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FULL PAPER

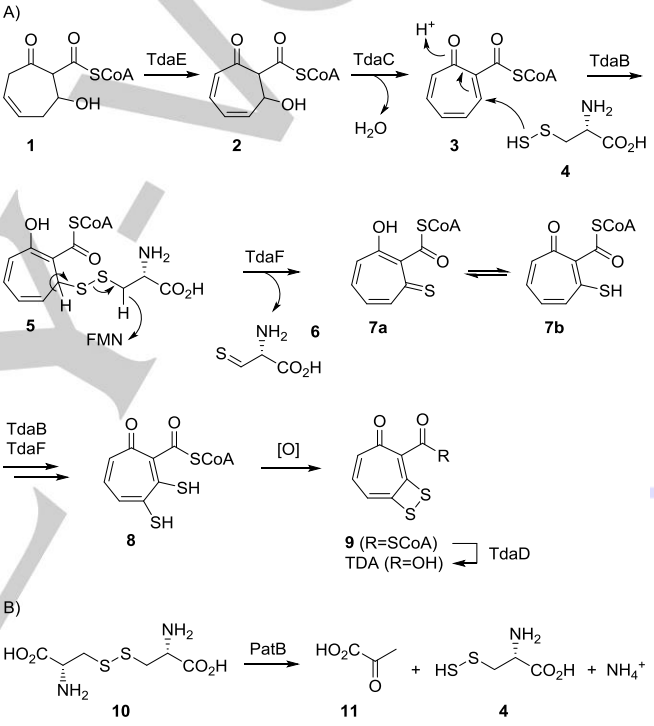
Characterisation of the L-cystine β-lyase PatB from *Phaeobacter inhibens*, an enzyme involved in the biosynthesis of the marine antibiotic tropodithietic acid

Jeroen S. Dickschat,*[a] Jan Rinkel,[a] Tim Klapschinski[a] and Jörn Petersen[b]

Abstract: The L-cystine β-lyase from *Phaeobacter inhibens* is involved in the biosynthesis of the sulfur containing antibiotic tropodithiethic acid. The recombinant enzyme was obtained by heterologous expression in *Escherichia coli* and biochemically characterised by unambiguous chemical identification of the products formed from the substrate L-cystine, investigation of the substrate spectrum, determination of the enzyme kinetics, sequence alignment with closely related homologs and site-directed mutagenesis to identify a highly conserved lysine residue that is critical for functionality. PatB from *P. inhibens* is a new member of the small group of characterised L-cystine β-lyases and the first example of an enzyme with such an activity that is required for the biosynthesis of an antibiotic. A comparison of PatB to previously reported enzymes with L-cystine β-lyase activity from bacteria and plants is given.

Introduction

The antibiotic tropodithietic acid (TDA, Scheme 1), initially described as its tautomer thiotropocin, was first isolated from *Pseudomonas* sp. CB-104 and was shown to exhibit significant activity against various Gram-positive and Gram-negative bacteria and ascomycete fungi at low micromolar concentrations.[1,2] The compound is also produced by marine alphaproteobacteria including *Phaeobacter inhibens* DSM 17395[3,4] and various isolates of *Pseudovibrio*,[5,6] besides several other bacteria from the *Roseobacter* group.[7-9] Notably, all these TDA producing bacteria are associated with eukaryotes such as sponges,[6] molluscs,[10] dinoflagellates[11] or marine green algae.[12] Since these symbionts may have beneficial effects for the host organisms by protecting them from infections with pathogens such as *Vibrio anguillarum*,[13-15] the compound or its bacterial producers are potentially interesting e. g. for applications in fish aquaculture. A recent mode of action study revealed that TDA acts as an electroneutral H^+/M^+ antiporter,[16] similar to polyether ionophores such as monensin.[17]



Scheme 1. TDA biosynthesis. A) Proposed biosynthetic pathway with suggested intermediates and bioinformatically assigned enzyme functions; B) cleavage of L-cystine (**10**) by PatB to pyruvate (**11**), the suggested sulfur precursor *S*-thio-L-cysteine (**4**) of TDA biosynthesis, and ammonium.

Feeding experiments with isotopically labelled precursors continue to be an effective method for studying biosynthetic pathways to secondary metabolites.[18] Using this approach, the biosynthesis of TDA was shown to proceed via degradation of L-phenylalanine to phenylacetic acid[19,20] which is further transformed by the phenylacetate catabolon.[21,22] Interestingly, a critical point mutation in the aldehyde dehydrogenase domain of the recombinant pathway enzyme PaaZ resulted in the spontaneous cyclisation of a reactive aldehyde intermediate to yield the seven-membered carbocycle **1** that has been suggested as the starting point for the further transformations to TDA (Scheme 1A).[22] Similarly, deletion of the gene for the related aldehyde dehydrogenase PacL resulted in the accumulation of

[a] Prof. Dr. Jeroen S. Dickschat, Jan Rinkel, Tim Klapschinski
Kekulé-Institut für Organische Chemie und Biochemie
Rheinische Friedrich-Wilhelms-Universität Bonn
Gerhard-Domagk-Straße 1, 53121 Bonn, Germany.
E-mail: dickschat@uni-bonn.de
[b] PD Dr. Jörn Petersen
Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
Inhoffenstraße 7b, 38124 Braunschweig, Germany.

