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A unique mechanism for methyl ester formation via an amide intermediate found in myxobacteria

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Kexwords

Myxobacteria, secondary metabolism, amide formation, hydrolase, methyl transferase, nonribosomal peptide biosynthesis

Abstract

Secondary metabolism involves a broad diversity of biochemical reactions resulting in a wide variety of biologically active compounds. Terminal amide formation during the biosynthesis of the myxobacterial electron transport inhibitor myxothiazol was analyzed by heterologous expression of the unique nonribosomal peptide synthetase MtaG and incubation with a synthesized substrate mimic. These experiments provide evidence that the terminal amide is formed starting from a carrier protein-bound myxothiazol acid thioesterified to MtaF. This intermediate is transformed to an amide by extension with glycine and subsequent oxidative cleavage by MtaG. The final steps of melithiazol assembly involve a highly similar protein-bound intermediate (attached to MelF, a homologue of MtaF), which is transformed to an amide by MelG (homologue of MtaG). In this study it is also shown that the amide moiety of myxothiazol A can be hydrolyzed *in vivo* to the formerly unknown free myxothiazol acid by heterologous expression of *melJ i*n the myxothiazol producer *Stigmatella aurantiaca* DW4/3-1. The methyl transferase MelK can finally methylate the acid giving rise to the methyl ester which is produced as the final product in the melithiazol A biosynthetic pathway. These experiments clarify the role of MelJ and MelK during melithiazol assembly.

Introduction:

Myxobacteria are a rich source of various biologically active secondary metabolites [1-3], e.g., antibacterial, antifungal or cytotoxic substances. These include the antifungal agents myxothiazol and melithiazol (see Figure 1), which represent very potent inhibitors of the electron transport via the cytochrome bc1-complex of the eukaryotic respiratory chain [4, 5]. Similar to the formation of most myxobacterial secondary metabolites that have been studied on the molecular level, melithiazol and myxothiazol are formed by large multifunctional and modular enzymes, termed polyketide synthases (PKSs) and/or nonribosomal peptide synthetases (NRPSs) [6-10]. The enzymology of these megasynthetases is subject to intensive studies and their modular logic has been reviewed recently [11-14].

Although both compounds are very similar in structure, myxothiazol and melithiazol formation involve some strikingly different biosynthetic steps including the employment of alternative starter units and the formation of a terminal methyl ester (in myxothiazol Z by *Myxococcus fulvus* Mx f333/8 and in melithiazol by *Melittangium lichenicola* Me l46; see Figure 1) instead of an amide (in myxothiazol A by *Stigmatella aurantiaca* DW4/3-1). Analysis of both biosynthetic gene clusters led to the proposed biosynthetic pathways depicted in Figure 2 [7, 8] which are based on PKS/NRPS assembly logic [14]. In this study, the question of the formation of the methyl ester via the hypothetical amide intermediate in melithiazol and myxothiazol biosynthesis is addressed.

Following standard PKS/NRPS enzymatic reactions, the intermediate myxothiazol/melithiazol acid bound as thioester to the acyl carrier protein (ACP) of MtaF/MelF is assumed to be formed. Subsequently, MtaG/MelG are believed to catalyze a series of uncharacterized reactions resulting in the formation of the amide structure, which may be formed by extension of the activated myxothiazol/melithiazol

