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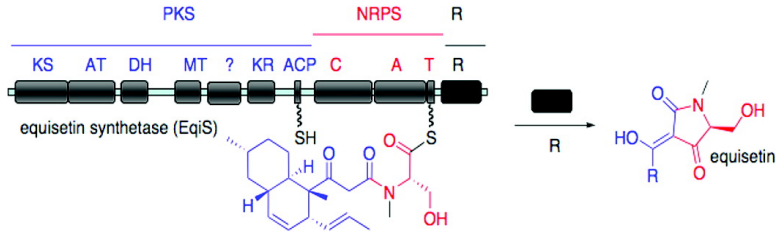

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Thioesterase-Like Role for Fungal PKS-NRPS Hybrid Reductive Domains

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Abstract: Fungal reduced polyketides possess diverse structures exploring a broad region of chemical space despite their synthesis by very similar enzymes. Many fungal polyketides are capped by diverse amino acid-derived five-membered rings, the tetramic acids and related pyrrolidine-2-ones. The known tetramic acid synthetase enzymes in fungi contain C-terminal reductive (R) domains that were proposed to release reduced pyrrolidine-2-one intermediates en route to the tetramic acids. To determine the enzymatic basis of pyrrolidine-2-one diversity, we overexpressed equisetin synthetase (EqiS) R domains and analyzed their reactivity with synthetic substrate analogs. We show that the EqiS R domain does not perform a reducing function and does not bind reducing cofactors. Instead, the EqiS R catalyzes a Dieckmann condensation, with an estimated $k_{cat} \approx 15\ s^{-1}$. This role differs from the redox reactions normally catalyzed by short chain dehydrogenase/reductase superfamily enzymes.

Introduction

Equisetin (**1**) is a polyketide tetramic acid from filamentous fungi that exemplifies a broad group of structurally diverse, biosynthetically related tetramic acids and derivatives (Figure 1).[1–4] Equisetin is active in an array of assays and inhibits HIV-1 integrase,[1,5–7] leading to its use as a model for the development of the clinically approved integrase inhibitor, raltegravir.[8,9] It is structurally and biochemically related to lovastatin, the canonical antihypercholesteremia agent that underlies the multibillion-dollar statin drug class.[10–12] In addition, equisetin is biosynthetically related to a broad array of active fungal tetramic acids and their derivatives, including fusarin C (**2**),[13] cytochalasins,[14] and relatives such as chaetoglobosin A (**3**),[15] tenellin (**4**),[16] and pseurotin A (**5**),[17] that are important in agriculture and medicine. These compounds contain either an intact tetramic acid ring or reduced and highly modified pyrrolidine-2-one rings.

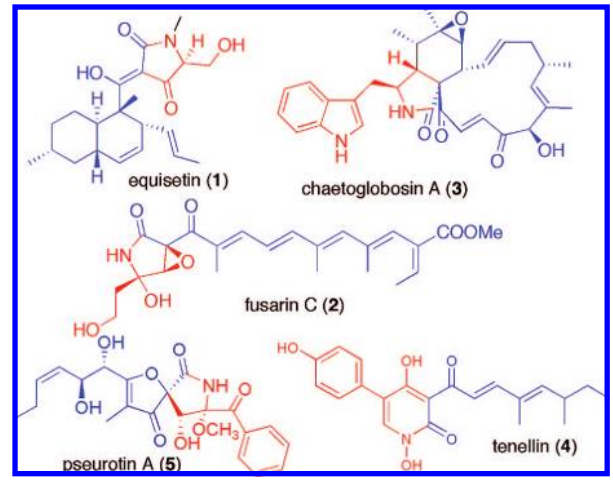

Figure 1. Representative polyketide-pyrrolidine-2-one metabolites from fungi. Polyketide-derived portions are blue, whereas amino acid-derived portions, including the pyrrolidine moieties, are red. Equisetin is the only true "tetramic acid" shown. All compounds shown are synthesized by homologous proteins.

The genetic basis of this structural diversity has been elusive. Genes have been identified for an array of different tetramic acid derivatives with variable carbon skeletons and pyrrolidine-2-one modifications.[18–24] In all of these cases, the predicted protein consists of a polyketide synthase (PKS)-nonribosomal peptide

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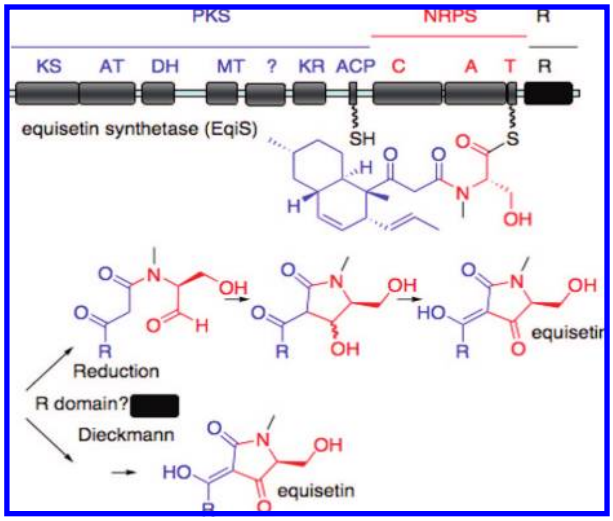

