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# Insights into Pyrroindomycin Biosynthesis Reveal a Uniform Paradigm for Tetramate/Tetronate Formation

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Supporting Information

**ABSTRACT:** The natural products pyrroindomycins (PYRs), active against various drug-resistant pathogens, possess a characteristic, cyclohexene ring spiro-linked tetramate moiety. In this study, investigation into PYR biosynthesis revealed two new proteins, both of which, phylogenetically distinct from but functionally substitutable to each other in vivo, individually catalyze a Dieckmann cyclization in vitro for converting an *N*-acetoacetyl-L-alanyl thioester into a tetramate. Their counterparts are commonly present in the biosynthetic pathways of spiro and polyether tetronates, supporting a uniform paradigm for tetronate/tetramate formation, which features an enzymatic way to generate the C−X (X = O or N) bond first and the C−C bond next in building of the 5-membered heterocycle.

3-Acyl tetramates, produced by a variety of terrestrial and marine organisms, constitute a large number of natural products that exhibit a wide range of biological activities.[1] Among them pyrroindomycins (PYRs) are unique (Figure 1),

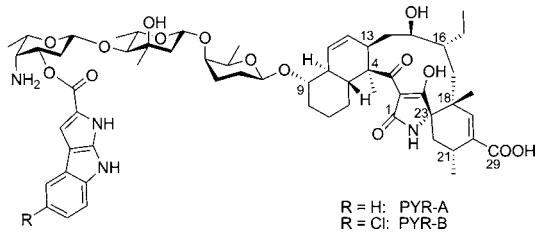


**Figure 1.** Structures of PYRs. The stereochemistry of the macrocyclic core, which has not been established, was predicted in this study from a biosynthetic view.

possessing the components A and B isolated from *Streptomyces rugosporus* LL-42D005 (NRRL 21084) in the screening of agents active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*.[2] PYRs represent the first spirotetramates found in nature, and overall, are structurally close to a family of spirotetronates including the members chlorothricin (CHL), tetrocarcin A (TCA), kijanimicin (KIJ), and abyssomicin (ABY),[3] showing that a cyclohexene ring spiro-linked tetramate/tetronate moiety exclusively conjugates with a *trans*-decalin system (Figure S1). This correlation suggests that they may share a similar biosynthetic strategy for affording the characteristic core structure.

In the past several years, we and others have established the biosynthetic pathways of the spirotetronates CHL, TCA, KIJ, and ABY, as well as a polyether tetronate tetronomycin (TMN), showing that the tetronate formation might be encoded by a highly conserved four or five gene-containing cassette (Figure S2).[4] The gene products, as those in CHL biosynthesis, include a $\beta$-ketoacyl-ACP synthase (KS) III-like protein ChlM, a bifunctional enzyme ChlD1 carrying the acyltransferase (AT) and phosphatase activities, a discrete acyl carrier protein (ACP) ChlD2, a hypothetical protein ChlD3 homologous to various AT E2 subunits of 2-oxoacid dehydrogenase complexes and an unknown protein ChlD4 belonging to the $\alpha/\beta$-hydrolase superfamily. The latter two can be in a fused version as exemplified by TcaD3 or KijE.[4b,c] The essentiality of these genes has been validated in vivo; by contrast, only ChlD1 and ChlD2-like enzymes were characterized in vitro, for loading glyceroyl from 1,3-biphosphoglycerate (1,3-BPG) onto ACP to provide the 3-carbon unit for tetronate formation.[4c,e] Recently, Leadlay and co-workers identified RkD,[4g] a ChlM-like KS III protein in the biosynthesis of a tetronate RK-682 (Figure S1), which alone catalyzes the condensation of $C_{18}$ 3-oxoacyl-ACP and glyceryl-ACP to construct the tetronate moiety. This produces two interesting queries: (i) given the fact that RK-682 synthesis requires no any counterpart of ChlD3 or ChlD4-like protein, the roles of such enzymes in the biosynthesis of above other tetronates remains elusive; and (ii) since tetronate formation involves the building of a C−O and a C−C bonds, which linkage formation is the first, and whether the second is generated spontaneously or enzymatically need to be determined. Herein, we report the investigation into the spirotetramate PYR biosynthesis, which led to identification and functional characterization of the counterparts of ChlD3 and ChlD4-like proteins in the pathway. These studies support a common tetramate/tetronate-forming chemistry and provide the insights into the timing of the catalytic steps to build the 5-membered heterocycle.

