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Concerted Action of P450 Plus Helper Protein To Form the Amino-hydroxy-piperidone Moiety of the Potent Protease Inhibitor Crocapeptin

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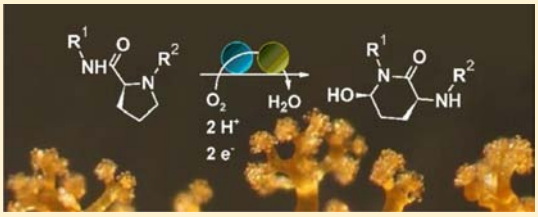
Supporting Information

ABSTRACT: The crocapeptins are described here as cyclic depsipeptides, isolated from cultures of the myxobacterium *Chondromyces crocatus*. Structure elucidation of the compounds revealed a cyanopeptolin-like skeleton, containing the characteristic amino-hydroxy-piperidone (Ahp)-heterocycle. Like the cyanopeptolins, the myxobacterial crocapeptins proved to be serine protease inhibitors. The nonribosomal origin of the peptide was confirmed by mutagenesis experiments, and the biosynthesis gene cluster was sequenced. It could be shown that the Ahp-heterocycle originates from a proline residue in the precursor molecule precrocapeptin, which is converted to crocapeptin by the tailoring enzymes CpnE and CpnF. Conversion of precrocapeptin isolated from a *cpnF* mutant into crocapeptin was achieved using recombinant CpnF, a cytochrome P450 enzyme responsible for hydroxylation of the proline residue in precrocapeptin. Addition of protein CpnE resulted in strongly increased conversion rates toward Ahp containing product. A mutant with 10-fold increased production of crocapeptin A was created through insertion of the P*npt*-promotor in front of the NRPS gene.



INTRODUCTION

Natural products from microorganisms continue to be important sources for bioactive compounds with potential for clinical, agricultural, or biotechnological use. Compounds with antimicrobial, antiviral, cytotoxic, immunosuppressive, and other biological activities are produced by bacteria and fungi. Myxobacteria are an established source of secondary metabolites,[1] and a large variety of novel chemical structures have been isolated, many of which possess novel modes of action.[2] Myxobacteria typically produce polyketides and nonribosomal peptides or hybrid molecules thereof, which are assembled in the cells by large multienzyme complexes, polyketide synthetases (PKS), or nonribosomal peptide synthethases (NRPS).[3] As in other prokaryotic producers, the genes encoding the enzymes necessary for biosynthesis of a compound family are usually found clustered in close vicinity in the genome of the producing organism, often together with genes necessary for product export, regulation, and self-resistance.

The myxobacterium *Chondromyces crocatus* Cm c5 has been found to produce an outstanding compound spectrum, comprising the ajudazols,[4] chondramids,[5] crocacins,[6] chondrochlorens,[7] and thuggacins,[8] which have been detected in activity-based screenings because of their antifungal, antibacterial, or cytotoxic qualities. Except for the chondramides, which are highly similar to the jaspamides isolated from a *Jaspis* sponge, they represent unique compound families. Partial genome sequencing of strain Cm c5 revealed the presence of more putative secondary metabolite gene clusters, which were not yet associated with a product. Thus, further bioactive compounds are expected to be produced by this strain under currently unknown conditions. In a combined genome and metabolite mining approach, noticeable UV peaks of Cm c5 extracts were investigated and a number of putative NRPS and PKS gene clusters were inactivated, to correlate compound peaks to genes and isolate novel bioactive metabolites.[9]

The crocapeptins presented in this Article belong to the cyanopeptolin-like compound family, cyanobacterial cyclic depsipeptides with over 100 congeners already isolated from various cyanobacteria.[10] Their unique structural feature is an amino-hydroxy-piperidone (Ahp)-heterocycle, which is crucial for the bioactivity as potent serine protease inhibitors. Different congeners show preference to different target enzymes, based on the amino acids incorporated at key positions within the peptide.[11] Cocrystallization of the elastase with scyptolin revealed that the compound fits into the active site of the enzyme and directly blocks the serine of the catalytic triad.[12] The rigidity of the compound, crucial for blocking the enzyme activity, is provided by the Ahp-heterocycle. Promising

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pharmaceutical potential may arise from this compound family as highly specific inhibitors for serine proteases, obtained either from a natural source or through modification of an existing scaffold. Because of the absence of toxicity, the cyanopeptolins are seen as lead structures with high potential in therapy, yet their development is hampered by the low amounts typically obtained from cyanobacteria. As an alternative approach, total synthesis of some congeners has been pursued.[13]

Since their first isolation,[14] the pharmaceutical potential of various cyanopeptolins has been investigated, because serine proteases are involved in a large variety of diseases, such as virus infections, chronic inflammations, and cancer. Scyptolin is a specific elastase inhibitor,[12] while the ichthyopeptins were found to block influenza virus replication in infected cells and to increase survival rates in infected mice.[15] Intriguingly, several cyanopeptolin and crocapeptin congeners were recently patented for specific inhibition of human kallikreins to treat chronic skin diseases such as psoriasis and atopic dermatitis.[29]

Cyanopeptolins are produced by NRPS enzymes in cyanobacteria, and four cyanopeptolin NRPS clusters from different cyanobacterial strains have been sequenced and published to date.[16] Despite the numerous variants isolated and studied, the origin and biosynthesis of the characteristic Ahp-heterocycle have not yet been elucidated. Because genetic tools for Cm c5 had been established previously, overproduction of the crocapeptins and the elucidation of the Ahp formation in *Chondromyces* were of particular interest. Detailed knowledge of the biosynthetic pathway and the responsible enzymes is often crucial for overproduction and further development of a promising compound and may provide options to generate non-natural derivatives by means of biotechnology. In this Article, the isolation and structure elucidation of the crocapeptins, the identification of the corresponding biosynthetic gene cluster, the isolation and structure elucidation of a precrocapeptin from a P450 mutant, and finally Ahp formation within this precursor molecule in vitro using purified P450 plus an unprecedented helper protein are presented.

RESULTS

Analysis and Isolation of Crocapeptins. The crocapeptins **1**–**3** were detected in extracts of *C. crocatus* Cm c5 by HPLC–UV–HR–ESI–MS. The molecular formulas of $C_{47}H_{66}N_8O_{12}$ for crocapeptin A_1 (**1**), $C_{48}H_{68}N_8O_{12}$ for crocapeptin A_2 (**2**), and $C_{49}H_{70}N_8O_{12}$ for crocapeptin A_3 (**3**) were calculated from the $[M + H]^+$ ion clusters *m*/*z* 935.4868, 949.5027, and 963.5186. Thus, **1** and **3** differ from **2** by the formal abstraction and addition of a methyl group, respectively.

The molecular formula of crocapeptin B (**4**), which is produced by *C. crocatus* Cm c2, was determined as $C_{50}H_{73}N_9O_{12}$ by $[M + H]^+$ ion cluster at *m*/*z* 992.5450. Additionally, in the extract of strain Cm c2, minor compounds with molecular formulas $C_{49}H_{71}N_9O_{12}$ and $C_{51}H_{75}N_9O_{12}$ were observed.

Structure Elucidation of Crocapeptins. The basic structure of the crocapeptins (Figure 1) was primarily elucidated with main component **2**. Using HMQC and DEPT NMR spectra, all protons were assigned to their respective carbon atoms. The remaining 8 protons were recognized from their chemical shifts as 7 amide protons and one hydroxyl proton. The ^{13}C NMR spectrum revealed nine carbon atoms within the amide/ester range. Following the interpretation of COSY, TOCSY, and HMBC data, eight partial

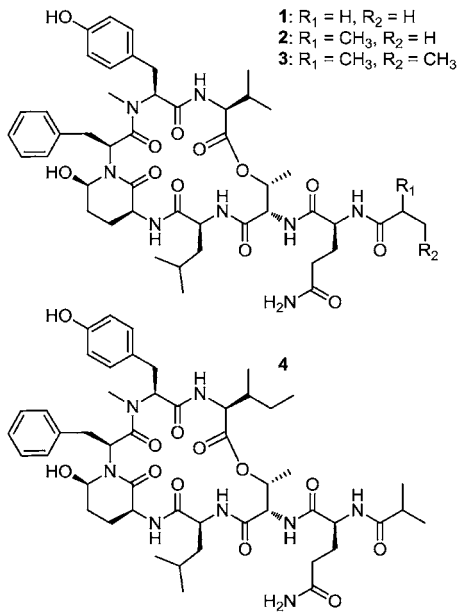

Figure 1. Crocapeptins A1–A3 (**1**–**3**) isolated from strain Cm c5 and crocapeptin B (**4**) isolated from strain Cm *c*2.

