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Perspective

Aminovinyl Cysteine Containing Peptides: A Unique Motif That Imparts Key Biological Activity

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Cite This: *JACS Au* 2021, 1, 1527–1540

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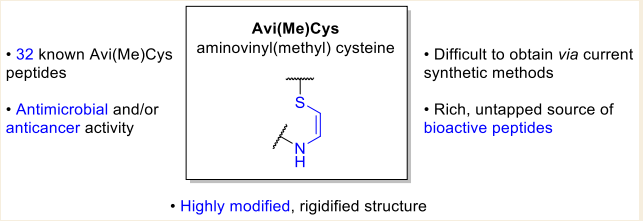
ABSTRACT: Natural products that contain distinctive chemical functionality can serve as useful starting points to develop Nature's compounds into viable therapeutics. Peptide natural products, an under-represented class of medicines, such as ribosomally synthesized and post-translationally modified peptides (RiPPs), often contain noncanonical amino acids and structural motifs that give rise to potent biological activity. However, these motifs can be difficult to obtain synthetically, thereby limiting the transition of RiPPs to the clinic. Aminovinyl cysteine containing peptides, which display potent antimicrobial or anticancer activity, possess an intricate C-terminal ring that is critical for bioactivity. To date, successful methods for the total chemical synthesis of such peptides are yet to be realized, although several advancements have been achieved. In this perspective, we review this burgeoning class of aminovinyl cysteine peptides and critically evaluate the chemical strategies to install the distinct aminovinyl cysteine motif.



KEYWORDS: *Lanthipeptides: aminovinyl cysteine, antimicrobials, cyclic peptides, biosynthesis, chemical synthesis*

1. INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a major class of diverse natural products that cover a broad range of bioactivities.[1,2] They are genetically encoded and expressed as large precursor peptides which are enzymatically processed into smaller modified bioactive peptides.[1] These enzymatic modifications are known as post-translational modifications (PTMs) and may include cyclization, halogenation, dehydration, and incorporation of D-amino acids.[3] These PTMs act to improve the peptides' bioactivity and increase their stability against proteolytic degradation.[3] One such PTM is the rigidifying C-terminal *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) or (2*S*,3*S*)-*S*-[(*Z*)-2-aminovinyl]-3-methyl-D-cysteine (AviMeCys) unit, herein jointly referred to as Avi(Me)Cys units (Figure 1).

To date, there are 32 peptides that contain this moiety. These are found among five major peptide families: lanthipeptides (Figures 2, 3, and 4), linaridins (Figure 5), thioamitides (Figure 6), lanthidins (Figure 7), and the lipolanthines (Figure 7). Gram-positive bacteria produce these compounds as a defense mechanism against competing bacterial species; therefore, many RiPPs have potent antibacterial activity against *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridia* species (Table 1).[4] Some Avi(Me)Cys-containing peptides, such as the thioamitides, exhibit cytotoxicity toward human cancer cell lines.[5]

These Avi(Me)Cys-containing peptides possess highly modified structures that give rise to desirable drug-like properties such as heat and pH stability, high target specificity, and resistance toward proteases.[6] These natural products also commonly contain other noncanonical amino acids, including

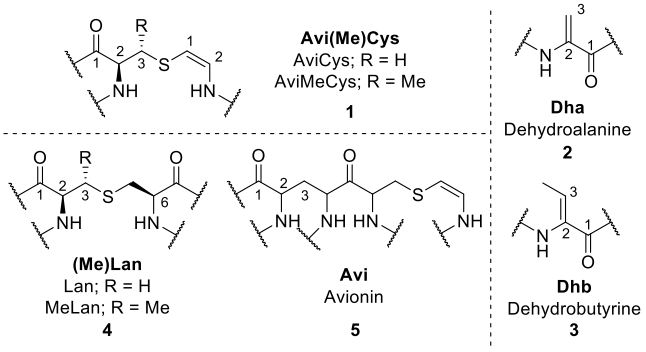
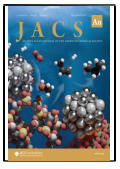


Figure 1. Chemical structures of the noncanonical amino acids commonly found in Avi(Me)Cys-containing peptides. (Me)Lan = (methyl)lanthionine (**4**), Avi = avionin (**5**), AviCys = *S*-[(*Z*)-2-aminovinyl]-D-cysteine (**1**), AviMeCys = (2*S*,3*S*)-*S*-[(*Z*)-2-aminovinyl]-3-methyl-D-cysteine, jointly referred to as Avi(Me)Cys (**1**).

Received: July 12, 2021
Published: September 15, 2021



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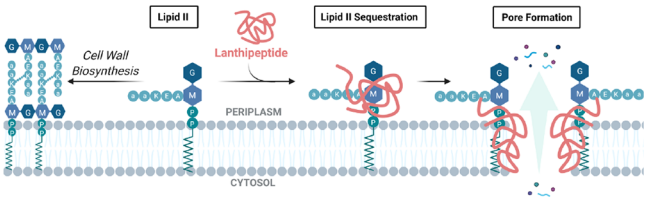

Figure 2. Sequestration of lipid II and concomitant pore formation by the lanthipeptide–lipid II complex, leading to bacterial cell lysis and death. This dual mechanism of action has been described for several lanthipeptides including mutacin-1140 and epidermin.[28,31] However, other lanthipeptides such as mersacidin are currently believed to only partake in sequestration of lipid II without pore formation.[32] Further investigation is required to identify other pore-forming lanthipeptides. A = L-alanine, a = D-alanine, K = lysine, E = glutamic acid, G = GlcNAc (*N*-acetylglucosamine), M = MurNAc (*N*-acetylmuramic acid), *P* = phospho group. Created with BioRender.com.

2,3-dehydroalanine (Dha, **2**), (*Z*)-2,3-dehydrobutyrine (Dhb, **3**), D-amino acids, and lanthionine (Lan, **4**), *β*-methyllanthionine ((Me)Lan (**4**), and avionin (Avi, **5**) (Figure 1).[3,4,7] These PTMs restrict the conformational freedom of the peptide, providing a dual function of (a) locking the peptide in an active conformation for target binding and (b) inhibiting proteolytic degradation.[6] In particular, unsaturated Dha, Dhb, and Avi(Me)Cys moieties increase structural rigidity by including sp^2 *α*-carbons in the peptide backbone.[8]

Despite the "drug-like" potential of Avi(Me)Cys-containing peptides, lack of viable synthetic routes to access the Avi(Me)Cys moiety limits their transition to the clinic. To date, there is no total synthesis of an Avi(Me)Cys-containing natural product peptide reported, although several routes have been investigated.[9−13] Therefore, these natural products present an intriguing synthetic target with important therapeutic applications and require the development of novel methodology. This perspective discusses in detail the different families of Avi(Me)Cys-containing peptides and the current attempts to install the Avi(Me)Cys unit by chemical methods.

2.0. LANTHIPEPTIDES

Over 100 lanthipeptides have been discovered and characterized since Rogers and Whittier discovered the lanthipeptide nisin in 1928.[14] This family encompasses RiPPs that contain one or more (Me)Lan thioether linkages and are further categorized into five classes (I−V) determined by their genetically encoded biosynthetic enzymes responsible for installing the Lan and (Me)Lan motifs.[15,16] The seven known Avi(Me)Cys-containing lanthipeptides are found only in classes I, II, and V. Class I comprises mutacin-1140,[17] microbisporicin,[18] gallidermin,[19] epidermin,[20] and clausin (Figure 3).[21] Class II and V contain the peptides mersacidin[22] and lexapeptide,[23] respectively (Figure 4). Daspyromycin (Figure 4), the most recently discovered Avi(Me)Cys-containing lanthipeptide, is yet to be categorized.[24]

The Avi(Me)Cys-containing peptides within the lanthipeptide family are the most well-known and studied of the Avi(Me)Cys natural products.[7] All display antimicrobial activity against Gram-positive bacterial species including

