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Unexpected enzyme-catalysed [4+2] cycloaddition and rearrangement in polyether antibiotic biosynthesis

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Abstract

Enzymes catalysing remarkable Diels-Alder-like [4+2] cyclisations have been previously implicated in the biosynthesis of spirotetronate and spirotetramate antibiotics. Biosynthesis of the polyether antibiotic tetronasin is not anticipated to require such steps, yet the tetronasin gene cluster encodes enzymes Tsn11 and Tsn15, homologous to authentic [4+2] cyclases. Here we show that deletion of Tsn11 led to accumulation of a late-stage intermediate, in which the two central rings of tetronasin, and four of its 12 asymmetric centres, remain unformed. In vitro reconstitution showed that Tsn11 catalyses an apparent inverse-electron-demand hetero Diels-Alder-like [4+2] cyclisation of this species to an unexpected oxadecalin compound, which is then rearranged by Tsn15 to form tetronasin. To gain structural and mechanistic insight into the activity of Tsn15, a 1.7 Å crystal structure of a Tsn15-substrate complex has been solved.

Introduction

The Diels-Alder reaction\(^1\), in which a 1,3-diene and an alkene (dienophile) undergo a concerted [4+2] cycloaddition to form a cyclohexene ring, is of central importance to synthetic organic chemistry\(^2\). Transformations during the biosynthesis of numerous cyclic microbial metabolites have been speculated to be catalysed by naturally-evolved [4+2] cyclase ("Diels-Alderase") enzymes\(^3,4\), and several enzymes have been shown to catalyse [4+2] cyclisations consistent with this mechanism\(^5–12\). Almost all examples studied so far involve the combination of an electron-rich 1,3-diene with an electron-poor dienophile to form cyclohexene-containing products. Important exceptions are the hetero-[4+2] cycloadditions catalysed by the pyridine synthases of
thiopeptide antibiotic biosynthesis\textsuperscript{13} and by LepI, which installs a dihydropyran
during leporin B biosynthesis\textsuperscript{14} (Fig. 1a). In the case of LepI, an electron-poor
(oxygen-based) diene reacts with an electron-rich dienophile in an inverse-electron-
demand hetero-Diels–Alder reaction\textsuperscript{14}.

\textbf{Fig. 1} | [4+2] cyclases in polyether tetronate biosynthesis. \textbf{a}, Structures of pyrroindomycin and leporin B, two polyketide natural products that
require a [4+2] cyclase in their biosynthesis to create the rings highlighted in red. \textbf{b}, Structures of the polyether tetronate antibiotics tetronasin (1) and tetronomycin. \textbf{c}, Proposed role of the [4+2] cyclase homologues Tsn11 and Tsn15 in tetronasin biosynthesis.

Tetronasin (1) (Fig. 1b) from \textit{Streptomyces longisporoflavus}, which has found use as
an antibiotic and antiparasitic agent, is an unusual polyether ionophore containing an
acyltetronic acid moiety\textsuperscript{15}. It bears a near mirror-image structural relationship to
tetronomycin (\textit{tmn}) from \textit{Streptomyces} sp. NRRL 11266\textsuperscript{16}, whose biosynthesis has
been shown to involve assembly of the polyketide backbone on a modular polyketide
synthase (PKS)\textsuperscript{16}, with release of the chain \textit{via} tetronic acid ring formation\textsuperscript{17}. Formation of the tetrahydrofuran ring occurs by epoxidation followed by ring-opening and concomitant cyclisation by an epoxide hydrolase\textsuperscript{16}. The origins and the timing of central cyclohexane and tetrahydropyran rings remain unknown, although a plausible metal-assisted cascade mechanism has been suggested in which both rings are formed in a single step following release of the polyketide chain from the PKS\textsuperscript{18,19}. Intrigued by the biosynthetic mystery of the origin of the two central rings, herein we show that the cyclohexane and tetrahydropyran rings of tetronasin are formed in a two-step enzymatic cascade reaction. The two enzymes responsible, Tsn11 and Tsn15, are homologues of [4+2] cyclases enzymes found in complex spirotetronate/tetramate pathways. Using a tetronasin precursor isolated from a \textit{S. longisporoflavus} Δtsn11 mutant (3), we show that Tsn11 catalyses an apparent inverse-electron-demand hetero-Diels-Alder reaction of this species to produce an unexpected oxadecalin intermediate (4). Tsn15 then catalyses a rearrangement that forms the tetrahydropyran ring and dismantles the oxadecalin moiety, producing tetronasin. To gain insight into the structure and mechanism of Tsn15, a 1.7 Å crystal structure of a Tsn15-substrate complex was solved. Mutagenesis experiments then indicated that, like its homologues that catalyse [4+2] cycloadditions, Tsn15 also uses a pericyclic mechanism for ring formation.
Results

The PKS-bound linear intermediate of tetronasin. We have re-analysed the previously sequenced tetronasin biosynthetic gene cluster (GenBank: FJ462704) \((tsn)\) (Supplementary Figure 1) and used domain analysis of the PKSs to predict the structure of the hypothetical full-length polyketide (Supplementary Figure 2). To investigate whether further enzymatic reactions tailor the structure before release from the PKS enzymes, we used a chemical probe (methyl 6-decanamido-2-fluoro-3-oxohexanoate \(6^{20}\) designed to intercept PKS-bound intermediates \(in vivo\) on the \(tsn\) PKS enzymes. In doing so we detected an incomplete undecaketide polyketide chain in which tetrahydrofuran ring formation, C-25 O-methylation and hydroxylation at C-30 had all apparently occurred, forming \(2\), the postulated PKS-bound intermediate (Fig. 1c, Supplementary Figure 3). While we cannot rule out that these enzymatic modifications occurred after being offloaded by \(6\), the detection of \(2\) strongly suggests that they occur before polyketide chain release from the PKS enzymes, as previously established for other polyethers\(^{21}\).

\([4+2]\) cyclase homologues present in the tetronasin biosynthetic gene cluster. The tetronasin cluster includes a gene, \(tsn11\), which is the homologue of \(tmn9\) (38% identity at the protein sequence level), previously shown to be essential for tetronomycin biosynthesis\(^{16}\). Intriguingly, both of these gene products show significant sequence similarity to the monooxygenase-like \([4+2]\) cyclases that catalyse dialkyldecalin formation in the biosynthetic pathways to the spirotetramate pyrroindomycin (PyrE3)\(^{11}\) and the spirotetronates versipelostatin (VstK)\(^9\) and chlorothricin (ChlE3)\(^{11}\) (42-44% protein sequence identity) (Fig. 2a, Supplementary
Like PyrE3, Tsn11 contains mutations in several of the four positively charged amino acids (typically arginine) involved in interacting with NADPH\textsuperscript{22,23}, immediately suggesting that it is not a functional monooxygenase (Supplementary Figure 4).

