Construction and *in vitro* analysis of a new bi-modular polypeptide synthetase for synthesis of *N*-methylated acyl peptides

Florian Schauwecker, Frank Pfennig, Nicolas Grammel and Ullrich Keller

Background: Many active peptides are synthesized by nonribosomal peptide synthetases (NRPSs), large multimodular enzymes. Each module incorporates one amino acid, and is composed of two domains: an activation domain that activates the substrate amino acid and a condensation domain for peptide-bond formation. Activation domains sometimes contain additional activities (e.g. *N*-methylation or epimerization). Novel peptides can be generated by swapping domains. Exchange of domains containing *N*-methylation activity has not been reported, however.

Results: The actinomycin NRPS was used to investigate domain swapping. The first two amino acids of actinomycin are threonine and valine. We replaced the valine activation domain of module 2 with an N-methyl valine (MeVal) activation domain. The recombinant NRPS (AcmTmVe) catalyzes the formation of threonyl–valine. In the presence of S-adenosyl-methionine, valine was converted to MeVal but subsequent dipeptide formation was blocked. When acyl-threonine (the natural intermediate) was present at module 1, formation of acyl-threonine–MeVal occurred. The epimerization domain of AcmTmVe was impaired.

Conclusions: A simple activation domain can be replaced by one with *N*-methylation activity. The same condensation domain can catalyze peptide-bond formation between *N*-methyl and nonmethylated amino acids. Modification of the upstream amino acid (i.e. acylation of threonine), however,

achieved with acylated threonine. Loss of the epimerase activity of AcmTmVe suggests *N*-methyltransferase and epimerase domains, not found together naturally, are incompatible.

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Introduction

Many pharmacologically important, low-molecular weight peptides are synthesized nonribosomally by nonribosomal peptide synthetases (NRPSs), large, multifunctional protein complexes. NRPSs are composed of highly conserved repeating units (modules) each with an average size of 120 kDa [1–6]. Each module catalyzes the incorporation of one specific substrate amino acid into the final peptide. The different catalytic activities of a single module can be assigned to distinct domains arranged in the following order. Firstly, the condensation domain, necessary for peptide-bond formation between the growing peptide chain and the substrate amino acid; secondly, the adenylation domain, necessary for recognition and adenylation of the substrate amino acid, and thirdly, the thiolation domain, required for covalently binding the substrate amino acid via a 4′-phosphopantetheine cofactor. Together, the adenylation and thiolation domains form an activation domain, which is the minimal NRPS unit capable of initiating peptide synthesis. A module might also contain accessory domains such as an epimerization

domain, located carboxy-terminal to the activation domain, that catalyzes epimerization of the substrate amino acid. In some of these extended modules, the substrate amino acid is epimerized after the condensation step. *N*-methyltransferase domains, which are located between the adenylation and thiolation domains, can catalyze *N*-methylation of the amino acid bound to the thiolation domain. Domains with homology to thioesterases, catalyzing release of peptide chains from NRPS, are found in the carboxyl-terminal region of some NRPSs. The number and sequential arrangement of modules in an NRPS determine the sequence and number of amino acids in the peptide formed by that NRPS.

Early enzymatic studies on the biosynthesis of the chromopentapeptide lactone antibiotic actinomycin [7] showed that some NRPS systems also involve autonomous domains such as the 4-methyl-3-hydroxy-anthranilic acid (4-MHA)-adenylating enzyme (ACMS I), which is an independent adenylation domain. This enzyme was the first of a number of similar adenylating enzymes later

found to operate in the biosyntheses of mikamycins, quinoxaline antibiotics, enterobactin and yersiniabactin [8–11]. These enzymes load their aromatic substrates onto specific thiolation domains, which are either fused to other proteins or exist as autonomous units similar in structure to the small acyl carrier proteins (ACPs) of some fatty acyl synthases and type II polyketide synthases (PKSs). The presence of enzymes that represent single domains in modular systems indicates that NRPS modules might have arisen from gene fusions of initially autonomous domains. This postulated system of domain fusion and switching may be the natural precedent of current engineering strategies to construct new enzymes using combinatorial approaches. The actinomycin synthetase system (Figure 1) combines nearly all features of nonribosomal systems, which can be used for anticipated domain shuffling. It has an adenylating enzyme (ACMS I) responsible for activation of the starter residue 4-MHA, a small 4-MHA carrier

enzyme (AcmACP) and two modular NRPSs, called actinomycin synthetase (ACMS) II and III, that consist of two and three modules, respectively, responsible for assembly of the five amino acids of the peptide lactone rings of actinomycin. This enzyme system synthesizes 4-MHA pentapeptide lactones, which in turn become converted to actinomycin in the presence of oxygen. The genes of ACMS I, AcmACP and ACMS II were previously identified by cloning and analyzing the actinomycin (*acm*) gene cluster from *Streptomyces chrysomallus* [12,13]. As shown here, the sequence analyses of the ACMS III gene (*acmC*) revealed, as expected, that ACMS III consists of three modules. The last two modules each possess an *N*-methyltransferase domain, which is consistent with the fact that the last two amino acids in the pentapeptide chain are *N*-methylated (sarcosine, Sar, and *N*-methyl-valine, MeVal, respectively). Interestingly, the three substrate amino acids (proline, glycine and valine), each activated as