structures accounting for all atoms of the molecule were detected, that is, the amino acids glutamine, threonine, leucine, phenylalanine, *N*-methyltyrosine, and valine together with 3-amino-6-hydroxy-2-piperidone (Ahp) and isobutyric acid (Ibu) moieties. Their linear sequence in **2** was determined from HMBC correlations as Ibu-Gln-Thr-Leu-Ahp-Phe-NMeTyr-Val (Supporting Information Table S1, Figure S1). The high field shift of the oxymethine H3 of Thr (δ 5.39) indicated an ester linkage at this position, which was confirmed by the HMBC correlation between H3 of Thr (δ 5.39) and C1 of Val (δ 171.9), thus completing the planar depsipeptide structure of **2**. Comparison of the NMR data of **1**, **2**, and **3** revealed the presence of different starter units: the isobutyrate moiety of **2** is replaced by propionate in the case of **1** and by 2-methylbutyrate in **3**. The absolute configurations of L-Gln, L-Thr, L-Leu, L-Phe, L-*N*MeTyr, and L-Val residues were assigned by Marfey's method.[17] However, initially an oxidation with CrO_3 was performed liberating glutamate from the unusual 3-amino-6-hydroxy-2-piperidone (Ahp) moiety.[18] Because no D-glutamate was detected, the 3*S*-configuration was deduced for Ahp. Furthermore, ROESY correlations within the Ahp residue between NH and H4a, H4a, and OH suggested a 6*R*-Ahp configuration (Supporting Information Figure S3). A strong NOE between α-protons of Phe and *N*MeTyr showed a cis-amide bond between these amino acids characteristic for structurally related compounds. Because 2-methylbutyrate biosynthetically derives from isoleucine, an *S*-configuration can be proposed for the branched-chain starter unit in **3**. The NMR spectra of **4** were very similar to those of **1**–**3**. They revealed the presence of isoleucine, *N*-methyltyrosine, phenylalanine, 3-amino-6-hydroxy-2-piperidone, leucin, threonin, citrullin (Cit), and isobutyric acid moieties. Their linear sequence in **4** was also determined by key HMBC correlations as Ibu-Cit-Thr-Leu-Ahp-Phe-*N*MeTyr-Ile (Supporting Information Table S2, Figure S2). Using Marfey's method, L-configurations were determined for all amino acids. The Ahp-configuration of **4** was deduced as 3*S*,6*R* similarly to **2**. Database searches resulted in one match for **4**, which refers to a

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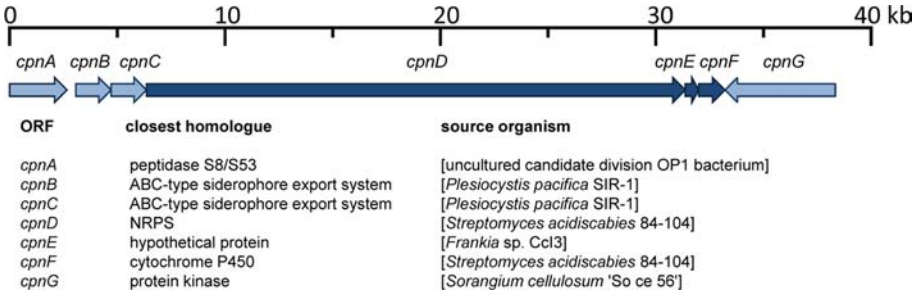

ORF	closest homologue	source organism
cpnA	peptidase S8/S53	[uncultured candidate division OP1 bacterium]
cpnB	ABC-type siderophore export system	[*Plesiocystis pacifica* SIR-1]
cpnC	ABC-type siderophore export system	[*Plesiocystis pacifica* SIR-1]
cpnD	NRPS	[*Streptomyces acidiscabies* 84-104]
cpnE	hypothetical protein	[*Frankia* sp. CcI3]
cpnF	cytochrome P450	[*Streptomyces acidiscabies* 84-104]
cpnG	protein kinase	[*Sorangium cellulosum* 'So ce 56']

Figure 2. The crocapeptin A biosynthetic gene cluster from *Chondromyces crocatus* Cm c5. The genes responsible for biosynthesis are indicated as dark blue arrows. The closest homologue found by BLAST sequence search against the nr database of the encoded protein sequence is shown below for each ORF.

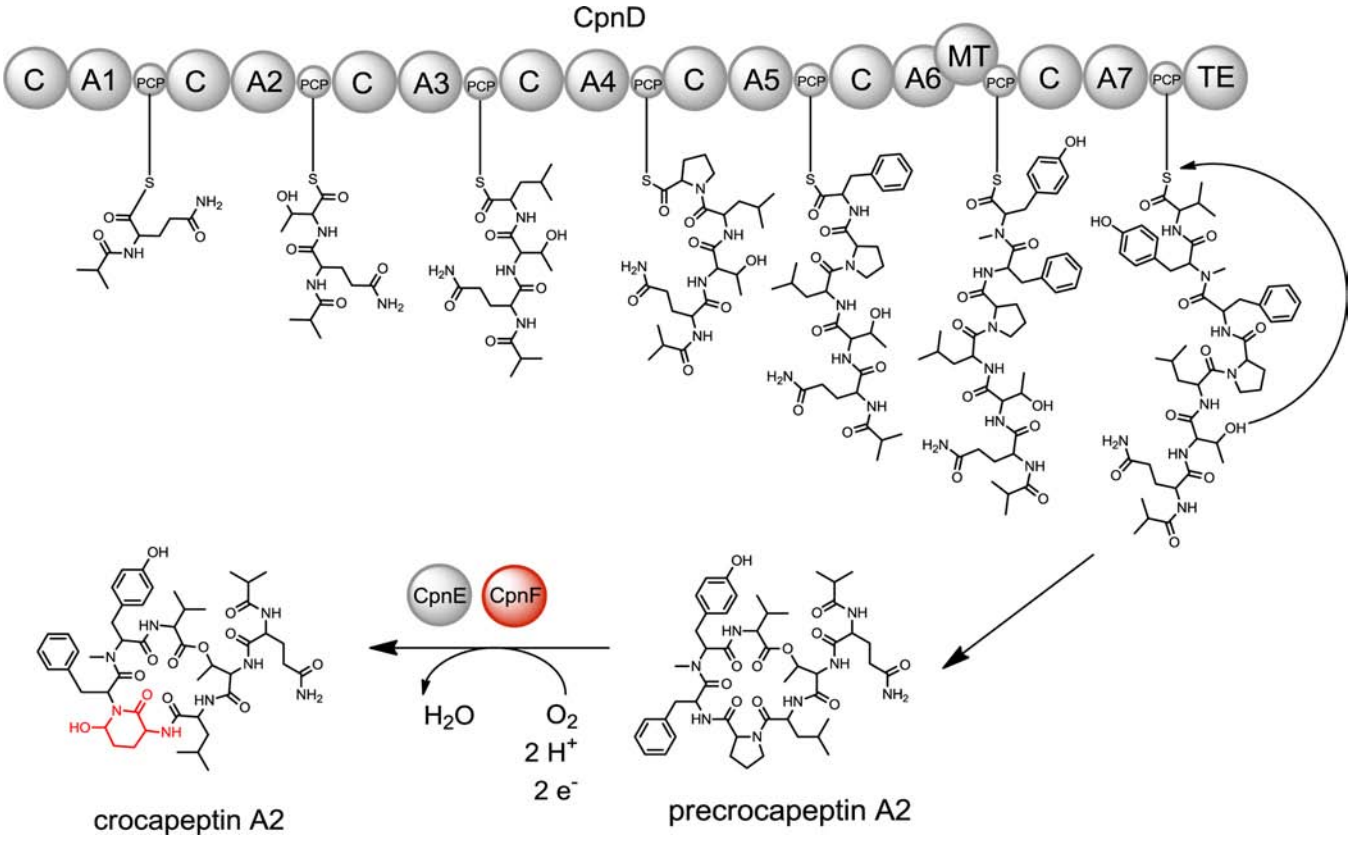

Figure 3. Biosynthesis of crocapeptin A2. The first condensation reaction defines the crocapeptin A derivative. The cyclic depsipeptide is released as precrocapeptin, which is then hydroxylated at the proline residue by the cytochrome P450 enzyme CpnF. Rearrangement of hydroxy-proline to Ahp is catalyzed by CpnE.

member of a depsipeptide patented because of submicromolar Kallikrein 7 inhibitory activity,[29,30] whereas **1**−**3** comprise new metabolites.