Co-located with \textit{tsn11} is a second, previously unannotated, gene \textit{tsn15} which also has a counterpart in the \textit{tmn} cluster (\textit{tmn8})\textsuperscript{16}. The products of the latter two genes resemble a second family of Diels-Alderase-like cyclases (VstJ, PyrI4, and AbyU) which create the spiro moiety in spirotetronates and spirotetramates\textsuperscript{9–11} (Fig. 2a, Supplementary Figure 5).
Tsn11 and Tsn15 are essential for tetronasin biosynthesis. To analyse the potential roles of Tsn15 and Tsn11 in the biosynthesis of tetronasin (which contains neither a dialkyldecalain nor a spirotetronate moiety), we specifically deleted each gene in S. longisporoflavus (Supplementary Figure 6). LC-MS analysis of fermentation extracts (Fig. 2b) showed that deletion of either gene abolished tetronasin production, and that complementation *in trans* using the wildtype gene restored production in both cases. In the Δtsn11 mutant a new metabolite, 3, with the same molecular weight as tetronasin 1 accumulated which was also produced by a Δtsn11 Δtsn15 double mutant (Fig. 2b, Supplementary Figure 6c), implying that Tsn11 acts before Tsn15. The new metabolite was isolated from the Δtsn11 mutant and its structure was determined using mass spectrometry and NMR spectroscopy to be 3 (Fig. 1c, Supplementary Figures 7a, 8, 9, 24-29, Supplementary Table 1, Supplementary Note 1). The new metabolite 3 differs from 1 in lacking both central six-membered rings of tetronasin, directly implicating Tsn11 and Tsn15 in catalysing ring formation as the final steps of the biosynthetic pathway.

*In vitro* reconstitution of the cyclohexane and tetrahydropyran rings of tetronasin. To attempt *in vitro* reconstitution of 1 from 3, Tsn11 and Tsn15 were individually expressed and purified from *Escherichia coli* (Supplementary Figure 10). Tsn11 was found to have flavin adenine dinucleotide (FAD) as a tightly bound cofactor (Supplementary Figure 10b). Incubation of 3 with Tsn11 *in vitro* led to its disappearance within 1 h (Fig. 2c) and HPLC-MS analysis showed the accumulation of a new compound 4. Tsn11 could not oxidise NADPH or NADH and assaying the activity of Tsn11 pre-incubated with sodium dithionite, to reduce the bound FAD, showed that change in its redox state did not inhibit the reaction of Tsn11 with 3.
(Supplementary Figure 11), consistent with previous reports\textsuperscript{11,22} that bound FAD is a passive spectator in the active site of flavocyclase PyrE3 in pyrroindomycin biosynthesis. The incubation of 3 with Tsn11 was scaled up to isolate larger amounts of 4, and its structure was determined by mass spectrometry and NMR spectroscopy analysis (Fig 1c, Supplementary Figures 7b, 12, 13, 30-35, Supplementary Table 2, Supplementary Note 2). Like 3, compound 4 still lacks the tetrahydropyran ring but the cyclohexane ring has closed within an unexpected oxadecalin hemiacetal moiety. The formation of an oxadecalin intermediate by Tsn11 is strikingly reminiscent of the formation of carbocyclic decalin rings catalyzed by its [4+2] cyclase homologs (PyrE3, ChlE3) in spirotetronate/spirotetramate biosynthesis\textsuperscript{11}.

Conversion of 4 into 1 requires dehydration of the hemiacetel, fragmentation of the oxadecalin ring, and a bond to form between the C17-OH and C13 to form the tetrahydropyran ring. Remarkably, incubation of 4 with Tsn15 alone, or of 3 with both Tsn11 and Tsn15, led to complete conversion to 1 within 10 minutes (Fig. 2c, Supplementary Figures 13, 14), Neither the Tsn11- nor the Tsn15-catalyzed reaction proceeded in the absence of enzyme. Despite the evident similarity between the tetronasin and tetronomycin pathways, purified Tmn9 could not substitute for Tsn11 in the assay, nor could purified Tmn8 substitute for Tsn15 (Supplementary Figure 16).

To probe the timing of cyclohexane and tetrahydropyran formation, the \(\Delta\)tsn11 and \(\Delta\)tsn15 mutant strains of \(S.\) longisporoflavus were treated \textit{in vivo} with the chemical probe 6\textsuperscript{20}. Exactly the same partially-assembled polyketide species were observed as from wildtype cells (Supplementary Figure 3). These data are consistent with the view that tetronate and tetrahydrofuran ring formation, C30-hydroxylation, and C25-O-
methylation may precede tetrahydropyran and cyclohexane formation. 3 and 4 are therefore likely true biosynthetic intermediates in vivo and the central six-membered rings of tetronasin are formed after polyketide chain release, as final steps in the pathway. Between them, these two enzymes establish the configuration at four asymmetric centres in tetronasin.

Cycloadditions of 1-oxa-1,3-butadienes via inverse-electron-demand hetero-Diels–Alder reactions are important transformations in synthetic chemistry, so an enzymatic counterpart like Tsn11 is of considerable interest. The formation of trans-oxadecalin 4 seems consistent with such a mechanism, but a stepwise process cannot be ruled out (Supplementary Figure 17). Likewise, alternative mechanisms are possible for the reaction catalysed by Tsn15 to convert 4 into 1 (Supplementary Figure 17). A previous isotope feeding study established that the ketone oxygen atom at C3 is derived from propionate rather than water, indicating that any mechanism in which the C3 oxygen is derived from water is unlikely (Supplementary Figure 17).

