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ACS Comb. Sci., **Just Accepted Manuscript** • DOI: 10.1021/acscombsci.7b00189 • Publication Date (Web): 12 Feb 2018

Solid-Phase Synthesis and Antibacterial Activity of Cyclohexapeptide Wollamide B Analogs

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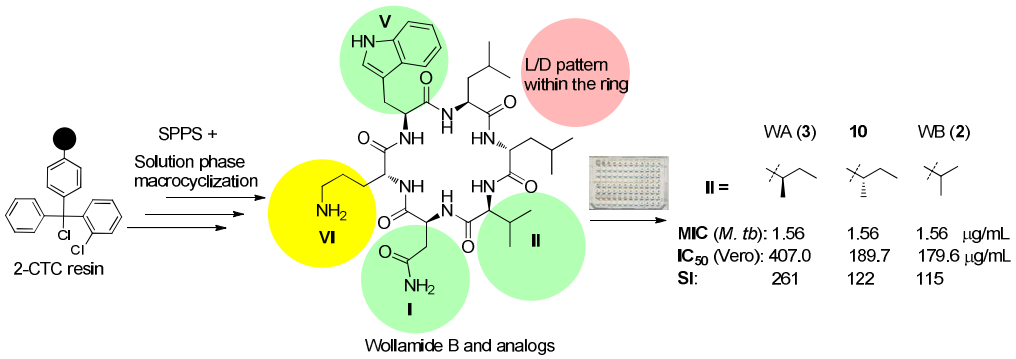
KEYWORDS: *cyclic hexapeptides, desotamide, solid-phase peptide synthesis, antibacterial, antituberculosis, structure-activity relationships, wollamide*



ABSTRACT: Herein we report the antibacterial structure-activity relationships of cyclic hexapeptide wollamide analogs derived from solid-phase library synthesis. Wollamide B, a cyclic hexapeptide natural product, has been previously found to have activity against *Mycobacterium bovis*. To further evaluate its antimycobacterial/antibacterial potential, 27 peptides including wollamides A/B, and desotamide B, were synthesized and subsequently tested against a panel of clinically significant bacterial pathogens. Biological evaluation revealed that the cyclic scaffold, amide functionality in position I, tryptophan residue in position V, and the original stereochemistry pattern of the core scaffold were key for antituberculosis and/or antibacterial activity. In addition, against *M. tuberculosis* and Gram-positive bacteria, residues in position II and/or VI greatly impacted antibacterial activity and selectivity. Wollamides A (**3**) and B (**2**) along with their corresponding II (L-Leu) analog **10** retained the most promising antituberculosis activity, with the lowest minimum inhibitory concentration (MIC) against virulent *M. tuberculosis* H37Rv (MIC = 1.56 μg/mL) as well as desirable selectivity indices (>100). Importantly, the antimicrobial activities of wollamides A and B do not result from disruption of the bacterial membrane, warranting further investigation into their mechanism of action.

INTRODUCTION

In the past several decades, many multi-drug resistant (MDR) pathogens have arisen by acquiring mechanisms to limit the effectiveness of many current antibiotic therapies.[1] Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), and penicillin-resistant *Streptococcus*[2] are especially problematic in clinical settings, as well as the Gram-negative extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[3] *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), has an increasing prevalence of MDR and extensively drug-resistant (XDR) tuberculosis strains and remains one of the top infectious killers worldwide.[4] TB is especially life-threatening in developing countries, particularly for patients who are immunocompromised with HIV.[5-7] This growing health and economic burden caused by drug-resistant infectious agents therefore demands a continuous search for new antibacterial and antitubercular drugs that can overcome the limitations of currently employed therapies.

The synthetic/semisynthetic enhancement of natural products derived from microorganisms has been a reliable source of novel antibacterial agents.[8] One such microorganism that has served as a source of bioactive secondary metabolites is *Streptomyces*, which began its history in the antibiotic field in the 1940s with the discovery of agents such as streptothricin and streptomycin.[9] Many antibiotics in current clinical use were originally sourced from *Streptomyces* species and it is estimated that this genus is capable of producing additional novel antibacterial metabolites for clinical development.[10] On the other hand, mining of these resources has also become more and more difficult to find unique antibiotic molecules; hence we turned to these rare secondary metabolite molecules.

The wollamides (Figure 1) are a rare class of cyclohexapeptides isolated from an Australian soil *Streptomyces* nov. sp. (MST-115088).[11] Wollamides (**2** and **3**) were reported to inhibit *M. bovis* with IC_{50}s of 2.8 and 3.1 μM, respectively.[11] These two wollamide cyclohexapeptides (**2** and **3**), as well as the related desotamide (**1**)[12] also displayed activity against Gram-positive bacteria (*S. aureus* and/or *Bacillus subtilis*). Notably, they were not cytotoxic (>30 μM) to mammalian cell lines, indicating selectivity for bacteria.[11] Structurally, wollamides A/B contain a characteristic basic D-Orn at the VI position, while desotamide B contains an achiral Gly. At position II, both desotamide B and wollamide B contain an L-Val, while wollamide A contains an L-*allo*-Ile.

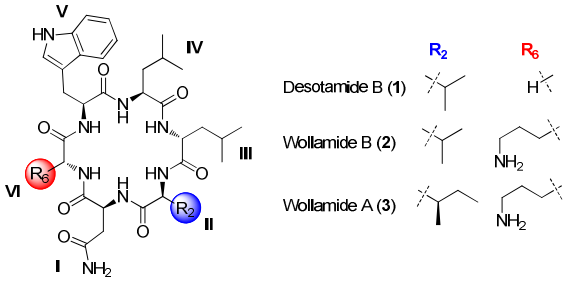


Figure 1. Chemical structures of cyclic hexapeptides desotamide B and wollamides A/B.

In this study, we sought to further evaluate these cyclohexapeptides for their antibacterial and antimycobacterial potential and to explore the structure-activity relationships (SARs) of this class of compounds. Recently, we reported the total syntheses and optimization studies of wollamides A/B and desotamide B using solid-phase peptide synthesis and solution phase cyclization.[13] Herein we describe the design, synthesis, and SAR of three series of wollamide B analogs: (i) amino acid substitutions; (ii) stereochemistry changes around the 18-membered core scaffold; (iii) chemical derivatization of the D-Orn residue.

RESULTS AND DISCUSSION

Library Design

Wollamide B (**2**) was selected as the most promising lead for further analog design as it displayed activity against mycobacteria that was not seen with desotamide B (**1**) and activity against *S. aureus* that was not seen with wollamide A (**3**).[11] Three series of compounds were designed based on this scaffold. *Series (i) - amino acid substitution (Figure 2)*. Analogs were designed and synthesized by substituting a residue at a single position (e.g., I, II, V, or VI) while keeping all other amino acids and stereochemistry (L-L-D-L-L-D) consistent with the original structure of wollamide B. In position I, L-Asn was substituted for L-Gln containing an additional methylene linker between the α-carbon and amide functionality (**8**), and for L-Asp to include a carboxylic acid side group (**9**). In position II, L-Val was replaced with other aliphatic residues including L-Ala (**11**), the non-proteinogenic α-Me-Ala (**12**), and the more bulky L-Leu (**10**) and L-*allo*-Leu (**3**). In position V, the L-Trp residue was substituted for aromatic L-Tyr (**13**) or L-Phe (**14**). In position VI, D-Orn was replaced with Gly (**1** and its II-Ile analog **4**), with D-Arg (**15**) containing a more basic guanidine moiety, as well as with D-Lys (**16**) containing an additional methylene linker.

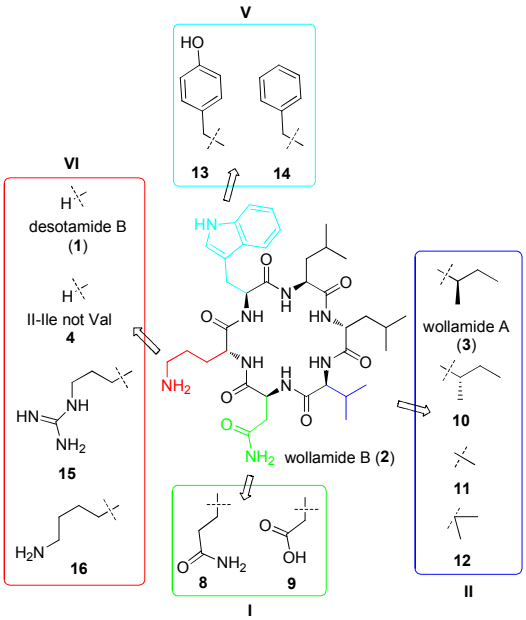


Figure 2. Structures of wollamide B and analogs (**1**-**4** and **8**-**16**) with amino acid substitutions.

Series (ii) - Stereochemical modifications to the core scaffold (Figure 3). Wollamide B contains two D-amino acid residues, Leu and Orn, at the III and VI position, respectively. Analogs in this series include either a single stereochemistry change (**17**-**19**), or multiple stereochemistry changes (**20**-**22**) containing up to five D-residues.

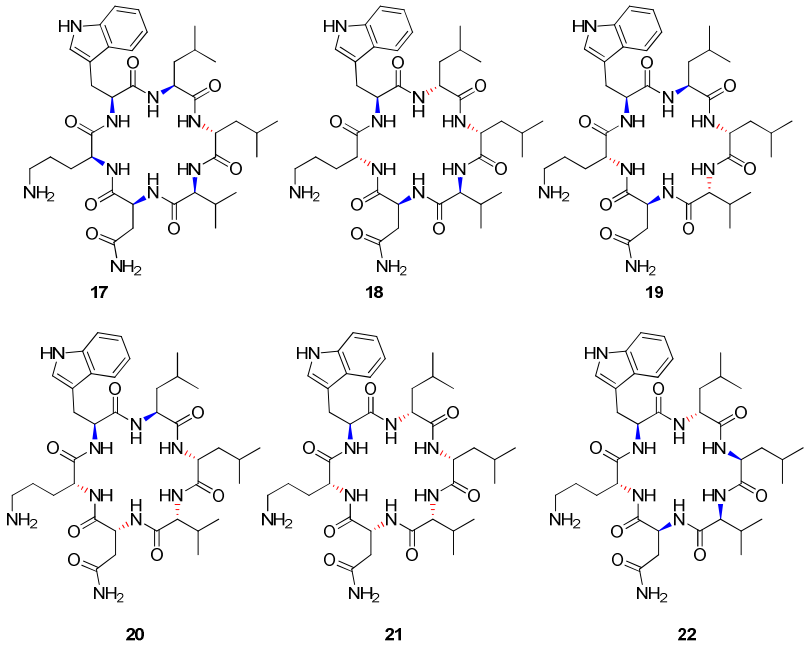


Figure 3. Structures of wollamide B analogs **17**-**22** with core stereochemistry changes.

