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The biosynthesis of tropodithietic acid was investigated using a combinatorial approach of feeding experiments, gene knockouts and bioinformatic analyses. The mechanism of sulfur introduction is distinct from known mechanisms in holomycin, thiomarinol A and gliotoxin biosynthesis.

Bacteria of the *Roseobacter* clade are associated with marine algae that can appear in massive blooms.¹ The bacteria are well known for their sulfur metabolism, especially for the degradation of the algal metabolite dimethylsulfoniopropionate into dimethyl sulfide.² Only a few secondary metabolites are known from the *Roseobacter* clade many of which contain sulfur. Structurally unique compounds from *Phaeobacter inhibens* include the antibiotics tropodithietic acid (TDA, **1**) and hydroxy-TDA (**2**),³ and the roseobacticide family of algicides, *e.g.* roseobacticide A (**3**, Fig. 1).⁴ In prosperous algal blooms **1** prevents bacterial infections, while lignin breakdown products that are released in fading blooms upregulate roseobacticide biosynthesis. Upon this switch the bacterial symbiont turns



Fig. 1 Metabolites from P. inhibens (1-3), holomycin and gliotoxin.

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[†] Electronic supplementary information (ESI) available: Alignment of PaaZ sequences, results from feeding experiments, experimental procedures, synthesis of ³⁴S-labeled amino acids, and NMR spectra of synthetic compounds. See DOI: 10.1039/c4cc01924e

Biosynthesis of the antibiotic tropodithietic acid by the marine bacterium *Phaeobacter inhibens*[†]

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into a pathogen that promotes the collapse of the bloom.⁴ The biosynthesis of **1–3** is largely unknown, but these tropone derivatives may have a common origin. A rare feature of **1** and **2** is the disulfide moiety that is also found in holomycin (**4**) from *Streptomyces clavuligerus*⁵ and gliotoxin (**5**) from *Aspergillus fumigatus*.⁶

Feeding of ¹³C-labeled phenylalanine (Phe) and phenylacetic acid (PAA) together with gene deletions showed that the carbon backbone of **1** arises *via* the PAA catabolon,^{7,8} in agreement with labeling studies of its tautomer thiotropocin in *Pseudomonas*.⁹ Transposon mutagenesis revealed several genes of the upper PAA catabolon and of primary sulfur metabolism as well as a TDA biosynthetic gene cluster (*tdaA-tdaF*, Fig. 2) to be relevant for TDA biosynthesis.^{7,10} TdaA is a positive regulator of the cluster,¹¹ while the functions of the other encoded enzymes are unknown. Here we report on the biosynthesis of **1** in *P. inhibens*.

During PAA catabolism (Scheme 1A) 6 is converted into phenylacetyl-CoA (7) by the CoA ligase PaaK. The aromatic ring oxidation complex PaaABCDE converts 7 into the epoxide 8 that is transformed into the oxepin 9 by the isomerase PaaG by a protonation-deprotonation mechanism. The subsequently acting PaaZ is a bifunctional enzyme with a C-terminal enoyl-CoA hydratase (ECH) and a N-terminal aldehyde dehydrogenase (ALDH) domain. The ECH domain catalyzes the water addition to 9 forming a hemiacetal that undergoes ring-opening and tautomerization to form 10. This is followed by oxidation to the corresponding carboxylic acid (11) by the ALDH domain. The next steps include a series of β-oxidations to yield two units of acetyl-CoA and one succinyl-CoA. A single mutation of a critical residue of E. coli PaaZ (E256Q) is sufficient to switch the function of its N-terminal domain, resulting in the conversion of 10 into 12 (Scheme 1B).8 In P. inhibens, besides paaZ1 of the PAA catabolon, a copy of this gene (paaZ2) is found



Fig. 2 TDA biosynthetic gene cluster of *P. inhibens* DSM 17395.



adjacent to *tdaF* (Fig. 2). While the C-terminal ECH domain of PaaZ2 is highly homologous to those of PaaZ1 and PaaZ of *E. coli*, the N-terminal ALDH domain shows only weak sequence homology. The highly conserved Cys-295, which covalently binds to the aldehyde function of **10** during catalysis and is critical for functionality,¹² is mutated to Arg in PaaZ2, while a Cys residue aligns with Glu-256 of *E. coli* PaaZ (Fig. S1 and S2 of ESI†). Thus, PaaZ2 cannot catalyze the conversion of **10** to **11**, but serves in the formation of the sevenmembered carbocycle **12** to install the carbon backbone of **1**.

