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Biocatalysts from Biosynthetic Pathways: Enabling Stereoselective, Enzymatic Cycloether-Formation on A Gram Scale

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Dedicated to Prof. Peter F. Leadlay on the occasion of his recent retirement.

ABSTRACT: Biosynthetic pathways of natural products contain many enzymes that contribute to the rapid assembly of molecular complexity. Enzymes that form complex structural elements with multiple stereocenters, like chiral saturated oxygen heterocycles (CSOH), are of particular interest for a synthetic application as their use promises to significantly simplify access to these elements. Here, the biocatalytic characterization of AmbDH3, an enzyme that catalyzes intramolecular oxa-Michael addition (IMOMA) is reported. This reaction essentially gives access to various types of CSOH with adjacent stereocenters, but is not yet part of the repertoire of preparative biocatalysis. An in-depth study on the synthetic utility of AmbDH3 was performed, which made extensive use of complex synthetic precursor surrogates. The enzyme exhibited stability and broad substrate tolerance in *in vitro* experiments, which was in agreement with the results of molecular modeling. Its selectivity profile enabled kinetic resolution of chiral tetrahydropyrans (THPs) under control of up to four stereocenters. After a systematic optimization of the reaction conditions, gram scale conversions became possible that led to preparative amounts of chiral THP. The synthetic utility of AmbDH3 was finally demonstrated by its successful application in the key step of a chemoenzymatic total synthesis to the THP-containing phenylheptanoid (–)-centrolobin. These results highlight the synthetic potential of AmbDH3 and related IMOMA cyclases as a biocatalytic alternative that further develops the available chemical-synthetic IMOMA methodology.

KEYWORDS: *biocatalyst discovery, cyclases, oxa-Michael addition, heterocycles, tetrahydropyrans, natural product (bio)synthesis, new enzymatic activity*

INTRODUCTION

Natural product biosynthetic pathways are a rich source of enzymes with unprecedented activities.^{1,2} Among them, enzymes that form complex molecular fragments with multiple stereocenters, like terpene cyclases, macrocyclizing thioesterases or heterocycle-forming cyclases^{3–5}, are particularly attractive for biocatalysis as their selectivity often significantly exceeds that of analogous chemical-synthetic methodology. Their biocatalytic application promises to enable efficient, step-economic access to complex natural products, synthetic drugs and high-value chemicals.^{1,6,7} Tapping this potential requires a thorough investigation of the synthetically relevant properties of such enzymes, like substrate specificity, stereoselectivity, stability and reaction scalability.⁸ Comprehensive studies on these enzymes are often complicated by high investment into the stereoselective synthesis of complex precursor surrogates and a significant number of analogs.

Chiral, saturated oxygen heterocycles (CSOH) are abundant, often pharmacophoric, structural elements of natural products with a strong influence on the molecular shape (Figure 1a).^{9,10} Their selective synthesis is often hampered by a multitude of densely packed stereocenters.¹¹ The intramolecular oxa-Michael addition (IMOMA) basically provides access to CSOH with adjacent stereocenters from α -branched precursors like **4** (Figure 1b).^{11–13}

Chemical IMOMA, often integrated into efficient tandem processes, gained growing attention for the stereoselective synthesis of tetrahydropyrans (THPs) during recent years.^{12–15} It has been shown that the *cis*- or *trans*-THP selectivity of IMOMA can be influenced by kinetic or thermodynamic reaction control, by the type of activation (acid, base or metal catalysis), by the configuration of the double bond and by the nature of the acceptor moiety.^{16–21} Although the reaction outcome is predictable for some systems (tandem-cross metathesis-IMOMA with vinylketone intermediates for

example reliably leads to *cis*-THPs with *d.e.* > 5:1¹⁵, low to moderate *d.e.s* are rather common and have only occasionally been improved by extensive reaction optimization or product re-equilibration (see for example^{22–27}). Important studies on the thioester IMOMA have provided valuable insight into the reasons for the high substrate-dependence of the *cis-trans* selectivity, along with the establishment of guidelines for diastereoselective THP cyclization in specific systems.^{16–19}