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tropone,[23] a compound that is also detected in headspace extracts of TDA producing bacteria,[24] and is likely formed from **1** by spontaneous and/or enzymatic degradation by the enzymes encoded in the TDA biosynthetic gene cluster. The further steps of the TDA biosynthesis depend on the TDA biosynthetic gene cluster[8] whose expression is under control of *N*-(3-hydroxydecanoyl)-L-homoserine lactone mediated quorum sensing in *P. inhibens*[25] and triggered in a positive feedback loop by its own metabolite in *Ruegeria* sp. TM1040,[26] while gene cluster regulation is different in *Pseudovibrio*.[6] A combination of bioinformatic analyses, gene knockouts and feeding experiments with isotopically labelled precursors resulted in tentatively assigned functions of the enzymes encoded in the TDA biosynthetic gene cluster and suggested structures for the pathway intermediates.[27] Specifically, the direct sulfur precursor in the TDA biosynthesis was suggested to be the notoriously instable compound *S*-thio-L-cysteine, because the deletion of a gene encoding PatB, an enzyme that exhibits high homology to L-cystathionine β-lyases, resulted in an abolished TDA production that could neither be complemented by the addition of L-cysteine nor L-homocysteine. However, if PatB would act on the L-cysteine dimer L-cystine (**10**, Scheme 1B), the product *S*-thio-L-cysteine (**4**) would be expected that fits into the biosynthetic mechanism for TDA (Scheme 1A).[27] Recently, Wang et al. have shown the involvement of PatB also in the biosynthesis of roseobacticides,[28] an algicidal class of compounds that is structurally and biosynthetically related to TDA and responsible for the switch from mutualistic to pathogenic behaviour of *P. inhibens* towards its algal symbionts.[29] Purified recombinant PatB, *N*-terminally fused to the solubility-enhancing domain NusA, was shown to convert **10** into a product with a free thiol group and with a mass corresponding to **4**, but L-cysteine or L-cystathionine were not accepted by the fused enzyme.[28] Here we present a detailed biochemical characterisation of recombinant PatB from *P. inhibens* that could be obtained as a His_6-tagged soluble protein, including the unambiguous identification of the products generated from **10** by comparison to standards, its pH optimum and substrate tolerance, and a mechanistic investigation by site-directed mutagenesis.

Results and Discussion

Identification of PatB as cystine β-lyase

The *patB* gene from *Phaeobacter inhibens* DSM 17395 (PGA1_c00860; accession no. WP014878874) was amplified by PCR from genomic DNA and cloned into the expression vector pYE-Express by homologous recombination in yeast.[30] The gene was expressed in *Escherichia coli* BL21(DE3) and the recombinant His_6-tagged protein was obtained in soluble form. Purification by Ni^{2+}-NTA affinity chromatography yielded the pure protein eluate (Figure S1) as a pale yellow solution that indicated co-purification with the PLP cofactor (Figure S2). Also MALDI-TOF measurements of the recombinant protein in comparison to the apo-enzyme prepared by treatment with $NaCNBH_3$[31] and hydroxylamine[32] showed a shift in the mass distribution approximately in line with the cofactor mass addition (Figure S3).

The characterisation of the expected product **4** during incubation experiments of PatB with **10** was significantly hampered by its known instability that leads to the rapid decomposition to L-cystine and elemental sulfur.[33] Therefore, the substrate **10** was incubated with PatB, followed by trapping of the product by the addition of dimethyl sulfate. Analysis by HPLC-ESI-MS revealed the presence of a compound with the molecular mass of methylated **4** (*m/z* = 168.0147, $[M+H]^+$) and an isotope pattern in agreement with the presence of two sulfur atoms (Figure 1). A synthetic reference compound was obtained by nucleophilic substitution of cysteine (**13**) at the sulfane sulfur of *S*-methyl methanethiosulfonate (**12**) under slightly acidic conditions (Figure 1C). The product *S*-(methylthio)-L-cysteine (**14**) was obtained in 53% yield, characterised by NMR spectroscopy and proved to be identical to the compound formed by PatB mediated lysis of **10** and subsequent methylation, both in terms of retention time and mass spectrum.