Figure 2. Proposed biogenesis of equisetin and possible roles of R domain. A polyketide portion (blue) is synthesized by the PKS, whereas serine (red) is appended by the NRPS. A covalently enzyme-bound thioester intermediate is the substrate for R, which would cleave the intermediate from the enzyme. R could catalyze a reduction, leading to an aldol reaction followed by oxidation to give equisetin. Alternatively, it could function as a Dieckmann cyclase. The timing of *N*-methylation is unclear.

synthetase (NRPS) hybrid, terminating in an apparently typical NRPS reductive (R) domain (Figure 2). The PKS proteins appear to be fungal highly reducing iterative synthases, which use the same set of domains in an iterative manner to cobble together carbon skeletons from acetyl- and malonyl-CoA.[11,12,25–27] Prediction of the resulting polyketide structure using gene sequences is beyond current art, as all of these PKS genes appear quite similar despite vastly different carbon skeletons. Subsequently, the NRPS module is proposed to append an amino acid, resulting in a beta-ketoamide aminoacetyl-thioester. This enzyme-bound thioester intermediate is primed for cleavage to yield the final pyrrolidine-2-one derivative. While the polyketide structural diversity is likely controlled by the PKS, the genetic basis for pyrrolidine-2-one diversity remained unclear.

Two proposals have been advanced to explain the diverse pyrrolidine-2-ones in this class of fungal polyketide metabolites. NRPS R domains have been characterized in a number of cases to reduce enzyme-bound thioesters using NAD(P)H as a cofactor, resulting in aldehydes or alcohols.[28–32] In the pyrrolidine-2-one family, it was possible that a reductive process would lead to a universal aldol intermediate (Figure 2). This aldol could undergo a variety of different fates, including oxidation back to the tetramic acid, oxidation at another site to yield pyrrolidine-2-one derivatives, or Diels–Alder cyclization to yield the cytochalasin family. Since the known NRPS R domains and virtually all of their relatives in the short chain dehydrogenase/reductase (SDR) superfamily catalyze redox reactions, this proposal appeared to be strongly supported.[33] Like these characterized reductive NRPS proteins, EqiS and other pyrrolidine NRPSs contain adenylation (A) and thiolation (T) domains, which activate amino acids as covalent enzyme-linked thioesters,[34] as well as condensation (C) domains, which catalyze peptide bond formation.[35] The NRPS proteins thus follow the standard domain order CATR. Recently, the tenellin PKS was heterologously expressed, resulting in tetramic acid formation.[36] This result supported a second possibility, that tetramic acids themselves are directly offloaded by this enzyme group via a Dieckmann condensation (Figure 2). In this scenario, the genetic basis for compound diversity would be less clear. However, as this result was obtained in vivo, it remained possible that a reduction–oxidation process occurred within whole fungal cells.

Since understanding the mechanism of tetramic acid formation would allow improved drug discovery and biosynthetic engineering in this important compound class, we set out to test these and other possibilities using the equisetin biosynthetic gene cluster *eqi* from *F. heterosporum*.[19] Synthesis of intermediate analogs and kinetic analysis using purified equisetin synthetase (EqiS) protein domains revealed that the EqiS R domain is a Dieckmann cyclase, not a reductase. The proposed chemical mechanism bears some similarity to that catalyzed by thioesterase enzymes in PKS and NRPS metabolism, indicating a new role for fungal NRPS R domains. These results have implications for the evolution of the diverse fungal polyketide–pyrrolidine-2-one compound family.

Experimental Methods

Expression of EqiS Domains. Detailed experimental methods for generating vectors, chemical synthesis, and other procedures can be found in the Supporting Information. C-terminal domain constructs of EqiS were expressed in *E. coli*, resulting in proteins that were purified to homogeneity using Ni-NTA resin followed by FPLC with a Superdex SD200 size exclusion column. This column was also used on a series of size standards, allowing the size of eluted proteins to be accurately estimated. Expressed proteins included ATR, TR, and R domains fused to N-terminal 6×-His tags. Purity of proteins was assessed by FPLC and SDS-PAGE. TR was analyzed by ESI–MS: ESI–MS [M + H_2O] 52784 ($C_{2341}H_{3707}N_{651}O_{709}S_{14}$ [M + H_2O] calcd 52783).

R Domain Putative Substrate and Product Synthesis. For mechanistic analysis, substrate analogs that were capable or incapable of undergoing a Dieckmann condensation were synthesized. Briefly, these consisted of acetoacetyl-alanine and acetyl-alanine, respectively. To imitate covalent enzyme attachment, these substrates were attached as thioesters to *N*-acetylcysteamine or coenzyme A (CoA) using the transthiolation method.[37] Thioester substrates were purified to homogeneity by HPLC and characterized by NMR and MS. Standards of putative products of enzymatic

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reactions were synthesized as follows: The tetramic acid resulting from acetoacetyl-Ala was synthesized by the conditions of Lacey,[38] purified to homogeneity, and characterized by NMR and MS. A putative aldehyde product of acetyl-Ala was synthesized by partial reduction using DIBALH. The unstable intermediate was characterized by TLC, staining with 2,4-dinitrophenylhydrazine. Detailed methods, including spectral data for reported compounds, are available in the Supporting Information.

