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A Tryptophan 6-Halogenase and an Amidotransferase Are Involved in Thienodolin Biosynthesis

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The biosynthetic gene cluster for the plant growth-regulating compound thienodolin was identified in and cloned from the producer organism *Streptomyces albogriseolus* MJ286-76F7. Sequence analysis of a 27 kb DNA region revealed the presence of 21 ORFs, 14 of which are involved in thienodolin biosynthesis. Three insertional inactivation mutants were generated in the sequenced region to analyze their involvement in thienodolin biosynthesis and to functionally characterize specific genes. The gene inactivation experiments together with enzyme assays with enzymes obtained by heterologous expression and feeding studies showed that the first step in thienodolin biosynthesis is catalyzed by a tryptophan 6-halogenase and that the last step is the formation of a carboxylic amide group catalyzed by an amidotransferase. The results led to a hypothetical model for thienodolin biosynthesis.

Introduction

A variety of natural compounds that show plant growth-regulating activities have been isolated from living organisms. Most, such as helminthosporol,[1] sclerin,[2] and spicifernin[3] are secondary metabolites isolated from fungi. During screening for new plant growth-regulating substances in microorganisms, such as actinomycetes, a secondary metabolite of *Streptomyces albogriseolus* MJ286-76F7 was isolated (thienodolin, **1**; Scheme 1).[4] The structure of this compound was elucidated in the early 1990s by Kanbe et al. as 6-chloro-8*H*-thieno[2,3-*b*]-indole-2-carboxamide (molecular formula $C_{11}H_7N_2OClS$, $M=$ 250.71 g mol^{-1}) by spectroscopic and X-ray crystallographic analyses.[5] Thienodolin has a unique secondary metabolite structure: a thienoindole skeleton chlorinated at the 6-position of the indole ring. The total chemical synthesis of thienodolin was accomplished in three steps starting from 6-chlorooxindole.[6]

The alkaloid thienodolin was the first actinomycete metabolite to show concentration-dependent growth-regulating activities in rice seedlings. Growth promotion occurred over the range 1.2–12 μM, whereas growth inhibition was evident above 40 μM.[4] It is not known whether the thienoindole skeleton of thienodolin is essential for its plant growth-regulating activity. A certain structural resemblance of the indole moiety to the auxin indole-3-acetic acid is obvious, but auxin activity could not be determined for thienodolin.[4] Recently, it was shown that thienodolin suppresses nitric oxide synthase and might thus be of pharmaceutical interest.[7] There is currently no information about the biosynthetic pathway of thienodolin. However, because of its structural similarity to tryptophan, it has been proposed that this amino acid could be its precursor, and that (as for other known halogenated secondary metabolites derived from tryptophan, like pyrrolnitrin,[8] pyrroindomycin,[9] and rebeccamycin)[10] the first biosynthetic step in thienodolin biosynthesis might be halogenation of the indole ring of tryptophan catalyzed by a tryptophan 6-halogenase. The identification of a gene coding for a $FADH_2$-dependent tryptophan 6-halogenase[11] in the genome of the thienodolin producer *S. albogriseolus* supported this hypothesis, but there was no experimental evidence for participation by this enzyme in thienodolin biosynthesis.

Here, we investigated the involvement of this tryptophan 6-halogenase in the biosynthesis of thienodolin; we identified the biosynthetic gene cluster, elucidated the last biosynthetic step of thienodolin biosynthesis, and suggest a biosynthetic pathway for thienodolin.

Results

Production of thienodolin and 6-bromothienodolin (6-bromothieno[2,3-*b*]indole-2-carboxamide) by *S. albogriseolus*

Thienodolin was successfully extracted from the culture broth and the mycelium of *S. albogriseolus* grown in the medium described by Kanbe et al.[4] Identification of thienodolin was achieved from its characteristic UV absorbance spectrum ($\lambda_{max}=$ 329.1 nm, 288.6 nm, 271.2 nm (shoulder), 235.7 nm, and by HPLC/MS analysis, which gave a mass (m/z 248.7/250.6 $[M-H]^-$; Figure 1B) that corresponded to the molecular mass of thienodolin (calculated mass $M=$ 250.00/251.99 g mol^{-1}) as well as the expected fragmentation pattern.

When grown in the presence of bromide, 6-bromothienodolin was detected in small amounts in ethyl acetate extracts of *S. albogriseolus*. HPLC/MS analysis gave m/z 292.9/294.9

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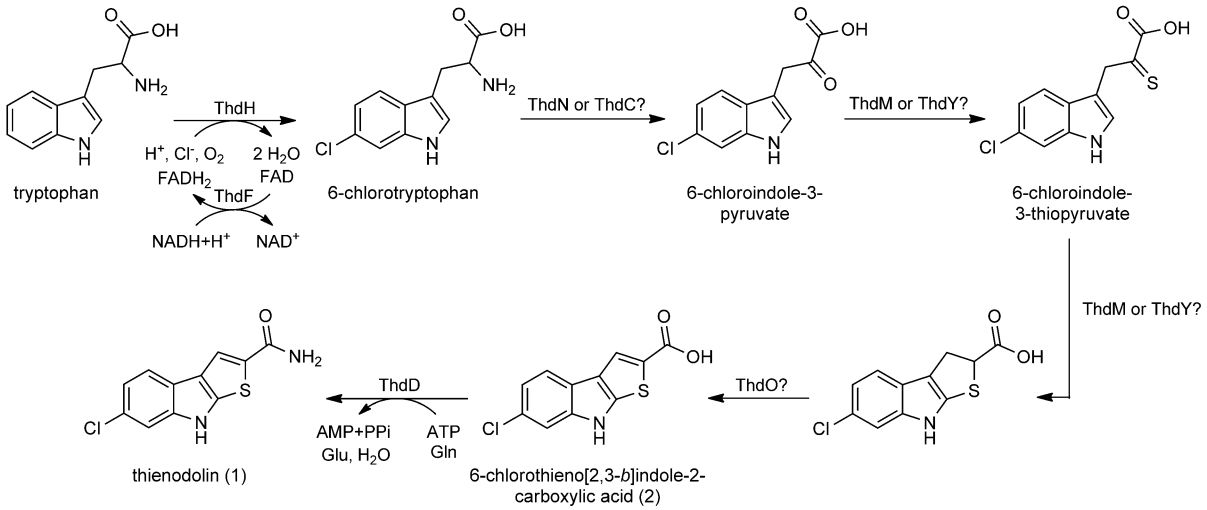

Scheme 1. Proposed biosynthetic pathway of thienodolin. The first step, halogenation of tryptophan catalyzed by the tryptophan-6-halogenase ThdH, and the last step, amidation of 6-chlorothieno[2,3-*b*]indole-2-carboxylic acid to thienodolin catalyzed by the amidotransferase ThdD were verified experimentally.

$[M-H]^-$ (calculated molecular mass of 6-bromothienodolin: $M = 293.95/295.94\ g\,mol^{-1}$) and showed the typical isotope distribution of monobrominated compounds (Figure 2 B).

