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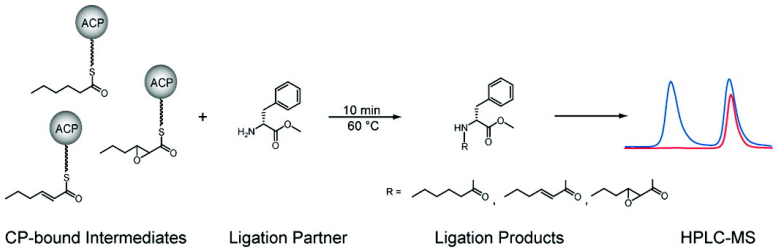

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Harnessing the Chemical Activation Inherent to Carrier Protein-Bound Thioesters for the Characterization of Lipopeptide Fatty Acid Tailoring Enzymes

Florian Kopp, Uwe Linne, Markus Oberthür, and Mohamed A. Marahiel*

Department of Chemistry, Philipps-University Marburg, Hans-Meerwein-Strasse, D-35032 Marburg, Germany

Received October 26, 2007; E-mail: marahiel@chemie.uni-marburg.de

Abstract: Here, we report a new experimental approach utilizing an amide ligation reaction for the characterization of acyl carrier protein (ACP)-bound reaction intermediates, which are otherwise difficult to analyze by traditional biochemical methods. To explore fatty acid tailoring enzymes of the calcium-dependent antibiotic (CDA) biosynthetic pathway, this strategy enabled the transformation of modified fatty acids, covalently bound as thioesters to an ACP, into amide ligation products that can be directly analyzed and compared to synthetic standards by HPLC-MS. The driving force of the amide formation is the thermodynamic activation inherent to thioester-bound compounds. Using this novel method, we were able to characterize the ACP-mediated biosynthesis of the unique 2,3-epoxyhexanoyl moiety of CDA, revealing a new type of FAD-dependent oxidase HxcO with intrinsic enoyl-ACP epoxidase activity, as well as a second enoyl-ACP epoxidase, HcmO. In general, our approach should be widely applicable for the in vitro characterization of other biosynthetic systems acting on carrier proteins, such as integrated enzymes from NRPS and PKS assembly lines or tailoring enzymes of fatty and amino acid precursor synthesis.

Introduction

Among the multitude of medicinally relevant natural products, nonribosomal lipopeptides such as daptomycin and the calcium-dependent antibiotic (CDA) have received considerable attention from basic and applied research. The last-resort antibiotic daptomycin, for example, is highly active against multidrug resistant pathogens and represents the first member of a new structural class of natural antimicrobial agents to be approved for clinical use in over 30 years.[1,2] A structural key feature of such lipopeptide antibiotics is the eponymous long chain fatty acid, which is invariably attached to the N-terminus of the cyclic peptide core. Moreover, straight and branched chain fatty acids that can significantly differ in the degree of saturation and the oxidation state (Figure 1) are frequently found and contribute to the remarkable structural diversity of this class of compounds. In particular, the lipid portion has a high impact on the biological properties of these molecules, since antimicrobial behavior and toxicity are dramatically affected by the nature of the incorporated fatty acid moiety.[2,3]

Intensive studies on modular biosynthetic assembly line machinery have provided researchers with a profound knowledge of how nonribosomal peptides (NRP) and polyketides (PK) used in different therapeutic areas are produced in nature.[4,5] However, today we still lack a detailed understanding of the biochemical mechanisms underlying fatty acid incorporation in nonribosomal lipopeptides and the dedicated tailoring steps for the generation of diverse fatty acid building blocks. Recently, important contributions from the Walsh laboratory elucidated the activation and tailoring processes involved in the formation of the β-amino fatty acid moiety of the mixed NRP/PK mycosubtilin.[6−8] The daptomycin and CDA nonribosomal peptide synthetases (NRPSs) do not contain integrated PKS elements; instead, the organization of the CDA biosynthetic gene cluster suggests a different type of fatty acid activation and modification mediated by stand-alone enzymes working *in trans* to the assembly line machinery.[9,10] Cloning and sequencing of the CDA biosynthesis genes revealed a putative *fab* operon adjacent to the NRPS, comprised of five open reading frames, which are predicted to be responsible for the synthesis and modification of the unique *trans*-2,3-epoxyhexanoyl moiety of CDA.[10]

Within this operon, the *hxcO* gene encodes for an enzyme that shows strong sequence homology to FAD-dependent acyl-

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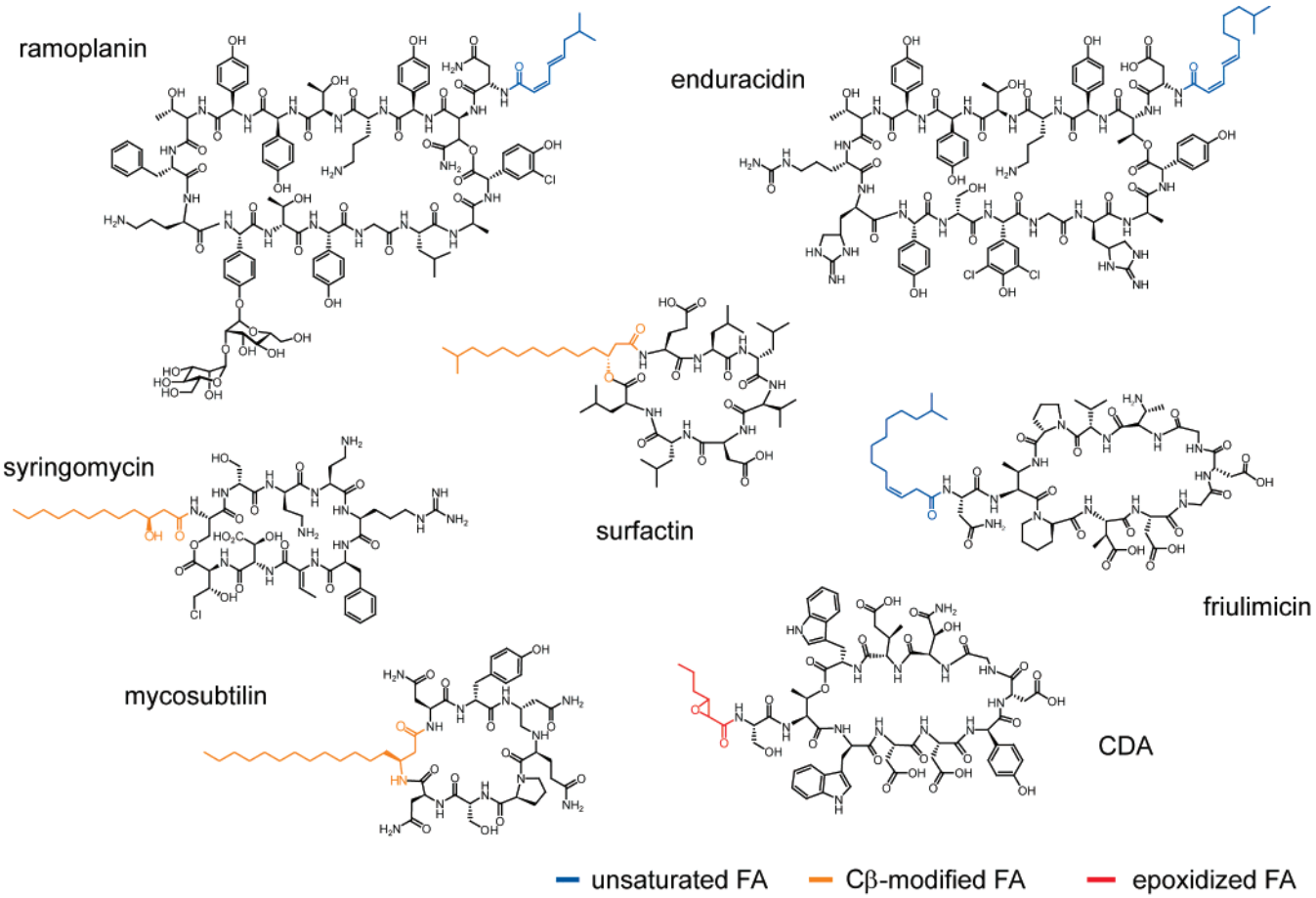

Figure 1. Chemical structures of nonribosomal peptides containing modified fatty acid building blocks. Unsaturated fatty acids (blue) and Cβ-modified fatty acids (orange) are predominantly found. The calcium-dependent antibiotic (CDA) contains a *trans*-2,3-epoxyhexanoyl moiety (red) with an unknown absolute configuration.

