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A Nonribosomal Peptide Synthetase Involved in the Biosynthesis of Ampullosporins in *Sepedonium ampullosporum*‡

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Abstract: Recently, the saprophytic ascomycete *Sepedonium ampullosporum* strain HKI-0053 was isolated from a basidiomycete on account of its premature induction of pigment formation in *Phoma destructiva*, a process often related to the neuroleptic activity of the inducing compound. The active substance was identified as the 15-membered peptaibol type peptide Ampullosporin. Although to date more than 300 peptaibols have been discovered, their biosynthetic machinery has not been characterized yet. By improving the culture conditions it was possible to grow *S. ampullosporum* in a submerged culture and to increase Ampullosporin production by more than three times to 33 mg/l at reduced fermentation times. The appearance of two high molecular weight proteins, HMWP1 (1.5 MDa) and HMWP2 (350 kDa) was closely related to the production of Ampullosporin during the course of fermentation. Both proteins showed a cross-reaction with antibodies against a core fragment of nonribosomal peptide synthetases (NRPSs). Biochemical characterization of the partially purified enzymes exhibited selectivity for the substrate amino acid α-aminoisobutyric acid (Aib), substantiating their involvement in Ampullosporin biosynthesis. Our data suggest that Ampullosporin synthetase has been isolated, and provides the basis for the characterization of the entire biosynthetic gene cluster. Furthermore, this knowledge will enable the manipulation of its NRPS template, in order to engineer mutant strains of *Sepedonium ampullosporum* which could produce more potent analogues of Ampullosporin.

Keywords: *Sepedonium ampullosporum*; nonribosomal peptide synthetase; Ampullosporin biosynthesis; peptaibol; neuroleptic activity

INTRODUCTION

Peptaibols are linear peptides of fungal origin, composed of 5–20 amino acid residues [1–3]. Their main characteristics are an acylated *N*-terminus, a high proportion of α-aminoisobutyric acid (Aib), and a *C*-terminal α-aminoalcohol group. Apart from Aib, other non-proteinogenic amino acids found in peptaibols include isovaline (Iva), hydroxyproline (Hyp) and ethylnorvaline (Etnor). Aib has a high tendency to form helices and this accounts for the helical structures of the peptaibols [4,5]. They usually occur as complex mixtures of homologues and derivatives whereby one major compound largely outweighs the other minor compounds. Up to now more than 300 peptaibols have been described and the crystal structures of seven molecules have been determined [2, 6–8]. Peptaibol type peptides

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DTE, dithioerythritol; FPLC, fast protein liquid chromatography; NBT, nitro blue tetrazolium; TFA, trifluoroacetic acid.

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exhibit antimicrobial activity most likely due to their ability to form pores in lipid bilayer membranes [9–11]. Based on their amino acid sequence, length and physical properties the peptaibols have been divided into nine subfamilies [2]. The main sources of the peptaibols known to date are soil fungi, ascomycetes of the genera *Trichoderma* and *Emericellopsis* [12,13]. In recent years a number of peptaibols have been isolated from another related genus, *Sepedonium*, a mycoparasite with a narrow host spectrum, basidiomycetes of the genus *Boletales* [1,14–16].

The presence of non-proteinogenic amino acids immediately suggests a nonribosomal biosynthesis of peptaibols following the thiotemplate mechanism [17]. According to this model, the number and organization of modules reflects the size and complexity of the synthesized peptide [18–20]. A typical module consists minimally of an adenylation domain, responsible for the activation of its cognate substrate amino acid as an aminoacyl adenylate, a peptidyl carrier protein (PCP) or thiolation domain, located downstream of the adenylation domain. PCPs carry a phosphopantetheinyl moiety attached to a conserved serine residue which subsequently binds the amino acid as a thioester [21]. A condensation domain, located upstream of the adenylation domain catalyses the peptide bond formation and directed translocation of the growing peptide. The order of modules of a nonribosomal peptide synthetase (NRPS) is collinear with the order of residues in its product. Additional domains have been found that catalyse the modification of the incorporated substrate amino acid, such as oxidation, methylation or epimerization [22–25]. If present these domains are located in the module responsible for the incorporation of the respective amino acid residue. Finally, the chain is usually released from the synthetase by action of an integrated thioesterase located at the *C*-terminus of the synthetase [26,27].

There is as yet no 'rule' as to how many modules can be housed by one protein. While in bacteria the NRPS machinery has been found to consist of either one or more proteins harbouring any number of modules from 1 to 6, all fungal NRPSs sequenced so far contain all the modules on only one polypeptide chain, regardless of their number [19,24]. Peptaibol synthetases could therefore be expected to consist of a single polypeptide chain. Considering that a minimal NRPS module has a M_r of approx. 120 kDa NRPSs catalysing the formation of long chain peptaibols with more than 16 residues can be expected to exceed molecular weights of 2 MDa. Indeed, recently Wiest *et al.* cloned and sequenced the first gene encoding a peptaibol synthetase, Tex1, from *Trichoderma harzianum* [28]. With a size of 62.8 kb the *tex1* gene encodes a deduced protein of more than 2.3 MDa and represents the largest NRPS gene known to date. It comprises 18 NRPS modules, a *C*-terminal reductase domain and an *N*-terminal polyketide synthase (PKS) starter module. Like NRPSs the integrated PKSs are arranged in a multimodular assembly line fashion [21]. They catalyse the sequential addition of small carboxylic acids. In analogy to NRPSs a minimal PKS module is composed of a ketosynthase (KS), an acyltransferase (AT) and an acyl carrier protein (ACP) domain [29]. Additional domains catalysing the reduction or methylation of the incorporated substrate acid can be present. Thus the deduced protein architecture of this mixed integrated polyketide/nonribosomal peptide synthetase immediately explains the origin of the *N*-terminal acyl-group and the *C*-terminal hydroxyl group of the peptaibol product. Interestingly, while modular polyketide synthases exist as parallel homodimers in solution it has been shown for all but one of the NRPSs investigated so far that they act as monomers [30]. Only VibF, an NRPS of unusual architecture that is involved in the biosynthesis of the iron chelator vibriobactin in *Vibrio cholerae*, seems to act as a dimer [31]. This raises the question as to how mixed PKS/NRPS systems such as peptaibol synthetases are organized. Knowledge of genetic information alone cannot answer this question. Rindfleisch and Kleinkauf reported the *in vitro* biosynthesis of the 20-membered peptaibol alamethicin by partially purified cell extracts of *Trichoderma viride* [32]. However, although the authors have performed a gel filtration experiment they do not show any SDS/PAGE gels nor do they provide information regarding the size of the active protein(s).