**The structure of Tsn15.** We propose that Tsn11, like its spirotetronate/spirotetramate homologs, catalyses a [4+2] cycloaddition even though the resulting ring is latent, that is, does not appear in the final antibiotic structure. The structure and mechanism of Tsn11 is currently under investigation. To gain insight into the specificity and mechanism of the unusual transformation catalysed by Tsn15, we have solved the crystal structure of Tsn15 at 1.8 Å using SAD phasing (Fig. 3), and also that of a Tsn15-substrate 1:1 complex, obtained by co-crystallisation of Tsn15 and 4, at 1.7 Å (Fig. 5).
Consistent with its quaternary structure in solution (Supplementary Figure 18), Tsn15 crystallised as a homodimer of two antiparallel-facing monomers (Fig. 3a). Each monomer chain consists of an N-terminal α-helical dimerisation region (α1-α3) followed by an eight-strand β-barrel (β1-β8, α4) enclosing a 388 Å³ hydrophobic internal cavity (Fig. 3b, Supplementary Figure 19, 20). Each Tsn15 monomer shows the conserved domain fold previously seen in the [4+2] cyclases of spirotetronate and spirotetramate biosynthesis (PyrI4 and AbyU)\textsuperscript{10,27}, despite low (ca. 20% sequence identity). In addition to these homodimeric [4+2] cyclases, Tsn15 also shares its fold with the homotrimeric allene oxide cyclase enzymes, which catalyse a 4π electrocyclisation (pericyclic) reaction in jasmonic acid biosynthesis\textsuperscript{28,29}, and PodA.
an unusual pyocyanin demethylase from *Mycobacterium fortuitum*. The different activities of these homologous enzymes demonstrate remarkable ability of this protein fold to catalyse a diverse range of reactions (Fig. 4).

![Fig. 4 | Structural homologues of Tsn15 and their respective reactions.](image)

The Cu chain of Tsn15 was aligned with its structural homologues AbyU, PyrI4, AOC2, and PodA. Beside each structure is its rmsd value to Tsn15, amino acid identity (%), and the reaction it catalyses.
There are no significant conformational differences between the Cα chains of the two crystal structures (Supplementary Figure 21). In the Tsn15-substrate co-crystal the hemiacetal C3 hydroxyl of 4 had undergone dehydration to produce 5 (Fig. 5a). The tetronate ring of 5 is fully inserted into the β-barrel cavity of Tsn15, with the tetrahydrofuran moiety protruding into the solvent (Fig. 5b). The sidechains of amino acid residues S142 and Q164 form hydrogen bonds to the tetronate of 5 (Fig. 5c) and R89 is hydrogen-bonded to the C30-OH (chain B only) (Supplementary Figure 22).

**Fig. 5 | Structure of Tsn15 and a Tsn15-substrate complex.**

a, F_o-F_c of 5 (contoured to 2.0 σ) and its chemical structure.
b, Tsn15 in complex with intermediate 5. The F_o-F_c map of 5 could be clearly assigned in the β-barrel cavity of Tsn15 chain A. Contouring to 2.0 σ. c, The detailed binding interactions between intermediate 5 and the internal β-barrel residues of Tsn15 (chain A) are shown. The dotted grey lines represent hydrogen bonding.
Our initial hypothesis was that Tsn15 might use acid/base catalysis to activate the C-17 hydroxyl and promote nucleophilic ring closure of the tetrahydropyran ring. However, mutagenesis of individual acidic and basic amino acid residues within the β-barrel (R89, E109, and D122) showed that none are essential for Tsn15 activity (Supplementary Figure 23). Further alanine mutagenesis highlighted W190 (essential) and Q164 (mutant shows ca. 30% wildtype activity) both of which are likely important for substrate binding and selectivity (Supplementary Figure 23). W190 appears to orient the oxadecalin moiety of 5 through π-stacking interactions (an equivalent tryptophan is conserved in Tmn8, suggesting a conserved role in binding oxadecalin polyether tetronate intermediates) (Fig. 5c, Supplementary Figure 5). The 12 N-terminal amino acids of Tsn15 do not form an essential substrate-enclosing lid26, as removing these residues did not abolish enzyme activity as in the case of PyrI427 (Supplementary Figure 23). The inability to identify essential general acid/general base catalytic residues suggests that the active site of Tsn15 is primarily a hydrophobic pocket that promotes a reactive conformation, possibly producing the tetrahydropyran ring via a pericyclic rearrangement (Fig. 6) similar to the mechanisms proposed for its homologue AOC228 and the [4+2] cyclases PyrI427 and AbyU10. One complication with this proposal is that the conformation of 5 in the active site appears to be inert for tetrahydropyran formation, with the atoms that must form a bond, C13 and the oxygen of the C17 hydroxyl, being 4.6 Å apart. A possible explanation for this could be that the conformational rigidity imposed on Tsn15 when it crystallised prevented 5 from adopting a near attack conformation. It is also notable that 5 is present in the active site of Tsn15 rather than 4 or tetronasin 1. We had thought it more likely that 4 would be converted by Tsn15 and tetronasin 1 would therefore cocrystallise in its active site, analogous to the PyrI4 cocrystallisation.
experiments. One explanation for this could be that 5 is an inert spontaneous dehydration product of 4, the true substrate. If 4 is the true substrate, however, then tetrahydropyran formation and dehydration at C3 must occur simultaneously (Supplementary Figure 17b), which would likely require an acidic residue to donate a proton to the hemiacetal hydroxyl, thereby creating a better leaving group. However, as demonstrated, the acidic groups in the active site are not essential and no acidic amino acid is adjacent to C3, so it is not clear how this could occur. While we cannot rule out 5 being inert, in our view 5 is more likely to be the Tsn15 substrate as the dehydration of 4 to form 5 should be a favourable first step for tetronasin formation. Dehydration of the hemiacetal hydroxyl (whether spontaneous or Tsn15 catalysed) would stabilise the anion that forms at the C3 oxygen during tetrahydropyran formation through conjugation with the C1 and C33 carbonyl groups (Supplementary Figure 17a, 17c, 17e). While the exact mechanism used by Tsn15 to convert 4 into tetronasin remains unknown, our data is more consistent with Tsn15 catalysing a pericyclic rearrangement rather than a nucleophilic acid/base reaction (Fig. 6). Regardless, the crystal structure of Tsn15 with 5 in its active site delineates the substrate-binding residues of Tsn15 and provides a robust foundation for conducting future mechanistic studies on this fascinating class of cyclase.
Fig. 6 | Proposed mechanism for formation of the cyclohexane and tetrahydropyran rings of tetronasin. Tsn11 catalyses an inverse-electron-demand hetero-Diels-Alder reaction to form an oxadecalin intermediate. The oxadecalin is hydrated to form the cyclic hemiacetal 4 which was purified from the Tsn11 + 3 assay and structurally characterised. Tsn15 then catalyses dehydration of 4 to form 5 (which crystallised in the active site of Tsn15), followed by a pericyclic rearrangement to form the tetrahydropyran ring and fragment the oxadecalin, producing tetronasin 1.