CoA oxidases and dehydrogenases, enzymes responsible for the desaturation of their thioester substrates in fatty acid catabolism.[11] In close proximity, *hcmO* encodes a putative flavin-dependent enoyl-CoA epoxidase, homologous to salicylate hydoroxylase and zeaxanthin epoxidase,[12] which was proposed to be responsible for the addition of the epoxy function to the enoyl-CoA substrate. Based on the sequence homologies, it was assumed that both enzymes act on CoA substrates.[10] In contrast to this, our in vitro experiments reported here reveal that the HxcO- and HcmO-catalyzed transformations occur on ACP-bound substrates.

In order to examine enzymatic epoxide formation in detail, we were confronted with several hurdles, since standard methodology for the cleavage and analysis of carrier protein (CP)-bound reaction products cannot be applied in this case. First, acyl carrier protein (ACP)-bound compounds are not recognized by the thioesterase II (TEII) for enzymatic cleavage,[13] and the use of ACP hydrolases is restricted by their high substrate specificity for native ACPs.[14,15] Second, although ACP-bound thioesters can be cleaved under alkaline conditions, the carboxylic acids obtained are often difficult to analyze, e.g. by HPLC, which makes additional derivatization steps necessary. Finally, cleavage of an ACP-bound epoxy acid under rather harsh basic conditions would obviously result in the racemization or opening of the relatively unstable epoxy function. To circumvent these problems, we developed a new experimental approach, utilizing an amide ligation reaction that allows a straightforward characterization of reaction intermediates and products of ACP-mediated pathways. By applying this strategy, we demonstrate that HxcO is a novel type of oxidase with intrinsic epoxidase activity, which suggests a different overall view of the synthesis of the 2,3-epoxyhexanoyl chain during CDA assembly. This approach allowed us to successfully characterize the biosynthesis of modified fatty acids in the CDA biosynthetic pathway and can in general be useful for the in vitro characterization of other biosynthetic processes occurring on CPs, such as fatty and amino acid precursor formation or modifications catalyzed by integrated enzymes from NRPS and PKS assembly lines.

Experimental Procedures

Cloning of hxcO, hcmO, and ACP (SCO3249) Genes. The hxcO, hcmO, and ACP (SCO3249) genes were amplified from genomic DNA of *Streptomyces coelicolor* A3(2) by PCR using the primer pairs listed in the Supporting Information (Table S1). PCRs were carried out with *Pfu* polymerase (Finnzymes) following the instructions of the manufacturer for GC-rich DNA templates. The resulting amplicons were digested with the enzymes indicated in Table S1 and cloned into

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appropriate restriction sites of a pET-28a(+) (Novagen) for hxcO and hcmO and a derivatized pQTev (Quiagen) for ACP, respectively. The resulting overexpression plasmids were checked by DNA sequencing and transformed into *E. coli* BL21(DE3) for protein production.

Heterologous Expression and Purification of HxcO, HcmO, and ACP. His-tagged proteins were overexpressed in *E. coli* BL21 (DE3) transformed with their respective overexpression plasmids. Cultures (0.5 L) in 2 L Erlenmeyer flasks were inoculated with 5 mL of an overnight culture and grown to an optical density of 0.5 at 37 °C. The cultures were cooled to 16 °C, and protein production was induced by the addition of IPTG to a final concentration of 0.1 mM. The cultures were incubated for another 14 to 16 h and then harvested by centrifugation.

For purification of ACP, HxcO, and HcmO proteins, cell pellets from 5 L of culture were resuspended in 50 mL of buffer A (50 mM Hepes, pH 8.0, 300 mM NaCl) and disrupted by the use of an EmulsiFlex-C5 High Pressure Homogenizer (Avestin). After centrifugation at 27 000 *g* for 30 min, the supernatant was carefully removed and applied to Ni-NTA chromatography on an FPLC system (Amersham Pharmacia Biotech). Briefly, the protein raw extract was run over a column filled with 500 μL of Ni-NTA superflow resin (Qiagen) with a flow rate of 0.5 mL/min. The bound proteins were washed with buffer A containing 2% buffer B (50 mM Hepes, pH 8.0, 300 mM NaCl, 250 mM imidazole) for 5 min and eluted by applying a linear gradient of 2−50% buffer B with a flow rate of 0.7 mL/min over 30 min.

Fractions containing the recombinant proteins were monitored by SDS-PAGE, pooled, and dialyzed against buffer C (25 mM Hepes, pH 7.0, 50 mM NaCl) using Hi-Trap desalting columns (GE Healthcare). For preparative expressions of HxcO and HcmO, buffer C was supplied with a 5-fold molar excess of FAD cofactor. The recombinant proteins could be stored at −20 °C for 3 months without significant loss of activity.

HPLC Analysis of HxcO and HcmO Flavin Cofactor. A 50 μL sample of purified HxcO (30 μM) or HcmO (30 μM) was boiled for 5 min, and denatured protein was removed by centrifugation. The flavin present in the supernatant was analyzed by HPLC on a C18ec Nucleodur column (Macherey and Nagel, 250/2, pore diameter of 100 Å, particle size of 3 μm) with the following gradient: 0−70% acetonitrile, 0.1% TFA in water, 0.1% TFA from 0 to 30 min at a flow rate of 0.2 mL/min. Product elution was monitored at 445 nm (Figure S2). Additionally, the identity of the cofactor was proven by mass spectroscopy (data not shown).

Synthesis of D-Phe-OMe Amides 3, 8, and 9. *N*-(Hex-2-enoyl)-D-Phe-OMe (3). To a solution of *trans*-hex-2-enoic acid (**1**, 230 mg, 2.0 mmol), DIPEA (0.77 mL, 4.4 mmol), and HOBt monohydrate (398 mg, 2.6 mmol) in dichloromethane (7 mL) was added EDCI (500 mg, 2.6 mmol) at 0 °C with stirring. After 5 min, D-Phe-OMe hydrochloride (**2**, 430 mg, 2.0 mmol) was added, and stirring was continued at room temperature for 16 h. The mixture was diluted with dichloromethane, washed with 1 N HCl, satd. aq. $NaHCO_3$ solution, and brine, dried ($MgSO_4$), filtered, and concentrated. Flash chromatography (SiO_2, petroleum ether/ethyl acetate 2:1 → 1:1) of the residue gave amide **3** (510 mg, 92%) as a colorless syrup. R_f = 0.44 (petroleum ether/ethyl acetate 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.16−7.24, 7.00−7.04 (2m, 3H and 2H, arom. H), 6.78 (dt, 1H, $J_{2,3}$ = 15.3, $J_{3,4}$ = 7.0 Hz, 3-H), 5.88 (bd, 1H, $J_{\alpha,NH}$ = 7.7 Hz, NH), 5.70 (dt, 1H, $J_{2,3}$ = 15.3, $J_{2,4}$ = 1.5 Hz, 2-H), 4.89 (dt, 1H, $J_{\alpha,\beta a} = J_{\alpha,\beta b}$ = 5.6, $J_{\alpha,NH}$ = 7.7 Hz, α-H), 3.65 (s, 3H, OMe), 3.11 (dd, 1H, $J_{\alpha,\beta}$ = 5.6, $J_{\beta,\beta}$ = 13.9 Hz, β-H_a), 3.06 (dd, 1H, $J_{\alpha,\beta}$ = 5.6, $J_{\beta,\beta}$ = 13.9 Hz, β-H_b), 2.07 (dq, 2H, $J_{2,4}$ = 1.5, $J_{3,4} = J_{4,5}$ = 7.0 Hz, 4-H_2), 1.40 (sext, 2H, $J_{4,5} = J_{5,6}$ = 7.0 Hz, 5-H_2), 0.85 (t, 3H, $J_{5,6}$ = 7.0 Hz, 6-H_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.1 (COOMe), 165.4 (CONH), 145.6 (C-3), 135.9, 129.3, 128.5, 127.1 (arom. C), 123.1 (C-2), 53.1 (C-α), 52.3 (OMe), 37.9 (C-β), 34.1 (C-4), 21.4 (C-5), 13.6 (C-6); ESI-MS calcd for $C_{16}H_{21}NO_3$: 275.15, found 275.1 $[M]^+$.

