Enzyme Catalysis

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A Dehydratase Domain in Ambruticin Biosynthesis Displays Additional Activity as a Pyran-Forming Cyclase**

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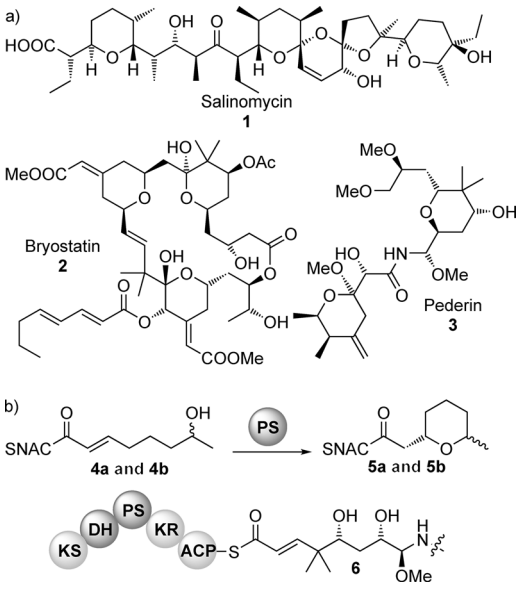
Abstract: *Hydropyran rings are a common structural motif in reduced polyketides. Information on their biosynthetic formation and particularly the biochemical characterization of the responsible enzymes has only been reported in few cases. The dehydratase domain AmbDH3 from the ambruticin polyketide synthase was investigated. Through in vitro assay of the recombinant domain with synthetically-derived substrate surrogates, it was shown that it has a second catalytic activity as a cyclase that performs oxa-conjugate addition. Probing AmbDH3 with synthetic substrate analogues revealed stereoselectivity and substrate tolerance in both substeps. This is the first characterization of a pyran-forming cyclase from a cis-AT PKS system and the first report of a polyketide synthase domain with this kind of dual activity. Finally, it was revealed that this domain shows potential for application in chemoenzymatic synthesis.*

Polyketides are important natural products with great potential for drug discovery. The products of reducing type I polyketide synthases (PKSs) are structurally highly diverse and represent ambitious synthetic targets. Bacterial type I PKSs are multimodular multi-enzyme complexes that conduct the standard processing of the polyketide backbones during biosynthesis.[1] They catalyze the elongation of a growing polyketide chain by decarboxylative Claisen-like condensations. In each module, a so-called reductive loop establishes the individual backbone functionalization pattern. Ketoreductase (KR) domains reduce 3-oxo-thioesters to 3-hydroxythioesters, dehydratase (DH) domains eliminate water, and enoylreductase (ER) domains reduce the resulting α,β-unsaturated thioesters to fully saturated chains. During the whole process, the substrate remains tethered to the PKS through the phosphopantetheine linker on the acyl carrier proteins (ACPs). PKS assembly lines can also contain domains that carry out unconventional transformations, which finally lead to the formation of alternative structural elements.[2] Although biosynthetic mechanisms for these processes have frequently been proposed based on gene cluster analysis, few have been confirmed by in vitro studies on the enzyme level.

Oxygen-containing heterocycles are present in many polyketides, including some with exceptional biological activity like salinomycin (**1**) and bryostatin (**2**; Scheme 1 a).[3] It is known that these rings are formed by oxidation/cyclization or epoxidation/epoxide-opening cascades.[4] In addition, domains with sequence similarities to Δ^5-3-ketosteroid isomerases, hydrolases, or dehydratases have been proposed to act as cyclases that perform oxa-conjugate addition, based on gene cluster analysis and bioconversion experiments.[5]



Scheme 1. a) Structures of pharmacologically relevant pyran-containing polyketides; b) In vitro activity of a PS domain from the pederin PKS was previously demonstrated with structurally reduced substrate surrogates **4a** and **4b**. These differ considerably from the natural precursor **6**.[2b,3,5h] PS = pyran synthase, SNAC = *N*-acetylcysteamine.