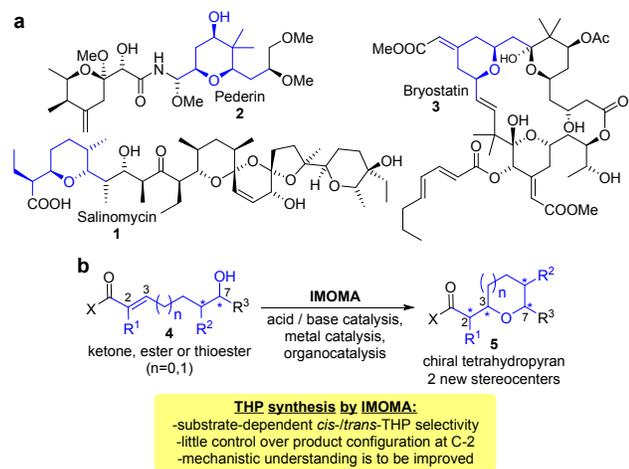
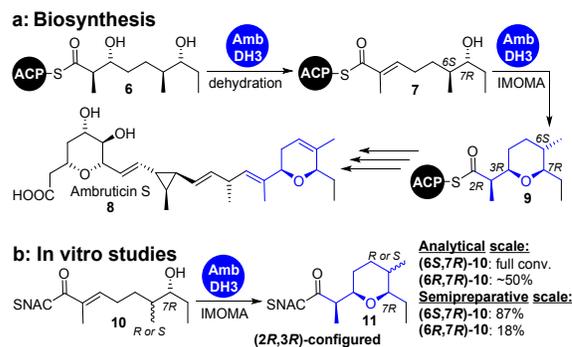


Figure 1. a) Pharmacologically relevant natural products containing THPs, which are biosynthesized by IMOMA. b) IMOMA enables stereoselective synthesis of chiral THPs and other CSOH. A C-2–C-6-bisbranched precursor is exemplarily shown.

Another important issue is the stereocontrol on C-2 of α -branched Michael acceptors. The product configuration at this position is highly dependent on the molecular environment.^{13,28–30} Selectivity has been realized in cases when C-2 was incorporated into rings containing existing stereocenters.^{31,32} The behavior of non-cyclic systems is scarcely described (nearly all of these systems in the literature are not α -branched). Tedious cascades, like IMOMA– α -halogenation–radical reduction, are thus currently used for the reliable synthesis of chiral THPs with adjacent stereocenters.^{33,34} Taken together, the modest stereoselectivity of IMOMA along with the harsh reaction conditions (e.g. strong base or acid) currently prevent the more frequent use of this reaction in organic synthesis.¹⁶ Novel variants that merge stereoselectivity and broad applicability are highly desirable. C-2 stereoselectivity and the potential to discriminate between C-7 enantiomers/epimers would for example add valuable new features to the existing IMOMA methodology. Enzymatic catalysis with its tight control over extended transition states is particularly well suited to provide a solution for the problems described above.

The biosynthesis of CSOH occurs by direct oxidation, by reduction of hemiacetals or by intramolecular addition of hydroxyls to electrophiles.^{5,35,36} The synthetic applicability of such cyclizing enzymes has been successfully studied in some cases. The bifunctional cytochrome P450 monooxygenase AurH introduces a tetrahydrofuran (THF) ring in (+)-aureothin biosynthesis by direct oxidation. Hertweck *et al.* have biochemically characterized this enzyme and successfully applied it in a total synthesis of the natural product as part of an elegant late-stage kinetic resolution process.^{37–39} The same group reported on a PKS domain for vinylogous chain branching that gives rise to a δ -lactone in rhizoxin biosynthesis.⁴⁰ This enzyme was extensively studied regarding

its synthetic properties and could for example be employed for the synthesis of hard to access medium sized lactones.^{41–43} Enzymes that catalyze IMOMA have been discovered in the secondary metabolism^{44,45,46–54} and a basic biochemical characterization has been reported for three enzymes from the ambroticin, the pederin and the sorangicin biosynthetic pathways.^{44,45,46,55} Despite their diverse sequence-based annotation, recent studies suggest that all these IMOMA cyclases share an enzymatic mechanism in which they activate the 7-hydroxyl group of the precursor by acid-base catalysis for attack onto the Michael acceptor.^{45,47,55} No in depth-studies on the biocatalytic potential of IMOMA cyclases are reported to date.