***N*-(Hex-2,3-epoxyhexanoyl)-D-Phe-OMe (8 (2*R*,3*S*) and 9 (2*S*,3*R*)).** The stereoselective preparation of **8** from (2*S*,3*S*)-2,3-epoxyhexanol (**4**), obtained by Sharpless epoxidation of hex-2-enol,[16] is described. (2*S*,3*S*)-Epoxy alcohol **4** (115 mg, 1.0 mmol) was dissolved in acetonitrile/carbon tetrachloride (1:1, 5 mL). To this solution, water (5 mL), ruthenium trichloride monohydrate (5 mg), and sodium periodate (1.0 g, 4.7 mmol) were added, and the mixture was stirred vigorously for 16 h at room temperature. Following the addition of dichloromethane (50 mL) and brine (50 mL), the emulsion was filtered through Celite, upon which phase separation occurred. The organic phase was dried ($MgSO_4$), filtered, and concentrated. Flash chromatography (SiO_2, dichloromethane/methanol/AcOH 10:1:0.1) of the residue gave the crude (2*R*,3*S*)-acid **6** (100 mg) as a dark, amorphous solid, which was used directly for the next step. To a solution of crude (2*R*,3*S*)-acid **6**, DIPEA (0.42 mL, 2.4 mmol), and HOBt monohydrate (185 mg, 1.2 mmol) in dichloromethane (3 mL) was added EDCI hydrochloride (230 mg, 1.2 mmol) at 0 °C with stirring. After 5 min, D-Phe-OMe hydrochloride (**2**, 260 mg, 1.2 mmol) was added, and stirring was continued at room temperature for 16 h. The mixture was diluted with dichloromethane, washed with 1 N HCl, satd. aq. $NaHCO_3$ solution, and brine, dried ($MgSO_4$), filtered, and concentrated. Flash chromatography (SiO_2, petroleum ether/ethyl acetate 2:1 → 1:1) of the residue gave amide **8** (125 mg, 43% over 2 steps) as a colorless syrup. R_f = 0.44 (petroleum ether/ethyl acetate 2:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.16−7.24, 6.98−7.01 (2m, 3H and 2H, arom. H), 6.43 (d, 1H, $J_{\alpha,NH}$ = 7.9 Hz, NH), 4.76 (ddd, 1H, $J_{\alpha,\beta a}$ = 5.8, $J_{\alpha,\beta b}$ = 7.0, $J_{\alpha,NH}$ = 7.9 Hz, α-H), 3.68 (s, 3H, OMe), 3.13 (dd, 1H, $J_{\alpha,\beta}$ = 5.8, $J_{\beta,\beta}$ = 14.0 Hz, β-H_a), 3.08 (d, 1H, $J_{2,3}$ = 2.1 Hz, 2-H), 2.94 (dd, 1H, $J_{\alpha,\beta}$ = 7.0, $J_{\beta,\beta}$ = 14.0 Hz, β-H_b), 2.52 (ddd, 1H, $J_{2,3}$ = 2.1, $J_{3,4a}$ = 4.7, $J_{3,4b}$ = 5.8 Hz, 3-H), 1.30−1.48 (m, 4H, 4-H_2, 5-H_2), 0.88 (t, 3H, $J_{5,6}$ = 7.1 Hz, 6-H_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.4 (COOMe), 168.3 (CONH), 135.7, 129.2, 128.6, 127.2 (arom. C), 59.4 (C-3), 55.1 (C-2), 52.4 (OMe), 52.2 (C-α), 37.8 (C-β), 33.5 (C-4), 18.9 (C-5), 13.6 (C-6); ESI-MS calcd for $C_{16}H_{21}NO_4$: 291.15, found 291.1 $[M]^+$.

HPLC retention time (gradient: 0−60% acetonitrile, 0.1% TFA in water, 0.1% TFA from 0 to 20 min at a flow rate of 0.4 mL/min on a ChiraDex Gamma column (Merck KGaA, Darmstadt, Germany, 250/4, particle size of 5 μm): t = 15.4 min.

For the synthesis of the diastereomeric mixture **8**/**9**, racemic 2,3-epoxyhexanol (**4**/**5**), obtained by *m*CPBA oxidation of hex-2-enol,[17] was used in an analogous fashion. As expected, HPLC analysis using the conditions described above showed two peaks of equal intensity (**8**: t = 15.4 min, **9**: t = 14.4 min). NMR data for **9**: ^{1}H NMR (300 MHz, $CDCl_3$): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.16−7.24, 7.00−7.04 (2 m, 3H and 2H, arom. H), 6.42 (d, 1H, $J_{\alpha,NH}$ = 8.2 Hz, NH), 4.77 (dt, 1H, $J_{\alpha,\beta}$ = 6.0, $J_{\alpha,NH}$ = 8.2 Hz, α-H), 3.63 (s, 3H, OMe), 3.13 (d, 1H, $J_{2,3}$ = 2.0 Hz, 2-H), 3.03 (t, 2H, $J_{\alpha,\beta}$ = 6.0 Hz, β-H_2), 2.87 (m, 1H, 3-H), 1.35−1.52 (m, 4H, 4-H_2, 5-H_2), 0.88 (t, 3H, $J_{5,6}$ = 7.0 Hz, 6-H_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.5 (COOMe), 168.4 (CONH), 135.5, 129.1, 128.7, 127.2 (arom. C), 59.4 (C-3), 55.0 (C-2), 52.3 (2x, OMe and C-α), 37.9 (C-β), 33.6 (C-4), 18.9 (C-5), 13.8 (C-6).

Synthesis of Fatty Acid-CoA Substrates. Coenzyme A trilithium salt (0.05 mmol), the fatty acid (0.10 mmol), PyBOP (0.08 mmol), and K_2CO_3 (0.20 mmol) were dissolved in 4 mL of THF/water (1:1) and incubated for 2 h at room temperature. After lyophilization, the resulting white solids were dissolved in water and purified by HPLC (Agilent, 1100 series) on a preparative Nucleodur C18ec column (Macherey and Nagel, 250/2, pore diameter of 100 Å, particle size of 3 μm) with a gradient of 5−70% acetonitrile, 0.1% TFA in water, 0.1% TFA over 30 min at a flow rate of 20.0 mL/min. Product elution was monitored at 215 nm, and the identity was confirmed by MALDI-TOF MS analysis (Table S2).

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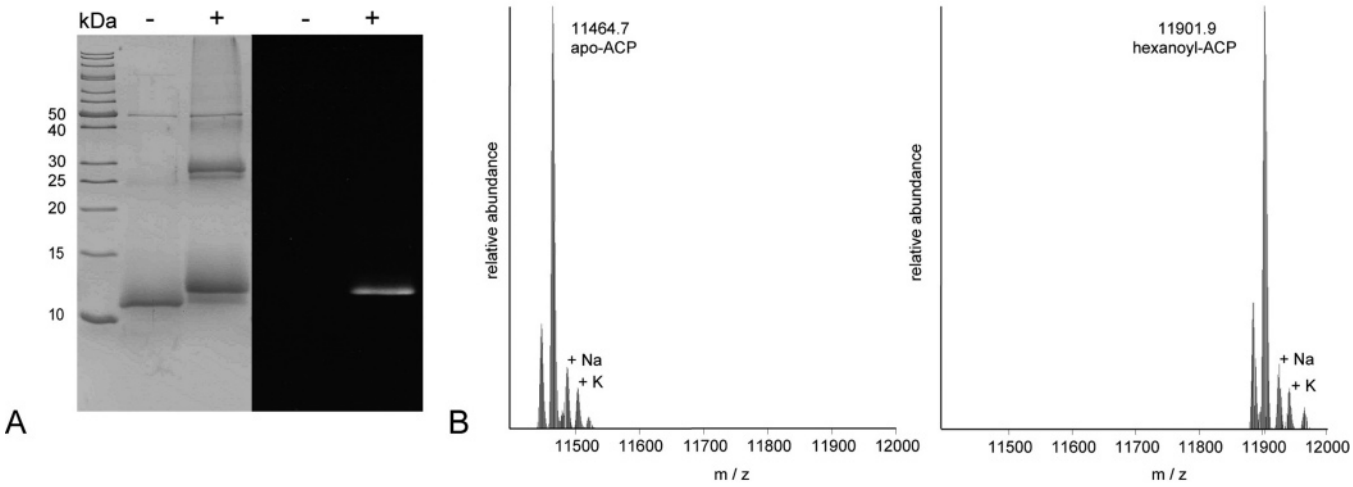

Figure 2. In vitro phosphopantetheinylation of apo-ACP. (A) Coomassie stained SDS-PAGE (left) and in-gel fluorescence (right) of the ACP laoding mixture with (+) and without (−) Sfp, the phosphopantetheine transferase, loading from *B. subtilis*. (B) FTMS broad band spectrum of ACP (c.m.: 11464.7) before and after loading with hexanoic acid. (c.m.: 11901.9).

Chemoenzymatic Synthesis of CDA Analogues. The chemoenzymatic synthesis of hexanoyl-CDA and hex-2-enoyl-CDA was carried out as described previously.[18] Briefly, linear peptidyl substrates were prepared on a 0.1 mmol scale via Fmoc solid-phase peptide synthesis and C-terminally activated with a thiophenol leaving group. After deprotection and preparative HPLC purification, the linear substrates were enzymatically cyclized by CDA TE domain in a semipreparative scale and purified by HPLC yielding macrocyclic hexanoyl-CDA and hex-2-enoyl-CDA. The identity of the products was confirmed by MALDI-TOF (Table S3).