Series (iii) - Chemical derivatization of the D-Orn residue (Figure 4). Khalil et al. observed that wollamides A and B (with a VI-D-Orn) displayed *M. bovis* inhibitory activity, but desotamides (with a VI-Gly) were inactive.[11] To further evaluate the importance of the ornithine residue to the antituberculosis activity of wollamide B, the primary amine of D-Orn was converted to secondary, (cyclic) tertiary, or quaternary amines. These transformations were achieved by selective chemical modifications (e.g., acylation or reductive alkylation) of the primary amine

group of the Orn residue of wollamide B, as the ornithine amine group is the most nucleophilic site of the molecule.[14]

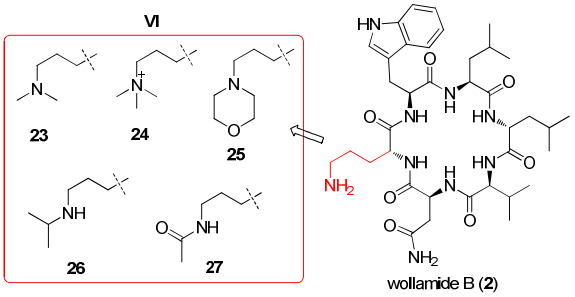


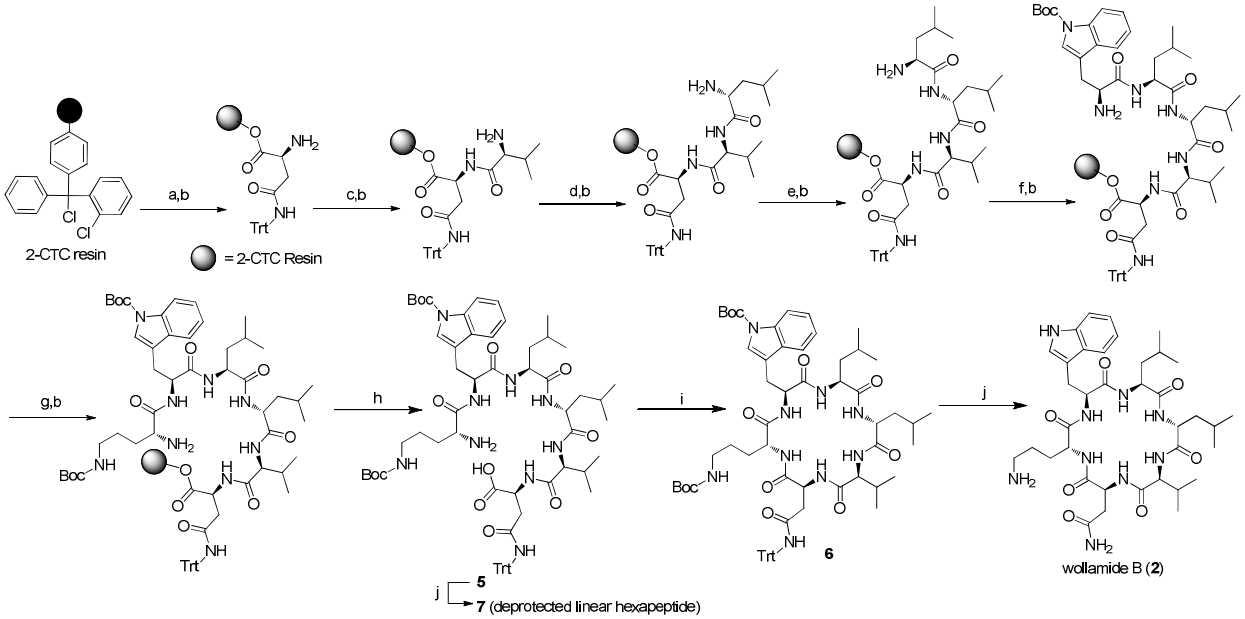
Figure 4. Structures of wollamide B D-Orn derivatives **23**-**27**.

In addition to these cyclic hexapeptide target molecules, and to enable comparison, the key intermediates of wollamide B, including the linear hexapeptide **5** with protecting groups, the linear hexapeptide **7** without protecting groups, and cyclic hexapeptide with protecting groups **6** (Scheme 1) were also evaluated to determine the importance of cyclization, as well as exposed side chains, on the antimicrobial profile of the wollamides.

Synthesis

Cyclohexapeptides were synthesized following our previously described solid-phase synthesis with solution phase cyclization.[13] Scheme 1 outlines the representative synthesis of wollamide B (**2**). Briefly, the solid-supported linear hexapeptide was built on 2-chlorotrityl chloride (2-CTC) resin following resin loading and sequential solid-phase peptide synthesis (SPPS) using standard Fmoc amino acid chemistry with appropriate orthogonally protected side-chains.[15, 16] After cleaving from the resin, the linear hexapeptide **5** with protected side chains was cyclized in solution to give **6**, which was then deprotected with a TFA solution to afford the final wollamide B (**2**). The synthesized compounds were characterized by ^{1}H, ^{13}C NMR and HRMS, and their

HPLC chromatograms are shown in Figure 5. To synthesize the linear precursor **7** without protecting groups, the same TFA deprotecting method was applied to **5**.



Scheme 1. Synthesis of wollamide B on 2-CTC Resin.[13] Reagents and Conditions: (a) Fmoc-Asn(trt)-OH, DIPEA, DCM, 3h; (b) 25% 4-methylpiperidine in DMF; (c) Fmoc-Val-OH, DIC, HOBt, DMF/DCM 1:1, 4h; (d) Fmoc-D-Leu-OH, DIC, HOBt, DMF/DCM 1:1, 4h; (e) Fmoc-Leu-OH, DIC, HOBt, DMF/DCM 1:1, 4h; (f) Fmoc-Trp(boc)-OH, DIC, HOBt, DMF/DCM 1:1, 4h; (g) Fmoc-D-Orn(boc)-OH, DIC, HOBt, DMF/DCM 1:1, 4h; (h) HFIP/DCM 1:4, 30 min; (i) HBTU, DIPEA, DMF, 30 min; (j) TFA/TIPS/DCM 50:5:45, 30 min.

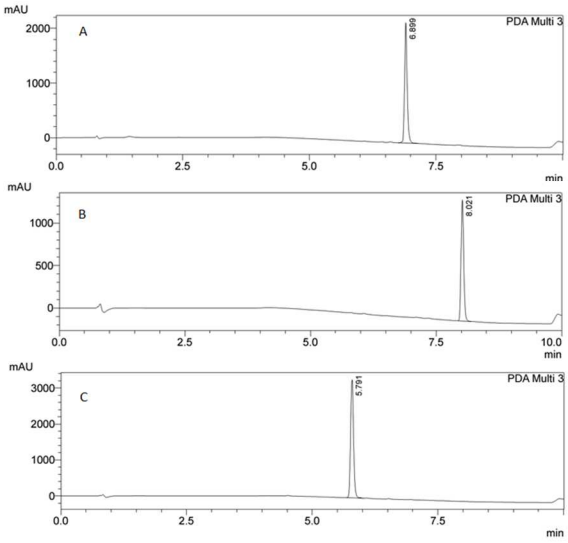
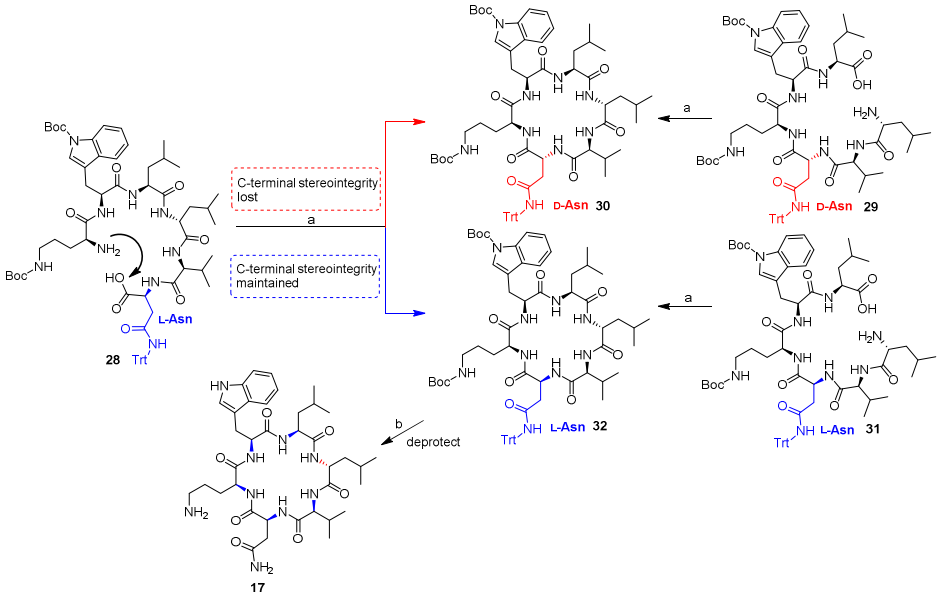


Figure 5. Representative HPLC chromatograms. A = Linear precursor **5**; B = Cyclized wollamide B (**6**) with protecting groups; C = Synthetic wollamide B (**2**).

Using the representative synthetic route/protocol outlined in Scheme 1, all of the other cyclohexapeptides (**1**, **3**-**4**, **8**-**16** in Figure 2 and **18**, **19**, **22** in Figure 3) were prepared accordingly and cyclized between position I and position VI without detected C-terminal epimerization. However, three cyclic hexapeptides **17**, **20**, and **21** from Series (ii) (Figure 3) were synthesized between position III and position IV to prevent epimerization.

Compounds in Series (ii) contained the same residues as wollamide B, but with different stereochemistry around the cyclic hexapeptide core. Based on the suggested importance of the D-Orn residue of wollamide B to the antimycobacterial activity,[11] an L-Orn epimer **17** was first synthesized to evaluate the stererospecific effect on antibacterial activity (Scheme 2). In the initially attempted synthesis, the linear peptide **28** was built starting from the C-terminal L-Asn to the N-terminal L-Orn (Scheme 2). Cyclization of **28** proceeded notably slower (1 h) than other structural analogs, and it was found that two products with the same mass could be detected by TLC, which closely eluted during column purification. This led to the revised and improved

synthesis of **17** from the linear hexapeptide **31** with L- and D-Leu as terminal residues and L-Orn at an internal position. This disconnection site was identified in our previous study to be an optimized cyclization alternative to access wollamide B.[13] Cyclization of **31** led to a sole product **32** as determined by TLC and NMR, and was found to be the slower eluting product from the first attempted synthesis. Deprotection of **32** afforded the L-Orn analog **17**. The C-terminal epimer **30** was also independently synthesized from the linear precursor **29** and was confirmed to be the faster eluting product from the first attempted synthesis from **28**.



Scheme 2. Synthesis of the L-Orn analog **17** of wollamide B. Reagents and Conditions: (a) HBTU, DIPEA, DMF, 30 min−1 h; (b) TFA/TIPS/DCM 50:5:45, 30 min.

Additional stereochemistry analogs prepared included **18-19** with a single stereochemistry change and **20-22** with multiple stereochemistry changes. Interestingly, we found that, in a comparison of wollamide B epimers obtained in the studies, greater TLC R_f values were seen with cyclic hexapeptides that had a balance of L and D residues. For example, in the

epimerization of the L-Orn precursor **28** (Scheme 2), the higher R_f product **30** contained a ratio of 2:4 D to L residues (entry 6, Table 1), while the lower R_f product **32** contained a ratio of 1:5 D to L residues (entry 5, Table 1). The cyclic peptides that contained five L or D residues (entries 5 and 8, respectively), yielded the lowest R_f values of 0.66 and 0.50, respectively. The same pattern was also observed for another two pairs of epimers **6** (L:D = 4:2; R_f = 0.85, entry 2) and **19** precursor (L:D = 3:3; R_f = 0.93, entry 3) as well as the precursors (L:D = 2:4; R_f = 0.78, entry 7) for **20** and (L:D = 1:5; R_f = 0.50, entry 8) for **21**.