Our results show that the standalone enzyme Tsn11 catalyses a previously unsuspected intramolecular [4+2] cycloaddition that, in formal terms, represents an inverse-electron-demand hetero-Diels-Alder reaction\textsuperscript{26} and which constitutes a latent but compelling mechanistic link with spirotetronate and spirotetramate biosynthesis. Likewise, Tsn15, structurally homologous to known Diels-Alderase-like cyclases, has evolved to catalyze a different, but possibly also a pericyclic, transformation. From a synthetic biology perspective, these enzymes could be useful for stereospecifically synthesising cyclohexane and tetrahydropyran moieties in synthetic polyether ionophore analogues. The tolerance of Tsn15 to mutations in its active site and the diverse range of reactions catalysed by its protein fold (Fig. 4) further suggests it may have some potential as a biotransformation catalyst. Meanwhile, these findings...
suggest that further examples of novel, and potentially latent, enzymatic pericyclic
reactions remain to be discovered in natural product biosynthetic pathways.
Methods

Material, bacterial strains and culture conditions

All chemicals and biomaterials were purchased from Merck (USA) or Thermo Fisher (USA) unless otherwise stated. All the enzymes were purchased from Thermo Fisher (USA) or New England Biolabs (USA). All *E. coli* strains were grown in liquid or on solid (1.5% agar) LB medium at 37 °C. For general maintenance of strain stocks, *S. longisporoflavus* was grown at 30 °C 220 rpm in TSBY (30 g/L tryptic soy broth, 5 g/L yeast extract, 103 g/L sucrose) or at 30 °C on SFM agar (20 g/L soy flour, 20 g/L D-mannitol, 20 g/L agar).

General DNA manipulation techniques

PCR amplification of DNA was performed using Q5 DNA polymerase (New England Biolabs). DNA cloning was performed using standard restriction digestion or isothermal DNA assembly. Sanger sequencing was used to confirm the sequence of all cloned DNA. Genomic DNA from *S. longisporoflavus* was isolated by chloroform extraction followed by isopropanol precipitation and washed using 70% ethanol. Mutagenesis of *tsn15* was performed for *tsn15*-E109A, *tsn15*-D122A, and *tsn15*-W190A using a Quikchange II mutagenesis kit (Agilent, USA). The remaining *tsn15* mutant constructs were ordered from GenScript (China).

Deletion and complementation of *tsn11* and *tsn15* in *S. longisporoflavus*
The deletions of tsn11 and tsn15 were made in the coding region to avoid introducing a frameshift that could have deleterious polar effects on downstream gene expression.

Genomic DNA of S. longisporoflavus was purified and used as a template for all PCR amplification necessary to create a tsn11 gene-deletion plasmid construct. 2 kbp stretches of the genomic DNA upstream and downstream of tsn11 were amplified using tsn11_Up_Fw/tsn11_Up_Rv and tsn11_Dn_Fw/tsn11_Dn_Rv primers, respectively. The primers contained additional nucleotides on their 5’ ends that enabled the two fragments to be joined and ligated into the Ndel site of pYH7 using isothermal DNA assembly, producing the recombinant construct pYH7-tns11 in which 1356 nt of the tsn11 coding frame was deleted. pYH7-tns11 was transformed into E. coli ET12657/pUZ8002 cells which were mixed into a fresh liquid culture of S. longisporoflavus. After mixing, the cell mixture was plated on SFM agar supplemented with 20 mM MgCl2 and incubated at 30 °C for 16 h. The SFM agar was then overlayed with 1 mL of sterilised water containing nalidixic acid and apramycin (0.875 mg of each per 35 mL plate). After several days of growth at 30 °C, apramycin-resistant S. longisporoflavus exconjugants containing a single homologous crossover event with pYH7-tns11 appeared. These single colonies were grown of SFM agar lacking antibiotics to promote a second recombination event via plasmid loss. S. longisporoflavus exconjugants that had undergone a double homologous crossover event (those that had regained apramycin sensitivity) were screened using tsn11_KO_Fw/tsn11_KO_Rv primers to detect the 572 bp PCR product indicative of the successful genomic deletion of tns11. The PCR product was sequenced to confirm the S. longisporoflavus Δtsn11 genotype.
For the deletion of Tsn15, genomic DNA of *S. longisporoflavus* was purified and used as a template for all PCR amplification necessary to create a *tsn15* gene-deletion plasmid construct. 2 kbp stretches of the genomic DNA upstream and downstream of *tsn15* were amplified using *tsn15* Up_Fw/*tsn15* Up_Rv and *tsn15* Dn_Fw/*tsn15* Dn_Rv primers, respectively. The primers contained additional nucleotides on their 5’ ends that enabled the two fragments to be joined and ligated into the *Nde*I site of pYH7 by Gibson assembly, producing the recombinant construct pYH7-*tsn15* in which the entire 621 bp *tsn15* coding frame was deleted. The protocol for deleting *tsn15* was then identical to that previously described for creation of *S. longisporoflavus* Δtsn11. Exconjugants that had undergone two homologous crossing over events with pYH7-*tsn15* were screened using *tsn15* KO_Fw/*tsn15* KO_Rv primers to detect the 713 bp PCR product indicative of the successful genomic deletion of *tsn15*. The PCR product was sequenced to confirm the *S. longisporoflavus* Δtsn15 genotype.

The *S. longisporoflavus* Δtsn11 Δtsn15 (double mutant) was created by deleting *tsn15* in *S. longisporoflavus* Δtsn11. The same protocol for deleting *tsn15* was followed, except that *S. longisporoflavus* Δtsn11 rather than wildtype *S. longisporoflavus* 83E6 was conjugated with pYH7-*tsn15*.

To complement the *S. longisporoflavus* Δtsn11 mutant, *tsn11* was amplified from the genomic DNA of *S. longisporoflavus* using *tsn11*-pIB139-Fw/*tsn11*-pIB139-Rv primers. The amplified *tsn11* was then cloned by isothermal DNA assembly at the *Xba*I site of pIB139, downstream of the *ermE* promoter, creating pIB139-*tsn11*. pIB139-*tsn11* was conjugated into *S. longisporoflavus* Δtsn11 using *E. coli*
Apramycin-resistant exconjugants were selected and grown for seven days before HPLC-MS analysis. To complement the *S. longisporoflavus* Δtsn15 mutant, *tsn15* was amplified from the genomic DNA of *S. longisporoflavus* using *tsn15*-pIB139-Fw/*tsn15*-pIB139-Rv primers. The amplified *tsn15* was then cloned by isothermal DNA assembly at the *Xba*I site of pIB139, downstream of the *ermE* promoter, creating pIB139-tns15. pIB139-tns15 was conjugated into *S. longisporoflavus* Δtsn15 using *E. coli* ET12657/pUZ8002.

