of product. Reaction conditions: [BNAH] = 30 mM, buffer (50 mM tris-SO_4, pH 7.0), catalase (650 U mL^{-1}), [FAD] = 50 μM, [*Sf*StyA] = 3 μM, [alkene] = 10 mM, [NaN_3] = 35 mM, final volume 10 mL, shaken in Erlenmeyer flasks on an incubator shaker at 180 rpm and 30 °C for 22 h.

see ESI†)[58,59] were screened towards racemic styrene oxide *rac*-**2a** (Table 3), using sodium azide for the selective epoxide ring opening,[58] giving two possible products, 1-azido-2-phenylethanol **3** or 2-azido-1-phenylethanol **4**. To minimize the uncatalyzed background reaction of sodium azide that leads to the α-azido alcohols, the reactions with HHDHs were performed using only one equivalence of sodium azide. In general, the most active HHDH towards the formation of β-azido alcohols **4** gives a higher ratio towards the desired product.

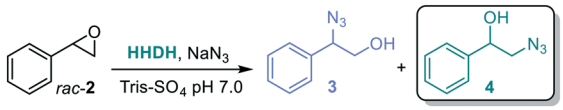
The results of the HHDH screening to obtain the β-azido alcohol were variable (Table 3): the enzymatic reaction was clearly outrun by the chemical one with HheD6 (entry 6), likely due to the low activity of the enzyme, as was the case with HheA3 (entry 1) and HheD3 (entry 4). The other HHDHs also showed mixed ratios (entries 2–3 and 6). HheE5 from *Gammaproteobacterium* strain IMCC3088 gave the best ratio of α : β 16 : 84 (entry 7).

The screened HHDHs lacked high selectivity for either the *S*- or *R*-enantiomer with racemic styrene oxide, the highest ee being reached with HheD3 (68% ee of (*R*)-**4**, entry 5), hence the importance of using a selective StyA in the asymmetric epoxidation step. In our case, the use of *Sf*StyA provided very good to excellent ee (Table 1), but other StyA enzymes could be used to obtain the best ee depending on the substituted substrate.

We set out to further screen selected HHDHs (Enzymicals screening kit, see ESI†) with (*S*)-styrene oxide as starting material (Table 4). HheD4 and HheF led to trace amounts of diol side product **5** in addition to low ratios (entry 1 and 4), and once again HheE5 stood out as the most active towards β-ring opening (entry 3, the higher ratio with respect to Table 3 is ascribed to the use of a different batch of lyophilized enzyme) and was selected for further reactions.

The substrates that gave highest conversion, ee and regioselectivity for the synthesis of α-azido alcohols were chosen for subsequent experiments. The bi-enzymatic cascade with *Sf*StyA and HheE5 was thus carried out for styrene **1a**, *trans*-β-methylstyrene **1c** and 4-fluorostyrene **1k**. The *trans*-β-methylstyrene **1c** led to a mixture of α- and

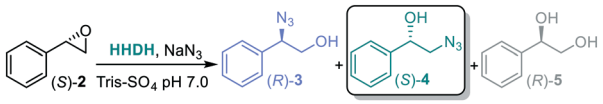
Table 3 HHDH-Catalyzed racemic styrene oxide ring opening[a]



Entry	HHDH	Ratio **3** : **4**	(*R*)-**3** ee[b] (%)	(*R*)-**4** ee[b] (%)
1[c]	HheA3	87 : 13	8	42
2	HheA5	54 : 46	43	67
3[c]	HheB5	33 : 67	44	27
4	HheD3	82 : 18	15	68
5[c]	HheD5	37 : 63	60	49
6	HheD6	95 : 5	<5	<5
7	HheE5	16 : 84	22	<5
8	None	98 : 2	<5	<5

[a] Reaction conditions: buffer (50 mM tris-SO_4, pH 7.0), [*rac*-styrene oxide] = 5 mM, [NaN_3] = 5 mM, lyophilized cell-free extract HHDH (10 mg mL^{-1}), final volume 1 mL in a 2 mL plastic tube shaken on a thermomixer at 900 rpm and 30 °C for 15 h 30 min. Full conversion was observed. [b] Determined by chiral GC-FID. [c] Diol side product observed in trace amounts (<2%).

Table 4 HHDHs-Catalyzed (*S*)-styrene oxide ring opening[a]



Entry	HHDH	Ratio **3** : **4**	(*R*)-**3** ee[b] (%)	(*S*)-**4** ee[b] (%)	(*R*)-**5** (%)
1	HheD4	63 : 37	>99	>99	8
2	HheE4	26 : 74	>99	>99	<1
3	HheE5	6 : 94	>99	>99	<1
4	HheF	60 : 40	>99	>99	5

[a] Reaction conditions: buffer (50 mM tris-SO_4 pH 7.0), HHDH (10 mg), [NaN_3] = 5 mM, [(*S*)-styrene oxide] = 5 mM, final volume 1 mL in a 10 mL glass vial shaken on an incubator shaker at 180 rpm and 30 °C for 15 h. Full conversion was observed. [b] Determined by chiral GC-FID.

β-azido alcohol **4c**, which can be ascribed to the poor activity of HheE5 towards the epoxide **3c**.[60] Further screening efforts with HHDHs did not display a more active enzyme, therefore there is room for enhancing HHDH enzymes with this type of substrate.