In 1996 the 15-membered peptaibol Ampullosporin A (Figure 1) was isolated from the mycelium of *Sepedonium ampullosporum* HKI-0053 on account of its premature induction of pigment formation in *Phoma destructiva*, a feature often associated with neuroleptic activity in vertebrates [1,33]. This compound displays moderate antibacterial and antifungal activity and in addition shows neuroleptic activity in mice. The mode of action for the latter feature shared by some, but not all other, peptaibols has not been identified to date. Nguyen *et al.*

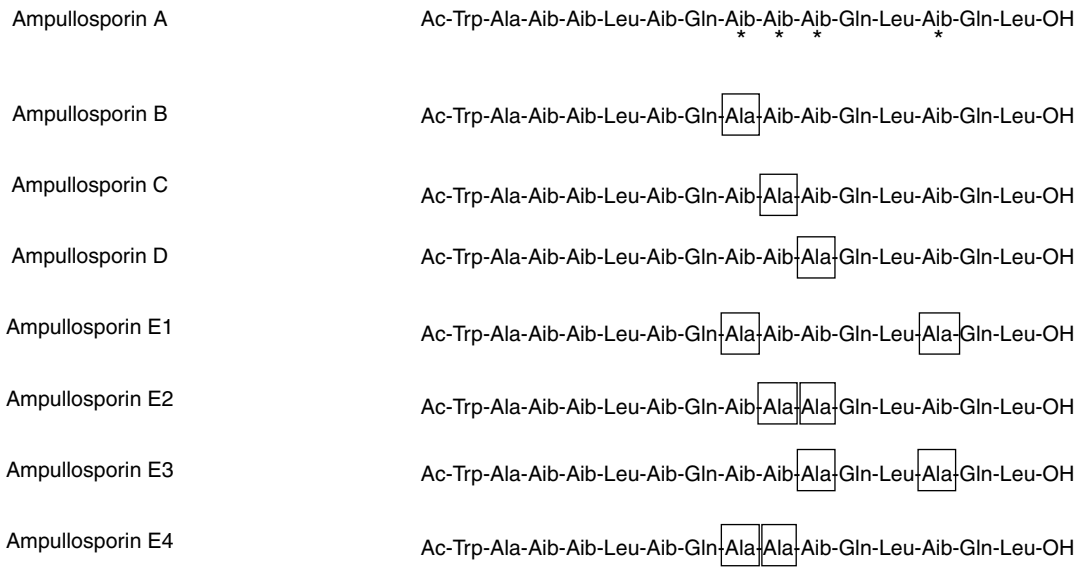

Figure 1 Ampullosporin A and derivatives. Positions in which an Aib residue in Ampullosporin A has been replaced with an Ala residue in the derivatives Ampullosporins B to E4 are labelled with an asterisk in the Ampullosporin A sequence and boxed in the respective derivatives.

[34] have shown that modifications at several positions of the molecule alter its antimicrobial as well as its neuroleptic effects. Hence Ampullosporin A may serve as a good lead substance for the development of potent psychotic drugs.

Our long-term interest is to manipulate the biosynthetic machinery for Ampullosporin in order to produce derivatives of the substance in high amounts by fermentation. Here we report on the partial purification and characterization of two high molecular weight proteins from *S. ampullosporum* strain HKI-0053 and present circumstantial evidence for their involvement in the biosynthesis of Ampullosporin. Furthermore, we suggest a model for the Ampullosporin synthetase based on our findings.

EXPERIMENTAL PROCEDURES

Strain, Growth Media and Culture Conditions

Sepedonium ampullosporum strain HKI-0053 was obtained from the culture collection of the Hans-Knöll-Institute of Natural Product Research, Jena (Germany). The strain was kept as agar-glycerol preserves (20% v/v glycerol) at −80 °C. Only the differentiating yellow to orange coloured strain represents the Ampullosporin producing clone. It was separated on agar-plates composed of 10 g/l glucose, 30 g/l glycerol, 5 g/l casein-peptone, 2 g/l NaCl, 1,5 g/l agar, pH 7.3, grown at 24 °C for 7–14 days. A slice of about 2 cm^2 of the agar-plate cultures was crushed and used to inoculate 100 ml of a liquid medium, composed of 8 g/l glucose, 3 g/l maltose, 4 g/l casein-peptone, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\ H_2O$, 0.008 g/l $ZnSO_4 \cdot 7\ H_2O$. After approx. 48 h 5–10 ml of the pre-culture was transferred into a main culture medium composed of 5 g/l maltose, 2 g/l casein-peptone, 2 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7\ H_2O$, 0.008 g/l $ZnSO_4 \cdot 7\ H_2O$. Fermentation was carried out at 90 rpm and 24 °C in 500 ml shake-flasks, containing 100 ml liquid medium.

Detection of Peptaibols

HPLC. Ampullosporin was detected quantitatively by HPLC as described previously [1,34,35] after extraction of culture broth with 1 volume of ethyl acetate. At least three shake flasks (300 ml) were combined and extracted.

Mass spectrometry. Matrix assisted laser desorption-time of flight (MALDI-TOF MS) mass spectra of Ampullosporin were obtained using a VOYAGER DE-PRO time of flight mass spectrometer from PerSeptive BioSystems, Framingham, MA, USA.