In Vitro 4′-Phosphopantetheinylation of ACP. A reaction mixture containing 200 μM fluorescein-CoA/acetyl-CoA, 200 μM ACP, 10 mM $MgCl_2$, and 50 μM recombinant *Bacillus subtilis* 4′-phosphopantetheine transferase (PPTase) Sfp in assay buffer (buffer C) was incubated at 30 °C for 30 min and analyzed directly by LC-ESI-MS. The loading of fluorescein-CoA, which was prepared as previously reported,[19] onto the ACP was monitored by measuring the in gel fluorescence.

Assays with ACP-bound Acyl Substrates. Acyl-phosphopantetheinylation of the ACP was achieved by incubating the carrier protein with Sfp and acyl-CoA substrates. The reaction mixture was incubated at 30 °C for 30 min and contained 200 μM ACP, 200 μM Acyl-CoA substrate, 2 mM $MgCl_2$, and 50 μM Sfp in a 25 mM HEPES buffer pH 7.5 (assay buffer). The solution was desalted using a Micro Bio-Spin 6 column (Bio-Rad).

HxcO Oxidation/Epoxidation Assay. A typical reaction mixture (150 μL) for the detection of HxcO epoxidation product was prepared using 100 μM loaded acyl-ACP substrate, 5 μM HxcO, and 250 μM FAD in assay buffer. After incubation at 25 °C for 30 min, 10 μg of trypsin (Promega) were added to the reaction mixture and incubated for 5 min at 30 °C. Finally, 15 μL of formic acid were added, and the samples were subjected to HPLC and high-resolution ESI-FTICR-MS analysis carried out with an LTQ-FT mass spectrometer (Finnigan, Bremen) using the following gradient: 25−50% acetonitrile, 0.045% formic acid in water, 0.05% formic acid over 30 min at a flow rate of 0.2 mL/min on a Jupiter C4 column (Phenomenex, 150 mm × 2 mm, 5 μm) at 40 °C. To achieve maximum sensitivity of the described ESI-FTICR-MS method, the ms experiment was carried out at single-reaction monitoring (SRM) mode of the LTQ mass analyzer. Masses selected for MS^2 were 1046.5 ($[M + H]^{2+}$ of hexenoyl-*S*-Ppan-fragment) and 1054.5 ($[M + H]^{2+}$ of 2,3-epoxy-hexenoyl-*S*-Ppan-fragment).

HcmO Epoxidation Assay. Typical incubations (150 μL) for the detection of HcmO epoxidation products were prepared using 100 μM loaded acyl-ACP substrate and 10 μM HcmO, 250 μM FAD, and 250 μM NAD(P)H in assay buffer. After 30 min at 25 °C, 10 μg of trypsin (Promega) were added and incubated for another 5 min at 30 °C. The tryptic digest was stopped by the addition of 15 μL of formic acid, and the reaction volumes were analyzed by ESI-FTICR-MS as reported for HxcO assays.

Analysis of ACP-Bound Products by Direct Amide Ligation. To further characterize the ACP-bound products of HxcO and HcmO reactivity the complete reaction mixtures were incubated with a 1000-fold excess of D-Phe-OMe (**2**) at 60 °C for 2 h. The precipitated proteins were removed by centrifugation. Protein pellets were washed twice with 25 μL of assay buffer, and the combined fractions were concentrated to 50 μL prior to HPLC-MS analysis.

For epoxide product detection, the reaction volumes were compared to the synthesized standard using the following gradient: 0−60% acetonitrile, 0.1% TFA in water, 0.1% TFA from 0 to 20 min at a flow rate of 0.4 mL/min on a ChiraDex Gamma column (Merck KGaA, Darmstadt, 250/4, particle size of 5 μm).

The HPLC-MS analysis of the 2,3-unsaturated fatty acid was carried out on a standard C18ec Nucleodur column (Macherey and Nagel, 250/2, pore diameter of 100 Å, particle size of 3 μm) with the following gradient: 0−60% acetonitrile, 0.1% TFA in water, 0.1% TFA from 0 to 20 min at a flow rate of 0.2 mL/min.

Assays with Acyl-CoA Substrates and Chemoenzymatically Derived CDA Analogues. HxcO and HcmO assays with acyl-CoA substrates and chemoenzymatically derived CDA analogs were carried out as reported in the Supporting Information.

Results

Characterization of SCO3249 as an Acyl Carrier Protein. Similar to the synthesis of NRPs and PKs, the assembly of fatty acids catalyzed by Fatty Acid Synthases (FASs) occurs on CPs, to which the substrates, reaction intermediates, and products are covalently tethered during biosynthesis.[20] SCO3249 of the CDA cluster, in the following referred to as ACP, showed strong sequence homology to CPs from NRPS and PKS and was likely to be involved in the biosynthesis of the CDA lipid portion.

The N-terminally His_7-tagged ACP was overproduced in *E. coli* BL21(DE3) and purified as described in the Experimental

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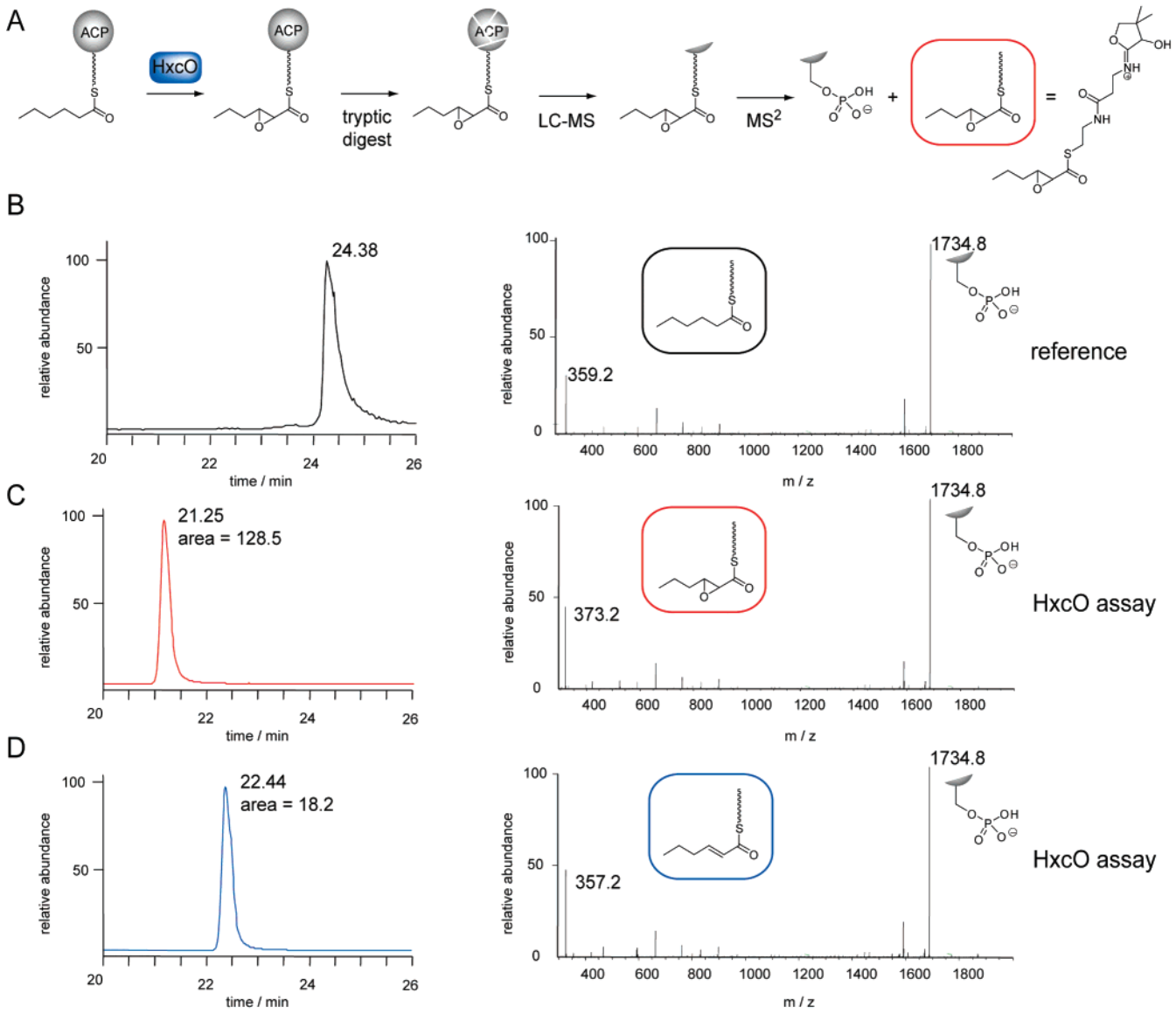


Figure 3. HxcO assay. (A) Experimental setup. HxcO reaction products were digested with trypsin and subjected to HPLC prior to tandem FTMS spectrometry. (B) Single reaction monitoring (SRM; 1046.5 → [1732.30−1737.30]) of hexanoyl-*S*-ACP (Lys^{57}-Arg^{71}) and MS^2 data of the ppan ejection assay resulting from a reference assay without HxcO. (C) SRM (1054.5 → [1732.30−1737.30]) of epoxyhexanoyl-*S*-ACP (Lys^{57}-Arg^{71}) and MS^2 data of the ppan ejection assay resulting from a HxcO assay. (D) SRM (1046.5 → [1732.30−1737.30]) of hex-2-enoyl-*S*-ACP (Lys^{57}-Arg^{71}) and MS^2 data of the ppan ejection assay resulting from a HxcO assay. This HxcO side product is formed in ∼15% compared to epoxyhexanoyl-*S*-ACP (indicated by areas in C and D).