Covalent Loading of T Using Sfp and Synthetic Substrate Analogs. The TR protein was characterized by loading the T domain with various Coenzyme A analogs via Sfp.[39,40] TR (180 μL, 16 μM) was incubated with Sfp (3 μL, 10 μM) in 50 mM Bis-Tris pH 6.0 or HEPES pH 6.0, 10 mM $MgCl_2$, and 0.4 mM substrate at 30 °C for 1 h. Loading of fluorescent analog was measured by an Odyssey Infrared Imaging System (Li-COR). Competition assays were performed under identical conditions, incubating the acetylalanine-CoA or acetoacetylalanine-CoA derivative (0.4 mM) for 1 h prior to addition of Alexafluor-CoA. To test reactivity, the pH was adjusted to 7 after 1 h and NADPH added. TR loaded with acetylalanine-pantetheine: ESI−MS [M + H_2O] 53236 ($C_{2357}H_{3733}N_{654}O_{717}PS_{15}$ [M + H_2O] calcd 53234).

Kinetic Analysis of R. The reductive domain was characterized using synthetic substrate mimics. Enzymatic assays were performed using 3.2 μM purified R or TR domains, 2−40 mM substrates, 1.5 mM NADPH, 10 mM $MgCl_2$, 10 μM $MnCl_2$, and 50 mM NaCl at 25 °C. Also, these reactions were reproduced in the presence and absence of 1.5 mM NADH or NADPH. The assays were quenched by addition of conc. H_2SO_4. The acidic aqueous mixture was extracted with a slight excess of $CHCl_3$ containing 5 ng/μL of limonene as internal standard. The $CHCl_3$ layer was analyzed by GC−MS for product identification, or by FID for kinetic analysis. Relative rates of reaction were determined by comparing the ratio of the tetramic acid product to the internal standard limonene. A standard curve was generated using synthetic tetramic acid at varying concentrations. Both enzyme and substrate concentrations were varied along with other controls to determine the rates of the reaction.

Control Reactions. R exhibited unusual behavior in that it was relatively thermostable (Figure S5, Supporting Information). To ensure that the observed reaction was due to enzymatic activity, Qiagen Protease was used in a series of control reactions. Qiagen Protease in water was either boiled for 20 min at 100 °C or left on ice. The R domain (16 μM) was treated with 8 μL (50 mU) of boiled or unboiled protease for 1 h at rt. These were subsequently used in enzyme assays as control reactions.

To test for cofactor binding, R (16 μM 20 μL) was mixed with urea (9 M, 80 μL) and incubated at rt for 1 h. The resulting mixture was analyzed on a HP UV−vis spectrophotometer and by ESI−MS in comparison to authentic standard of NADPH and $NADP^+$. The limits of NADPH detection were estimated to be <30 nM with similar injection volumes, providing excellent sensitivity.

Product Analysis. Chemical products from enzymatic reactions were detected in a number of ways. Ketoamide derivatives were readily observed on TLC plates following staining with ferric chloride, while aldehydes could be detected on TLC using 2,4-dinitrophenylhydrazine. Volatile standards could be readily observed by GC−MS and confirmed on the basis of the parent ion mass. Thus, several different, sensitive detection methods were used to observe the products of enzyme reactions. These methods were used with many different enzyme reaction conditions, and only tetramic acid products were detected. In addition, data concerning degradation of synthetic intermediates was obtained both via kinetic analysis and by examining ^{1}H NMR spectra following different treatments. Formation of tetramic acid from thioester derivatives could be clearly observed by NMR. Thus, conditions for enzyme reactions were determined following numerous chemical and enzymatic preliminary investigations.

NADPH Consumption. The reaction mixture used to test for NADPH consumption was identical to that used in reductive domain characterization, which has been previously described above. Consumption was analyzed by monitoring loss of absorbance at 340 nm in 40 μL reactions in a 96 well plate in triplicate on a Vector3V automated UV plate reader (Perkin−Elmer) for 2 h with a 5 min delay between measurements. Reactions of 100 μL were observed using a HP UV−vis spectrophotometer. Every 5 min, an aliquot of the reaction was diluted to 1:100 and measurements were taken. Following loading of TR, the pH was increased to 7.0 and 1.5 mM NADPH was added. This mixtures was immediately placed in SpectraMax M5 (Molecular Devices) automated plate reader, monitoring change in absorbance 340 nm for 1 h. A buffer only sample, lacking protein, served as a negative control.