Recently, the first in vitro characterization of a so-called pyran synthase (PS) domain was reported.[2b,5e] The PS domain from module 7 of the pederin PKS-NRPS hybrid (PedPS7) converted simple substrate surrogates **4a** and **4b** into the respective cyclized forms **5a** and **5b** with *ee* values of 67 and 89% in favor of the natural 3-L-configuration (Scheme 1 b; NRPS = nonribosomal peptide synthetase). Since the proposed natural PedPS7 precursor **6** differs considerably from the surrogate substrates **4a** and **4b**, this

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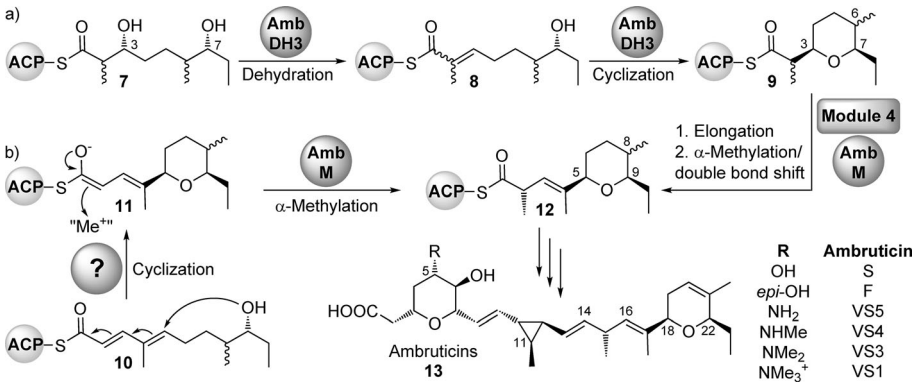
result demonstrates substrate tolerance of PedPS7, but also leaves open questions about its actual stereoselectivity. Further experiments with more realistic substrate surrogates are thus required.

Gene cluster analyses revealed that PS domains are usually coded in DH-containing modules and act downstream of the latter domain by cyclizing its α,β-unsaturated product (Scheme 1b). All PS-containing systems known to date belong to the class of *trans*-AT PKS. For the much more abundant *cis*-AT PKS, nature seems to have developed alternative strategies, which have not yet been extensively studied.[5h,6]

Although the gene clusters of several pyran-containing *cis*-AT PKS products, for example, the ionophoric polyethers salinomycin (**1**) and nigericin, have been sequenced, a unifying proposal for the responsible catalytic entity is still missing.[5h,6a]

The ambruticins (**13**) are important lead structures for the development of antimycotic agents because of their unique interaction with the high-osmolarity glycerol protein kinase pathway (Scheme 2).[6b,7] They are assembled by a type I *cis*-AT PKS and contain a dihydropyran ring in the eastern part of the molecule. This ring was proposed to be formed by an oxa-conjugate addition, followed by dehydrogenation by the iron-sulfur Rieske protein AmbO at a later point of the biosynthesis. The configuration of the intermediately formed stereocenter at C-8 (in **12**) is unknown. The respective 20,21-dihydroambruticins were isolated from an *ambO* deletion mutant and structurally analyzed. However, both epimers of 20,21-dihydroambruticin were shown in the respective manuscript and the analytical data were not presented. A clear assignment of the absolute configuration at C-21 was thus not possible.[6b]



Scheme 2. Two hypothetical pathways for hydropyran ring formation in the eastern part of the ambruticins as proposed by us (a) and Reeves et al. (b).[6]

Gene cluster analysis suggested that the pyran cyclization must occur during processing by PKS modules 3 or 4.[6b] Both modules contain ACP, KS, AT, KR, and DH domains, consistent with twofold ketide elongation and ultimate processing to a 2,4-dienoyl thioester. Reeves et al. suggested a biosynthetic route to **12** that proceeds through a 1,6-conjugate addition on the vinylic Michael acceptor **10** and subsequent trapping of the resulting enolate **11** with an electrophilic methyl group originating from *S*-adenosyl-L-methionine (Scheme 2b).[6b] The latter step was proposed to be catalyzed by the C-methyltransferase AmbM. Usually, standard PKS domains process intermediates only on C-2 and C-3. Thus we favored a mechanisms in which oxa-1,4-conjugate addition in module 3 leads to tetrahydrofuran **9** (Scheme 2a). Elongation to the α,β-unsaturated system in module 4, followed by methylation and a double-bond shift would then yield the β,γ-unsaturated intermediate **12**.[8] Since the ambruticin gene cluster does not harbor a PS-like domain, we hypothesized that the DH domain of module 3 (AmbDH3) is responsible for carrying out both transformations: dehydration and the following cyclization.