Figure 1
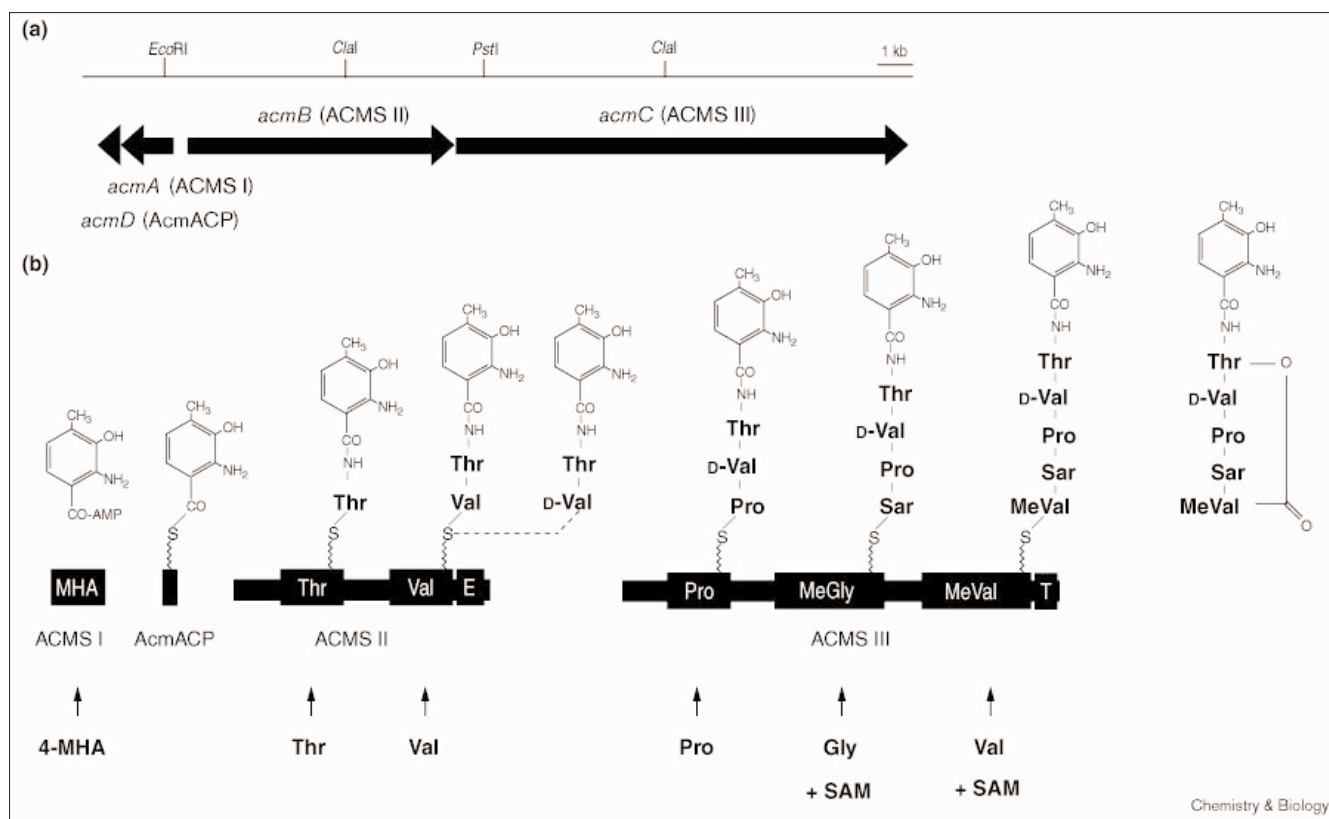
Synthesis of the actinomycin pentapeptide lactone in *Streptomyces chrysomallus*. **(a)** The genomic arrangement of the four genes, coding for the enzymes AcmACP and ACMS I–III, is shown at the top. The encoded enzymes catalyze the ATP-dependent, nonribosomal synthesis of the aryl pentapeptide from substrates 4-MHA (4-methyl-3-hydroxy-anthranilic acid), threonine, valine, proline, glycine and S-adenosyl-methionine (SAM) as indicated. **(b)** 4-MHA is first adenylated by ACMS I, bound by AcmACP and stepwise condensed with the amino acids residing at the activation

domains of NRPSs ACMS II and III. Glycine and valine are *N*-methylated prior to condensation (MeVal, *N*-methyl-valine; Sar, sarcosine). The NRPS activation domains, the thioesterase-like domain (T) and the epimerization domain (E) are shown as thick blocks and the NRPS condensation domains shown as slender blocks. The 4′-phosphopantetheine co-factors for covalent substrate binding are indicated on top of the domains. The dotted line indicates that epimerization of valine to D-Val occurs only after formation of the aryl-dipeptide.

its thioester by ACMS III, are finally condensed in the form of an imino acid (proline, Sar and MeVal), whereas the modules of ACMS II exclusively condense α-amino acids (threonine and valine). In view of recent reports concerning the specificity of condensation domains, which discriminate between various substrates [14,15], we asked whether the condensation domains of ACMS II are specific for α-amino acids or whether they also condense *N*-methyl amino acids. Moreover, from primary sequence comparisons of numerous condensation domains, a system for determining the specificity of condensation domains was recently postulated [16]. We therefore designed experiments to exchange the valine activation domain of ACMS II for the MeVal activation domain of ACMS III and to test the resulting enzyme for its ability to catalyze the formation of *N*-methylated peptides. If successful, these experiments would answer the question of whether activation domains with *N*-methyltransferase activity can replace minimal activation domains in foreign NRPSs. Moreover, by using ACMS II as a scaffold, the experiment would also determine whether such elongated activation domains are compatible with an adjacent epimerase domain. To date, no NRPS gene is known to code for this kind of domain arrangement.

Results
Sequencing the gene of ACMS III (*acmC*)
As reported previously, we located the ACMS III gene (*acmC*) immediately downstream of the ACMS II gene (*acmB*) in the *acm* gene cluster of *S. chrysomallus* [12]. Sequencing this region revealed that *acmC* is 12,744 bp, has a start codon overlapping the stop codon of *acmB* and codes for a deduced protein of 462 kDa. This is consistent with the determined size of 480 kDa for ACMS III [17]. The analysis of the deduced amino acid (aa) sequence revealed the presence of three amino acid activation domains (aa 426–1051, 1488–2525 and 2964–3983), each with an accompanying, upstream condensation domain. In the last two modules, the adenylation and thiolation domains are separated by an *N*-methyltransferase domain (aa 1978–2391 and 3444–3849), similar to those found in related activation domains of the cyclosporine (CysA), enniatin (ESYN) and pristinamycin I (SNBD) synthetases [18–20], which also catalyze *N*-methylation of substrate amino acids. According to the degree of sequence identities, the various known *N*-methyltransferase domains of NRPSs can be classified into those of bacterial origin (ACMS III and SNBD; three domains) and those of fungal origin (CysA and ESYN; eight domains). Sequence identities of *N*-methyltransferase domains are 41–69% within each group but range only between 21–27% when bacterial and fungal sequences are compared. Another recently identified *N*-methyltransferase domain of a microcystin NRPS from *Microcystis aeruginosa* [21] shares 31–34% and 21–23% identity to the other bacterial and fungal sequences, respectively. A thioesterase-like domain was

identified in the carboxy-terminal part of ACMS III (the typical GxSxG motif at aa 4066), probably used for release/cyclization of the synthesized peptide lactone.

Construction and heterologous gene expression of engineered ACMS II derivatives
The set of five different activation domains (threonine, valine, proline, Sar, and MeVal) of the ACMS system provides a collection of functionally compatible modules for combinatorial *in vitro* experiments. The *in vivo* and *in vitro* catalytic activity of the two-modular ACMS II (threonine and valine) is well characterized [12,22,23]. We therefore used this NRPS as a scaffold to investigate whether a condensation domain, which naturally catalyzes peptide-bond formation between nonmethylated amino acids, can also condense *N*-methylated substrates and whether a *N*-methyl amino acid activation domain is compatible with the other domains present in this scaffold. To address this question, we replaced the ACMS II valine domain with the MeVal domain of ACMS III. As both domains can activate valine, possible differences in the catalytic activity of the chimeric protein should only result from the presence of the additional *N*-methylation domain or the altered reactivity of *N*-methyl-valine compared with that of valine. To swap the valine domain of ACMS II (Figure 2) we used a natural *Cla*I site at basepair (bp) 4519 in the ACMS II gene (*acmB*). This region contains a less conserved amino acid motif that is found at the borders between the carboxy-terminal ends of the condensation domains and the downstream activation domains (see the Materials and methods section). In fact, the amino termini of some NRPSs, which lack an amino-terminal condensation domain (e.g. GrsA, PhsA, TycA or BacA) [24–27], map very closely to this border. To create a suitable fusion point at the carboxy-terminal end of the valine domain, we introduced an *Eco*RV site in *acmB* at bp 6250, which corresponds to a position 20 amino acids carboxy-terminal to the serine of the 4′-phosphopantetheine binding site in the thiolation domain. As this fusion point is still located within the highly conserved carboxy-terminal end of the thiolation domains, the border to the downstream ACMS II epimerase domain remained unchanged after domain swapping. The region in *acmB* between *Cla*I and *Eco*RV was then replaced by a polymerase chain reaction (PCR)-generated 2.9 kb *Cla*I–*Eco*RV fragment obtained from *acmC*. This PCR fragment codes for the MeVal domain of ACMS III, and the restriction sites were introduced in a manner that exactly fitted the fusion points described for ACMS II (see the Materials and methods section). To compare this new enzyme (AcmTmVe; 330 kDa) with appropriate controls, we constructed an ACMS II derivative with a swapped MeVal domain in a similar manner, but the epimerase domain was deleted (AcmTmV; 260 kDa). We also back-constructed ACMS II by inserting the original valine domain, using the fusion points described above (AcmTVe; 280 kDa). We performed this