Results

Heterologous EqiS NRPS Domains Are Stably Expressed and Folded. To assess R domain function three protein constructs of EqiS were expressed and purified as soluble, N-terminal 6xHis-tagged proteins, including ATR, TR, and R. Unfortunately, ATR existed in several oligomeric states that complicated analysis (Figure S3, Supporting Information). By contrast, both TR and R proteins were monomers that were more uniform and stable than ATR (Figure S5, Supporting Information), so they were used for further analysis. Proper folding of TR was tested by enzymatically loading a fluorescent-CoA derivative using Sfp, a broad substrate pantetheinyltransferase. TR treated with this derivative was brightly fluorescent, indicating that TR contained a competent thiolation domain (Figure 3). Both TR and R were further analyzed by CD and mass spectrometry. HPLC−ESI−MS analysis of the tryptic digest of TR and R showed that these were the predicted proteins (data not shown). Both pantetheine-modified TR and unmodified TR were also analyzed as intact proteins by ESI−MS, confirming that TR was pantetheinylated to afford nearly 100% holoprotein (Figure 3). CD spectra of the TR and R show that both are structured proteins with melting temperatures of >90 and 75 °C, respectively (Figure S5, Supporting Information). Thus, TR and R were used to test the mechanism of tetramic acid formation in equisetin.

EqiS R Domain Produces Tetramic Acids and Not Reduced Intermediates. On the basis of several considerations, there were three likely possibilities for the formation of equisetin (Figure 2). First, R could catalyze a reduction, yielding an aldehyde that would aldol cyclize. Subsequent reoxidation by R or another protein would give the tetramic acid. Second, R could directly catalyze a Dieckmann condensation to yield the tetramic acid. Third, R may be uninvolved in tetramic acid formation. Synthetic substrate analogs were designed to tease apart these mechanistic possibilities (Figure 4). All of the analogs are thioesters, resembling the presumed enzyme-linked thioester substrate of wild-type R.[31,32] Ala was used in place of Ser for ease of synthesis. Finally, Ala was modified by *N*-acetylation or *N*-acetoacetylation. The acetyl derivative was designed so that it could in principle be reduced by R, but the pK_a of the acetate methyl is too high to allow aldol condensation in this

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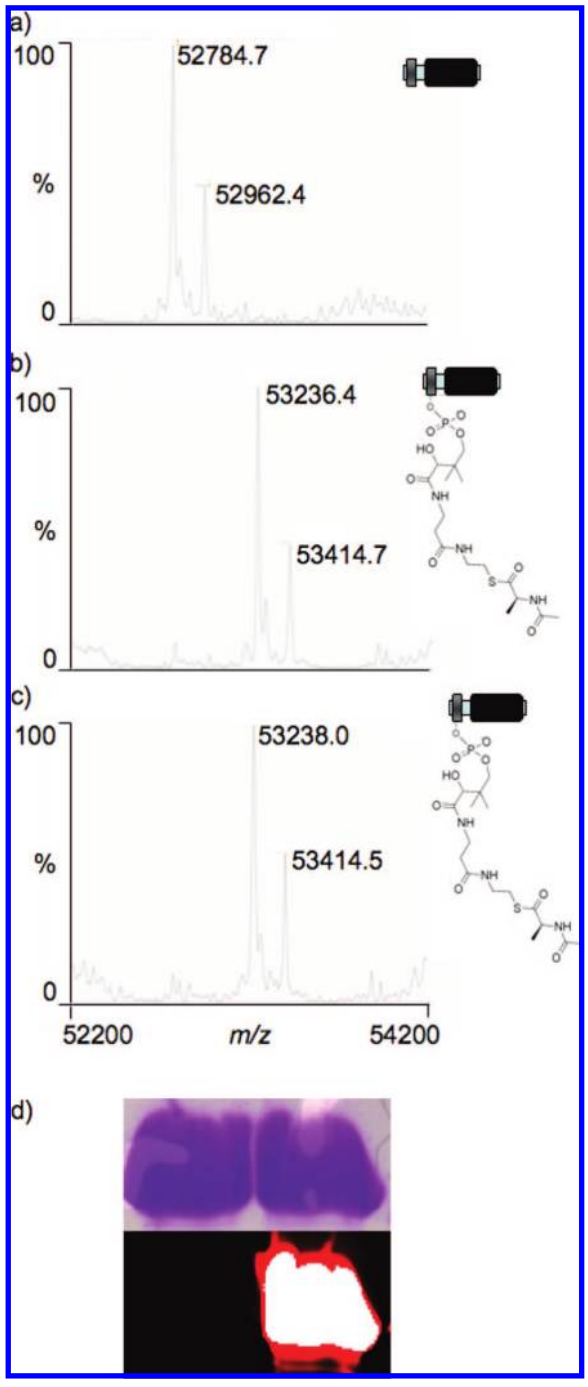

Figure 3. Covalent modification of T domain. a−c are ESI−MS of whole proteins, *y*-axis is % abundance, *x* axis is *m*/*z* from 52200 to 54200 Da. Smaller peaks at +178 are likely due to gluconoylation of the His-tag.[41,42] (a) ESI−MS of purified T−R didomain. (b) ESI−MS of T−R loaded with acetylalanine-CoA using Sfp pantetheinyltransferase. (c) ESI−MS of acetylalanine-loaded T−R following incubation with NADPH at pH 7. No reductive cleavage occurs, and the shift of 2 Da for 1 of the 2 peaks is within error. (d) Coomassie staining (top) and fluorescence analysis of T−R competition loading experiments. The protein in the left lane was treated with acetylalanine-CoA and Sfp for 1 h, followed by treatment with fluorescent CoA. The protein at right was treated with buffer and Sfp for 1 h, then with fluorescent CoA. This shows that T−R is ∼100% covalently loaded by Sfp.

context. Thus, this substrate would allow direct monitoring of a thioester reduction process. The acetoacetyl derivative could be reduced, yielding aldol products, or alternatively it is also a substrate for the Dieckmann condensation.