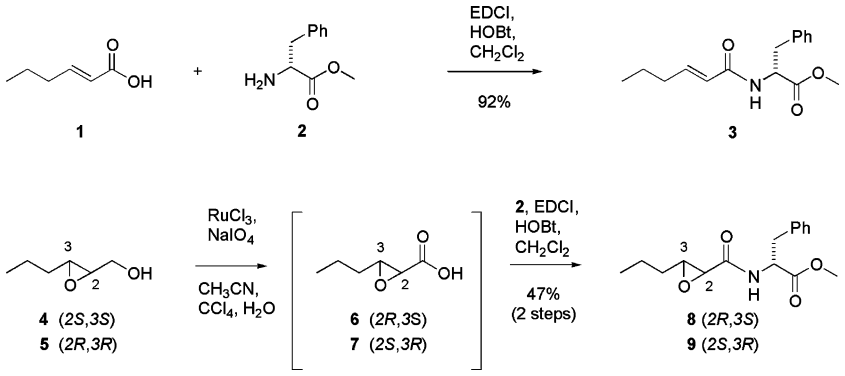
Procedures yielding 8 mg of protein per liter of culture. The identity of the expressed protein was proven by mass spectrometry and SDS-PAGE (Figure S1). To test whether ACP could be loaded in vitro, the recombinant protein was incubated with the 4′-phosphopantetheine transferase (PPTase) (Sfp) from *Bacillus subtilis* and fluorescein-CoA. The 4′-phosphopantetheinylation of ACP was monitored by the in-gel fluorescence of the loading mixture (Figure 2 A); shown are positive and negative control experiments. Additionally, the loading with fatty acid CoA-substrates to the ACP was verified by mass spectroscopy (Figure 2 B).

Characterization of HxcO as a Fatty Acid-*S*-ACP Oxidase with Intrinsic Epoxidase Activity. HxcO shows sequence homology in the range of 30% to several members of the acyl-CoA dehydrogenase/oxidase superfamily that catalyze double bond formation between C-2 and C-3 of their thioester substrates.[11] Based on the sequence homology, it was also postulated that HcmO is responsible for the oxidation of a hexanoyl-CoA substrate to 2,3-hexenoyl-CoA.[10] Alternatively, different scenarios with an ACP-bound HxcO substrate or the possibility that the oxidation occurs at the stage of the already assembled CDA molecule are conceivable. Accordingly, in vitro assays to examine all three possible routes were conducted.

The N-terminally His_7-tagged HxcO was overproduced in *E. coli* BL21(DE3) and purified yielding ∼1.0 mg of protein per liter of culture. The identity of the protein was confirmed by mass spectrometry and SDS-PAGE (Figure S1). HxcO was colorless and showed no spectral absorbance typical for flavin cofactors. However, HPLC analysis of HxcO supernatant after heat denaturation revealed the presence of FAD as the electron accepting cofactor (Figure S2). This observation suggests that HxcO utilizes FAD as a free coenzyme.

To test if acyl-CoAs are the physiological substrates of HxcO, the purified enzyme was assayed with synthetic hexanoyl-CoA

Scheme 1. Synthesis of Chemical Standards[a]



[a] EDCI = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole monohydrate.

under a variety of assay conditions typical for acyl-CoA dehydrogenases and oxidases.[21,22] No oxidation products were detected by HPLC-MS. Additional substrates with a chain length from 4 to 10 C-atoms (Figure S3) did not reveal the enzymatic introduction of a double bond. Analogously, experiments with chemoenzymatically generated hexanoyl-CDA showed no fatty acid oxidation, indicating that HxcO does not act on the mature CDA molecule.

Next, Sfp, which had previously been shown to be promiscuous for the in vitro phosphopantetheinylation of various acyl- and peptidyl-CoA substrates onto carrier proteins,[23] was employed to assay the alternative ACP-mediated pathway. The required hexanoyl-ACP substrate was prepared and incubated with HxcO. After tryptic digest, the samples were subjected to HPLC high-resolution Fourier-transform mass spectrometry (FTMS) to analyze product formation (Figure 3).

The ppan ejection assay[24] of samples obtained from negative control reaction volumes without the enzyme resulted in the observation of a 1734.8 Da signal corresponding to the active site ACP fragment (Lys^{57}-Arg^{71}) without the phosphopantetheine (ppan) arm that was cleaved off during gas-phase fragmentation. Additionally, a fragment ion of 359.2 Da was detected, which is consistent with the calculated mass of the hexanoyl-ppan MS−MS fragmentation product (Figure 3B). After incubation with HxcO, a fragment ion of 373.2 Da (Figure 3C) and a second signal of 357.2 Da (Figure 3D) with ∼15% intensity compared to the 373.2 signal could be detected. These masses are consistent with the calculated masses of the hex-2-enoyl-ppan (357.2 Da) and the epoxyhexanoyl-ppan (373.2 Da) fragmentation products. HxcO showed no conversion of the unsaturated fatty acid-*S*-ACP in the presence of FAD and NAD(P)H cofactors, which are commonly used for the generation of $FADH_2$ by flavin dependent monooxygenases. This suggests that HxcO generates $FADH_2$ in situ by the oxidation of the saturated fatty acid substrate. The reduced cofactor is then the reactive species for the epoxidation reaction.

To prove whether the unexpected epoxyhexanoyl-product, which was predominantly found (∼85%), is of enzymatic origin or caused by H_2O_2 formed in vitro during the reoxidation of $FADH_2$, catalase was added to the assay in different concentrations. Further, reaction buffers with different pH values in the range from 5 to 9 were tested to decompose potentially formed H_2O_2. No influence on product formation was observed. Therefore, epoxyhexanoyl-*S*-ACP was clearly identified to be the main product of HxcO reactivity. HxcO displayed epoxide formation (Figure S4) with a $k_{obs} = 0.3\ min^{-1}$, whereas double bond formation did not increase over the examined period of time.

The substrate specificity of HxcO was evaluated with additional linear fatty acids (4−10 C-atoms) loaded onto an ACP (Figure S3). As with the physiological substrate, these reactions revealed the formation of the expected enoyl product paired with the most apparent species (∼85%), a fragment ion with a mass of +16 compared to the enoyl product.

To further characterize the ACP-bound product formed by HxcO, we envisioned an HPLC-based comparison with synthetic standards. We therefore had to establish conditions that would allow the efficient transformation of the modified fatty acid-*S*-ACPs into derivatives of smaller size, which would then in turn be analyzed by HPLC. Initial experiments using an excess of a simple amine showed that this approach was indeed valid. For example, after incubation of an HxcO reaction with benzylamine, LC-MS analysis of the reaction mixture showed the formation of the expected mass for the amide ligation products (203.2 and 219.2 Da, data not shown). However, we were unable to determine the stereochemistry of the epoxide formed by HxcO by comparison with a synthetic standard as the two enantiomeric benzyl amides (2*S*,3*R* and 2*R*,3*S*) showed the same retention time using a chiral cyclodextrin-based column (data not shown). Therefore, we turned our attention to chiral ligation partners. In this case, the possible products are diastereomeric instead of enantiomeric and therefore hopefully easier to distinguish by HPLC analysis. Accordingly, we oxidized a racemic mixture of 2,3-epoxyhexanols **4**/**5** with $RuCl_3/NaIO_4$[25] and coupled the resulting carboxylic acids **6**/**7** with different chiral amines using EDCI/HOBt. Best results in terms of separation of the reaction products were obtained using the amino acid derivative D-Phe-OMe (**2**, Scheme 1) as a ligation partner and a chiral column for the HPLC analysis of the diastereomeric mixture of amides **8** and **9** (Scheme 1). Phenylalanine derivative **2** could also be used successfully for the analysis of the HxcO reaction. Under optimized conditions, HxcO reaction volumes containing the reactive fatty acid-*S*-ACP thioester products were incubated with a 1000-fold molar excess of D-Phe-OMe (**2**), which afforded