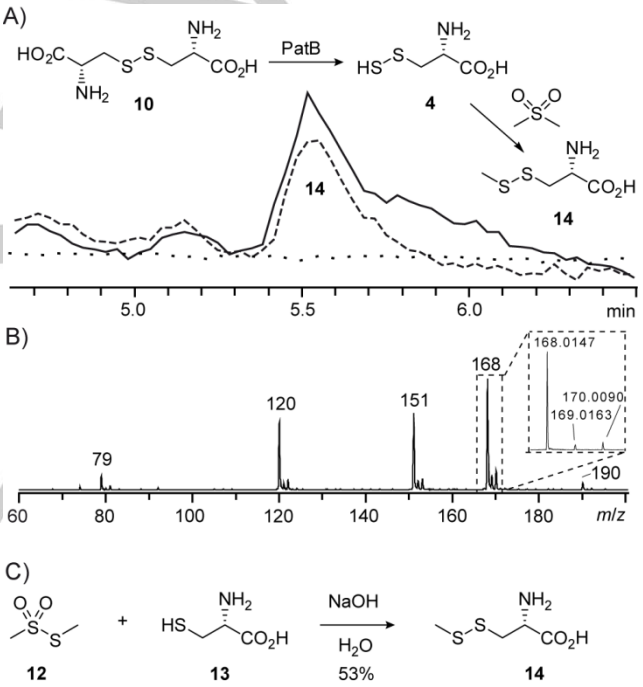


Figure 1. Identification of the product obtained by cleavage of L-cystine (**10**) with PatB. A) HPLC-ESI-MS total ion chromatogram of the enzyme product obtained from **10** after methylation with dimethyl sulfate (dashed line), of the synthetic standard *S*-(methylthio)-L-cysteine **14** (full line) and of a negative control without the enzyme (dotted line); B) ESI mass spectrum of the methylated enzyme product eluting at a retention time of 5.5 min ($[M+H]^+$: *m/z* = 168.0147, calcd. for $C_4H_{10}NO_2S_2^+$: *m/z* = 168.0147); C) synthesis of reference compound **14**.

The second product of the lysis of **10** by PatB was trapped by the addition of 4-fluorophenylhydrazine (**15**) that reacts with pyruvate (**11**) upon gentle warming to a mixture of the *E* and *Z* hydrazones **16** that were detected by HPLC-MS (Figure 2). For comparison, an authentic sample of pyruvate was treated with **15** under the same conditions, yielding the hydrazones **16** that had an identical elution time with the trapped products from the enzyme incubation.

Finally, addition of Nessler's reagent to the enzyme reaction produced an auburn precipitate which pointed to the detection of ammonium in the PatB reaction mixture. Taken together, these results established the conversion of L-cystine (**10**) into *S*-thio-L-cysteine, pyruvate and ammonium by PatB, thus identifying this enzyme as an L-cystine β-lyase.

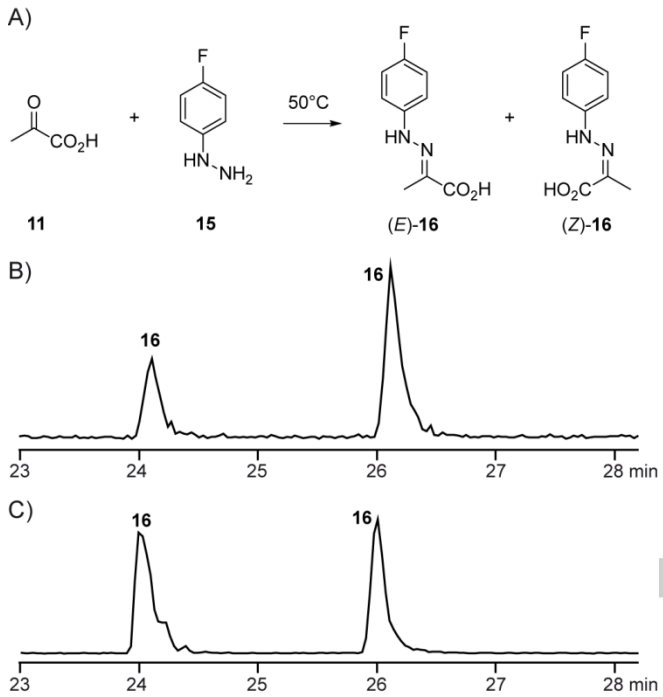
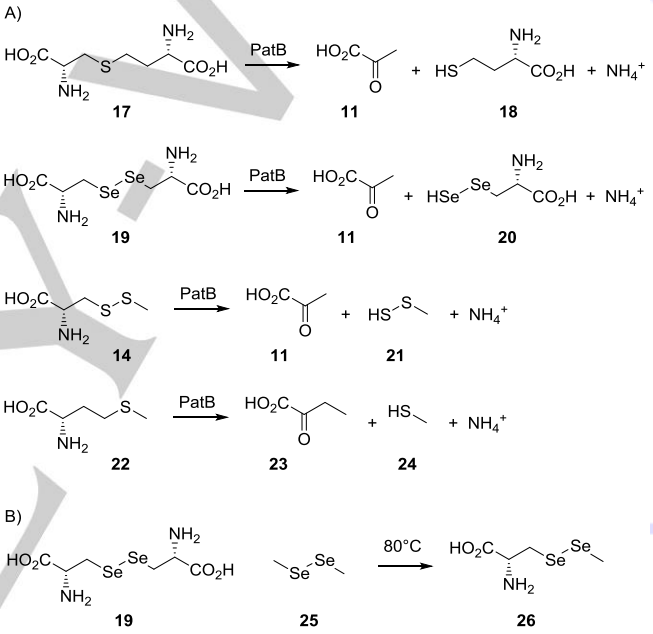


