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Enzymatic Basis of 'Hybridity' in Thiomarinol Biosynthesis

Zachary D. Dunn,
Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599

Walter J. Wever,
Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599

Dr Nicoleta J. Economou,
Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599

Prof Albert A. Bowers, and
Division of Chemical Biology and Medicinal Chemistry, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599

Prof Bo Li*
Department of Chemistry University of North Carolina at Chapel HillChapel Hill, NC, 27599

Abstract

Thiomarinol is a naturally occurring double-headed antibiotic that is highly potent against methicillin-resistant *Staphylococcus aureus*. Its structure comprises two antimicrobial sub-components, marinolic acid and holothin, linked by an amide bond. TmlU was thought to be the sole enzyme responsible for this amide bond formation. Contrary to this idea, we show that TmlU acts as a CoA ligase that activates marinolic acid as its thioester before it is processed by the acetyltransferase HolE to catalyze the amidation. TmlU prefers complex acyl acids as substrates, whereas HolE is relatively promiscuous, accepting a range of acyl-CoA and amine substrates. Our results provide detailed biochemical information on thiomarinol biosynthesis, and evolutionary insight regarding how the marinolic acid and holothin pathways converge to generate this potent hybrid antibiotic. This work also demonstrates the potential of TmlU/HolE enzymes as engineering tools to generate new "hybrid" molecules.

Keywords

dithiolopyrrolone; bioengineering; antibiotics; evolution; adenylating enzyme

The rapid rise of antibiotic resistance creates an urgent need for new antibiotics. One strategy to meet the diminishing return on traditional antibiotics is to covalently link combinations of existing antibiotics to produce novel hybrids. These hybrid antibiotics exhibit enhanced bioactivity and pharmacology compared to the parent compounds. This

*boli@email.unc.edu.

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strategy has a synergistic effect because it improves activity against drug-resistant bacteria, expands the spectrum of the individual compounds, and reduces the potential for new resistance.[1] A drawback of synthetic hybrid antibiotics is that the partner compound or the covalent linker may hinder target binding. Furthermore, the effective concentration of both species may be reduced if each compound targets disparate cellular sites, thus creating a "bi-localization" dilemma.[2] Naturally occurring hybrid antibiotics, like the marine natural product thiomarinols (**1** and **2**) from *Pseudoalteromonas spp.* SANK73390,[3] have already been honed by Nature for selectivity and biological activity. Thus, these natural hybrids can provide valuable insight into useful therapeutic combinations and linker strategies.

Thiomarinol combines the monic acid warhead of the FDA-approved agent mupirocin (pseudomonic acids, **3**, Bactroban® - GlaxoSmithKline)[4] and the compact holothin (**4**) core of the dithiolopyrrolones (DTPs) holomycin (**5**)[5] and thiolutin (**6**) (Figure 1A).[6] These fragments, which in thiomarinol are linked by a fatty acyl amide bridge, exhibit broadly different antibiotic activities: mupirocin exhibits high specificity for the bacterial isoleucyl-tRNA synthetase and subsequent inhibition of protein synthesis,[7] whereas the proposed holomycin mechanism of action involves inhibition of bacterial transcription.[8] Importantly, despite these differences, the hybrid is more potent than its constituents, with greatly enhanced activity against many drug resistant pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA).[3a]

Thiomarinol's hybrid structure generates at least two thought provoking questions. How did nature come to couple the two distinct moieties? What are the mechanistic benefits of combining these seemingly unrelated antibiotic motifs? Here, we begin to answer the first question via detailed characterization (and functional reassignment), of the TmlU, and HolE gene products.