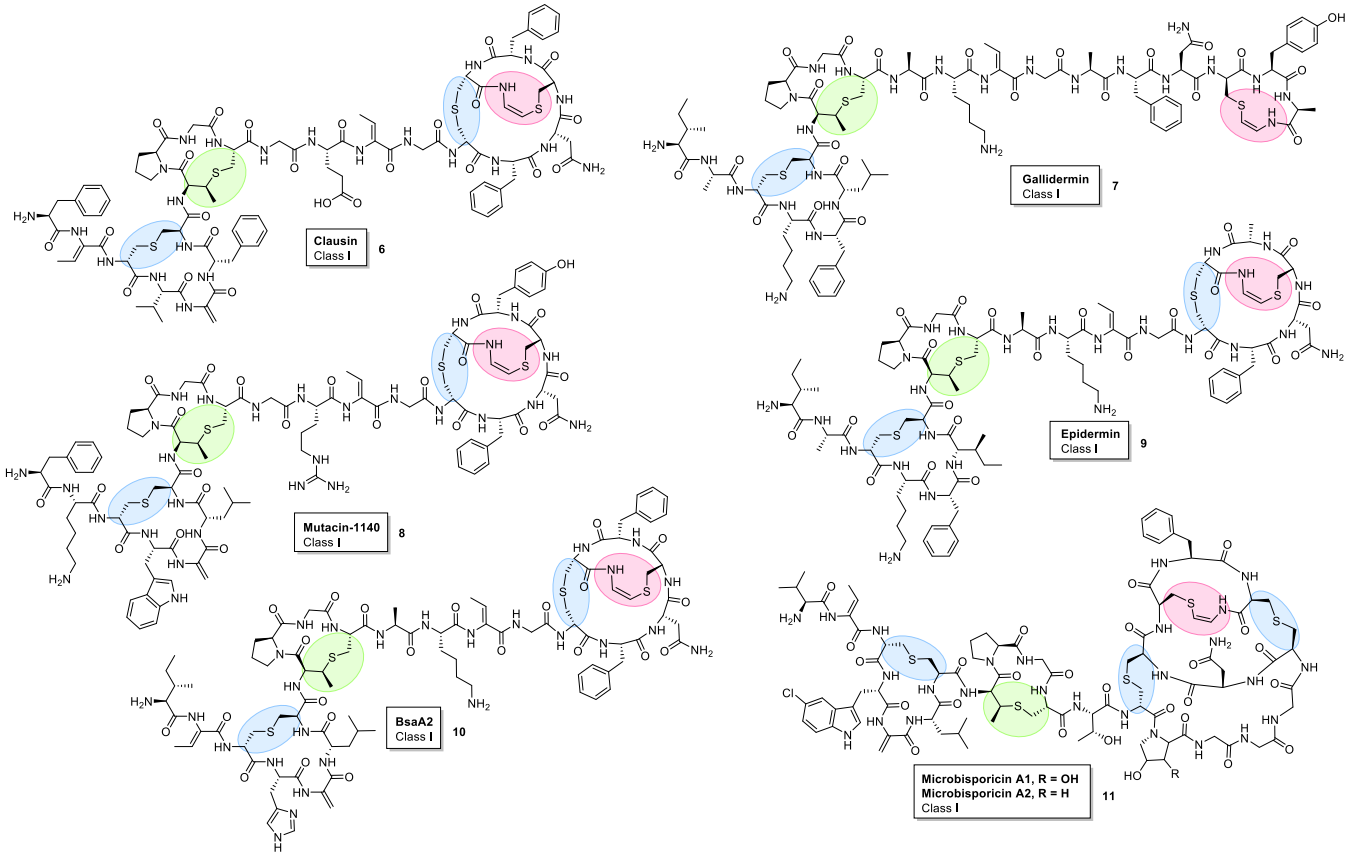

Figure 3. Structures of Avi(Me)Cys-containing class I lanthipeptides; clausin (**6**),[21] gallidermin (**7**),[19] mutacin-1140 (**8**),[53] epidermin (**9**),[20] BsaA2 (**10**),[37] and microbisporicins A1 and A2 (**11**).[18] Pink = Avi(Me)Cys. Blue = Lan. Green = MeLan.

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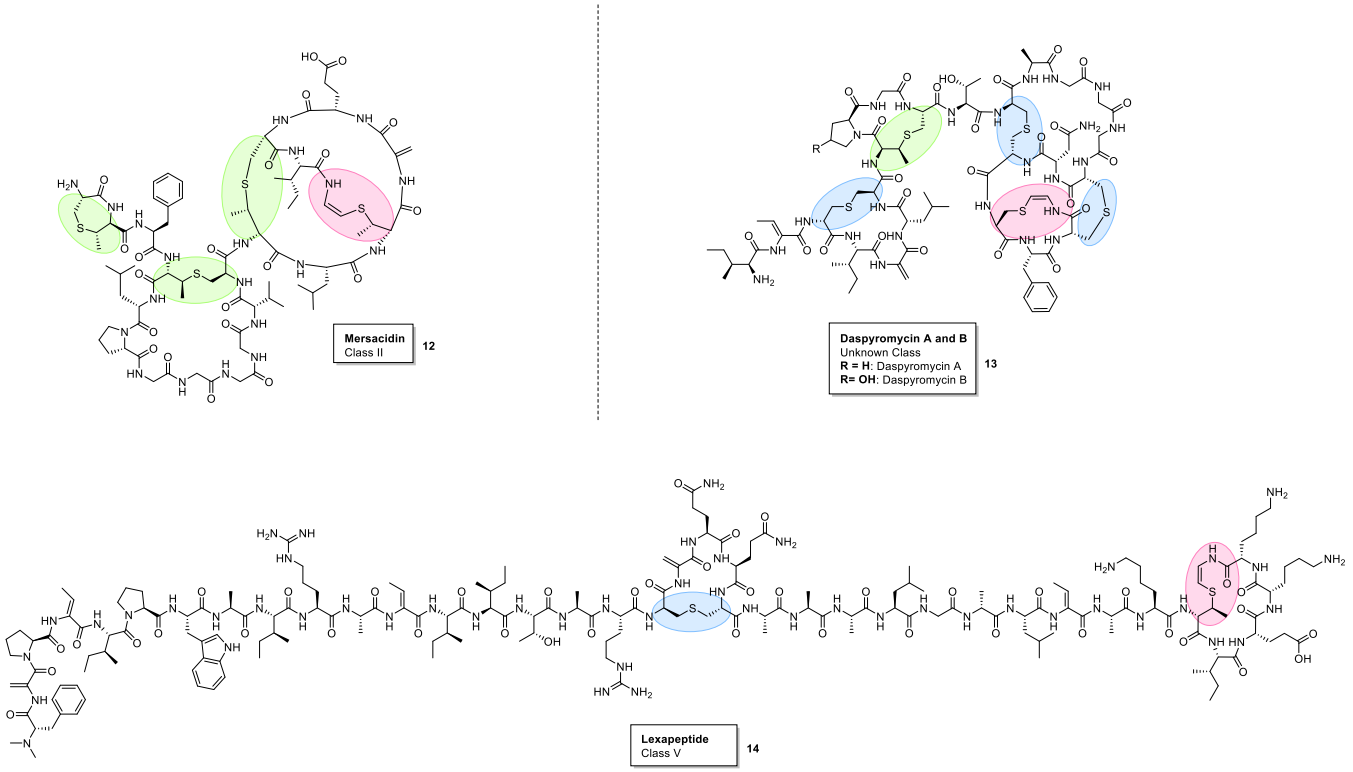

Figure 4. Structures of Avi(Me)Cys-containing class II and class V lanthipeptides; mersacidin (**12**) (class II),[22] daspyromycins A and B (**13**) (unknown class),[24] and lexapeptide (**13**) (class V).[23] Pink = Avi(Me)Cys. Green = MeLan. Blue = Lan.

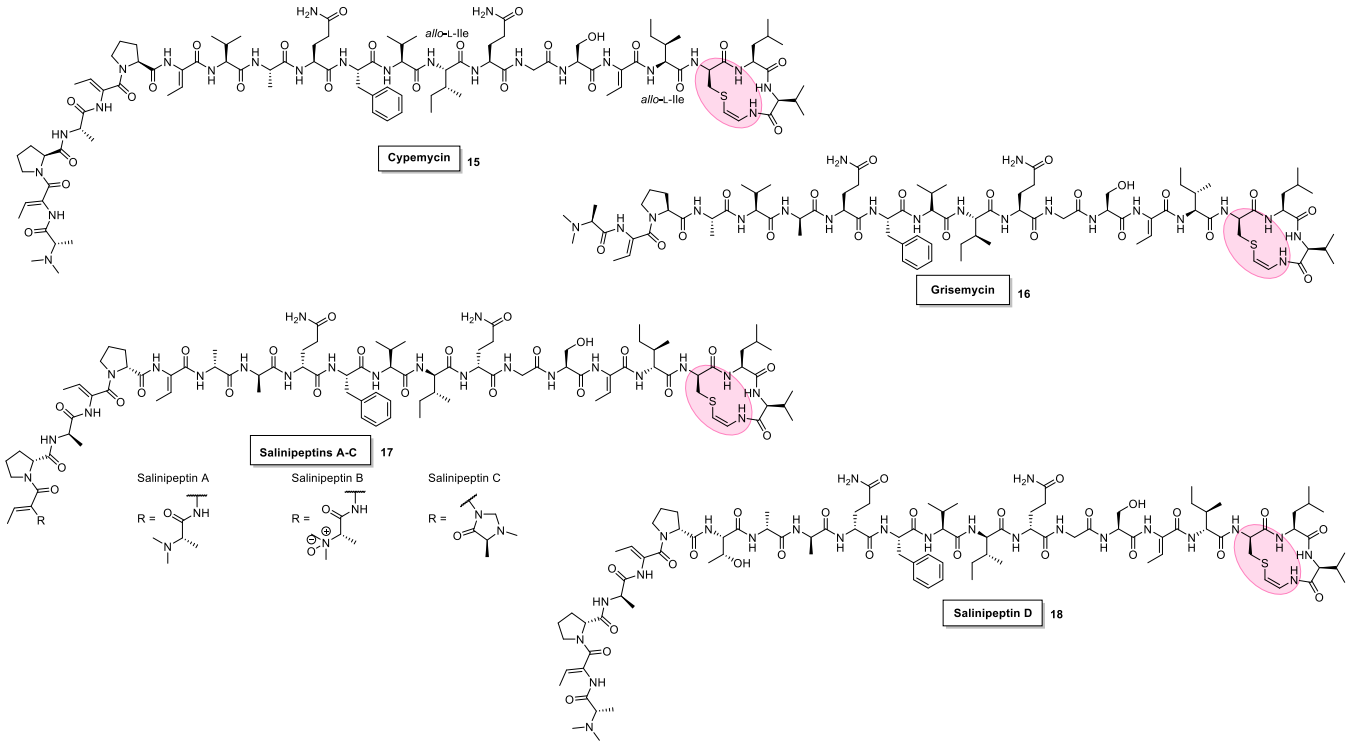

Figure 5. Chemical structures of Avi(Me)Cys-containing peptides of the linaridin family; cypemycin (**15**),[40] grisemycin (**16**),[41] and salinipeptins A–D (**17** and **18**).[42] Pink = AviCys.