Protease Inhibition Assays. Ahp-containing cyclic depsipeptides frequently possess serine protease inhibitory activity; the amino acids adjunct to Ahp determine the enzyme specificity.[12] With Phe and Leu in these positions, the crocapeptins belong to the class of chymotrypsin inhibitors. Consequently, their inhibitory activity against Chymotrypsin was determined and resulted in IC_{50} values of 0.1 μM for **1**−**3** and 0.2 μM for **4**.

Analysis of the Crocapeptin A Gene Cluster and Proposed Biosynthesis. Because of the apparent similarity to the nonribosomally synthesized cyanopeptolins, partial genome data of crocapeptin A producing strain Cm c5 were screened for the presence of NRPS genes to identify the corresponding biosynthetic gene cluster. Using prediction of substrate specificity of adenylation domains from sequence data[19] allowed one to reduce the number of target sequences to two contigs, containing an NRPS gene fragment with expected adenylation domain specificity for Thr-Leu-Pro-Phe and Tyr. Inactivation mutants for each contig were found unable to produce crocapeptin. Thus, both identified NRPS gene fragments were proven to be part of the crocapeptin biosynthetic gene cluster. To complete the cluster sequence, a cosmid gene bank covering the Cm c5 genome[20] was screened with DNA probes directed against parts of the two crocapeptin NRPS gene fragments, which are present on cosmids B:E7, C:O19, and D:J18. Cosmid end-sequencing, primer walking, and inverse PCR were applied to complete the gene cluster sequence. In Figure 2, a schematic drawing of the gene cluster is shown, with the closest homologues to the

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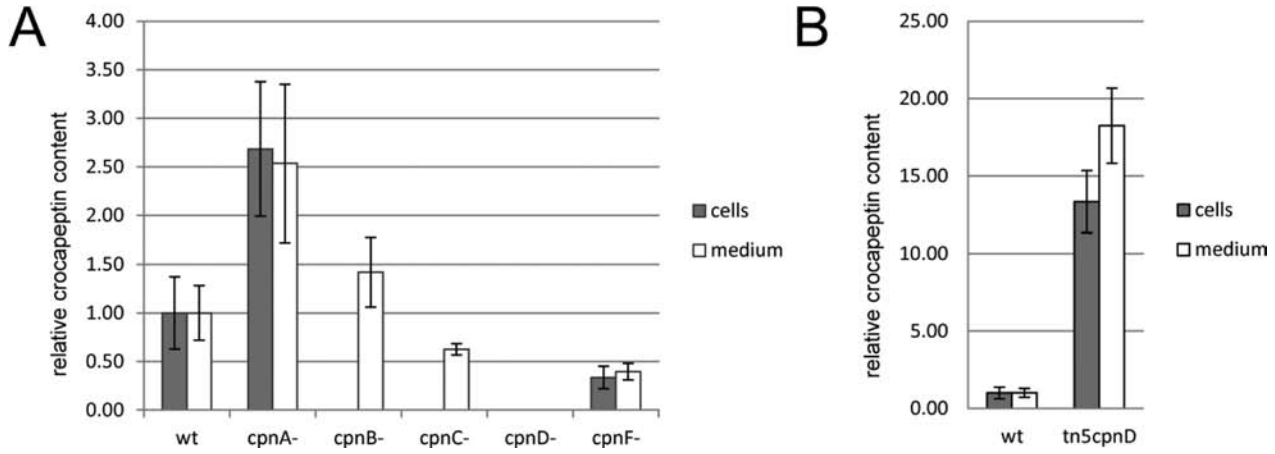

Figure 4. (A) Effect of gene inactivation in Cm c5 by insertional mutagenesis. For consistency, relative crocapeptin A content of extracts from Cm c5 wild type and mutants is given relative to the chondramide content in extracts of cells and growth medium. Crocapeptin A content was related to chondramid B content in each extract by division of the integrated MS-signal intensities of crocapeptins A by chondramid intensities, respectively. (B) Comparison of crocapeptin production in Cm c5 wild type and P*npt-cpnD* mutant cells. Extracts of cells and medium showed a 10-fold increased crocapeptin content in the mutant.

identified ORFs listed below. The NRPS gene *cpnD* is 24.5 kb in size and forms an operon with the hypothetical protein gene *cpnE* and cytochrome P450 encoding gene *cpnF*. Upstream of the NRPS gene, a putative serine protease gene *cpnA* and two ABC transporter genes *cpnB* and *cpnC* are located. Downstream of *cpnF*, a protein kinase is encoded by gene *cpnG* on the opposite strand. Because of their arrangement and predicted gene product function, ORFs *cpnA* to *cpnG* were chosen for knockout experiments to test their involvement in crocapeptin biosynthesis, transport, or regulation.

The catalytic domain sequence of CpnD and the substrate specificity of its adenylation domains were obtained from the prediction based on the amino acid sequence, according to the NRPS predictor.[19] As depicted in Figure 3, biosynthesis is expected to start with incorporation of glutamine by domain A1, to which one of the observed short chain fatty acids is bound by domain C1, creating the crocapeptins A_1–A_3. The lipopeptide chain is then elongated by incorporation of threonine, leucine, proline, phenylalanine, tyrosine (which becomes *N*-methylated in trans), and valine. The TE domain finally releases precrocapeptin by intramolecular lactonization via the hydroxyl group of threonine and the carboxy-terminus of valine. Free precrocapeptin is hydroxylated by CpnF at the proline residue, which is then transformed to the bioactive Ahp-containing crocapeptin. This biosynthesis scheme implies that each crocapeptin derivative must have its distinct precursor, as the variable short fatty acid chain is introduced during the start of biosynthesis. No function could be assigned to the gene product of *cpnE*, which has high sequence similarity to ORFs annotated as "hypothetical proteins" in various prokaryotic genomes. The closest sequence homologue with an assigned protein function is an Aha1 activator protein of the Hsp90 ATPase.[21] The function of CnpE in crocapeptin biosynthesis is described below. The adenylation domain A4 of the crocapeptin assembly line, responsible for incorporation of the amino acid that would be converted to Ahp, is predicted to incorporate proline, as deduced from the motif "DVQFAAH-VAK" by using the nonribosomal code.[22] To verify this prediction, $^{13}C_5{}^{15}N$-proline was amended to Cm c5 wild-type liquid culture, and cultivation was continued for 2 days. After extraction of cells and supernatant, crocapeptins were detected by HR–MS. $[M + H]^+$, $[M + NH_4]^+$, and $[M - H_2O + H]^+$ molecular ion clusters of crocapeptins A_1–A_3 exhibiting a m/z that increased by 6 units were clearly detected, however only at a low percentage, making quantification hardly possible. In the negative control experiment, these ions were not detected. Thus, proline is indeed incorporated into the crocapeptins, and all proline N and C atoms remain in the metabolite after conversion of this residue to Ahp.

Gene Inactivation Studies in Cm c5. To investigate the function of the genes identified, targeted insertional mutagenesis was applied. Mutant Cm c5 clones exhibiting hygromycin B resistance were grown in liquid culture for isolation of genomic DNA, and insertion was verified by PCR experiments (for genetic verification, see Supporting Information section 3). At least three independent verified mutants of genes *cpnA*, *cpnB*, *cpnC*, *cpnD*, and *cpnF* were obtained and grown in liquid culture to enable secondary metabolite analysis. For *cpnE* and *cpnG*, no mutants could be obtained. As two of the genes that were inactivated are predicted to encode ABC-transporters, mutant cells and culture supernatant were extracted separately, to observe differences in crocapeptin content between cells and growth medium. Quantification of crocapeptin production was performed by dividing the summarized $[M + H]^+$ signal peak areas from MS analysis regarding crocapeptins A_1–A_3 by the chondramide B $[M+H]^+$ signal peak area in each chromatogram, because chondramid B production was known to be very stable in Cm c5 by experience.[23] UV data were not used because of overlapping peaks with other compounds. The average ratio of crocapeptins to chondramide was normalized to wild-type levels and is shown in Figure 4A.