acid with glycine followed by an oxidative cleavage of the extension product by the unusual monooxygenase (Mox) found in MtaG/MelG (see Figure 2). The thioesterase present in MelG/MtaG would subsequently cleave the glyoxylate bound to the peptidyl carrier protein (PCP). As feeding experiments have indeed shown that the amide nitrogen is derived from glycine [8], we assumed that melithiazol is formed in a similar fashion *via* an amide intermediate [8]. According to this hypothesis, two more enzymes are required to catalyze the conversion of the amide to the methyl ester - a hydrolase and a methyltransferase, the latter of which probably needs S-adenosyl methionine (SAM) as cofactor. Indeed, the candidate genes, *melK* and *melJ,* were found in close proximity to the melithiazol biosynthetic gene cluster and the translated proteins MelK and MelJ exhibit similarities to the expected types of enzymes. MelJ shows the characteristics of an amidase belonging to the nitrilase superfamily [15] including the catalytic triad responsible for the hydrolysis of amides [15]. In contrast to the *O*-methyltransferase domains (O-MT) found in MelE *and* MelF, MelK presumably represents a new subclass of SAM-dependent methyltransferases [8]. MelK contains a highly conserved primary structure for *O*-methyltransferases but a poorly preserved SAM binding site. Additionally, a feeding experiment employing [^{13}C]-labeled methionine clearly established SAM as the origin of the methyl moiety of the methyl ester. Since the natural melithiazol producer exhibits resistance to all antibiotics tested and therefore a protocol for genetic manipulations does not exist, we chose *Stigmatella aurantiaca* DW4/3-1 - a myxothiazol A producing strain - to characterize the conversion of the amide to the methyl ester [16]. This strain does not produce any myxothiazol Z. The involvement of *melK* and *melJ* in methyl ester formation was proven by the heterologous expression of both genes in mutant *S. aurantiaca* ESW602 resulting in the production of myxothiazol A and Z [8].

In previous work, two alternative mechanisms were proposed for methyl ester formation in melithiazol biosynthesis (Figure 3). First, an iminoester intermediate [17] may be established from the amide (presumably by MelK-dependent methylation) and then hydrolyzed to form the methyl ester (catalysed by MelJ). Alternatively, one could assume that MelJ hydrolyzes the amide giving rise to the free acid, which is then methylated by MelK.

This article reports the effect of heterologous expression of *melK* and *melJ* when integrated separately into the chromosome of *S. aurantiaca* DW4/3-1. We established myxothiazol-acid as the intermediate of methyl ester formation in myxothiazol *Z* and melithiazol biosyntheses. Myxothiazol acid, purified from the fermentation broth of a mutant expressing the hydrolase MelJ, was subsequently used as a precursor to synthesize the presumed MtaG substrate mimic, *N*-acetylcysteamine (SNAC)-ester of myxothiazol acid. MtaG was heterologously expressed in *E. coli* and the recombinant protein was shown to catalyze the conversion of this substrate mimic into myxothiazol.

Experimental procedures:

Bacterial strains and culture conditions:

Escherichia coli and *Stigmatella aurantiaca* DW4/3-1 were cultivated as described previously [7, 18].

Construction of the plasmids for the single cross over experiments:

Cosmid M1 [8] was digested with *Stu*I to yield four blunt end DNA fragments with sizes between 3.1 and 3.2 kb. The target DNA fragment harbours part of *melH* (309 bp), the complete genes *melK* and *melJ,* and their presumed promoter region (the latter was shown to be active in *S. aurantiaca* DW4/3-1 [8]). The DNA fragment mixture was cloned into pCR-XL-TOPO (Invitrogen) after addition of a single 3´ adenine overhang using Taq DNA polymerase. The resulting clones (pMSW13; see figure 4) were verified by restriction mapping and sequencing.

For heterologous expression of only *melK* in *S. aurantiaca* DW4/3-1, pMSW13 was digested with *Bam*HI and religated resulting in a construct (pMSW13-2) harbouring a truncated *melJ* gene shortened by 320 bp (*melJ*Δ1-687), which ensures that the gene product is inactive.

For homologous recombination via *mtaH*, the insert of pESW26 [7] was isolated after digestion with *Not*I/*Spe*I and cloned into plasmid pMSW13-2 predigested with *Not*I/*Xba*I (Figure 4) to give pMSW20. The latter plasmid was transferred into *S. aurantiaca* DW4/3-1 by electroporation followed by a homologous recombination process as described previously [8], resulting in mutant strain ESW637.