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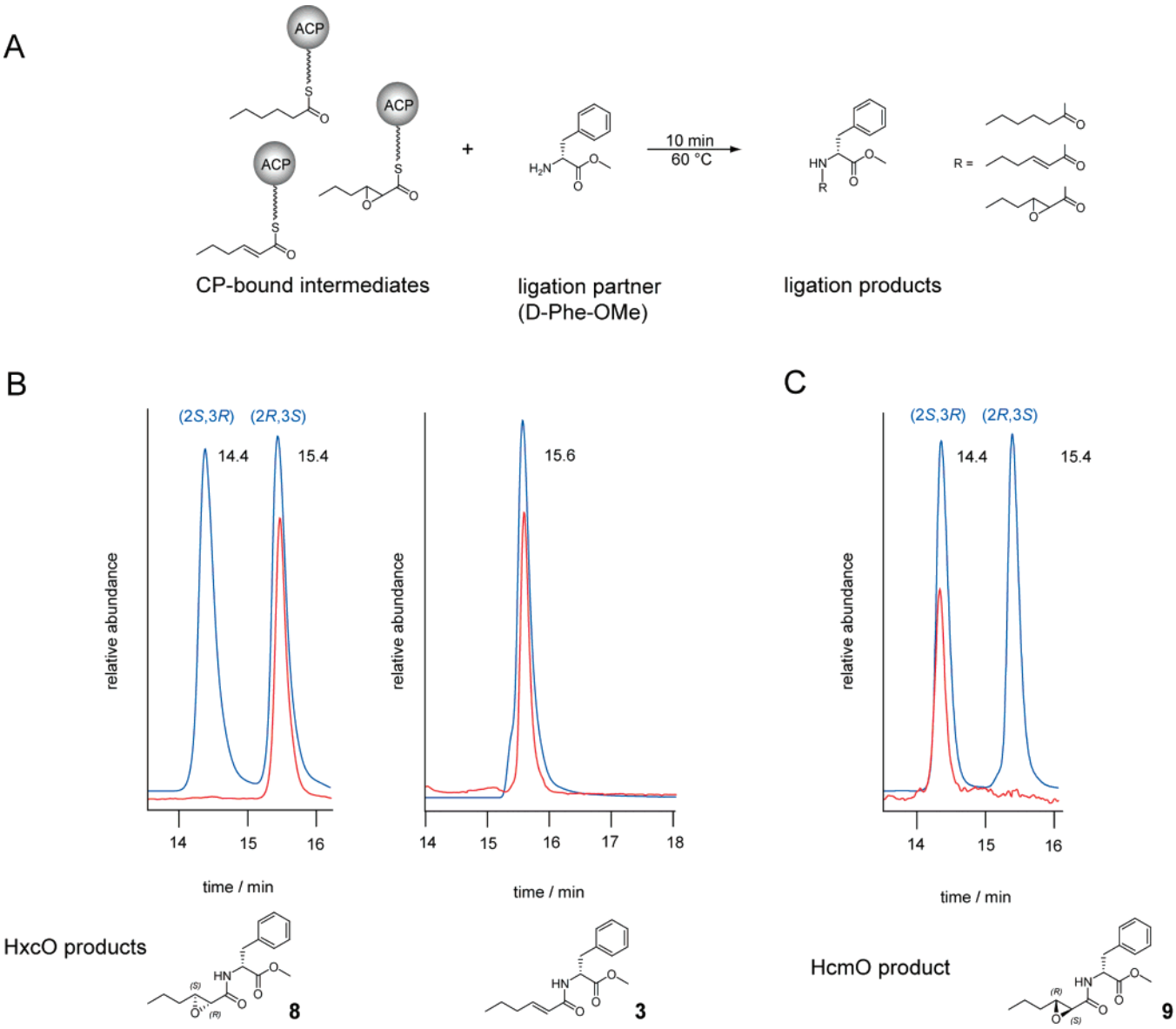

Figure 4. Characterization of HxcO and HcmO reaction products via direct carrier protein amide ligation. (A) Cleavage of ACP-bound thioesters with a nucleophile (D-Phe-OMe) allows the comparison of enzymatic reaction products bound to an ACP with synthetic standards by HPLC-MS. (B) Amide ligation products of HxcO reactions coelute with the (2*R*,3*S*)-2,3-epoxyhexanoyl-D-Phe-OMe standard **8** ($[M + H]^+ = 291.1$). The HxcO side product was proven to be identical with the synthetic hexenoyl amide **3** ($[M + H]^+ = 275.1$). (C) The reaction product of HcmO coelutes with the (2*S*,3*R*)-2,3-epoxyhexanoic acid **9** ($[M + H]^+ = 291.1$). Shown are extracted ion chromatograms (EICs).

amide ligation products suitable for LC-MS analysis. The chromatograms clearly evidenced that the epoxide generated by HxcO is formed as a single enantiomer (Figure 4B). In order to establish the exact stereochemistry of the enzyme product, we next synthesized one of the stereoisomers, i.e., amide **8**, selectively. For this purpose, enantiomer **4**, obtained by Sharpless epoxidation of hex-2-enol,[16] was oxidized[25] and coupled with D-Phe-OMe **2** to provide the (2*R*,3*S*)-configured **8** (>90% ee, Scheme 1). Comparison of the HPLC retention time of stereoisomer **8** with the two peaks obtained from the diastereomeric mixture **8**/**9** then led to the structural assignments shown in Figure 4B and C. The HPLC trace of a HxcO reaction established that the HxcO reaction product has the (2*R*,3*S*)-configuration (Figure 4B). The formation of the 2,3-hexenoic acid side product, which occurred only to a minor extent, was also proven by comparison with the synthetic standard **3**, which was obtained by EDCI/HOBt-mediated coupling of hex-2-enoic acid (**1**) and D-Phe-OMe (**2**, Scheme 1).

Characterization of HcmO as a Fatty Acid-S-ACP Epoxidase. HcmO shows amino acid homology to the diverse class of flavin-dependent monooxygenases, particularly salicylate hydroxylases[26] and the zeaxanthin epoxidases[12] in the range of 40% and 30%, respectively. To determine whether HcmO acts on ACP-bound hexenoyl-substrates or on coenzyme A thioesters, both possibilities were assayed.

The N-terminally His_7-tagged HcmO was produced in *E. coli* BL21(DE3) and purified with a yield of ~1.0 mg protein per liter of culture. The identity of the protein was proven by mass

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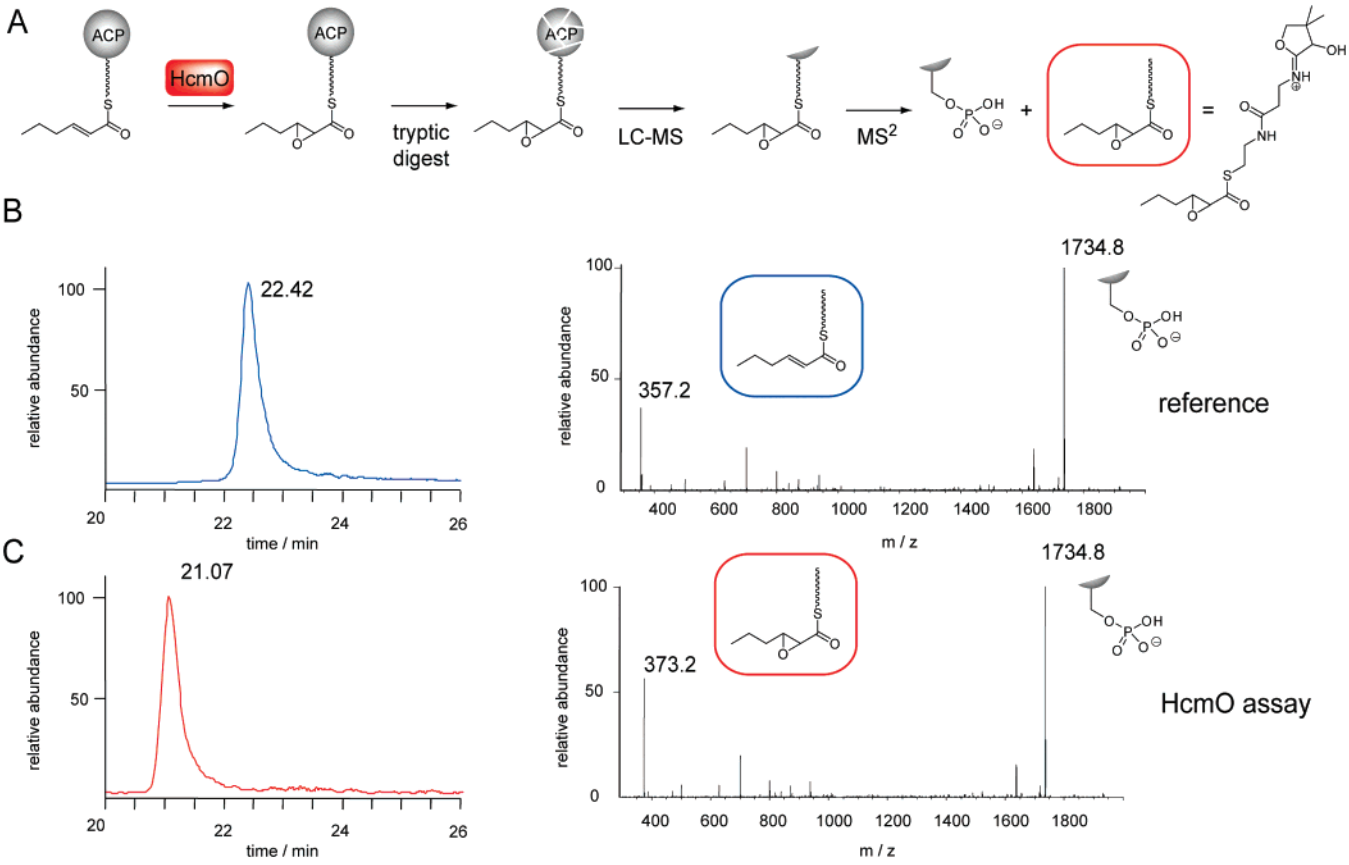