A few μg of fungal mycelium from an agar plate were transferred to the MALDI-TOF MS sample

plate. 1 µl of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.3% TFA) was added and mixed with each sample. After airdrying for 5 min, the samples were used in MALDI-TOF MS analysis. Desorption of analyte molecules was achieved using a pulsed nitrogen laser at 337 nm. Ions were accelerated with a voltage of 20 kV to perform a time of flight separation. Measurements were performed in the delayed extraction mode, allowing the determination of monoisotopic mass values. A low mass gate of 800 Da improved the measurement by filtering out the most intensive matrix ions. The mass spectrometer was used in the positive ion detection mode. In order to enhance the spectral resolution, all spectra were run in the reflector mode. Post source decay (PSD) analysis takes advantage of the metastable decay of the precursor ions occurring in the field-free region of the flight tube and mass analysis of the fragment ions using reflector mode to generate complete sequence information from linear peptides, and such fragmentation can be enhanced with collision induced dissociation (CID).

PSD measurements were performed with the same probes on the template as used for peptide mass determination. Using a timed ion selector, different precursor ions were selected and subjected to fragmentation. Spectra were obtained by accumulating data from 100 to 200 shots. To obtain complete PSD spectra, a series of reflector TOF spectral segments was acquired, each optimized to focus fragment ions within different m/z ranges. Segments were stitched together using Biospectrometry Workstation software to generate a composite PSD spectrum. For CID experiments, the collision cell was filled with air until the pressure in the source chamber reached $\sim 6 \times 10^{-6}$ Torr.

Protein purification. Cells were harvested by centrifugation and washed twice in 100 mM Tris, pH 7.5, 150 mM KCl. The washed mycelia was lyophilized and stored at −20° to −40 °C. Cell disruption, protein extraction and fractionation were carried out as follows: the dried cells were suspended in 100 mM Tris, pH 7.5, 50 mM NaCl, 30 mM DTE, 20 mM EDTA and 30% (w/v) glycerol, containing 10 mg/ml DNase, 10 mg/ml RNase and a spatula tip of PMSF. Cell disruption was performed either by two passages through a french press at 600–700 bar or by high pressure homogenization in a Gaulin-homogenizer for 5–10 min at 700–900 bar. Protein purification steps were performed at 4 °C. Cell debris was removed by centrifugation at 40 000 × **g** for 30 min. The clear supernatant was diluted with 1 volume 100 mM Tris, pH 7.5, 50 mM NaCl, 30 mM DTE, 20 mM EDTA to dilute the glycerol. Protein was precipitated by stepwise addition of solid ammonium sulphate to 60% saturation. The following column purification steps were performed using an FPLC apparatus (Pharmacia, Uppsala, Sweden). The protein pellet was resuspended in a small volume of buffer A (50 mM Tris, pH 7.5, 200 mM NaCl, 3 mM DTE, 1 mM EDTA and 10% (w/v) glycerol) and subjected to gel permeation chromatography on a Superdex 200 column equilibrated with the same buffer. Fractions containing the the two HMWPs were combined and mixed with 1 vol. of buffer A containing 1 M ammonium sulphate. The solution was centrifuged at 30 000 × **g** for 10 min to remove particulate matter and applied to a phenyl-sepharose previously equilibrated with buffer B (buffer A containing 500 mM ammonium sulphate). The column washed with 1 vol. of buffer B, followed by 1 vol. of buffer A. Subsequently 2 vol of a gradient from 0 to 40% ethylene glycol in buffer A was applied. Both proteins eluted in the same fractions at approx. 35% ethylene glycol.

SDS/PAGE and immunoblotting. Proteins were separated on 5% SDS/PAGE and blotted onto Hybond C extra membrane (Amersham Lifescience) following standard techniques. Protein blotting was carried out as described by Pfeifer *et al.* [36]. Protein gels were stained with Coomassie brilliant blue R-250. Polyclonal antibodies against the decapeptide SGTTGRPKGV, a highly conserved core sequence of known NRPSs, were generated as described [39]. Polyclonal antibodies against the putative Ampullosporin synthetase were raised in a rabbit. 460 µg protein from a gel filtration containing both HMWPs in 3 ml buffer A were mixed with 1 ml adjuvants (MPL + TDM + CWS Emulsion, Sigma) and administered in a divided dose by intradermal, hypodermic, intramuscular and intraperitoneal injection. Secondary injections (300 µg protein in 0.1 ml adjuvants) were given subcutaneously 3 weeks after the first injections. Blood was taken 3 weeks after the booster inoculation. 40 ml serum were applied onto a protein A column. Following a washing step with 0.5 M NaCl the IgG was eluted with 25 ml acetic acid (1 M, pH 3.0), precipitated with 85% ammonium sulphate and redissolved in deionized water. The pH was adjusted to 5.0. The resulting solution was used at 1 : 2000 dilution in western blotting. Antigen–antibody complexes were detected using

goat anti-rabbit IgG conjugated to alkaline phosphatase and visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) as substrate.

Enzyme Assays

Adenylate formation. ATP/PP_i exchange reactions were carried out essentially as described [37]. All incubations contained 50 µl enzyme solution and 50 µl reaction mix composed of 2 mM ATP, 1 mM substrate amino acid, 30 mM magnesium acetate, 1 mM DTE, 0.1 mM EDTA, 0.1 mM PP_i and 0.05 µCi [^{32}P]PP_i. The reaction mixtures were incubated at 25 °C for 60 min. Reactions were stopped by addition of 1% activated charcoal in 3% perchloric acid, and the [^{32}P]ATP content was determined.

In vitro biosynthesis. 100 µl of a gel filtration fraction were incubated for 60 min at 25 °C with 0.5 mM Trp, 0.5 mM Ala, 1.5 mM Leu, 1.5 mM Gln, and 3.5 mM Aib, 5 mM ATP, 25 mM $MgCl_2$, 1 mM DTE and 0.1 mM EDTA in a total volume of 200 µl. Ampullosporin was extracted from the incubation mixture with 2 × 1 vol. ethylacetate. The ethylacetate was dried and the Ampullosporin was taken up in 50% MeOH and subjected to MALDI-TOF MS analysis.