Plasmid pESW243 [8] was constructed earlier to generate the mutant producing myxothiazol Z (ESW602). This plasmid was digested with *Cla*I and religated resulting in a 165 bp in frame deletion of *melK* in plasmid pESW243-2 (*melK*Δ165) (Figure 4).

The construct thus lacks a functional methyl transferase gene and was also transferred into the chromosome of *S. aurantiaca* DW4/3-1 resulting in mutant strain IMUHd1. The correctness of the integration in the resulting mutants from both experiments was analyzed and confirmed by Southern hybridization (data not shown).

Production and analysis of secondary metabolites in S. aurantiaca *DW4/3-1 and its decendants:*

The cultivation of the strains, the preparation of culture extracts from them and the conditions for the analysis of the spectrum of secondary metabolites using diode array coupled HPLC were described previously [19, 20]. Solvents used for HPLC were: A) acetonitrile (5%), water (95 %), ammonium acetate (5 mM), acetic acid (0.003 %), and B) acetonitrile (95%), water (5%), ammonium acetate (5 mM), acetic acid (0.003 %); isocratic conditions were applied in the first 5 min (50 % solvent B), then a gradient up to 70 % solvent B at 20 min, followed by isocratic conditions of 100 % B for 3 min switching back to 50% B for 7 min; flow rate was 0.5 mL min^{-1} and detection was performed at 254 nm.

Isolation and structure elucidation of myxothiazol-acid

To a three-day culture of *S. aurantiaca* IMUHd1 (1 L) Amberlite XAD-2 resin (5 g) was added. The culture was grown for another 24 h and centrifuged. The solid pellet was extracted with acetone until the orange red color was completely removed. The extract was concentrated *in vacuo* and passed through a silica gel column with ethyl acetate, ethyl acetate:methanol (1:1), and methanol, respectively. The fractions were checked with ESI-MS for the presence of the m/z 489 $(M+H)^+$ peak. Fractions containing myxothiazol acid were pooled, concentrated, and subjected to HPLC

column chromatography (Symmetry C_{18}, acetonitrile (0.2% AcOH): water (0.2% AcOH) = 75:25, flow rate 5 ml/min, detection wavelength 210 nm) to yield pure myxothiazol acid (0.3 mg).

^{1}H-NMR-spectra were recorded on a AM-400 spectrometer (Bruker) at 400 MHz and in [$^2H_4$]methanol as solvent. Chemical shifts δ are given in ppm and coupling constants are given in Hz.

^{1}H-NMR (CD_3OD, 400MHz): δ 5.05 (1H, s, H-2), 4.25 (1H, dq, *J*= 8.5, 6.5 Hz, H-4), 3.83 (1H, dd, *J*= 8.5, 8.0 H-5), 6.41 (1H, dd, *J*= 16, 8, H-6), 6.62 (1H,d, *J*= 16, H-7), 7.4 (1H, s, H-9), 8.05 (1H, s, H-12), 3.97 (1H, dq, *J*= 7.5, 7, H-14), 5.83 (1H, dd, *J*= 15, 7.5, H-15), 6.26 (1H, dd, *J*= 15, 10, H-16), 6.08 (1H,dd, *J*= 15, 10, H-17), 5.73, (1H, dd, *J*= 15, 7, H-18), 2.38 (1H, m, H-19), 1.06 (3H, d, *J*=6.5, H-20), 1.06 (3H, d, *J*= 6.5, H-21), 3.65 (3H, s, H-22), 1.26 (3H, d, *J*= 6.5, H-23), 3.37 (3H, s, H-24), 1.57 (3H, d, *J*= 7, H-25).

