VIP Polyketide Biosynthesis Very Important Paper

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An Enzymatic Domain for the Formation of Cyclic Ethers in Complex Polyketides**

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Five- and six-membered cyclic ether moieties are structural features of many bioactive natural products. Examples are the bacteria- and sponge-derived polyketides shown in Figure 1 A as well as many dinoflagellate toxins. Since such heterocycles can be important pharmacological determinants, much effort has been invested in understanding their formation in nature[1] and by stereoselective synthesis.[2] Considerable biosynthetic detail is known for polyether ionophores[3] that are matured by means of intriguing cyclization cascades catalyzed by free-standing epoxide hydrolases. However, as these enzymes act on complex substrates at the post-polyketide synthase (PKS) stage, their development as more general tools in chemoenzymatic synthesis might be challenging.

Many polyketide shown (**1**–**4**) or suspected (e.g., **5**) to be generated by a PKS group termed *trans*-acyltransferase PKS (*trans*-AT PKS)[4] contain ether rings that are likely introduced during chain elongation. *trans*-AT PKSs are giant modular enzymes, in which each module typically elongates the growing polyketide chain by one building block. The minimal domain set is a ketosynthase (KS) for chain elongation and an acyl carrier protein (ACP), to which acyl intermediates are attached by a thioester bond. Optional further processing at this stage is controlled by additional domains that may be present on the module. An important characteristic of *trans*-AT PKSs is the great diversity of module variants as a result of unusual domains and domain combinations.[4,5] In addition to the canonical ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains that also occur in other type I polyketide and fatty acid synthase systems, a wide range of further domains are found, many of which have poorly understood functions. For *trans*-AT PKS modules that introduce building blocks attached to cyclic ethers, a characteristic feature is the presence of a unique domain that exhibits moderate protein homology to DH domains, but features aberrant active-site motifs (Figure S1) and falls into a distinct phylogenetic clade (Figure S2).[6] This PKS component, provisionally termed pyran synthase (PS) domain,[6c] was proposed to catalyze ring closure via oxa-conjugate cyclization as part of the reaction sequence shown in Figure 1 B.[6a,c] All known modules of this type feature the same overall architecture, suggesting a general biosynthetic strategy used by PKSs to install diverse cyclic moieties exhibiting different ring sizes, stereochemistry, and substitution patterns.

To investigate the function of this domain, we expressed the PS of the biosynthetic pathway of pederin (**1**) from an as-yet unculturable symbiotic *Pseudomonas* sp. bacterium associated with *Paederus* spp. rove beetles.[6a,7] Pederin (**1**) is a potent cytotoxin and blistering agent that is used by these beetles as chemical defense.[8] The PS was hypothesized to participate in the generation of the eastern tetrahydropyran (THP) moiety with an *anti* substitution pattern at the ether α positions.[6a] Domain boundaries were determined by alignment to other homologues, including DH domains (Figure S1). After amplification by polymerase chain reaction (PCR) and cloning into pET29a, the PS was expressed and purified as a C-terminally His_6-tagged protein (Figures S3–S5). As test substrates, we synthesized thioesters **6** and **7** along with standards for potential products **8** and **9**, using the routes shown in Scheme 1. These compounds harbor *N*-acetylcysteamine thioester (SNAC) units as simplified surrogates of the ACP-bound 4′-phosphopantetheinyl thioesters in polyketide biosynthesis. The *E*-configured double bond of the natural substrate was assumed on the basis of the stereospecificity motif of the KR located within the same module, which usually correlates with the olefin configuration after dehydration.[9] HPLC (Figure 2) and NMR analyses showed that the conjugated thioesters undergo negligible spontaneous ring closure.

Enzyme assays were performed by incubating **6** and **7** in separate reactions with the expressed PS and monitored by HPLC. New product peaks were detected for both test reactions, but not in controls containing boiled enzyme (Figure 2), indicating conversion of the substrates. For test substrate **6** with a primary alcohol function, HPLC profiles exhibited a single additional peak (Figure 2 A), while for the secondary alcohol **7**, two new peaks (peaks I and II in Figure 2 B) were observed. High-resolution MS analysis suggested that all new peaks belong to compounds with molecular formulae identical to that of the respective test substrate (for the product of **6**: $m/z = 246.1158$, calcd 246.1158, corresponding to $C_{11}H_{20}NO_3S$; products of **7**: $m/z = 282.1139$ and 282.1144, calcd 282.1140, corresponding to $C_{12}H_{21}NO_3SNa$). The UV spectra of all products had