Figure 5. HcmO assay. (A) Experimental setup. HcmO reaction products were digested with trypsin and subjected to HPLC and tandem FTMS spectrometry. (B) Single reaction monitoring (SRM; 1046.5 → [1732.30−1737.30]) corresponding to the hex-2-enoyl-*S*-ACP (Lys^{57}-Arg^{71}) and MS^2 data of the ppan ejection assay from a reference without HcmO. (C) SRM (1054.5 → [1732.30−1737.30]) of the HcmO reaction product 2,3-epoxyhexanoyl-*S*-ACP (Lys^{57}-Arg^{71}) and MS^2 data of the ppan ejection assay.

spectrometry and SDS-PAGE (Figure S1). In contrast to HxcO, HcmO is bright yellow in color, and shows absorption maxima at 377 and 450 nm, typical of a flavin cofactor (Figure S2). HPLC analysis of HcmO supernatant after heat denaturation identified FAD as the flavin cofactor.

The putative physiological substrate of HcmO, a hexenoyl-moiety, was loaded onto the ACP with Sfp and subsequently incubated with HcmO at a 25-fold molar excess of NAD(P)H and FAD. Subsequent FTMS of the proteolytic mixture resulting from tryptic digest was used to characterize HcmO reactivity (Figure 5A). The ppan ejection assay[24] of samples obtained from negative control reaction volumes without the enzyme resulted, in addition to the 1734.8 Da signal corresponding to the active site ACP fragment (Lys^{57}-Arg^{71}) without the ppan arm, in a fragment ion of 357.2 Da, which is consistent with the calculated mass of the hex-2-enoyl-ppan MS−MS fragmentation product (Figure 5B). The HcmO reaction revealed the formation of the fragment ions of the active site ACP(Lys^{57}-Arg^{71}) without the ppan arm (1734.8) accompanied by the epoxy acid ppan product (373.2 Da), both resulting from MS^2 fragmentation (Figure 5C). A hexanoyl-*S*-ACP substrate could not be converted into the epoxide product by HcmO that shows only 10% amino acid sequence similarity with HxcO. Further, the ensuing application of the described amide ligation strategy enabled the comparison of the epoxy fatty acid with a synthetic standard (Figure 4C) and proved the identity of the HcmO reaction product to be (2*S*,3*R*)-2,3-epoxyhexanoic acid.

The substrate specificity of HcmO was assessed with additional enoyl-ACP substrates (Figure S3). Whereas variations of the position and the configuration of the double bond were not tolerated, a crotonyl moiety with a chain length shorter than that of the physiological C_6 substrate was accepted. HcmO was able to epoxidize the hexenoyl-substrate utilizing both NADH and NADPH cofactors. In contrast, the hexenoyl-CoA substrate and chemoenzymatically generated hex-2-enoyl-CDA could not be epoxidized by incubation with HcmO.

Discussion

Herein, we report the biochemical characterization of three enzymes of the CDA biosynthetic gene cluster, HxcO, HcmO, and ACP (SCO3249), and their mechanistic role in the biosynthesis of the unique 2,3-epoxyhexanoyl moiety of CDA.

The obtained results are surprising in two aspects: First, predictions based on sequence similarities favored HxcO and HcmO to be a fatty acid oxidase and epoxidase, respectively, both acting on acyl-CoA substrates. However, our experiments with a set of acyl-CoA, acyl-ACP, and chemoenzymatically derived CDA substrates clearly demonstrate that only fatty acids loaded onto the recombinant ACP (SCO3249) are accepted by HxcO and HcmO. In addition to the physiological substrate, hexanoyl-*S*-ACP, HxcO accepted a range of ACP-bound substrate analogues, namely linear fatty acids of 4−10 C-atoms. HcmO epoxidation activity, instead, showed a more restricted substrate specificity and was limited to hex-2-enoyl- and

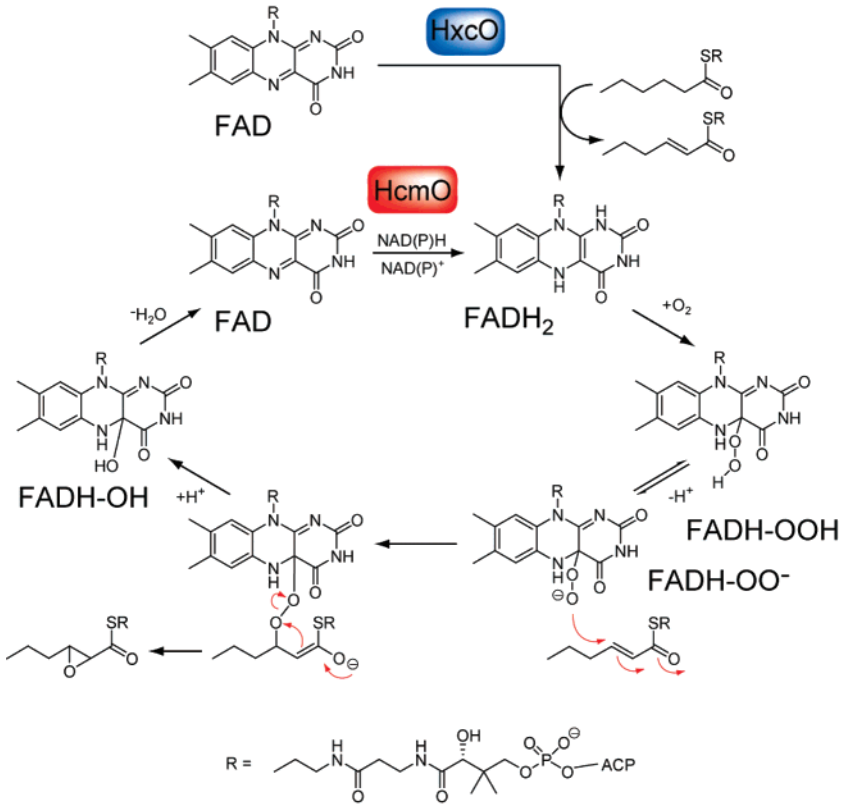

Figure 6. Proposed mechanism of epoxidation catalyzed by HxcO and HcmO. During fatty acid oxidation (HxcO) or by the consumption of NAD(P)H (HcmO) $FADH_2$ is generated. The reaction with molecular oxygen affords the flavin C4a-hydroperoxide intermediate, which acts as a nucleophile in the attack of the 2,3-hexenoyl-*S*-ACP substrate originating from either HxcO reactivity or FAS enzymes. Finally, the C4a-hydroxy flavin intermediate dehydrates to regenerate the oxidized FAD.

crotonyl-*S*-ACP substrates. Second, whereas HcmO showed the expected epoxidase activity, the characterization of the putative dehydrogenase HxcO showed the time dependent formation of 2,3-epoxyhexanoyl-*S*-ACP as the main product and only minor amounts of the expected hex-2-enoyl-*S*-ACP product.

To further characterize the identity of HxcO and HcmO enzymatic products by comparison to synthetic standards, we were confronted with the problem that standard methodology for the cleavage and analysis of CP-bound substances could not be applied in this case. Enzymatic cleavage by TEII is only possible for peptidyl carrier protein (PCP)-bound substrates,[13] and the use of ACP hydrolases is restricted by their high specificity for native ACPs.[14,15] Relatively harsh alkaline treatment, on the other hand, could hydrolyze the epoxy function and lead to racemization. To establish alternative conditions that would allow the direct transformation of the ACP-bound enzyme products into derivatives of smaller size appropriate for standard HPLC-MS analysis, we envisioned cleaving the reactive acyl-*S*-ACP thioesters by incubation with amine nucleophiles, thereby taking advantage of the chemical activation already inherent to thioester-bound substrates. Initial experiments with hydrophobic amines, e.g., benzylamine, showed indeed the formation of the desired amide ligation products suitable for HPLC analysis and comparison with synthetic standards. However, we were unsuccessful in determining the absolute stereochemistry of the epoxides generated by HxcO and HcmO, respectively, because enantiomeric synthetic standards were not well resolved by HPLC using a chiral column.