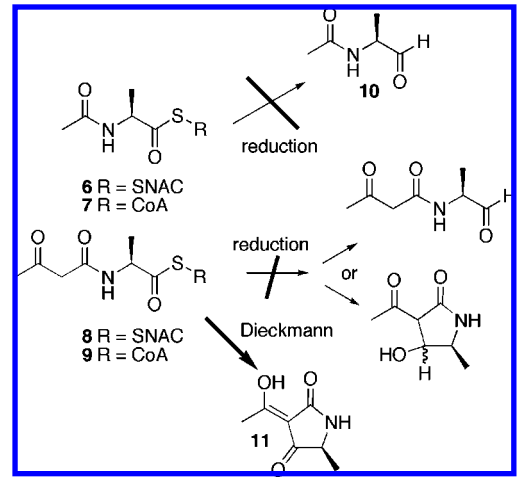

Figure 4. Synthesis of substrate analogs to test mechanistic possibilities. Analogs **6**−**9** were synthesized, as were standards **10** and **11**. If R operates as a typical NRPS reductase, reduced products should be observed. R catalyzes the Dieckmann condensation to give **11**, which was the only observed product in any enzymatic reaction with R.

Acetyl- and acetoacetyl-Ala thioesters were synthesized, using both CoA and SNAC thiols (Figure 4 and Figure S7, Supporting Information). The acetylated derivatives were relatively stable, while the acetoacetyl derivatives were unstable at pH > 7.5. Standards of predicted products were synthesized, including a tetramic acid and an acetyl-alaninyl aldehyde (data not shown). The tetramic acid was stable and readily purified to homogeneity, while the unstable aldehyde was only transiently present in solution and was detected by TLC.

Both the TR and R domains were analyzed for reactivity with substrate analogs. The purified R domain was treated with the acetoacetylalanine-CoA substrate, both with and without NADPH. The reactions were monitored by TLC and GC−MS (Figure 5). Under these conditions, tetramic acid was produced, as verified by comparison with an authentic standard. At pH 8, nonenzymatic background formation of tetramic acid was rapid, while at pH 6, no tetramic acid was formed either enzymatically or nonenzymatically. Experiments to determine the pH sensitivity are described in the enzyme kinetics section. Therefore, all further reactions were performed at pH 7.0, a condition giving negligible background but rapid enzymatic turnover to give the tetramic acid. Addition of either CoA or SNAC thioesters of acetoacetylalanine afforded tetramic acid at approximately equivalent rates. However, the acetylalanine derivatives were not substrates for the reaction.

No aldehyde or aldol products were observed by GC−MS or by TLC under any condition, using either the acetyl or acetoacetyl analogs. With the acetyl aldehyde analog, a synthetic standard was available that clearly stained with DNP on TLC plates. No such standard was available for the acetoacetyl analog. However, no aldehyde was detected by TLC. In addition, the acetoacetyl moiety could be readily observed by TLC staining with ferric chloride. No peak, aside from starting material or tetramic acid, was observed. Moreover, no peak corresponding to a reduced product or a reduced product from which water was eliminated was ever detected by GC−MS. Instead, we only observed tetramic acids by both TLC and GC−MS. Addition of NADPH was not required for production of tetramic acid, but tight binding of NADPH could not be ruled out. Thus, two likely mechanisms remained for the role of the reductive domain: either this enzyme catalyzes a Dieckmann

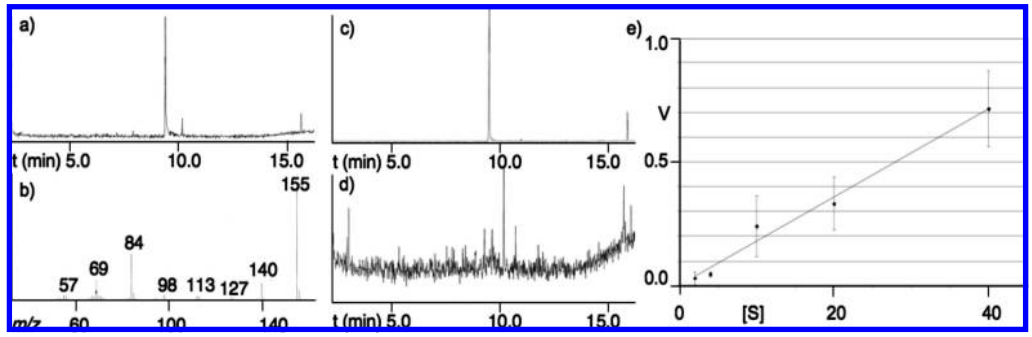

Figure 5. Dieckmann condensation catalysis by R. (a) GC−MS trace of tetramic acid standard **11**, extracting ions at $m/z = 155$. (b) Mass pattern of **11**. (c) GC−MS extracted for $m/z = 155$ of R reaction with **9**. Tetramic acid is produced and exhibited the same spectrum as in (b). (d) GC−MS extracted for $m/z = 155$ of a negative control lacking enzyme. (e) Velocity of tetramic acid formation. *x*-axis: substrate concentration in mM; *y*-axis: rate of product formation in mM min^{-1}.