multi-drug-resistant *Staphylococcus epidermidis* and *Staphylococcus aureus*.[25] The antibacterial activity of Avi(Me)Cys-containing lanthipeptides arises from targeting lipid II, an essential precursor for the cell wall of Gram-positive bacteria (Figure 2).[4,26] Lipid II consists of a bactoprenol "carrier lipid" coupled to a peptidoglycan building block (*N*-acetylmuramyl pentapeptide-*N*-acetylglucosamine) via a pyrophosphate bridge.[27] The lanthipeptide nisin and the Avi(Me)Cys-

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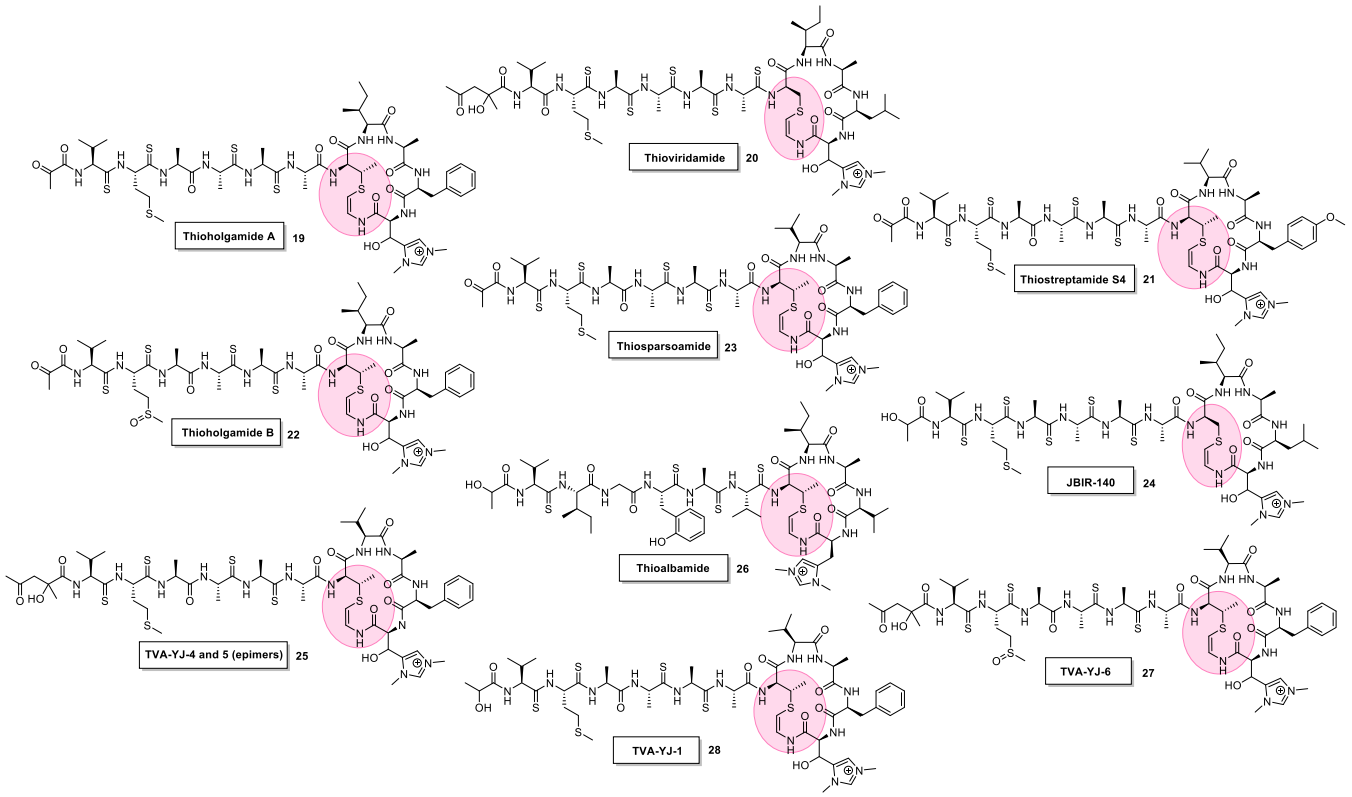

Figure 6. Chemical structures of Avi(Me)Cys-containing peptides of the thioamitide family; thioholgamide A (**19**),[44] thioviridamide (**20**),[5] thiostreptamide S4 (**21**),[45] thioholgamide B (**22**),[44] thiosparsoamide (**23**),[46] JBIR-140 (**24**),[48] TVA-YJ-4 and 5 (epimers at α-hydroxyamide) (**25**),[43] thioalbamide (**26**),[47] TVA-YJ-6 (**27**),[43] and TVA-YJ-1 (**28**).[43] Pink = Avi(Me)Cys.

Table 1. Producing Bacterial Strain and Bioactivity of Known Avi(Me)Cys-Containing Peptides (G+ = Gram-Positive, G− = Gram-Negative)

family	peptide	producing strain	notable bioactivity
lanthipeptide	mutacin-1140[17] (class I)	*Streptococcus mutans*	*Neisseria gonorrhea* (G−), *Enterococcus faecalis* (G+), *Staphylococcus epidermidis* (G+), *Helicobacter pylori* (G−)
	microbisporicins[18] (class I)	*Microbispora corallina*	*Staphylococcus aureus* (G+), *Streptococcus pneumoniae* (G+)
	gallidermin[19] (class I)	*Staphylococcus gallinarum*	*Mariniluteicoccus flavus* (G+), *Staphylococcus simulans* (G+)
	epidermin[20] (class I)	*Staphylococcus epidermidis*	*Mariniluteicoccus flavus* (G+), *Staphylococcus simulans* (G+)
	clausin[21] (class I)	*Bacillus clausii* O/C	*Clostridium difficile* (G+)
	BsaA2[37] (class I)	*Staphylococcus aureus*	antibacterial (G+)
	mersacidin[22] (class II)	*Bacillus amyloliquefaciens*	*Staphylococcus aureus* (G+), MRSA (G+)
	lexapeptide[23] (class V)	*Streptomyces rochei*	MRSA (G+), MRSE (G+), *Micrococcus luteus* (G+), *Bacillus subtilis* (G+)
	daspyromycin A and B[38] (unknown class)	*Actinokineospora diospyrosa* NBRC 15665	MRSA (G+), VRE (G+)
	cypemycin[39,40]	*Streptomyces* sp. OH-4156	cytotoxic against P388 leukemia cells, antibacterial against *Micrococcus luteus*
linaridin	grisemycin[41]	*Streptomyces griseus*	not reported
	salinipeptins A−D[42]	*Streptomyces* sp. strain GSL-6C	group A *Streptococcus pyogenes* M1T1
thioamitide	thioviridamide[5]	*Streptomyces olivoviridis*	apoptosis inducer (anticancer)
	TVA-YJ-4, 5, and 6[43]	*Streptomyces* sp. NRRL S-87	cytotoxic (anticancer)
	thioholgamides A and B[44]	*Streptomyces malayseiense*	antiproliferative and cytotoxic (anticancer)
	thiostreptamide S4[45]	*Streptomyces* sp. NRRL S-4	
	thiosparsoamide[46]	*Streptomyces sparsogenes*	
	thioalbamide[47]	*Amycolatopsis alba*	anticancer (specific activity yet to be reported)
	JBIR-140[48]	*Streptomyces avermitilis*	
	TVA-YJ-1[49]	*Streptomyces laurentii*	
lipolanthine	microvionin[50]	*Microbacterium arborescens*	MRSA (G+), *Streptococcus pneumoniae* (G+)
	nocavionin[50]	*Nocardia terpenica*	not reported
	goadvionins[51]	*Streptomyces* sp. TP-A0584	*Staphylococcus aureus* (G+), *Bacillus subtilis* (G+), *Micrococcus luteus* (G+)
lanthidin	cacaoidin[52]	*Streptomyces cacaoi*	MRSA (G+), *Clostridium difficile* (G+), *Staphylococcus simulans* (G+)