Pleasingly, substrates **1a** and **1k** afforded the corresponding β-azido alcohols **4a** and **4k** with good to excellent conversion, enantio- and regioselectivity (Fig. 6). With the discovery and engineering of HHDHs, we can expect variants with higher selectivity in the near future.

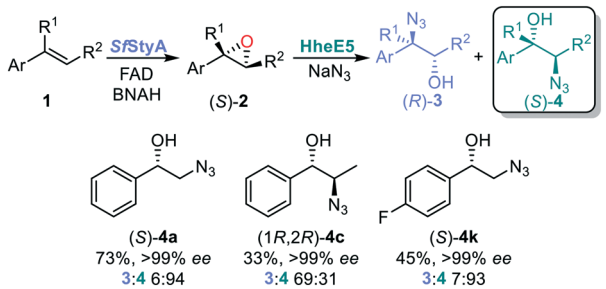


Fig. 6 One-pot bi-enzymatic cascade to β-azido alcohols. % conversions. Reaction conditions: [BNAH] = 15 mM, buffer (50 mM tris-SO_4 pH 7.0), catalase = 650 U mL^{-1}, [FAD] = 50 μM, [alkene] = 5 mM, [*Sf*StyA] = 3 μM, [NaN_3] = 5 mM, HheE5 (20 mg), final volume 1 mL in a 10 mL glass vial shaken on an incubator shaker at 180 rpm and 30 °C for 22 h.

Conclusions

In conclusion, we explored the substrate scope of a new two-component flavoprotein styrene monooxygenase *Sf*StyA for the asymmetric oxidation of styrene derivatives, leading mostly to high conversions and ee. This study further demonstrates the use of the oxygenase component of two-component flavoprotein monooxygenases with a practical flavin reduction system. Through this biocatalytic asymmetric epoxidation with *Sf*StyA, we established a linear artificial one-pot chemo- and bi-enzymatic cascade to achieve the asymmetric azidohydroxylation of styrenes. The epoxide ring opening was achieved with sodium azide either chemically, or catalyzed by a halohydrin dehalogenase, leading to high purity of either azidoalcohol isomers with retention of the StyA selectivity. The latest discovery of (*R*)-selective StyA oxygenases allows access to the other isomers.[23,61,62]

We anticipate our biocatalytic cascade to expand the use of oxygenase StyA for the production of amino alcohols[63] and

styrenyl aziridines. Furthermore, the use of alternative nucleophiles, such as halides, cyanate, *etc.*,[64] can expand the portfolio of chiral enantiopure products obtained. In the future we expect the use of peroxygenases will be attractive once evolved to achieve high enantioselectivity.[65]

Experimental details

All biocatalytic reactions were performed on an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany) and followed by gas chromatography with flame ionization detection (GC-FID). Analyses were carried out on a Shimadzu GC-2010 GC-FID. Products were confirmed by reference standards and GC-MS. Product concentrations were obtained with a calibration curve equation using 5 mM dodecane as an internal standard.

StyA-Catalyzed epoxidations

Stock solutions were made fresh in buffer: catalase from bovine liver (6500 U mL^{-1}), FAD (5 mM), alkene **1** (2.5 M in DMSO). Reaction conditions: 2 mL microcentrifuge plastic tube, BNAH (15 mM), buffer (50 mM tris-SO_4 pH 7.0), catalase (650 U mL^{-1}), FAD (50 μM), *Sf*StyA (3 μM), alkene **1** (5 mM, final 2% v/v DMSO), final volume 1 mL. The reaction mixtures were agitated on a thermomixer at 30 °C and 900 rpm for 1 h. Product concentration and ee were determined by GC-FID with a chiral column.

Chemo-enzymatic cascade

Chemo-enzymatic cascade reactions (1 mL in volume) were performed in buffer (50 mM tris-SO_4 pH 7.0) containing the styrene derivative **1** (5 mM), *Sf*StyA (3 μM), FAD (50 μM), BNAH (15 mM), NaN_3 (35 mM) catalase (650 U mL^{-1}). The reaction mixtures were agitated on a thermomixer at 30 °C and 900 rpm. Comparison of retention times with authentic standards and GC-MS allowed product identification. Conversion and enantiomeric excess were determined by GC-FID analysis.

Bi-enzymatic cascade

Lyophilized HHDH (10 mg, Enzymicals AG) was rehydrated in buffer for 30 min before use. Bi-enzymatic cascade reactions (1 mL in volume) were performed in buffer (50 mM tris-SO_4 pH 7.0) containing the styrene derivative **1** (5 mM), *Sf*StyA (3 μM), FAD (50 μM), BNAH (15 mM), NaN_3 (5 mM), catalase (650 U mL^{-1}) and HHDH (10 mg mL^{-1}). The reaction mixtures were agitated on a thermomixer at 30 °C and 900 rpm. Conversion and enantiomeric excess were determined by GC-FID analysis.

Experimental details for the synthesis of compounds, characterization, enzyme production and reactions, GC-FID analyses and more detailed experiments are available in the ESI.†

Conflicts of interest

There are no conflicts to declare.

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