**Analysis of metabolites from *S. longisporoflavus***

To test for the production of either tetronasin 1 or intermediate 3, a colony of *S. longisporoflavus* (or of a *S. longisporoflavus* deletion mutant/complementation strain) was inoculated into tsn-medium-A (30 g/L tryptic soy broth, 3 g/L CaCO₃, trace elements: 4 mg/L FeSO₄, 4 mg/L ZnSO₄, 0.6 mg/L CuSO₄, 0.4 mg/L MnSO₄, 0.4 mg/L KMoO₄) and grown for two days at 30 °C 200 rpm. The culture was then plated onto petri dishes containing tsn-medium-B (30 g/L tryptic soy broth, 3 g/L CaCO₃, 100 g/L dextrin, 20 g/L agar, trace elements: 4 mg/L FeSO₄, 4 mg/L ZnSO₄, 0.6 mg/L CuSO₄, 0.4 mg/L MnSO₄, 0.4 mg/L KMoO₄) and grown at 30 °C for seven days. The agar and cells were then extracted using ethyl acetate. The ethyl acetate was evaporated under reduced pressure and the organic extract was dissolved in methanol for analysis HPLC (Hewlett Packard, Agilent Technologies1200 series) coupled to a mass spectrometer (Thermo Finnigan MAT LTQ). The HPLC-MS was fitted with a 250 mm x 4.6 mm 5µm C18 reverse-phase column (5µ OSD3, 100Å. Phenomenex, USA). The mobile phase comprised of 20 mM ammonium acetate and increasing methanol at a flow rate of 0.7 mL/min: 0-5 min, 5-75% methanol; 5-30 min, 75-95%
methanol, 30-34 min, 95% methanol 35-36 min, 95-5% methanol. Normalised collision energy of 35% was used for the molecular fragmentation of tetronasin. High-resolution mass spectra were obtained using a Vion IMS QTOF (Waters, USA) operated by the mass spectrometry service of the Department of Chemistry, University of Cambridge.

Heterologous expression of cyclase genes in *E. coli*

The *tsn15*, *tsn11*, *tmn9*, and *tmn8* genes were each amplified by PCR. The PCR products were individually digested using *Nde*I and *Xho*I and cloned between the *Nde*I and *Xho*I sites of pET28a(+), in-frame with the *N*-terminal polyhistidine tag (MGSSHHHHHHSSGLVPRGSH). Each expression plasmid was individually used to transform chemically competent *E. coli* BL21 (DE3) cells (Thermo Fisher, USA). A single colony from each *E. coli* BL21 (DE3) transformant was cultured at 37 °C, 200 rpm in lysogeny-broth (LB) containing 50 µg/mL kanamycin. Once the culture had reached an *A*<sub>600</sub> = 0.5, protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was then incubated for 16 h at 20 °C, 200 rpm. The cells were harvested by centrifugation and resuspended in 30 mL of 20 mM Tris-Cl pH 7.9 buffer, 500 mM NaCl). Cells were lysed using an Emulsiflex C5 (Avestin, Canada) according to the manufacturer’s instructions. The insoluble fraction was removed by centrifugation and the soluble fraction was passed though Ni-affinity resin (Qiagen, Germany) to bind the recombinant protein. Lysis buffer containing increasing imidazole concentrations (50-400 mM) was used to remove contaminants and subsequently elute pure recombinant protein (monitored by SDS-PAGE). Fractions containing the purified recombinant
proteins were concentrated using Amicon Ultra-15 centrifugal filter units (Merck
Millipore, USA) and the buffer was exchanged into 20 mM Tris-Cl, 100 mM NaCl,
10% glycerol pH 7.9 using a PD-10 column (GE Healthcare, USA) according to the
manufacturer’s instructions. Protein concentration was determined by the Bradford
assay using a BSA standard curve. Protein aliquots were then snap frozen in liquid
nitrogen and stored at -80°C.

Protein quaternary structure analysis by analytical ultracentrifugation

Analytical ultracentrifugation (AUC) was used to measure the molecular weight of
Tsn11 and Tsn15 in solution. Tsn15 and Tsn11 were purified and individually
concentrated to 0.75 mg/mL in a final volume of 800 µL. When Tsn11 and Tsn15
were sedimented together, each was present in the same chamber at a concentration of
0.75 mg/mL. AUC experiments were performed using an Optima XL-I (Beckman
Coulter, USA) centrifuge fitted with an An60 Ti four-hole rotor. Absorbance and
interference data were acquired in the continuous mode at time intervals of 170 s and
at a rotor speed of 40,000 rpm, at 20 °C with systematic noise subtracted, but without
averaging. The density and viscosity of the buffer (20 mM Tris-Cl pH 7.9, 100
mM/150 mM NaCl) and the partial specific volume of the protein were both
calculated using Sednterp\textsuperscript{32}. The multi-component sedimentation coefficient
distributions were obtained from 128 scans by direct boundary modeling of the Lamm
Isolation of Intermediate 3 for NMR analysis

Intermediate 3 was isolated from *S. longisporoflavus Δtsn11*. Well-grown *S. longisporoflavus Δtsn11* colonies were inoculated into 5 mL of tsn-medium-A and grown for two days at 30 °C and 200 rpm. The cultures were then inoculated into flasks containing 50 mL of tsn-medium-A and grown for an additional two days at 30 °C 200 rpm, or until a thick mycelial culture had grown. These cells were plated on 400 12 cm x 12 cm agar plates each containing 50 mL of tsn-medium-B agar (30 g/L tryptic soy broth, 3 g/L CaCO₃, 100 g/L dextrin, 20 g/L agar, trace elements: 4 mg/L FeSO₄, 4 mg/L ZnSO₄, 0.6 mg/L CuSO₄, 0.4 mg/L MnSO₄, 0.4 mg/L KMoO₄) and grown for seven days at 30 °C. The agar was then cut into small squares and combined in a large glass flask where it was extracted three times by submerging in 2 L of ethyl acetate. The ethyl acetate was evaporated under reduced pressure to yield 7 g of a brown crude organic extract. The crude extract was dissolved in 8 mL of methanol and loaded onto 8 10g/70 mL C18 reverse-phase Isolute cartridges (Biotage, Sweden) according to the manufacturer’s instructions. Fractions were eluted from the columns using a mixture of 20 mM ammonium acetate and increasing methanol. Fractions containing intermediate 3 were identified by HPLC-MS and pooled together (1.15 g in total) before a second round of SPE purification. The final purification step was performed using an Infinity II semipreparative HPLC (Agilent, USA) fitted with a Phenomenex (USA) C18 Prodigy column (5 µm ODS-3 100Å, 250 x 10 mm). Gradient elution of intermediate 3 was achieved using a mobile phase of 5 mM ammonium acetate and methanol with a flow rate of 3 mL/min: 0-5 min, 5-75% methanol; 5-15 min, 75-85% methanol; 15-19 min, 85-100% methanol; 19-20 min, 100-5% methanol. Fractions containing intermediate 3 were identified by detecting its
characteristic chromophore ($\lambda_{\text{max}} = 236$) nm and pooled together, followed by solvent evaporation under reduced pressure. The dried extract was dissolved in ethyl acetate to remove the ammonium acetate, followed by freeze-drying to yield purified 3 (12.5 mg). For structural determination, 3 was dissolved in deuterated chloroform and analysed using a 500 MHz DCH Cryoprobe Spectrometer (Bruker, USA).