Figure 2

Construction and expression of recombinant NRPS genes. **(a)** The ACMS II gene (*acmB*) and 1.1 kb downstream region; important restriction sites are indicated (S, *Sst*I; Cl, *Cla*I; Bm, *Bam*HI; EV, *Eco*RV). The region between a natural *Cla*I site and a PCR-generated *Eco*RV site (EV*) was replaced by PCR fragments, coding for the valine activation domain (Val) of ACMS II or the *N*-methyl-valine activation domain (MeVal) of ACMS III. In the construct coding for AcmTmV, the region between a PCR-introduced *Sst*I site (S*) and a natural *Sst*I site at the end of *acmB* was deleted in order to remove the epimerization domain. **(b)** The SDS–PAGE (5%; coomassie stained) shows formation of gene products (arrow) after heterologous gene expression in *S. lividans* from the melanin promotor in pIJ702 derivatives. Size markers were wild-type ACMS II (280 kDa) and SDS-6H from Sigma (205–29 kDa).
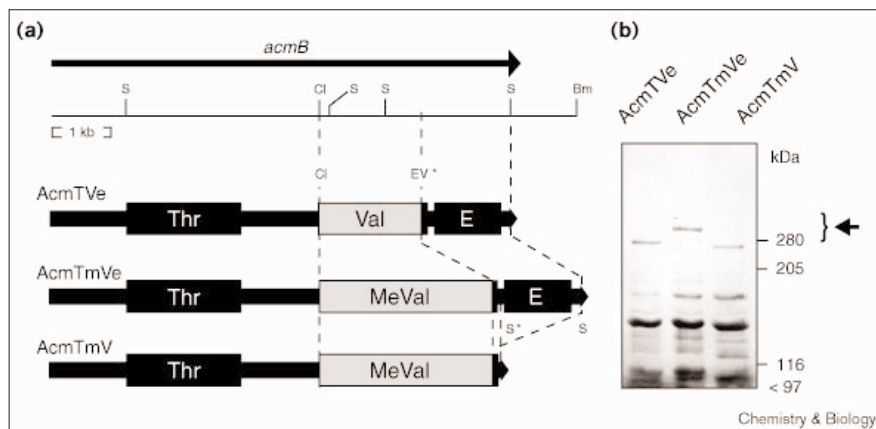
control to verify that the amino acid changes to the ACMS II sequence did not disturb catalytic activity and that the scaffold, which was partially assembled from PCR generated fragments, encodes a functional NRPS. The NRPS genes were then heterologously expressed in *Streptomyces lividans* under the control of the melanin promotor on pIJ702 derivatives, as previously described for heterologous expression of the ACMS II gene [12]. Proteins of the expected sizes were detected using sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 2) and the enzymes were partially purified for further enzymatic studies.

In vitro formation of an *N*-methylated acyl dipeptide after co-incubation of engineered NRPS with ACMS I and AcmACP

The aromatic precursor 4-MHA in actinomycin biosynthesis is first adenylated by ACMS I, then bound by AcmACP and finally condensed with the threonine bound at the threonine domain of ACMS II. With p-toluic acid as the precursor (which can replace the natural but highly oxidation sensitive 4-MHA), ACMS II can catalyze the *in vitro* formation of p-toluyl-Thr–L-Val and p-toluyl-Thr–D-Val [22,23]. Because ACMS II lacks a thioesterase domain, products or intermediates are not released from the enzyme *in vitro* but remain covalently bound through a thioester linkage. They can, however, be analyzed after chemical thioester cleavage and extraction, as described in the Materials and methods section. Accordingly, the products formed by the engineered NRPS were also analyzed after co-incubation with ACMS I, AcmACP, p-toluic acid and ^{14}C-labelled substrate amino acids. The reactions were performed in the presence or absence of S-adenosyl methionine (SAM), which is the methyl group donor for *N*-methylation of substrate amino acids.

In the absence of SAM and by labelling with either ^{14}C-p-toluic acid, ^{14}C-Thr or ^{14}C-Val, thin-layer chromatography (TLC) revealed that all engineered NRPS, similar to wild-type ACMS II, can synthesize both p-toluyl-Thr and p-toluyl-Thr–Val (the latter shown for AcmTmVe with ^{14}C-Val as label; compound **i** in Figure 3). This result was not unexpected as the *in vitro* formation of nonmethylated peptides in the absence of SAM was also reported for the peptide synthetase ESYN, which also possesses a *N*-methyltransferase domain [28]. Amino acid hydrolysis of the formed p-toluyl-Thr–Val and analyzing the configuration of valine revealed, however, that AcmTmVe did not synthesize the expected p-toluyl-Thr–D-Val diastereomer (data not shown). This indicated the epimerization domain in this enzyme was not functioning properly.

Strikingly, in the presence of SAM, when ^{14}C-Thr or ^{14}C-Val were used as labels two new radioactive compounds were formed by the enzyme constructs AcmTmVe and AcmTmV but not by ACMS II or AcmTVe (shown for AcmTmVe with ^{14}C-Val as label; compounds **ii** and **iii** in Figure 3). From these two SAM-dependent compounds, only **ii** was also labelled when ^{14}C-p-toluic acid was used as label (data not shown). Analysis of **ii** by amino acid hydrolysis revealed that ^{14}C-Val was converted into ^{14}C-labelled *N*-methyl-L-valine. That **ii** was p-toluyl-Thr–MeL-Val was confirmed by TLC and high-performance liquid chromatography (HPLC) co-chromatography with authentic p-toluyl-Thr–MeL-Val (Figure 3). The amino acid hydrolysis of **iii** revealed that it contained MeVal in both optical configurations (the ratio of MeL-Val to MeD-Val was 3:2). Compound **iii** did not contain ^{14}C-p-toluic acid and TLC co-chromatography with authentic cyclo-(Thr–MeVal)

showed that it was not identical to that diketopiperazine. The structure of **iii**, therefore, remains unknown.