Synthesis of myxothiazol acid SNAC ester

Myxothiazol acid (1 mg, 2 μmol), 1-ethyl-3-(3´-dimethylaminopropyl) carbodiimide (0.8 mg, 4 μmol), and a catalytic amount of *N*,*N*-dimethylaminopyridine were mixed under argon atmosphere. *N*-Acetylcysteamine (1.2 mg, 10 μmol) in dichloromethane (150 μL) was added drop-wise while stirring. The reaction mixture was stirred overnight at room temperature and filtered through a 0.2 μm PVDF W/GMF Whatman filter and washed with methanol. The reaction mixture was concentrated and the compound purified by HPLC [Symmetry C18, acetonitrile (0.2% acetic acid) : water (0.2% acetic acid) 75:25 to give myxothiazol acid SNAC-ester (0.8 mg, 65%).

Myxothiazol acid SNAC-ester: ESI-MS *m/z* 590 $[M+H]^+$, 612 $[M+Na]^+$.

HR-MS (TOF-ESI-MS): Calculated for $C_{29}H_{40}N_3O_4S_3$ $[M+H]^+$ 590.2181, Found: 590.2202. Chemical shifts δ are given in ppm and coupling constants are given in Hz.

^{1}H NMR (CD_3OD, 600 MHz): δ 4.84 (overlapped with solvent peak, H-2), 4.13-4.08 (1H, m, H-4), 3.82-3.79 (1H, m, H-5), 6.37 (1H, dd, *J* = 8*,* 16*,* H-6), 6.60 (1H, d, *J* = 16, H-7), 8.03 (1H, s, H-9), 7.39 (1H, s, H-12), 3.96-3.94 (1H, m, H-14), 5.82 (1H, dd, *J* = 7.5, 15, H-15), 6.25 (1H, dd, *J* = 10, 15, H-16), 6.07 (1H, dd, *J* = 10*,* 15, H-17), 5.71 (1H, dd, *J* = 7*,* 15, H-18), 2.37-2.34 (1H, m, H-19), 1.04 (6H, d, *J* = 7, H-20, 21), 3.85 (3H, s, H-22), 1.24 (3H, d, *J* = 7, H-23), 3.67 (3H, s, H-24), 1.57 (3H, d, *J* = 7, H-25), 3.28 (overlapped with solvent peak, C*H*$_2$S),.3.01-2.97 (2H, m, -C*H*$_2$NH-), 1.92 (3H, s, -COC*H*$_3$),

Cloning of mtaG

mtaG was amplified from a cosmid harbouring part of the myxothiazol biosynthetic pathway (data not shown) as template using the oligonucleotide primers gfow (5`-CAGAAGAA**CATATG**GCAGAGCTGTCCAAGC-3`) and grev (5`-ACCTCGGG**GAATTC**CCGCGCGGATGCGGCT-3`), introducing the restriction sites *Nde*I in the forward primer and *Eco*RI in the reverse primer (exchanged nucleotides are shown in bold). The PCR was carried out using PWO (Expand High Fidelity System (Roche Diagnostics)) according to the manufacturer´s protocol with the addition of 5% DMSO. Conditions for amplification using a Eppendorf mastercycler gradient (Eppendorf, Germany) were as follows: denaturation for 30 s at 95° C, annealing for 30 seconds at 54° C, and extension for 5 minutes and 30 seconds at 72° C (30 cycles and a final extension of 10 min at 72° C). The resulting 5251bp amplificate, after extraction from an agarose gel, was cloned into pCR-2.1-TOPO (Invitrogen). The clones were analyzed by restriction analysis and sequenced to verify the correctness of the insert of pMtaGTOPO11. This plasmid was digested with *Nde*I and *EcoR*I and the excised fragment was ligated into the expression vector

pTYB1 (NEB), which resulted in pMtaGTYB1-27. The plasmid was transferred into *E. coli* ER2566 (NEB) for protein expression.