Cloning and organization of the thienodolin biosynthetic gene cluster

To elucidate the pathway for thienodolin biosynthesis, a cosmid library (3500 clones) of *S. albogriseolus* MJ286-76F7 was constructed. Based on the hypothesis that *thdH*, the gene that encodes tryptophan 6-halogenase ThdH (formerly, Thal),[11] is part of the thienodolin biosynthetic gene cluster, the cosmid library was screened with a digoxigenin (DIG)-labeled *thdH* gene. Three overlapping cosmids (cos2B8, cos3A8, and cos3G10; Figure 3) were identified.

The sequenced genomic region represented by the partially sequenced cosmid cos2B8 encompasses 26.7 kb and contains according to BLAST!! analysis (http://ncbi.nlm.nih.gov/blast/), one incomplete and 21 complete open reading frames (ORFs, Figure 3). All show high G+C content and a G+C bias at the third codon position (characteristic of Streptomyces genes). General knowledge of the organization of secondary metabolite gene clusters and the structural features of thienodolin suggested that 14 of the identified ORFs are involved in the biosynthesis of this alkaloid. Among them, nine probable structural genes were identified, as well as five genes possibly involved in regulation and transport. BLAST bioinformatics analysis of predicted gene products with the proposed functions is presented in Table 1.

The suggested borders of the cluster are the two-component regulatory system *thdK*/*thdL* (left) and the transcriptional regulator *thdR* (right; K, L, and R in

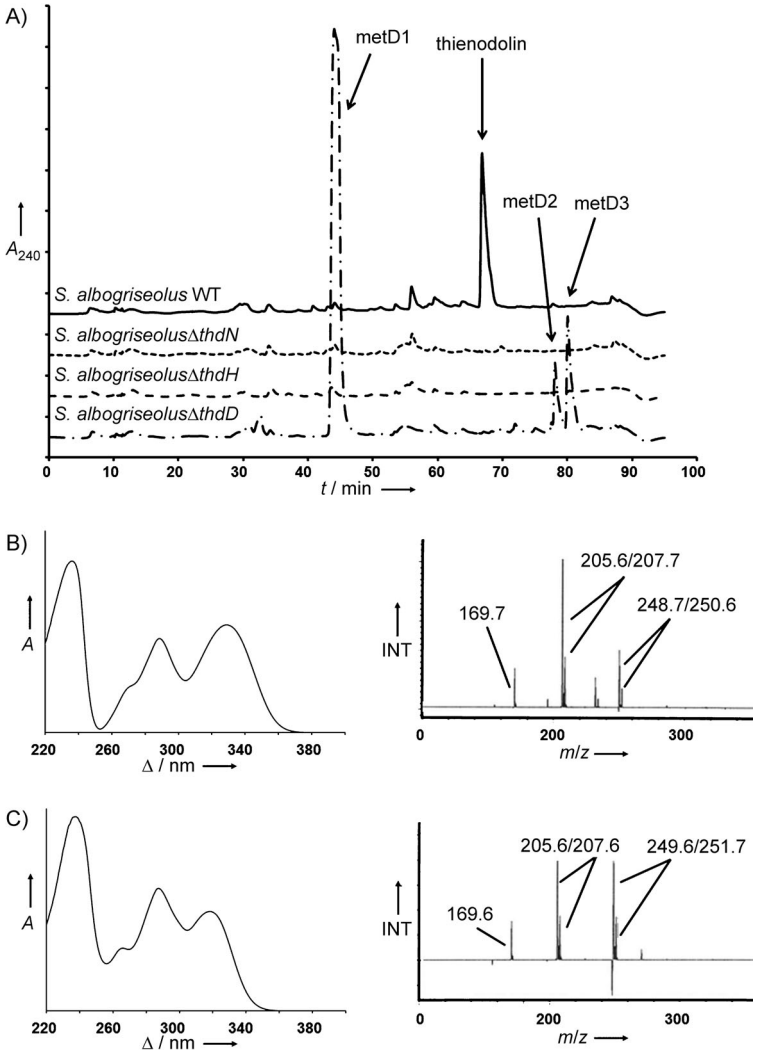

Figure 1. Analysis of *S. albogriseolus* wild-type and mutant strains. A) HPLC analysis of ethyl acetate extracts of *S. albogriseolus* wild-type and mutant strains. B) UV absorption and mass spectra of thienodolin. C) UV absorption and mass spectra of 6-chlorothieno-[2,3-*b*]indole-2-carboxylic acid (metD1).

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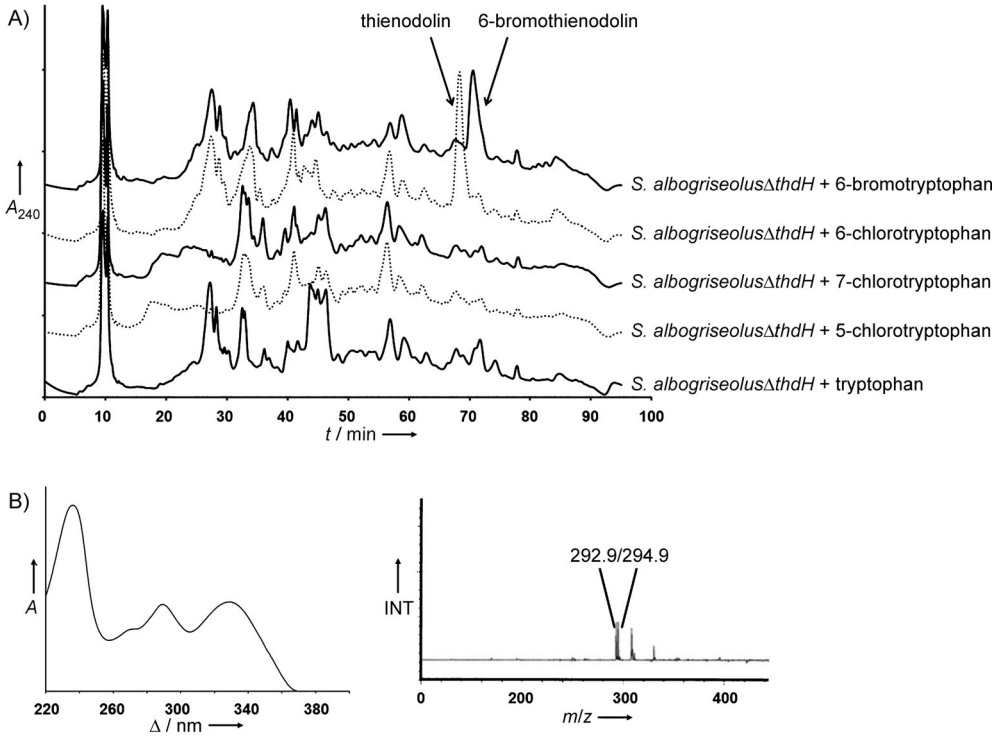

Figure 2. Analysis of ethyl acetate extracts of *S. albogriseolus* Δ*thdH* grown in the presence of different halogenated tryptophans. A) HPLC analyses of ethyl acetate extracts of *S. albogriseolus* Δ*thdH* grown in the presence of tryptophan, 5-chlorotryptophan, 7-chlorotryptophan, 6-chlorotryptophan, or 6-bromotryptophan. B) UV absorption and mass spectra of 6-bromothienodolin.