RESULTS AND DISCUSSION

Since our initial attempts to detect nonribosomal peptide synthetases in *S. ampullosporum* and in a number of peptaibol producers of the genus *Trichoderma* were not successful, it was decided to increase the peptaibol production rates of these wild type strains. While addition of fungal cell wall preparations to the growth medium did increase production of peptaibols in *Trichoderma* strains as reported earlier by Geremia *et al.* [38], it was not possible to identify high molecular weight proteins in crude cell extracts, either by SDS/PAGE or by immunoblot using antibodies raised against a core motif of known NRPSs [39] (data not shown). Therefore attention was focused on *S. ampullosporum*. This ascomycete initially only produced peptaibols in emers fermentation conditions on highly viscous complex media. Fermentation times of 1 to 2 weeks typically yielded maximally 8–10 mg/l Ampullosporin. In order to increase the production rate as well as to reduce fermentation times a protocol was developed for submerged fermentation. Using variations of the HK-0* medium with a number of different carbon and nitrogen sources as well as different aeration rates and shearing forces, growth conditions were established that typically lead to the production of up to 33 mg/l Ampullosporin after 5 to 6 days. For optimized ampullosporin production the cells were grown at 90 rpm in 500 ml non-buffered shake flasks, containing 100 ml HK0* medium supplemented with 2 g/l peptone and 5 g/l maltose, pH 7.5.

At several time points during fermentation samples were taken and subjected to HPLC analysis in order to follow the production of Ampullosporin. Crude protein extracts of the cells harvested at the respective time points were separated on 5% SDS/PAGE. As shown in Figure 2 the occurrence of two high molecular weight protein (HMWP) bands correlated with the production of Ampullosporin during fermentation. While it is usually difficult to judge the size of very large proteins in gel electrophoresis due to the lack of appropriate marker proteins a relatively precise judgement was made

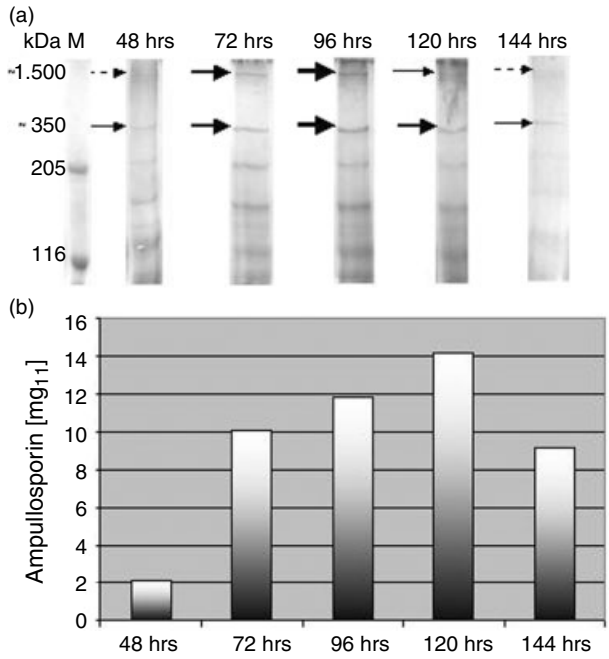

Figure 2 Correlation of Ampullosporin formation in *S. ampullosporum* with the occurrence of two high molecular weight proteins, HMWP1 (approx. 1.5 MDa) and HMWP2 (approx. 350 kDa). Mycelium from 1 l culture medium was collected at the times indicated. Cells were broken as described in Experimental Procedures. About 40 µg of protein per time point was subjected to 5% SDS/PAGE. M: high molecular weight markers (Sigma). The marker proteins cyclosporin synthetase (1.68 MDa) and enniatin synthetase (350 kDa) are not shown in this figure.

in this case as both proteins migrated very close to two marker proteins. While the upper of the two *S. ampullosporum* protein bands, HMWP1, migrated a little further into the gel than cyclosporin synthetase (1.68 MDa) [40] indicating a M_r of about 1.5 MDa, the lower one, HMWP2, almost exactly co-migrated with enniatin synthetase (346 kDa) [23] indicating a similar mass.

As these two proteins were equal in intensity and time of appearance during fermentation, it was plausible to assume that both might be involved in the biosynthesis of Ampullosporin. This was a somewhat surprising finding as a putative Ampullosporin synthetase housed on only one polypeptide chain was expected. It was decided to purify these two proteins from *S. ampullosporum* to further investigate their possible role in the biosynthesis of Ampullosporin.

Mycelium was harvested after 48 h, washed and broken by two passages through a french press as described in Experimental Procedures. The crude protein extract was precipitated by addition of solid ammonium sulfate to a concentration of 60% solubility. Precipitated proteins were subjected to gel filtration chromatography on Sephadex S 200 material. As shown in Figure 3 both high molecular weight proteins eluted in the same fractions, suggesting either a similar size or a tight association of the native proteins.