Scheme 1. a) AmbDH3 catalyzes THP formation in ambroticin biosynthesis. c) Enzymatic in vitro studies on AmbDH3. *N*-Acetylcysteamine (SNAC) mimics the natural acyl carrier protein (ACP) attachment.^{44,45}

We have previously characterized the unusual bifunctional polyketide synthase (PKS) domain AmbDH3 by a combination of enzymatic in vitro experiments, X-ray crystal structure analysis and site-selective mutagenesis (Scheme 1a).^{44,45} It catalyzes a cascade of dehydration and IMOMA of **6** to the chiral tetrahydropyran (THP) **9** *via* **7**. In vitro conversion experiments of the purified domain with synthetic substrate surrogates showed quantitative, stereospecific transformation of the natural precursor surrogate (6*S*,7*R*)-**10** into (2*R*,3*R*,6*S*,7*R*)-**11** in an analytical scale experiment and 87% conversion on the semipreparative scale (8 mg / 27 μ mol starting material, Scheme 1b). The C-6 epimer (6*R*,7*R*)-**10** was partially converted into (2*R*,3*R*,6*R*,7*R*)-**11**, suggesting substrate tolerance of the enzyme.

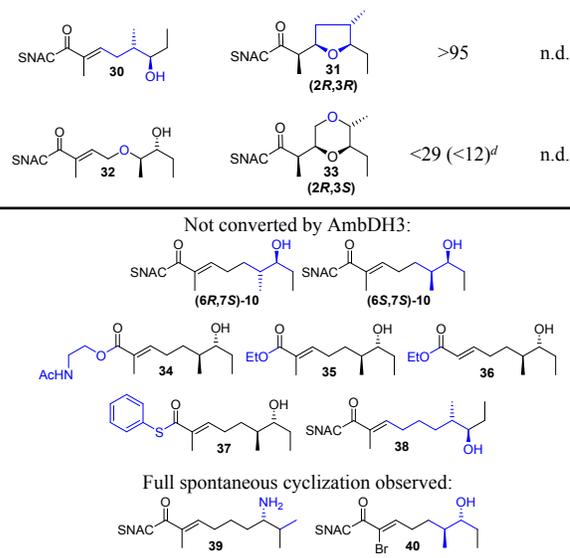
RESULTS AND DISCUSSION

Substrate specificity of AmbDH3. A library of precursor derivatives was stereoselectively synthesized *via* multistep sequences (see supporting information). For probing the substrate selectivity of AmbDH3, the members were individually incubated with the purified enzyme under the reported conditions (8–10 mg \approx 30 μ mol starting material, Table 1, Figures S1–S26).⁴⁴ Enzyme-free reference incubations determined the spontaneous background cyclization. The 7*S*-configured compounds (6*R*,7*S*)-**10** and (6*S*,7*S*)-**10** were not accepted, showing the crucial nature of the stereochemistry at C-7. Various alterations of the polyketide backbone substitution pattern were tolerated by the enzyme with the conversion increasing with the steric bulk of the residues on C-6/C-7 (results for (6*S*,7*R*)-**10**, **12**, **14** and **22**) and in the absence of electron-donating substituents on the acceptor (results for (6*S*,7*R*)-**10**, **18**, **20**, **22** and **24**). The low solubility of **16** and **18** in aqueous buffer prevented their reaction under standard assay

conditions. Modest conversion was however observed in small-scale experiments of these compounds that were carried out at lower concentration. The enzyme strongly differentiated between various types of carbonyls in the acceptor (results for **(6*S*,7*R*)-10**, **26** and **28** as well as **34-37**). Neither the dethia-oxo analog **34** nor the other oxo esters **35** and **36** were competent substrates of AmbDH3. This underlines the aforementioned dependence of the enzymatic reaction on the electron-density of the acceptor. Accordingly, the little soluble *S*-ethyl-enethioate **26** and vinylketone **28** were cyclized by the enzyme by approximately 40% (see footnotes c, d and f in Table 1). Phenylthioester **37** was not a competent substrate, showing that structural aspects play a role, even if the acceptor is sufficiently activated (Figure S27 and Table S1). While precursor **30** was fully converted by AmbDH3 into homochiral tetrahydrofuran (THF) **31**, oxepane precursor **38** was not a competent substrate (Figure S27 and Table S1). The decarba-oxo analog **32** was partially converted into the 1,4-dioxane **33**. Substrates **39** and **40** underwent complete spontaneous cyclization under all tested reaction conditions, avoiding their assessment in this study.