Overexpression, purification and posttranslational activation of MtaG

Post-translational phosphopantetheinylation of apo-MtaG was achieved by co-expression of MtaA in *E. coli* [21, 22]. To achieve this, pSUMtaA was introduced into ER2566/pMtaGTYB1-27 and a freshly grown overnight culture harbouring both plasmids was used to inoculate one liter LB-medium supplemented with ampicillin and chloramphenicol to final concentrations of 100 µg/ml and 35 µg/ml, respectively. The cells were grown to an optical density of 0.5 to 0.7. Expression of the gene was induced by addition of 0.1 mM Isopropyl-ß-D-thiogalactopyranoside (IPTG) and the temperature was immediately shifted to 16°C. After overnight growth the cells were harvested by centrifugation for 15 min at 5000 rpm. The pellet (from 1L culture) was resuspended in 2.5 mL of cell lysis buffer (20 mM Tris-HCl; 500 mM NaCl 0.5 mM EDTA; 0.1% Triton-X-100) and the cells were disrupted by two passages through a French Press (SLM Amico) at 13000 psi. After centrifugation (10000 rpm, 25 min, 4°C) the supernatant was loaded onto chitin beads. Purification of the target protein was performed as described [22] according to the manufacturer´s manual (IMPACT One Step Protein Purification System (NEB)) using 6 ml of chitin beads for the affinity chromatography. After overnight cleavage of the target protein from the intein chitin-binding domain using 50 mM DTT, MtaG was eluted with 50 ml of cleavage buffer (20 mM Tris-HCl; 500 mM NaCl; 0.5 mM EDTA). The protein was concentrated 100 fold with a centricon plus 20 (Millipore) to a final volume of 0.5 ml. Detection of the proteins was achieved after separation using 10% SDS gel electrophoresis [23] and staining with Coomassie blue. Protein concentrations were determined by the Bradford method [24].

In vitro assay for myxothiazol formation

Assays were performed using either crude cell extracts or partially purified protein. Incubations contained 20 mM Tris-HCl, 50 mM NaCl, 0.5 mM EDTA 1.5 mM $MgCl_2$, 0.001 mM glycine; 8.5 µM myxothiazol acid SNAC-ester, 0.015 mM ATP, 0.015 mM $FMNH_2$ and 500 µg protein from crude cell extracts (alternatively 250 µg of "purified" MtaG) in 1.0 mL. Negative control experiments were performed by replacing the purified protein with water (the crude extract of *E. coli/*pMtaGTYB1-27/pSUMtaA was replaced with the crude extract of *E. coli*/pTYP/pSUMtaA). In additional assays other cofactors (0.015 mM), e.g., FMN, FAD^+, NAD^+, $NADP^+$ and their reduced forms ($FMNH_2$; $FADH_2$; $NADH_2$ $NADPH_2$), were tested in various combinations. Assays were incubated at 30 °C overnight and subsequently extracted 3 times with 1 ml ethyl acetate. The extracts were dried in a speed vac (Eppendorf) and redissolved in 100 µl methanol. Aliquots of 20 µl were analyzed using high performance liquid chromatography coupled to a mass spectrometer (HPLC-MS).

HPLC-MS analysis:

The assays were analyzed using HPLC-MS on a Bruker HCTplus coupled to an Agilent 1100 series HPLC system using a 125x2 mm Nucleodur C18/3 µm column. Detection was performed in positive ionization mode. The solvent system consisted of H_2O (A) and acetonitrile (B), each containing 0.1% formic acid. Using a gradient of 19 minutes length and running from 50% to 95% solvent B, separation of products was achieved (retention time for myxothiazol A and myxothiazol acid SNAC-ester were 11.5 and 13.0 min, respectively).