The thiomarinol gene cluster was discovered by whole genome sequencing of *Pseudoalteromonas* spp. SANK73390.[9] The genes are present on a 97 kb plasmid comprising a polyketide synthase (PKS) portion and a nonribosomal peptide synthetase (NRPS) portion, which are responsible for synthesizing the marinolic acid and holothin segments, respectively (Figure 1B). Subsequent genetic deletions by Simpson and co-workers identified TmlU as a key enzyme responsible for coupling the holothin and marinolic acid moieties.[9-10] TmlU was initially assigned as an amide ligase, based on its significant sequence identity with the amide ligases NovL (20.7%), CouL (21.1%), CloL (19.7%) and SimL (18.5%), which catalyze amide bond formation in the biosynthesis of aminocoumarin antibiotics novobiocin, coumermycin, chlorobiocin, and simocyclinone, respectively (Scheme 1A, Figure S1).[11]

We tested whether TmlU could similarly act as a stand-alone amide ligase by generating recombinant TmlU in *E. coli*. The proposed amine donor for TmlU-mediated amide coupling, holothin, could be readily accessed *via* total synthesis in five steps with 13% overall yield.[13] The 7-carbon fatty acyl monic acid, marinolic acid (**8**), was not readily available. The 8-carbon fatty acyl monic acid, pseudomonic acid A (PAA), which is commercially available (Sigma), was used instead. The epoxy-group in PAA was reduced in

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three steps to give pseudomonic acid C (PAC, **9**) with a C-*10,11 trans*-olefin, similar to marinolic acid.

Despite investigating a large number of conditions, TmlU failed to yield the anticipated product with the substrates holothin and either PAA or PAC. This observation led us to reexamine the assignment of TmlU. A Phyre2 homology model (Figure S2) indicated close structural similarity to the SrfA-C termination module of the non-ribosomal peptide synthetase responsible for surfactin biosynthesis.[14] Moreover, we found that TmlU has 14% sequence identity to MupU, a putative acyl-CoA ligase in the mupirocin biosynthetic pathway (Figure S1 and S3). As a result, we wondered whether TmlU might activate marinolic acid by linking to CoA or an acyl carrier protein, which could then be transferred onto the acceptor holothin through tandem action of another enzyme from the cluster, and speculated that the putative acyl-transferase HolE might play this auxiliary role.

HolE is a homolog of HlmA, which is present in the holomycin pathway from *Streptomyces clavuligerus*, catalyzing the terminal acylation of holothin with acetyl-CoA.[15] It was hypothesized that HolE was solely responsible for the "background" acylation observed in isolates from *Pseudomonas* spp. SANK73390, giving rise to a series of short-chain xenorhabdins-like molecules.[16]

HolE was expressed in *E. coli* and purified to homogeneity. For *in vitro* reconstitution of enzyme activities, we treated PAC and holothin with 1 μM purified TmlU and 1 μM HolE in the presence of CoASH, $MgCl_2$, and ATP. We detected a significant peak for the Pseudomonic acid C-holothin (PAC-holothin, **10**) conjugate, which displayed the same molecular weight and retention time as a synthetic standard (Figure 2A, 2B and 2G; see Supporting Information for synthesis). Omitting CoA abolished PAC-holothin production, suggesting that CoA is necessary for efficient conversion (Figure 2C). Assays carried out in the absence of TmlU or ATP failed to yield the expected product (Figure 2D and S6). Furthermore, when HolE was omitted, the substrate PAC was consumed, but the final PAC-holothin product was not observed (Figure 2E). Instead a PAC-CoA adduct accumulated (Figure 2F and S7-8). These results demonstrate that TmlU is an acyl-CoA ligase and that HolE catalyzes the subsequent acyl-transfer step required for thiomarinol biosynthesis. With this understanding, we assessed the kinetics and promiscuity of this two-step enzymatic process.

Kinetic parameters for TmlU were measured using saturating concentrations of the co-substrates CoA and ATP with PAC as a substrate. The formation of the PAC-CoA product was measured by a coupled assay with saturating concentrations of HolE and 3-aminocoumarin. 3-Aminocoumarin was used instead of holothin, because we found it to be well accepted by HolE, more stable than holothin, and not susceptible to substrate inhibition. Under these conditions, TmlU displays a K_m of 6 ± 1 μM for PAC and a k_{cat} of 3.2 ± 0.1 s^{-1} (Figure 3A). These parameters are consistent with those reported for other acyl-CoA ligases, such as 4-chlorobenzoate-CoA ligase.[17] Interestingly, use of PAA as substrate yielded similar values to PAC (Figure 3B), suggesting that the presence of the epoxy-group does not affect TmlU activity. Thus, the lack of the epoxide moiety in thiomarinols is likely due to the absence of epoxide forming enzymes in thiomarinol biosynthesis rather than the

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substrate selectivity of TmlU or HolE. We additionally investigated the substrate scope against a number of other carboxylic acids. Under high enzyme concentration, TmlU was capable of activating acetic, octanoic, 2,4-dodecadienoic, and 2,4-decadienoic acids, albeit to a lesser extent (Figure 4A). In particular, we measured the k_{cat}/K_m of TmlU for octanoic acid and found it to be 50,000 fold less than those for PAC or PAA (Figure 3C). Overall, TmlU appears selective for long and relatively complex fatty acyl carboxylates.