Fractions of the gel filtration containing the HMWPs were further purified by hydrophobic interaction chromatography on phenylsepharose material. Interestingly, this step also did not result in a separation of the two large proteins, nor did it remove a protein of about 90 kDa, indicating their tight association under the conditions employed (Figure 4). For further experiments protein was used from the gel filtration step. Fractions containing the target proteins were subjected to SDS/PAGE, transferred onto an Immobilon PVDF membrane, and probed using polyclonal antibodies raised against the decapeptide SGTTGRPKGV, a highly conserved core sequence of known NRPSs [39]. Both HMWPs showed a specific cross-reaction to these antibodies (Figure 5). Likewise polyclonal antibodies raised against the two partially purified HMWPs showed a cross reaction with several NRPSs, cyclosporin synthetase from *Tolypocladium niveum*, enniatin synthetase from *Fusarium scirpi*, and siderophore synthetase (Sid1) from *Ustilago maydis* (data not shown). In order to verify the suspected involvement of both HMWPs in peptaibol biosynthesis it was decided to perform several bioassays. To check for *in vitro* biosynthesis, the partially purified enzymes were incubated for several time periods with the five constituent amino acids of Ampullosporin and ATP as the energy source. In addition NADPH was added to serve as a cofactor for the suspected reductase domain that is thought to reside at the *C*-terminus of the Ampullosporin synthetase and to catalyse the release of the newly formed peptaibol from the enzyme. After incubation at 24 °C samples were extracted with ethylacetate as described in Experimental Procedures and subjected to MALDI-TOF MS analysis. This technique enables a direct determination of peptaibols in a few microlitres of extract without prior treatment of the sample. To our surprise even a sample taken at time point t_0 of the incubation already contained a substance with a mass of 1622 Da, corresponding to protonated Ampullosporin A. A PSD analysis clearly established that this substance was indeed Ampullosporin A (Figures 6, and Table 1). No Ampullosporin was detected in fractions of the gel filtration that did not contain the HMWPs. Apparently the partially purified proteins still had intracellularly produced Ampullosporin tightly bound in a non-covalent manner, most likely because of the high hydrophobicity of the peptaibol. Since MALDI-TOF MS results are not quantitative, it was not possible to determine whether incubation *in vitro* resulted in newly synthesized Ampullosporin. To circumvent this problem it will be necessary to use either a radioactively labelled precursor or to replace substrate amino acids with chemically related analogues of different

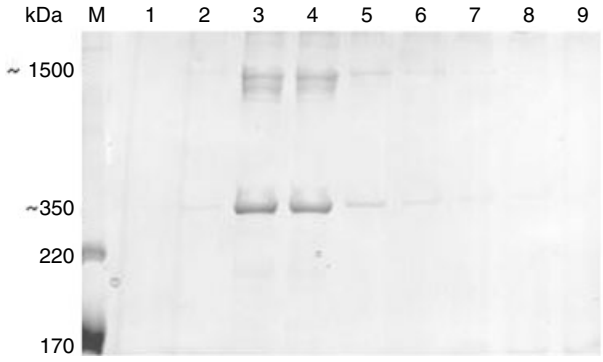

Figure 3 5% SDS/PAGE gel of fractions of a gel filtration of *S. ampullosporum* proteins. A crude protein extract of *S. ampullosporum* cells harvested after 96 h of fermentation was precipitated with a final concentration of 60% ammonium sulphate as described in Experimental Procedures. The redissolved pellet was subjected to gel filtration chromatography on Superdex S 200 material. Lanes 1–9 correspond to fractions 9–17.

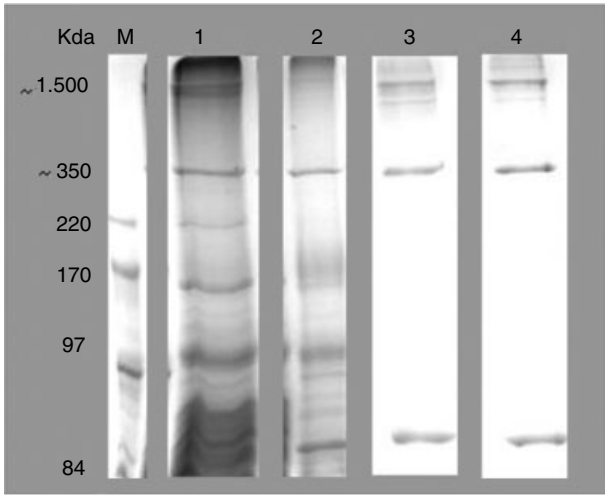

Figure 4 5% SDS/PAGE gels of different purification steps of HMWP1 and HMWP2. M: high molecular weight marker. Lane 1: crude protein extract. Lane 2: 60% ammonium sulphate precipitation. Lane 3: combined fractions of gelfiltration chromatography. Lane 4: peak fraction of hydrophobic interaction chromatography on phenylsepharose material.

masses. This should allow for a differentiation of the newly formed Ampullosporin analogues from those molecules already present at the start of the *in vitro* experiment. Rindfleisch and Kleinkauf showed an *in vitro* biosynthesis of the 20-membered peptaibol alamethicin by a partially purified protein preparation of the producer *Trichoderma viride* [32]. Interestingly, their assay did not contain acetyl-CoA. This compound should be required as the starter for the biosynthesis according to the thiotemplate mechanism if the alamethicin synthetase does contain a PKS starter module as suggested by the deduced sequence of the Tex1 protein. It remains unclear whether the alamethicin synthesized *in vitro* was not acetylated or whether the partially purified enzyme preparation did contain traces of acetyl-CoA.

Even though *de novo* biosynthesis of Ampullosporin *in vitro* was not shown, its presence only in NRPS containing fractions of a gel filtration chromatography, but not in others, provides further evidence that the partially purified HMWPs represent the Ampullosporin synthetase.

Since biosynthesis *in vitro* of Ampullosporin could not be demonstrated ATP-pyrophosphate exchange was performed to probe for the formation of aminoacyladenylates of substrate amino acids. As four of the five substrate amino acids, alanine, glutamine, leucine and tryptophan are proteinogenic