Isolation of Intermediate 4 for NMR analysis

Intermediate 4 was isolated from an in vitro reaction of 3 and Tsn11. The assay mixture (60 mL in total) contained: 20 mM Tris-Cl, 100 mM NaCl, pH 7.9 containing 50 µM Tsn11, 400 µM of 3, and 5% v/v methanol. After incubation at 30 °C for 1.5 h, the reaction mixture was extracted six times with 30 mL of ethyl acetate. The ethyl acetate was evaporated, and the organic residue was dissolved in methanol. The extract was chromatographed on semipreparative HPLC as described above. Fractions containing intermediate 4 were identified by detecting its characteristic chromophore ($\lambda_{\text{max}} = 254$) nm and pooled together for solvent evaporation. The dried residue was dissolved in ethyl acetate to remove the ammonium acetate, followed by freeze-drying to yield purified 4 (3.3 mg). For structural determination, 4 was dissolved in deuterated methanol and analysed using a 500 MHz DCH NMR Cryoprobe Spectrometer (Bruker, USA).

In vitro assays
In vitro activity assays of Tsn11 and Tsn15 were performed in 20 mM Tris-Cl buffer, 100 mM NaCl, 5% v/v methanol pH 7.9. Typically, reaction volumes were 100 µL and contained 200 µM of 3 or 4. Tsn11 and/or Tsn15 were added to a final concentration of 5 µM. The reactions were carried out at 30 °C for 1 h unless stated otherwise. The reactions were terminated by adding 400 µL of methanol before being completely dried under reduced pressure. The dried extract was redissolved in 100 µL methanol and analysed by HPLC fitted with a Phenomenex C18 Prodigy column (5 µm ODS-3 100Å, 250 x 10 mm) using a gradient program of 20 mM ammonium acetate and increasing methanol at a flow rate of 3 mL/min: 0-5 min, 5-75% methanol; 5-15 min, 75-85% methanol; 15-19 min, 85-100% methanol; 19-20 min, 100-5% methanol. For all in vitro assays, the identity of intermediates 3 and 4 were confirmed by their unique UV chromophores and MS^3 fragmentation patterns. For analysis of the Tsn11 sodium dithionite assays and the Tsn15 point mutation assays, a Poroshell 120, EC-C18, 27 µM, 46 x 100 mm (Agilent, USA) column eluted with 5 mM ammonium acetate and methanol at 1 mL/min: 0-5 min, 5-75% methanol; 5-15 min 75-95% methanol; 15-19 min, 95% methanol; 19-20 min, 95-5% methanol.

When the Tsn11 activity assay was conducted in the presence of sodium dithionite, 100 µM of 3 was used with 1 µM of Tsn11 and 1 mM of sodium dithionite. Parallel reactions were carried out and terminated at 0 min, 2 min, 4 min, and 8 min, respectively.

Protein crystallisation

For protein crystallisation, tsn15 was expressed in E. coli BL21 (λDE3) and purified by nickel-affinity chromatography as previously described. The proteins were further
purified using a preparative gel-filtration ÄKTA (GE Healthcare Lifescience, USA) connected to a Hiload 16/60 column packed with Superdex 200 resin. Fractions containing Tsn15 were pooled and concentrated. Tsn15 was concentrated to 15 mg/mL (20 mM Tris-Cl pH 7.9 buffer, 100 mM NaCl) and crystallised in 0.1 M PCTP (0.1 M each sodium propionate, sodium cacodylate trihydrate, and bis-Tris propane) pH 6.0, 25% PEG 1500 using the sitting-drop, vapour-diffusion method at 19 ºC. The Tsn15-substrate complex was crystallised using hanging-drop, vapor-diffusion method at 18 ºC. To obtain the protein complex, a solution composed of Tsn15 protein (15 mg/mL in 20 mM Tris-Cl pH 7.9 buffer, 0.5 M NaCl) and 10 mM of 4 was incubated for 10 minutes and then crystallised in 0.1 M PCTP pH 6.0 buffer, 27% PEG 1500. To solve the phases for the Tsn15 structure, a selenomethionine labeled version of Tsn15 (SeMet-Tsn15) was created and the structure was determined using the Single Anomalous Dispersion (SAD) method. To achieve this, E. coli BL21 (λDE3) cells transformed with pET28a(+)-tsn15 were cultured in M9 medium. Once the culture had reached A600 = 0.5, 0.1 g/L L-lysine, 0.1 g/L L-threonine, 0.1 g/L L-phenylalanine, 0.05 g/L L-leucine, 0.05 g/L L-isoleucine, 0.05 g/L L-valine were added to the growing culture, followed by 0.06 g/L of L-selenomethionine. SeMet-Tsn15 was purified and exchanged into buffer containing 20 mM Tris-Cl pH 7.9, 100 mM NaCl, 2 mM EDTA, and 2 mM tris(2-carboxyethyl)phosphine (TCEP), then crystallised as before. Crystals were harvested from the crystallisation drop and transferred into a cryoprotecting solution containing the crystallisation solution and 25-27% ethylene glycol. The crystals were then flash-frozen in liquid nitrogen using nylon loops. X-ray diffraction data for SeMet-Tsn15 were collected at the Diamond Light Source (DLS) I04 beamline, Oxford, UK, while for the complex Tsn15-substrate data were collected at PETRAIII, beamline 13,
Hamburg, Germany. The X-ray data processing was performed using XDS\textsuperscript{33} and scaled using Aimless\textsuperscript{34} from CCP4 suite\textsuperscript{35}. The phases for SeMet-Tsn15 was solved using AutoSol\textsuperscript{36} from Phenix suite\textsuperscript{37}. The structural model was initially built by AutoBuild\textsuperscript{38} also from PHENIX suite\textsuperscript{37}. The phases for the Tsn15-substrate complex was determined by molecular replacement using the program Phaser\textsuperscript{39} from Phenix suite\textsuperscript{37}. The SeMet-Tsn15 structure was used as the search model for molecular replacement. Both SeMet-Tsn15 and Tsn15-substrate complex models were refined using Phenix.refine\textsuperscript{40} and further visual inspection and real space refinement was performed by COOT\textsuperscript{41}. The stereochemical quality of the models was assessed using MolProbity\textsuperscript{42}. The protein structure figures were prepared using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Revised sequence of Tsn11