The products formed by AcmTmVe in the presence of SAM were monitored by TLC over a period of 45 minutes (as described in the Materials and methods section). Kinetic analysis revealed that the reaction was linear for 5 minutes and approached saturation after 20 minutes. Interestingly, the reaction rates of p-toluyl-Thr–Val and p-toluyl-Thr–MeVal formation were identical, indicating that valine and *N*-methyl valine are equally reactive for AcmTmVe-catalyzed condensation with acyl-threonine. Formation of **iii** showed the same time dependency but it was synthesized in larger amounts. The time course of p-toluyl-Thr–Val formation in the absence of SAM was fairly equal to that of p-toluyl-Thr–Val formation in the presence of SAM, but nearly double the amount was formed.
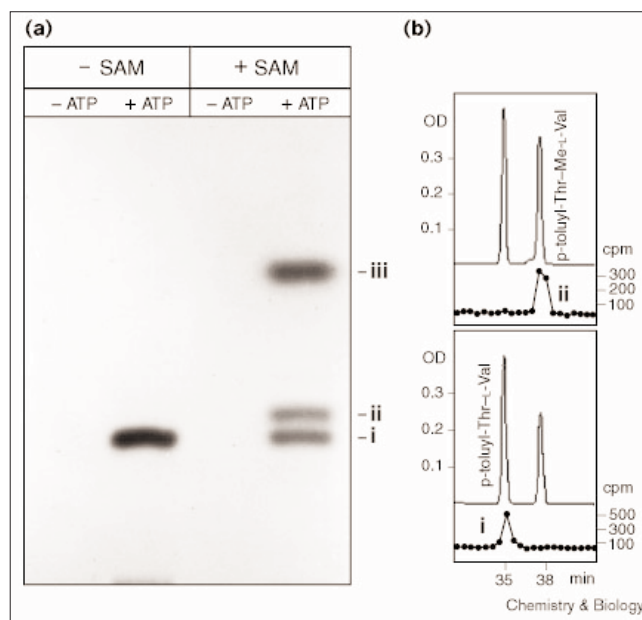
In summary, the identification of p-toluyl-Thr–MeVal shows that the MeVal domain can function in the ACMS II scaffold and that an *N*-methyl amino acid can be condensed with p-toluyl-threonine by the ACMS II condensation domain.

Condensation of *N*-methyl-valine depends on the prior acylation of threonine

The finding that AcmTmV and AcmTmVe can both catalyze formation of p-toluyl-Thr–L-Val and p-toluyl-Thr–MeL-Val, with AcmTmVe being incapable of epimerizing these dipeptides, prompted us to investigate the mechanism of dipeptide formation in more detail. It is known that ACMS II can also catalyze the *in vitro* synthesis of the nonacylated dipeptide Thr–Val along with its diastereomer Thr–D-Val in the absence of p-toluic acid, ACMS I or AcmACP [23]. AcmTmV and AcmTmVe were then analyzed under the same conditions for their ability to synthesize respective *N*-methylated dipeptides. Enzymes were incubated with ^{14}C-labelled substrates and products were analyzed by TLC after thioester cleavage (shown for AcmTmVe in Figure 4). After incubation with valine alone, valine was released unmodified from the enzyme but was converted into MeVal in the presence of SAM. The MeVal formed was identified exclusively to be MeL-Val. This was not unexpected as ACMS II catalyzes valine epimerization only after dipeptide formation. Incubation with valine in the presence of threonine, however, yielded only the nonepimerized dipeptide Thr–L-Val. In contrast, the ACMS II, as with the control construct AcmTVe, retained its epimerase activity (data not shown).

The results of the analyses of AcmTmV and AcmTmVe in the presence of valine, threonine and SAM were remarkable. It was observed from labelling with ^{14}C-valine that nearly all of the valine was converted into MeVal, but formation of the expected Thr–MeL-Val was not observed. Only a very faint signal indicated formation of only a small

Formation of p-toluyl-threonyl–*N*-methyl-valine. **(a)** An autoradiogram of TLC analysis of products formed by AcmTmVe. Products were released from the enzyme by chemical thioester cleavage after incubation with ACMS I, AcmACP, p-toluic acid, threonine and ^{14}C-valine. ATP and S-adenosyl-methionine (SAM) were added or omitted as indicated above the lanes. Formation of p-toluyl-threonyl–[14]C-valine **i** was dependent on ATP, formation of p-toluyl-threonyl–*N*-methyl-^{14}C-valine **ii** and an uncharacterized compound **iii** were both dependent on ATP and SAM. **(b)** Labelled compounds **i** and **ii** (not UV-detectable) were isolated from silica plates and mixed with chemically synthesized p-toluyl-threonyl–valine and p-toluyl-threonyl–*N*-methyl-valine (OD 0.2–0.4 at 260 nm) for HPLC analysis as described in the Materials and methods section. Fractions were collected and ^{14}C-labelled compounds were identified by liquid scintillation counting (dotted curve).

amount of nonmethylated Thr–L-Val, resulting from a condensation reaction prior to the *N*-methylation of valine. To verify the absence of Thr–MeL-Val, which would migrate very close to Thr–L-Val in TLC, we also used ^{14}C-SAM as a label (Figure 4a, right panel). In the presence of valine, the labeled methyl group of SAM was incorporated into MeVal. No formation of *N*-methylated peptides was detected after addition of threonine, which confirmed that the MeVal was not condensed with threonine. It appears that condensation of MeVal with threonine, catalyzed by AcmTmVe and AcmTmV, is dependent on the prior acylation of threonine as revealed by the analyses in the presence of ACMS I and AcmACP. The results are schematically summarized in Figures 5 and 6.

Discussion

Our previous work on the biosynthesis of *N*-methylated acyl-peptide lactones with aromatic side groups, such as the actinomycins, quinoxaline antibiotics and mikamycins, has revealed that these compounds are synthesized by similar
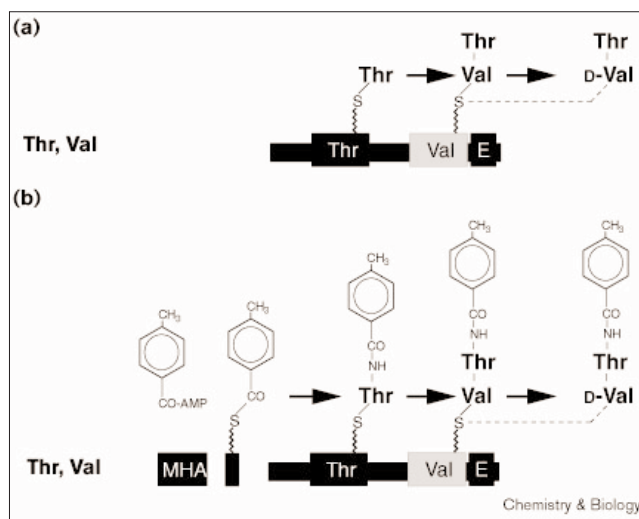
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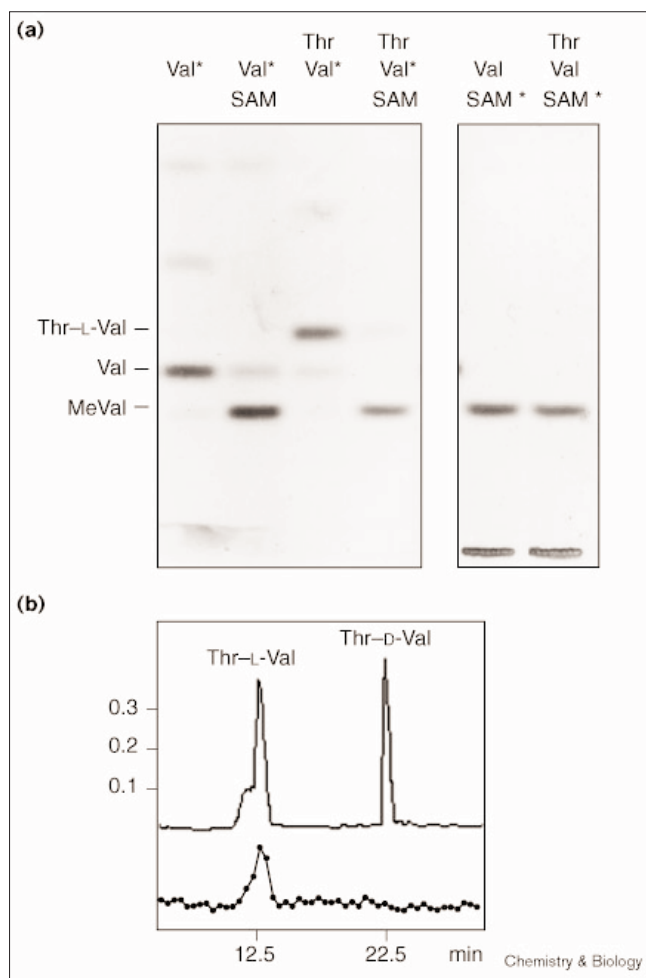
Dipeptide formation and synthesis of *N*-methyl-valine catalyzed by AcmTmVe. **(a)** Autoradiograms of TLC analyses of products synthesized by AcmTmVe that were released from the enzyme after chemical thioester cleavage. The enzyme was incubated with the substrate combinations indicated at the top of the lanes in the presence of ATP. ^{14}C-labelled substrates are indicated by an asterisk. In the presence of S-adenosyl-methionine (SAM), valine is converted into *N*-methyl-valine and formation of *N*-methylated dipeptides was not detectable with ^{14}C-valine (left panel) nor with ^{14}C-SAM (right panel). **(b)** The configuration of the synthesized threonyl–^{14}C-valine (not UV-detectable) was verified to be exclusively the L-form by HPLC co-chromatography with chemical synthesized threonyl–L-valine and threonyl–D-valine (OD 0.3 at 205 nm) as described in the Materials and methods section.