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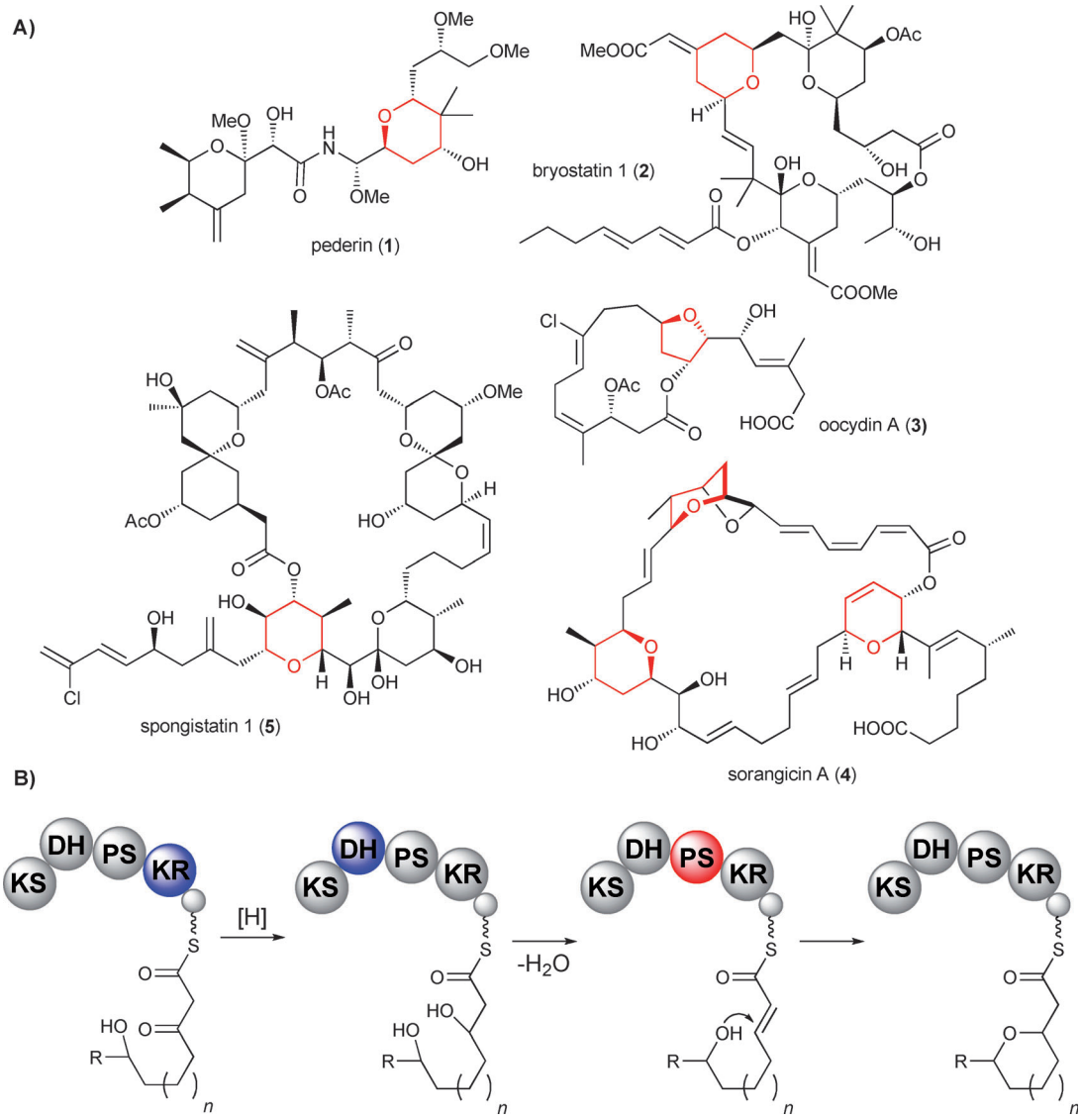
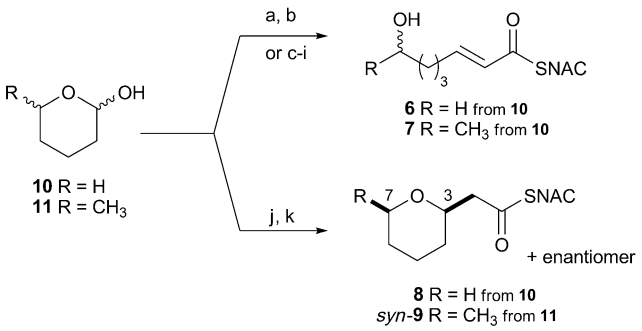