condensation, or it recycled NADPH to both reduce and oxidize the substrate. While the recycling mechanism seems convoluted, sugar epimerase enzymes use this mechanism and are homologous with R.[43–45]

NAD(P)H is Not Required for Tetramic Acid Synthesis. The role of the cofactor was further analyzed by monitoring for consumption by UV spectroscopy. Both reactions containing acetoacetylalanine-CoA or acetylalanine-CoA were monitored on an automated plate reader. Reactions were performed in triplicate with varying substrate concentrations, from 0 to 4 mM, with and without enzyme. All rates of consumption of NADPH were equivalent, indicating that enzymatic consumption of NADPH was negligible. However, this left the possibility that NADPH was bound tightly enough to coelute with the protein upon purification, again as seen in some representatives of the related sugar epimerase family. To determine if this was the case with EqiS, R was treated with 8 M urea or Qiagen Protease for an hour, and the resulting mixtures were analyzed by MALDI−MS and DAD−HPLC in comparison to standards. In no case was NAD(P)(H) observed, which is inconsistent with a tightly bound cofactor hypothesis. As our detection limit was 30 nM, we would have observed the cofactor even if only ~5% of the protein was cofactor-loaded. Also, when TR was submitted for ESI−MS analysis, only the apoprotein was observed, with no shift for any tightly bound cofactor. Thus, EqiS R does not copurify with redox cofactors, ruling out the recycling mechanism for tetramic acid biosynthesis.

Covalently Loaded TR Does Not Catalyze Reduction. The above experiments were done using substrate analogs that were not covalently loaded onto T. It remained possible that R could reduce covalently loaded substrates that more closely resemble the natural pathway intermediates. To test this possibility, TR was covalently loaded with acetyl- and acetoacetyl-Ala-CoA derivatives using the broad-substrate pantetheinyltransferase, Sfp (Figure 3 and Figure S6, Supporting Information). A competition experiment was performed to show that this reaction is specific and complete. Acetylalanine-CoA or buffer only was preincubated with TR and Sfp, followed by treatment with Alexafluor-CoA. The resulting proteins were visualized by SDS-PAGE followed by fluorescence analysis, revealing that the buffer-only control was highly fluorescent, but no fluorescence could be detected in TR that was pretreated with acetylalanine-CoA. Therefore, the phosphopantetheine attachment site was completely blocked with acetyalaninyl-phosphopantetheine. This reaction was repeated with the acetoacetylalanine-CoA compound, also demonstrating complete conversion to the holoprotein. ESI−MS of the intact protein further established that these proteins were modified as predicted. In addition to providing a rapid assay for apoprotein loading, the fluorescent competition assay was useful to quickly assess Sfp functionality, revealing that the enzyme was functional from pH 5 to 9. This pH range was critical to avoid conditions amenable to tetramic acid synthesis or thioester hydrolysis.

The loaded TR domains were used to define the effects of NAD(P)H cofactors using ESI−MS. The loading reactions with Sfp were done at pH 6.0, since we had previously determined that R did not react with acetoacetyl derivatives at pH < 6.5. (Experiments to determine pH sensitivity are described in the next section.) The acetylalanine-CoA loaded TR showed a shift in mass of 453 AMU, corresponding to modification with a phosphopantetheinyl-acetylalanine. After increasing the pH to 7 and incubating with equimolar NADPH or excess or NADPH, no significant change in mass was observed by ESI−MS (Figure 3; Figure S9, Supporting Information). When excess NADH was used, a mass was observed consistent with no modification of substrate and binding to one equivalent of NADH. Consumption of NAD(P)H was also monitored by UV, which showed no change in absorbance at 340 nm greater than background. Thus, R was incapable of reducing this thioester.

When the acetoacetylalanine-CoA substrate was used in similar experiments, the acetoacetylalanine group was lost even at pH 6, and only the pantetheine-bound enzyme was observed by ESI−MS. This result strongly indicates that the protein likely catalyzes tetramic acid synthesis even at much reduced pH when the substrate is covalently enzyme-bound for the following reason. When using noncovalent substrates (SNAC or CoA derivatives), the thioesters are stable for hours with no detectable degradation by NMR. Thus, the acetoacetyl group would not normally be lost under these conditions. Covalent attachment of a substrate to enzyme is known to greatly accelerate reactions, since this covalent attachment much more closely imitates the natural substrate and much of the K_m factor is removed. Thus, the fact that the acetoacetyl group is lost at this low pH when covalently linked to enzyme further supports this as an enzyme-catalyzed reaction. Because these are by necessity single-turnover experiments, it is technically demanding to detect the small molecule products of the reaction. However, in numerous chemical analyses of the acetoacetyl derivatives, we in no case detected the hydrolysis product instead of the tetramic acid.

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Moreover, NAD(P)H was not consumed, as measured by extremely sensitive UV techniques.