Figure 2. Identification of the product obtained by cleavage of L-cystine (**10**) with PatB. A) Reaction of pyruvate (**11**) and 4-fluorophenylhydrazine (**15**) to the diastereomeric mixture of *E* and *Z* hydrazones **16**; B) HPLC-MS analysis (extracted ion chromatogram for *m*/*z* = 195) of the products obtained from the incubation of **10** with PatB and **15**; C) HPLC-MS analysis of the products obtained from **11** and **15** under the same conditions. The (*E*)- and (*Z*)-isomers of **16** could not be assigned to the individual peaks in the HPLC-MS chromatograms.

Substrate tolerance of PatB

The substrate scope of PatB was investigated by incubation experiments with several compounds that are structurally related to L-cystine (Scheme 2A). L-Cystathionine (**17**) has a central function in the primary sulfur metabolism and is cleaved by L-cystathionine β-lyases into L-homocysteine, pyruvate and ammonium. In comparison to L-cystine one sulfur atom is exchanged by a methylene group in **17**. This compound was included to study the L-cystathionine β-lyase activity of PatB. In a recent study on three different dimethylsulfoniopropionate (DMSP) lyases (DddW, DddP and DddQ) from the marine bacterium *Ruegeria pomeroyi* we have shown that the selenium analog of DMSP, dimethylseleniopropionate (DMSeP), was also accepted and efficiently converted into the lysis products dimethyl selenide and acrylate.[34] For a similar investigation on the tolerance of PatB towards an exchange of sulfur against selenium, L-selenocystine (**19**) was included in this study. Furthermore, the synthetic compound *S*-(methylthio)-L-cysteine (**14**) and L-methionine (**22**) were tested as substrates.

The incubation of **17** with PatB resulted in the efficient conversion into L-homocysteine (**18**) that was identified trough HPLC-MS by comparison to a commercially available standard (Figure S4). The bis-selenium analog of **10**, L-selenocystine (**19**), was also accepted as substrate. As discussed above for the conversion of **10** into **4**, the initial product **20** proved to be instable and was only detectable after its methylation by the addition of dimethyl sulfate to the enzyme reaction. A compound with the same chromatographic behaviour, tentatively identified from its exact mass as compound **26**, was obtained in a metathesis reaction between **19** and dimethyl diselenide (**25**) that was achieved through gentle heating of the compound mixture (Figure 3).



Scheme 2. A) Substrate spectrum of PatB. B) Metathesis reaction of **19** and **25**.

The lysis of **14** by PatB resulted in the formation of sulfur volatiles that were captured by use of a solid phase micro-extraction (SPME) fibre that allows for the solvent free analysis of volatiles by direct insertion of the fibre into the injection port of a GC/MS.[35,36] Besides the expected product **21** that was found as a trace compound several other sulfur volatiles incuding methanethiol (MeSH, **24**), dimethyl disulfide (**27**), dimethyl trisulfide (**28**) and dimethyl tetrasulfide (**29**) were detected (Figure 4A). All four compounds **24** and **27** – **29** are known as headspace constituents of many bacteria and fungi[37,38] and their mass spectra were included in our mass spectral libraries which allowed for their instantaneous and unambiguous identification. Only for compound **21** no mass spectrum was available, but the recorded mass spectrum showed a molecular ion, isotope and fragmentation pattern that were in line with the structure of **21** (Figure 4B). However, the isomeric structure to **21**, methanedithiol, cannot be completely ruled out based on the obtained MS data,

although its formation would be difficult to understand. The formation of the other observed products can be understood by the oxidative dimerisation of **21** in the presence of air to **29**, while subsequent metathesis reactions that occur under the thermal impact of the GC analysis can explain the generation of **24**, **27** and **28** (Figure 4C). No cleavage of L-methionine into sulfur volatiles by PatB was observed, showing that the enzyme has no γ-lyase activity.