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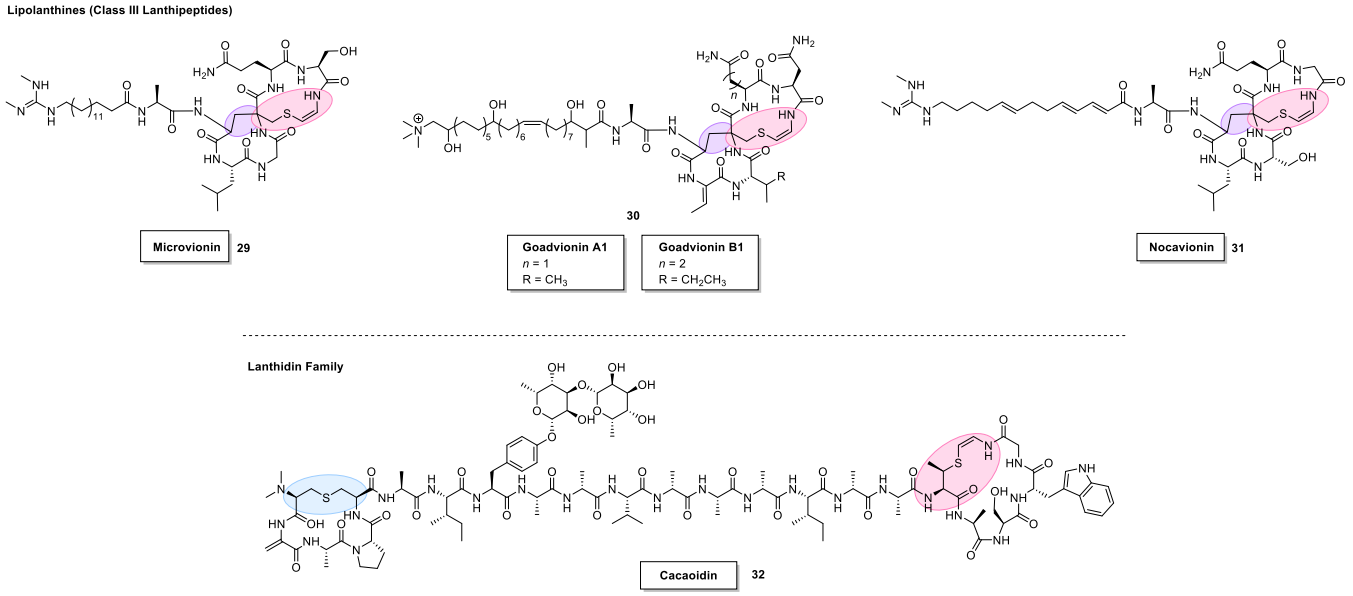


Figure 7. Chemical structures of Avi(Me)Cys-containing peptides of the lipolanthine family (since reclassified as class III lanthipeptides); microvionin (**29**),[50] goadvionins A1/B1 (**30**)[51] and nocavionin (**31**),[50] and the lanthidin family; cacaoidin (**32**).[52] Pink = Avi(Me)Cys. Purple = avionin (Avi). Blue = Lan.

containing microbisporicin sequester lipid II by forming lanthipeptide−lipid II complexes.[28,29] Sequestration of lipid II blocks cell wall biosynthesis, resulting in poor structural integrity and lysis due to high intracellular osmotic pressure (Figure 2).[27] Additionally, some lanthipeptide−lipid complexes can form pores in the bacterial cell membrane by insertion of the complexed lanthipeptide into the phospholipid bilayer (Figure 2).[27,28,30] These pores provide a permeable channel through which cellular components may leak through into the periplasm, causing loss of osmotic pressure and cell death.[28] This pore-forming mode of action remains poorly understood, with only a handful of lanthipeptides known to exhibit this behavior, notably the Avi(Me)Cys-containing peptides epidermin and mutacin-1140.[28,31]

Glycopeptide antibiotics such as vancomycin typically target the peptidyl side chain (D-Ala-D-Ala) of lipid II.[33] In vancomycin-resistant bacteria, the D-Ala-D-Ala sequence is mutated to prevent vancomycin from binding, therefore reducing its efficacy.[34,35] Another mechanism of resistance is a "false target" wherein vancomycin-resistant bacteria produce excess peptidoglycan containing the D-Ala-D-Ala unit, which trap and sequester vancomycin.[35] Lanthipeptides typically do not target the D-Ala-D-Ala sequence of lipid II and instead bind via the lipid II pyrophosphate bridge (Figure 8).[30] Microbisporicin, an Avi(Me)Cys-containing lanthipeptide, interacts electrostatically with the negatively charged lipid II pyrophosphate bridge, an interaction that is also favored by the association of the hydrophobic ring system in microbisporicin with the cell membrane.[36] The pyrophosphate bridge of lipid II is less susceptible to mutation than the D-Ala-D-Ala sequence, thereby reducing the probability of antibiotic resistance toward microbisporicin developing compared to vancomycin.[36] The Avi(Me)Cys-containing lanthipeptides lexapeptide and microbisporicin exhibit potent activity against vancomycin-resistant bacteria, indicating their potential for therapeutic use to combat antimicrobial resistance.[18,23]

Microbisporicins A1 and A2 (Figure 3), produced in a 60:40 ratio by fermentation of the actinomycete *Microbispora*

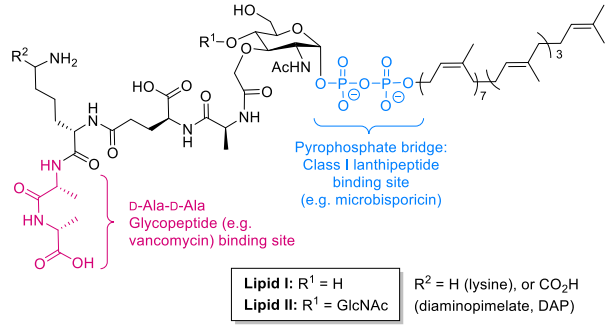


Figure 8. Generic chemical structure of lipid I and lipid II, precursors for the biosynthesis of the peptidoglycan cell wall.[27] GlcNAc = *N*-acetylglucosamine, postulated binding site of mersacidin. Pink = D-Ala-D-Ala vancomycin binding site;[33] blue = pyrophosphate bridge of lipid II, the postulated microbisporicin binding site.[18]

corallina, were previously being developed by Naicons SRL and Sentinella Pharmaceuticals for the treatment of multi-drug-resistant bacterial infections.[54,55] Known as NAI-107,[56] both peptides exhibit potent antibacterial activity against both Gram-positive and Gram-negative bacteria, including against vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *Enterococcus faecalis*.[54,57] Microbisporicins A1 and A2 are both 24-mer pentacyclic lanthipeptides, identical except at Pro14, wherein A1 contains 3,4-dihydroxyproline, and A2 contains 4-hydroxyproline.[56] With promising activity in preliminary in vivo studies,[58,59] NAI-107 was in preclinical development by Naicons SRL and Sentinella Pharmaceuticals for treatment of multi-drug-resistant Gram-positive infections; however, the current status of NAI-107 development is unknown.[54,55]

In contrast to lexapeptide and microbisporicin, the class II Avi(Me)Cys-containing lanthipeptide mersacidin (Figure 4) does not target the pyrophosphate bridge of lipid II.[22,60] Instead, mersacidin exhibits affinity for the lipid II GlcNAc (*N*-acetylglucosamine) unit, which is absent in lipid I (Figure 8).[61] As such, mersacidin does not show affinity for lipid I, unlike

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

class I lanthipeptides which can bind to lipid I via the pyrophosphate bridge.[30] The mersacidin−lipid II complex does not lead to the formation of pores in the bacterial membrane, and therefore, the primary mechanism of action of mersacidin is by the sequestration of lipid II and inhibition of cell wall biosynthesis.[60]

Mersacidin is also unusual in that it does not possess any positively charged amino acids. Typically, antimicrobial peptides (AMPs) have a net positive charge, which facilities association of the peptide with the negatively charged bacterial membrane.[62] Mersacidin, however, contains a negatively charged glutamate at position 17 (Figure 4). Mutation of this residue by amidation or substitution for an alanine greatly reduces the activity of mersacidin, which indicates that Glu17 is essential for activity.[63] It has been postulated that Glu17 interacts with calcium ions (Ca^{2+}) to form a salt bridge with negatively charged moieties on the bacterial cell membrane.[32]

Mersacidin has previously displayed moderate in vitro antimicrobial activity; however, preliminary in vivo antibacterial activity studies have shown promise.[64,65] Antimicrobial assays have shown low to moderate activity against some Gram-positive bacteria and negligible activity against Gram-negative strains, with mersacidin having significantly less activity compared to that of the glycopeptide class overall.[66] However, work by Kruszewska et al.[65] found that mersacidin eliminated methicillin-resistant *Staphylococcus aureus* (MRSA, strain 99308) from the nasal cavity in mouse rhinitis models. A twice daily treatment of mersacidin (1.66 mg/kg per dose) over 3 days effectively cured mice infected with MRSA in the nasal cavity (n = 12) compared to mice that did not receive mersacidin.[65] Additionally, cytotoxic effects such as morphological changes, mucosal lesions, and cytokines related to MRSA infection were not detected in mouse models when treated with mersacidin intranasally.[65]