EqiS R Catalyzes a Dieckmann Condensation. A reductive role for R was ruled out by the above experiments, strongly suggesting a Dieckmann mechanism to form the tetramic acid. However, a caveat to these experiments is that the substrates are analogs of the natural ones, and thus it was possible that the natural substrates would be reduced. It was thus important to obtain kinetic measurements to validate the Dieckmann condensation enzyme activity. To provide further evidence that this is indeed an enzyme catalyzed Dieckmann condensation, studies on the rate of reaction were done (Figure 5). All reactions were run as independent experiments in triplicate, using acetoacetylalanine-SNAC as the substrate. The SNAC derivative was selected because it was substantially easier to synthesize and purify and had approximately the same reactivity as the CoA.

Experiments were performed to determine the pH dependence of this process. Many similar thioesterase-catalyzed reactions affect substrates with similar or identical pK_a's to those of our synthetic substrates, and thus it was known that there may be some pH sensitivity to this reaction. For example, the "Claisen cyclase" subclass of thioesterases catalyzes a reaction involving groups with very similar pH-based activity to those reported here,[46] and even "normal" thioesterase macrocyclization reactions are somewhat similar when amines are involved in terms of their pH dependence. As alluded to above, the enzyme was not reactive at pH 6 down to the detection limit (250 ng after 1 h reaction), whereas at pH 8, reactions in duplicate indicated that the background rate in the absence of enzyme was ~50% of the enzyme-catalyzed rate at that pH. At pH 7, the background rate was measured in a time-course experiment to be <5% of the enzyme-catalyzed rate (Figure S9, Supporting Information). A background rate was also obtained using protease-digested enzyme, as described below and used in the calculation of the enzyme-catalayzed rate.

An additional problem was the thermotolerance exhibited by R. It was possible to kill the enzyme activity after extensive boiling for >15 min, but 5 min at 100 °C did not completely abolish the activity. Therefore, the stability of the enzyme was measured by circular dichroism (Figure S5, Supporting Information). R began to lose structure at 75 °C, whereas TR did not become unstructured even at 90 °C. Therefore, both constructs were relatively thermotolerant in comparison to many fungal enzymes. Such thermotolerance is a common occurrence in proteins even from mesophilic organisms, including fungi.[47] In order to obtain a relevant control, we chose instead to digest R with Qiagen Protease. In this control, all of the buffers and the amino acid content would be identical to the actual reaction, but R would be fully degraded by the protease. Duplicate reactions were set up in which protein was treated either with Qiagen Protease or with a boiled control of Qiagen Protease. By SDS-PAGE, the proteolyzed R domain was completely degraded, while R treated with boiled protease was intact. The control protein still produced the tetramic acid while the proteolyzed solution did not. This showed unequivocally that the reaction is protein catalyzed.

The rates of reaction were monitored by GC−FID using an internal standard of limonene for increased accuracy. Using constant enzyme concentrations and varying substrate from 2 mM to 40 mM, a linear slope was observed, indicating that saturation could not be achieved for this enzyme. A total of 60 independent measurements were used to obtain a reaction velocity slope. Assuming linearity, we calculated a V/K_m of 0.018 min^{-1} using the Michaelis−Menten approximation. Because saturating substrate concentrations could not be achieved, the lower limit for the rate of reaction could only be estimated at $k_{cat} \approx 15\ s^{-1}$ using a Lineweaver−Burk plot. This rate estimate falls within an order of magnitude of the rates of reduction observed with sugar epimerases and NRPS R domains, indicating a robust catalysis of tetramic acid formation. We could not approach the K_m limit in these experiments because it was very high, exceeding our substrate saturation limit. These are unnatural substrate analogs, and moreover, they are not covalently linked to enzyme as natural substrates would be. Thus, it can be expected that K_m for the enzyme-tethered, natural substrates would be much lower than that for the noncovalent, unnatural substrates. The overall catalytic rate of reaction should be much faster for the natural substrates. These experiments were not designed to measure this natural rate, but rather to show that the process is enzyme catalyzed. These experiments indeed clearly indicate that the R domain catalyzes a Dieckmann condensation, that cofactor is not required, and that an aldehyde intermediate is not produced en route to the tetramic acid equisetin.

Discussion

NRPS R domains are found in a wide range of biosynthetic pathways to natural products of biomedical and agricultural importance.[18–24] EqiS synthesizes equisetin from polyketide and peptide building blocks, forming the tetramic acid ring via a Dieckmann condensation catalyzed by R. By contrast, all of the previously characterized NRPS R domains from bacteria and fungi are reductases, catalyzing release of an enzyme-bound thioester to yield an intermediate aldehyde. Within bacteria, the myxochelins are produced from an aldehyde intermediate that is produced by the R domain of MxcG in a reaction requiring NAD(P)H.[29] Lyngbyatoxin is synthesized by a 4-electron reductive cleavage, requiring 2 equiv of NADPH, to yield an alcohol.[32] The nostocyclopeptide precursor is reductively cleaved from the enzyme by NADPH, resulting in the formation of an imine macrocycle.[31] The fungal NRPS R domains have been rigorously examined only in the case of Lys2 in fungal lysine biosynthesis.[28] This enzyme contains the ATR domain order, activating and then reducing α-aminoadipic acid to the semialdehyde with the aid of NADPH. Using in vivo expression, evidence was obtained that the fungal PKS-NRPS proteins might catalyze a Dieckmann condensation.[36] Here, we obtained in vitro evidence using purified proteins, confirming this reaction and demonstrating that it takes place on the NRPS R domain.