As mentioned above, inactivation of the NRPS gene *cpnD* was found to abolish crocapeptin production. Inactivation of the putative serine protease encoding gene *cpnA* led to a 2-fold increase of crocapeptin levels in extracts from cells and medium, but high variation was observed within the individual cultures. Inactivation of the ABC transporter genes *cpnB* and *cpnC* led to cell extracts devoid of crocapeptins, while these metabolites were still present in the medium in similar amounts as compared to the wild type. Inactivation of *cpnF* did not abolish crocapeptin production completely, but reduced

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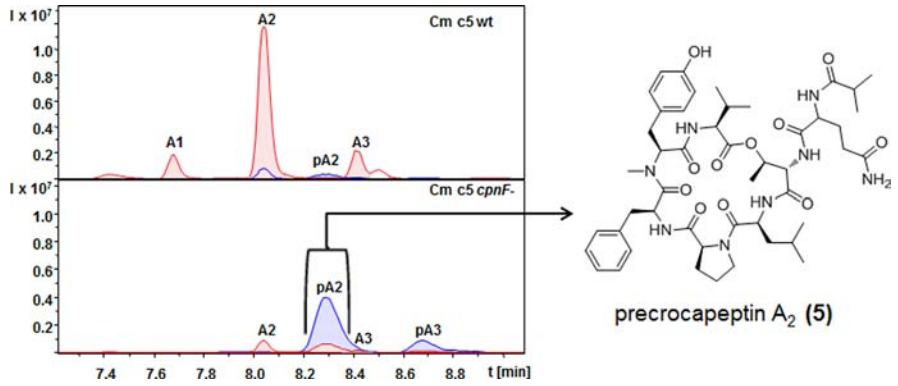

Figure 5. Detection and structure elucidation of precrocapeptin A_2 (**5**). Extracted ion chromatogram for crocapeptin masses (red areas) and precrocapeptin masses (blue areas). The putative crocapeptin precursors were found in extracts of *cpnF*$^-$ mutants of strain Cm c5. Subsequent isolation and NMR analysis of **5** confirmed the structure shown on the right, a cyclic depsipeptide exhibiting L-proline incorporated instead of Ahp.

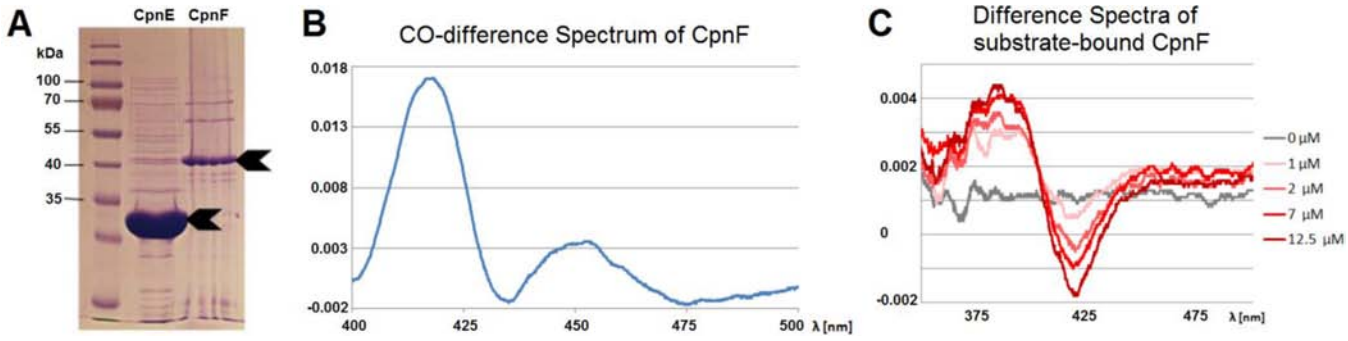

Figure 6. (A) SDS-PAGE of purified proteins CpnE and CpnF. The target proteins are indicated by black arrows. (B) CO difference spectrum of purified CpnF. The peak characteristic for cytochrome P450 enzymes at 450 nm can be observed. (C) Difference spectra of CpnF with increasing concentrations of precrocapeptin A_2 show type I binding.

abundance to less than one-half of wild-type levels in the respective mutants. The effects of the inactivation experiments are discussed below.

Overproduction of Crocapeptins in Cm c5 by Promotor Insertion. The tn*5*-derived *npt*-promotor P*npt* had been shown to enable overexpression of genes for secondary metabolite production of strain Cm c5 previously, where replacing the native thuggacin promotor by P*npt* led to significantly increased levels of thuggacins.[24] To test whether a similar effect could be induced with crocapeptins, the promotor was placed in front of the NRPS gene *cpnD* by insertional mutagenesis, leading to a disrupted short copy of *cpnD* and a P*npt*-driven intact *cpnD* in the mutant. Extracts of mutant cells and medium were prepared and analyzed as described previously, and crocapeptin content was measured as described above. An increase of approximately 10-fold crocapeptin content was found in the cells and 15-fold in the medium, as shown in Figure 4B. As compared to the wild type, the overproducer mutants did not show altered growth or swarming behavior in liquid culture or on agar plate.

Detection of Precrocapeptins in *cpnF*$^-$ Mutants of Cm c5. The proposed crocapeptin biosynthetis scheme implies that the Ahp-heterocycle is derived from a proline residue, which is converted to Ahp either in trans or after release of a depsipeptide precursor molecules by the tailoring cytochrome P450 oxidase CpnF. These crocapeptin precursors would be expected to have a L-proline residue instead of Ahp in the respective position. Extracts of Cm c5 *cpnF*$^-$ mutants, in which the tailoring P450 was inactivated, were therefore screened for masses that would correspond to the postulated precursors.

Indeed, new $[M + H]^+$ mass signals were detected in the extracts of the growth medium from *cpnF*$^-$ mutants. These masses were later found to be detectable in the supernatant of wild-type cultures, but only in trace amounts. Because of their retention times and m/z values, the new compounds were designated precrocapeptins pA_1 m/z = 919.49, pA_2 m/z = 933.52, and pA_3 m/z = 947.52. To verify the identity of the newly observed mass signals, Cm c5 *cpnF*$^-$ was grown in liquid culture, and the putative precursors were isolated from culture supernatant. The purified precrocapeptin A_2 (pA_2) with a mass of 932.3 Da was analyzed by NMR, which confirmed the expected lipopeptide lactone, corresponding to crocapeptin A_2 with L-proline replacing the Ahp heterocycle (Figure 5).

Establishing the Role of CpnE and CpnF in the Conversion of Precrocapeptin to Crocapeptin in Vitro. To elucidate the reaction mechanism and the role of the gene product of *cpnE* and *cpnF*, corresponding proteins CpnE and CpnF were overexpressed in *E. coli* and purified, as shown in Figure 6A. CO-difference spectrometry was applied to verify that CpnF is expressed as active Cytochrome P450, but revealed that only a fraction of the purified CpnF is active holoenzyme, while a larger fraction is apoenzyme, as indicated by the peak at 420 nm (Figure 6B). Difference spectrometry of CpnF coincubated with precrocapeptin A_2 led to concentration-dependent type I binding, as indicated by the reduction of the Soret absorption band at 420 nm, as shown in Figure 6C. This confirms that that free pA_2 is indeed a substrate for CpnF.

Cytochrome P450 enzymes catalyze electron-dependent redox reactions, typically hydroxylations and epoxidations, and thus depend on electron carrier proteins to deliver the

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necessary electrons. Because the corresponding native electron carriers for CpnF are not known, the versatile recombinant ferredoxin Adx 4-108 was utilized instead, as it had been shown to be able to interact with several myxobacterial CYPs.[25,26] To transfer electrons to CpnF, Adx was used together with its native electron donor, adrenodoxin reductase, a flavoenzyme that reduces oxidized adrenodoxin by oxidizing NADPH to $NADP^+$. To keep NADPH levels elevated during the reaction, a NADPH regeneration system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase was used, which restores NADPH from $NADP^+$ by conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone.[27] Purified CpnE was also included in this assay, to test its involvement in this conversion reaction. Enzymes CpnE and CpnF were tested separately and in combination in three separate reactions A, B, and C, each containing precrocapeptin A_2 as substrate, as shown in Figure 7. Reaction A contained only CpnE, B contained only CpnF, and in C both proteins were present. After incubation for 30 min, the extracts were subjected to MS analysis and compared to a reference sample of crocapeptin A_2 (**2**). Unaltered substrate precrocapeptin A_2 was detected in reaction A. Complete hydroxylation of precrocapeptin A2 took place in reactions B and C where two isomeric products were detected with m/z 949.5 by ESI MS with retention times of 7.2 and 7.8 min. Comparison with the reference confirmed the identity of the peak at 7.8 min as crocapeptin A_2. The role of CpnE in the conversion is shown by comparing the ratio of crocapeptin A_2 (peak 2) and the presumed intermediate (peak 6) in reactions B and C. Without CpnE, mainly the isomer with a retention time of 7.2 min was formed, while in the presence of CpnE a much more efficient conversion into crocapeptin A_2 was observed. Nevertheless, in the course of several days, a slow rearrangement of the supposed intermediate into crocapeptin A_2 could also be observed.