To determine the presence of ChlD3 and ChlD4-like proteins specific in the PYR biosynthetic pathway, we set out to identify the PYR gene cluster from the producing strain *S. rugosporus*, by PCR amplification according to the conserved motifs of known ChlD4-like proteins in spirotetronate biosynthesis (Figure S3).[5] Consequently, we identified a 103.5 kb contiguous DNA region (Figure S4), in which the *chlD4* homologue *pyrD4* (57% identity) is closely clustered

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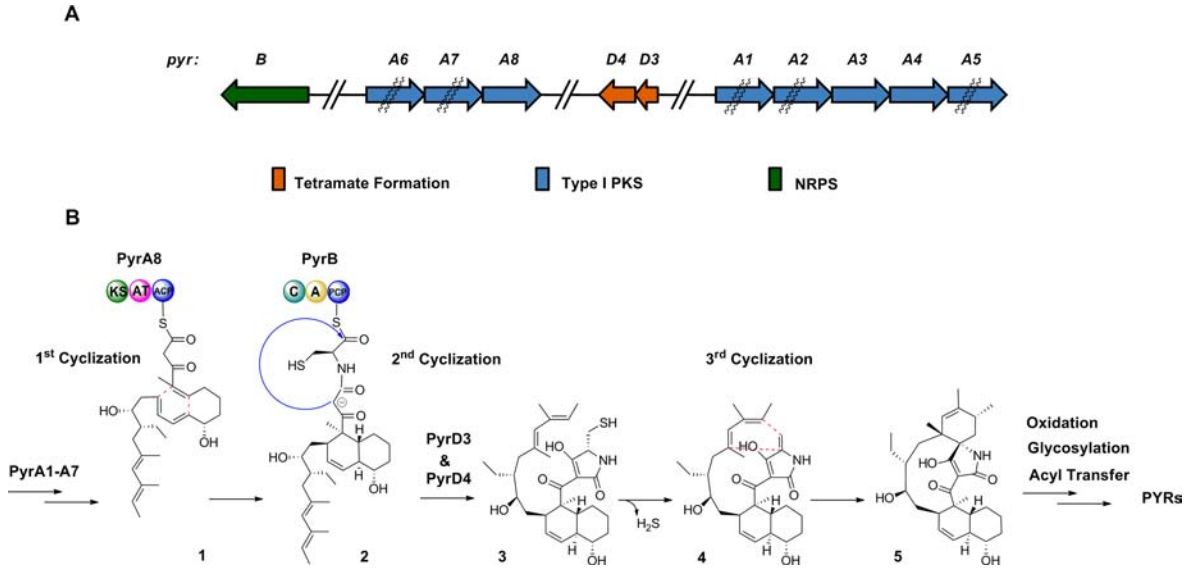


**Figure 2.** Biosynthetic genes and proposed pathway for the macrocycle formation in PYR biosynthesis. (A) Organization of relevant genes (Table S3), encoding tetramate formation (yellow), type I PKSs (blue) and NRPS (green). (B) Skeleton assembly and cyclizations. The functional domains of the modules of selected PKS or NRPS are indicated in color: green for KS, red for AT, purple for ACP or PCP, blue for C, and yellow for A.

with the *chlD3* homologue *pyrD3* (61% identity). They are central to eight polyketide synthase (PKS) genes (*pyrA1*−*A8*) and one non-ribosomal peptide synthetase (NRPS) gene (*pyrB*), as well as those in deoxysugar synthesis, pyrroloindole formation, oxidoreduction, chlorination (by PyrH, previously characterized),[5] and others (Figure 2A and Figure S5A). To correlate the locus with PYR production, we chose the *nrps pyrB* for gene inactivation. As anticipated, the corresponding mutant strain completely lost the ability to produce PYRs (Figure S6), validating that this *chlD3* and *chlD4* homologues-containing gene cluster encodes the biosynthesis of PYRs.

The presence of the *pkss pyrA1*−*A8* suggests a type I PKS system to program the assembly of a linear polyketide intermediate that constitutes the main backbone of the PYR aglycone (Figure S5). We analyzed the functional domains of the PKSs, for the extender unit selection and incorporation, and particularly, for reductive *β*-oxo processing to predict the stereo-configurations of the corresponding functionalities (SI Results). Consequently, these analyses supported that PyrA1-A8 may catalyze 10 Claisen condensations to form a nascent docosanoyl-*S*-ACP (**1**, Figure 2B) in a collinear way. During the process the octahydronaphthalene ring can be closed via the first intramolecular cylcization to give the configurations at C-4, C-5, C-10, and C-13 in PYRs (Figure 1), assumed to be same as those in CHL, TCA, KIJ, or ABY.