Closer examination of the \textit{tsn11} gene compared to the deposited sequence revealed the start codon to be an additional 12 bp 5′ of the previously annotated start codon. The new start codon was a methionine (ATG) rather than a leucine (TTG) and was six bp downstream from a likely Shino-Delgarno sequence (GGAAGAA). The revised protein sequence of the updated Tsn11 is presented below.
Tsn11:

MEIPLTGTVVIAGAPGVLFLASERLLAGVEAVVLSRSPKANEHTVGGTLHARTDL
FDQRGIMDTRLAQNPLPWRLHASYWLDLAPHEDEEYSLPLLQPYTEEMLEAHATE
LGADIRRGHTCVSTQDAGGTVGVRADGAYELRGLAVLGCDGDSTVRELAAFPV
QESGPRWYGLADVESISGNDHPGNYPGQFAVIRSPHEGGPSRIMTLEFNETTQPP
PADQPSVVEVIERTHTGPVGEQWHLRYNTTREANYRGVRGVDAAH
LHVAFAAGHGLSTGLHDAAANLGLAVLGRAPDSLLLTDYDEERRPGVHRACVFTQS
QWILLTQGQLDILRQLFLTDLVLPEVNHHLHIITTVTREDAGAEKEDTHPLLGDP
VPNQLVKGADVQATAVAILRAGRGVLIDLTGAAAAALPDTSGRGRHLDVSRGCAPA
VDATALLVRPGFVWAATAADTGNDGEPALRWRFGTA

Revised Sequence of Tmn9

During cloning of \( tmn9 \) it was noted that the sequence differed from that previously published\(^{15} \) (confirmed by sequencing independent PCR reactions). The revised Tmn9 protein sequence is as follows:

Tmn9:

MSEPVVVAGPAGLMLACELAMRDVPAVLVDIHTQRACEAPAMAINAGTLEMLDQR
GLAAGLREGTFTPPEVRFADELRRLAFLKVQEGPREPHMLQSRLKEKLVNDRAVEQVD
LRWATRTLGFEEAADSGVTMTASDAEEQRLCRYLVGCDGREGSIVBAGIDYVG
DDWMVVRGIVGDVAINEDVAPEQGYLSYTDNQPLGAFLPSDVRVFSAEFSTEP
PEFEDGPATLEQLGDANTLTGELKATEAHWQHYTSIYTRNAEQYRKGVRFAIGDA
AHVHPYNYGQLGTAGDANLGNLWIAEAVHGWAAPDLDSYHERHLARGCNMI
QAQLALLYPRLARYMREMMEGFLKPEDEVNVLAEIVTNGPVIPAYEGVEPVEG
DRLLGRLPKVQIKTADSDMGVAETLQSSGRVLDDLDSGDAQAQSESGWADRDVDVRA
QPVPLPGLTLLRPDGCVAWHDONGWQQDELRTALRTWFGAPGT

Sequence of Tsn15

Prior to the submission of this manuscript a different ORF in the tetronasin gene cluster, an ABC transporter, was named Tsn15. The Tsn15 described in this paper was not annotated. As such, the ABC transporter has been renamed Tsn15b and Tsn15 now refers to the [4+2] cyclase homologue. For clarity the DNA and protein sequence of Tsn15 are presented below:

\( tsn15 \):
ATGACCACTTCCCATCGATCCGACGACCCCGCTGACCTACAACCCCGTCATCGACGCG
CTCGTCGGCTCGTGGCGCCAGATCATCGACGCCGACTACTCGGCGGACGACCCGG
CTGCCCGATCTCGCCGTGCTGGCCCGCTCCACCGCGCGGGCGGTCGCGGCTGCCGTA
CCCCGTCCGCTCGCGGAGATCTCGGCCCCGGACGCGCCGGACGAGCGCGGCGAACTC
GTGCTGCTGGAGAAGGTGATCCAGGAAGTGGCCGACCGCGAGTACACCCCGCTGAGC
CCCGAGGGGCCGAGCGTCGGGGACCTCGTCCTGGTGACGGAGAAGATCTACAACTCC
GACCGCGAGGAGATCGGCGCGGACACCGGGCGGCTGCGGATCATCCGCAAGGGG
GAGACCGGGCACCACTTC
CTGTTCGCCCTTCGGCTACACCGAGATGGAGGCCCAGCTCGCCGGGGGCCGCACCACC
ATCCAGGTGCTCCGTGGAATGGCCACCCGCTGAGCCTGAACTCCATGACGGCCGCCGAGTCGCGGTACGAGCTGCGCCGCTGA

Tsn15:
MTTSLDPTTPPLTYNPVIDALVGSRQIDADYSADDTRLPDLAVLARSTARVA
PRPLAEISAPDPADERGELVLLEKVIQEVAETPLEPSGVDLVLVT
DREEIGADTGRLRIIRKDPETGHHFTVSTVQGNKLFAFGYTEMEALAGGRT
IQVACWDGPWAGMSGTLSWVINSMATAESRYELR

Interception and detection of PKS-bound polyketide intermediates

All S. longisporoflavus strains were grown in 10 mL tsn-medium A for 2 days at 30 °C at 200 rpm. Seed cultures (100 µl) were used to inoculate tsn-medium A (10 mL of liquid culture, in duplicate, in 50 mL Erlenmeyer flasks with springs). They were then incubated at 30 °C for 5 days. After the first day of incubation, the probe (methyl 6-decanamido-2-fluoro-3-oxohexanoate (6), final concentration: 1 mM) was dissolved in 80 µl of MeOH and added portionwise over 4 days (20 µl each day, days 2-5). Control liquid cultures omitting methyl 6-decanamido-2-fluoro-3-oxohexanoate were also prepared (in duplicate copy). After 5 days of fermentation, the liquid cultures were extracted with ethyl acetate (20 mL). The extracts were concentrated and the residues were redissolved in HPLC-grade methanol (1 mL) for mass spectrometry analysis.