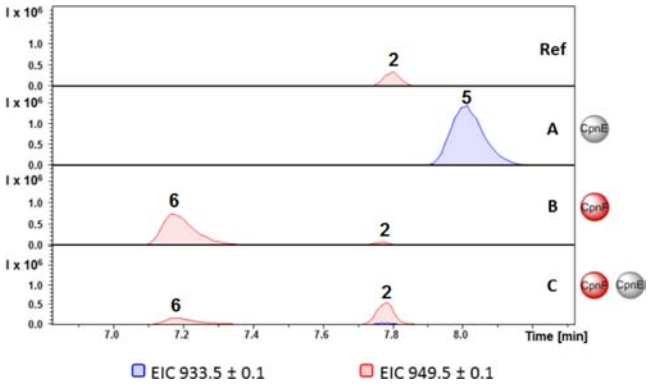

Figure 7. In vitro conversion of precrocapeptin A_2 (peak 5) to crocapeptin A_2 (peak 2). The result of incubation of precrocapeptin A2 (933.5 Da) with different enzyme combinations A, B, and C is compared to a reference of crocapeptin A2 (949.5 Da). Extracted ion chromatograms for the masses 933.5 Da (blue) and 949.5 Da (red) are shown. Peak 6 is a crocapeptin isomer that forms mostly in the absence of CpnE.

Using pA_3 as substrate for in vitro hydroxylation leads to the same effects, that is, one intermediate peak corresponding to peak 6 in Figure 7 and one product peak. For corroboration of the identity of the rather instable intermediate, the in vitro hydroxylation reaction was repeated in a larger scale, using 400 μg of pA_3 as substrate. The reaction products were separated chromatographically (HPLC) and analyzed by NMR individually. From this analysis, formation of a 5-OH proline moiety is strongly indicated, as discussed below. Using the same type of analysis, also nonenzymatic conversion from 5-OH-proline to Ahp could be proven, as shown in Supporting Information Figure S7-4.

DISCUSSION

The Myxobacterium *Chondromyces crocatus* Produces Potent Protease Inhibitors: The Crocapeptins. The crocapeptins, isolated from the myxobacterium *Chondromyces crocatus*, were found to belong to the diverse compound class of cyanopeptolin-like peptides, and mutagenesis experiments in strain Cm c5 confirmed their nonribosomal origin. The Ahp-heterocycle is a structural feature crucial for protease inhibiting bioactivity of the cyanopeptolins. Here, the previously unknown biosynthesis of this structural part was elucidated by mutagenesis and biochemical studies. These investigations led to the identification of the precrocapeptins as precursor molecules released from the NRPS assembly line, featuring an L-proline instead of the Ahp heterocycle. These precursors are then converted into the bioactive crocapeptins by the hydroxylation via the tailoring P450 CpnF and the Aha1-domain containing helper protein CpnE. Comparison of the crocapeptin gene cluster with published cyanopeptolin gene clusters from cyanobacteria shows that the cyanobacterial gene clusters contain neither a predicted P450 gene nor a gene with homology to *cpnE*. Furthermore, in the cyanopeptolin NRPS genes, the predicted substrate for the A-domain corresponding to the Ahp-position is not proline. Instead, all cyanopeptolin A-domains at the Ahp-position have the same DVENAGVV-motif, of which the substrate is not known yet. Consequently, further studies are required to unravel the biosynthesis of the Ahp herterocycle in cyanobacteria.

The function of the ORFs in the crocapeptin gene cluster was investigated by knockout experiments. The putative serine protease gene *cpnA* is considered to be part of the gene cluster, possibly involved in regulatory mechanisms or otherwise linked to crocapeptin production, because of elevated crocapeptin content in the respective mutant. The results of *cpnB* and *cpnC* inactivation are contrary to the initially expected function as crocapeptin export proteins and point toward a function as importer proteins. Another possibility is a rather complex export process involving the bacterial secretory pathway, which cannot be investigated by simple separation of cell clumps and media. We suggest that crocapeptins are actively secreted into the extracellular slime of the *Chondromyces* biofilm by CpnB and CpnC to serve as feeding deterrent, whereas crocapeptin dissolves in the medium by diffusion. *CpnF* mutants are still able to produce crocapeptins, so either the disrupted *cpnF* gene is still translated into a protein with residual activity, or another P450 in the genome is complementing its function.

CpnE Assists in Ahp Formation after Hydroxylation by Cytochrome P450 Enzyme CpnF. Because of the small size of the gene and its location, inactivation of *cpnE* via insertional mutagenesis was not possible, and therefore the function of a hypothetical CpnE could not be elucidated by mutagenesis. A *cpnE* mutant is expected to exhibit a phenotype similar to a *cpnF*-mutant, because transcription of *cpnF* would be impaired due to the pSUP_*hyg* insertion. However, because *cpnE* was found encoded between *cpnD* and *cpnF*, it was supposed to play a role in crocapeptin biosynthesis. To shed light on this hypothesis, *cpnE* and *cpnF* were heterologously expressed in *E. coli* and purified.

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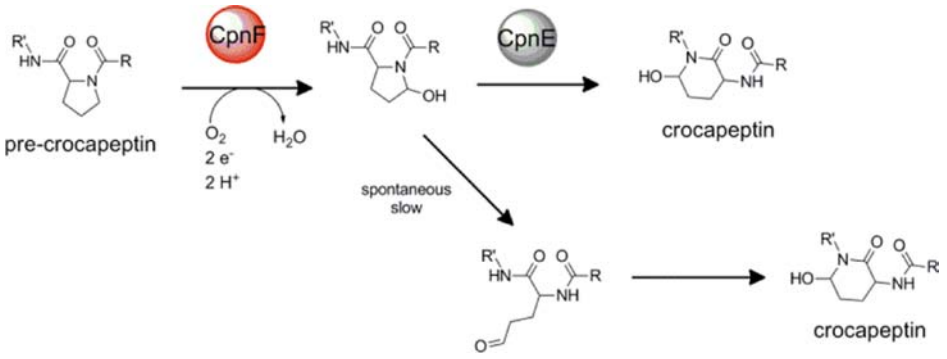
Scheme 1. Proposed Formation of the Amino-hydroxy-piperidone (Ahp) Heterocycle



Ahp formation is expected to occur as shown in Scheme 1. Hydroxylation at C5 of the proline residue in precrocapeptins gives rise to a cyclic hemiaminal corresponding to peak 6 in Figure 7. This hydroxy-proline derivative is then converted to Ahp via a glutamate-semialdehyde intermediate, which is highly reactive and forms the more stable six-membered heterocycle via the amide bond of the adjacent phenylalanine residue. From synthetic efforts of the cyanobacterial congener symplocamide, spontaneous Ahp formation has been reported starting from a L-glutamic acid semialdehyde residue,[13] which can be obtained by hydroxylation of proline at C5 and subsequent ring-opening. Indeed, a hydroxylated precrocapeptin with either hydroxyproline or glutamic-acid-semialdehyde does exhibit a mass identical to that of the respective Ahp-containing crocapeptin. Hydroxylation of proline at positions other than C5 is not expected, as this would not lead to a hemiaminal able to undergo ring-opening. Proline residues with hydroxylations at C3 and C4 have been described for the lantibiotic microbiosporin, and hydroxylation at these positions does not lead to Ahp formation.[28]

Activity of Purified Recombinant Proteins. CpnE and CpnF were tested with isolated precursor precrocapeptin A_2 (**5**) as substrate. Hydroxylation was achieved in vitro with CpnF, as shown by HPLC−MS analyses of the reaction extracts, as shown in Figure 7. Incubation with CpnF alone mostly leads to an unstable isomer (peak 6) exhibiting shorter retention time, which slowly converts to crocapeptin A_2 (peak 2). Incubation of precrocapeptin A_2 with CpnE alone does not indicate any reaction. However, coincubation of precrocapeptin with CpnF and CpnE results mostly in crocapeptin formation, while formation of the unstable isomer (peak 6) is detected in much smaller quantities.