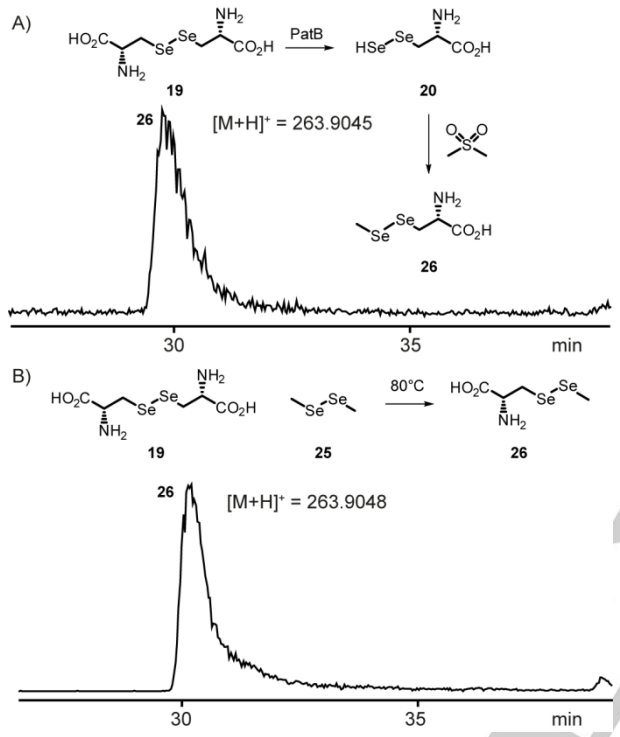


Figure 3. Identification of the lysis product obtained with PatB from **19** and by subsequent methylation with dimethyl sulfate. HPLC-MS analysis of A) the methylated enzyme product ([M+H]$^+$: *m/z* = 263.9045) and B) the synthetic reference compound **26** ([M+H]$^+$: *m/z* = 263.9048, calcd. for $C_4H_{10}NO_2Se_2^+$: *m/z* = 263.9036).

Enzyme kinetics of PatB

The enzyme kinetics of PatB were monitored by the addition of an excess of Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid))[39] to the enzyme incubations.[28] This reagent allows for the detection of free thiol groups as formed during the lysis of L-cystine and its substrate analogs by PatB. The reaction of Ellman's reagent with free thiols results in the release of the 2-nitro-5-thiobenzoate dianion that exhibits a yellow colour and can be quantified by the UV/Vis absorbance at λ = 412 nm. The reagent also enables recording of kinetic data for enzymes that produce free thiols, if the absorbance is measured in a time resolved manner with different substrate concentrations. In a first set of experiments the pH optimum for PatB was determined in a pH range that does not affect the molar absorption coefficient of the 2-nitro-5-thiobenzoate dianion (ε = 14150 L mol^{-1} cm^{-1}).[40] Therefore, the initial reaction rates for the conversion of L-cystine (**10**) by PatB at a substrate concentration of 200 μmol L^{-1} were determined, revealing optimal conditions for pH = 8.75 (Figure 5A, conditions above pH = 9 resulted in the non-enzymatic decomposition of **10** and are not recommended for Ellman's reagent). The temperature optimum at pH = 8.75 was found at 30 °C (Figure 5B).

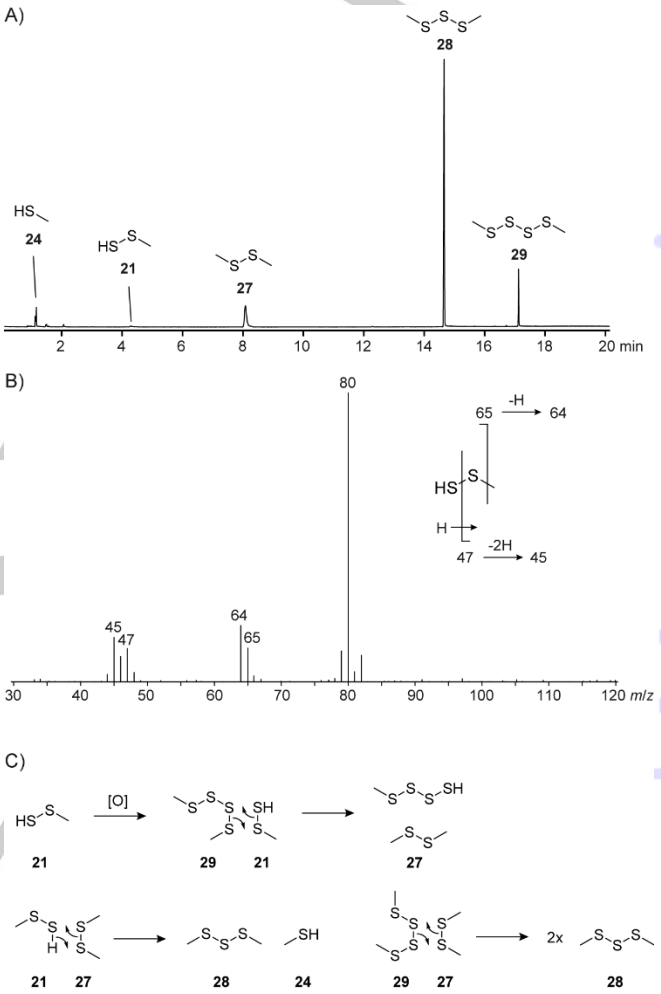


Figure 4. Products generated by incubation of **14** with PatB. A) Total ion chromatogram of the volatiles released from the enzyme reaction and captured by SPME, B) EI mass spectrum of the putative initial product **21**, C) reactions of **21** in the presence of air and under the thermal impact of the GC/MS analysis that explain the observed product portfolio.