To assess the kinetics of HolE, the substrate pseudomonic acid C CoA (PAC-CoA) was generated using TmlU under the conditions of full conversion. HolE and holothin were subsequently added and the conversion of PAC-CoA to PAC-holothin was observed. For a fixed concentration of PAC-CoA (100 μM), holothin displayed inhibitory activity against HolE at concentrations above 20 μM (Figure S5). This prevented determination of k_{cat}/K_m. Efforts instead turned to assessing its basal promiscuity at a fixed concentration of holothin and in the presence of several different, commercially available acyl-CoA substrates. HolE accepted linear CoA substrates of different lengths, including propionyl-, hexanoyl-, octanoyl-, oleoyl- and dodecanoyl-CoA, readily converting all to the corresponding acyl-holothin adducts (Figure 4B and S12). This finding is consistent with our observation regarding the substrate tolerance of HlmA, the acetyltransferase in the holomycin biosynthetic pathway.[15] The promiscuity of HolE with respect to fatty acyl CoAs suggests that it is likely responsible for the formation of the xenorhabdins (**7**), which were seen as pathway by-products by Thompson et al.[10]

Given the potential for new and useful hybrid antibiotics from this pathway, we explored the promiscuity of the HolE/TmlU pair by supplying the reaction with various amine donors and measuring conversion over fixed time (Figure 4C and S10). The HolE/TmlU pair could readily ligate PAC to a variety of primary amines including 3-aminocoumarins, but was less effective with a series of aryl amines. Overall, an adjacent substrate carbonyl appears useful or important for recognition of the amine donor. This promiscuity stands in contrast to the related enzyme systems, SimL and CouL from simocyclinone and coumermycin biosynthesis, respectively. The latter two amide ligases reportedly show strong preference for native aminocoumarin derived substrates.[11b, 12, 18] The biological activities of PAC-aminocoumarin and other analogs generated in this study will be the subject of future studies.

TmlU/HolE points to an intriguing convergent strategy for creating PKS/NRPS hybrid molecules. While many known hybrid linkages are installed on the PKS/NRPS assembly line (Figure 5A and 5B),[19] TmlU/HolE makes use of a fully mature PKS product, which is reactivated by a standalone acyl-CoA ligase (TmlU) and transferred to a fully mature NRPS product by a standalone acyltransferase (HolE) (Figure 5C). This strategy does not require direct participation of assembly proteins or attachment to a carrier protein and is highly amenable to engineered biosynthesis, in particular of glycosylated natural products using glycosyltransferases.[20]

It is intriguing to consider the implications for the evolution of the pathway and the future of thiomarinol or similar hybrid molecules in light of this new understanding. Both the biosynthetic logic and the antibiotic mechanism must have dictated the role of the TmlU/

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HolE pair. MupU, the TmlU homolog in mupirocin biosynthesis, loads the fatty acyl carboxylate in pseudomonic acid B onto a stand alone acyl carrier protein (ACP) MacpE for additional tailoring.[22] However, thiomarinol biosynthesis lacks a MacpE-like standalone ACP. Instead, an additional ACP and ketosynthase domain appear to have been incorporated into the large PKS subunits, possibly replacing MacpE and MupU, respectively. TmlU would then be free to facilitate alternative post-PKS tailoring (i.e., ligation to holothin *via* HolE), taking advantage of the promiscuity of HolE, which provides a branch point for further molecular diversity.