To test our biosynthetic hypothesis, we aimed to assay the recombinant domain AmbDH3 in vitro with suitable substrate surrogates.[9] In order to learn about mechanistic details and to assign the exact constitution and configuration of the biosynthetic precursor, we envisaged chemically synthesizing all potential precursors, intermediates, and products of the process. The number of putative substrates could gratifyingly be reduced by bioinformatic analysis.[10] The KR domains of the ambruticin PKS modules 1 and 3 both showed the characteristic LDD motif and thus indicated a 3-D,7-D-configuration of precursor **7**.[10a,b] Application of another recently reported algorithm, however, did not give a sufficiently clear prediction for the configurations at C-2 and C-6, so we decided to synthesize all possible configurational isomers.[10c]

Surrogates for 3-hydroxy-ACP thioester **7**, enoate-ACP thioester **8**, and tetrahydropyran-ACP thioester **9** were prepared in the form of *N*-acetylcysteamine (SNAC) thioesters, which are common surrogates for the ACP-bound form of DH substrates (Figure 1a).[9] Two series of compounds with 6-L,7-D-configuration (**14**–**18**) and 6-D,7-D-configuration (**19**–**22**) were synthesized and their absolute configurations were determined by single-crystal X-ray analysis and NMR spectroscopy (Scheme S1, Figure S16–18, and Table S2 in the Supporting Information).[11] The domain boundaries of AmbDH3 were determined through alignment to previously characterized DH domains.[9a,b] The identified gene was cloned into a pET28a(+) vector, heterologously expressed in *E. coli*, and used in the assays after affinity purification (Figure S1).

HPLC–MS analysis after individual overnight incubations of the 3-hydroxy thioesters **14** and **15** with AmbDH3 showed conversion only for the 2-D,3-D-configured precursor **15** (Figure 1b, Figure S3, S4). This finding is in agreement with previous reports of a conserved high substrate specificity for DH domains regarding the configurations at C-2 and C-3.[9] The two more hydrophobic products of this reaction co-eluted with the synthetic standards **16** and **17**, thus suggesting a dehydration–cyclization cascade (Figure 1b,c). To confirm

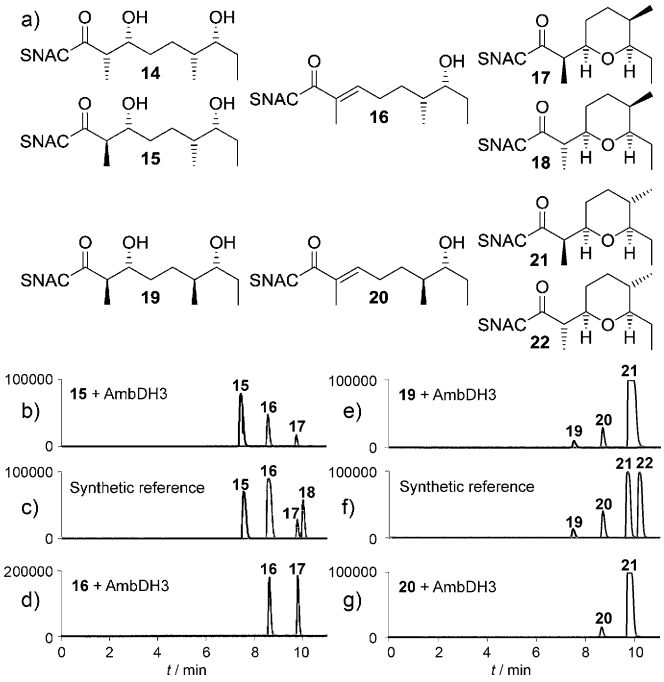


Figure 1. a) Structures of the synthetic substrate and product surrogates **14**–**22** that were applied in the assays. HPLC–MS analyses are shown for the AmbDH3 overnight incubations with **15** (b) and **16** (d), as well as with compounds **19** (e) and **20** (g). The individual traces for the synthetic reference compounds **15**–**22** are combined in panels (c) and (f) for clarity (for unprocessed data, see Figure S5–9).

the nature of *E*-configured olefin **16** as a competent intermediate, we also individually incubated this compound with the AmbDH3 domain. We observed the formation of only one product, which also co-eluted with the 2D,3D-configured pyran **17** (Figure 1d and Figure S5). Usually, DH-catalyzed reactions exist in an equilibrium between the hydrated and dehydrated forms. Interestingly, in our case no 3-hydroxy thioester like **15** was obtained, thus suggesting that the position of the equilibrium was located far on the side of the unsaturated (**16**) and cyclized (**17**) forms.[9]