The enzyme kinetics of PatB were determined for **10** and the other accepted substrates discussed above at pH = 8.75 and 30°C with different substrate concentrations ranging from 200 μM to 3 μM and Lineweaver-Burk analysis. Substrate **10** exhibited a k_{cat}/K_M = (3.6 ± 0.3) μM^{-1} min^{-1} (Table 1). An addition of PLP to the reaction did not result in any enhancement of this value. The synthetic compound **14** showed a slightly increased enzyme affinity (indicated by a moderately decreased K_M) as observed for **10**, suggesting that one fully established cysteinyl moiety in the substrate is sufficient for substrate binding, which is in agreement

with the substrate binding determined from structural data of the L-cystine β-lyase from the cyanobacterium *Synechocystis*.[41] The significantly lower turnover number (k_{cat}) for substrate analog **14** in comparison to **10** resulted in an overall lower performance of PatB towards **14**. The k_{cat}/K_M indicated that L-cystathionine (**17**) was converted with the lowest efficiency of the tested substrates. The selenium analog **19** showed a decreased turnover number in comparison to **10**, while the enzyme affinity was almost the same, which reflects the similar electronic states and sizes of sulfur and selenium atoms (covalent radii of sulfur and selenium are 104 pm and 117 pm, respectively). The overall kinetics revealed that **19** is converted by PatB with lower catalytic efficiency compared to the natural substrate **10** and substrate analog **14**, but faster than **17**.

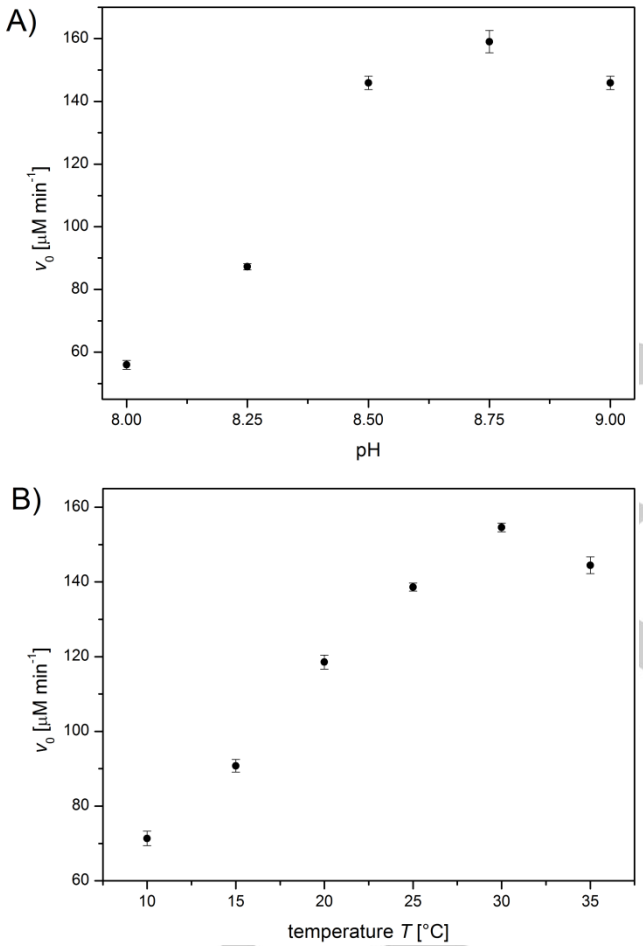


Figure 5. Optimum conditions for PatB. A) pH optimum and B) temperature optimum determined from initial reaction rates with L-cystine (200 μmol L^{-1}).

Interestingly, although the k_{cat} and K_M data determined for PatB from *P. inhibens* deviated from those reported for the enzyme from *Synechocystis*, the overall kinetics in terms of the k_{cat}/K_M matched very well (reported for the *Synechocystis* enzyme: k_{cat}/K_M = 36 L mmol^{-1} s^{-1} = 2.16 L μmol^{-1} min^{-1}). Similar to our findings, the substrate analog **17** was also converted with much lower efficiency by the enzyme from *Synechocystis* (k_{cat}/K_M = 0.36 L μmol^{-1} min^{-1}).[42]

Table 1. Kinetic data for the enzymatic lysis of substrate analogs by PatB at pH = 8.75 and 30 °C.

substrate	k_{cat} [min^{-1}][a]	K_M [μM][a]	k_{cat}/K_M [μM^{-1} min^{-1}]
10	306 ± 7	85 ± 7	3.6 ± 0.3
14	101 ± 15	68 ± 12	1.5 ± 0.3
17	57 ± 6	107 ± 15	0.5 ± 0.1
19	117 ± 11	104 ± 16	1.1 ± 0.2

[a] Determined from kinetic data summarised in Tables S5–S8.