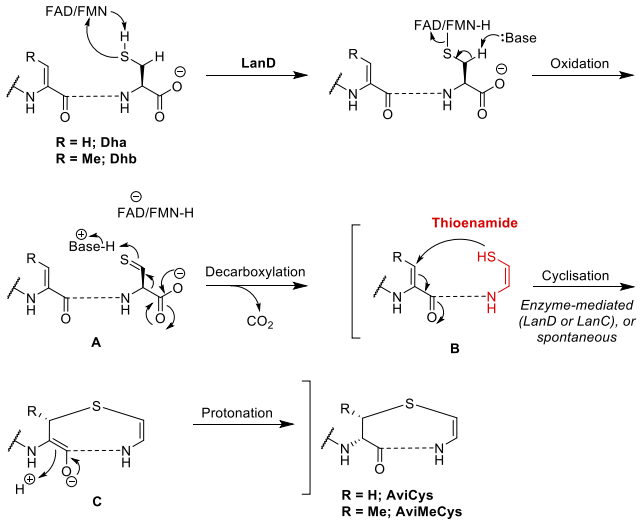
2.1. Biosynthesis of Avi(Me)Cys in Lanthipeptides

Within the lanthipeptide family, the incorporation of Avi(Me)Cys units across the relevant lanthipeptides is catalyzed by structurally homologous flavoprotein decarboxylase (LanD) enzymes.[67] These flavoproteins require a redox-active flavin cofactor, such as flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), for catalytic activity.[67]

LanD enzymes are part of the homo-oligomeric flavoprotein cysteine decarboxylase (HFCD) family.[68] These proteins form complex homo-oligomeric quaternary structures with active site(s) at the central interface of the complex.[69] LanD enzymes form dodecameric oligomers with a substrate binding clamp that is highly disordered in the absence of a peptide substrate. The flavin cofactor (FMN or FAD) is anchored in a structurally conserved region of the active site.[67,69,70]

For the biosynthesis of Avi(Me)Cys-containing peptides, the C-terminal Cys-sulfhydryl of the LanA prepeptide undergoes flavin-mediated oxidation to give a corresponding thioaldehyde **A** (Scheme 1).[67,69,70] Spontaneous decarboxylation of the C-terminal carboxylate then occurs, driven by tautomerization of the thioaldehyde to enethiol **B** to install the Cα−Cβ double bond of Avi(Me)Cys (Scheme 1).[67,69,70] Thioether cyclization then occurs by nucleophilic attack of the sulfhydryl upon the β-carbon of an α,β-unsaturated amino acid (Dha or Dhb), and the resulting enolate **C** is protonated to afford the (2*S*)-AviCys or (2*S*,3*S*)-AviMeCys moiety (Scheme 1).[15,67] To date, this thioether cyclization step during Avi(Me)Cys formation is not fully understood and may be enzyme-catalyzed (e.g., by LanC or LanD) or a spontaneous process.[15,23]

Scheme 1. Oxidation and Decarboxylation of a C-Terminal Cysteine-Bearing Peptide, Catalyzed by a Decarboxylase (LanD)[68] (Reactive Thioenamide (Red) Then Undergoes Thiol-Michael Cyclization with an Unsaturated Amino Acid (Dha/Dhb) to Provide the Avi(Me)Cys Unit Following Protonation)



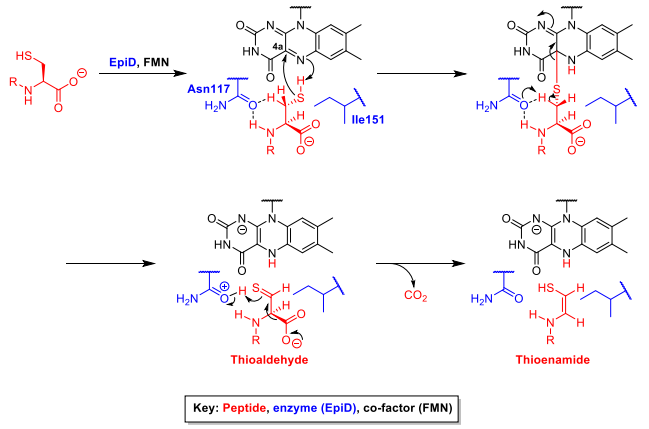
Oxidative decarboxylation of C-terminal cysteine peptides by the epidermin decarboxylase, EpiD, has been reported to occur in the absence of a Michael acceptor residue (Dha/Dhb), giving the linear thioenamide-containing products.[71] Kupke et al.[70] demonstrated the decarboxylase activity of EpiD on C-terminal Cys-sulfhydryl substrates and the absence of activity on S-alkylated peptides, confirming that the decarboxylation step occurs prior to cyclization.[67] Furthermore, EpiD has been successfully applied to the formation of Avi(Me)Cys linkages on non-epidermin peptides from 4 to 52 amino acids in length with the C-terminal tripeptide sequence AA_1-AA_2-Cys, where AA_1 = Val, Ile, Leu, Met, Phe, Tyr, or Trp and AA_2 = Ala, Ser, Val, Thr, Cys, Ile, or Leu.[67,71] However, the leader peptide sequence is seemingly nonessential for the enzymatic activity of EpiD.[71] Combined with its broad substrate scope, the use of EpiD for the decarboxylation of synthetic peptides may be a viable strategy for the efficient production of bioactive compounds.[67]

The X-ray crystal structures of the LanD enzymes EpiD[70] and MrsD (mersacidin decarboxylase)[69] have been determined. The X-ray crystal structure of EpiD complexed with a C-terminal cysteine containing a pentapeptide substrate revealed a contact between the FMN cofactor and the Cys-sulfhydryl but not the Cys-carboxylate, supporting the idea that decarboxylation occurs indirectly as a result of sulfhydryl modification.[70] Obtaining this X-ray crystal structure was achieved by mutation of EpiD-His67 to an asparagine (Asn) residue, which abolished decarboxylase activity without preventing substrate binding.[70] Therefore, His67 of EpiD plays an essential role in the oxidative decarboxylation of C-terminal cysteines, proposed to occur by increasing acidity of the Cys-sulfhydryl proton.[68−70]

In the proposed mechanism (Scheme 2), nucleophilic attack of the Cys-sulfhydryl on the C4a position of flavin initiates the oxidative decarboxylation reaction of a C-terminal cys-

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

Scheme 2. Postulated Mechanism of Oxidation and Subsequent Decarboxylation Catalyzed by EpiD[70] (Peptide Substrate Is Indicated in Red, Enzyme (EpiD) in Blue, and Enzyme Cofactor (FMN) in Black)



teine.[67,70] H-bonding interactions between the side-chain carbonyl of Asn117 in EpiD and the peptide Cys-Cβ protons facilitate Cβ-deprotonation to afford the thioaldehyde intermediate.[70] The subsequent decarboxylation step is favored over Cα-deprotonation as the Cα-proton is blocked by Ile151 of EpiD (Scheme 2), and decarboxylation to give the C−C double bond of Avi(Me)Cys is further promoted through exposure of the Cys-carboxylate to solvent, allowing carbon dioxide to exit the active site.[70]

The observed interaction of Asn117 with the Cys-Cβ protons provides insight to the selective formation of the (*Z*)-alkene in Avi(Me)Cys, as these contacts enable rotation of the Cys-sulfhydryl toward a *syn* conformation with respect to the Cys-Nα (Scheme 2).[70] When the peptide substrate binds in the enzyme active site, the C-terminal Cys sits above the flavin *re* face, with the sulfhydryl orientated downward toward flavin-C4a and -N5 (Scheme 2).[70] Formation of thioaldehyde then positions the sulfur atom toward the protonated Asn117 oxygen, and decarboxylation generates the (*Z*)-alkene (Scheme 2).[70] The role of EpiD Asn117 in Avi(Me)Cys formation is further supported by the presence of Asn residues at structurally similar sites across other LanD enzymes, such as Asn125 in MrsD.[69]

2.2. Lipolanthines

In 2018, Wiebach et al. isolated microvionin (Figure 7) from a culture of *Microbacterium arborescens*. This peptide natural product exhibits an unusual triaminodicarboxylic acid moiety named avionin, as well as an Avi(Me)Cys macrocycle and N-terminal guanidino fatty acid.[50,72] It displayed potent antibacterial activity against both MRSA and *Streptococcus pneumoniae*, which makes it a promising candidate for the treatment of antibiotic-resistant bacteria.[50] Nocavionin (Figure 7), from *Nocardia terpenica*, was also isolated by Wiebach et al. in 2018 and contains the same defining structures as microvionin yet with a shortened and unsaturated N-terminal fatty acid.[50] In 2020, Kozakai et al. characterized eight new lipolanthines and coined the name goadvionins.[51] Bioactivity assays established antibacterial activity of the goadvionins against Gram-positive bacteria, the exact mechanism of which is currently unknown.[51]