Table 1. Substrate surrogates that were reacted with purified AmbDH3.

Substrate ^a	Product (abs. configuration ^b)	conversion [%] ^b	Yield [%]
		87	70 ^b
		18	n.d.
		>95	n.d.
		89	52 ^g
		13 ^c	n.d.
		17 ^c	n.d.
		>90 (32) ^d	72 ⁱ
		37	24 ^b
		72 (<6) ^{d,e}	58 ^b
		43 ^c	25 ^b
		>95 (>95) ^d 39 (0) ^{d,f}	n.d.



^aCarried out under non-optimized conditions (see supporting information). ^bDetermined by ¹H NMR spectroscopy. ^cConducted on the 1-4 mg-scale with a substrate concentration of 2-4 mM due to solubility issues. ^dIf applicable, the conversion of the spontaneous IMOMA under standard reaction conditions is shown in brackets. ^eAn e.e. of >95% was determined by ¹H NMR spectroscopy after reduction and esterification with (*S*)-Mosher's acid chloride. ^fConducted in an Eppendorf tube under shaking to suppress spontaneous cyclization of **28** that occurred during the stirred reaction. The *d.e.* and absolute configuration of the components in the crude reaction product could not be determined. ^gpurified by semipreparative HPLC. ^hpurified by flash chromatography on silica gel. ⁱcrude yield.

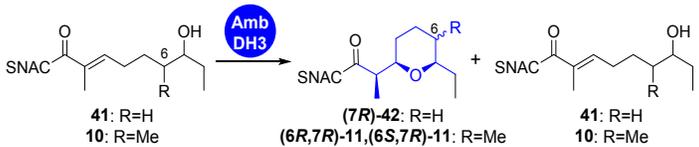
Taken together, these results demonstrate a relaxed substrate specificity of AmbDH3. This might be explained by its origin as a modular type I PKS domain. The high relevance of protein-protein interactions in these systems goes along with lower substrate affinity and lower reaction rates of their individual domains compared to free enzymes.⁵⁶⁻⁵⁹ Indeed, the kinetic parameters for the reaction between AmbDH3 and **(6*S*,7*R*)-10** were determined to be in the range of previously observed values for reductive loop domains from modular type I PKS ($K_m = 8.2 \pm 3.1$ mM, $k_{cat} = 230 \pm 83$ s⁻¹, $k_{cat}/K_m = 28.2 \pm 14.8$ s⁻¹*M⁻¹; Figure S28).⁵⁶⁻⁵⁹ A semipreparative scale time course experiment showed a steady and finally quantitative conversion in an overnight incubation, confirming that the rather slow reaction is not an issue at all for the use of the enzyme in biocatalytic reactions (Figures S29-S33). Only *2R,3R*- / *3R*-configured products (or equivalent, like **(2*R*,3*S*)-33**) were observed in the substrate specificity study, showing that the stereoselectivity of AmbDH3 is conserved among a broad range of substrates. The enzymatic cyclization gives access to various types of heterocycles, such as THFs (**31**) and 1,4-dioxanes (**33**). The low solubility of substrates like **16**, **18**, and **26** in aqueous buffer was identified as a cause for their low to modest conversion. This problem might be overcome by adaption of the reaction conditions (*vide infra*).

Molecular modeling. After we had gained a comprehensive overview of the AmbDH3 substrate specificity, we turned to link this knowledge with structural data. Some members of the precursor library were modeled into the active site of the previously determined X-ray structure of AmbDH3 (PDB ID: 5O15)⁴⁵ and the distances between C-3, 7-OH and the catalytic

residues were put in relation to the conversion (Figure S27, Table S1). A good correlation of the latter with the D215–7-OH distance was observed. Complex model structures of AmbDH3 with the non-competent substrates **(6*S*,7*S*)-10**, **37** and **38** showed distances of >5 Å and a bent backbone conformation that orients 7-OH away from D215 so that its activation for a nucleophilic attack is prevented. In the complex models of all accepted substrates, these values were in the range of 1.9–3 Å. Among these, a strict proportionality between distance and in vitro conversion was not observed, because other effects, like the low solubility of **16**, **18** and **26**, interfered. These results suggest that molecular modeling can be used to estimate the principle acceptance of substrates by AmbDH3. They also set the stage for a structure-guided directed evolution of AmbDH3 features.