Site-directed mutagenesis

A BLAST search using the sequence of PatB from *Phaeobacter inhibens* revealed that closely related enzymes are widespread particularly in bacteria of the *Roseobacter* group. The 500 closest hits showed a strongly conserved sequence length between 370 and 418 amino acid residues. An amino acid sequence alignment of the 500 sequences returned 18 identical residues that occur in all PatB homologs that are marked in the PatB sequence of *P. inhibens* in Figure S9. Among these residues a highly conserved Lys is found (K235 in the enzyme from *P. inhibens*) that was assumed to be responsible for binding of the PLP cofactor, similar to the findings reported for the L-cystathionine β-lyase from *Arabidopsis thaliana*.[43] Site-directed mutagenesis of K235 yielded the two variants K235Q and K235M that were both incubated with **10**. Both variants co-purified with PLP (Figure S2), probably due to non covalent binding, but did not show any detectable conversion of this substrate which established the critical role of K235 for enzyme functionality.

Feeding experiments

The importance of PatB for TDA biosynthesis in *P. inhibens* DSM 17395 has been shown previously in a combined knockout and feeding study with isotopically labelled (^{34}S)-L-cysteine.[27] Interestingly, the labelling from (^{34}S)-L-cysteine is efficiently incorporated into TDA not only by *P. inhibens*, but also by *P. gallaeciensis* DSM 26640, while labelling from the algal sulfur metabolite (^{34}S)DMSP[44] is not incorporated into TDA at all (Figure 6). *P. gallaeciensis* encodes a PatB homolog with 95% identical amino acid residues in its genome (WP_024095761) that likely also functions as a L-cystine β-lyase for the formation of the sulfur precursor *S*-thiocysteine for TDA biosynthesis. The finding that sulfur from DMSP is not incorporated into TDA is rather surprising, because it has been well documented that this compound is taken up and degraded to small sulfur volatiles in *P. inhibens* that derive from MeSH.[45] However, this finding is in line with the reported incorporation of labelling from [^{35}S]DMSP into proteins via [^{35}S]MeSH by marine bacterioplankton. Specifically,

these investigations demonstrated that the main portion of labelling in the proteins occurs in methionine, but not in cysteine.[46] These and our results indicate that the labelling from methionine is not quickly distributed to cysteine and TDA.