Although there is no clear evidence that the mupirocin pathway preceded the thiomarinol pathway in terms of evolution, the observations that these late stage modifications have been subsumed by larger pathway enzymes and that other enzymes (TmlU) are repurposed suggests that thiomarinol is the more advanced of the two. The pronounced biological activity and reduced antibiotic resistance of the hybrid molecule would further substantiate this idea. It remains to be determined how adding holothin to a monic acid aids its mechanism of action: the crystal structure of Ile-tRNA synthetase bound pseudomonic acid A shows the carboxylate jutting from the active site, uninvolved in the key inhibitory binding event.[23] Although the cyclic disulfide in holomycin was shown to be important for the antimicrobial action,[24] additional mechanistic studies are needed to reveal the role that holothin could play in the Ile-tRNA synthetase steric space. The combination of the two molecules is a potentially fortuitous evolutionary event, not easily predicted by a modern structure-based approach. We anticipate that our characterization of TmlU and HolE will aid efforts to gain insight into the evolution and confluence of biosynthetic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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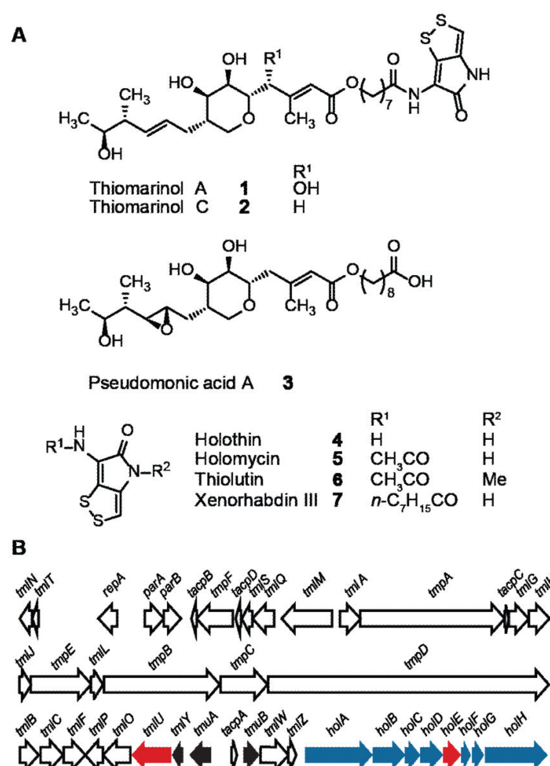

Figure 1.

A) Structures of thiomarinols, pseudomonic acids, and dithiopyrrolones. B) Gene cluster for thiomarinol. Open arrows indicate ORFs with homology to the mupirocin pathway; blue ORFs are homologous to DTP biosynthetic genes; black ORFs are unique to the thiomarinol pathway; red ORFs, TmlU and HolE, the targets of this study, have counterparts in the mupirocin and holomycin pathway, respectively. They are represented in red arrows instead of open and blue arrows for clarity.

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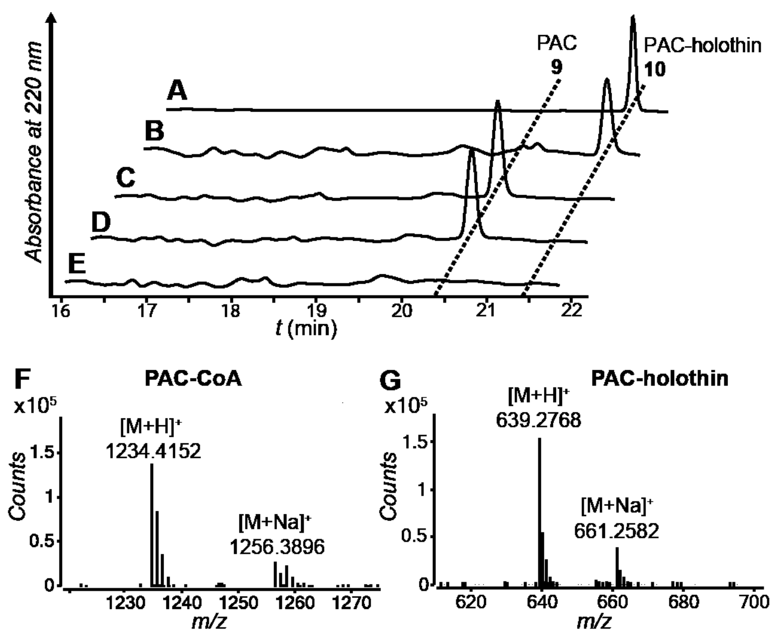

Figure 2.