Figure 1. Pyran synthases (PSs) and polyketide heterocyclization. A) Selected polyketides with moieties (shown in red) suspected to be generated by PS domains. B) Biosynthetic model for cyclic ether formation. $n = 0, 1$. Domains acting at the respective stages are highlighted in color.

maxima shifted from 260 and 224 nm to 230 nm as compared to the substrates. These data were in agreement with isomerization resulting from conjugate addition rather than *E*/*Z* isomerization. Further support for ring closure was provided by product HPLC retention times, which were identical to those of the cyclized standards **8** and **9** (*syn* diastereomer only available for the latter).

For detailed characterization, the assay reaction was repeated for **7** at preparative scale for NMR analysis. This yielded 6.4 and 8.3 mg of product eluting at earlier (Figure 2B, peak I) and later (peak II) retention times, respectively. Spectral data for peak II were in agreement with those of the synthetic standard *syn*-**9** and indicated formation of the tetrahydropyran moiety. NMR analysis of peak I was consistent with the *ant* -diastereomer (namely, NOESY cross peak between the methyl doublet and oxygenated methine at C3).

These data raised questions about the stereochemical details of the cyclization. Since the secondary alcohol **7** was used as a racemic mixture in the assay, formation of the two diastereomers could be the consequence of 1) high substrate, but low product selectivity of the PS, 2) low substrate, but high product selectivity, or 3) overall low selectivity. In scenario (1) this would yield two enantiomerically enriched products that exhibit the same configurations at C7 of the THP ring, in (2) two enriched diastereomers with the same configuration at C3, and in (3) mixtures containing significant amounts of all four diastereomers. To distinguish these possibilities, the purified *syn* and *anti* thioesters of peaks I and II were analyzed by HPLC on a chiral stationary phase (Phenomenex, Lux Amylose-2, Figure 3). For each case, the presence of two isomers was observed, with one enantiomer dominating at an *ee* value of 88.6 % for the *anti* and of 66.8 % for the *syn* enantiomeric mixture, thus excluding scenario (3).



Scheme 1. Synthesis of substrates (**6** and **7**) and potential products (**8** and **9**): a) $CH_2(CO_2H)_2$, piperidine, pyridine, overnight at RT→100 °C, 4 h, 43 % b) NAC, EDC-HCl, 4-DMAP, CH_2Cl_2, 0 °C→RT, overnight, 7 %, c) MeMgBr, THF, 0 °C to RT, overnight, 69 %, d) TBSCl, imidazole, CH_2Cl_2, RT, overnight, quant., e) HF, pyridine, THF, RT, overnight, 68 %, f) oxalylchloride, DMSO, CH_2Cl_2, −78 °C, 1.15 h, then Ph_3PCHCO_2Et, NEt_3, RT, overnight, 81 %, g) LiOH, THF, MeOH, H_2O, RT, overnight, 79 %, h) NAC, EDC-HCl, HOBt, 4-DMAP, CH_2Cl_2, 0 °C→RT, overnight, 45 %, i) HF, pyridine, THF, RT, 6 h, 68 %, j) $CH_2(CO_2H)_2$, piperidine, AcOH, DMSO, RT→100 °C, 4.5 h, 71 % (R = H), 75 % (R = CH_3) k) NAC, EDC-HCl, 4-DMAP, CH_2Cl_2, 0 °C→RT, overnight 68 % (**8**), 53 % (**9**). 4-DMAP = 4-(dimethylamino)pyridine, EDC-HCl = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, NAC = *N*-acetylcysteamine, TBSCl = *tert*-butyldimethylsilyl chloride.