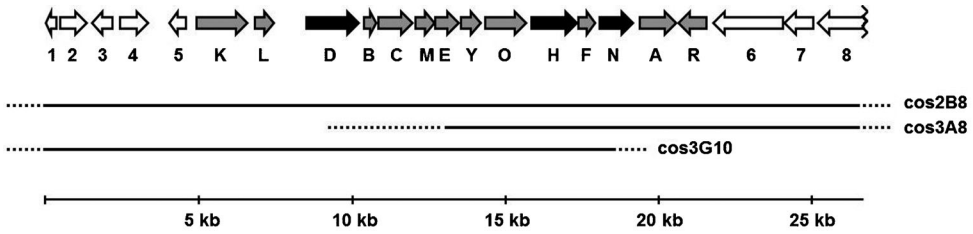

Figure 3. Organization of the thienodolin biosynthetic gene cluster in *S. albogriseolus* MJ286-76F7. White arrows indicate genes not involved in thienodolin biosynthesis. Black and gray arrows indicate genes involved in thienodolin biosynthesis. Black arrows indicate genes deleted in the mutants Δ*thdD*, Δ*thdH*, and Δ*thdN*; cosmids: cos2B8, cos3A8, and cos3G10.

Figure 3). The gene products of *thdK* and *thdL* show high similarity to the histidine kinase AbsA1 and its cognate response regulator AbsA2, respectively, from *Streptomyces lividans* TK24. The gene *thdR* is transcribed in the opposite direction to that of the other putative genes of the thienodolin biosynthetic gene cluster and shows significant homology to LysR-type transcriptional regulators. The other genes of the thienodolin biosynthetic gene cluster are located between these two types of regulatory systems.

The translation product of one gene, *thdA*, might be involved in thienodolin transport or a resistance mechanism. ThdA resembles ion exchangers found in many secondary metabolite gene clusters and might therefore be responsible for efflux of thienodolin and/or the transport of protons into the cell. *thdH* encodes tryptophan 6-halogenase, which has been suggested to catalyze the regioselective chlorination of tryptophan to 6-chlorotryptophan during thienodolin biosynthesis.[11] This flavin-dependent halogenase requires the participation of a flavin reductase, which seems to be encoded by *thdF*; ThdF shows high similarity to flavin reductases from a number of known biosynthetic gene clusters for halogenated secondary metabolites. ThdD shows high similarity to amidotransferases, thus suggesting that it might be responsible for the formation of the carboxylic amide group. ThdN (384 amino acids) contains a PLP-binding domain and shows significant homology to aminotransferases from various organisms. The products of several genes have no obvious function in thienodolin biosynthesis. ThdM and ThdY are putative proteins with only poor homology to several other putative proteins. Based on sequence analysis alone, it was not possible to suggest roles for ThdM and ThdY in the biosynthesis of thienodolin. For ThdM, a role in sulfur incorporation was suggested, as it showed some homology to a hypothetical protein from *Nostoc punctiforme* PCC 73102 (formerly annotated as a putative 2-methyl-thioadenine synthetase). Four putative gene products showed significant homology to known proteins, but without a clear function in thienodolin biosynthesis. Genes *thdB*, *thdC*, *thdE*, and *thdO* were predicted to encode a DNA-binding protein, a cytochrome P450 protein, a short-chain dehydrogenase/reductase, and a FAD-dependent oxidase, respectively.

ORFs 1–5 and 6–8 (left and right flanks of the gene cluster) do not seem to have any role in thienodolin biosynthesis. ORFs 1–5 code for three different types of transcriptional regulators (ORF1, -3, and -4), an alcohol dehydrogenase (ORF2), and a hypothetical protein of unknown function (ORF5); ORFs 6–8 code for proteins involved in sugar metabolism.

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Table 1. Genes identified in and near the thienodolin biosynthetic gene cluster and putative functions.

Gene	Protein (aa)[a]	(Proposed) function in thienodolin biosynthesis	Highest similarity with Protein, origin	Similarity/ identity [%]	Accession no.
orf1	126	–	HxlR family transcriptional regulator, *Streptomyces violaceusniger* Tu 4113	94/96	YP_008412347
orf2	310	–	alcohol dehydrogenase zinc-binding domain-containing protein, *Streptomyces violaceusniger* Tu 4113	95/98	YP_004812346
orf3	246	–	TetR family transcriptional regulator, *Streptomyces coelicolor* A3(2)	68/79	NP_631583
orf4	326	–	AraC family transcriptional regulator, *Streptomyces violaceusniger* Tu 4113	58/72	YP_004816445
orf5	199	–	hypothetical protein SZN_21996, *Streptomyces zinciresistens* K42	61/73	ZP_08805441
thdK	606	regulatory protein	two-component sensor kinase, *Streptomyces lividans* TK4	57/67	ZP_06532709
thdL	220	regulatory protein	two-component system response regulator, *Streptomyces griseoflavus* Tu 4000	87/91	ZP_07313681
thdD	605	amidotransferase	asparagine synthetase, *Thermobispora bispora* DSM 43833	62/74	YP_003653656
thdB	153	DNA binding protein	DNA binding protein, *Salinispora arenicola* CNS-205	56/75	YP_001536821
thdC	397	cytochrome P450	hypothetical protein, cytochrome P450 Sros_9048, *Streptosporangium roseum* DSM 43021	45/60	YP_003344420
thdM	238	unknown	hypothetical protein Npun_R4134, *Nostoc punctiforme* PCC 73102	48/67	YP_001867455
thdE	274	dehydrogenase/ reductase	short-chain dehydrogenase/reductase SDR, *Serratia proteamaculans* 568	49/61	YP_001479951
thdY	238	unknown	hypothetical protein MXAN_2178, *Myxococcus xanthus* DK 1622	44/62	YP_630401
thdO	468	oxidoreductase	putative FAD-dependent oxido-reductase, *Bacillus subtilis* BSn5	41/58	YP_004206890
thdH	531	tryptophan 6-halogenase	tryptophan 6-halogenase, *Streptomyces* sp. C	70/84	ZP_07289786
thdF	193	flavin reductase	flavin reductase, *Streptomyces uncialis*	55/79	AEO12709
thdN	383	aminotransferase	aminotransferase, *Francia* sp. Ccl3	53/65	YP_480967
thdA	420	integral membrane transporter	Na/H antiporter, *Streptomyces viridochromogenes* DSM 40736	51/69	ZP_07302117
thdR	318	regulatory protein	substrate binding protein LysR, *Nocardioides* sp. JS614	66/79	YP_921458
orf6	811	–	β-D-xylosidase, *Streptomyces ghanaensis* ATCC 14672	70/79	ZP_06576083
orf7	298	–	binding-protein-dependent transport protein, *Streptomyces roseosporus* NRRL 11379	78/89	ZP_04713223
orf8	[b]	–	putative binding-protein-dependent transport protein, *Streptomyces* sp. Tü 6071	80/92	ZP_08456041

[a] If more than one possible start codon was identified, the longest deduced protein is given and was used for BLAST analysis/homology search. [b] Incomplete.