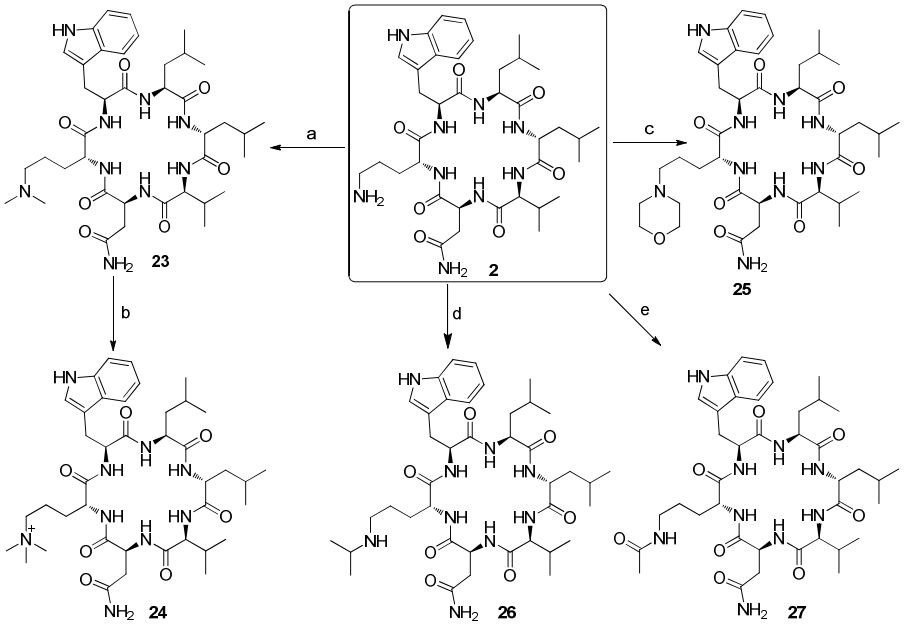
Table 1. Thin-Layer Chromatography (TLC) R_f values for wollamide B epimers

Entry	Linear Peptide	Cyclic Peptide	R_f (EtOAc/MeOH/DCM 65:5:30)	L:D Ratio
1	HO-Val-D-Leu-Leu-Trp(boc)-D-Orn(boc)-Asn(trt)-NH_2	a mixture of epimers[a]	0.85/0.93	4:2/3:3
2	HO-Asn(trt)-Val-D-Leu-Leu-Trp(boc)-D-Orn(boc)-NH_2	*cyclo*[Asn(trt)-Val-D-Leu-Leu-Trp(boc)-D-Orn(boc)] (**6**)	0.85	4:2
3	HO-Asn(trt)-D-Val-D-Leu-Leu-Trp(boc)-D-Orn(boc)-NH_2	*cyclo*[Asn(trt)-D-Val-D-Leu-Leu-Trp(boc)-D-Orn(boc)] (for **19**)	0.93	3:3
4	HO-Asn(trt)-Val-D-Leu-Leu-Trp(boc)-Orn(boc)-NH_2 (**28**)	a mixture of epimers[a]	0.66/0.90	5:1/4:2
5	HO-Leu-Trp(boc)-Orn(boc)-Asn(trt)-Val-D-Leu-NH_2 (**31**)	*cyclo*[Asn(trt)-Val-D-Leu-Leu-Trp(boc)-Orn(boc)] (**32**)	0.66	5:1
6	HO-Leu-Trp(boc)-Orn(boc)-D-Asn(trt)-Val-D-Leu-NH_2 (**29**)	*cyclo*[D-Asn(trt)-Val-D-Leu-Leu-Trp(boc)-Orn(boc)] (**30**)	0.90	4:2
7	HO-Leu-Trp(boc)-D-Orn(boc)-D-Asn(trt)-D-	*cyclo*[D-Asn(trt)-D-Val-D-Leu-Leu-Trp(boc)-D-	0.78	2:4

	Val-D-Leu-NH_2	Orn(boc)] (for **20**)		
8	HO-D-Leu-Trp(boc)-D-Orn(boc)-D-Asn(trt)-D-Val-D-Leu-NH_2	*cyclo*[D-Asn(trt)-D-Val-D-Leu-D-Leu-Trp(boc)-D-Orn(boc)] (for **21**)	0.50	1:5

[a]Entries 1 and 4 resulted in a mixture of epimers after solution-phase macrocyclization. For Entry 1, the cyclization products were identified to be the desired product **6** (Entry 2) and its corresponding C-terminal epimer (Entry 3). For Entry 4, the cyclization products were identified to be the desired product **32** (Entry 5) and the C-terminal epimer **30** (Entry 6).

The Series (iii) Orn-derivatized analogs were prepared directly from synthetic wollamide B (Scheme 3). To synthesize the dimethylated analog **23**, wollamide B (**2**) was treated with aq. formaldehyde and sodium cyanoborohydride in ethanol to afford **23** in 32% yield. Accordingly, the trimethylated quaternary amine derivative **24** was synthesized from **23** using excess methyl iodide in high yield (86%).[17] Similarly, the isopropyl secondary amine **26** was prepared from **2** in 28% yield using acetone and sodium cyanoborohydride.[18] To afford morpholine compound **25**, **2** was treated with sodium iodide, 2-chloroethyl ether and triethyl amine in acetonitrile and **25** was obtained in 27% yield. Finally, the N-acylated compound **27** was prepared in moderate yield (58%) with potassium carbonate and acetic anhydride in DMF/acetonitrile.[18]



Scheme 3. Synthesis of D-Orn modified analogs **23**-**27** from synthetic wollamide B (**2**). Reagents and conditions: (a) Aq. HCHO, $NaBH_3CN$, EtOH, 1.5 h; (b) MeI, EtOH, overnight; (c) 2-chloroethyl ether, NaI, Et_3N, CH_3CN, overnight; (d) Acetone, $NaBH_3CN$, EtOH, 2 h; (e) Acetic anhydride, K_2CO_3, DMF/CH_3CN 1:1, overnight.

Biological Activity

All synthesized compounds were evaluated against *M. tuberculosis* (H37Rv), and a panel of Gram-positive and -negative pathogens including: *S. aureus*, *Clostridium difficile*, *Staphylococcus epidermis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus cereus*, and *Escherichia coli* (K12 and Δ*tolC*). The antitubercular and antibacterial activities are summarized in Table 2. The activities of the analogs are discussed with respect to that of wollamide B (**2**), the lead for this series, unless otherwise specified.

Cytotoxicity of the synthesized peptides was determined with mammalian Vero kidney epithelial cells. The selectivity index (SI) of each compound was calculated using the cytotoxic IC_{50} value divided by the MIC against *M. tuberculosis*, and is shown in Table 2.

Table 2. Antibacterial activities (MIC, μg/mL) and cytotoxicity (IC_{50}, μg/mL) of linear and cyclic hexapeptides

Compound Class	Compound No.	*M. tb*[a]	*S. a.*[b]	*C. d.*[c]	*S. e*[d]	*E. f*[e]	*E. f*[f]	*B. c.*[g]	*E. c.* (K12)[h]	*E. c.* (*ΔtolC*)[i]	IC_{50} (Vero)	Selectivity Index (SI)[j]
Desotamide	**1**	>200	100	128	200	100	100	100	>200	>200	246.7	<1.2
	4	>200	12.5	64	25	12.5	12.5	12.5	>200	>200	90.8	<0.5
Wollamide	**2**	1.56	25	64	25	25	25	50	100	50	179.6	115
	2(TFA salt)	3.13	50	Nd[k]	50	100	100	50	100	100	405.9	130
	3	1.56	12.5	32	12.5	12.5	12.5	25	100	50	407.0	261
Precursor	**5**	>200	>200	>256	>200	>200	>200	>200	>200	>200	187.0	<0.9
	6	>200	>200	>256	>200	>200	>200	>200	>200	>200	127.2	<0.6
	7	>200	>200	>256	>200	>200	>200	>200	>200	>200	223.0	<1.1
I	**8**	12.5	100	256	100	100	100	100	>200	100	404.3	32
	9	>200	>200	>256	>200	>200	>200	>200	>200	>200	407.6	<2.0
II	**10**	1.56	25	Nd	12.5	12.5	12.5	25	100	50	189.7	122
	11	200	>200	>256	>200	>200	>200	>200	>200	>200	242.7	1.2
	12	>200	>200	>256	>200	>200	>200	>200	>200	>200	406.2	<2.0
V	**13**	200	>200	>256	>200	>200	200	>200	>200	>200	403.5	2.0
	14	100	>200	>256	>200	>200	>200	>200	>200	200	403.4	4.0
VI	**15**	6.25	50	32	50	50	50	50	>200	200	193.7	31
	16	3.13	25	64	25	25	25	25	100	100	402.6	129
Stereochemistry	**17**	200	>200	>256	200	>200	>200	>200	>200	>200	73.2	0.4
	18	100	>200	Nd	>200	200	200	>200	>200	>200	405.4	4.1
	19	>200	>200	Nd	>200	>200	>200	>200	>200	>200	398.6	<2.0
	20	200	>200	Nd	>200	200	>200	>200	>200	>200	400.9	2.0
	21	200	>200	>256	>200	>200	>200	>200	>200	>200	401.9	2.0
	22	>200	>200	Nd	>200	>200	>200	>200	>200	>200	403.2	<2.0
D-Orn modified	**23**	6.25	100	256	100	50	50	100	>200	200	105.7	16.9
	24	6.25	>200	>256	>200	>200	200	>200	>200	>200	89.2	14.3

	25	12.5	25	Nd	50	50	50	50	>200	>200	190.5	15.2
	26	12.5	200	256	200	200	200	200	>200	200	42.2	3.4
	27	>200	200	128	200	100	100	200	>200	>200	86.4	<0.4
	Ampicillin	-	0.39	-	6.25	12.5	12.5	200	25	12.5		

[a]*M. tuberculosis* (H37Rv) (Positive control ethambutol inhibited *M. tuberculosis* at 1.6 μg/mL); [b]*S. aureus* (ATCC 29213); [c]*C. difficile* (R20291) (Positive control vancomycin inhibited *C. difficile* at 0.25 μg/mL); [d]*S. epidermis* (ATCC 14990); [e]*E. faecalis* (ATCC 33186); [f]*E. faecium* (ATCC 19434); [g]*B. cereus* (ATCC 14579); [h]*E. coli* (K12); [i]*E. coli* (Δ*tolC*); [j]Selectivity Index (SI) = cytotoxic IC_{50}/MIC against *M. tuberculosis*; [k]Nd = Not determined.

Antituberculosis Activity.

Evaluation of compounds against *M. tuberculosis* H37Rv demonstrated that wollamide B (**2**) and wollamide A (**3**) exhibited antituberculosis inhibitory activity (MIC = 1.56 μg/mL) whereas desotamide B (**1**) and its II-Ile analog (**4**) were inactive (MIC > 200 μg/mL). The L-Ile analog (**10**) of wollamide A, as well as the TFA salt form of wollamide B, were also active with MICs of 1.56 and 3.13 μg/mL, respectively. Therefore, replacement of the Gly at position VI (present in desotamide B) with the basic D-Orn (present in wollamides A/B) considerably enhances *M. tuberculosis* activity, as previously reported for *M. bovis*.[11]

Both linear synthetic precursors (**5** and **7**), and the side chain protected wollamide B (**6**) were inactive against *M. tuberculosis* (H37Rv), suggesting that the cyclic hexapeptide scaffold, in addition to the exposed functional groups of Orn, Trp, and/or Asn, are important for activity.

The amide functionality at position I plays a notable role in antituberculosis activity as evident from the comparison of **2** (I-Asn, MIC = 1.56 μg/mL) to **9** (I-Asp, MIC > 200 μg/mL). Compound **8** (I-Gln, MIC = 12.5 μg/mL) with an additional methylene group maintained activity but with reduced potency.

Notably, the lipophilic and bulky residues in position II dramatically influenced antituberculosis activity. While more steric bulky groups (e.g., **2**, II-Val; **3**, II-*allo*-Ile; and **10**, II-Ile) at position II produced compounds with excellent antitubercular activity (MIC = 1.56 μg/mL), **11** (II-Ala, MIC = 200 μg/mL) and **12** (II-MeAla, MIC > 200 μg/mL) were inactive. These data strongly suggest that the aliphatic group of the residue in this position must extend beyond one carbon length. In addition, by comparing wollamide A (**3**, II-*allo*-Ile) and its side chain epimer (**10**, II-Ile), both compounds showed antituberculosis activity with a MIC of 1.56 μg/mL, indicating that the stereochemistry of the side chain at position II does not influence antitubercular activity.

To investigate the impact of the Trp residue in position V, other aromatic residues such as Phe and Tyr were introduced and evaluated. However, both groups were not well tolerated, as **13** (V-Tyr, MIC = 200 μg/mL) and **14** (V-Phe, MIC = 100 μg/mL) produced very weakly active compounds compared to **2** (V-Trp, MIC = 1.56 μg/mL).

At position VI, although replacement of the D-Orn residue with Gly resulted in complete loss of activity in H37Rv, several other modifications at this position were tolerated. Increasing the linker chain from 3 carbons in **2** (VI: D-Orn, MIC = 1.56 μg/mL) to 4 carbons in **16** (VI: D-Lys, MIC = 3.13 μg/mL) increased the MIC by only 2-fold. Other modifications resulted in moderately active compounds with 4-8 fold increases in the MIC. The Arg analog **15** with the more basic guanidine motif increased the MIC by 4-fold (MIC = 6.25 μg/mL). Tri and dimethylated analogs **23** and **24** (MIC = 6.25 μg/mL) also showed 4-fold increase in the MIC relative to wollamide B (**2**), while the cyclic tertiary amine morpholine derivative **25** and the secondary isopropyl amine analog **26** showed moderate antitubercular activity and increased the MIC by 8-fold (MIC = 12.5 μg/mL). The only modification that was not tolerated at the VI position in this amine derivatization series, was the acylation analog **27** (MIC > 200 μg/mL).