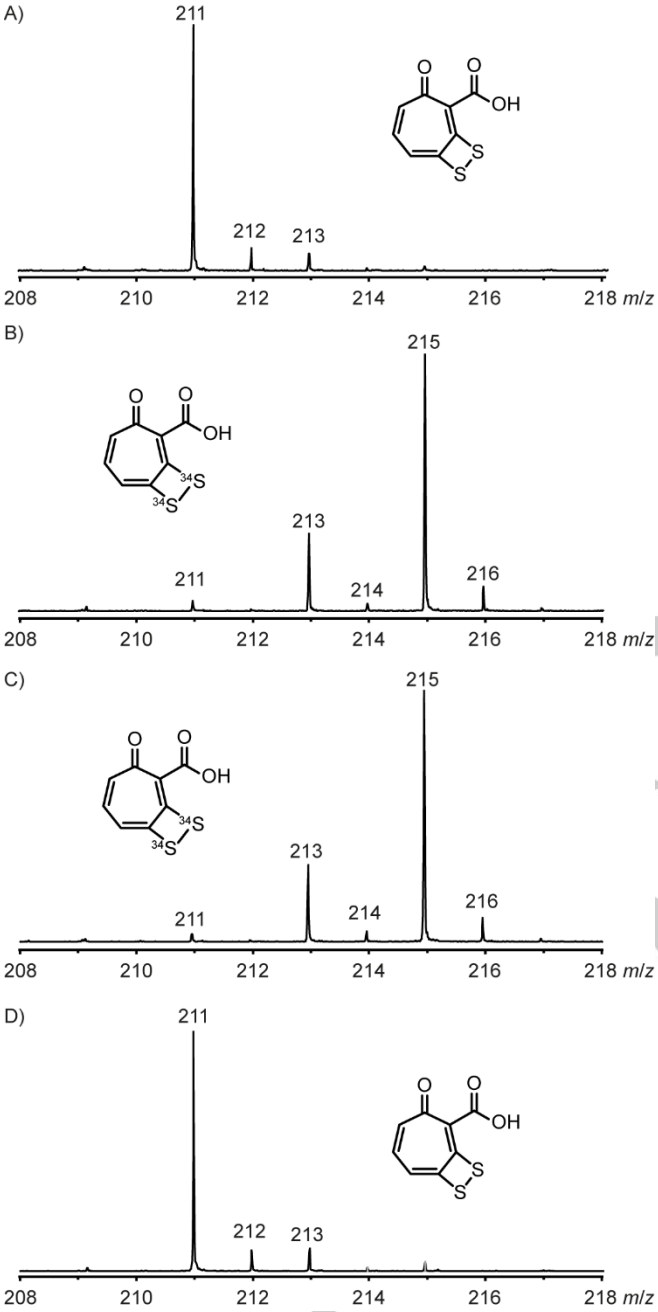


Figure 6. Feeding experiments with ^{34}S-labelled precursors. Partial ESI mass spectrum showing the molecular ion $[M+H]^+$ of A) unlabelled TDA, B) ^{34}S-labelled TDA after feeding of (^{34}S)-L-cysteine to *P. inhibens*, C) ^{34}S-labelled TDA after feeding of (^{34}S)-L-cysteine to *P. gallaeciensis*, and D) TDA with no incorporation of labelling after feeding of (^{34}S)DMSP.

Comparison to functionally related enzymes

A few previously described enzymes catalyse the same reaction as described here for PatB from *P. inhibens*. As mentioned above, another L-cystine β-lyase has been reported from the cyanobacterium *Synechocystis*.[41,42] This enzymes shows only a weak sequence identity of 10% to the enzyme from *P. inhibens*, but its substrate tolerance is similarly broad. Furthermore, two L-cystine β-lyases from plants, one from *Brassica oleracea* and one from *Arabidopsis thaliana*,[43,47] are known. The amino acid sequences of these two enzymes also strongly deviate from the sequence of PatB from *P. inhibens*. Notably, also the L-cystine β-lyases from *Brassica* accepts alternative substrates that are structurally related to L-cystine, while this has not been investigated in detail for the *Arabidopsis* enzyme. Finally, the L-methionine γ-lyase from *Brevibacterium linens* has been described that also accepts L-cystine and catalyses its β-lysis.[48]

Conclusions

The L-cystine β-lyase from *P. inhibens* was biochemically characterised in this study. The enzyme cleaves L-cystine into *S*-thio-L-cysteine, pyruvate and ammonium, but also accepts structurally related substrate analogs with lower efficiency. Closely related homologs of this enzyme occur in many marine bacteria, particularly those of the *Roseobacter* group. An amino acid sequence alignment revealed a highly conserved lysine residue that is critical for enzyme functionality and most likely involved in binding of the PLP cofactor. A few enzymes with similar function have been characterised from other organisms, but all these enzymes show no homology to PatB from *P. inhibens* The previously reported involvement of PatB in the biosynthesis of TDA is further supported by the findings obtained during the course of this study. Notably, the unambiguous identification of the product *S*-thio-L-cysteine is in line with the suggested mechanism of sulfur incorporation into TDA that is fundamentally different from other sulfur incorporation mechanisms reported for holomycin and thiomarinols (from cysteine),[49,50] gliotoxin (from glutathione),[51] or thiotetronate antibiotics (from cysteine).[52] Future experiments in our laboratory will address the further characterisation of intermediates and enzymes of the unique TDA biosynthetic pathway in *P. inhibens*.

Acknowledgements

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Keywords: antibiotics • biosynthesis • *Roseobacter* • marine natural products • sulfur metabolism

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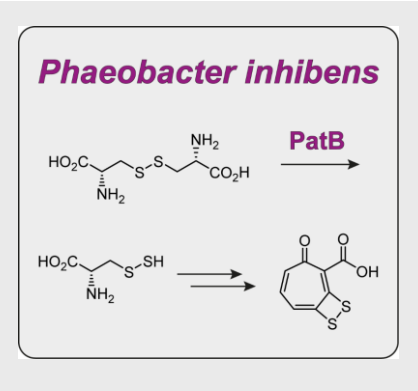
FULL PAPER

The L-cystine β-lyase from *Phaeobacter inhibens* was biochemically characterised in terms of the identification of products from the natural substrate, its substrate scope and enzyme kinetics, and by site-directed mutagenesis. The obtained results together with feeding experiments with ^{34}S-labelled substrates corroborate a previously suggested mechanism for sulfur incorporation into the antibiotic tropodithietic acid.



Jeroen S. Dickschat, Jan Rinkel, Tim Klapschinski and Jörn Petersen*

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Characterisation of the L-cystine β-lyase PatB from *Phaeobacter inhibens*, an enzyme involved in the biosynthesis of the antibiotic tropodithietic acid