Kinetic resolution. The ability to efficiently differentiate between various presented stereoisomers forms the basis for DKR and DR.^{60,61} AmbDH3 was incubated with various stereoisomeric mixtures of **41** and **10** (Table 2). Of *rac*-**41**, AmbDH3 cyclized only **(7*R*)-41**, while **(7*S*)-41** remained unreacted (entry 1, Figures S34, S41 and S42). In incubations of AmbDH3 with various stereoisomer mixtures of **10**, only **(6*S*,7*R*)-10** and **(6*R*,7*R*)-10** were converted into the expected *cis*-THPs, albeit to a strongly varying degree (entries 2–6, Figures S35–S40 and S43–S48). **(6*S*,7*R*)-10** was converted in all reactions by at least 80%, irrespective of the ratio of competing stereoisomers. **(6*S*,7*R*)-11** was the major stereoisomer formed in all reactions with **(6*S*,7*R*)-11**:**(6*R*,7*R*)-11** ratios of up to 90:10.

Table 2. Reaction of AmbDH3 with stereoisomeric mixtures of 41 and 10.



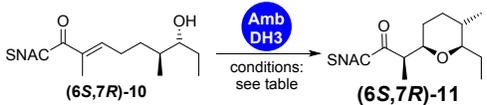
R=H		Inserted stereoisomers of 41 [%]		Conversion into 42 ^[b]		Product ratio ^[b]			
Entry ^[a]		7 <i>R</i>	7 <i>S</i>	S[(7 <i>R</i>)+(7 <i>S</i>)]		(7 <i>R</i>):(7 <i>S</i>)			
1		50	50	47%		>95:<5			
R=Me		Inserted stereoisomers of 10 [%]				Conversion into 11 ^[b]		Product ratio ^[b]	
Entry ^[a]		6 <i>S</i> ,7 <i>R</i>	6 <i>R</i> ,7 <i>R</i>	6 <i>S</i> ,7 <i>S</i>	6 <i>R</i> ,7 <i>S</i>	Σ[(6 <i>S</i> ,7 <i>R</i>)+(6 <i>R</i> ,7 <i>R</i>)]		(6 <i>S</i> ,7 <i>R</i>):(6 <i>R</i> ,7 <i>R</i>)	pr/sr ^[c]
2		50	0	0	50	41%		>95:<5	n.d.
3		13	37	37	13	15%		73:27	8.1
4		30	30	30	10	32%		90:10	9.1
5		10	30	30	30	12%		74:26	8.4
6		26	24	24	26	23%		90:10	8.5

^aThe summed up concentration of the precursor stereoisomers was 2 mM for **41** and 4 mM for **10**. ^bThe conversion of the individual stereoisomers of **10** was qualitatively estimated by analysis of the chiral HPLC chromatograms. The overall conversion and the ratio of the cyclic stereoisomers in the conversions of **41** and **10** were quantified by ¹H NMR analysis in a separate experiment. ^cpr: ratio of **(6*S*,7*R*)-11**:**(6*R*,7*R*)-11** in the crude product; sr: ratio of **(6*S*,7*R*)-10**:**(6*R*,7*R*)-10** in the starting material

This selectivity opens the possibility to develop novel types of DKR or DR processes to chiral CSOH that would provide control over up to four stereocenters. Dynamic epimerization of the secondary alcohol could be achieved by adapting

established systems for chemoenzymatic DKR using transfer hydrogenation catalysts or alcohol dehydrogenase-catalyzed DR.^{62–70} Under ketone-epimerizing conditions, the stereocenter at C-6 could additionally be included.

Table 3. Optimization of the reaction between (6*S*,7*R*)-10 with AmbDH3.