Enzymatic production of PAC-holothin, a thiomarinol analogue, *in vitro* by TmlU and HolE, in the presence of 1 mM ATP, 2 mM $MgCl_2$, and 1 mM CoASH, at pH 7.5. A) Synthetic PAC-holothin standard, B) *In vitro* reconstitution of TmlU and HolE activity generating PAC-holothin, C) Control lacking CoA, D) Control lacking TmlU, E) Control lacking HolE, F) Mass spectrum of the PAC-CoA product generated by TmlU (calculated $[M+H]^+$, 1234.4155). G) Mass spectrum of PAC-holothin product generated enzymatically by TmlU and HolE (calculated $[M+H]^+$, 639.2768).

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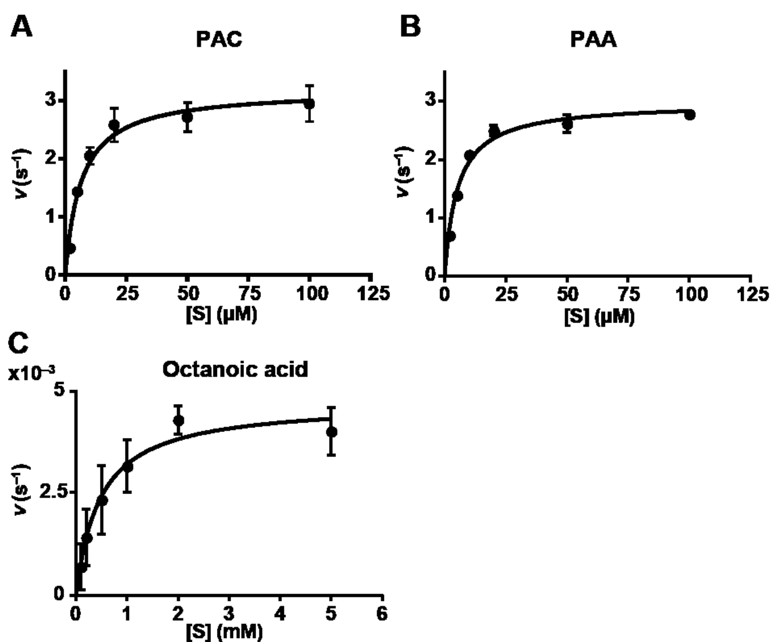

Figure 3.

TmlU Kinetic Curves of different substrates. A) PAC, $K_m = 6 \pm 1$ μM and $k_{cat} = 3.2 \pm 0.1$ s^{-1} B) PAA, $K_m = 5.2 \pm 0.5$ μM, $k_{cat} = 3.0 \pm 0.1$ s^{-1} C) octanoic acid, $K_m = 0.5 \pm 0.1$ mM and $k_{cat} = (5.0 \pm 0.3) \times 10^{-;3}$ s^{-1}.

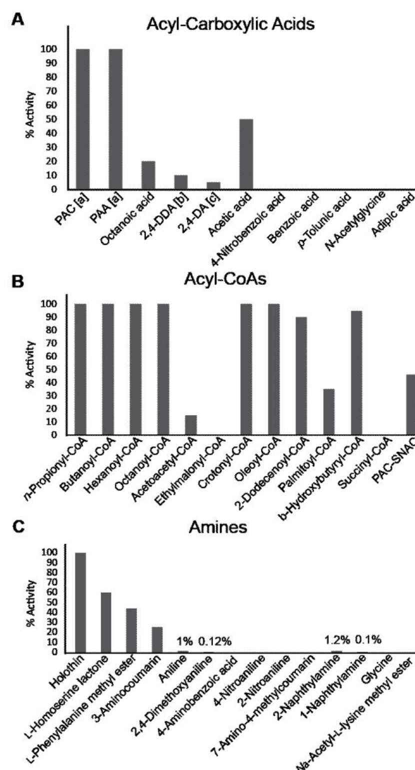

Figure 4.