In vitro reactions of CpnF with precrocapeptin A_3 as substrate were scaled up to isolate the presumed intermediate 5-hydroxy-proline form of crocapeptin A_3 for structure elucidation. First, the mixture of both isomers was used for NMR spectroscopic analysis; afterward, the isomers were separated and analyzed individually (Supporting Information Figure S7-1). The NMR spectrum of isomeric crocapeptin A_3 clearly shows shifted ^{1}H signals in the area of interest as compared to crocapeptin A_3 (Ahp-ring, Figure 8). A chemical shift of δ 3.81 can be detected for the α-proton in Ahp. The signal of the α-proton exhibits TOCSY correlations with two CH_2-groups at δ 2.61, 1.69 and δ 1.81, 1.68, respectively, additional to correlating with a CHOH-group at δ 5.19 and a NH at δ 7.58 (Supporting Information Figure S7-2). In contrast, the α-proton of the isomer shows a chemical shift of δ 4.30 with TOCSY correlations to CH_2 groups at δ 1.71, 1.61

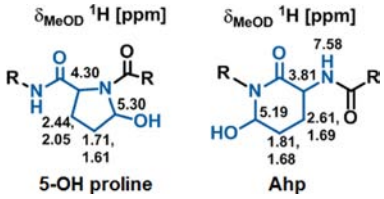


Figure 8. ^{1}H NMR shifts in cyclic hemiaminals after in vitro hydroxylation of proline in precrocapeptin A_3: Ahp-containing crocapeptin A_3 (right) and its unstable isomeric intermediate containing 5-OH proline instead (left).

and δ 2.44, 2.05 (Supporting Information Figure S7-3). Correlation to the CHOH group at δ 5.30 cannot be detected with the applied parameters, but integrity of the spin system can be assigned via correlations of the signal of CHOH with both CH_2 groups and the α-proton. A strong indication for the presence of the hydroxy-proline is the missing correlation to an NH proton in this spin system in addition to the existence of the NH-signal belonging to the phenylalanine spinsystem at δ 8.86. Furthermore, the shifts of N-Me-Tyr aromatic protons in the intermediate (δ 7.09, 6.50) are exactly the same as for precrocapeptin (Supporting Information Table S4-4), whereas these proton signals in the product are significantly shifted to δ 7.14 and 6.85, indicating a conformational change of the structure probably due to rearrangement from a five- to a six-membered ring. Thus, Ahp formation is likely initiated by opening of the five-membered ring hemiaminal to glutamic-acid semialdehyde (Scheme 1). Efforts to indirectly detect the glutamic-acid semialdehyde intermediate were carried out by incubating the reaction mixture with an excess of ethanolamine to capture the aldehyde. A fraction of the short-lived semialdehyde would then favor nucleophilic addition to the primary amino group of the ethanolamine over the adjacent peptide bond. A mass signal corresponding to the imine form of the ethanolamine adduct could indeed be detected, but is also formed when pure crocapeptin is treated with ethanolamine. We therefore propose that the crocapeptins exist in equilibrium between the six-membered Ahp and the five-membered 5-OH-proline heterocycles, with Ahp being the more preferred state. Incubation of precrocapeptin with ethanolamine did not result in any detectable products. Details for the aldehyde trapping experiments are provided in Supporting Information section S6.

Because the glutamic acid intermediate in the crocapeptin conversion is highly reactive, a function of CpnE, which is shown here to significantly enhance Ahp formation, could be to promote formation of Ahp and shield the reactive glutamic-acid semialdehyde intermediate from unfavorable cross-reactions.

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Whether CpnE interacts directly with CpnF or with the free substrate remains to be elucidated.

The ability of CpnF to receive electrons from the broad-range ferredoxin Adx4-108 also disclosed a route for biotechnological production of Ahp-containing molecules for targeted protease inhibitor design. From the cyanobacterial cyanopeptolins, a large variety of structures with different target specificities is known, depending mostly on the amino acids incorporated at the positions adjacent to Ahp.[11] Further studies should investigate the substrate range of CpnF and CpnE to investigate whether this enzyme pair could be used to generate Ahp-containing peptides of a specific and even non-natural structure, to create highly specific/selective protease inhibitors. For heterologous expression of the compounds, coexpression of Adx4-108 in the same host might be an option to obtain a functional cytochrome in a host that naturally has no compatible ferredoxin for CpnF.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. *C. crocatus* Cm c5 was cultivated in 70 L of Pol 0.3 medium (Probion 3 g/L, soluble starch 3 g/L, $MgSO_4 \times 7\ H_2O$ 2 g/L, $CaCl_2 \times 2\ H_2O$ 0.5 g/L) in a biofermenter Biostat UE100 (Braun Melsungen, Melsungen, D) at 30 °C for 11 days; *C. crocatus* Cm c2 was cultivated in 100 L of pol medium in a biofermenter P150 (Bioenineering AG, Wald, CH) at 30 °C for 10 days. After 3 days, 1% Amberlite XAD-16 was added to the fermentation broths. For metabolite analysis of Cm c5 mutants, 1 mL of cell clumps was used to inoculate 20 mL of Pol 0.3 medium with 50 mg/L Hyg. At least three verified clones from each inactivation construct were used. Cm c5 wild-type cells were cultivated without Hyg. After 4 days, cell clumps and medium supernatant were separated. Medium supernatant was extracted with 25 mL of ethyl acetate, and cell clumps were extracted with 10 mL of ethyl acetate and 40 mL of methanol. Extracts were dried in vacuo and dissolved in 1 mL of methanol.

Isolation and Purification of Crocapeptins. XAD-16 and cell mass of *C. crocatus* Cm c5 were harvested by centrifugation. Cells were separated from XAD-16 by flotation and discarded. The XAD was washed with water and 50% methanol, and subsequently eluted by methanol and acetone. All fractions were analyzed by HPLC–UV–MS for the presence of target compounds. Consequently, the methanol extract was subjected to solvent partition using 85% aqueous methanol, which was extracted twice with heptane. Subsequently, the aqueous methanol was adjusted to 70% methanol and extracted twice with dichloromethane. The dichloromethane fraction containing the main amount of **1**–**3** was fractionated by RP MPLC [column 480 × 30 mm, ODS/AQ C18 (Kronlab), gradient 30–100% methanol in 60 min, flow 30 mL/min, UV peak detection at 210 nm]. Fractions containing **1**–**3** were combined and further purified by preparative RP HPLC (column 250 × 21 mm, VP Nucleodur 100-10 C18 EC, gradient 40–80% methanol in 25 min, 50 mM sodium acetate, flow 20 mL/min). Finally, preparative RP HPLC (column 250 × 21 mm, VP Nucleodur C18 Gravity 5 μm, gradient 45–60% methanol in 25 min, 0.2% acetic acid, flow 20 mL/min) provided 6.2 mg of **1**, 8.9 mg of **2**, and 6.7 mg of **3**.

XAD-16 and cells of *C. crocatus* Cm c2 were washed stepwise with water (2 L) and 30% methanol (4 L), and extracted with methanol (8 L). The crude methanol extract was evaporated and partitioned three times between ethyl acetate and water. After evaporation of the ethyl acetate, the residue was dissolved in 85% aqueous methanol and extracted twice with heptane. The methanol extract was fractionated in two batches by RP MPLC (column 480 × 30 mm, ODS/AQ C18 (Kronlab), gradient 37–100% methanol in 60 min, flow 30 mL/min, UV detection 210 nm). The fraction containing **4** was separated by preparative RP HPLC (column 250 × 21 mm, VP Nucleodur C18 Gravity 5 μm, gradient 45–60% methanol/0.2% acetic acid in 25 min, flow 20 mL/min) providing 10.8 mg of **4**.

Isolation and Purification of Precrocapeptins. Cm c5 mutant *cpnF*$^-$ was grown in shake flasks with Pol 0.3 medium containing Hyg. Medium was replaced regularly, and used medium was extracted with Amberlite XAD-16 adsorber resin under sterile conditions. Pooled XAD batches were extracted with ethyl acetate, dried in vacuo, and dissolved in methanol. The crude extract was separated on a Sephadex DAE20 column (80 cm length, 2.5 cm diameter) using methanol, and precrocapeptin containing fractions were identified by LC–MS. Precrocapeptin was purified by RP-HPLC [HPLC system (Dionex) Famos autosampler, P680 pump, TCC100 thermostat, and PDA100 detector; Phenomenex Luna C18 column, 250 × 4.6 mm, 5 μm dp; solvent A = H_2O, solvent B = ACN, gradient from 20% B to 60% B in 30 min; flow rate 2.5 mL/min; temp 30 °C; UV detection 254 and 280 nm]. The sample was injected by μL-pick-up technology with a water/methanol (50:50 v/v) mixture as supporting solvent. Up to 50 μL of sample was injected for manual fraction collection. For precrocapeptin A_2 NMR data, see Supporting Information Table S3. Fractions containing 0.3 mg of precrocapeptin A1, 0.9 mg of precrocapeptin A_2, and 0.3 mg of precrpcapeptin A_3 were obtained.