Results and discussion:

In vivo *analysis of the final steps in melithiazol biosynthesis*

The methyl ester in melithiazol is thought to be derived from a hypothetical amide intermediate in a two-step process. Although MelK and MelJ were shown to be required for this transformation, it is not clear whether the pathway proceeds via an iminoester or a free acid intermediate (see Figure 3). We have addressed this question by expression of either *melJ* or *melK* in the myxothiazol producer *S. aurantiaca* DW4/3-1. The mutant ESW637, carrying the intact *melK* gene, produces myxothiazol A at wild type levels. However, no iminoester intermediate could be detected in the extract (as judged by HPLC-MS analysis, data not shown). On the other hand, a new peak was observed in extracts of the mutants harbouring the *melJ* gene (mutant IMUHd1; Figure 5). Further analysis of the corresponding compound using HPLC-MS revealed a mass of 489 $(M+H)^+$ which is in agreement with the structure of the free myxothiazol acid (cp. Figure 1). The ^{1}H-NMR spectral data for this novel metabolite showed the expected change in chemical shifts in comparison to myxothiazol A (myxothiazol acid H-2: δ 5.05 ppm; myxothiazol A H-2: δ 4.94 ppm), whereas chemical shifts of all other protons were identical to those reported for myxothiazol A. These data provide clear evidence that myxothiazol acid is indeed the intermediate in the pathway to the methyl ester (see Figure 3). The iminoester intermediate proposed in an earlier study based on relatively low incorporation rates of labelled compound [17] most likely does not exist. Our results, on the other hand, show that the amide moiety of myxothiazol A is hydrolyzed by MelJ resulting in the formation of myxothiazol acid which is subsequently methylated by MelK to the methyl ester (Figure 3, pathway B). Interestingly, a similar mechanism of methyl ester formation is described for bacterial chemotaxis transmembrane factors in *Salmonella typhimurium* [25, 26]. Theoretically, free melithiazol acid might alternatively be

released by the hydrolase from the ACP of MelF and thus MelG might not be required for melithiazol biosynthesis. However, this compound has never been found in strains producing the methyl ester and the presence of the *mtaG* homologue *melG* in the melithiazol producer does not support this speculation.

The experiments described above reveal a unique and unexpected mechanism of methyl ester formation found in myxobacteria. If MelG is involved in the biosynthesis as shown in figure 2, the biosynthetic sequence is of energy ineffectiveness, because melithiazol acid is available during earlier steps of the biosynthesis as a thioester intermediate coupled to the ACP of MelF. Simple thioesterase-catalysed cleavage and subsequent methylation would represent a much more efficient way to produce the same compound. These results also lead to the conclusion that melithiazol A is produced via a hypothetical amide intermediate and a free acid. The work reported here is the prerequisite for further detailed biochemical investigations of this unusual mechanism to clarify the role of MelG and MtaG in the assembly line. The heterologous expression of *melJ* and *melK* in *E. coli* and the determination of kinetic parameters for these enzymes are currently in progress.

In vitro *investigation of terminal amide formation*

Having myxothiazol acid established as the correct biosynthetic intermediate, which is produced by mutant IMUHd1, it seemed possible to also investigate the unusual formation of the amide catalyzed by MtaG/MelG. After purification of myxothiazol acid an experimental approach was chosen which is based on the heterologous expression of MtaG and its incubation with the *N*-acetylcysteamine (SNAC)-thioester of myxothiazol acid. The latter compound represents a substrate mimic of the presumed enzyme bound form of the compound that is thioesterified to the phosphopantetheinylated ACP of MtaF during the biosynthetic process (see figure 2).