This Dieckmann condensation was unanticipated, since bioinformatic analysis predicts EqiS and related R domains to be similar in structure and function to fungal and bacterial NRPS R domains that are redox enzymes (Figure S8, Supporting Information). The fungal hybrid PKS-NRPS R domains are approximately 40% similar to characterized fungal Lys2 proteins. The N-terminal NADPH binding site is well conserved; however, homology essentially disappears in the C-terminal substrate-binding region. On the basis of BLAST and conserved domain searches, the tetramic acid R domains clearly fall into the short-chain dehydrogenase/reductase (SDR) protein family, which almost universally depends upon NAD(P)(H) cofactors.[33]

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(47) For example, see: Pasamontes, L.; Haiker, M.; Wyss, M.; Tessier, M.; van Loon, A. P. *Appl. Environ. Microbiol.* **1997**, *63*, 1696–1700.

Crystal structures for SDR proteins from different subfamilies indicate that they are structurally similar, with all members containing a Rossman fold and a catalytic triad composed of Ser, Tyr, and Lys.[48–51] These enzymes function in diverse redox reactions on substrates including sugars, alcohols, steroids, aromatic compounds, and amino acids.[33] The catalytic triad and NADPH binding site are all apparently found in the NRPS R domains as well, based upon sequence alignment and analysis. Critically, they are found in EqiS and its relatives.

Some of the best characterized SDR proteins are UDP-D-galactose epimerase (UDP-epimerase) and ADP-L-glycero-D-mannoheptose 6-epimerase (AGME). Both enzymes use NAD^+ in a catalytic fashion to epimerize sugar hydroxyls.[52] A catalytic triad of Lys, Tyr, and Ser generate an acidic Tyr with a pK_a of 6.08.[43–45] This drives the general acid−base mechanism by removing a proton from the sugar hydroxyl, facilitating oxidation by NAD^+ to give NADH. The resulting ketone is then reduced from the opposite face by the same NADH, which regenerates cofactor.[43] This catalytic triad and the cofactor-binding pocket are also found in NRPS R domains. Cofactor binds extremely tightly to UDP-epimerase resulting in a coelution with protein,[53] and it is used in a catalytic fashion. In contrast, EqiS R does not coelute with cofactor and does not need cofactor to catalyze tetramic acid formation. In the epimerases, the catalytic Tyr participates in acid−base catalysis. Since this triad is conserved in NRPS R domains, including EqiS R, it is possible that this residue is involved in a general acid−base mechanism to catalyze Dieckmann condensation, generating tetramic acids.

The family of tetramic acids and derivatives produced by fungi is highly variable. Five-membered rings resulting from these pathways have variable redox states and are often subject to further modifications, generating an enormous chemical diversity from similar starting points.[54–58] An attractive hypothesis would be the formation of an aldol intermediate, formed by the R domain, which could be enzymatically modified to the oxidized tetramic acids and other derivatives (Figure S10, Supporting Information). On the basis of the work presented here, at least EqiS R does not perform a reductive mechanism, disproving the universal aldol hypothesis. It remains unclear if the other pathways, especially those leading to more reduced pyrrolidine-2-one derivatives, go via a Dieckmann condensation or arise through a reduction on R. In recent years, the genes responsible for several tetramic acids and more modified relatives have been characterized. Sequence and phylogenetic analysis show that the fungal hybrid PKS-NRPS R domains form a distinct branch separate from other SDR proteins (data not shown), and thus these R domains can be easily recognized and differentiated from their reducing relatives. Unfortunately, there are no readily observable sequence cues that enable the differentiation of R domains leading to the tetramic acids versus the relatively reduced pyrrolidine-2-ones. It is possible that some of the R domains catalyze reduction, whereas others catalyze Dieckmann condensation, or the tetramic acid itself could be the universal intermediate en route to fungal pyrrolidine-2-ones. Further experiments are needed to differentiate these possibilities. Like fungal PKSs, similar R domains generate diverse chemical structures by evolutionary mechanisms that may subtly different than those found in bacterial pathways.

Recently, genes for two bacterial tetramic acids have been sequenced.[59,60] These hybrid PKS-NRPS genes terminate with a thioesterase (TE) domain in place of the fungal R. TE domains are known to catalyze acid−base chemistry, and they likely are the catalysts of the Dieckmann condensation in the bacterial group. Here, we have identified and SDR superfamily member, EqiS, that also functions as a thioesterase rather than a reductase. This represents a new class of thioesterase domain in NRPS metabolism. These results will be useful in the genetic synthesis of new tetramic acids, which are often potently bioactive, and in the discovery of new potential pharmaceuticals by genome mining.

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Supporting Information Available: Complete experimental details for all procedures, NMR spectra and spectral data, mass spectra and data, and pyrophosphate assay results. This material is available free of charge via the Internet at http://pubs.acs.org.

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