Construction of *S. albogriseolus* gene inactivation mutants *Δ thdH*, *Δ thdD*, and *Δ thdN*, and analysis of an intermediate of thienodolin biosynthesis

Tryptophan 6-halogenase is encoded by *thdH*; *thdD* and *thdN* putatively encode an amidotransferase and an aminotransferase, respectively. These genes were inactivated by deleting parts of the genes and inserting the apramycin resistance cassette *acc(3)IV* into the gene to be inactivated (see Table S1 and Figure S1 in the Supporting Information). Organic extracts of five-day-old cultures of wild-type *S. albogriseolus* and Δ*thdH*, Δ*thdD,* and Δ*thdN* mutants were prepared and analyzed by HPLC. Thienodolin was produced by wild-type *S. albogriseolus* (t_R = 68 min), but no thienodolin was detected in ethyl acetate extracts from the mutant strains. HPLC-analysis of organic extracts of *S. albogriseolus* Δ*thdD* indicated the presence of three compounds that were absent from wild-type *S. albogriseolus*: metD1, metD2, and metD3, with retention times (t_R) of 45, 78, and 82 min, respectively (Figure 1 A). The major compound, metD1 (t_R = 45 min), showed an UV absorbance spectrum very similar to that of thienodolin (Figure 1) with λ_{max}(metD1) = 318.4, 287.8, 266.3, and 237.4 nm (Figure 1 C). The UV spectra of the minor compounds, metD2 and metD3, did not show any similarity to those of thienodolin or tryptophan (data not shown). Thus, metD2 and metD3 were not analyzed in detail. MetD1 was purified by preparative HPLC and further analyzed by HPLC/MS and ^{1}H NMR. HPLC/MS analysis of metD1 (*m/z* 249.6/251.7 $[M-H]^-$) indicated that the molecule contains an odd number of nitrogen atoms and furthermore showed an isotope distribution typical of a monochlorinated compound (Figure 1 C). ^{1}H NMR analysis ([D$_6$]DMSO, 500.13 MHz) gave four signals for aromatic protons: δ_H = 7.92 ppm (s, 1 H, Ar*H*); 7.78 ppm (d, *J* = 8.3 Hz, 1 H, Ar*H*), 7.48 ppm (d, *J* = 1.8 Hz, 1 H, Ar*H*), 7.16 ppm (dd, *J* = 8.4, 1.8 Hz, 1 H, Ar*H*). Comparison of these data with ^{1}H NMR signals obtained for thienodolin[4] together with the similar UV absorption spectra of the two compounds (Figure 1 B and C) led to the conclusion that thienodolin and metD1 have high structural similarity and that metD1 very likely has a thienoindole skeleton. Given that ThdD might catalyze an amidotransferase reaction, compound metD1 was proposed to be 6-chlorothieno[2,3-*b*]indole-2-carboxylic acid (**2**; Scheme 1) with a molecular mass of *M* = 250.98/252.98 g mol^{-1} (calculated mass *M* = 250.98 g mol^{-1}).

To test this hypothesis, **2** was synthesized in five steps starting from 6-chlorooxindole. The final product was characterized by HPLC/MS, ^{1}H NMR, and ^{13}C NMR spectroscopy (Figures S1 and S2) and by its UV absorption spectrum (HPLC/MS: *m/z* 249.6/251.7 $[M-H]^-$; ^{1}H NMR (500.13 MHz, [D$_6$]DMSO): δ_H = 12.20 ppm (br. s, 1 H, NH), 7.79 ppm (s, 1 H, Ar*H*), 7.70 ppm (d,

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J = 8.4 Hz, 1H, Ar*H*), 7.52 ppm (s, 1H, Ar*H*), 7.04 ppm (dd, J = 8.4, 1.8, 1H, Ar*H*); ^{13}C NMR (125.75 MHz, [D$_6$]DMSO): δ_C = 167.5 ppm (s, -COOH), 144.2 ppm (s, >C=), 142.0 ppm (s, >C=), 137.8 ppm (s, multiplicity, >C=), 126.2 ppm (s, >C=), 122.9 ppm (s, >C=), 120.8 ppm (s, >C=), 119.9 ppm (d, -C=), 119.6 ppm (d, -C=), 119.0 ppm (d. -C=), 111.3 ppm (s -C=); UV (MeOH), λ_{max} = 317.9 nm, 287.6 nm, 266.0 nm, and 236.8 nm. The data obtained for synthesized **2** correspond to those obtained for metD1. When the chemically synthesized compound was co-injected with metD1 and analyzed by HPLC, only a single peak appeared. Thus, metD1 was verified as 6-chlorothieno[2,3-*b*]indole-2-carboxylic acid (**2**).

Conversion of compound 2 to thienodolin in vivo and in vitro

Chemically synthesized **2** was used for in vivo and in vitro analysis of the amidotransferase activity of ThdD. For this purpose, *thdD* was ligated into the *Escherichia coli–Pseudomonas* shuttle vector pCIB0 and and transferred into *Pseudomonas fluorescens* BL915 ΔORF1–4 to create strain ΔORF1–4 pCIB0*thdD*. Translation was achieved by attaching the ribosome binding site from *prnA* of *P. fluorescens* BL915, and expression was analyzed by SDS-PAGE (Figure 4A).

Feeding of *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD* with **2** revealed that this compound was converted to thienodolin by ThdD. Thienodolin was isolated both from *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD* cells and from the medium after cultivation in the presence of **2**. No thienodolin was obtained from the control strain, *P. fluorescens* BL915 ΔORF1–4 pCIB0 (Figure 4B).

In vitro analysis of amidotransferase activity of ThdD was performed with cell-free crude extract of *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD* obtained by sonication. Thienodolin was detected by HPLC analysis when using glutamine as an amino group donor (Figure 4C). Almost identical results were obtained when ammonium acetate was used as the amino group donor (results not shown). Thus, ThdD was identified as the enzyme that catalyzes the last step in thienodolin biosynthesis: formation of the carboxylic amide group.