Substrate promiscuity of TmlU and HolE. A) Carboxylic acids tested for incorporation into thiomarinol by 5 μM TmlU in the presence of saturating concentrations of HolE and holothin. Activity was measured by the formation of the acyl-holothin products at $UV_{360\ nm}$ and normalized to activity with PAC as substrate. [a] 1 μM TmlU was used in this assay. [b] 2,4-DDA, (2*E*,4*E*)-2,4-dodecadienoic acid. [c] 2,4-DA, (2*E*,4*E*)-2,4-decadienoic acid. B) Acyl-CoAs as substrates for HolE to generate acyl-holothin products. Activity was measured as described in A. C) Amines as substrates for HolE. Co-substrate PAC-CoA was generated by TmlU. Activity was measured by integration of ion intensities in mass spectra.

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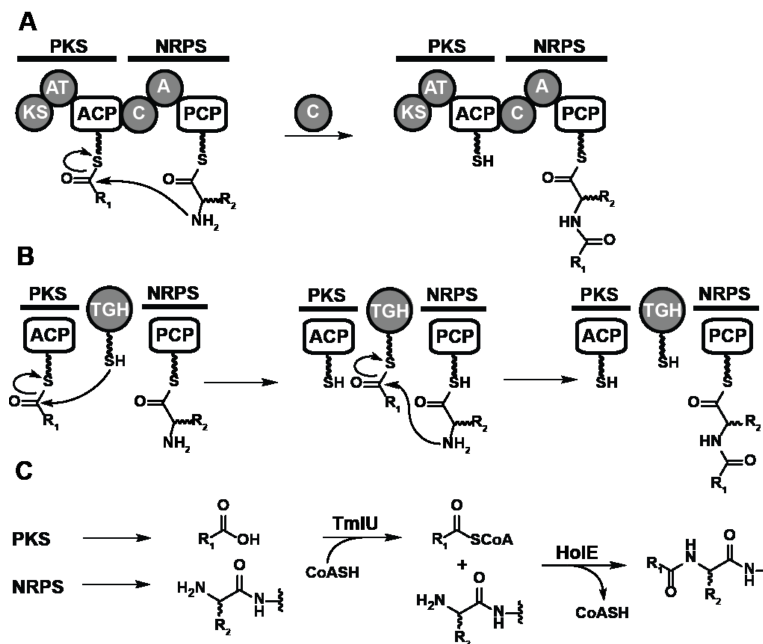

Figure 5.

Mechanism of thiomarinol production by TmlU and HolE compared to assembly-line tethered mechanisms to create PKS/NRPS hybrids. A) Condensation domain of a NRPS directly adds a PKS product to a growing peptide chain. Chemistry occurs on the assembly line. B) A standalone transglutaminase-like domain (TGH) catalyzes the transfer between tethered PKS and NRPS products in the biosynthesis of andrimid.[21] C) Tailoring enzymes TmlU and HolE create a NRPS/PKS hybrid with released products of discrete PKS and NRPS pathways. KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein; C, condensation domain; A, adenylation domain; PCP, peptidyl carrier protein.

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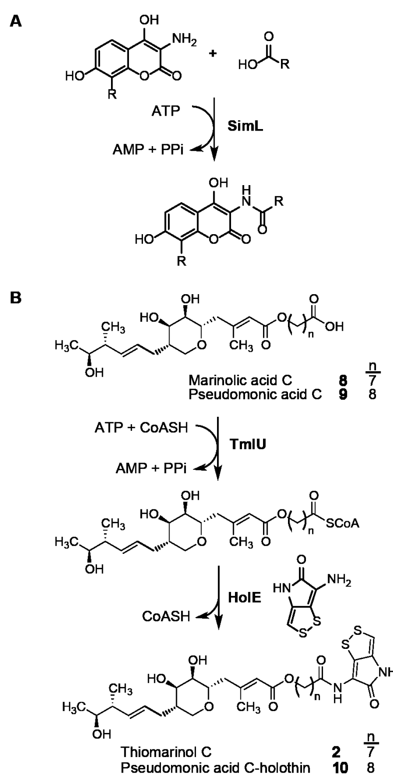

Scheme 1.
A) Reported mechanism of amide formation catalysed by SimL.[12] B) Our proposed mechanism of thiomarinol formation catalysed by TmlU/HolE.