Figure 5

Steps in peptide synthesis catalyzed by ACMS II or AcmTmVe. The grey region indicates the part of ACMS II that was replaced in the construction of AcmTmVe. It differs from ACMS II only in two amino acids at the carboxy-terminal border. NRPS substrates are indicated in the left of the figure. Enzyme-bound products and intermediates are shown in **(a)** the absence or **(b)** the presence of the 4-MHA-adenylating enzyme ACMS I and the ACP AcmACP (small box), which provide the aryl moiety for acylation of threonine when p-toluic acid is added. Catalyzed reaction steps (e.g. condensation of threonine with valine) are indicated by arrows. The dotted line indicates that the epimerized dipeptide remains covalently bound to the second module; other symbols and abbreviations are the same as in Figure 1.

always separated by condensation domains in these multi-modular NRPS arrays.

sets of modular NRPSs that harbour additional *N*-methyl-transferase and epimerase activities. NRPSs are arrays of highly conserved modules, each ~1000–1400 amino acids in length, and each module is responsible for the activation and incorporation of one amino acid into the final peptide product [1–6]. A typical NRPS module consists of an acti-vation domain, which determines substrate specificity, and an amino-terminally located condensation domain, which catalyzes peptide-bond formation. Activation domains are

We recently cloned the actinomycin (*acm*) gene cluster of *S. chrysomallus* and sequenced the ACMS II gene (*acmB*) [12]. This NRPS consists of two modules with activation domains for threonine and valine, respectively, and an epimerization domain located at the carboxy-terminal end of the enzyme. In conjunction with the adenylating enzyme ACMS I and the small ACP AcmACP, ACMS II attaches threonine and valine to the aromatic starter unit of actinomycin biosynthesis and also epimerizes valine to form the aryl dipeptide [22,23]. The data presented here show that the ACMS III gene (*acmC*) codes for the remaining three modules required for pentapeptide lactone ring assembly in actinomycin synthesis. These modules are responsible for the incorporation of proline, sarcosine and *N*-methyl-valine; a carboxy-terminally located thioesterase domain might be responsible for product release. The last two modules of ACMS III each carry a *N*-methyltransferase domain inserted into their activation domains, which is necessary for the *N*-methyla-tion of glycine and valine prior to condensation. The genetic arrangement of the encoded ACMS modules, as in most NRPS systems, therefore dictates the sequence and

modifications of the amino acids in the synthesized peptide product.

The high level of conservation between NRPS modules and the precise constant locations of activation domains within the modules suggested that targeted alterations of modules by swapping activation domains could create a NRPS with altered substrate specificity. Theoretically, this could yield a new NRPS that contains altered modules and catalysing the incorporation of new or modified amino acids into a peptide product. Attempts to achieve this by chromosomal gene replacement have been reported in the cases of the mono- and tri-modular NRPS components of the surfactin synthetase system, and the production of new products *in vivo* has been demonstrated [29–31]. Altering the specificity of the activation domain in order to design new or modified NRPSs might, however, be constrained by interfering with the inter-action between the new modules because of spatial reasons, by a change in the reactivity of the new peptidyl intermediates, or simply by inappropriate folding of these intermediates, which might in turn lead to undesired reactions or complete loss of activity in peptide-chain elongation. Moreover, the fact that modules can contain additional domains (e.g. for *N*-methylation of covalently bound amino acids or for epimerization of reaction intermediates) might provoke adverse effects in the achievement of successful new module combinations. A further obstacle in the proper interaction of foreign modules could lie in the correct positioning and in the substrate specificity of the condensation domains. The essential role of the condensation domain for peptide-bond formation has been demonstrated previously *in vitro* for the condensation between D-phenylalanine (activated and bound by gramicidin synthetase I, GrsA) and proline (activated and bound by a truncated derivative of tyrocidine synthetase II, TycB) [15]. The amino acids, bound on the two separate enzymes, were condensed by the amino-terminal condensation domain of the TycB derivative. Further analysis with this system [14] revealed that the condensation domain appears to discriminate between various foreign amino acids that were introduced into the activation domain of GrsA and TycB via chemically synthesized aminoacyl-coenzyme A thioesters. From the same data, however, one can also speculate that the non-natural substrates, synthetically introduced into the activation domains, are not properly juxtaposed for subsequent condensation by the activation domains themselves. In any case, when designing new NRPSs, possible structural constraints arising from the intrinsic nature of the module or its domains must be taken into account.