Chemical complementation of *S. albogriseolus* Δ*thdH* with 6-chlorotryptophan and other halogenated tryptophans

Upon feeding of the *S. albogriseolus* Δ*thdH* mutant with chemically synthesized 6-chlorotryptophan, thienodolin production was restored. When this strain was cultured in the presence of 6-bromotryptophan, 6-bromothienodolin was obtained (Figure 2). The biosynthetic products were detected by HPLC analysis in ethyl acetate extracts of five-day-old cultures after cultivation for 4 days in the presence of the respective substrate, and these were identified by their characteristic UV absorbance spectra and HPLC/MS analysis. The latter gave masses of m/z 248.7/250.6 $[M-H]^-$ for thienodolin and m/z 292.9/294.9 $[M-H]^-$ for 6-bromothienodolin, and showed isotope distributions typical for chlorine and bromine, respectively (Figures 1B and 2B). Feeding of *S. albogriseolus* Δ*thdH* with 5-chlorotryptophan and 7-chlorotryptophan did not result in the production of the 5- and 7-chloro-substituted analogues of thienodolin (Figure 2A).

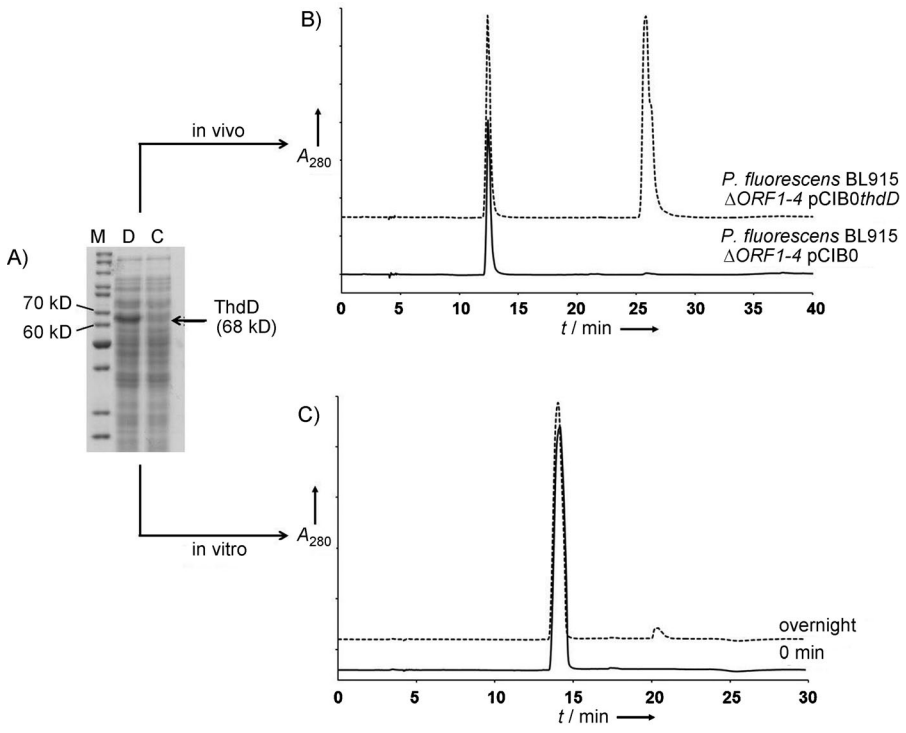

Figure 4. In vivo and in vitro conversion of **2** into thienodolin by the amidotransferase ThdD. A) SDS-PAGE of the amidotransferase ThdD overproduced by *P. fluorescens*; M: Marker, D: crude extract of *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD*, C: crude extract of *P. fluorescens* BL915 ΔORF1–4 pCIB0. B) HPLC analysis of ethyl acetate extracts of *P. fluorescens* BL915 ΔORF1–4 and *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD* after growth in the presence of **2** (gradient: grad3); 12.5 min: **2**, 26.5 min: thienodolin. C) HPLC analysis of in vitro enzyme assays performed with crude extract of *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD* (gradient: grad2); 14.5 min: **2**, 21 min: thienodolin.

Discussion

In many cases, the bromo analogues of chlorinated metabolites produced by soil bacteria can be obtained when chloride in the culture broth is substituted with bromide.[12] This is also the case for the biosynthesis of thienodolin, and is in accordance with previous results obtained with purified tryptophan 6-halogenase ThdH, which was shown to catalyze the regioselective chlorination, as well as bromination, of tryptophan in the 6-position.[11] Feeding of the deletion mutant *S. albogriseolus* Δ*thdH*

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with 6-chlorotryptophan and 6-bromotryptophan led to the production of thienodolin and 6-bromothienodolin, respectively, thus indicating that substitution of chlorine with bromine does not have any influence on the enzymes along the biosynthetic pathway, as has been observed for other biosynthetic pathways.[13,14] In contrast, halogenation of tryptophan at the 6-position of the indole ring seems to be a prerequisite for thienodolin biosynthesis, as neither unhalogenated thienodolin derivatives nor 5- or 7-chloro-substituted thienodolin analogues were detected in the Δ*thdH* mutant after feeding with the respective chlorotryptophans.

ThdH is a flavin-dependent halogenase; it requires a flavin reductase for the formation of the reduced FAD required by the halogenase. A putative flavin reductase gene, *thdF*, was found in the cluster. However, it has been shown that ThdH, like other flavin-dependent halogenases, accepts free $FADH_2$, and there seems to be no specific association between these two components of the halogenase system.[15,16] Thus, in vivo, other flavin reductases, which are always present in organisms, can provide reduced flavin for the halogenase.[17] This is consistent with the fact that a number of halometabolite biosynthesis gene clusters have been identified without a flavin reductase gene.[18,19] Therefore, it is possible that ThdF is part of a two-component monooxygenase system. The oxygenase part of such a system could be ThdC (an enzyme with homology to cytochrome P450 enzymes) or ThdO (homologous to FAD-dependent oxidoreductases).

Further downstream in the biosynthetic pathway, activation of the α-carbon of 6-chlorotryptophan is necessary. For this activation and the subsequent sulfur incorporation, different reactions can be imagined. Activation of the α-carbon of 6-chlorotryptophan could be achieved by oxidative deamination to 6-chloroindole-3-pyruvate, catalyzed by a cytochrome P450 enzyme.[20] A gene coding for such a protein was found in the biosynthetic gene cluster of thienodolin. The protein ThdC shows homology to several cytochrome P450 monooxygenases from other Gram-positive bacteria and might catalyze this oxidative deamination. A second route for 6-chloroindole-3-pyruvate formation is an oxidation reaction analogous to the reaction catalyzed by the amino acid oxidases StaO (staurosporine biosynthesis in *Streptomyces clavuligerus* ATCC 27064)[21] and RebO (rebeccamycin biosynthesis in *Lechevalieria aerocolonigenes*).[10] These enzymes catalyze the formation of indole-3-pyruvate from tryptophan and 7-chloroindole-3-pyruvate from 7-chlorotryptophan, respectively, in monooxygenase reactions that release ammonia and hydrogen peroxide. A similar reaction, generating 6-chloroindole-3-pyruvate from 6-chlorotryptophan, seems unlikely for thienodolin biosynthesis, as no gene coding for a protein similar to RebO or StaO was detected in the thienodolin biosynthetic gene cluster.