*pyrB* encodes a typical NRPS composed of the condensation (C), adenylation (A), and peptidyl carrier protein (PCP) domains, which could then take over the polyketide intermediate for amino acid incorporation (Figure 2B). To determine the substrate specificity, we expressed PyrB in and purified it from *Escherichia coli*. The PyrB-catalyzed adenylation was measured by using a well-developed, ATP-based PPi exchange assay method,[6] showing the highest activity for L-cysteine (Figure S8). Thus, according to known NRPS assembly logic, PyrB may catalyze the aminoacyl extension by building the C−N bond, to complete the PYR skeleton for providing an oxoacyl-cysteinyl-*S*-PCP intermediate **2** (Figure 2B). Distinct from glycerate in tetronate biosynthesis, cysteine, with a *α*-amino group instead of a *α*-hydroxyl group, could serve as the three carbon unit for tetramate formation.

To form the C−C bond of the tetramate moiety of **3** (Figure 2B), the second intramolecular cyclization may take place on **2** via a Dieckmann reaction. To validate the hypothesis that the process potentially involves the uncharacterized PyrD3 or PyrD4, we overexpressed these two proteins in maltose-binding protein (MBP)-tagged forms, and subsequently yielded the soluble PyrD3 and PyrD4 by removal of MBP for assaying the activities in vitro (Figure S7B). For the substrate, we first prepared a stand-alone, N-terminally 6x His-tagged PyrB-PCP (Figure S7C) protein from *E. coli* in an apo-form (**15**, *m*/*z* [M+H]$^+$ calcd 11637.85, found 11637.80), near 100% of which can be converted into the holo-form (**16**, *m*/*z* [M+H]$^+$ calcd 11977.93, found 11978.03) upon the incubation with CoA-SH and a widely used phosphopantetheinyl transferase Sfp[7] (Figure S9). Second, we synthesized *N*-acetoacetyl-L-alanyl-*S*-CoA (**17**) and -*S*-(*N*-acetyl) cysteamine (-SNAC, **18**) (SI), the mimics of the acylpeptidyl thioester intermediate. These compounds were utilized by Schmidt and co-workers to characterize the C-terminal reductase* (R*)-like domain of a fungal hybrid PKS/NRPS EqiS,[8a] which functions as an unusual Dieckmann cyclase in the biosynthesis of the tetramate equisetin. Third, the CoA thioester **17** was pre-incubated with the apo-form **15** and Sfp, yielding *N*-acetoacetyl-L-alanyl-*S*-PCP (**19**, *m*/*z* [M+H]$^+$ calcd 12133.18, found 12133.14) as the substrate. The PCP thioester **19** was relatively stable, only a trace of which was spontaneously cyclized to give tetramate **20** (using the synthetic standard as a control) during a 2-h incubation period (Figure 3B, III). Finally, we carried out a time course analysis of the reactions by adding PyrD3 or PyrD4 into the **19**-containing reaction mixture, to examine the products upon HPLC-MS. Intriguingly, in the presence either of PyrD3 or of PyrD4, the production of tetramate **20** remarkably increased in a time-dependent manner (Figure 3B, I and II, and Figure S10). Meanwhile, we evaluated the change of the PyrB-PCP form, showing that the consumption of acyl-*S*-PCP **19** was consistent with the generation of holo-PCP **16** (Figure 3). These findings indicated that both of PyrD3 and PyrD4 catalyze a Dieckmann


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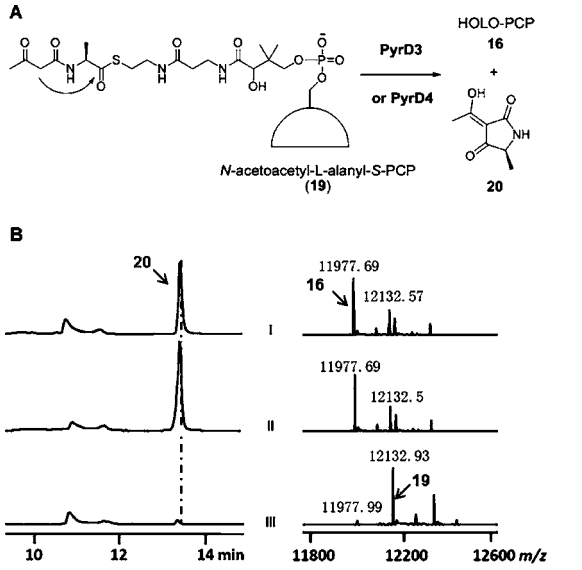