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer 241 instrument; UV spectra were recorded with a Shimadzu UV–vis spectrophotometer UV-2450; IR spectra were measured with a Spectrum 100 FTIR spectrometer (Perkin-Elmer); NMR spectra were recorded with Bruker AM 300 (^{1}H 300 MHz, ^{13}C 75 MHz), Bruker ARX 600 (^{1}H 600 MHz, ^{13}C 150 MHz), and Bruker Ascend 700 with a 5 mm TXI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz) spectrometers; HRESIMS mass spectra were obtained with an Agilent 1200 series HPLC–UV system combined with an ESI–TOF–MS (Maxis, Bruker) [column 2.1 × 50 mm, 1.7 μm, C18 Acquity UPLC BEH (Waters), solvent A, H_2O + 0.1% formic acid; solvent B, AcCN + 0.1% formic acid, gradient, 5% B for 0.5 min increasing to 100% B in 19.5 min, maintaining 100% B for 5 min, FR = 0.6 mL min^{-1}, UV detection 200–600 nm].

Crocapeptin A_1 (1). Colorless, amorphous powder, $[\alpha]_D$ −34° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 230 (sh), 277 nm (3.49); IR (neat) 3380.9, 2962,2, 2934.1, 1736.0, 1655.4, 1534.4, 1518.1, 1445.4, 1384.5 cm^{-1}; ^{1}H NMR, ^{13}C NMR data (DMSO-d_6), see Supporting Information Table S3; HR–ESI–MS *m*/*z* 935.4868 $[M + H]^+$ (calcd for $C_{47}H_{67}N_8O_{12}$, 935.4873).

Crocapeptin A_2 (2). Colorless, amorphous powder, $[\alpha]_D$ −31° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 229 (sh), 277 nm (3.45); IR (neat) 3401.0, 2961,5, 2931.4, 1735.0, 1659.7, 1540.4, 1517.4, 1453.1, 1384.5 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, ROESY data (DMSO-d_6), see Supporting Information Table S1; HR–ESI–MS *m*/*z* 949.5032 $[M + H]^+$ (calcd for $C_{48}H_{69}N_8O_{12}$, 949.5027).

Crocapeptin A_3 (3). Colorless, amorphous powder, $[\alpha]_D$ −25° (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 228 (sh), 275 nm (3.43); IR (neat) 3376.6, 2961.7, 2932.6, 1735.4, 1663.7, 1535.1, 1518.2, 1452.3, 1384.4 cm^{-1}; ^{1}H NMR, ^{13}C NMR data (DMSO-d_6), see Supporting Information Table S3; HR–ESI–MS *m*/*z* 963.5186 $[M + H]^+$ (calcd for $C_{49}H_{71}N_8O_{12}$, 963.5186).

Crocapeptin B (4). Colorless, amorphous powder, $[\alpha]_D$ −44° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 229 (sh), 276 nm (3.68); IR (neat) 3377.0, 2964,1, 2933.8, 1735.2, 1648.2, 1536.1, 1518.1, 1452.4, 1384.4 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, ROESY data (DMSO-d_6), see Supporting Information Table S2; HR–ESI–MS *m*/*z* 992.5450 $[M + H]^+$ (calcd for $C_{50}H_{74}N_9O_{12}$, 992.5451), 990.5305 $[M - H]^-$ (calcd for $C_{50}H_{72}N_9O_{12}$, 990.5306).

Precrocapeptin A_2 (5). ^{1}H NMR, ^{13}C NMR, COSY, HMBC data (MeOH-d_4), see Supporting Information Table S4; HR–ESI–MS *m*/*z* 933.5070 $[M + H]^+$ (calcd for $C_{48}H_{69}N_8O_{11}$, 933.508).

Amino Acid Stereochemistry Determination. Crocapeptin A1–A3 and B were hydrolyzed in 6 N HCl at 90 °C for 17 h, conditions that resulted in the conversion of Gln to Glu. The hydrolysate was evaporated to dryness and dissolved in H_2O (100 μL). 1 N $NaHCO_3$ (20 μL) and 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (100 μL in acetone) were added, and the mixture was heated at 40 °C for 40 min. After being cooled to room temperature, the solutions were neutralized with 2 N HCl (20 μL) and evaporated to dryness. The residues were dissolved in MeOH and analyzed by

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LC−MS. Retention times in minutes of FDAA-derivatized amino acids were Glu 6.6; Thr 5.5; Leu 8.5; Phe 8.4; *N*MeTyr 5.8; Val 7.6 for **2** and Cit 5.5; Thr 5.5; Leu 8.5; Glu 6.6; Phe 8.4; *N*MeTyr 5.8; Ile 8.3 for **4**. Retention times of the FDAA-derivatized authentic standards were L-Val 7.5, D-Val 8.5 m/z 368.123 $[M - H]^-$; L-Thr 5.4, D-Thr 6.2 m/z 370.100 $[M - H]^-$; L-allo-Thr 5.3, D-allo-Thr 5.7 m/z 370.100 $[M - H]^-$; L-*N*MeTyr 5.8, D-*N*MeTyr 8.4 m/z 446.131 $[M - H]^-$; L-Phe 8.5, D-Phe 9.2 m/z 416.120 $[M - H]^-$; L-Glu 6.7, D-Glu 7.4 m/z 412.110 $[M - H]^-$; L-Leu 8.5, D-Leu 9.4 m/z 382.138 $[M - H]^-$; L-Ile 8.3, D-Ile 9.3 m/z 382.138 $[M - H]^-$; L-allo-Ile 8.4, D-allo-Ile 9.4 m/z 382.138 $[M - H]^-$; L-Cit 5.5, D-Cit 5.6 m/z 426.138 $[M - H]^-$. Oxidation of the compounds was performed as previously described.[18] **2** and **4** (0.3 mg) were dissolved in glacial acetic acid (0.3 mL) and mixed with CrO_3 (2.0 mg). After being stirred at room temperature for 20 h, the reaction mixture was separated using a C18 SPE cartridge eluting with H_2O followed by MeOH. The resulting oxidized material from the MeOH fraction was treated with FDAA as described above; L-Glu was detected at t_R 6.6 min.

Mutagenesis of *Chondromyces crocatus* Cm c5. An internal fragment of the target gene was amplified by PCR and cloned into the suicide plasmid pSUP_*hyg*,[20] yielding the respective pSUP_*hyg* derivative. After restriction analysis, the plasmid was introduced into the donor strain *E. coli* ET12567/pUB307. *E. coli* ET12567/pUB307 clones containing a pSUP_*hyg* plasmid derivative were selected on LB-Agar (low salt) at 37 °C supplemented with 50 mg/L Kanamycin sulfate (Kan), 30 mg/L Chloramphenicol (Cm), and 50 mg/L Hyg. Clones were grown in liquid LB medium (low salt), supplemented with the same antibiotic concentrations at 37 °C. Introduction of the plasmid into Cm c5 was done through biparental conjugation with *E. coli* strain ET12567/pUB307 as described elsewhere.[6] Mutant clones of Cm c5 were selected on Pol03-Agar containing 100 mg/L Hygromycin B (Hyg) and 60 mg/L Spectinomycin, followed by cultivation in liquid Pol03 medium containing 50 mg/L Hyg. Hyg-resistant clones of Cm c5 were verified genetically by PCR as described in Supporting Information S1.