Conversion optimization ^a					Yield optimization						
Entry	Scale [mg]	Enzyme ^b	Co-solvent ^c	Conversion [%] ^d	Entry	Scale [mg]	PhMe [vol%]	T [°C]	c (substrate) [mM]	Workup ^e	Yield [%] ^f
1	10	purified	-	87	1 ^[g]	50	10%	37	8	A	26
2	30	purified	-	<50	2	50	10%	37	4	A	60
3	10	lysate	-	>95	3	50	10%	37	2	A	54
4	30	lysate	-	93	4	50	5%	37	4	A	66
5	50	lysate	-	<50	5	50	2%	37	4	A	68
6	10	lysate	PhMe	quant.	6	50	1%	37	4	A	76

7	30	lysate	PhMe	>95	7	100	1%	37	4	A	62
8	50	lysate	PhMe	94	8	100	1%	30	4	A	66
					9	100	1%	25	4	A	64
					10	50	1%	30	4	B	80
					11	50	1%	30	4	C	80
					12	50	1%	30	4	D	76

^aReaction conditions: “enzyme”, 8 mM of (**6S,7R**)-**10**, HEPES buffer (pH 6.8), 37 °C, 16 h. ^b“purified”: 5 mg/mL of affinity-purified enzyme. “lysate”: The equivalent amount of cell pellet as for “purified” (2 g pellet / 50 mg substrate) was sonicated and the supernatant used directly after centrifugation. ^cThe substrate was dissolved in the organic co-solvent and added to the cell lysate (10vol% co-solvent). ^dThe conversion was determined by ¹H NMR spectroscopy. ^eWorkup comprised triple extraction of the crude reaction mixture with EtOAc (A), accompanied by previous treatment with CaCl₂ and proteinase K (B), CaCl₂ and papain (C) or CaCl₂, proteinase K and papain (D), respectively. ^fThe yield was determined after column chromatography on silica gel. ^gIdentical conditions as in entry 8 of “Conversion optimization”.

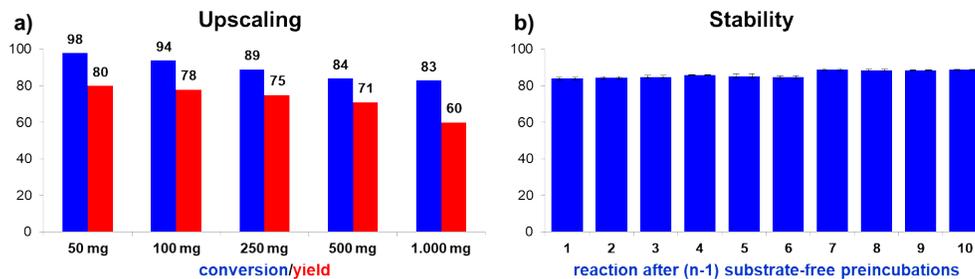


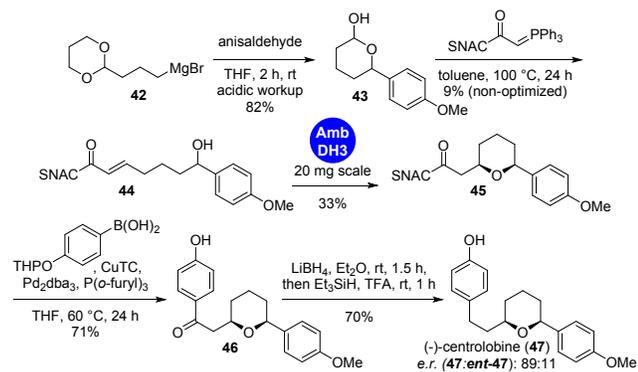
Figure 2. a) Upscaling of the reaction to the preparative scale (conditions as in Table 3, right, entry 10). b) Conversion of (**6S,7R**)-**10** by AmbDH3 after various substrate-free preincubations of the enzyme.

Upscaling of the enzymatic reaction. Lack of scalability is a common problem of enzymatic transformations. In order to enable the preparative-scale synthesis of chiral THP building blocks we performed an optimization of the cyclase reaction conditions (Table 3). Scaling up the reaction between AmbDH3 and (**6S,7R**)-**10** under the previously described conditions led to a significant decline in conversion for reaction batches above 10 mg (33.3 μmol) starting material (entries 1 and 2).⁴⁴ An upscaling to 50 mg batches with a conversion of 94% became possible by using the cell-free extract instead of the isolated enzyme and by adding 10% of toluene to the reaction (entries 3–8). Reducing the substrate concentration from 8 to 4 mM and fine tuning of the solvent amount gave 76% yield on the 50 mg scale (Table 3, right, entries 1–6). At higher quantities, increased precipitation of the enzyme led to lower yield (entry 7). This problem was addressed by reducing the reaction temperature and by treating with a proteinase before extractive workup (entries 7–12). An isolated yield of 80% was reproducibly obtained on the 50 mg scale along with nearly complete recovery of the inserted organic material (entries 10 and 11). The established conditions allowed a further upscaling to batches of 1 g (3.32 mmol) starting material (Figure 2a, Figure S48). 83% Conversion and 60% isolated yield (15% recovery of (**6S,7R**)-**10**) after one round of workup were observed. Further recovery of organic material was possible after repetitive proteinase treatment of the precipitate. The scalability and clean course of the reaction shows that this enzymatic transformation is suitable for producing preparative batches of chiral heterocyclic building blocks under operationally simple conditions.