**Figure 3.** Characterization of PyrD3 and PyrD4 *in vitro*. (A) PyrD3 or PyrD4-catalyzed reaction. (B) Product examination after 2-h incubation in the presence of PyrD3 (I) or PyrD4 (II), or in the absence of each of them (III, control). Left, for compound **20** generation by HPLC analysis; and right, for conversion of *N*-acetoacetyl-L-alanyl-*S*-PCP **19** into holo-PCP **16** by MS analysis.

reaction to release *N*-acetoacetyl-L-alanyl from PCP by cyclization. To produce tetramate **20** they also accepted the SNAC thioester **18** as the substrate (Figure S11), reminiscent of the observation of EqiS-R*.[8a]

To further verify the substitutability between PyrD3 and PyrD4 in function, we performed the in vivo investigations by individually inactivating *pyrD3*, *pyrD4*, and both of them. As shown in Figure 4, while the Δ*pyrD3* or Δ*pyrD4* single mutant strain still produced PYRs in an apparently decreased yield, only the deletion of both *pyrD3* and *pyrD4* completely abolished PYR production. Accordingly, single complementation of *pyrD3* or *pyrD4* to the Δ*pyrD3*−*D4* double mutant strain led to the partial restoration of PYR production, whereas introduction of the both genes resulted in the yield of PYRs comparable to that of the wild type strain. These findings are well in agreement with those observed from in vitro studies, strongly supporting that PyrD3 and PyrD4 participate in the tetramate formation to generate intermediate **3** (Figure 2B). **3** may undergo the desulfurization-coupled (to yield **4**), third intramolecular cyclization to provide the spiral cyclohexene ring of intermediate **5**. The C-20 methyl group oxidation, C-9 doxytrisaccharide side chain attachment, and subsequent pyrroloindolic acid moiety transfer may then take place to eventually generate PYRs (Figure S5).

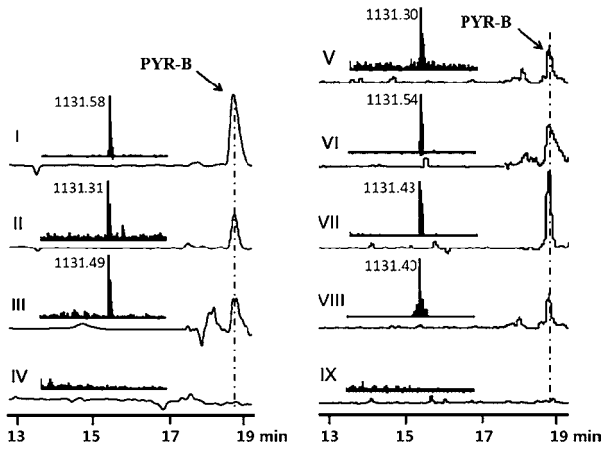


**Figure 4.** HPLC-MS analysis of PYR production in *S. rugosporus*. I, wild-type strain; II, *WL2002* (Δ*pyrD3*); III, *WL2003* (Δ*pyrD4*); IV, *WL2004* (Δ*pyrD3*−*D4*); V, *WL2005* (*WL2004* derivative carrying *pyrD3 in trans*); and VI, *WL2006* (*WL2004* derivative that carries *pyrD4 in trans*); VII, *WL2007* (*WL2004* derivative that carries *pyrD3*−*D4 in trans*); VIII, *WL2009* (*WL2004* derivative that carries *chlD4 in trans*) and IX, *WL2008* (*WL2004* derivative that carries *chlD3 in trans*).

The finding that two phylogenetically distinct proteins share the function to catalyze a Dieckmann cylcization is surprising. Recently, the pathways of a few tetramates have been shown to involve a hybrid PKS/NRPS system.[8,9] In bacteria, this system is terminated by a discrete or cognate thioesterase (TE) domain of NRPS, which, falling into the $\alpha/\beta$-hydrolase superfamily, was predicted to cyclize acylpeptidyl for tetramate formation rather than for well-known hydrolysis or macrolization.[10] In fact, it has been reported that certain TE-like protein/domains have the ability to catalyze the Claisen cyclization.[11] Typically, TE-catalyzed reaction requires transfer of the full-length acyl/peptidyl onto a conserved serine residue to give an oxoester. PyrD4 has a residue, S199, corresponding to this (Figure S15). Taking advantage of the above developed complementation system, we evaluated the essentiality of S199 for the PyrD4 activity in vivo. Consequently, a *pyrD4* variant for a S199A mutation was introduced into the Δ*pyrD3*−*D4* double mutant strain, resulting in a recombinant that produced a trace of PYRs (Figure S13). Thus, S199 is not indispensible for PyrD4 in PYR biosynthesis; however, it awaits further evidence to conclude whether or not PyrD4 operates on the serine-channeled oxoester intermediate. On the other hand, PyrD3 contains the residues S182 and H231 (Figure S16), which are conserved in the C-terminal AT domains of E2 core enzymes of 2-oxoacid dehydrogenase complexes. For such kind of enzymes, they often serve as the active sites to catalyze the acyl transfer from the thioester substrates. Similar to these enzymes and perhaps the terminal R* domains of PKS/NRPSs in fungi,[8] PyrD3 may directly act on the PCP-tethered thioester intermediate. We subsequently mutated these two residues in PyrD3 for in vitro assay. The S182A mutation had no apparent effect on the cyclase activity of PyrD3, which, however, can be significantly lowered by the H231A variation (Figure S12). Consistently, in the Δ*pyrD3-D4* strain, complementation by a *pyrD3* variant encoding a S182A mutant partially restored the PYR production (as by the native *pyrD3*), whereas introduction of that encoding a H231A mutant did not (Figure S13). These results validated the essentiality of H231 in PyrD3 catalysis.