Previously understood to be a family of their own, lipolanthines have since been classified as class III lanthipeptides, owing to the utilization of class-III-type lanthipeptide synthetases in biosynthesis. Formation of the Avi(Me)Cys unit in microvionin is catalyzed by FAD-dependent cysteine decarboxylases, MicD and MicKC, which resemble class III lanthipeptide-modifying enzymes.[50,73,74] Mechanistic studies show both enzymes function in a mutual regulatory manner, wherein the activity of MicKC is enhanced by MicD through substrate binding.[73]

3.0. LINARIDINS

In 1993, Komiyama et al. isolated a new peptide natural product from *Streptomyces* sp. OH-4156 called cypemycin, displaying antibacterial activity against *Micrococcus luteus* and cytocidal activity against mouse P388 leukemia cells.[39] Cypemycin possesses four Dhb residues, two L-*allo*-isoleucines, an *N*α-dimethylated N-terminal alanine, and an AviCys ring at the C-terminus (Figure 5).[39] Due to the presence of the Dhb and AviCys moieties, cypemycin was initially classified as a lanthipeptide until in 2010, when Claesen and Bibb identified the cypemycin biosynthetic gene cluster which revealed that there was no lanthipeptide dehydratase-like enzyme encoded. Therefore, cypemycin was reclassified as a linaridin.[40,75]

Linaridin natural products are a small but growing family of linear dehydrated peptides, and to date, there are 10 characterized members: cypemycin, grisemycin, legonaridin, salinipeptin A−D, mononaridin, and pegvadin A and B.[39,41,42,76−78] Of these 10, six contain the Avi(Me)Cys moiety (Figure 5); however, only two of them have shown antimicrobial and anticancer properties, cypemycin and salinipeptin A, with the latter displaying modest levels of activity against *Streptococcus pyogenes*, U87 glioblastoma and HCT-116 colon carcinoma cells.[39,42] The mechanism of their antimicrobial activity is unclear, but for cypemycin, it is postulated that it inserts itself into the bacterial membranes and forms pores, thereby causing cell lysis.[41]

Biochemically, incorporation of the Avi(Me)Cys moiety into these natural products is achieved via a decarboxylation using a homo-oligomeric flavin-containing Cys decarboxylase known as LinD followed by Michael-type addition of the thioenol to a Dha residue formed prior to decarboxylation.[79] Unlike most other Avi(Me)Cys-containing peptides, the Dha intermediate is derived from a Cys rather than a Ser.[80] The cyclization step to form Avi(Me)Cys is still unclear for linaridins as their biosynthetic gene clusters (BGCs) do not encode any cyclase enzymes like those found in lanthipeptide and lipolanthines (LanC).[80] It is thought that a LinD enzyme (e.g., CypD) catalyzes both decarboxylation and cyclization, although further investigation is required.[80]

4.0. THIOAMITIDES

Thioviridamide (Figure 6), the first thioamitide, was characterized by Hayakawa et al. in 2005.[5] This unusual natural peptide was isolated from *Streptomyces olivoviridis* and was revealed to contain a 2-hydroxy-2-methyl-4-oxopentanoyl (HMOP) group, a β-hydroxy-N^1,N^3-dimethylhistidinium, and an AviCys ring.[5] However, the most unique part of the structure was that it contained thioamide bonds in the peptide backbone.[2] This natural product displayed potent cytotoxic properties against rat fibroblasts transformed with adenovirus oncogenes with an IC_{50} = 3.9 ng/mL.[5] Since then, nine other Avi(Me)Cys-containing thioamitides have been discovered, with all of them displaying anticancer activity across multiple

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

cell lines.[5,45,46,49,81] Thioviridamide acts as an apoptosis inducer by targeting the F_1F_0-ATP synthase and inducing the integrated stress response, a cellular process that down-regulates protein synthesis in response to internal or environmental stresses such as amino acid deprivation or endoplasmic reticulum stress.[82] Thioholgamide A, which was discovered in 2020 by Kiemer et al., also targets the ATP synthase which causes antiproliferative and cytotoxic effects on cancer cells.[44] The antiproliferative nature of these compounds is due to the initiation of the integrated stress response which causes the cancer cells to enter a resting state, thereby preventing proliferation before they undergo apoptosis.[44]

Much like the other Avi(Me)Cys-containing peptide families, formation of the Avi(Me)Cys motif involves the dehydration of serine/threonine residues, cysteine decarboxylation, and Avi(Me)Cys macrocyclization. Apart from that, little is known about the biosynthetic pathway, although it has been postulated that enzymes similar to those found in the class III lanthipeptides ($LanKC_t$) are responsible for forming the Avi(Me)Cys structure. These enzymes dehydrate the precursor peptide and form a complex with the cysteine decarboxylase to produce the Avi(Me)Cys macrocycle.[46]

5.0. LANTHIDINS

Lanthidins are the most recently discovered family of Avi(Me)Cys-containing peptides, currently only encompassing one member, cacaoidin (Figure 7).[52] Cacaoidin was characterized in 2020 by Ortiz-López et al. and found to contain a dimethylated N-terminus, a lanthionine, an Avi(Me)Cys ring, and a rare *O*-glycosylated tyrosine residue.[52] It was isolated from *Streptomyces cacaoi* CA-170360 and demonstrated potent antibacterial activity against MRSA and moderate activity against *Clostridium difficile*.[52] Cacaoidin was observed to trigger the induction of the lipid II cycle interfering with the antibiotic response regulator and sensor (LiaRS) bioreceptor, indicating interference with the lipid II biosynthesis cycle.[52] This suggests that cacaoidin targets lipid II in the bacterial membrane, resulting in poor structural integrity and lysis. Formation of the Avi(Me)Cys moiety is believed to be facilitated by a protein homologous to the cypemycin decarboxylase CypD.[52]

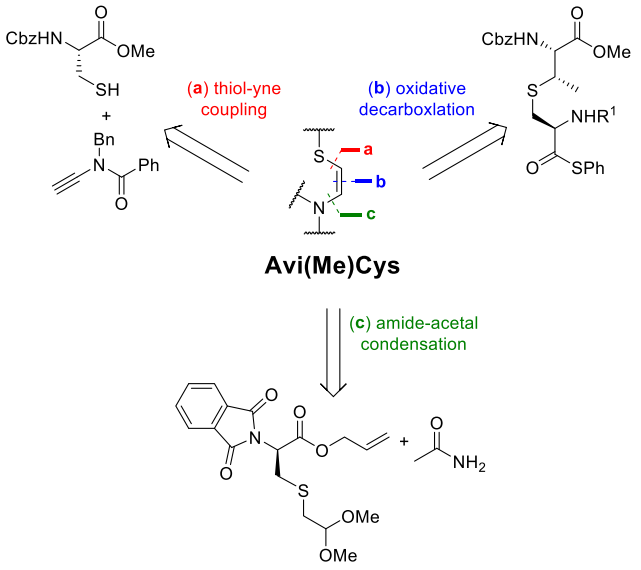
6.0. CHEMICAL SYNTHESIS OF Avi(Me)Cys UNITS

The preparation of multicyclic peptides containing dehydrated residues (Dha, Dhb) and (Me)Lan rings has been researched extensively;[83,84] however, the total chemical synthesis of Avi(Me)Cys-containing natural product peptides has remained elusive. Several research groups have investigated different chemical methods for forming the Avi(Me)Cys moiety on building-block-type compounds and peptide fragments including thiol-yne conjugation by Castle et al.,[9] oxidative decarbonylation by VanNieuwenhze et al.,[10] and condensation of amines with acetals by Taylor et al. (Scheme 3).[11,12] However, each route only generated a low to moderate yield of the Avi(Me)Cys and in *E*/*Z* mixtures. Further modification of these peptides is yet to be reported, perhaps due to the chemical instability of the Avi(Me)Cys unit. Therefore, a total chemical synthesis of any bioactive Avi(Me)Cys-containing natural product has yet to be reported.

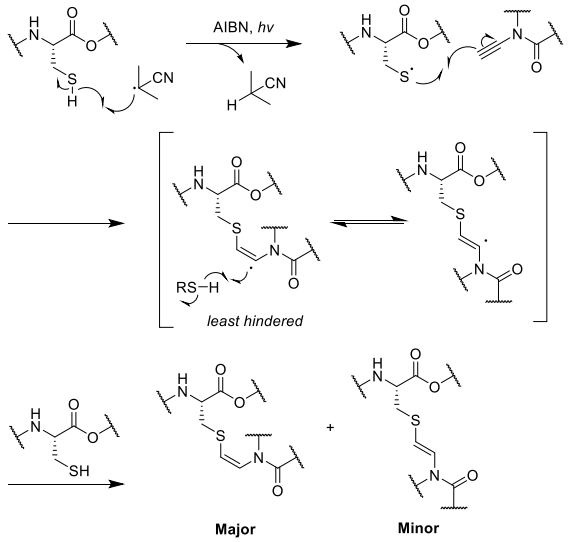
6.1. Radical Thiol-Yne Couplings

In a study toward the synthesis of thioviridamide, Castle et al. prepared building block analogues of AviCys using a radical thiol-yne reaction, where backbone-protected cysteine derivatives were coupled with various ynamides.[9] In this synthesis, the radical initiator, 2,2′-azobis(2-methylproionitrile) (AIBN), first abstracts the sulfhydryl proton to yield a thiyl radical, which propagates across the electron-rich alkyne (Scheme 4).