SNAC-thioesters have successfully been used as mimics of the phosphopantetheine arm of ACPs as they can serve as surrogates for the natural ACP-bound substrates [27, 28]. Myxothiazol acid SNAC-ester was synthesized by coupling *N*-acetylcysteamine with myxothiazol acid and the purified compound was shown to be free of myxothiazol A by HPLC-MS (see figure 6B). To gain a better understanding of the mechanism of amide formation, the megasynthetase MtaG was subsequently expressed heterologously in *E. coli*. MtaG represents a NRPS including five domains: condensation (C), adenylation (A), monooxygenase (Mox), PCP and a thioesterase (TE) (see Figure 2). The protein was expressed as a C-terminal intein-chitin fusion protein approximately 250 kDa in size and the internal PCP of MtaG was posttranslationally activated by co-expression of MtaA as described previously [21, 22]. Because glycine had been shown to be the nitrogen source of the amide [8] (see Figure 6C), partially purified MtaG was incubated with this amino acid together with the substrate mimic myxothiazol acid SNAC-ester. Despite numerous efforts and although all protein isolation buffers were stabilized with proteinase inhibitors, degradation of MtaG during the purification could not be avoided (see Figure 6A). Nevertheless, a fusion protein of the expected size could clearly be detected in the protein mixture. The conversion of myxothiazol acid SNAC-ester into myxothiazol A was low but clearly detectable after overnight incubations at 30°C (see figure 6B). Control reactions were carried out using heat-denatured MtaG of the partially purified sample or using crude cell extracts (see below), which resulted in no detectable product formation. The MS^2 fragmentation pattern of the reaction product showed the occurrence of characteristic fragments (m/z values of 456 and 439, data not shown) identical to those found when using an authentic myxothiazol A standard. We tried to increase the efficiency of the assay by varying several parameters and by changing the expression system. However, these changes did not result in higher conversion

rates (data not shown). For this reason no further time course experiments were performed. However, these results clearly show that MtaG is capable of catalyzing the conversion of myxothiazol acid SNAC-ester to myxothiazol A and thus provide evidence for the hypothetical pathway shown in Figure 6. A further purification, e. g., via size exclusion chromatography of MtaG is not possible, because of the instability of the enzyme. We conclude that the size of the expressed protein is critical and that presumably only a very small amount of MtaG is folded correctly and activated after expression in *E. coli.* Expression problems might also be due at least in part to the difference in codon usage in comparison to high GC myxobacteria. Additionally, myxothiazol acid SNAC-ester might not be accepted very well as a substrate mimic by MtaG (low conversion rates have been reported for other SNAC-mimics). Because of the problems during purification of MtaG, crude cell extracts of the *E. coli* cells expressing the protein (as judged by SDS page analysis, Figure 6A) were analyzed in parallel resulting in similar results (data not shown).

As shown in Figure 6, the model for the biosynthesis of the terminal amide involves the activation of glycine by the A domain in an ATP and magnesium dependent reaction and the attachment of the amino acid to the PCP. Subsequently, the C domain catalyzes the nucleophilic attack of the free α-amino group to the activated substrate mimic myxothiazol acid SNAC-ester resulting in a glycine extended myxothiazol acid derivative bound to the PCP. The monooxygenase found inserted into the A domain presumably hydroxylates glycine giving rise to an unstable α-hydroxylated intermediate, degradation of which (either spontaneous or enzyme dependent) leads to the release of myxothiazol A. The last step in this reaction cascade involves the release of glyoxylate, which is performed by the thioesterase found at the C-terminus of MtaG. In this cascade the Mox domain is also of special interest because it is unusually located between the core motifs 4 and 5 of the

adenylation domain of MtaG. To the best of our knowledge, a similar mechanism for amidation has never been described in bacteria. However, the overall reaction sequence is highly reminiscent of the formation of terminal amides in peptide hormones [29, 30] or the biosynthesis of nicotinamide from nicotinuric acid by peptidylglycine α-amidating monooxygenase (PAM) [31]. During these biosyntheses, glycine extension products are hydroxylated in α-position by copper or zinc dependent enzymes, whereas MtaG most likely employs FMN as cofactor (see below). The Mox domain of MtaG shows no significant homology to enzymes involved in the reactions mentioned above, such as PAM [31]. In figure 7, the protein sequence of the Mox domain is compared to those of bacterial luciferases, and the Mox domains of MelG and CtaG (CtaG represents a functionally identical homologue of MelG from a different myxobacterium [32]). Bacterial luciferases catalyze the oxidation of aldehydes to acids employing reduced FMN and releasing energy in form of bioluminescense [33]. To our knowledge, a specific FMN binding motif has not been defined and all known luciferase structures were determined in their apoforms without cofactor. Luciferases are known to employ reduced FMN as a cosubstrate rather than a tightly bound cofactor [34]. Based on the alignment shown in figure 7 which demonstrates significant homology of luciferases and Mox domains, we assume that these domains of MelG, MtaG and CtaG require FMN/$FMNH_2$ as cosubstrate. However, the cofactor requirement could not be clarified in the in vitro experiments. Although different combinations of cofactors were analyzed, no clear cofactor preference could be assigned.