Stability of AmbDH3. In order to investigate the stability of the enzyme, we reacted AmbDH3 with (**6S,7R**)-**10** after repetitive, substrate-free exhaustion cycles (Figure 2b, Figures S49–S58). The enzyme was incubated up to nine times in the

absence of substrate for 16 h at 30 °C, interrupted by resting periods of 8 h at 4 °C. In the respective ultimate cycle, (**6S,7R**)-**10** was added and the conversion determined after 16 h reaction. The conversion remained on a high level independent of the number of substrate-free preincubations. Lyophilization or freeze-thawing of AmbDH3 also had no detrimental effect on the conversion (Figures S59–S68 and Tables S2–S3).

Chemoenzymatic total synthesis. Pyran-containing diarylheptanoids are plant natural products with antibiotic, antiproliferative and antioxidative activity.^{71,72} (–)-Centrolobine (**47**) is a member of this class that shows no further structural similarities to the ambruticins and thus represents an optimal model system to demonstrate the suitability of AmbDH3 for general chemoenzymatic synthesis (Scheme 2, for reported total syntheses see for example^{73–76}). Racemic **44** was synthesized in two steps from bromoacetal **42**. Enzymatic cyclization of **44** on a reaction scale of 20 mg (57 μmol) starting material resolved the enantioenriched *syn*-THP with a conversion of 40% and an isolate yield of 33%. The SNAC moiety was exchanged with a 4-hydroxyphenyl group by Liebeskind-Srogl coupling. Ketone reduction using LiBH₄ followed by EtSiH₃ and TFA treatment led to a mixture of (–)-centrolobine (**46**) and (+)-centrolobine (*ent*-**46**). The enantiomeric ratio of 89:11 in favor of **46** suggests that AmbDH3 exhibits comparable selectivity for the precursor (*7R*)-**44** as for the natural substrate surrogate (see section “Kinetic resolution”). The substrate tolerance and stereoselectivity as well as the scalability of the AmbDH3 reactions, positively impact the outcome of this enantioselective synthetic sequence so that the need for stereoselective chemical transformations is avoided.



Scheme 2. Chemoenzymatic total synthesis of (-)-centrolobine using AmbDH3 for *cis*-THP-formation in the key step.

CONCLUSION

We report our investigations on AmbDH3, a member of the recently discovered, but biocatalytically not exploited group of IMOMA cyclases. AmbDH3 exhibits relaxed substrate specificity and conserved stereoselectivity along a broad range of substrates, like enethioates and vinylketones, providing access to various types of CSOH like THPs, THFs and 1,4-dioxanes. The high stereoselectivity of AmbDH3 on C-2 and the efficient discrimination between individual C-6 and C-7 stereoisomers go beyond the scope of the current synthetic methodology for IMOMA. These features were exploited in the resolution of chiral THPs with up to four asymmetric centers from racemic precursor mixtures and might form the basis for the development of multienzyme or hybrid catalytic systems for DKR or DR to CSOH. Upscaling of the enzymatic cyclization succeeded, providing access to preparative amounts of chiral THP. The enzyme was successfully applied in the enantioselective total synthesis of the THP natural product (-)-centrolobine.