Scheme 3. Retrosynthetic Scheme Depicting the Different Avi(Me)Cys Synthesis Pathways Reported to Date[9−13]



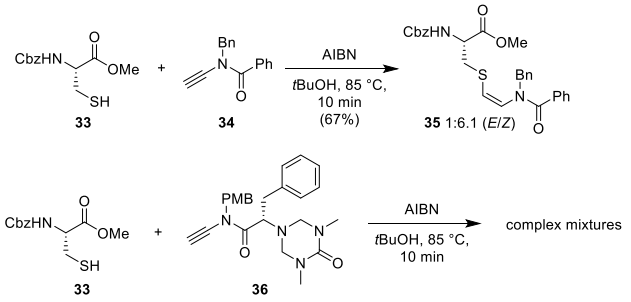
Scheme 4. Postulated Mechanism for Radical Thiol-Yne Reaction for the Synthesis of an AviCys Derivative by Castle et al.[9] (AIBN = 2,2′-Azobis(2-methylpropionitrile))



The resulting carbon-centered alkene radical is then quenched by hydrogen atom transfer (HAT) from a second equivalent of the sulfhydryl. Preliminary studies suggested the (*Z*)-alkene dominates as the kinetically favored product, due to rapid quenching of the alkene radical by HAT on the least hindered side (Scheme 4). Under these conditions, the reaction proceeded well with small substrates, successfully affording **35** as the desired isomer in good yield (Scheme 5).[9] However, when these methods were applied to more complex and potentially useful ynamide building blocks, such as **36**, no desired product was obtained. Therefore, although this procedure proved to be effective for small molecules, it may

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

Scheme 5. Radical Thiol-Yne Reaction for the Synthesis of an AviCys Derivative and Attempted Radical Thiol-Yne Coupling of Cysteine Derivative with Ynamides by Castle et al.[9] (AIBN = 2,2′-Azobis(2-methylpropionitrile), Cbz = Carboxybenzyl, PMB = *para*-Methoxybenzyl)



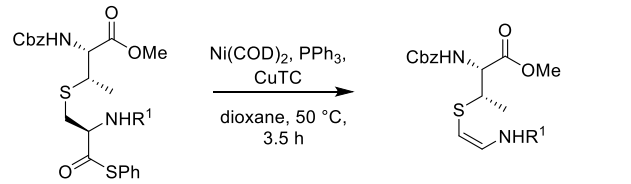
not be practical for the installation of AviCys into more complex substrates.

6.2. Oxidative Decarboxylation/Decarbonylation

In 2012, VanNieuwenhze et al.[10,13] published two back-to-back research articles investigating decarbonylation/decarboxylation strategies toward the synthesis of mersacidin. Their work focused on mimicking the biosynthetic route to Avi(Me)Cys-containing peptides to create building blocks and peptide fragments.

Decarbonylation of model cysteine-containing thioester derivatives afforded the corresponding Avi(Me)Cys derivatives selectively using a $Ni(COD)_4$ and copper(I) thiophene-2-carboxylate (CuTC) catalyst system (Scheme 6). Upon

Scheme 6. Decarbonylation of Thioesters to Give AviMeCys Derivatives and Building Blocks[10] ($Ni(COD)_2$ = Bis(1,5-cyclooctadiene)nickel(0), CuTC = Copper(I) Thiophene-2-carboxylate, Cbz = Carboxybenzyl)



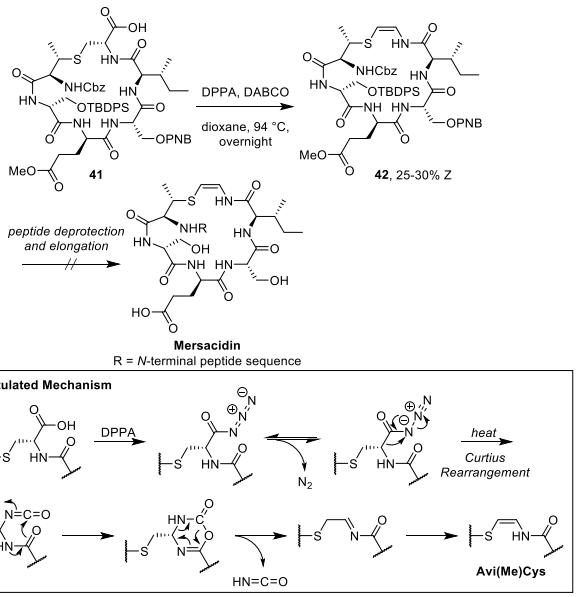
37; R^1 = Fmoc-
38; R^1 = Fmoc-Ile-

39; R^1 = Fmoc-, 75%, *Z* only
40; R^1 = Fmoc-Ile-, 40%, *Z* only

application of the optimized method to building block **37**, complete *Z*-selectivity successfully afforded **39** in 75% yield. However, they found that the yield decreased with increasing substrate complexity, illustrated by the low to moderate yield of **40** from **38**.[10] Furthermore, incorporation of Avi(Me)Cys-containing amino acids such as **39** or **40** into a peptide has not yet been reported, suggesting that a building block approach may not be viable for the synthesis of Avi(Me)Cys-containing peptides.

VanNieuwenhze et al.[13] then reported a similar approach to form a protected fragment of the C-terminal D-ring fragment of mersacidin. The cyclic substrate **41** was prepared by solution-phase peptide synthesis utilizing protecting groups that are orthogonal to TFA-mediated deprotection of the C-terminal carboxylic acid. The MeLan building block was prepared by literature procedures and incorporated into the growing peptide chain, followed by lactamization to yield **41** (Scheme 7). Preliminary attempts at decarboxlation of **41** to

Scheme 7. Oxidative Decarboxylation/Decarbonylation of 41 to Afford 42,[a] the C-Terminal Ring of Mersacidin by VanNieuwenhze et al.[13]



[a]Inset: Curtius rearrangement and postulated mechanism of intramolecular isocyanate trapping and decomposition to give the AviMeCys unit. To date, there has been no further publication utilizing this strategy toward the total synthesis of mersacidin or other Avi(Me)Cys-containing peptides. Cbz = carboxybenzyl, TBDPS = *tert*-butyldiphenylsilyl, PNB = *para*-nitrobenzyl, DPPA = diphenylphosphoryl azide, DABCO = 1,4-diazabicyclo[2.2.2]octane.

afford **42** using diphenylphosphoryl azide (DPPA) with Et_3N in toluene gave promising but low-yielding results.[13] Optimization of this route afforded *Z*-isomer **42** in 25−30% yield using DPPA and 1,4-diazabicyclo[2.2.2]octane (DABCO) in dioxane at reflux overnight (Scheme 7).[13]

This method employed by VanNieuwenhze et al.[13] is presumed to undergo a Curtius rearrangement followed by intramolecular trapping of the isocyanate and collapse of the resulting six-membered ring (Scheme 7, inset). The resulting protected analogue **42** remains the largest Avi(Me)Cys-containing substrate synthesized to date; however, deprotection and/or ligation of this fragment for the total or partial synthesis of mersacidin is yet to be reported.

6.3. Condensation of Amides with Acetals

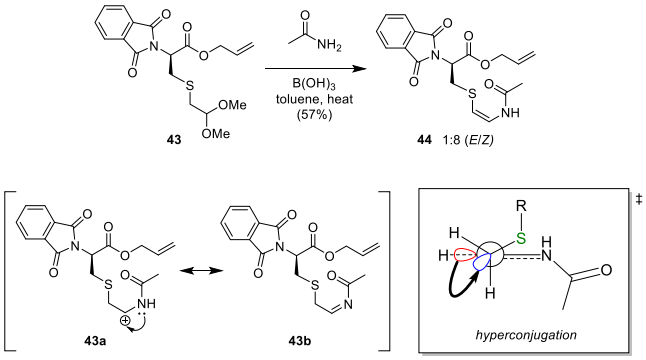
Taylor et al.[11] published a unique strategy toward (*Z*)-thioenamide moieties and AviCys derivatives based on condensation of amides with acetals, followed by β-hydride elimination. The optimized reaction used the mild Lewis acid, $B(OH)_3$, in toluene at reflux to catalyze the condensation of cysteine derivative **43** with acetamide, affording (*Z*)-AviCys derivative **44** in moderate yield with high stereoselectivity.[11]

The presence of the sulfur atom directed the *E*/*Z* selectivity of the reaction.[11] Electronic structure calculations were performed for the reaction of butanal dimethyl acetal with acetamide, with a methylene unit replacing the sulfur atom. The calculations were performed using the Gaussian 09 suite of programs, and it was found that the (*E*)-enamide was the favored product. Conversely, the *Z*-isomer was favored in all reactions with sulfur-containing substrates. This strongly supported the idea that the sulfur atom was favoring the formation of the desired *Z*-isomer; hence computational