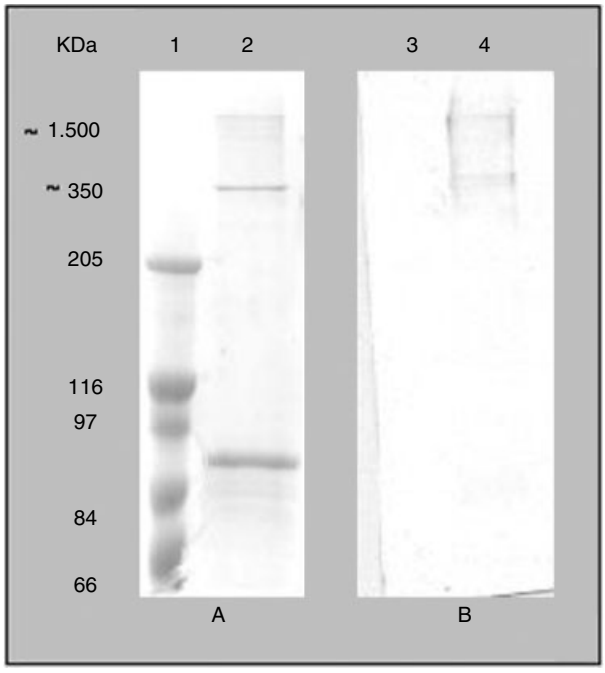

Figure 5 Western blot analysis of the two HMWPs from *S. ampullosporum*. Proteins were separated on SDS/PAGE, blotted onto nitrocellulose membrane and probed with antibodies against a core motif of authentic NRPSs as described in Experimental Procedures. A: SDS-PAGE. Lane 1: marker. Lane 2: fraction of gel filtration of *S. ampullosporum* with HMWP1 and HMWP2. B: Western blot. Lane 3: marker. Lane 4: fraction of gel filtration of *S. ampullosporum* with HMWP1 and HMWP2.

it was expected that their respective tRNA synthetases would be present in *S. ampullosporum*. These enzymes also catalyse an ATP-pyrophosphate exchange. Since a partially purified enzyme was involved the possibility could not be excluded that these enzymes were still present in the extract. The fifth substrate, Aib, the main component of peptaibols, is a non-proteinogenic amino acid. As shown in Figure 7, the HMWPs containing fractions catalysed an Aib-dependent ATP-pyrophosphate exchange showing that a peptaibol synthetase had been isolated. The partially purified proteins were found to activate the substrate amino acids Aib and Ala as the respective amino acyladenylates. It cannot be excluded that the adenylation of alanine was at least partially catalysed by the respective alanine : tRNA synthetase which may have been present in the partially purified enzyme preparation. However, as there is no Aib-specific tRNA synthetase the formation of Aib-adenylates strongly suggest that this reaction is indeed performed by the NRPS.

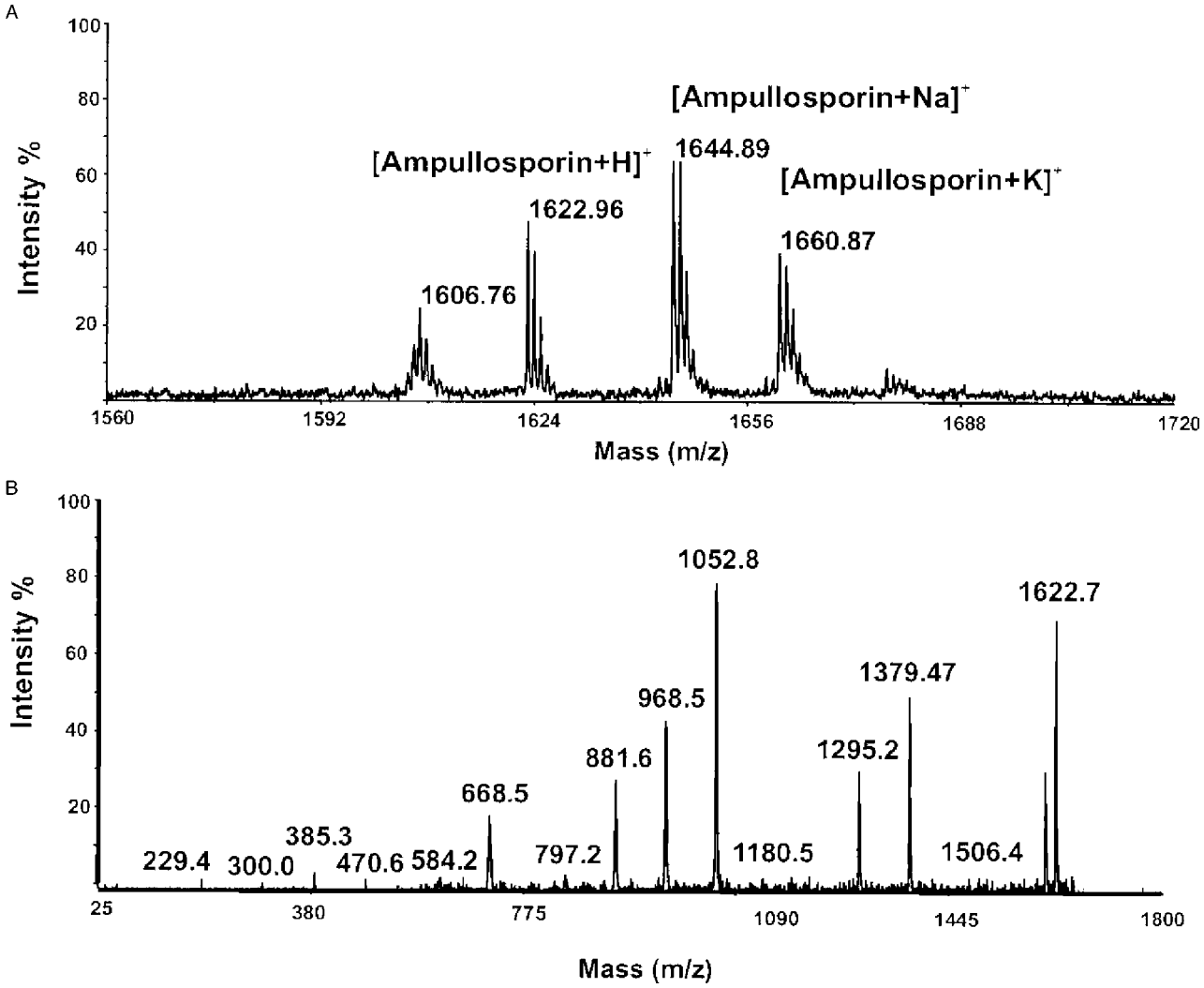

Figure 6 MALDI-TOF MS analysis of Ampullosporin. Fractions of a gel filtration containing the two HMWPs were incubated as described in Experimental Procedures with the substrates presumably required for *in vitro* biosynthesis of Ampullosporin. At different time intervals aliquots of the assay mix were extracted twice with 1 volume of ethylacetate. After evaporation of ethylacetate the residual was dissolved in 5 µl 50% MeOH and subjected to MALDI-TOF MS analysis. A: reflector spectrum of a sample taken at t_0 of the *in vitro* biosynthesis experiment showing peaks corresponding to the masses of protonated, sodium- and potassium-complexed molecular ions of Ampullosporin. B: post source decay analysis of the peak at *m/z* 1622. The fragments are listed and described in Figure 7.