From a comparison of these results, a primary amine functionality generally enhanced the inhibitory activity against *M. tuberculosis* as opposed to secondary, (cyclic) tertiary or quaternary amines.

Any stereochemical modification to the core scaffold was not tolerated. Of the six compounds in this series, only **18** displayed weak antituberculosis activity (MIC = 100 μg/mL). Notably, this compound contained just a single stereochemistry change from L-Leu to D-Leu at the IV position. The lack of activity for all other stereochemistry analogs suggests that the original stereochemistry pattern of this cyclic hexapeptide scaffold is of crucial importance, potentially due to flexibility or conformation for binding[19] and/or associated drug-like properties.[20] This finding is also consistent with a recent report by Imming and colleagues.[21]

Antibacterial activity.

Wollamide A (**3**) and Wollamide B (**2**) were moderately active against Gram-positives, with **3** (MIC = 12.5 μg/mL) generally being 2-fold more potent than **2** (MIC = 25 μg/mL). It should be noted that, contrary to the previous report,[11] compound **3** showed higher anti-*S. aureus* activity than **2** in our antibacterial MIC assay. The wollamide A Ile epimer **10** (II-Ile, MIC = 12.5-25 μg/mL) had comparable Gram-positive activity to **2** and **3**, suggesting that L-*allo*-Ile, L-Ile, and L-Val were all well tolerated at the II position when D-Orn is present on the VI position. The Ala (**11**) and MeAla (**12**) substitutions at the II position were not tolerated for all strains tested. Interestingly, the VI-Gly substituted desotamide B (**1**) displayed weak activity (MIC = 100 μg/mL) against Gram-positive strains, but replacement of the L-Val with L-Ile (**4**) at position II restored activity (MIC = 12.5 μg/mL). This finding was consistent with a very recent study by Chen et al. that also found an improvement in anti-MRSA activity with the replacement of Val to

Ile at position II.[22] Taken together, the data indicates that the residue in position II may play a more important role in the Gram-positive antibacterial profile.

At position I, **8** (I-Gln) displayed weak activity (MIC = 100 μg/mL) against the Gram-positive pathogens, while **9** (I-Asp) was inactive against the entire test panel, demonstrating that the carboxylic acid functionality is detrimental to antibacterial activity. Compound **8** also showed 4-fold less potent MIC values compared to wollamide B (**2**, I-Asn, MIC = 25 μg/mL), suggesting that the amide functionality and its distance from the macrocyclic ring system is important for antibacterial activity. At position V, both Tyr (in **13**) and Phe (in **14**) substitutions were not tolerated by Gram-positive organisms, indicating Trp is essential at this position.

Modifications to position VI revealed some modification to this residue may be possible for future design and that this residue may play an important role in the antibacterial activity and selectivity of these compounds. The D-Lys analog **16** had a nearly identical antibacterial profile to wollamide B (**2**, VI-D-Orn). The D-Arg **15** and morpholine ring **25** analogs were active against the same pathogens, but generally with a 2-fold reduction in potency. Dimethyl analog **23**, secondary amine isobutyl derivative **26**, and the acylated **27** showed weak activity (MIC = 50-200 μg/mL) against all Gram-positive strains. Notably, the quaternary trimethylated amine derivative **24** had no activity against the panel of strains, except *M. tuberculosis* (MIC = 6.25 μg/mL). Conversely, combining VI-Gly with II-Ile (**4**) resulted in no activity against *M. tuberculosis* (MIC = >200 μg/mL), while enhancing activity 2-fold in Gram-positives (MIC = 12.5 μg/mL).

All stereochemical analogs **17**-**22** did not display MICs below 100 μg/mL against any of the test organisms, suggesting that like *M. tuberculosis,* the original stereochemistry pattern in

wollamide B is also important for activity against Gram-positives. Very recently, Chen et al. also found that the replacement of D-Orn to L-Orn resulted in a cyclic peptide that was devoid of activity against the test panel of bacteria,[22] consistent with our findings that the stereochemical pattern is an important determining factor for activity.

Activities were also determined against the Gram-positive spore-forming *C. difficile*[23] that now poses an urgent threat to healthcare in the United States.[24] Against *C. difficile,* compounds had a similar profile as the other Gram-positives. Desotamide compounds **1** and **4**, wollamides **2** and **3**, and position VI substituted derivatives **15**, **16** and **27** showed moderate to weak activity. The Ile analog **4** (MIC = 64 μg/mL) was 2-fold more potent than desotamide B (**1**, MIC = 128 μg/mL). Wollamide A (**3**) was 2-fold more potent than wollamide B (**2**) with MICs of 32 and 64 μg/mL, respectively. This suggests that, similar to the SAR pattern toward other Gram-positives, an *allo*-Ile or Ile substituent at position II was more favorable for antidifficile activity. At position VI, D-Arg and Lys substitutions were tolerated. Analog **15** with the Arg motif was 2-fold more potent than wollamide B (MIC = 32 μg/mL), whereas the corresponding lysine analog **16** was equipotent to wollamide B (MIC = 64 μg/mL). In addition, N-acylated derivative **27** exhibited weak inhibitory activity (MIC = 128 μg/mL). Finally, compounds were generally inactive against Gram-negative organisms, with only wollamides A/B, **10**, and **16** having weak activity (MIC = 50-100 μg/mL).

A summary of antitubercular and antibacterial SAR defined in this study is represented in Figure 6.

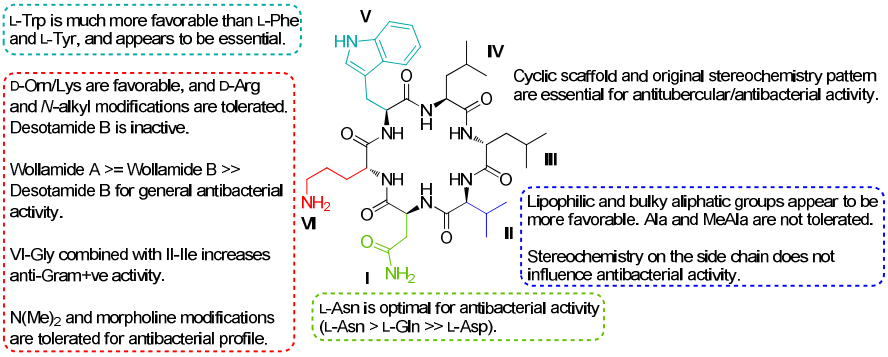


Figure 6. General antitubercular and antibacterial SARs of cyclic hexapeptide analogs.

The progression of promising antibacterial agents is often curtailed by lack of a specific antibacterial mode of action, as hit molecules often act non-specifically by rupturing the bacterial membrane or by inhibiting cellular respiration.[25] Such molecules can be readily detected, as they dissipate the membrane potential. Using a classic fluorescent assay to measure effects on the membrane potential of *S. aureus* NRS70, neither of the wollamides **2** or **3** dissipated the membrane potential (Figure 7). This indicates that wollamides do not target the bacterial membrane or act as respiratory poisons and in light of stereochemistry requirements for activity, wollamides may have a specific drug target. This finding warrants future mechanistic studies to discover the cellular target(s) for the wollamides in order to provide a rationale and future guidance on the design and development of more potent wollamide B analogs.

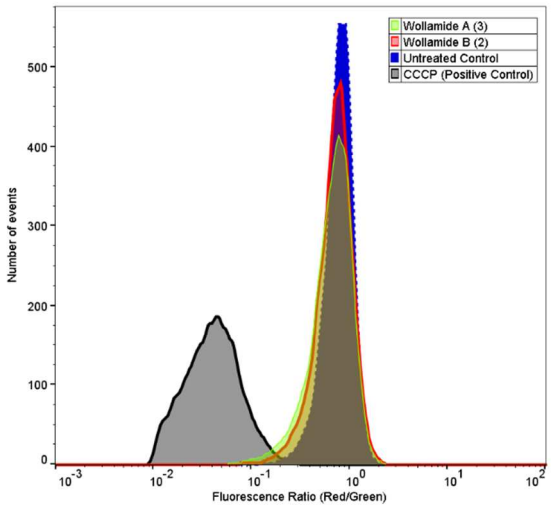


Figure 7. Effects of wollamides **2** and **3** on staphylococcal membrane potential. Both wollamides A and B at 100 μM did not dissipate the membrane potential of *S. aureus* NRS70. As expected, the ionophore CCCP (black) at 10 μM dissipated the membrane potential, causing a red-to-green shift in fluorescence.

CONCLUSIONS:

In summary, natural products wollamides A/B, desotamide B, and analogs of wollamide B were synthesized and evaluated against *M. tuberculosis* and a select panel of Gram-positive and -negative bacterial pathogens. This study revealed that antibacterial activities for wollamide and desotamide cyclohexapeptides depend on their cyclic scaffold, amide functionality in position I, a tryptophan residue in position V, and retention of the original stereochemistry pattern. In addition, residues in position II and VI greatly impacted activity and bacterial selectivity, with position II having more relevance for Gram-positives and position VI more importance for mycobacteria. Position VI was found to be somewhat tolerable for modification. Despite the activity displayed by some of the chemically modified Orn derivatives (**23**, **24**), wollamides A

(**3**) and B (**2**) along with the II-L-Leu analog **10** remained the most promising as antitubercular agents (MIC = 1.56 μg/mL). Importantly, these compounds also showed desirable selectivity indices (SI > 100), with minimal toxicity to mammalian cells. Collectively, this work represents one of the most extensive antibacterial assessments of wollamides A/B and desotamide B along with wollamide B analogs against *M. tuberculosis* and a select panel of Gram-positive and -negative bacteria. Future studies will focus on the systematic medicinal chemistry optimization at position II and VI, as well as pharmacokinetic and in vivo efficacy studies of our promising antitubercular lead candidates.

EXPERIMENTAL PROCEDURES

General Methods. Protected L-amino acids, 2-CTC resin, and HOBt were obtained from AAPPTec LLC (Louisville, KY, USA). Fmoc-L-*allo*-Ile was purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). Protected D-amino acids were purchased from ChemPep Inc. (Wellington, FL, USA). DIPEA, DIC, 4-methylpiperidine and all solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monitoring of reaction progress in solution-phase and final purity of compounds were determined by analytical HPLC (Shimadzu LC-20A series) using a Gemini, 3 μm, C18, 110 Å column (50 mm × 4.6 mm, Phenomenex) and a flow rate of 1.0 mL/min. The gradient conditions used were solvent A (0.1% trifluoroacetic acid in water) and solvent B (acetonitrile): 0-2.0 min 100% A, 2.0-7.0 min 0-100% B (linear gradient), 7.0-8.0 min 100% B, with UV detection at 254 and 220 nm. Compounds were purified by flash column chromatography on silica gel using a Biotage Isolera One system using Biotage SNAP KP-NH 11g or SNAP HP-Ultra 10g columns. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 (400 MHz) spectrometer (Bruker, Billerica, MA, USA) using TopSpin 3.2 software. NMR solvent peaks were referenced as follows: (^{1}H NMR) DMSO-d_6: 2.50 ppm; (^{13}C

NMR) DMSO-d_6: 39.52 ppm. High-resolution mass spectra were measured on an Agilent 6530 QTOF-MS with ESI probe. Lyophilization was done with a LABCONCO FreeZone Plus 12 Liter Cascade Console Freeze Dry System (Kansas City, MO, USA).