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

studies were carried out. By determining the relative energies of each reagent, transition state, and product, it was found that C−C bond rotation occurs, leading to a defined transition state exhibiting an *s-cis* orientation of the heteroatom substituents (**43a** and **43b**, Scheme 8, inset).[11] This transition state has a

Scheme 8. Synthesis of AviCys Derivatives[a] via Condensation of Acetamide upon Acetal 43 in the Presence of a Mild Lewis Acid[11]



[a]Inset: (left) transition state **43a** and **43b** occurring with sulfur-containing substrates; (right) illustration of hyperconjugation occurring with sulfur-containing substrates leading to the *Z*-isomer **44**.

stabilization energy of 4.04 kcal mol^{-1} associated with the hyperconjugative donation from the σ-orbital of the adjacent C−H bond (red) into the σ^*-orbital of the C−S bond (blue) (Scheme 8).[11] This does not occur with substrates lacking sulfur, as the intermediate cation loses a proton resulting in the rapid formation of the (*E*)-enamide.[11] This was consistent with their original hypothesis that a rotational barrier to elimination exists, which favors the arrangement of the electronegative substituents to maximize hyperconjugative stabilization.[11] Additionally, their calculations found that the sulfur atom of the final product possesses a partial positive charge enabling electrostatic attraction with both the oxygen and the nitrogen, thus stabilizing the *Z*- over the *E*-isomer by 1.8 kcal mol^{-1} (Figure 9).[11]

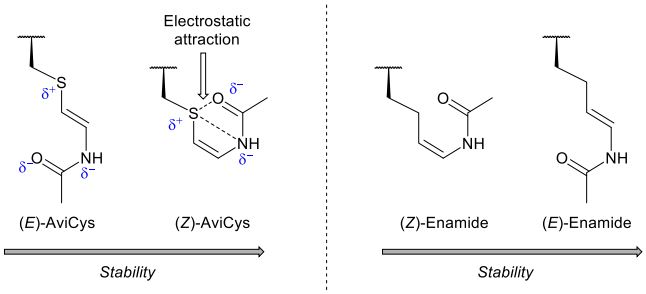
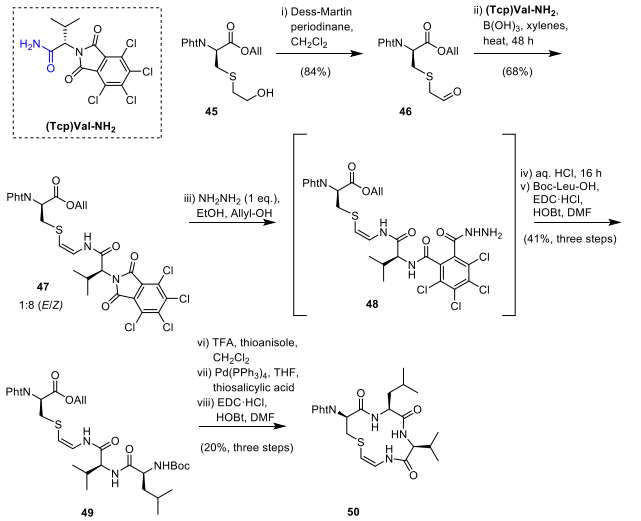


Figure 9. *E*- and *Z*-isomers of AviCys derivatives and enamides prepared by Taylor et al.[11] Left: Due to an electrostatic interaction between the sulfur and both the nitrogen and oxygen atoms, the (*Z*)-AviCys is thermodynamically more stable than the corresponding (*E*)-AviCys.[11] Right: Synthesis of enamides (i.e., substrates lacking a sulfur atom) using the same methods as used for AviCys formation favor the *E*-isomer.[11] Without an electrostatic interaction stabilizing the *Z*-configuration, the (*E*)-enamide predominates as it is less sterically hindered than the (*Z*)-enamide.

Recently, Taylor et al. successfully applied this methodology to the synthesis of the AviCys C-terminal macrocycle of all known members of the linaridin family (Scheme 9).[12] First, the

Scheme 9. Synthesis of the AviCys-Containing Ring of Cypemycin via Condensation of Aldehyde 46 with Amide (Tcp)Val-NH$_2$, Followed by Elongation of the Peptide Chain and Lactamization to Give 50 in 4.6% Yield from 46[12] (Tcp = 3,4,5,6-Tetrachlorophthalimide, Pht = Phthalimide)



primary alcohol of **45** was oxidized to an aldehyde with Dess−Martin periodinane to afford aldehyde **46** in high yield (84%).[12] Condensation of **46** with (Tcp)Val-NH$_2$ (Tcp = 3,4,5,6-tetrachlorophthalimide) in the presence of the Lewis acid B(OH)$_3$ afforded a 1:8 *E*/*Z* mixture of isomers of the AviCys-containing compound **47** in good yield (68%).[12] Previous work had demonstrated that the Tcp group in **47** could be removed in the presence of a Pht (Pht = phthalimide) group using ethylenediamine; however, for Tcp removal of **47**, this led to complex mixtures.[12] It was then found that treatment of **47** with 1 equiv of hydrazine led to a single product: the ring-opened acyl hydrazide intermediate **48**, which was hydrolyzed in aqueous HCl to afford a free amine.[12] Boc-Leu-OH was then coupled using EDC·HCl and HOBt to afford **49** (Scheme 9).

The final step was deprotection of the N- and C-termini with required modifications to the standard reaction conditions due to the sensitivity of the thioenamide.[12] The N-terminal deprotection of **49** was achieved using TFA and thioanisole. Thioanisole was used instead of the more common triethylsilane due to partial reduction of the thioenamide double bond when triethylsilane was used.[12] For deprotection of the C-terminus, initial deallylation conditions using palladium(0) with barbituric acid as an allyl acceptor were unsuccessful and resulted in oxidation of the thioenamide sulfur to the corresponding sulfoxide.[12] Deallylation of the C-terminus was then successfully achieved using thiosalicylic acid as both the allyl acceptor and sacrificial reductant.[12] Finally, lactamization with EDC/HOBt afforded the cyclic AviCys-containing peptide **50** as a single isomer (4.6% overall yield from **45**) (Scheme 9).[12]

https://doi.org/10.1021/jacsau.1c00308
JACS Au 2021, 1, 1527−1540

7.0. CONCLUSION

RiPPs are a large class of diverse natural peptides that contain a number of PTMs, including the structurally distinct Avi(Me)-Cys macrocycle. Nearly all peptides that contain this unusual motif exhibit antimicrobial or anticancer activity, suggesting a biological importance of this scaffold. This is further highlighted by the fact that the biosynthesis of Avi(Me)Cys is conserved across all families, involving the dehydration of serine/threonine or cysteine residues, cysteine decarboxylation, and final macrocyclization to fashion the Avi(Me)Cys unit.

Despite the clinical potential these of natural products, difficulties in synthesizing this important structural moiety hinder drug development. Several synthetic strategies toward Avi(Me)Cys moieties have been attempted; however, the application of these methods to the total synthesis of an Avi(Me)Cys-containing peptide is yet to be reported. Nevertheless, the successes of VanNieuwenhze et al.[13] and Taylor et al.[12] in the syntheses of the Avi(Me)Cys-containing rings of mersacidin and the linaridins, respectively, offer encouragement.

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Funding

The authors wish to acknowledge the Ministry of Business, Innovation and Employment (MBIE Endeavor Grant No. UOAX2010) for generous financial support and the Maurice Wilkins Centre for Molecular Biodiscovery.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AIBN, 2,2′-azobis(2-methylproionitrile); AMP, antimicrobial peptide; AviCys, *S*-[(*Z*)-2-aminovinyl]-D-cysteine; AviMeCys, (2*S*,3*S*)-*S*-[(*Z*)-2-aminovinyl]-3-methyl-D-cysteine; Cbz, carboxybenzyl; CuMeSal, copper(I) 3-methylsalicylate; CuTC, copper(I) thiophene-2-carboxylate; DABCO, 1,4 diazabicyclo[2.2.2]octane; Dha, 2,3-didehydroalanine; Dhb, (*Z*)-2,3-didehydrobutyrine; DPPA, diphenylphosphoryl azide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GlcNAc, *N*-acetylglucosamine; HAT, hydrogen atom transfer; HFCD, homo-oligomeric flavoprotein cysteine decarboxylase; HMOP, 2-hydroxy-2-methyl-4-oxopentanoyl; Lan, lanthionine; LiaRS, lipid II cycle interfering antibiotic response regulator and sensor; (Me)Lan, *β*-methyllanthionine; MRSA, methicillin-resistant *Staphylococcus aureus*; $Ni(COD)_2$, bis(1,5-cyclooctadiene)nickel(0); PMB, *para*-methoxybenzyl; PNB, *para*-nitrobenzyl; RiPP, post-translationally modified peptide; TBDPS, *tert*-butyldiphenylsilyl; Tcp, 3,4,5,6-tetrachlorophthalimide; VISA, vancomycin-intermediate *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus faecalis*

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