The most probable route for α-C activation is an aminotransferase reaction. The enzyme might be encoded by *thdN*. ThdN shows significant homology to aminotransferases and contains a pyridoxal-5′-phosphate binding motif. In an aminotransferase reaction, the α-amino group of the substrate is transferred to the α-carbon of an α-keto acid (α-ketoglutarate, oxaloacetate, or pyruvate), thus forming a new α-keto acid from the substrate and an amino acid (glutamate or aspartate). In the context of the biosynthesis of thienodolin, the putative aminotransferase ThdN would convert 6-chlorotryptophan to 6-chloroindole-3-pyruvate, which could then be the substrate for sulfur insertion at the activated α-carbon atom by nucleophilic attack of a sulfur donor, such as a thiocarboxylic acid, a persulfide or a hydrogen sulfide anion[22,23] catalyzed by another enzyme. After sulfur incorporation leading to 6-chloroindole-3-thiopyruvate, ring-closure to form the thienoindole skeleton would be necessary; however, no gene coding for an enzyme with putative cyclase activity was found in the thienodolin biosynthetic gene cluster. Candidates for sulfur insertion and ring closure are ThdM and ThdY (homologous to putative proteins, but with no indication for their function). The subsequent oxidation of the new ring might be catalyzed by the potential FAD-dependent oxidase ThdO.

The last step in thienodolin biosynthesis is catalyzed by the 605-residue enzyme ThdD. This shows high homology to various proteins of the amidotransferase family, which is a special form of the asparagine synthetase family. Asparagine synthetase family proteins are found in primary metabolism (mainly in asparagine biosynthesis), but some of them, especially the amidotransferases, also appear in secondary metabolism. A well described example of an amidotransferase in secondary metabolism is the *Streptomyces rimosus* enzyme OxyD involved in the formation of the malonamate starter unit during oxytetracycline biosynthesis.[24] This shows high homology (74% similarity, 60% identity) to ThdD. Analysis of the primary structure of ThdD for conserved motifs revealed affiliation to type II asparagine synthetases. ThdD shows the two-domain architecture typical for enzymes of this family: an N-terminal glutaminase domain and a C-terminal synthase domain.[25] Similarly to guanosinemonophosphate synthetase (EC 6.3.5.2)[26] and glucosamine 6-phosphate synthetase (EC 2.6.1.16),[27] the two domains of ThdD are on a single polypeptide. An example of an amidotransferase with the two domains on separate subunits is carbamoylphosphate synthetase (EC 6.3.5.5).[28] The glutaminase domain contains a nucleophilic cysteine residue C2, which is highly conserved in all proteins of this family and is essential for the glutamine-hydrolyzing activity of the enzymes.[29] In this part of the enzyme, glutamine is converted to glutamate and ammonia, which is then transported to the synthetase domain where it is used to aminate the substrate in an ATP-dependent reaction. Corresponding to the mechanism common among asparagine synthetases, the substrate is probably activated as a substrate–AMP intermediate.[30] An assay developed to characterize *E. coli* asparagine synthetase B[31] could be applied to verify in vitro ThdD-catalyzed conversion of **2** to thienodolin. ThdD accepts glutamine as well as free ammonium ions (in the form of ammonium acetate) as the amino group donor. In both cases, thienodolin could be detected by HPLC-UV analysis (characteristic UV absorption spectrum), thereby verifying that ThdD catalyzes the last step in the biosynthesis of thienodolin.

Any downstream effects of the three mutations can be ruled out, as addition of 6-chlorotryptophan to the halogenase mutant led to the formation of thienodolin. Thus all the en-

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zymes required for thienodolin biosynthesis after formation of 6-chlorotryptophan by the halogenase ThdH as the first step in thienodolin biosynthesis are functional. In the case of the aminotransferase mutant, any downstream effects on other biosynthetic genes can be ruled out because *thdN* is the last gene in the biosynthetic gene cluster. Any effect of the destruction of *thdD* can be ruled out as ThdD catalyzes the last step in thienodolin biosynthesis, and all the enzymes required for formation of the last intermediate were still functional in the amidotransferase mutant.

In analogy to the biosynthesis of rebeccamycin, where the ion exchanger RebU was found to be involved in efflux of the secondary metabolite,[10] a role for ThdA in thienodolin efflux is now proposed. Furthermore, it can be hypothesized that ThdA is necessary to establish and maintain concentration gradients between the extracellular matrix and the cytosol. These gradients and established membrane potentials are the basis for many cellular processes, especially Na^+- or H^+-dependent secondary transport systems important for uptake or efflux of metabolites and other ions.[32] They are important for maintaining the intracellular pH, intracellular ion concentrations and the cell volume of actively metabolizing cells.[33] Besides RebU (rebeccamycin biosynthesis), transporters of this class can be found in the biosynthetic gene clusters of many halogenated secondary metabolites, such as PrnF, AviJ, ComF, and SimEx2 from the biosynthesis of pyrrolnitrin, avilamycin A, complestatin, and simocyclinone D8, respectively.[34–37]

For regulation of thienodolin production, two different types of regulatory systems are proposed: the two-component system ThdK/ThdL (genes *thdK* and *thdL* at the left of the cluster) and the LysR-type transcriptional regulator ThdR (*thdR* at the right).

Conclusions

In this paper, we describe the biosynthetic gene cluster of thienodolin, the first growth-regulating compound identified in actinomycetes. Parts of the pathway of thienodolin biosynthesis were elucidated. Feeding a halogenase gene disruption mutant with 6-chloro- and 6-bromotryptophan verified that the tryptophan 6-halogenase gene *thdH* (identified in the genome of *S. albogriseolus* in 2006)[11] belongs to the biosynthetic gene cluster of thienodolin and that ThdH catalyzes the first step in thienodolin biosynthesis. Identification of ThdD as an amidotransferase catalyzing the conversion of the carboxylic acid group of 6-chlorothieno[2,3-b]indole-2-carboxylic acid to the amido group of thienodolin elucidated the last step of the biosynthesis. ThdD amidotransferase activity was shown both in vivo and in vitro. ThdD is one of the very few examples of an amidotransferase in secondary metabolism. The detection of the thienodolin biosynthetic gene cluster should assist the detection of the enzymes involved in the very unusual sulfur incorporation step and the concomitant formation of the thienoindole ring.