Because it was possible to express *acmB* as a functionally active ACMS II in the foreign host *S. lividans* [12] and test this enzyme in conjunction with its cohorts, ACMS I and AcmACP, we decided to use this enzyme as a scaffold to

Figure 6
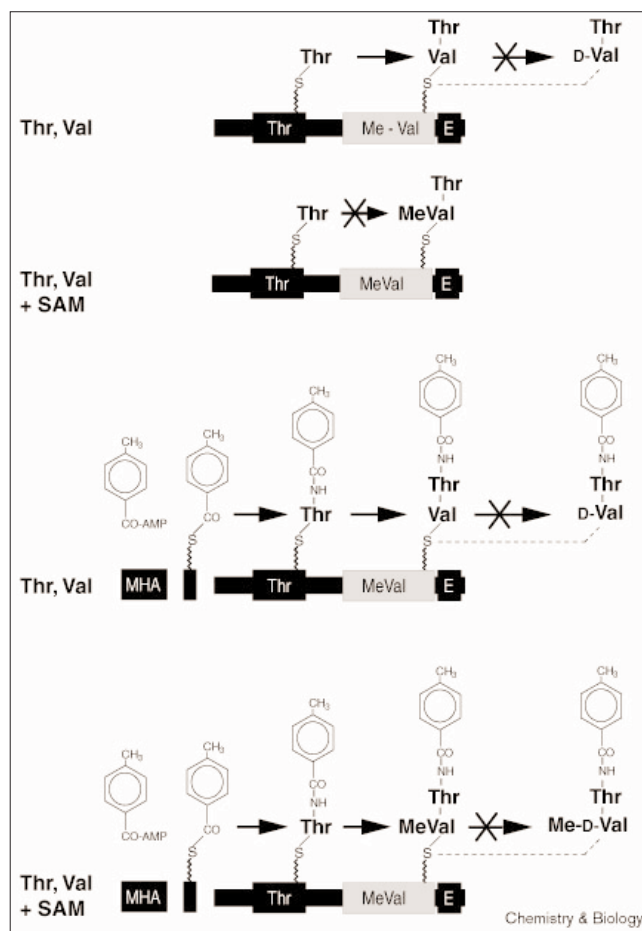
Steps in peptide synthesis catalyzed by AcmTmVe. The ACMS-III-derived activation domain, which activates and *N*-methylates valine (MeVal), is indicated by a grey bar, other symbols and abbreviations are the same as in Figure 1. Noncatalyzed reaction steps (e.g. condensation of threonine with MeVal) are crossed out.

test the functional compatibility of foreign domains. Because the ACMS III sequence is available, it was tempting to introduce an activation domain from ACMS III, which possesses an integrated *N*-methyltransferase domain, into the ACMS II scaffold. It is important to note that, in ACMS III, all of the three modules are processing imino acid residues (proline, Sar or MeVal) during peptide-chain synthesis. This raises the question of whether their condensation and activation domains are destined to interact exclusively with imino acids or if they can be combined freely with domains from modules catalyzing peptide-bond formation between α-amino acids. Another crucial point is that the integrated *N*-methyltransferase domain elongates the activation domain for some 450 amino acids and, hence, separates the thiolation domain from the condensation domain by that additional distance. Although it is anticipated that the *N*-methyltransferase domain is organized in a globular fold, it is not clear, *a priori*, whether

such an enlarged activation domain fits neatly into the ACMS II scaffold and yields a fully active enzyme. Furthermore, the right choice of the condensation domain might be critical, as can be concluded from the results of the D-Phe–Pro condensation system [14,15] described above. In contrast to this condensation system, the two modules in the ACMS II scaffold are on one polypeptide chain and cannot, therefore, achieve all degrees of freedom of their mutual interactions as two independent modules potentially could.

The data presented here show that the valine activation domain in ACMS II can, in principle, be replaced by the MeVal activation domain of ACMS III. The two engineered ACMS II derivatives AcmTmVE and AcmTmV, both containing the MeVal domain from ACMS III in place of the Val domain, were heterologously expressed in *S. lividans* at appreciable levels. The two recombinant enzymes were efficiently modified by host 4′-phosphopantetheine transferases, revealed by their ability to bind threonine and valine covalently as thioesters, and bind to N-methylate, the thioester-bound valine, in the presence of SAM. To test the ability of AcmTmVe and AcmTmV to catalyze peptide-bond formation between threonine and MeVal (or valine), two basic approaches were followed. When the chimeric enzymes were tested in the presence of ACMS I and AcmACP, together with p-toluic acid as starter substrate, AcmTmVe and AcmTmV catalyzed both formation of p-toluyl-Thr and p-toluyl-Thr–Val in the same manner as ACMS II. This result sheds some light on the spatial organisation of the N-methyltransferase domain in module 2 or on activation domains in general. Clearly, formation of p-toluyl-Thr and p-toluyl-Thr–Val catalyzed by AcmTmVe or AcmTmV proceeds as if the N-methyltransferase domain were absent. It is further presumed that the distance of the 4′-phosphopantetheine cofactor in the second module to the condensation domain between modules 1 and 2 is the same as in the natural ACMS II. One has to conclude, therefore, that the N-methyltransferase domain does not affect the spacing between the reactive centers of covalent substrate binding and that this domain might be located peripherally to the site where the condensation reaction between the two amino acids, thioesterified to their carrier arms, takes place.

In contrast to ACMS II, AcmTmVe did not catalyze expected epimerization of p-toluyl-Thr–Val to p-toluyl-Thr–D-Val. It has to be stressed that in the absence of SAM the valine is not N-methylated and that this acyl peptide is accepted as substrate for the ACMS II epimerase domain. The observed failure of epimerization did not result from a mutation in the epimerization domain, which was demonstrated by reconstructing ACMS II in the same way AcmTmVe was constructed and using the same epimerization-domain-encoding DNA

fragment. The epimerization domain was first subcloned and sequenced and the obtained ACMS II-like construct AcmTVe was fully active in peptide-bond formation and epimerization. A further crucial point in domain swapping is in choosing the correct domain borders. The epimerization domain was fused to the MeVal domain within the highly conserved end of the activation domain (the fusion point 20 amino acids carboxy-terminal to the serine in the 4′-phosphopantetheine-binding site). The adjacent variable interdomain region is the same, therefore, as the region that lies upstream of the epimerization domain in wild-type ACMS II. This positioning ensures that this region is as intact as possible in the recombinant enzyme. It is important to note that in the case of the type I PKSs, module swapping experiments have revealed a crucial role for interdomain linkers in the assembly of functional PKS modules [32]. The loss of epimerase activity in AcmTmVe might, therefore, indicate that the N-methyltransferase domain does not fit neatly into the ACMS II scaffold. The inactivation of the epimerase domain could either result from distortion of its structure by an unfavourable contact with the N-methyltransferase domain or by displacement of the epimerase domain from the radius of the 4′-phosphopantetheine arm in module 2, resulting from an altered architecture of the multienzyme. From the known structures of natural, low molecular peptides, only a few contain N-methyl amino acids in the D-configuration. As yet, there are no NRPS sequences available showing the implied NRPS domain arrangement in which an epimerase domain is located carboxy-terminal to an activation domain with N-methylation activity as in AcmTmVe. Interestingly, the NRPS genes of chloroeremomycin [33], which belongs to the vancomycin group of antibiotics, do not contain any integrated N-methyltransferase domains, although vancomycins contain N-methyl-D-leucine at the first position of their peptide backbones. Moreover, from recent work of Pelzer *et al.* [34] it can be concluded that N-methylation occurs after synthesis of the vancomycin peptide backbone, as revealed by the analysis of intermediates formed by mutants impaired in steps of balhimycin production. It remains still unclear, therefore, whether the construct AcmTmVe represents a 'naturally forbidden' domain arrangement or would function, with respect to epimerization, with domains from other NRPS systems or in another scaffold.

The catalytic activity of the N-methyltransferase domain in the constructs AcmTmV and AcmTmVe was demonstrated by their ability to form enzyme-bound MeVal from valine in the presence of SAM. More importantly, in the presence of SAM, the constructs also catalyzed formation of the N-methylated aryl dipeptide p-toluyl-Thr–MeVal as predicted. According to the above mentioned putative dislocation of the epimerization domain, caused by the additional N-methyltransferase domain, this N-methylated aryl dipeptide was not epimerized.