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Figures

Figure1: Structures of myxothiazols and melithiazol A

Figure 2: Proposed models of melithiazol A and myxothiazol A biosynthesis. PKS modules are shown in grey, NRPS modules are shaded, and unusual domains are shown in black. ACP, acyl carrier protein; KS, ß-ketoacyl-ACP synthase; KR, ß-ketoacyl-ACP reductase; AT, acyl transferase; DH, ß-hydroxy-acyl-ACP dehydratase; O-MT, *O*-methyltransferase; ER, enoyl reductase; S, spacer region; PCP, peptidyl carrier protein; C, condensation domain; HC, heterocyclization domain; A, adenylation domain; Ox, oxidation domain; Mox, monooxygenase domain; TE thioesterase

Figure 3: Alternative models for methyl-ester formation in melithiazol biosynthesis

Figure 4: Construction strategies of plasmids used in the heterologous expression of *melK* (A) and *melJ* (B) in *S.aurantiaca* DW4/3-1. Functional genes are shown as arrows; truncated genes are shown as blocks

Figure 5: HPLC analysis of secondary metabolite production in *S. aurantiaca* DW4/3-1 (A), mutant IMUHd1 (B), and mutant ESW602 (C).

Figure 6 : (A) Heterologous expression of MtaG in *E. coli* as intein-chitin fusion protein; MtaG: Protein(s) eluted after affinity purification (see text); pTYB1: Extract of *E. coli* harbouring the control vector pTYB; pMtaGTYB1-27: crude cell extract of *E. coli* harbouring the MtaG expression plasmid pMtaGTYB1-27. The arrow indicates the expressed fusion protein of the expected size.

(B) Extracted ion chromatograms (EIC) of the myxothiazol acid –SNAC-ester and myxothiazol A formed in incubations using MtaG (right) or control reactions (left) employing myxothiazol acid –SNAC-ester. A magnification of the EIC (m/z = 488) showing myxothiazol A is provided in the inset.
(C) Model for terminal amide formation in myxothiazol and melithiazol biosynthesis as analyzed by the *in vitro* formation of myxothiazol A from the myxothiazol acid –SNAC-ester. Active domains in each step are shown in white.

Figure 7: Multiple sequence alignment: Identical amino acids are marked with asterisks. Similar amino acids are marked with colons. Clustal W was used to align the sequences. Corresponding accession numbers from top to bottom: *Melitangium lichenicola* Me l46 (MelG) (CAD 89778.1); *Cystobacter fuscus* strain AJ-13278 (CtaG) (AAW 03330.1) *Stigmatella aurantiaca* DW4/3-1 (MtaG) (AAF19815.1); *Crocosphaera watsonii* WH 8501 (2Lc) (ZP 00514743.1); *Burkholderia ambiferia* (Luci) (ZP00685175); *Xenorhabdus luminescens* HW (Lxa) (HM38525).

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How to generate a methyl ester from a thioester? This study shows that in myxobacteria producing the electron transport inhibitor melithiazol most likely a nonribosomal peptide synthetase generates an amide in a multistep reaction starting from the respective thioesterified acid. This is achieved using a mechanism similar to peptide hormone biosynthesis in eukaryotes. Subsequently, two more enzymes hydrolyze the amide to the free acid and a methyl transferase finally generates the methyl ester.

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