General Procedure for the Solid-Phase Synthesis of Cyclic Hexapeptides. The SPPS synthesis used a similar standard coupling protocol that has been previously reported.[15, 16] 2-Chlorotrityl (2-CTC) resin (250 mg, 0.21 mmol, 0.84 mmol/g loading capacity) was treated with a mixture of the first FMOC protected amino acid (1.5 equiv., 0.315 mmol), 3 mL of DMF, and *N,N*-diisopropylethylamine (DIPEA) (2 equiv., 0.42 mmol, 0.073 mL) and the reaction was agitated for 3 h. The reaction was filtered and then washed three times with DCM, MeOH, DMF, and then DCM again (3 mL, 1 min each). After the removal of the first FMOC group with 25% 4-methylpiperidine in DMF (3 mL, 30 min), the resin was washed with DMF (3 ×) and then DCM (3 mL, 1 min each). The remaining FMOC protected amino acids were coupled using a mixture of amino acid (3 equiv., 0.63 mmol), HOBt (3 equiv., 0.63 mmol, 96.4 mg), and DIC (3 equiv., 0.63 mmol, 97.5 μL) in 3 mL DCM/DMF (1:1, v/v) that was pre-activated for 10 min before the addition to the resin. After 4 h, the resin was washed three times with DMF, and then DCM (3 mL, 1 min each) and the cycle of deprotection, washing (DMF, DCM), coupling, and washing was repeated for the remaining amino acid sequence. Between couplings, syringes were stored at -4ºC. Following the SPPS synthesis of the linear peptide, the final FMOC deprotection proceeded as previously described. The resin was then treated with a solution of HFIP in DCM (1:4, v/v) for 30 min[26] and the filtrate was concentrated *in vacuo*. Macrolactamization was performed using HBTU (1.02 equiv.) and DIPEA (3 equiv.) in DMF to give a 10^{-3} M solution.[27] After 30 min, the crude cyclohexapeptide was concentrated *in vacuo* and purified with a SNAP-HP Sphere column (gradient of 50 to 80% EtOAc in Hexanes).

General Procedure for Global Deprotection and Purification. A cleavage cocktail of trifluoroacetic acid/triisopropylsilane/DCM solution (50:5:45, v/v/v) was added to the cyclic hexapeptide intermediate and the reaction was stirred for 30 min. The reaction was then concentrated *in vacuo* before purifying by the following: To produce the TFA salt form of the product, the reaction was concentrated *in vacuo* and then a precipitate was formed by trituration with a mixture of hexanes and EtOAc (1:1). The precipitate was collected and lyophilized. The free base form of the cyclohexapeptide was obtained from the purified TFA salt form by dissolving it in 20% MeOH in DCM and then stirring with 0.5-1g of base-modified silica to scavenge the TFA. The filtrate was then concentrated *in vacuo*. Alternatively, the free base form can be obtained by purifing the reaction mixture using a Biotage SNAP KP-NH 11g cartridge (gradient of 0 to 10% MeOH in DCM). All purified peptides were lyophilized in a freeze-dryer and characterized by ^{1}H and ^{13}C NMR, HRMS, and HPLC analyses.

Synthesis and characterization data of **1**-**3** and **5**-**6** were reported previously.[13]

Cyclo[Asn-Ile-D-Leu-Leu-Trp-Gly] ***4***. Yield: 38% (two steps). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.31 (d, J = 7.7 Hz, 1H), 8.26 (d, J = 7.9 Hz, 1H), 8.16 (d, J = 6.3 Hz, 1H), 7.88 (t, J = 4.9 Hz, 1H), 7.72 (s, 2H), 7.60 (s, 1H), 7.51 (d, J = 7.8 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.15 (d, J = 2.4 Hz, 1H), 7.09 – 7.04 (m, 2H), 7.02 – 6.95 (m, 1H), 4.50 (dd, J = 13.1, 5.5 Hz, 1H), 4.41 – 4.30 (m, 3H), 4.04 (dd, J = 7.2, 5.4 Hz, 1H), 3.89 (dd, J = 16.2, 5.6 Hz, 1H), 3.65 – 3.55 (m, 1H in water peak), 3.14 (dd, J = 14.8, 4.7 Hz, 1H), 2.98 (dd, J = 14.8, 9.3 Hz, 1H), 2.79 (dd, J = 16.2, 6.3 Hz, 1H), 2.65 (dd, J = 16.2, 5.6 Hz, 1H), 1.99 – 1.88 (m, 1H), 1.63 – 1.41 (m, 6H), 1.38 – 1.17 (m, 2H), 0.93 – 0.77 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.5, 172.5, 172.1, 171.5, 171.4, 171.2, 169.6, 136.6, 127.5, 124.0, 121.4, 118.8, 118.6, 111.8, 110.5, 58.7, 55.9, 52.0, 51.2, 49.9, 43.7, 41.7, 40.6, 37.0, 35.8, 27.7, 24.9, 24.6 (×2), 23.1, 23.0, 22.8, 22.6,

16.0, 12.0. HRMS *m/z* $[M+H]^+$ calc for: $C_{35}H_{53}N_8O_7^+$: 697.4032; found: 697.4033. *m/z* $[M+Na]^+$ calcd for $C_{35}H_{52}N_8NaO_7^+$:719.3851; found: 719.3856. HPLC purity: 96.7% (254 nm), t_R: 6.47 min; 97.3% (220 nm), t_R: 6.47 min.

*HO-Asn-Val-D-Leu-Leu-Trp-D-Orn-NH$_2$ **7**.* The compound was prepared from **5** using the general procedure for global deprotection and purification. Yield: 91%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.93 (s, 1H), 8.97 (s, 1H), 8.64 (d, *J* = 7.0 Hz, 1H), 8.23 (d, *J* = 8.7 Hz, 1H), 8.17 (d, *J* = 7.3 Hz, 1H), 7.68 – 7.59 (m, 2H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.19 (d, *J* = 2.2 Hz, 1H), 7.04 (dd, *J* = 11.0, 4.1 Hz, 1H), 6.97 (dd, *J* = 10.9, 4.0 Hz, 1H), 6.76 (s, 1H), 4.48 – 4.37 (m, 2H), 4.28 (dd, *J* = 13.8, 6.9 Hz, 1H), 4.19 (dd, *J* = 12.9, 6.1 Hz, 1H), 4.01 (dd, *J* = 8.8, 6.3 Hz, 1H), 3.20 – 3.12 (m, 2H), 3.06 (dd, *J* = 14.9, 8.8 Hz, 1H), 2.76 – 2.69 (m, 2H), 2.60 (dd, *J* = 15.3, 6.6 Hz, 1H), 2.38 (dd, *J* = 15.3, 6.0 Hz, 1H), 2.16 – 2.09 (m, 1H), 1.59 – 1.44 (m, 9H), 1.34 – 1.23 (m, 1H), 0.93 – 0.76 (m, 18H). HRMS *m/z* $[M+H]^+$ calcd for: $C_{37}H_{60}N_9O_8^+$: 758.4559; found: 758.4554. HPLC purity: 98.7% (254 nm), t_R: 5.37 min; 98.3% (220 nm), t_R: 5.37 min.

Cyclo[Gln-Val-D-Leu-Leu-Trp-D-Orn] ***8***. Yield: 75%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.54 (d, *J* = 8.4 Hz, 1H), 8.38 (dd, *J* = 13.6, 7.2 Hz, 3H), 7.54 (d, *J* = 7.8 Hz, 1H), 7.33 – 7.30 (m, 4H), 7.16 (s, 1H), 7.08 – 7.03 (m, 1H), 7.00 – 6.96 (m, 1H), 6.74 (s, 1H), 4.53 – 4.46 (dd, *J* = 14.6, 7.1 Hz, 1H), 4.37 (dd, *J* = 14.2, 7.0 Hz, 2H), 4.30 (ddd, *J* = 11.6, 8.2, 3.1 Hz, 1H), 4.06 (dd, *J* = 8.7, 4.4 Hz, 1H), 3.97 (t, *J* = 7.3 Hz, 1H), 3.36 – 3.29 (m, 1H in water peak), 2.89 (dd, *J* = 14.7, 11.5 Hz, 1H), 2.38 – 2.25 (m, 3H), 2.08 – 1.94 (m, 2H), 1.87 – 1.71 (m, 2H), 1.63 – 1.20 (m, 8H), 1.09 – 0.96 (m, 1H), 0.97 – 0.77 (m, 19H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 173.3, 172.5, 171.53, 171.49, 171.2, 170.8, 136.6, 127.5, 124.3, 121.3, 118.7, 118.6, 111.8, 110.9, 58.5, 54.9, 54.0, 52.2, 52.1, 51.2, 43.1, 41.4, 39.5, 31.8, 29.7, 29.4, 29.2, 28.1, 27.7,

24.8, 24.6, 23.3, 23.0 (×2), 22.6, 19.7, 17.5. HRMS *m/z* $[M+H]^+$ calcd for: $C_{38}H_{60}N_9O_7^+$: 754.4610; found: 754.4608. $[M+Na]^+$ calcd for: $C_{38}H_{59}N_9NaO_7^+$: 776.4430; found: 776.4408. HPLC purity: 96.6% (254 nm), t_R: 5.76 min; 98.4% (220 nm), t_R: 5.76 min.

Cyclo[Asp-Val-D-Leu-Leu-Trp-D-Orn] ***9***. Yield: 67%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1H), 10.50 (d, *J* = 8.6 Hz, 1H), 9.02 (d, *J* = 4.6 Hz, 1H), 8.61 (d, *J* = 7.5 Hz, 1H), 8.55 (d, *J* = 10.4 Hz, 1H), 7.55 (d, *J* = 7.7 Hz, 1H), 7.48 (d, *J* = 9.3 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.08 – 6.94 (m, 3H), 6.86 (d, *J* = 6.1 Hz, 1H), 4.71 – 4.64 (m, 1H), 4.62 – 4.52 (m, 1H), 4.28 – 4.14 (m, 3H), 3.78 (t, *J* = 4.6 Hz, 1H), 3.06 (dd, *J* = 14.7, 6.4 Hz, 1H), 2.99 (dd, *J* = 14.7, 8.7 Hz, 1H), 2.74 – 2.61 (m, 2H), 2.65 (dd, *J* = 13.8, 2.9 Hz, 1H), 2.33 – 2.18 (m, 2H), 1.84 – 1.32 (m, 10H), 1.03 – 0.70 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 175.6, 174.8, 172.4, 172.1, 172.0, 170.5, 170.1, 136.5, 127.7, 123.8, 121.2, 118.80, 118.76, 111.6, 110.4, 61.6, 56.4, 52.0, 51.9 (×2), 50.8, 42.7, 40.7, 39.5, 39.2, 29.4, 28.8, 27.6, 25.1, 24.8, 24.6, 23.9, 23.6, 22.6, 21.5, 19.4, 17.7. HRMS *m/z* $[M+H]^+$ calcd for: $C_{37}H_{57}N_8O_8^+$: 741.4294; found: 741.4295. $[M+Na]^+$ calcd for: $C_{37}H_{56}N_8NaO_8^+$: 763.4113; found: 763.4119. HPLC purity: 95.9% (254 nm), t_R: 5.82 min; 95.9% (220 nm), t_R: 5.82 min.

Cyclo[Asn-Ile-D-Leu-Leu-Trp-D-Orn] ***10***. Yield: 93%. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.86 (s, 1H), 8.35 – 8.29 (m, 3H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.51 – 7.38 (m, 4H), 7.34 (d, *J* = 8.1 Hz, 1H), 7.16 (s, 1H), 7.06 (t, *J* = 7.5 Hz, 1H), 7.02 – 6.94 (m, 2H), 4.57 (dd, *J* = 13.9, 6.2 Hz, 1H), 4.43 (dd, *J* = 14.8, 6.6 Hz, 1H), 4.37 – 4.25 (m, 2H), 4.08 – 4.01 (m, 2H), 3.23 (dd, *J* = 14.6, 3.9 Hz, 1H), 2.92 (dd, *J* = 14.6, 10.7 Hz, 1H), 2.58 (d, *J* = 6.4 Hz, 2H), 2.40 – 2.32 (m, 2H), 2.03 – 1.91 (m, 1H), 1.64 –1.21 (m, 10H), 1.03 – 0.70 (m, 20H). ^{13}C NMR (DMSO-d_6, 101 MHz) δ 173.2, 171.8, 171.6, 171.5, 170.9, 170.5, 170.4, 136.2, 127.0, 123.8, 120.9, 118.4, 118.1, 111.4, 110.1, 58.1, 55.0, 52.8, 51.7, 50.7, 49.4, 41.8, 40.7, 37.4, 35.5, 28.3, 27.4, 27.1, 24.5, 24.1, 23.9,

22.60, 22.57, 22.54, 22.53, 22.0, 15.7, 11.8. HRMS *m/z* $[M+H]^+$ calcd for: $C_{38}H_{60}N_9O_7^+$: 754.4610; found: 754.4613. HPLC purity: 96.2% (254 nm), t_R: 5.91 min; 95.1% (220 nm), t_R: 5.91 min.