To explore the formation of the dithiet moiety of **1** feeding experiments with ³⁴S-labeled compounds were performed. Inorganic sulfur sources $(Na_2^{34}SSO_3 \text{ and } NaH^{34}SO_4)$ were obtained as reported previously.¹³ For the synthesis of labeled cysteine (**16**) ³⁴S₈ was treated with a hot aqueous KCN solution to obtain K³⁴SCN (Scheme 2). Nucleophilic substitution at methyl *N*-(*tert*butoxycarbonyl)-*O*-tosyl-L-serinate (**13**) yielded the thiocyanate **14** that was cleaved into the thiol **15** with SmI₂, followed by acid deprotection to form [³⁴S]-**16**. Its dimer [³⁴S₂]-**17** was obtained by oxidation with NaI and H₂O₂.¹⁴ Feeding of NaH³⁴SO₄ to *P. inhibens*, diluted to a 4% labeling due to the sulfate content in the medium, resulted in the incorporation into both sulfur atoms of **1** with 4% incorporation rate that was hence completely recovered (Fig. S3 of ESI†).







Incorporation into both sulfur atoms of **1** was also observed from $[^{34}S]$ -**16** (87%) and $[^{34}S_2]$ -**17** (45%, this lower incorporation rate is likely due to the poor water solubility of **17**), demonstrating that sulfur amino acid metabolism is involved in the biosynthesis of **1**. The incorporation of labeling from NaH³⁴SO₄ proceeds by reduction to H³⁴S⁻ and reaction with *O*-acetyl serine (**18**) to form $[^{34}S]$ -**16** by the cysteine synthase CysK (Scheme 3). Incorporation of Na₂³⁴SO₃ was less efficient (30%) and is explained by oxidation to sulfate *via* the Sox pathway.¹⁵ Transposon mutation³ or deletion of *patB* encoding a cystathionine- β -lyase completely abolished the production of **1** (Fig. S4 of ESI†) that was not restored by cysteine or homocysteine addition. PatB also acts on **17** to yield *S*-thiocysteine (**21**),¹⁶ thus pointing to **17** as a direct precursor of **1**. Incorporation of ³⁴S-labeling was also observed in **2** in all feeding experiments (Fig. S5 of ESI†).

A plausible mechanism for further elaboration of the carbon backbone of **12** and sulfur introduction into **1** was deduced from a bioinformatic analysis of the enzymes of the TDA gene cluster (Table S1 of ESI[†]). Oxidation of **12** to **22** by the acyl-CoA dehydrogenase TdaE and water elimination by the dehydratase TdaC may yield the CoA ester **23** (Scheme 4). Intermediates **22** and **23** are reflected by the occurrence of tropone (**25**) and its hydrate **24**¹⁷ in headspace extracts of *P. inhibens* that arise by thioester hydrolysis and decarboxylation.

The flavoprotein TdaF has a high homology to phosphopantothenoylcysteine decarboxylases. These enzymes are involved in coenzyme A biosynthesis and catalyze the flavine mononucleotide (FMN) dependent oxidation of phosphopantothenoylcysteine (26) to the thioaldehyde 27, followed by decarboxylation and reduction with FMNH₂, without net consumption of FMN (Scheme 5A).¹⁸ TdaB, homologous to glutathione-S-transferases, may catalyze the addition of 21 to 23 that can be oxidized by TdaF and FMN to yield 32 (Scheme 5B). The byproduct thioaldehyde 31 can undergo decarboxylation and FMNH₂ reduction to cysteamine, similar to the respective steps in CoA biosynthesis. Addition of



21 to the vinylogous Michael acceptor in 32 and oxidation can introduce the second sulfur to yield 33 that may undergo spontaneous oxidation to 34 in air. Finally, the thioesterase TdaD catalyzes thioester hydrolysis to yield 1.

We also investigated the biosynthetic origin of the additional oxygen in 2, assuming two alternatives: (i) formation of 1 from tyrosine *via p*-hydroxy-PAA, and (ii) oxidation of 1 or a late stage intermediate. Feeding experiments with [*phenol*-¹⁷O]tyrosine and ¹⁸O₂ proceeded only with incorporation of labeling from ¹⁸O₂



(Fig. S6 of ESI[†]), in full agreement with assumption (ii). Feeding of ${}^{18}O_2$ also resulted in the incorporation into one oxygen of the shunt product **24** (Fig. S7 of ESI[†]), in agreement with the pathways shown in Schemes 1 and 4.

We have investigated the biosynthesis of **1** and **2** by a combined strategy of gene knockouts, bioinformatic analyzes, and feeding experiments with labeled precursors. The delineated pathway is in full agreement with all results from labeling, mutation and complementation experiments, corroborated by the presence of the shunt products tropone and tropone hydrate in *P. inhibens* headspace extracts, and plausible in light of the knowledge about characterized homologous enzymes. Particularly interesting is the mechanism of sulfur introduction that is distinct from recently unraveled mechanisms in holomycin, the related thiomarinol A, and gliotoxin biosynthesis.^{19–21} Further studies on the enzymes of TDA biosynthesis are now possible.

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