We thus reason that the tetramate-forming chemistry of PYRs can be extended into tetronate biosynthesis (Figure 5)—that is, the incorporation of glycerate may proceed in an enzymatic way characteristic of the C−O bond generation prior to the C−C bond formation. Their difference may exist in the three-carbon unit preparation. While The NRPS activates an amino acid residue to give an oxoacyl-aminacyl-*S*-PCP for tetramate, ChlM, ChlD1, and ChlD2-like proteins are functionally associated to provide oxoacyl-glyceryl-*S*-ACP for tetronate. Since previous studies have shown the absence of *chlD3* and *chlD4* homologues in the RK-682 gene cluster and complete blocking of CHL or TMN production by inactivating each single gene (*chlD3/tmn7* or *chlD4/tmn17*), we considered


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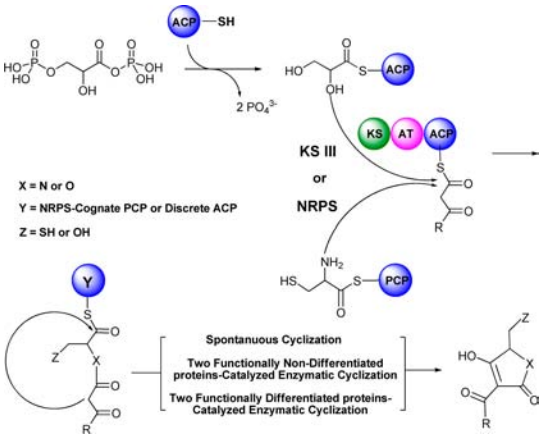


**Figure 5.** Proposed mechanism in tetramate/tetronate formation.

an evolution strategy in microorganisms regarding the construction of the tetramate/tetronate biosynthetic machinery: (1) for the tetronate such as RK-682, ChlM-like protein is responsible for forming a linear oxoacyl-glyceryl thioester, release of which from ACP by cyclization may occurs spontaneously; and (2) for the tetramate/tetronates such as PYR, CHL, TCA, KIJ, and TMN, because their thioester intermediates bear certain highly functionalized groups (including the *trans*-decalin system or the polyether moiety) that may sterically hinder the chain release, nature recruits two PyrD3/ChlD3 and PyrD4/ChlD4-like new proteins to catalyze this. To support the relevance, we carried out the heterologous expression of *chlD3* or *chlD4* in the Δ*pyrD3*−*D4* double mutant *S. rugosporus* strain. While the complementation of *chlD3* failed to produce PYRs, introduction of *chlD4* indeed partially restored the PYR production (Figure 4). This finding supported the functional identity between *pyrD4* and *chlD4*, and implied that ChlD3 might be further evolved to be distinct from ChlD4, either specific for loading of the oxoacyl-glyceryl from ACP to ChlD4 as a transferase or for releasing and cyclizing the ChlD4-based oxoester intermediate as a Dieckmann cyclase (in this case ChlD4 may serve as a carrier protein).

In conclusion, based on in vitro and in vivo characterization of the PyrD3 and PyrD4 in PYR biosynthesis, we have indicated a common paradigm for tetramate/tetronate biosynthesis, which likely proceeds via an enzymatic assembly logic to afford a C−X (X = N or O) bond first and a C−C bond next in the incorporation of a three-carbon unit to form the five-membered heterocycle. Their intrinsic functions remain to be dissected; however, the available gene cluster and proposed pathway now pave the way for the on-going experimental validation.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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