Experimental Section

Strains, culture conditions and plasmids: The following bacterial strains were used: thienodolin producer *S. albogriseolus* MJ278-7F,[4,5] *E. coli* TG1[38] for subcloning, *E. coli* ET12567 (pUB307)[39,40] for inter-species transfer of pHZ1358-based constructs to *S. albogriseolus*, *E. coli* XL1-Blue MRA (Stratagene) for propagation of the cosmid library, *Pseudomonas fluorescens* BL915 ΔORF1–4[8] for heterologous expression of genes. The growth medium for *S. albogriseolus* and mutants for DNA isolation was tryptic soy broth (TSB); the medium described by Kanbe et al.[4] was used for the production of thienodolin, biosynthetic intermediates, and 6-bromothienodolin. Medium A[41] was used as the sporulation medium. Conjugation of *Streptomyces* spores was performed by standard procedures[42] in mannitol soybean flour medium.[43] *E. coli* and *P. fluorescens* were cultivated in lysogeny broth (LB).[44] If necessary for selective growth, the medium was supplemented with the appropriate antibiotics. *Streptomyces* strains were cultivated in the presence of apramycin (5 $\mu g\,mL^{-1}$ for liquid cultures, 25 $\mu g\,mL^{-1}$ for solid medium) or nalidixic acid (25 $\mu g\,mL^{-1}$); for *E. coli* strains ampicillin (100 $\mu g\,mL^{-1}$), apramycin (100 $\mu g\,mL^{-1}$), chloramphenicol (25 $\mu g\,mL^{-1}$), kanamycin (25 $\mu g\,mL^{-1}$), and tetracycline (15 $\mu g\,mL^{-1}$) were used; for *Pseudomonas* strains, tetracycline (30 $\mu g\,mL^{-1}$) and kanamycin (50 $\mu g\,mL^{-1}$) were used. *E. coli* strains were grown at 37 °C, *Streptomyces* and *Pseudomonas* strains were grown at 30 °C. Liquid cultures were grown under continuous agitation at 150 rpm. For the construction of specific plasmids, the vectors pBluescript II SK(+) (Stratagene), pLitmus28[45] and pEFBA[46] were used. For gene replacement, the pHZ1358 vector[47] was used, and for the construction of the cosmid library pOJ446[48] were employed, respectively. For gene expression in *Pseudomonas*, pCIB0[49] was used.

General genetic manipulation and PCR: Standard molecular biology procedures were performed according to protocols described for *E. coli*[44] and *Streptomyces*.[42] Plasmid DNA preparation, DNA restriction/dephosphorylation/ligation, PCR, and Southern hybridization were performed by following the enzyme and reagent manufacturers' protocols: MBI Fermentas (Vilnius, Lithuania), Roche Diagnostics, Amersham Pharmacia Biotech, PeQLab Biotech (Erlangen, Germany). PCR conditions were 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 3 min, and final extension at 72 °C for 5 min. *Pfu* polymerase (Thermo Scientific) and DMSO (10%) were used for all amplifications. Oligonucleotide primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

Construction and screening of *S. albogriseolus* MJ286-76F7 cosmid library: DNA fragments obtained from a partial digestion with Sau3AI were ligated into cosmid pOJ446 digested with BamHI and in vitro packed by using the Gigapack III Gold Packaging Extract Kit (Stratagene) according to the manufacturer's instructions. The resulting *E. coli* transductants were picked and transferred to 96-well microtiter plates containing LB medium and apramycin. Clones were replicated on LB agar plates containing apramycin. After overnight growth at 37 °C, colonies were transferred to nylon membranes for colony hybridization analysis according to described methods.[44] The screening of the cosmid clones was performed with a digoxigenin (DIG)-labeled tryptophan 6-halogenase probe, which was obtained by PCR amplification from *S. albogriseolus* with primers ThdH_probe_for (5′-CTGGA TGACT GCGGC GTATC TCGGC AAGGC-3′) and ThdH_probe_rev (5′-CGTGC AACTG ACGCA GCAGG TCATA GGTCG-3′). For labeling of the probe and detection of signals the DIG DNA Labeling and Detection kit (Roche) was used.

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DNA Sequencing and computer-assisted sequence analysis: DNA sequencing was performed by Eurofins MWG Operon with the dideoxy chain termination method on ABI 3730XL sequencer. Sequence analysis and comparison of deduced proteins with other known proteins was carried out by using BLAST (http://ncbi.nlm.nih.gov/blast/). For translation of nucleotide into amino acid sequences and calculation of protein properties such as molecular mass, programs at the ExPASy Proteomics Server (http://www.expasy.ch/tools/) were used. Sequences were aligned with the program MultAlin (http://multalin.toulouse.inra.fr/multalin/)[50] and colored with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The sequences *thdH*, *thdD*, and *thdN* have been deposited in GeneBank with accession numbers KC182553, KC182554, and KC182555, respectively.

Generation of *S. albogriseolus* mutant strains: Involvement of the genes *thdD*, *thdH*, and *thdN* in thienodolin biosynthesis was demonstrated by gene disruption. For the generation of chromosomal mutants of the thienodolin producer *S. albogriseolus* MJ286-76F7 by homologous recombination, the gene disruption plasmids pHZ1358 MutH, pHZ1358 MutD and pHZ1358 MutN were constructed (Figure S3). PCR primers, lengths of the generated fragments, and restriction sites for cloning (Table S1) are provided in the Supporting Information. The inactivation plasmids were used to conjugate *Streptomyces* spores. The conjugation was carried out by biparental conjugation[42] using *E. coli* ET12567 (pUB307), which contains all genes required for horizontal gene transfer. Twenty hours after conjugation, the plates were overlaid with nalidixic acid to kill *E. coli* and apramycin to select for *S. albogriseolus* crossover mutants. After 7 to 10 days, apramycin resistant transformants were obtained. To differentiate double from single crossover mutants, apramycin-resistant colonies were replated on solid medium containing thiostreptone (25 mg mL^{-1}). Thiostreptone-resistant transformants (single crossover events) were neglected; some thiostreptone-sensitive colonies were selected for analysis. Verification of the correct gene replacement was carried out by Southern hybridization with the apramycin resistance cassette and the plasmid pHZ1358 as DIG-labeled probes.

Analysis of thienodolin production by HPLC: Thienodolin production in wild-type *S. albogriseolus* and mutants was monitored by HPLC analysis of culture extracts. Precultures were grown for 3 days in the medium described by Kanbe et al.[4] (50 mL). In the case of mutants, apramycin (5 μg mL^{-1}) was added. An aliquot (2 mL) of preculture was transferred to fresh Kanbe medium (125 mL) and cultured for 5 days. After separation of cells from culture medium by centrifugation, both culture broth and cells were extracted twice with equal volumes of ethyl acetate. Subsequently, the organic phases were evaporated, then the respective residues were dissolved in methanol (300 μL) and analyzed by HPLC by using gradient grad1 (see below).

Heterologous expression of the amidotransferase gene *thdD*: The gene *thdD* was heterologously expressed in *Pseudomonas fluorescens* BL915 ΔORF1–4. For this, the gene was amplified with primers ThdD_for (5′-ATCGA CTAGT CTC**AG GAGA**T TCCAC CATGT GCGGT ATCGC AGGCT GGGTA GATTT C-3′) and ThdD_rev (5′-ATCGT CTAGA TTCAG ACGTC GACGC GCAGC TCCTG GGACT CCAGC CAGG-3′). Restriction sites SpeI and XbaI are underlined; the ribosome binding site (shown in bold) is from the *P. fluorescens* BL915 tryptophan 7-halogenase gene *prnA* (8 nucleotides upstream of the translation initiation codon of *thdD*). The gene was amplified by PCR, double-digested with SpeI and XbaI, and ligated into vector pCIB0 (linearized with XbaI and dephosphorylated with calf intestine alkaline phosphatase). After verification of the correct orientation of the gene in the resulting plasmid (pCIB0*thdD*) by restriction digest, the plasmid was transferred into *P. fluorescens* BL915 ΔORF1–4 by electroporation (12.5 kV cm^{-1}, 5 ms). Cells were grown on LB agar containing kanamycin and tetracycline at 30 °C for 48 h, and some of the colonies were chosen for protein production analysis. One clone showed significant ThdD production and was selected for further experiments such as protein accumulation and in vivo assays/feeding experiments.