The presence of detectable quantities of starting material **15**, intermediate **16,** and product **17** (amounts decreasing in this order) following long-term incubation of AmbDH3 with compound **15** points towards low specificity of the domain for the applied substrate, particularly in the cyclization step. By contrast, incubation of 2-D,3-D,6-D,7-D-configured compound **19** with AmbDH3 gave only minor amounts of the starting material **19** and the α,β-unsaturated intermediate **20**, but high quantities of tetrahydropyran **21** (Figure 1e,f and Figure S7). Incubation of intermediate **20** gave nearly complete conversion into compound **21** and no rehydration to **19** (Figure 1g and Figure S8). On the basis of the differences in conversion, we concluded that the natural intermediates of the pathway must be 2-D,6-D-**7**, (2*E*)-6-D-**8**, 2-D,6-D-**9** and 8-D-**12** (Scheme 2).

To definitely assign the cyclase activity to AmbDH3, we performed a series of control experiments. Enzyme-free overnight incubations of the synthetic surrogates **14**–**16** and **19**–**22** under assay conditions showed no conversion, thus excluding spontaneous, non-enzymatic side reactions (Figure S3a,b–8a,b,S9). To ensure that the observed enzymatic conversion was not caused by trace amounts of another protein that was purified along with AmbDH3, we generated a specific mutant in which the active site histidine was replaced with an alanine residue. No conversion was obtained if **19** or **20** were incubated with this mutant, thus showing that AmbDH3 was indeed responsible for dehydration (Figure S10). Finally, we aimed to show that the cyclization activity of AmbDH3 is an intrinsic feature of the domain and not just an artifact from applying this particular kind of substrate. Therefore, we incubated the competent substrate **19** with the recombinant domain BorDH3 from the borrelidin PKS. We had shown before that this domain accepts 2-D,3-D-configured precursors and transforms them stereoselectively into *E*-configured enoates with broad substrate specificity.[9a,b] BorDH3 performed the expected dehydration to the *E*-configured enoate **20**, but no further cyclization to tetrahydrofuran rings like **21** or **22** was observed (Figure S11).

In order to unambiguously prove the configurational assignment of the products and furthermore explore the potential of AmbDH3 for synthetic purposes, we repeated all of the assays with substrates **15**, **16**, **19**, and **20** on a semi-preparative scale (>8 mg substrate in each case) and analyzed the outcome by NMR spectroscopy (Figure 2 and Figure S12–15). In all cases, we observed similar results to the assays analyzed by HPLC–MS. AmbDH3 formed products **17** and **21** from substrates **16** and **20**, respectively, with conversions of 18 and 87% (determined from the NMR spectra). Incubation of AmbDH3 with 8.0 mg of the precursor **20,** followed by extractive workup gave virtually pure product **21**. This result is a promising starting point for the further development of AmbDH3 as a useful chemoenzymatic tool for the stereoselective synthesis of tetrahydropyran rings.

Known PS domains show relevant protein sequence homology to DH domains, but fall into a distinct phylogenetic clade.[2b] Furthermore, the DH-characteristic active-site motif His-Ser-Asp is mutated to His-Ser-His/Asn, in agreement with the fact that an acidic residue located close to C-3 of the α,β-unsaturated PS precursor would be counterproductive for the proposed conjugate addition mechanism. We aligned the AmbDH3 amino acid sequence against a collection of PS domains and DH domains from both *cis*- and *trans*-AT PKSs and found that AmbDH3 bears the DH-characteristic His-Ser-Asp active-site motif (Figure S19). Phylogenetic analysis also revealed that AmbDH3 falls into a clade with typical DH domains from *cis*-AT PKS systems (Figure S20).

In summary, we describe the unprecedented discovery of a PKS domain that shows two catalytic activities: as a dehydratase and as a cyclase that performs oxa-conjugate 1,4-addition. Our work is the first characterization of a pyran-forming cyclase from a *cis*-AT PKS system and the first report of a PKS domain with this kind of dual activity.[2b] Stereoselective synthesis of surrogate substrates allowed us to interrogate the AmbDH3 domain with realistic precursor, intermediate, and product analogues. As a result, we were able to show that both sub-steps naturally proceed from 2-D,6-D-**7** with high stereoselectivity, thereby leading to the intermediate (2*E*),6-D-**8** and finally to the tetrahydropyran product