Cyclo[Asn-Ala-D-Leu-Leu-Trp-D-Orn] ***11***. Yield: 65%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 8.72 (d, *J* = 6.7 Hz, 1H), 8.37 (d, *J* = 5.9 Hz, 1H), 8.24 (d, *J* = 8.0 Hz, 1H), 7.78 (s, 1H), 7.59 – 7.48 (m, 3H), 7.39 – 7.29 (m, 2H), 7.14 (s, 1H), 7.09 – 6.94 (m, 3H), 4.54 (dd, *J* = 13.8, 5.9 Hz, 1H), 4.47 – 4.39 (m, 1H), 4.31 (ddd, *J* = 10.6, 8.6, 4.4 Hz, 1H), 4.16 (dt, *J* = 9.2, 5.8 Hz, 1H), 4.10 – 3.98 (m, 2H), 3.21 (dd, *J* = 14.6, 4.1 Hz, 1H), 2.91 (dd, *J* = 14.6, 10.5 Hz, 1H), 2.66 (dd, *J* = 15.6, 5.4 Hz, 1H), 2.57 (dd, *J* = 15.6, 6.4 Hz, 1H), 2.55 – 2.50 (m, 1H, overlapping with DMSO-d_6 peak), 2.36 (td, *J* = 6.8, 2.8 Hz, 2H), 1.65 – 1.31 (m, 8H), 1.26 (d, *J* = 7.4 Hz, 3H), 1.18 – 1.06 (m, 2H), 0.94 – 0.81 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.2, 172.3, 172.2, 171.93, 171.90, 171.4, 171.0, 136.6, 127.5, 124.2, 121.3, 118.8, 118.6, 111.8, 110.5, 55.5, 53.2, 52.3, 51.2, 49.8, 49.7, 42.2, 41.4, 39.5, 37.7, 29.3, 27.7, 25.0, 24.6, 23.2, 23.1 (×2), 22.9, 22.1, 17.6. HRMS *m/z* $[M+H]^+$ calcd for: $C_{35}H_{54}N_9O_7^+$: 712.4141; found: 712.4142. *m/z* $[M+Na]^+$ calcd for: $C_{35}H_{53}N_9NaO_7^+$: 734.3960; found: 734.3959. HPLC purity: 95.6% (254 nm), t_R: 5.64 min; 97.3% (220 nm), t_R: 5.64 min.

Cyclo[Asn-MeAla-D-Leu-Leu-Trp-D-Orn] ***12***. Yield: 51%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.86 (s, 1H), 8.71 (s, 1H), 8.06 (d, *J* = 6.1 Hz, 1H), 7.93 (d, *J* = 8.6 Hz, 1H), 7.81 (s, 2H), 7.66 (d, *J* = 5.4 Hz, 1H), 7.51 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.23 (d, *J* = 6.0 Hz, 1H), 7.16 (s, 1H), 7.11 (s, 1H), 7.09 – 7.04 (m, 1H), 7.01 – 6.96 (m, 1H), 4.47 – 4.33 (m, 3H), 4.23 (dt, *J* = 9.2, 6.1 Hz, 1H), 4.07 (dd, *J* = 10.1, 4.4 Hz, 1H), 3.10 (dd, *J* = 14.6, 5.3 Hz, 1H), 2.97 – 2.85 (m, 2H), 2.60 (dd, *J* = 16.0, 4.6 Hz, 1H), 2.42 (td, *J* = 6.9, 1.8 Hz, 2H), 1.68 – 1.56 (m, 2H), 1.54 – 1.38 (m, 4H), 1.35 (s, 3H), 1.33 (s, 3H), 1.29 – 1.08 (m, 4H), 0.93 – 0.78 (m, 12H).^{13}C

NMR (101 MHz, DMSO-d_6) δ 174.0, 173.5, 173.0, 171.8 (×2), 171.7, 171.6, 136.6, 127.5, 124.1, 121.4, 118.8, 118.6, 111.8, 110.1, 56.6, 56.0, 52.7, 52.1, 51.1, 50.4, 41.5, 41.4, 40.3, 36.8, 29.5, 27.8, 27.6, 27.4, 25.1, 24.6, 23.4, 23.2, 22.91, 22.89, 22.2. HRMS *m/z* $[M+H]^+$ calcd for: $C_{36}H_{56}N_9O_7^+$: 726.4297; found: 726.4295. $[M+Na]^+$ calcd for: $C_{36}H_{55}N_9NaO_7^+$: 748.4117; found: 748.4117. HPLC purity: 96.2% (254 nm), t_R: 5.68 min; 96.5% (220 nm), t_R: 5.68 min.

Cyclo[Asn-Val-D-Leu-Leu-Tyr-D-Orn] ***13***. Yield: 73%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.40 – 8.25 (m, 3H), 7.88 (s, 1H), 7.49 – 7.37 (m, 3H), 7.02 (d, *J* = 8.5 Hz, 2H), 6.95 (s, 1H), 6.64 (d, *J* = 8.5 Hz, 2H), 4.59 (dd, *J* = 14.2, 6.4 Hz, 1H), 4.44 (dd, *J* = 14.6, 6.8 Hz, 1H), 4.35 (dd, *J* = 13.8, 7.2 Hz, 1H), 4.17 (ddd, *J* = 11.6, 8.0, 4.0 Hz, 1H), 4.06 – 3.98 (m, 2H), 3.02 (dd, *J* = 13.7, 3.7 Hz, 1H), 2.64 (dd, *J* = 13.7, 11.1 Hz, 1H), 2.57 (d, *J* = 6.3 Hz, 2H), 2.44 – 2.35 (m, 2H), 2.32 – 2.22 (m, 1H), 1.61 – 1.36 (m, 8H), 1.16 – 0.95 (m, 2H), 0.93 – 0.82 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.6, 172.2, 172.0, 171.8, 171.0, 170.8, 170.7, 156.3, 130.4, 128.5, 115.4, 58.9, 56.3, 53.5, 52.2, 51.1, 49.8, 42.5, 41.4, 38.2, 36.6, 29.3, 29.2 (×2), 28.1, 24.9, 24.6, 23.1, 23.03, 22.95, 22.6, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calcd for: $C_{35}H_{57}N_8O_8^+$: 717.4294; found: 717.4293. $[M+Na]^+$ calcd for: $C_{35}H_{56}N_8NaO_8^+$: 739.4113; found: 739.4112. HPLC purity: 92.5% (254 nm), t_R: 5.57 min; 97.4% (220 nm), t_R: 5.57 min.

Cyclo[Asn-Val-D-Leu-Leu-Phe-D-Orn] ***14***. Yield: 87%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.48 (d, *J* = 8.3 Hz, 1H), 8.36 (d, *J* = 6.0 Hz, 1H), 8.31 (d, *J* = 8.0 Hz, 1H), 7.49 (d, *J* = 8.1 Hz, 1H), 7.42 (d, *J* = 7.6 Hz, 2H), 7.30 – 7.16 (m, 6H), 6.96 (s, 1H), 4.60 (dd, *J* = 14.1, 6.4 Hz, 1H), 4.46 (dd, *J* = 14.8, 6.9 Hz, 1H), 4.35 (dd, *J* = 13.9, 7.6 Hz, 1H), 4.27 (ddd, *J* = 11.8, 8.1, 3.7 Hz, 1H), 4.06 – 3.95 (m, 2H), 3.16 (dd, *J* = 13.8, 3.8 Hz, 1H), 2.75 (dd, *J* = 13.8, 11.3 Hz, 1H), 2.56 (d, *J* = 6.5 Hz, 2H), 2.41 – 2.23 (m, 3H), 1.62 – 1.34 (m, 8H), 1.12 – 0.97 (m, 2H), 0.96 – 0.78 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.6, 172.2, 172.0, 171.8, 170.82, 170.81, 170.7,

138.5, 129.5, 128.6, 126.7, 58.8, 55.9, 53.5, 52.2, 51.2, 49.8, 42.6, 41.3, 38.3, 37.3, 29.3, 29.1 (×2), 28.0, 24.9, 24.6, 23.1 (×2), 22.9, 22.6, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calcd for: $C_{35}H_{57}N_8O_7^+$: 701.4345; found: 701.4361. $[M+Na]^+$ calcd for: $C_{35}H_{56}N_8NaO_7^+$: 723.4164; found: 723.4160. HPLC purity: 88.4% (254 nm), t_R: 5.71 min; 98.1% (220 nm), t_R: 5.71 min.

Cyclo[Asn-Val-D-Leu-Leu-Trp-D-Arg] ***15***. Yield: 82%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 8.36 (s, 3H), 7.79 (d, J = 4.5 Hz, 1H), 7.57 – 7.40 (m, 5H), 7.35 (d, J = 7.3 Hz, 1H), 7.15 (s, 1H), 7.09 – 6.94 (m, 5H), 4.65 – 4.57 (m, 1H), 4.50 – 4.42 (m, 1H), 4.36 – 4.26 (m, 2H), 4.15 – 4.08 (m, 1H), 4.06 – 3.97 (m, 1H), 3.23 (d, J = 14.1 Hz, 1H), 3.03 – 2.87 (m, 3H), 2.73 – 2.57 (m, 2H), 2.33 – 2.18 (m, 1H), 1.62 – 1.21 (m, 9H), 1.11 – 1.00 (m, 1H), 0.96 – 0.75 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 172.2, 172.0, 171.8, 171.3, 171.1 (×2), 157.1, 136.6, 127.4, 124.2, 121.4, 118.8, 118.5, 111.9, 110.5, 59.1, 55.5, 52.9, 52.3, 51.0, 50.0, 42.4, 40.8, 38.7, 37.7, 29.2 (×2), 27.6, 27.5, 25.0, 24.9, 24.6, 23.0, 22.9, 22.6, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calcd for: $C_{38}H_{60}N_{11}O_7^+$: 782.4672; found: 782.4680. $[M+Na]^+$ calcd for: $C_{38}H_{59}N_{11}NaO_7^+$: 804.4491; found: 804.4482. HPLC purity: 96.6% (254 nm), t_R: 5.79 min; 99.2% (220 nm), t_R: 5.79 min.

Cyclo[Asn-Val-D-Leu-Leu-Trp-D-Lys] ***16***. Yield: 89%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.86 (s, 1H), 8.37 – 8.26 (m, 3H), 7.75 (d, J = 5.8 Hz, 1H), 7.52 (d, J = 7.9 Hz, 1H), 7.48 – 7.38 (m, 3H), 7.33 (d, J = 8.0 Hz, 1H), 7.15 (s, 1H), 7.06 (t, J = 7.0 Hz, 1H), 7.00 – 6.94 (m, 2H), 4.59 (dd, J = 14.3, 6.3 Hz, 1H), 4.45 (dd, J = 14.9, 7.0 Hz, 1H), 4.37 – 4.24 (m, 2H), 4.00 (dd, J = 7.6, 4.4 Hz, 2H), 3.24 (dd, J = 14.8, 3.6 Hz, 1H), 2.91 (dd, J = 14.8, 10.8 Hz, 1H), 2.58 (d, J = 6.1 Hz, 2H), 2.38 (t, J = 7.0 Hz, 2H), 2.31 – 2.20 (m, 1H), 1.65 – 1.33 (m, 8H), 1.27 – 1.02 (m, 4H), 0.96 – 0.79 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.8, 172.2, 172.01, 171.95, 171.3, 170.9 (×2), 136.6, 127.4, 124.2, 121.3, 118.8, 118.5, 111.8, 110.6, 59.0, 55.4, 53.5, 52.2, 51.1,

49.9, 42.4, 41.6, 40.6 38.0, 33.0, 30.3, 29.3, 27.6 (×2), 24.9, 24.6, 23.03, 22.97, 22.6, 22.5, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calcd for: $C_{38}H_{60}N_9O_7^+$: 754.4610; found: 754.4619. $[M+Na]^+$ calcd for: $C_{38}H_{59}N_9NaO_7^+$:776.4430; found: 776.4414. HPLC purity: 96.0% (254 nm), t_R: 5.77 min; 97.9% (220 nm), t_R: 5.77 min.