HPLC-HR-ESI-MS analyses of S. longisporoflavus extracts were carried out on an LTQ-T Orbitrap Fusion instrument. Reverse phase chromatography was used to
separate the mixtures prior to MS analysis. Two columns were utilised: an Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5 µm 100 Å and an Acclaim PepMap RSLC 75 µm x 15 cm 2 µm 100 Å (Thermo Scientific). The columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was 0.1% aqueous formic acid and mobile phase B was composed of 100% acetonitrile containing 0.1% formic acid. Samples were loaded onto the µ-precolumn equilibrated in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid for 8 min at 10 µL min⁻¹. After which compounds were eluted onto the analytical column following a 75 min gradient for which the mobile phase B concentration was increased from 50% B to 80% over 15 min, then maintained at 80% B for 50 minutes, then decreased to 50% over 1 min, followed by a 9 min wash at 50% B. Species were analysed by electrospray ionisation mass spectrometry. Survey scans of precursors from 150 to 1500 m/z were performed at 60K resolution (at 200 m/z) with a $4 \times 10^5$ ion count target. Tandem MS was performed by isolation at 1.6 Th with the quadrupole, HCD fragmentation with normalised collision energy of 32, and rapid scan MS analysis in the ion trap. The MS/MS ion count target was set to $2 \times 10^5$ and the maximum injection time was 50 ms. A filter targeted inclusion mass list was used to select the precursor ions.

**Data availability**

The tetronasin biosynthetic gene cluster sequence is available on GenBank under the accession number: FJ462704. Crystal structure data is available on the PDB database under the accession numbers: 6NOI (Tsn15) & 6NNW (Tsn15-substrate complex). All other data that supports the findings of this study is available from the corresponding author upon reasonable request.
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Author contributions

R.L., P.F.L., F.J.L., M.T. and M.V.B.D. developed the hypothesis and designed the study. Y.D., Y.S., and M.S. cloned, sequenced and analyzed gene clusters; R.L., Y.S., H.H., carried out gene deletions, M.T. and R.J. carried out and analysed experiments with chain-terminating probes; R.L. carried out protein expression and purification, in vitro experiments and compound isolation; R.L. and F.J.L. performed compound characterisation. F.C.R.P., R.L. and M.V.B.D. solved crystal structures. All authors analysed and discussed the results. P.F.L., R.L., and F.C.R.P. prepared the manuscript.

Competing Interests

The authors declare no competing interests

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Figure 1. [4+2] cyclases in polyether tetronate biosynthesis. a. Structures of pyrroindomycin and leporin B, two polyketide natural products that require a [4+2] cyclase in their biosynthesis to create the rings highlighted in red. b. Structures of the polyether tetronate antibiotics tetronasin (1) and tetronomycin. c. Proposed role of the [4+2] cyclase homologues Tsn11 and Tsn15 in tetronasin biosynthesis.
Figure 2. Functional characterisation of the Diels-Alderase homologues Tsn11 and Tsn15 in tetronasin biosynthesis. a, The biosynthetic gene clusters of tetronomycin (tmn) and tetronasin (tsn) encode a VstJ-like (red) and PyrE3-like (yellow) [4+2] cyclase characteristic of spirotetrate and spirotetramate biosynthesis pathways including abyssomicin (aby), chlorothricin (chl), veripelostatin (vst), and pyrroindomycin (pyr). Only partial biosynthetic gene clusters are displayed and individual genes are not shown to scale. b, HPLC-MS analysis of the production of tetronasin 1 (m/z = 625) and intermediate 3 (m/z = 625) from wildtype S. longisporoflavus, S. longisporoflavus Δtsn11, Δtsn15, and Δtsn11 Δtsn15, gene deletion mutants, and genetically complemented S. longisporoflavus deletion mutants. Data are representative of three independent experiments. c, HPLC analysis of the in vitro conversion of intermediate 3 into tetronasin 1 using purified Tsn11 and Tsn15. Data are representative of three independent experiments.
Figure 3. Crystal structure of Tsn15. The crystal structure of Tsn15 was solved to 1.8 Å resolution using SAD phasing. **a.** Ribbon and surface representation of the homodimeric Tsn15. **b.** The active-site cavity within the β-barrel of Tsn15. Right: surface charge representation contoured at ±5 kT/eV; blue/red.
Figure 4 – Structural homologues of Tsn15 and their respective reactions. The Cα chain of Tsn15 was aligned with its structural homologues AbyU, PyrI4, AOC2, and PodA. Beside each structure is its rmsd value to Tsn15, amino acid identity (%) to Tsn15, and the reaction it catalyses.
Fig. 5. Structure of Tsn15 and a Tsn15-substrate complex.
a, \( F_o - F_c \) of 5 (contoured to 2.0 \( \sigma \)) and its chemical structure. 
b, Tsn15 in complex with intermediate 5. The \( F_o - F_c \) map of 5 could be clearly assigned in the \( \beta \)-barrel cavity of Tsn15 chain A. Contouring to 2.0 \( \sigma \). c, The detailed binding interactions between intermediate 5 and the internal \( \beta \)-barrel residues of Tsn15 (chain A) are shown. The dotted grey lines represent hydrogen bonding.
Fig. 6. Proposed mechanism for formation of the cyclohexane and tetrahydropyran rings of tetronasin. Tsn11 catalyses an inverse-electron-demand hetero-Diels–Alder reaction to form an oxadecalal intermediate. The oxadecalal is hydrated to form the cyclic hemiacetal 4 which was purified from the Tsn11 + 3 assay and structurally characterised. Tsn15 then catalyses dehydration of 4 to form 5 (which crystallised in the active site of Tsn15), followed by a pericyclic rearrangement to form the tetrahydropyran ring and fragment the oxadecalal, producing tetronasin 1.
Tetronasin biosynthetic gene cluster

Tsn11, Tsn15

[4+2] cyclase homologues

in vitro reconstitution of cyclohexane and tetrahydropyran biosynthesis

Open ring intermediate → Chlorodecalin intermediate → Tetronasin

Tetronasin biosynthetic gene cluster

Str. longisporoflavus