Synthesis of 6-chlorothieno[2,3-*b*]indole-2-carboxylic acid (2): The synthesis of **2** was carried out in five steps starting from 6-chlorooxindole. The conversion of 6-chlorooxindole to 6-chlorothieno[2,3-*b*]indole-2-methylcarboxylic acid in four steps was previously described.[6] The final conversion to **2** was achieved by alkaline hydrolysis with LiOH. For this, 6-chlorothieno[2,3-*b*]indole-2-methylcarboxylic acid (1.6 mmol) was dissolved in THF/MeOH (4:1, 114 mL) and refluxed for 7 days. After adjusting the pH to 5–6 with HCl (2 N) the reaction mixture was extracted three times with CH_2Cl_2 (120 mL). The organic phases were combined and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the resulting solid was dissolved in hexane/ethyl acetate (2:8) and purified by chromatography on silica gel. After elution of unreacted 6-chlorothieno[2,3-*b*]indole-2-methylcarboxylic acid with hexane/ethyl acetate (2:8), **2** was eluted in methanol.

Confirmation of the chemical structure by NMR: Samples for NMR spectroscopy were prepared by dissolving the compound (1 mg for ^{1}H NMR, 5 mg for ^{13}C NMR) in [D_6]DMSO (1 mL) and transferred into 3 mm NMR tubes. All NMR experiments were recorded at room temperature on an Avance 600 spectrometer (Bruker). Proton and carbon chemical shifts were referenced to the TMS signal. Coupling constants and chemical shifts are given in Hz and ppm, respectively.

Feeding experiments: Three feeding experiments were performed. For the formation of 6-bromothienodolin by *S. albogriseolus*, sodium chloride in the medium described by Kanbe et al.[4] was substituted with sodium bromide. Thienodolin and 6-bromothienodolin production in the deletion mutant *S. albogriseolus* Δ*thdH* was achieved by feeding with 6-chlorotryptophan and 6-bromotryptophan, respectively. For the in vivo conversion of **2** to thiendolin by the amidotransferase produced by *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD*, cultures were fed with chemically synthesized **2**.

To produce the brominated thienodolin analog 6-bromothienodolin, *S. albogriseolus* was precultured as described above for the production of thienodolin, but with sodium bromide (5 mM) instead of sodium chloride in the main culture. After 5 days, the culture broth was extracted twice with the same volume of ethyl acetate, then the organic phases were combined and evaporated to dryness under reduced pressure. The residue was dissolved in methanol (300 μL) and analyzed by HPLC/MS.

For chemical complementation of the deletion mutant *S. albogriseolus* Δ*thdH* by feeding, the strain was precultured for 3 days as described above for the production of thienodolin. The main culture (125 mL) was inoculated with preculture (2 mL) and grown at 28 °C. After 24 h, 6-chlorotryptophan (16 μM) or 6-bromotryptophan (16 μM) was added, and cells were cultured for a further 4 days. The culture broth was extracted twice with an equal volume of ethyl acetate, and the combined organic phases were evaporated under reduced pressure. The residue was dissolved in methanol (500 μL) and analyzed by HPLC and HPLC/MS.

For the conversion of **2** by *P. fluorescens* BL915 ΔORF1–4 pCIB0*thdD*, the strain was precultured in LB medium (10 mL) con-

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taining the appropriate antibiotics and grown overnight at 30 °C with shaking (150 rpm). Preculture (1 mL) was transferred into fresh LB (30 mL) containing the appropriate antibiotics. After cultivation for 24 h, **2** (5 μg) was added to the culture and cultivated for further 48 h. After separation by centrifugation, both cells and culture broth were extracted twice with equal volumes of ethyl acetate. The organic phases were evaporated to dryness under reduced pressure, then the residue was dissolved in methanol (200 μL) and analyzed by HPLC (gradient grad3). As a control, *P. fluorescens* BL915 ΔORF1–4 pCIB0 was treated identically.

In vitro activity assay of ThdD: Analysis of the in vitro amidating activity of ThdD was carried out based on an enzyme assay for *E. coli* asparagine synthetase B (AS-B).[31] With the exception of substrate **2**, which was dissolved in methanol, all components were dissolved in Tris·HCl (100 mM, pH 8.0). The assay mixture (160 μL) consisted of **2** (0.5 mM), L-glutamine or NH_4OAc (0.5 mM), ATP (0.5 mM), $MgCl_2$ (8 mM) and protein solution (100 μL). All components were combined and incubated at 30 °C. Subsequently, the reaction was stopped by adding triacetic acid (32 μL, 20%), precipitated proteins were removed by centrifugation, and the supernatant was extracted twice with one volume of ethyl acetate. The combined extracts were dried under reduced pressure, then the residue was dissolved in methanol (130 μL) and analyzed by HPLC (gradient grad2).

HPLC and HPLC/MS: Analytical and preparative HPLC were performed on a preparative HPLC system (Knauer, Berlin, Germany) by using reversed-phase columns at room temperature, and with Eurochrom 2000 software (Knauer). For analytical HPLC, a LiChrospher 100 column (RP18, 5 μm, 250 mm × 4.6 mm) was used. Preparative HPLC was performed with a Eurospher 100 column (C18, 5 μm, 250 mm × 20 mm). In both cases, eluent A was water containing NH_4OAc (0.5 mM), and eluent B was methanol containing NH_4OAc (0.5 mM). For preparative HPLC, a gradient program over 110 min with a flow rate of 5 mL min^{-1} was used. The column was equilibrated in 5% methanol. For elution, the following gradient was applied: 5% B (7.5 min), 5–50% B (7.5 min), 50% B (15 min), 50–70% B (45 min), 70% B (15 min), 70–100% B (7.5 min), 100% B (12.5 min). Three different gradients were used for analytical HPLC: grad1: flow rate 0.4 mL min^{-1}, 5% B (5 min), 5–100% B (60 min), 100% B (10 min); grad2: flow rate 0.8 mL min^{-1}, 5% B (2.5 min), 5–100% B (10 min), 100% B (12.5 min); grad3: flow rate 0.8 mL min^{-1}, 5% B (2.5 min), 5–100% B (30 min), 100% B (7.5 min).

HPLC/MS was performed on a model 1100 liquid chromatography system (HewlettPackard) linked with a Esquire Ion Trap (ESI/APCI) mass spectrometer (Bruker) running with DataAnalysis 3.0 software (Bruker). Solvents were water and methanol, containing NH_4OAc (0.5 mM).

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