In the second approach to test their catalyzing ability, AcmTmVe and AcmTmV were analyzed in the absence of ACMS I and AcmACP. Wild-type ACMS II was found to have a relaxed specificity in the initiation reaction of peptide lactone-ring formation _in vitro_. ACMS II is unusual in that in the absence of ACMS I, AcmACP or aromatic carboxylic acid substrate, it can also condense threonine with valine, albeit with less efficiency. The formed dipeptide is further epimerized _in vitro_ to its diastereomer Thr–D-Val. Testing AcmTmVe or AcmTmV in that way showed that both enzymes catalyzed the formation of Thr–Val from threonine and valine. The finding that AcmTmVE was impaired in dipeptide epimerization is consistent with its inability to epimerize the aryl dipeptide as described above. More surprising then was that the additional presence of SAM had an adverse effect on peptide-bond formation. Instead of the expected formation of Thr–MeVal, nothing happened except that the thioester-bound valine in module 2 became _N_-methylated.

Taken together these findings clearly show that the new enzymes can condense valine with both threonine and p-toluyl-threonine, but that the enzymes strictly distinguish between threonine and p-toluyl-threonine when valine is _N_-methylated. The reasons for this substrate discrimination are not clear. It is known that hydrolysis or aminolysis of carboxylic acid esters is greatly favored when there are electronegative substituents at α C due to a minus I effect. From this, one would expect that threonyl–enzyme thioester would be much more prone to aminolysis than the p-toluyl-threonine–enzyme thioester. The opposite was observed, however, as MeVal reacted exclusively with p-toluyl-threonine (and not with threonine). Measurements of dipeptide and p-toluyl-dipeptide synthesis with wild-type ACMS II [22,23] have shown that condensation between threonine and valine is more efficient when threonine is first acylated with p-toluic acid. The acylation of threonine probably triggers or enables the optimal juxtaposition of the thioester-bound p-toluyl-threonine in the active center of the condensation domain. The relaxed specificity of ACMS II might be explained by assuming that the nonacylated threonine can also be located somehow beside or near this active center although not optimally juxtaposed. This could be sufficient for condensation with an α-amino acid residing on module 2, but not for condensation with a _N_-methylated amino acid, which is a sterically more complex reaction. The sterical distortion, caused by the methyl group, is thought to be the reason why in chemical peptide synthesis _N_-methylated amino acids are less reactive in peptide-bond formation than the related α-amino acids.

The findings presented here indicate that it is possible, in principle, to introduce _N_-methyl amino acids into nonribosomally synthesized peptides by swapping activation domains with integrated _N_-methyltransferase domains in

NRPS modules. The interference of the _N_-methyltransferase domain with other domains, such as epimerization domains, has to be taken into account. Furthermore, the substrate specificity in the peptide-bond formation appears to be dictated by steric constraints in the upstream module, rather than by the nature of the _N_-methyl amino group itself.

Significance

Modular nonribosomal peptide synthetases (NRPSs) can be engineered by exchanging or recombining modules to achieve synthesis of new or altered peptides. As presented here, substitution of the valine activation domain in the bimodular actinomycin synthetase II by an _N_-methyl valine activation domain (which contains an additional _N_-methylation activity) resulted in a NRPS that catalyzes the synthesis of both the unmethylated and _N_-methylated acyl dipeptides. Peptide-bond formation in this case is catalyzed by a condensation domain, derived from the parent NRPS, that does not distinguish between the _N_-methylated and nonmethylated forms of valine. In contrast, condensation of the _N_-methylated valine was dependent on the substrate amino acid of the preceding module, threonine or acylthreonine. Both threonine and acyl-threonine will be condensed with valine, whereas _N_-methyl valine is only condensed with acyl-threonine. One reason for this might be that only the acylated threonine, which is the natural intermediate, is positioned correctly for the nucleophilic attack of _N_-methyl-valine. This implies that the substrate positioning by the activation domain might be important for peptide-bond formation, rather than an assumed specificity of condensation domains. The epimerase domain in this new NRPS was inactive, indicating an incompatibility between an activation domain harbouring _N_-methylation activity with an adjacent epimerase domain in this NRPS scaffold. In summary, these results show that synthesis of _N_-methylated peptides can be achieved by swapping activation domains within NRPS modules.

Materials and methods

Strains and growth of organisms
Streptomyces lividans TK 64 was maintained at 30°C on R5 plates [35]. Submerged growth took place for 3 days in 100 ml of YEME liquid medium [35] in 300 ml flasks equipped with steel springs and shaken at 200 rpm. _S. lividans_ was transformed as described by Hopwood _et al._ [35] and transformants were grown in the presence of 5 µg ml⁻¹ thiostrepton.

Cloning and expression of engineered NRPS genes
For swapping the ACMS II valine activation domain, a single _Cla_I site in _acmB_ at bp position (pos) 4519 and a PCR-generated _Eco_RV site at pos 6250 were used (Figure 2). The _Eco_RV site was generated with primer M1, and reverse primer M2 binds downstream to an _acmB_ internal _Sst_I site (pos 7780) near the end of the gene. The PCR product was digested with _Eco_RV and _Sst_I and the obtained 1.5 kb fragment was subcloned for control sequencing. The end of _acmB_ and some _acmB_ downstream region was added by ligating a 1.1 kb genomic _Sst_I–_Bam_HI fragment to the _Sst_I site of the PCR fragment.

The obtained 2.6 kb EcoRV−BamHI fragment therefore contains the complete 3′ region of acmB, starting from the EcoRV site at pos 6250. The 5′ region of acmB, from the start of the gene up to the ClaI site at pos 4519, was obtained as a 4.6 kb PstI−ClaI fragment from pACM5 (see below). The two 5′ and 3′ regions were subsequently linked via a 1.8 kb ClaI−EcoRV fragment, encoding the Val domain of ACMS II and amplified from acmB (pos 4519−6250) with primer M3 and M4. This yielded the gene coding for AcmTVe. Similarly, joining the 5′ and 3′ regions via a 2.9 kb ClaI−EcoRV fragment, encoding the MeVal-domain of ACMS III and amplified from acmC (pos 8911−11872) with primer M5 and M6, yielded the gene encoding AcmTmVe. To delete the epimerase domain of AcmTmVe (generating AcmTmV), a new SstI site (marked by an asterisk in Figure 2) was generated using PCR with primers M7 and M8, 48 bp downstream of the EcoRV site and with the AcmTmVe gene as template. The obtained PCR fragment spans the EcoRV site, which was used to assemble the AcmTmV gene as described above, and the new SstI site was directly fused with the natural SstI site in acmB (bp 7780) next to the end of the gene. This in-frame fusion deletes the epimerase domain and retains the last 18 amino acids of the carboxyl terminus unmodified. All three recombinant NRPS genes were heterologously expressed in Streptomyces lividans from the melanin (mel) promotor of plasmid pIJ702 [36] as described for the expression of acmB in the pIJ702 derivative pACM5 [12]. The start of acmB was modified to enable fusion to the mel promotor by partial replacement of the pIJ702 melanin gene melC1. As the 5′ regions of all recombinant NRPS genes were derived from pACM5, the same fusion point to the mel promotor was achieved.