Accordingly, we turned our attention to chiral ligation partners, such as D-α-methyl benzylamine, (*S*)-2-phenyl-1-propylamine, or amino acid methyl esters, e.g., D-Phe-OMe (**2**). The rationale for this change was the now diastereomeric relationship of the two possible epoxyhexanoyl amide ligation products, which was anticipated to lead to a better separation by HPLC. Synthesis of 2,3-epoxyhexanoyl amides using racemic 2,3-epoxyhexanoic acid (**6**/**7**, Scheme 1) and the amines mentioned above and analysis of the diastereomeric product mixtures by HPLC using a chiral column showed that, only in the case of the *N*-acylated phenylalanine esters **8** and **9** (Scheme 1), a sufficient peak separation was obtained. Gratifyingly, phenylalanine derivative **2** could also successfully be used as a nucleophile in the amide ligation reaction, where it efficiently cleaved the ACP-bound thioesters. Using this strategy, we could clearly establish that the epoxide generated by HxcO has the (2*R*,3*S*)-configuration (Figure 4B). Interestingly, the 2,3-epoxyhexanoyl moiety generated by the second putative tailoring enzyme studied here, HcmO, was shown to be of opposite configuration (2*S*,3*R*) based on HPLC analysis of the ligation product. This experimental approach was therefore essential for this study, since it enabled the detailed analysis of the CP-bound HxcO and HcmO enzyme products as well as the unambiguous assignment of product stereochemistry by comparison to synthetic standards.

Taken together, it turned out that HxcO is a new type of FAD-dependent acyl-ACP oxidase with intrinsic epoxidase activity, responsible for the desaturation and subsequent epoxidation of the initially formed enoyl-ACP product. Regarding the oxidation mechanism, it is likely that, similar to the case of related acyl-CoA oxidases, flavin C4a-hydroperoxide (FADH-OOH) is formed during the reoxidation of $FADH_2$ by molecular oxygen

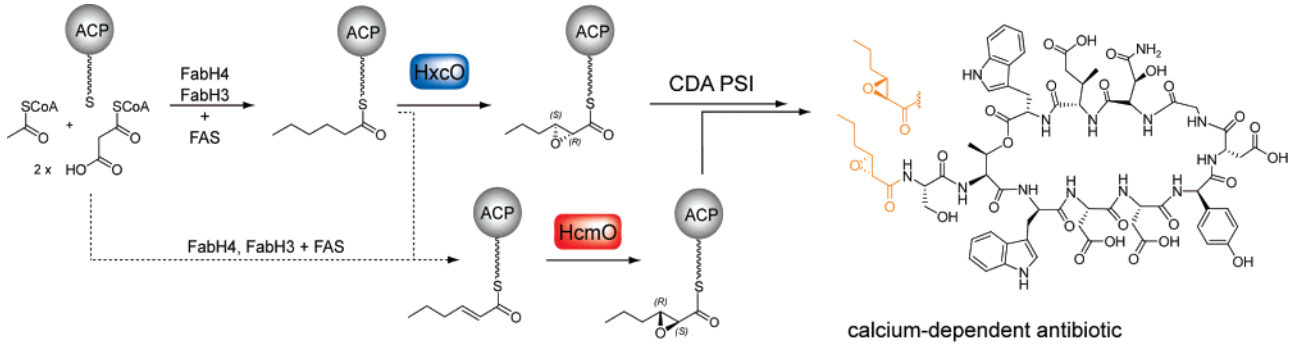

Figure 7. Proposed model for the ACP-mediated biosynthetic pathway of the unique *trans*-2,3-epoxyhexanoyl moiety found in CDA. The two epoxidases HxcO and HcmO are responsible for the generation of the two shown 2,3-epoxyhexanoyl enantiomers. Either FAS enzymes from primary metabolism together with FabH3 and FabH4, or HxcO are likely to produce the hex-2-enoyl substrate for HcmO. The absolute configuration of the epoxyhexanoyl side chain in the CDA antibiotics is unknown up to now. CDA PSI = CDA Peptide Synthetase I.

(Figure 6). Instead of decomposing to FAD and H_2O_2, FADH-OOH could be deprotonated and serve as a nucleophile in the subsequent epoxidation of the electron deficient double bond. Therefore, the enoyl substrate can undergo conjugated addition with the reactive FAD-OO^- species following a mechanism that has been proposed for the Baeyer−Villiger type oxidation of cyclic ketones by FAD-dependent epoxidases (Figure 6).[27] The biochemical characterization of HcmO as a flavin-dependent epoxidase revealed the formation of the 2,3-epoxyhexanoyl residue while acting on the ACP-bound hexenoyl-substrate. In this case, $FADH_2$ is generated by the oxidation of NAD(P)H, and as described for HxcO, epoxidation is also likely to occur with FAD-OO^- as the nucleophilic species (Figure 6).

The existence of two epoxidases within the CDA gene cluster that display different stereoselectivity gives rise to speculations on the molecular logic underlying the CDA epoxyhexanoic acid biosynthesis. A total of five genes comprise the putative *fab* operon that is involved in fatty acid biosynthesis and tailoring of the CDA epoxyhexanoyl moiety. The hexanyol-ACP substrate for HxcO is likely to be produced by the interplay of FabH4 and FabH3, putative β-ketoacyl-ACP synthases encoded within the *fab* operon, together with enzymes from primary metabolism. Subsequently, fatty acid tailoring by HxcO occurs on the ACP-bound hexanoyl moiety and results in the (2*R*,3*S*)-2,3-epoxyhexanoyl product and minor amounts of the hex-2-enoyl-ACP, as demonstrated in this study. This "side product" of HxcO could then serve as the substrate for subsequent epoxidation by HcmO. Alternatively, FAS enzymes from primary metabolism could directly produce the hex-2-enoyl-ACP substrate for HcmO during fatty acid synthesis on the ACP. Based on the finding that two epoxidases exist in the CDA cluster, one might speculate that HxcO has gained its epoxidation activity during evolution and produces now together with HcmO the two possible 2,3-epoxyhexanoyl enantiomers. However, it remains to be seen whether both enantiomers are then transferred from the ACP to the first module of the CDA peptide synthetases (Figure 7). NMR studies allied with a total synthesis approach are currently underway to investigate the stereochemistry of the 2,3-epoxyhexanoyl moiety in the natural product.

Overall, the data presented here support the view that fatty acid tailoring during NRPS synthesis occurs on ACP-bound substrates. The biosynthetic gene clusters of other nonribosomal lipopeptides, e.g., enduracidin,[28] ramoplanin,[29] and friulimicin,[30,31] also contain stand-alone fatty acid tailoring enzymes. Most particularly, gene knock-outs in the friulimicin NRPS system confirmed the role of an acyl-CoA-dehydrogenase to be involved in the formation of the *cis* double bond of the N-terminal lipid moiety.[30] In analogy to the examined CDA fatty acid epoxidation, ACP-mediated tailoring pathways seem now to be likely for the above-mentioned examples. This hypothesis is additionally supported by the fact that in close proximity to the genes of the tailoring enzymes ACP encoding segments were found for each biosynthetic system.

Conclusions

Here, we have shown that the described amide ligation strategy is a robust system for the rapid and sensitive detection of CP-bound substances. Using this powerful approach new tailoring enzymes of CP-mediated pathways could be stereochemically characterized, which was not possible via established methodology. Within the CDA biosynthetic system, HxcO was shown to be a novel type of enzyme with a dual function as an FAD-dependent fatty acid-*S*-ACP oxidase paired with intrinsic epoxidase activity. A second epoxidase HcmO was shown to be responsible for the production of another 2,3-epoxyhexanoic enantiomer during CDA biosynthesis. It remains to be shown whether both enantiomers are then incorporated into the natural product. In addition to this, fatty acid tailoring during CDA biosynthesis was determined to occur on ACP-bound substrates. Taken together, the obtained biochemical data provide new insights into fatty acid tailoring of nonribosomal lipopeptides and may facilitate future projects aiming to manipulate the lipid portion of these compounds. Moreover, the herein presented approach holds great potential for the detailed analysis of other biochemical processes involving CP-bound intermediates, a central paradigm in secondary metabolism.

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Supporting Information Available: Oligonucleotide primers, SDS-PAGE of studied enzymes, HxcO and HcmO cofactor analysis, synthesized acyl-CoA substrates, experimental details and kinetic data. This information is available free of charge via the Interment at http://pubs.acs.org.

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