This work is one of the rare examples in which isolated PKS domains were investigated regarding their performance in (semi)preparative enzymatic synthesis. Future work will aim to improve the reaction conditions to expand the scope to hydrophobic substrates and more common Michael acceptors like *S*-alkylthioesters and vinylketones. The reported AmbDH3 structure will form a solid base for improving relevant properties by enzyme engineering.⁷⁷ Our work paves the way for the characterization of IMOMA cyclases from other biosynthetic pathways to expand the scope of biocatalysts and accessible CSOH types. Incorporation of IMOMA cyclases into (chemo)enzymatic cascades might expand the product spectrum to other types of chiral heterocycles like piperidines. Finally, the combined results on the AmbDH3 structure-function relationship and its substrate specificity profile are an important milestone on the way to a rational engineering of cyclase activities into existing multimodular assembly lines in the frame of directed biosynthesis approaches.⁴⁵

EXPERIMENTAL SECTION

Enzyme expression and purification. The plasmid *ambDH3 pET28a(+)* was transformed into chemically competent *E. coli* BL21(DE3) cells. A single colony was used for inoculation in dYT medium with kanamycin (final concentration 0.1 mM) for plasmid selection. Cultures were grown to an OD₆₀₀ of 0.5 at 37 °C, induced with 0.1 mM IPTG and shaken at 15 °C for 22 h. Cells were harvested by

centrifugation (5,000 g, 4 °C, 15 min). For purification, 1 g of cells was suspended in 10 mL HEPES buffer (pH 6.8, 25 mM HEPES, 100 mM NaCl) and cell disruption was performed on ice by sonication (45% amplitude, 10 cycles, 30 s sonication, 30 s pause). After centrifugation (10,000 g, 4 °C, 30 min) the obtained crude lysate was filtered (0.45 μm, cellulose acetate) and the target protein purified by FPLC equipped with a HisTrap™ FF Ni-NTA column. The column was washed with washing buffer (30 mM Tris, 500 mM NaCl, 10 mM imidazole, 10% glycerol, pH 7.5). Elution of the target protein could be observed during a linear gradient elution with elution buffer (30 mM Tris, 500 mM NaCl, 500 mM imidazole, 10% glycerol, pH 7.5) from 0 to 100% in 25 mL. The protein-containing fractions were united and the elution buffer replaced by HEPES buffer (25 mM HEPES, 100 mM NaCl, pH 6.8) using PD-10 desalting column. If necessary, the purified enzyme was concentrated and immediately used for activity assays.

Semipreparative scale conversions. Assays for determination of substrate tolerance were carried out in a total volume of 4 mL containing 8–10 mM of substrate and 5.0–5.4 mg/mL of AmbDH3 in HEPES buffer (pH 6.8, 25 mM HEPES, 100 mM NaCl). After incubation at 37 °C for 16 h, the sample was extracted with 3 * 5 mL EtOAc and the solvent was removed under reduced pressure. The product was dissolved in CDCl₃ and analyzed by ¹H NMR spectroscopy to determine the conversion.

Preparative scale enzymatic conversions. For preparative scale assays, 2.0 g cell pellet per 50 mg substrate were resolved in HEPES buffer (1 mL per 0.1 g pellet, 25 mM HEPES, 100 mM NaCl, pH 6.8) and cells were disrupted by sonication (10 cycles, amplitude 45%, 0.02 kJ/s, 30 s output, 30 s break). After centrifugation (10,000 g, 30 min, 4 °C) the crude lysate was directly used for the enzyme assays without further purification. The substrate was dissolved in toluene (1 vol%), the volume filled up to give a final substrate concentration of 4 mM and the crude lysate was added. After stirring at 30 °C and 175 rpm for 16 h proteinase K (4 mg per 50 mg substrate) and CaCl₂ (finale concentration 1 mM) were added and the reaction was stirred at 37 °C for 2 h. The phases were separated and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with saturated NaCl solution, dried over MgSO₄ and concentrated under reduced pressure. The conversion was determined by integration of the appropriate signals in the ¹H NMR spectrum. Purification by flash chromatography on silica gel (CH₂Cl₂:EtOAc / 2:1 → 0:1) gave the product (**6S,7R**)-**11** as colorless solid. Variations of this procedure were implemented as described in Table 3.

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Notes

The authors declare no competing financial interest

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information material is available free of charge at the ACS website.

1. Methods and materials, Synthetic procedures, Studies on the substrate tolerance of AmbDH3, Docking calculations, Kinetic analysis, Time course experiment, Kinetic resolution, Upscaling of the enzymatic reaction, Stability studies.
2. NMR spectra for all synthetic compounds and assay products.

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