Another strain of *Sepedonium ampullosporum*, HKI-0117, when grown under identical conditions has been shown to produce two peptaibols, Ampullosporin as well as the five-membered peptaibolin [16]. MALDI-TOF MS analysis performed on the mycelia of *S. ampullosporum* HKI-0053 from which the protein was purified did not reveal the presence of peptaibolin or any other peptaibol apart from Ampullosporin A. Hence it can be concluded from these findings that the Ampullosporin synthetase was detected and that this enzyme consists of two polypeptide chains rather than one.

PROPOSED MODEL

Small amounts of the seven derivatives of Ampullosporin A shown in Figure 1 have been found in fermentations of *S. ampullosporum* HKI-0053 [41]. These are most likely synthesized by the same synthetase as Ampullosporin A. Many NRPS systems have been shown to possess a relaxed specificity for some of their modules, leading to the production of a number of derivatives of the main component [42,43]. Usually some modules are more

Table 1 Fragments of Ampullosporin generated by post source decay in MALDI-TOF MS analysis. Numbers in the left column refer to the fragments shown in Figure 6B

Observed mass (m/z)	Corresponding fragment	Mass difference to Ampullosporin A	Fragment corresponding to the mass difference
1622	**Ampullosporin A**	—	
1506	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln	−116	Leuol
1379	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib	−243	Gln-Leuol
1295	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu	−327	Aib-Gln-Leuol
1180	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln	−442	Leu-Aib-Gln-Leuol
1052	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib	−570	Gln-Leu-Aib-Gln-Leuol
968	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib	−654	Aib-Gln-Leu-Aib-Gln-Leuol
881	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln-Aib	−741	Aib-Aib-Gln-Leu-Aib-Gln-Leuol
797	AcTrp-Ala-Aib-Aib-Leu-Aib-Gln	−825	Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
668	AcTrp-Ala-Aib-Aib-Leu-Aib	−954	Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
584	AcTrp-Ala-Aib-Aib-Leu	−1038	Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
470	AcTrp-Ala-Aib-Aib	−1152	Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
385	AcTrp-Ala-Aib	−1237	Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
300	AcTrp-Ala	−1322	Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol
229	AcTrp	−1393	Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Leuol

permissive than others and amino acids are generally substituted at the respective positions in the derivatives with those of similar size and charge, most likely because the non-cognate amino acid still has to fit into an active site binding pocket of the respective adenylation domain. Notably, all minor Ampullosporin A derivatives have Aib (methylalanine) to Ala substitutions. No minor components have been identified that differ in more than two positions from Ampullosporin A. However, it cannot be concluded from these findings that those modules responsible for the incorporation of amino acids into positions for which no alterations have yet been found are less permissive. It is conceivable that the processivity of the Ampullosporin synthetase decreases upon incorporation of a non-cognate amino acid into the growing chain. Components with more than two changes in the peptide chain or even a single substitution at a position

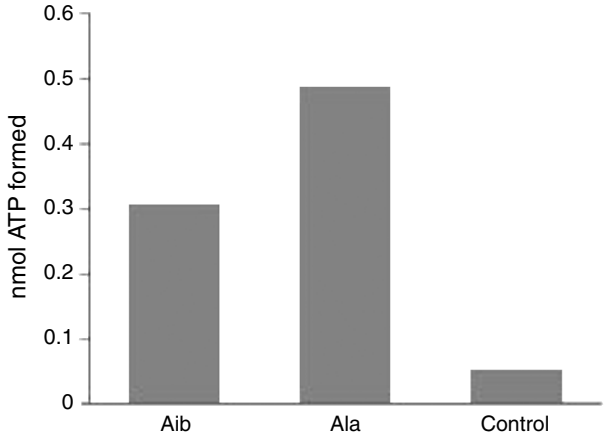

Figure 7 ATP/PP_i exchange reaction. Combined fractions of a gel filtration containing the two HMWPs were incubated as described in Experimental Procedures with Ala and Aib as substrate amino acids. No amino acid was added to the negative control.

other than those found so far may not be produced at a detectable level. Furthermore, as the production of the minor compounds was found only towards the end of a fermentation after 5 to 6 days it is assumed that at that time the Aib supply may have dropped significantly, favouring a substitution with alanine at the respective positions. Two high molecular weight proteins (~1.5 MDa and 350 kDa resp.) were detected by SDS/PAGE from cell extracts of *S. ampullosporum*. These proteins were only detectable in cells grown under conditions in which Ampullosporin was produced. Their appearance and intensity during fermentation correlated with the amount of Ampullosporin produced. Antibodies raised against a core motif (SGTTGRPKG) of the adenylation domain of NRPS showed specific crossreaction with both proteins.

Both proteins occurred in roughly the same amounts during fermentation and co-purified in gel filtration and hydrophobic interaction chromatography, indicating a very strong association. Their combined masses of approx. 1.8 to 1.9 MDa would fit very well with the expected size for a 15-modular peptaibol synthetase, taking into account that the Tex1 protein containing 18 modules has a deduced mass of 2.2 MD with an average size of approx. 120 kDa per module.