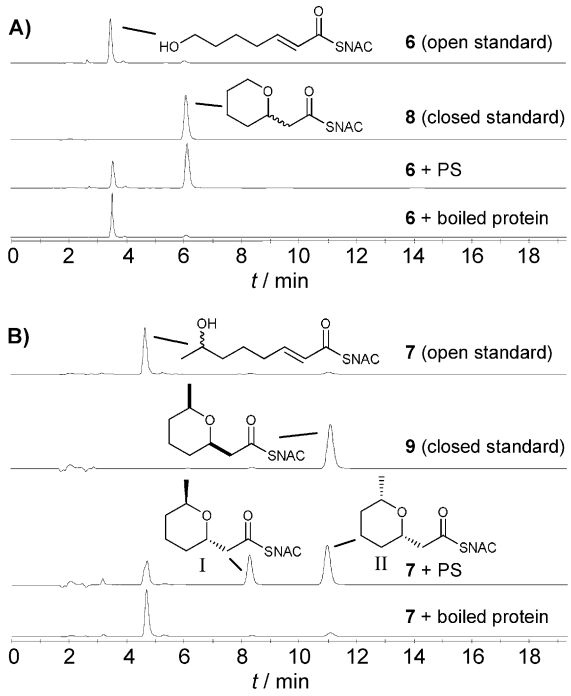


Figure 2. HPLC analysis of the PS-catalyzed reaction. A) Assays with the primary alcohol **6**. The upper two profiles are measurements of the standards, the lower two are analyses of the assay mixture and the negative control, respectively. B) Assays with the secondary alcohol **7**. The order of profiles is as for (A).

To determine the absolute configuration of the products, the compounds corresponding to peaks I and II were converted to the corresponding acids, for which published optical rotation

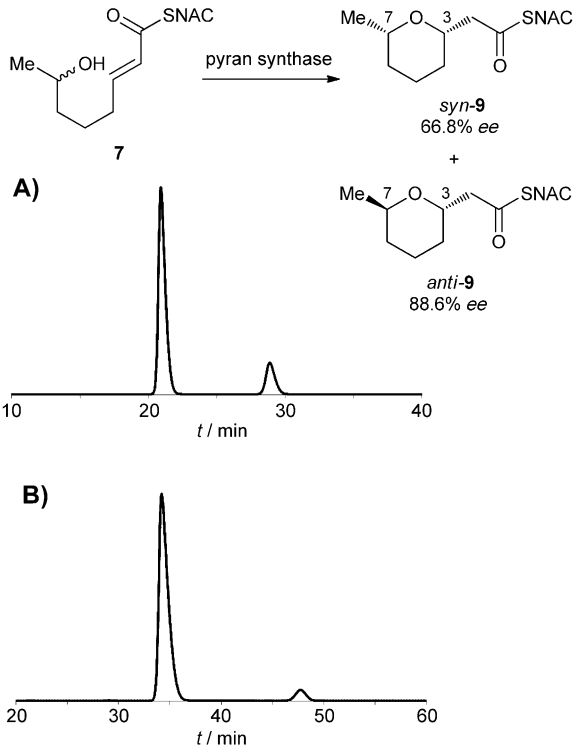


Figure 3. Products of pyran synthase and HPLC analysis of the products using a chiral stationary phase (Phenomenex, Amylose-2, 10 % *i*PrOH/hexane). A) Chromatogram for *syn*-**9**. B) Chromatogram for *anti*-**9**.

values exist.[4,5] This analysis (see the Supporting Information) showed that the main enantiomers of both peaks possess the 3*S* configuration at the newly formed C–O bond, consistent with the *S* configuration at C14 in pederin. The data therefore suggest that the pederin PS exhibits relaxed specificity for the C7 configuration and the attached substituent, but closes the ring in a stereoselective fashion.

In conclusion, we provide evidence for oxa-conjugate addition as a new reaction type catalyzed during chain elongation by a dedicated modular PKS domain. This reaction is likely relevant for the biosynthesis of many further potent natural products from bacterial and invertebrate sources. In addition to uncharacterized transformations that do not seem to involve PS domains,[10] identified examples of ether-forming enzymes in polyketide pathways are epoxide hydrolases,[3] chalcone isomerases,[11] nonactate synthase,[12] and the P450 monooxygenase involved in aureothin biosynthesis,[13] which are all isolated enzymes that are evolutionarily unrelated to PSs and seem to act after formation of the polyketide carbon chain. In contrast to most of these enzymes, our data suggest that PS domains stereoselectively convert simple as well as complex precursors with diverse substitution patterns to a broad range of five- and six-membered products. This feature and the fact that the pederin PS did not require other PKS parts to be active in vitro provides an attractive tool for the chemoenzymatic synthesis of stereodefined oxacyclic products. Current studies on the substrate tolerance, product range, and mechanism of PSs will further assess their preparative potential and biosynthetic scope.

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