Heterologous Expression and Purification of CpnE and CpnF. CpnE was expressed in *E. coli* BL21 DE3 as an N-terminal his-tagged protein using the pET28b plasmid (Novagen). Cultivation, induction of expression, and protein purification were done according to standard procedures, and CpnE purity was confirmed by SDS-PAGE. Protein concentration was determined 10 mg/mL using Bradford measurement. CpnF was expressed in *E. coli* BL21 DE3 as a SUMO-Fusion protein using pSUMO plasmid (TaKaRa). Expression and purification were done according to protocols adapted for optimized heterologous expression of cytochromes.[29] *E. coli* BL21 DE3/pSUMO_CYP clone was grown in 200 mL of TB medium supplemented with 50 mg/L Kan. As the culture reached OD_{600} = 0.6, 5-amino-levulinic acid was added to a final concentration of 1 mM, $FeCl_3$ was added to 0.5 mM, and the culture was incubated for 30 min at 26 °C before addition of IPTG to 0.2 mM. The culture was then further incubated for 63 h until cell harvest. Cleared *E. coli* lysate was separated with the ÄKTA HPLC system using a 5 mL HisTrap Nickel IMAC column. A linear gradient from 25 to 500 mM Imidazol was applied, and absorption was monitored at 280 and 430 nm. Fractions containing the absorption peak at 430 nm were subjected to SDS-PAGE, and purity of SUMO-CpnF was judged by Coomassie staining. Fractions containing the 55 kDa SUMO-CpnF bands were pooled, concentrated, and cleaved with 20 u SUMO2-protease. The cleaved SUMO-tag was separated from CpnF in a second IMAC step. Buffer was exchanged four times with 10 mM potassium phosphate buffer pH 7.4, and protein was concentrated to a volume of 0.5 mL. Protein concentration was determined 7.5 mg/mL using Bradford measurement. SDS-PAGE of the protein fraction revealed a strong band at 40 kDa, and two distinct bands at 55 and 70 kDa. These bands were excised, and subjected to in-gel-digestion and MALDI-MS analysis according to standard protocols. The signal at 40 kDa is CpnF, the signal at 55 kDa is SUMO-CpnF, and the signal at 70 kDa is the *E. coli* chaperone DnaK.

In Vitro Conversion of Precrocapeptin to Crocapeptin. Precrocapeptin was isolated from *cpnF*$^-$ mutants as described above and used as substrate for purified CpnF. As electron transporters, recombinant bovine Adx 4-108 and adrenodoxin reductase were used. In vitro conversion was performed in 10 mM potassium phosphate buffer pH 7.4 or 50 mM Tris/HCl buffer at pH 8.0 in 0.5 mL volumes. Substrate concentration was 100 μM, and enzyme concentration was 0.25 μM for CpnF and CpnE, 2 μM for AdR, and 20 μM for Adx. To regenerate NADPH, 5 mM glucose-6-phosphate, 1 mM $MgCl_2$, and 2 u/mL glucose-6-phosphate dehydrogenase were in the reaction mixture. All ingredients were mixed on ice; the reaction was started by adding 200 μM NADPH and incubated at 30 °C for 30 min. The reaction was stopped by adding 500 μL of ethyl acetate and strong mixing. After brief centrifugation, 50 μL of the organic phase was subjected to ESI−MS for detection of the reaction products.

■ CONCLUSIONS

The crocapeptins, cyanopeptolin-like cyclic depsipeptides with kallikrein and protease inhibiting bioactivity, have been isolated from the terrestrial myxobacterium *Chondromyces crocatus*. The previously unknown biosynthesis of the Ahp-heterocycle, the main structural feature crucial for bioactivity of this compound class, was elucidated biochemically. The formation of Ahp in crocapeptin A was achieved in vitro using purified precrocapeptin and recombinant tailoring enzymes CpnE and CpnF. The established ability of cytochrome P450 enzyme CpnF to receive electrons from the broad-range ferredoxin Adx4-108 opens a route for biotechnological production of Ahp-containing molecules for selective protease inhibitor design. A function could be assigned to the previously uncharacterized protein CpnE, encoding a SBPRCC-domain superfamily protein that catalyzes the rearrangement of a hydroxyproline residue to Ahp.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectroscopic data, NMR spectra of crocapeptins, details for a chymotrypsin inhibition assay, genetic verification of Cm c5 mutants, and aldehyde-trapping experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Wenzel, S. C.; Müller, R. *Mol. BioSyst.* **2009**, *5*, 567.
(2) Weissman, K. J.; Müller, R. *Nat. Prod. Rep.* **2010**, *27*, 1276.
(3) Wenzel, S. C.; Müller, R. *Nat. Prod. Rep.* **2007**, *24*, 1211.

(4) Kunze, B.; Jansen, R.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **2004**, *57*, 151.
(5) Kunze, B.; Jansen, R.; Sasse, F.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **1995**, *48*, 1262.
(6) Kunze, B.; Jansen, R.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **1994**, *47*, 881.
(7) Jansen, R.; Kunze, B.; Reichenbach, H.; Höfle, G. *Eur. J. Org. Chem.* **2003**, 2684.
(8) Steinmetz, H.; Irschik, H.; Kunze, B.; Reichenbach, H.; Höfle, G.; Jansen, R. *Chemistry* **2007**, *13*, 5822.
(9) Cortina, N. S.; Krug, D.; Plaza, A.; Revermann, O.; Müller, R. *Angew. Chem., Int. Ed.* **2012**, *51*, 811.
(10) Welker, M.; von Döhren, H. *FEMS Microbiol. Rev.* **2006**, *30*, 530.
(11) Yamaki, H.; Sitachitta, N.; Sano, T.; Kaya, K. *J. Nat. Prod.* **2005**, *68*, 14.
(12) Matern, U.; Schleberger, C.; Jelakovic, S.; Weckesser, J.; Schulz, G. E. *Chem. Biol.* **2003**, *10*, 997.
(13) Stolze, S. C.; Meltzer, M.; Ehrmann, M.; Kaiser, M. *Chem. Commun. (Cambridge, U.K.)* **2010**, *46*, 8857.
(14) Martin, C.; Oberer, L.; Ino, T.; Konig, W. A.; Busch, M.; Weckesser, J. *J. Antibiot.* **1993**, *46*, 1550.
(15) Zainuddin, E. N.; Mentel, R.; Wray, V.; Jansen, R.; Nimtz, M.; Lalk, M.; Mundt, S. *J. Nat. Prod.* **2007**, *70*, 1084.
(16) Rounge, T. B.; Rohrlack, T.; Kristensen, T.; Jakobsen, K. S. *BMC Microbiol.* **2008**, *8*, 141.
(17) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.
(18) Itou, Y.; Ishida, K.; Shin, S. J.; Murakami, M. *Tetrahedron* **1999**, *55*, 6871.
(19) Bachmann, B. O.; Ravel, J. *Methods Enzymol.* **2009**, *458*, 181.
(20) Rachid, S.; Krug, D.; Kunze, B.; Kochems, I.; Scharfe, M.; Zabriskie, T. M.; Blocker, H.; Müller, R. *Chem. Biol.* **2006**, *13*, 667.
(21) Koulov, A. V.; LaPointe, P.; Lu, B.; Razvi, A.; Coppinger, J.; Dong, M. Q.; Matteson, J.; Laister, R.; Arrowsmith, C.; Yates, J. R., III; Balch, W. E. *Mol. Biol. Cell* **2010**, *21*, 871.
(22) Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. *Chem. Biol.* **1999**, *6*, 493.
(23) Buntin, K.; Irschik, H.; Weissman, K. J.; Luxenburger, E.; Blocker, H.; Müller, R. *Chem. Biol.* **2010**, *17*, 342.
(24) Khatri, Y.; Hannemann, F.; Ewen, K. M.; Pistorius, D.; Perlova, O.; Kagawa, N.; Brachmann, A. O.; Müller, R.; Bernhardt, R. *Chem. Biol.* **2010**, *17*, 1295.
(25) Khatri, Y.; Girhard, M.; Romankiewicz, A.; Ringle, M.; Hannemann, F.; Urlacher, V. B.; Hutter, M. C.; Bernhardt, R. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 485.
(26) Ewen, K. M.; Hannemann, F.; Khatri, Y.; Perlova, O.; Kappl, R.; Krug, D.; Huttermann, J.; Müller, R.; Bernhardt, R. *J. Biol. Chem.* **2009**, *284*, 28590.
(27) Foulston, L. C.; Bibb, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 13461.
(28) Hussain, H. A.; Ward, J. M. *Appl. Environ. Microbiol.* **2003**, *69*, 373.
(29) Krastel, P.; Liechty, B.-M.; Meingassner, J. G.; Schmitt, E.; Schreiner, E. P. Cyclic Depsipeptides. WO Patent WO 2009/024527 A1, February 26, 2009.
(30) Krastel, P.; Liechty, B.-M.; Meingassner, J. G.; Moore, C.; Schmitt, E. Nucleic Acid of a Biosynthetic Cluster Encoding Non Ribosomal Peptide Synthases and Uses Therof. WO Patent WO 2010/092109 A2, August 19, 2010.