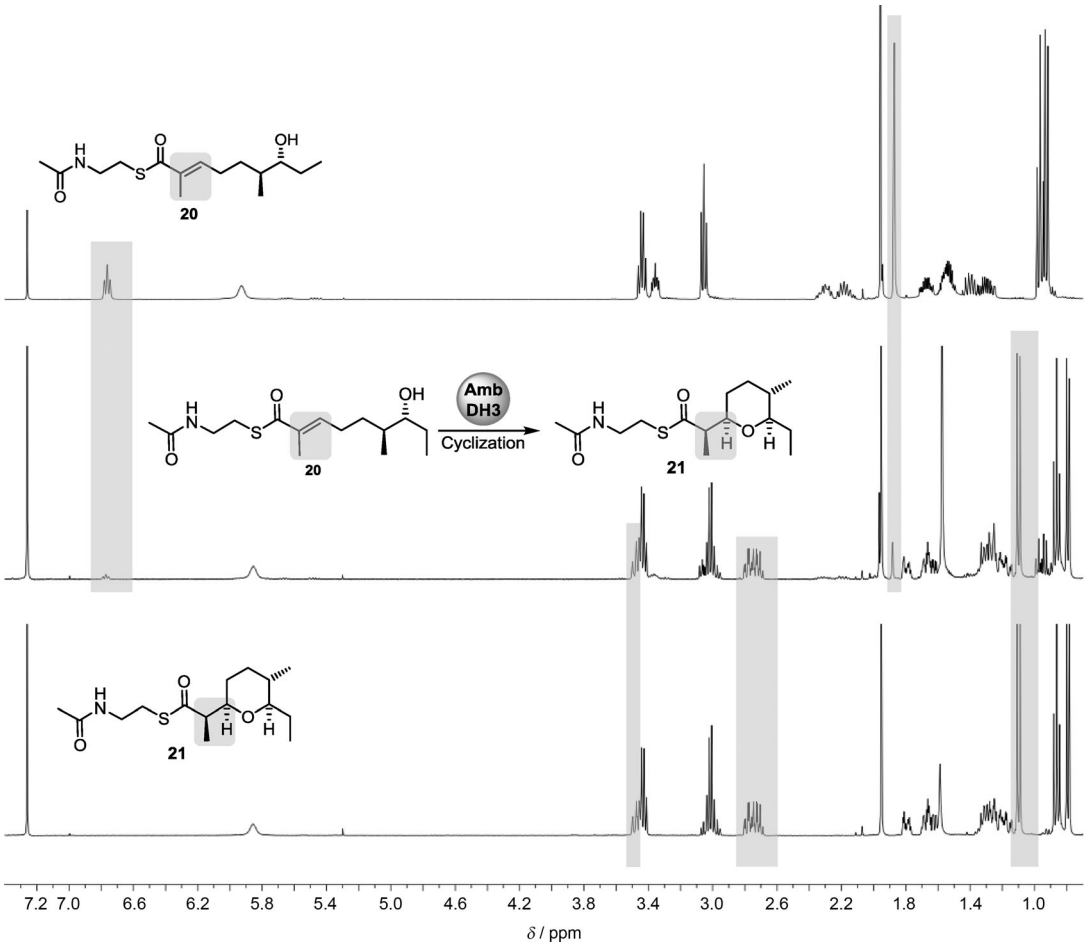


Figure 2. ^{1}H NMR analysis of a semipreparative-scale conversion of compound **19** with AmbDH3 (middle panel), as well as for starting material **19** (upper panel) and product **21** (lower panel; all spectra recorded in $CDCl_3$ at 400 MHz). Characteristic signals are highlighted with a gray background.

2-D,6-D-**9**. The substrate specificity of AmbDH3 is narrow regarding the configurations at C-2 and C-3, but configurational changes at C-6 are tolerated. Pronounced differences in the conversion of the diastereomeric substrates **15** and **19**, as well as **16** and **20**, clearly indicate that the previously uncertain configuration at C-8 in the biosynthetic intermediate **12** is D.

Enzymes from the biosynthetic pathways of secondary metabolites have great potential to find application as tools for the chemoenzymatic synthesis of natural products.[12] We have shown that AmbDH3 is able to conduct clean, semipreparative-scale reactions under neutral conditions. Thus, the stage is set for a further investigation of AmbDH3 regarding its applicability to the chemoenzymatic synthesis of pyran-containing natural products. Extensive investigation of the substrate specificity of AmbDH3 will be the next step, as well as the further elucidation of mechanistic and structural details in order to understand how two catalytic activities are combined in one PKS domain.

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[11] CCDC 1017520 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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