Amino acid sequences at domain borders

The amino acid sequences at the ClaI and EcoRV fusion sites (indicated by slashes) or at the corresponding positions in wild-type enzymes are: (1503)PLSRI/DVLTP and (2080)FSVRD/VFEQR for wild-type ACMS II; (2967)PLSRI/DVLTP and (3954)LGLRS/LFEAP for wild-type ACMS III; PLSRI/DVLTP and FSVRD/IFEQR for AcmTVe; PLSRI/DVLTP and LGLRD/IFEQR for AcmTmVe and AcmTmV. The fusion sequence in AcmTmV, after deletion of the ACMS II epimerase domain (deletion of aa 2101−2594 in ACMS II) is VVAEE/LSQHD.

Protein purification and unit definition

Recombinant NRPSs were partially purified from 10 g of mycelium (wet weight) of S. lividans transformants as previously described for ACMS II [12]; basic steps were cell disruption by French press, DNase I treatment, 60% ammonium sulfate precipitation and size fractionation on Ultrogel-AcA-34 (Biosepra). In this work, Ultrogel-AcA-34 fractions with proteins larger than 200 kDa were pooled and applied to a Q-Sepharose-FF (Pharmacia) anion exchanger column. Bound proteins were eluted with a step gradient of 150, 250 and 500 mM NaCl in 15% (w/v) glycerol, 100 mM Tris−HCl (pH 8), 4 mM dithiothreitol, 1 mM benzamidine. All recombinant NRPS eluted with 250 mM NaCl and were used at this purification stage for enzymatic studies. One unit of NRPS is the amount of enzyme that covalently binds 1 nmol of threonine as determined by the thioester formation assay [12]. ACMS I and AcmACP were isolated after heterologous expression in S. lividans and E. coli, respectively, as described previously [13].

Isolation of reaction intermediates from NRPS

For peptide synthesis, about 0.05 units of NRPS in 0.5 ml Q-Sepharose elution buffer (see above) were mixed with 130 µl reaction mixture containing 10 µmol ATP, 15 µmol MgCl$_2$, 2 µmol threonine, 2 µmol valine and 2 µmol S-adenosyl-methionine (SAM). For aryl-peptide synthesis, the reaction mixture further contained 2 µmol p-toluic acid, 0.8 µmol ACMS I and 1.6 µmol AcmACP. For ^{14}C-labelling, 1.5 µCi of amino acids, 0.5 µCi of SAM or 3 µCi of p-toluic acid were used. After incubation for 30 min at 30°C, the reaction was stopped with 1.5 ml of 10% trichloracetic acid (TCA) and proteins were precipitated for 30 min on ice. Proteins were washed first with 2 ml TCA (5%), then with 2 ml EtOH and then air dried. Peptides were released from the enzyme by thioester cleavage with 0.4 ml performic acid for 4 h at 20°C, vacuum dried, resuspended in formic acid and directly analyzed

by TLC. Aryl peptides were released from the enzyme with 0.3 ml NaOH (0.5 M) for 5 min at 20°C. After neutralizing with HCl (0.5 M), they were extracted twice with 2 ml ethylacetate. In order to detect spontaneously released aryl-intermediates during enzymatic synthesis, control reactions were extracted with ethyl acetate omitting TCA precipitation and thioester cleavage. Extracts were vacuum dried and resuspended in a small volume of ethylacetate for TLC analysis.

Time course of acyl-peptide formation

^{14}C-valine labelled reaction intermediates were isolated from reaction mixtures as described above after 2, 3, 5, 7, 10, 15, 30 and 45 min of incubation. Labelled compounds were separated by TLC (as shown for t = 30 min in Figure 3) and quantified with a TLC-linear analyser Tracemaster 20 (Berthold).

Thin-layer chromatography and HPLC analysis

Peptides and amino acids were analyzed on silica 60 F$_{254}$ TLC plates (Merck) with the solvent system n-propanol/acetic acid/H$_2$O (4:1:1 [vol/vol/vol]); aryl-peptides with the solvent system ethylacetate/n-Hexan/acetic acid (5:5:1 [vol/vol/vol]). Labelled compounds were detected by autoradiography, nonlabelled standards with ninhydrin (amino acids and peptides), UV detection (aryl peptides) or chlorine/o-tolidin (diketopiperazines). For further HPLC analysis and peptide hydrolysis, labelled compounds were scraped from silica plates and extracted with 50% EtOH (amino acids and peptides) or MeOH (aryl-peptides). Configuration of amino acids, obtained from peptide hydrolysis with HCl (8 M) for 20 h at 110°C, was determined on TLC chiral plates (Macherey-Nagel) with solvent system MeOH/acetonitrile/H$_2$O (1:4:1 and 5:3:5 [vol/vol/vol]). For HPLC analysis, about 200−500 cpm of a labelled compound (not UV-detectable) was mixed with synthetic standards (UV absorbance of 0.3−0.5 at 205 nm for peptides and 260 nm for aryl-peptides) and applied on a reversed-phase column (SuperPac Pep-S, Pharmacia). Chromatography was performed at a flow rate of 0.5 ml/min with solvent A (0.1% trifluoric acid) and solvent B (acetonitrile with 0.1% trifluoric acid) with the following gradient profiles: 5 min, 0% B; 40 min, 20% B; 45 min, 100% B for peptides and 5 min, 0% B; 65 min 55% B; 70 min, 100% B for aryl-peptides. Fractions of 0.5 ml were collected and labelled compounds were identified by liquid scintillation counting.

Radioisotopes and chemicals

S-adenosyl-L-[methyl-^{14}C] methionine (57 Ci/mol, 25 µCi/ml) and p-toluic acid [carboxy-^{14}C] (8.3 Ci/mol, 83 µCi/ml) were from Amersham and Sigma, respectively; L-threonine [U-^{14}C] (224 Ci/mol, 250 µCi/ml) and L-valine [U-^{14}C] (227 Ci/mol, 250 µCi/ml) were from ICN. Authentic dipeptides used as standards were either from Bachem (Thr−Val) or were synthesized (Thr−D-Val) as described previously [23]. Boc-Thr(OBzl)MeValOMe served as the starting material for the synthesis of the reference peptides Thr−MeVal, cyclo(Thr−MeVal) and p-toluyl-Thr−MeVal by standard procedures. It was obtained by coupling of Boc-Thr(OBzl)-OH with MeValOMe in the presence of PyBrOP (Novabiochem). The identities of all compounds were verified by amino acid analysis and mass spectrometry.

PCR oligonucleotides

PCR primers were: M1: 5′-TCTCCGTCCGGGATATCTTCGAGCAG-CGCACg-3′; M2: 5′-GCAGGATGAATTCGCATGCCGACGACGTC-ATTTCTGAAT-3′; M3: 5′-CAACCCGAGGATCCGCTCAGCCGTAT-CGAT-3′; M4: 5′-TGCGGAATTCGAAGATATCCCGGACGGAGAA-ACCGAT-3′; M5: 5′-CTCAGCCGCATCGATGTCCTCA-3′; M6: 5′-CGCCTCGAAGATATCGCGCAGGCCCA-3′; M7: 5′-AACTCCG-TGGTCCAGGAATTCCCGAA-3′; M8: 5′-ACGCGGTGAGCTCCTC-GGCGACCA-3′; As described above, some primer combinations resulted in PCR products from which only internal subfragments were used. Additional restriction sites were used for subcloning.

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