Considering the high degree of conservation among NRPSs, the architecture of the Ampullosporin synthetase is most probably very similar to that of the deduced Tex1 synthetase from *T. virens*, containing a PKS module at its *N*-terminus. If the *N*-terminus of the Ampullosporin synthetase is located on the smaller polypeptide, HMWP2, it could account for the formation of a homodimer carrying in addition the first two NRPS modules while the larger polypeptide, HMWP1, housing the remaining 13 NRPS modules and a *C*-terminal reductase would exist as a monomer. The average size of an

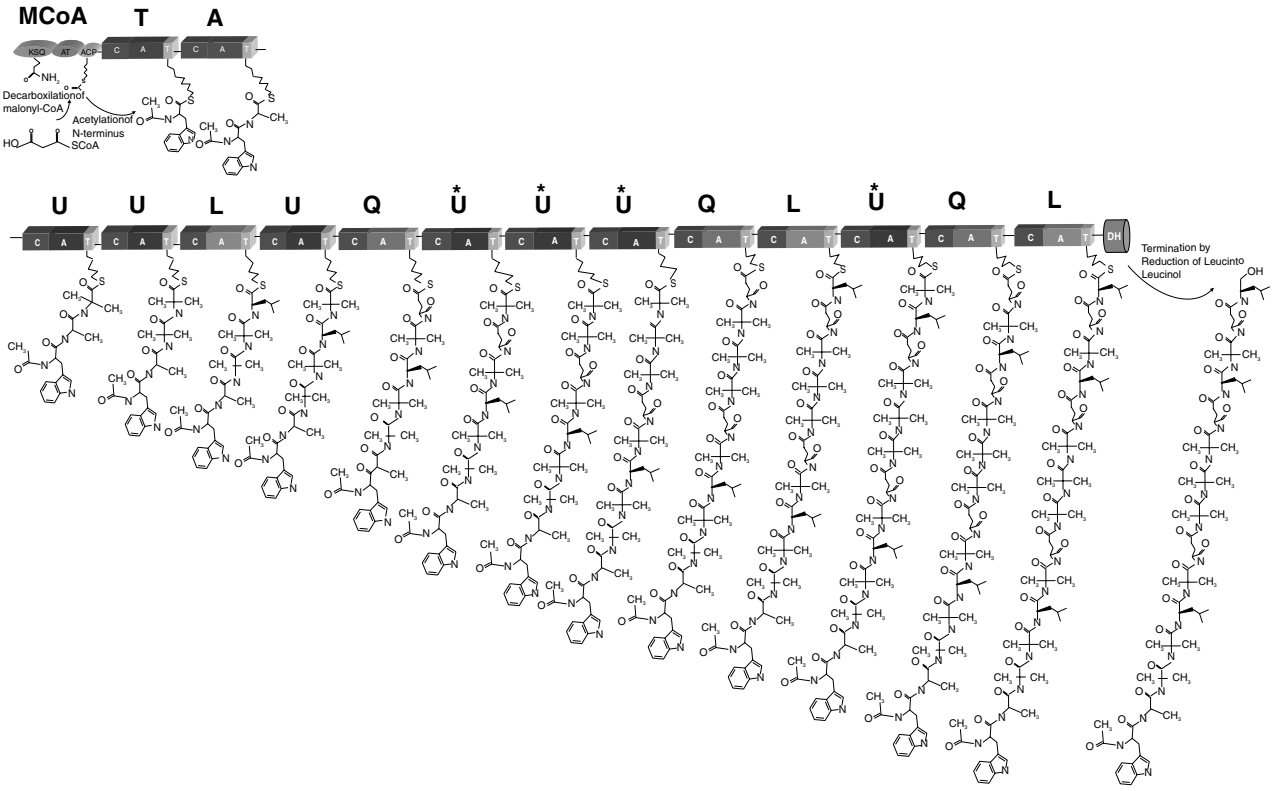

Figure 8 Proposed model for the Ampullosporin synthetase. Shown here is the biosynthesis of Ampullosporin A. Amino acids incorporated by each module are shown in single letter code. U, Aib; MCoA, malonyl-CoA. The smaller polypeptide, HMWP2, (~350 kDa) is thought to accommodate the PKS starter module responsible for the *N*-terminal acetate residue and two modules of NRPS incorporating a tryptophan and an alanine residue, respectively. The rest of the required activities, 13 NRPS modules and a reductase catalysing the release of the product, are proposed to be housed on the larger polypeptide (~1.5 MDa).

NRPS module of the deduced Tex1 protein is about 121 kDa. The PKS starter module of that protein has a M_r of about 100 kDa. This corresponds to a M_r of about 340 kDa for a mixed PKS/NRPS carrying one PKS and two NRPS modules and fits precisely the size determined here for HMWP1 from *S. ampullosporum*. On the other hand, a polypeptide housing two or three NRPS modules and the *C*-terminal reductase would have a M_r of about 285 or 407 kDa, respectively.

As is the case for ketosynthases in loading modules of bacterial type 1 PKS systems the ketosynthase domain of Tex1 does not contain a cysteine residue in its active site. It has been suggested by Bisang *et al.* that these domains, while unable to perform a condensation reaction might work as a decarboxylase [44]. The actual substrate for the starter module for Tex1, and presumably the Ampullosporin synthetase, may thus be malonyl-CoA rather than acetyl-CoA. This substrate would be bound by the acyltransferase and decarboxylated prior to chain elongation. Based on the results presented in this report and assuming a similar setup of modules and domains as shown for the Tex1 peptaibol synthetase a model for Ampullosporin synthetase is proposed (Figure 8). While this model explains the production of the 15mer derivatives of Ampullosporin A detected in *S. ampullosporum* by a relaxed specificity of some of the modules, it could not easily explain the production of peptaibols with either more or less than 15 residues. If peptaibols of different lengths were to be found in *S. ampullosoporum* they would have to be synthesized by a different synthetase. Interestingly, Wiest *et al.* reported that disruption of the *tex1* gene in *T. virens* abolished production not only of the 18mer but of all peptaibols produced by the wildtype strain, including the 12mer and 15mer compounds [28]. As the mutant strains also contained ectopic integrations of the vector used for disruption it is assumed that in addition to *tex1* the genes responsible for the production of the 12 and 14mer peptaibols in their strain were also disrupted.

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