Cyclo[Asn-Val-D-Leu-Leu-Trp-Orn] ***17****.* Yield: 73%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 8.66 (d, J = 5.1 Hz, 1H), 8.31 (d, J = 8.8 Hz, 2H), 8.21 (d, J = 8.7 Hz, 1H), 7.87 (d, J = 9.4 Hz, 1H), 7.68 (s, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.41 (d, J = 5.9 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.25 (s, 1H), 7.08 – 7.03 (m, 2H), 6.98 (dd, J = 14.7, 0.7 Hz, 1H), 4.69 (dt, J = 9.5, 6.0 Hz, 1H), 4.42 – 4.34 (m, 1H), 4.31 (dd, J = 13.1, 7.3 Hz, 2H), 4.09 (dd, J = 9.2, 4.6 Hz, 1H), 3.58 (t, J = 6.5 Hz, 1H), 3.12 – 3.07 (m, 2H), 2.94 (dd, J = 15.3, 5.7 Hz, 1H), 2.72 (dd, J = 15.3, 5.7 Hz, 1H), 2.39 – 2.26 (m, 3H), 1.59 – 1.40 (m, 8H), 1.12 – 1.03 (m, 2H), 0.96 – 0.81 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.0, 172.9, 172.2, 171.9, 171.8, 171.7, 171.0, 136.6, 127.7, 123.6, 121.3, 118.8, 118.6, 111.8, 111.1, 58.6, 56.2, 55.7, 52.8, 52.1, 49.0, 42.4, 41.3, 40.0, 38.3, 28.8, 28.7, 28.2, 27.9, 25.0, 24.6, 23.8, 22.8, 22.7, 22.4, 19.8, 17.4. HRMS *m/z* $[M+H]^+$ calc for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4487. *m/z* $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$: 762.4273; found: 762.4273. HPLC purity: 98.2% (254 nm), t_R: 5.82 min; 100% (220 nm), t_R: 5.82 min.

Cyclo[Asn-Val-D-Leu-D-Leu-Trp-D-Orn] ***18***. Yield: 32%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 8.41 (d, J = 7.9 Hz, 1H), 8.22 (d, J = 7.0 Hz, 2H), 8.18 (d, J = 6.5 Hz, 1H), 8.03 (d, J = 6.5 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.42 (s, 1H), 7.37 – 7.25 (m, 2H), 7.11 (s, 1H), 7.05 (t, J = 7.5 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 6.82 (s, 1H), 4.51 – 4.37 (m, 3H), 4.34 – 4.22 (m, 1H), 4.07 – 3.93 (m, 1H), 3.83 (t, J = 6.5 Hz, 1H), 3.06 (dd, J = 14.1, 8.8 Hz, 1H), 2.90 (dd, J = 14.1, 6.7 Hz, 1H), 2.76 (dd, J = 15.7, 4.8 Hz, 1H), 2.56 (dd, J = 15.7, 7.7 Hz, 1H), 2.48 – 2.40 (m, 2H), 2.06 – 1.94 (m, 1H), 1.71 – 1.60 (m, 1H), 1.56 – 1.43 (m, 4H), 1.42 – 1.18 (m, 4H),

1.13 – 1.01 (m, 1H), 0.90 – 0.79 (m, 12H), 0.71 (d, J = 6.6 Hz, 3H), 0.58 (d, J = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.7, 172.6, 172.2, 171.8, 171.5, 171.1, 170.6, 136.6, 127.5, 124.2, 121.3, 118.7 (×2), 111.7, 109.9, 60.2, 54.8, 52.6, 52.3, 51.7, 50.8, 42.0, 41.6, 40.1, 36.2, 30.1, 29.7, 29.4, 27.2, 24.5, 24.3, 23.5, 23.2, 23.1, 21.2, 19.6, 18.5. HRMS *m/z* $[M+H]^+$ calc for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4480. $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$: 762.4273; found: 762.4291. HPLC purity: 87.4% (254 nm), t_R: 5.63 min; 96.8% (220 nm), t_R: 5.63 min.

Cyclo[Asn-D-Val-D-Leu-Leu-Trp-D-Orn] ***19***. Yield: 34%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.23 (dd, J = 10.7, 7.8 Hz, 2H), 8.11 (d, J = 7.2 Hz, 1H), 7.91 (s, 1H), 7.58 (d, J = 7.7 Hz, 1H), 7.53 (d, J = 7.2 Hz, 1H), 7.45 (d, J = 8.1 Hz, 1H), 7.38 – 7.27 (m, 2H), 7.14 (s, 1H), 7.12 – 7.01 (m, 1H), 7.02 – 6.95 (m, 1H), 6.90 (s, 1H), 4.52 – 4.46 (m, 1H), 4.40 – 4.32 (m, 1H), 4.27 (dd, J = 14.2, 7.3 Hz, 1H), 4.14 (ddd, J = 10.7, 8.2, 5.0 Hz, 1H), 4.09 – 4.02 (m, 2H), 3.16 (dd, J = 14.6, 4.8 Hz, 1H), 2.99 (dd, J = 14.6, 10.0 Hz, 1H), 2.42 (m, 2H), 2.35 (t, J = 7.0 Hz, 2H), 2.06 – 1.94 (m, 1H), 1.68 – 1.32 (m, 8H), 1.12 – 0.94 (m, 2H), 0.92 – 0.77 (m, 18H).^{13}C NMR (101 MHz, DMSO-d_6) δ 172.6, 172.0, 171.7, 171.6, 171.5, 171.1, 170.8, 136.6, 127.6, 124.1, 121.3, 118.7 (×2), 111.8, 110.6, 58.0, 55.6, 53.4, 53.1, 52.1, 50.2, 41.4, 41.1, 41.0, 36.8, 30.6, 29.2, 29.0 (×2), 24.7, 24.6, 23.5, 23.1, 23.0, 21.4, 19.6, 18.8. HRMS *m/z* $[M+H]^+$ calcd for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4488. $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$: 762.4273; found: 762.4289. HPLC purity: 97.4% (254 nm), t_R: 5.75 min; 99.5% (220 nm), t_R: 5.76 min.

Cyclo[Leu-Trp-D-Orn-D-Asn-D-Val-D-Leu] ***20***. Yield: 51%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.24 (d, J = 6.0 Hz, 1H), 8.18 (d, J = 7.7 Hz, 1H), 8.11 (d, J = 8.5 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H), 7.73 (d, J = 6.6 Hz, 1H), 7.67 (s, 1H), 7.62 (d, J = 7.8 Hz, 1H), 7.46 (s, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.18 (s, 1H), 7.09 – 7.04 (m, 1H), 7.01 – 6.97 (m, 2H), 4.41 – 4.25 (m, 3H), 4.20 (dd, J = 13.8, 7.1 Hz, 1H), 4.10 – 4.04 (m, 1H), 3.97 – 3.92 (m, 1H), 3.22 (dd, J =

14.8, 5.2 Hz, 1H), 2.95 (dd, *J* = 14.8, 10.2 Hz, 1H), 2.70 – 2.55 (m, 2H), 2.48 – 2.39 (m, 2H), 2.31 – 2.20 (m, 1H), 1.67 – 1.49 (m, 6H), 1.49 – 1.37 (m, 2H), 1.26 – 1.13 (m, 2H), 0.94 – 0.79 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.7, 172.2, 171.8 (×2), 171.7, 171.4, 171.3, 136.6, 127.6, 124.3, 121.3, 118.8, 118.7, 111.8, 110.6, 59.7, 55.1, 53.6, 52.8, 52.1, 51.8, 40.8, 40.4, 39.5, 36.8, 30.0, 28.7, 28.0, 27.2, 24.8, 23.6, 23.0, 22.9, 21.5, 20.1, 18.7, 18.3. HRMS *m/z* $[M+H]^+$ calcd for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4490. *m/z* $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$: 762.4273; found: 762.4286. HPLC purity: 96.5% (254 nm), t_R: 5.75 min; 98.6% (220 nm), t_R: 5.75 min.

Cyclo[D-Leu-Trp-D-Orn-D-Asn-D-Val-D-Leu] ***21***. Yield: 39%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.84 (s, 1H), 8.69 (d, *J* = 4.7 Hz, 1H), 8.41 (d, *J* = 8.5 Hz, 1H), 8.17 (d, *J* = 6.7 Hz, 1H), 8.09 (d, *J* = 3.5 Hz, 1H), 7.74 (d, *J* = 9.0 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.40 (s, 1H), 7.31 (d, *J* = 8.1 Hz, 1H), 7.25 (s, 1H), 7.15 (s, 1H), 7.07 – 7.01 (m, 1H), 7.00 – 6.96 (m, 1H), 6.89 (s, 1H), 4.49 – 4.41 (m, 1H), 4.36 (dd, *J* = 13.4, 7.7 Hz, 1H), 4.29 (td, *J* = 7.7, 4.2 Hz, 1H), 4.20 – 4.14 (m, 1H), 4.02 – 3.94 (m, 1H), 3.50 (dd, *J* = 7.0, 3.9 Hz, 1H), 3.09 (dd, *J* = 14.2, 8.8 Hz, 1H), 2.94 (dd, *J* = 14.2, 6.8 Hz, 1H), 2.73 (dd, *J* = 15.5, 7.8 Hz, 1H), 2.55 – 2.53 (m, 2H), 2.39 (td, *J* = 6.8, 2.2 Hz, 1H), 2.02 – 1.89 (m, 1H), 1.84 – 1.43 (m, 6H), 1.38 – 1.22 (m, 2H), 1.15 – 1.01 (m, 2H), 0.96 – 0.83 (m, 12H), 0.72 (d, *J* = 6.6 Hz, 3H), 0.55 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.8, 172.7, 172.5, 171.6 (×2), 171.24, 171.20, 136.6, 127.5, 124.2, 121.3, 118.8, 118.6, 111.6, 109.7, 62.4, 55.7, 53.7, 52.0, 51.5, 50.2, 41.6, 40.5, 39.7, 36.7, 29.8, 29.0 (×2), 26.7, 24.8, 24.4, 23.9, 23.6, 21.2, 21.0, 19.8, 19.4. HRMS *m/z* $[M+H]^+$ calcd for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4467. *m/z* $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$:762.4273; found: 762.4279. HPLC purity: 98.4% (254 nm), t_R: 5.62 min; 99.2% (220 nm), t_R: 5.63 min.

Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn] ***22***. Yield: 34%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 9.13 (s, 1H), 8.69 (d, J = 6.8 Hz, 2H), 8.07 (d, J = 6.0 Hz, 1H), 7.99 (d, J = 7.6 Hz, 1H), 7.58 (d, J = 7.7 Hz, 1H), 7.43 (s, 2H), 7.31 (d, J = 8.0 Hz, 1H), 7.09 – 7.02 (m, 2H), 7.01 – 6.91 (m, 2H), 4.55 (dd, J = 14.2, 7.8 Hz, 1H), 4.36 (dd, J = 8.7, 6.6 Hz, 1H), 4.20 – 4.04 (m, 4H), 3.12 (dd, J = 14.1, 8.2 Hz, 1H), 2.92 (dd, J = 14.1, 6.1 Hz, 1H), 2.69 (dd, J = 15.9, 5.3 Hz, 1H), 2.58 – 2.52 (m, 2H), 2.45 – 2.37 (m, 1H), 2.08 – 2.00 (m, 1H), 1.82 – 1.55 (m, 5H), 1.45 – 1.20 (m, 5H), 0.91 – 0.73 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.6, 172.2, 171.9, 171.8, 171.6, 171.13, 171.10, 136.4, 132.8, 127.9, 123.6, 121.2, 118.6, 111.6, 110.8, 56.8, 54.2, 52.8, 52.7, 52.4, 52.2, 41.7, 40.7, 40.3, 36.3, 31.9, 29.3, 28.9, 27.8, 24.7, 24.6, 23.7, 23.0, 22.5, 21.0, 19.6, 18.3. HRMS *m/z* $[M+H]^+$ calc for: $C_{37}H_{58}N_9O_7^+$: 740.4454; found: 740.4485. $[M+Na]^+$ calcd for $C_{37}H_{57}N_9NaO_7^+$:762.4273; found: 762.4291. HPLC purity: 96.1% (254 nm), t_R: 5.77 min; 97.2% (220 nm), t_R: 5.77 min.

Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn(dimethyl)] ***23***. Wollamide B (**2**) (29.6 mg, 0.04 mmol) was added to ethanol (3 mL). Aq. formaldehyde (16 µL, 0.12 mmol) and sodium cyanoborohydride (5.5 mg, 0.088 mmol) were added and the resulting mixture was stirred for 1.5 h at room temperature. Then, one drop of concentrated hydrochloric acid was added to the reaction mixture and the solvent was removed *in vacuo*. The crude product was purified with a SNAP-NH column (gradient of 0 to 3% MeOH in DCM). Yield: 32%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 8.36 – 8.25 (m, 3H), 7.75 (d, J = 6.9 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.48 – 7.38 (m, 3H), 7.33 (d, J = 8.1 Hz, 1H), 7.15 (d, J = 2.1 Hz, 1H), 7.08 – 7.04 (m, 1H), 7.00 – 6.94 (m, 2H), 4.58 (dd, J = 14.0, 6.3 Hz, 1H), 4.45 (dd, J = 15.1, 7.4 Hz, 1H), 4.38 – 4.27 (m, 2H), 4.08 (dd, J = 14.2, 6.8 Hz, 1H), 3.99 (dd, J = 7.6, 4.4 Hz, 1H), 3.22 (dd, J = 14.9, 4.2 Hz, 1H), 2.93 (dd, J = 14.9, 10.8 Hz, 1H), 2.61 (d, J = 6.8 Hz, 2H), 2.32 – 2.23 (m, 1H), 2.08 (s,

8H), 1.66 – 1.35 (m, 8H), 1.27 – 1.13 (m, 1H), 1.08 – 0.97 (m, 1H), 0.97 – 0.81 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 172.1 (×2), 172.0, 171.3, 170.93, 170.89, 136.6, 127.4, 124.1, 121.4, 118.8, 118.6, 111.8, 110.6, 59.1, 58.5, 55.5, 53.2, 52.2, 51.1, 49.9, 45.0, 42.3, 39.9, 37.8, 29.2, 28.1, 27.6, 24.9, 24.6, 23.1, 23.05, 23.00, 22.8, 22.5, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calc for: $C_{39}H_{62}N_9O_7^+$: 768.4767; found: 768.4757. $[M+Na]^+$ calcd for $C_{39}H_{61}N_9NaO_7^+$: 790.4586; found: 790.4552. HPLC purity: 97.3% (254 nm), t_R: 5.79 min; 96.9% (220 nm), t_R: 5.79 min.

Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn(trimethyl)] ***24***. Compound **23** (15.3 mg, 0.02 mmol) was added to ethanol (1 mL). Methyl iodide (10 μL, 0.16 mmol) was added and the resulting mixture was stirred overnight at room temperature. The crude product was evaporated to a residue and triturated with ethyl acetate and hexanes to give a precipitate. Yield: 86%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 8.45 (d, J = 6.0 Hz, 2H), 8.28 (d, J = 8.1 Hz, 1H), 7.67 (d, J = 7.9 Hz, 1H), 7.61 (s, 1H), 7.56 – 7.50 (m, 2H), 7.42 (d, J = 8.2 Hz, 1H), 7.34 (d, J = 8.1 Hz, 1H), 7.16 (d, J = 2.2 Hz, 1H), 7.10 – 7.04 (m, 2H), 7.02 – 6.96 (m, 1H), 4.60 (dd, J = 14.0, 6.1 Hz, 1H), 4.45 (dd, J = 15.0, 7.2 Hz, 1H), 4.38 – 4.27 (m, 2H), 4.26 – 4.19 (m, 1H), 4.00 (dd, J = 7.3, 4.5 Hz, 1H), 3.24 – 3.10 (m, 4H), 2.95 (s, 9H), 2.76 – 2.62 (m, 2H), 2.34 – 2.21 (m, 1H), 1.66 – 1.37 (m, 10H), 0.96 – 0.82 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.3, 172.3, 172.2, 171.44, 171.35, 171.3, 171.2, 136.6, 127.5, 124.0, 121.5, 118.9, 118.7, 111.9, 110.6, 65.2, 59.4, 55.8, 52.6, 52.5, 52.0, 50.9, 50.3, 42.4, 40.1, 37.3, 29.3, 27.7, 27.0, 25.0, 24.6, 23.1, 23.0, 22.9, 22.6, 19.6, 18.9, 17.6. HRMS *m/z* $[M+H]^+$ calc for: $C_{40}H_{64}N_9O_7^+$: 782.4923; found: 782.4961. HPLC purity: 96.3% (254 nm), t_R: 5.82 min; 96.0% (220 nm), t_R: 5.82 min.

Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn(morpholine)] ***25***. Wollamide B (**2**) (29.6 mg, 0.04 mmol) was added to acetonitrile (3 mL). Sodium iodide (39 mg, 0.26 mmol), 2-chloroethyl ether (18.8

μL, 0.16 mmol) and triethyl amine (36.2 μL, 0.26 mmol) were added and the resulting mixture was stirred overnight at 80°C. The crude product was purified with a SNAP-NH column (gradient of 0 to 3% MeOH in DCM). Yield: 27%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 8.36 – 8.26 (m, 3H), 7.73 (d, J = 6.7 Hz, 1H), 7.53 (d, J = 7.9 Hz, 1H), 7.47 (d, J = 8.7 Hz, 2H), 7.39 (d, J = 8.0 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.15 (d, J = 2.3 Hz, 1H), 7.09 – 7.04 (m, 1H), 7.01 – 6.95 (m, 2H), 4.58 (dd, J = 13.7, 6.3 Hz, 1H), 4.45 (dd, J = 14.7, 6.8 Hz, 1H), 4.37 – 4.27 (m, 2H), 4.06 (dd, J = 15.3, 7.8 Hz, 1H), 3.96 (dd, J = 7.5, 4.4 Hz, 1H), 3.47 (t, J = 4.5 Hz, 4H), 3.23 (dd, J = 14.5, 3.8 Hz, 1H), 2.92 (dd, J = 14.5, 10.1 Hz, 1H), 2.60 (d, J = 7.3 Hz, 2H), 2.32 – 2.21 (m, 1H), 2.20 – 2.09 (m, 4H), 2.09 – 1.97 (m, 2H), 1.62 – 1.34 (m, 8H), 1.26 – 1.12 (m, 1H), 0.95 – 0.81 (m, 19H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 172.13, 172.06, 172.0, 171.4, 170.9, 170.8, 136.6, 127.4, 124.1, 121.3, 118.8, 111.8, 118.6, 110.6, 66.7, 58.0, 55.5, 53.6, 53.4, 52.2, 51.1, 49.9, 42.3, 40.5, 39.6, 37.8, 29.2, 28.3, 27.6, 24.9, 24.6, 23.1, 23.00, 22.96, 22.6, 22.1, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calc for: $C_{41}H_{64}N_9O_8^+$: 810.4872; found: 810.4882. $[M+Na]^+$ calcd for $C_{41}H_{63}N_9NaO_8^+$: 832.4692; found: 832.4685. HPLC purity: 96.6% (254 nm), t_R: 5.80 min; 96.0% (220 nm), t_R: 5.80 min.

Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn(isopropyl)] ***26****.* Wollamide B (**2**) (29.6 mg, 0.04 mmol) was added to ethanol (3 mL). Excess acetone (30 μL) and sodium cyanoborohydride (5.5 mg, 0.088 mmol) were added and the resulting mixture was stirred for 2 h at room temperature. Then, one drop of concentrated hydrochloric acid was added to the reaction mixture and the solvent was removed *in vacuo.* The crude product was purified with a SNAP-NH column (gradient of 0 to 3% MeOH in DCM). Yield: 28%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H), 8.31 (t, J = 7.6 Hz, 3H), 7.77 (d, J = 5.7 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.46 – 7.40 (m, 2H), 7.32 (d, J = 8.0 Hz, 1H), 7.15 (d, J = 1.9 Hz, 1H), 7.09 – 7.02 (m, 1H), 7.00 – 6.95 (m, 2H), 4.59 (dd, J = 14.0,

6.4 Hz, 1H), 4.44 (dd, *J* = 14.8, 6.8 Hz, 1H), 4.38 – 4.27 (m, 2H), 4.06 – 3.97 (m, 2H), 3.23 (dd, *J* = 14.6, 3.7 Hz, 1H), 2.93 (dd, *J* = 14.6, 10.8 Hz, 1H), 2.62 – 2.55 (m, 3H), 2.34 – 2.22 (m, 3H), 1.63 – 1.37 (m, 8H), 1.20 – 0.95 (m, 2H), 0.95 – 0.75 (m, 24H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.8, 172.2, 172.03, 172.97, 171.3, 170.9, 170.8, 136.6, 127.5, 124.2, 121.3, 118.8, 118.6, 111.8, 110.6, 59.1, 55.5, 53.5, 52.2, 51.1, 49.9, 48.4, 46.6, 42.3, 40.3, 38.0, 29.2, 28.5, 27.6, 26.0, 24.9, 24.6, 23.4, 23.3, 23.02, 22.96, 22.5, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calc for: $C_{40}H_{64}N_9O_7^+$: 782.4923; found: 782.4951. $[M+Na]^+$ calcd for $C_{40}H_{63}N_9NaO_7^+$:804.4743; found: 804.4734. HPLC purity: 88.5% (254 nm), t_R: 5.87 min; 94.5% (220 nm), t_R: 5.87 min.

*Cyclo[Asn-Val-Leu-D-Leu-Trp-D-Orn(acyl)] **27**.* Wollamide B **2** (29.6 mg, 0.04 mmol) was added to DMF/acetonitrile (1:10, 3 mL). Potassium carbonate (11 mg, 0.08 mmol) and acetic anhydride (4.2 µL, 0.044 mmol) were added and the resulting mixture was stirred overnight at room temperature. The crude product was purified with a SNAP-NH column (gradient of 0 to 5% MeOH in DCM). Yield: 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (s, 1H), 8.39 – 8.28 (m, 3H), 7.77 (d, *J* = 6.8 Hz, 1H), 7.63 (t, *J* = 5.4 Hz, 1H), 7.53 (d, *J* = 7.9 Hz, 1H), 7.48 – 7.39 (m, 3H), 7.33 (d, *J* = 8.1 Hz, 1H), 7.15 (d, *J* = 2.0 Hz, 1H), 7.09 – 7.03 (m, 1H), 7.01 – 6.92 (m, 2H), 4.60 (dd, *J* = 13.9, 6.2 Hz, 1H), 4.44 (dd, *J* = 14.6, 6.8 Hz, 1H), 4.38 – 4.26 (m, 2H), 4.11 – 3.99 (m, 2H), 3.23 (dd, *J* = 14.4, 3.8 Hz, 1H), 2.99 – 2.88 (m, 2H), 2.87 – 2.79 (m, 1H), 2.60 (d, *J* = 6.3 Hz, 2H), 2.33 – 2.22 (m, 1H), 1.77 (s, 3H), 1.62 – 1.37 (m, 8H), 1.27 – 1.16 (m, 1H), 1.09 – 0.96 (m, 1H), 0.95 – 0.81 (m, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.9, 172.10, 172.06, 172.0, 171.3, 171.0, 170.9, 169.5, 136.6, 127.5, 124.1, 121.4, 118.8, 118.6, 111.8, 110.6, 59.0, 55.5, 53.1, 52.2, 51.1, 49.9, 42.3, 39.8, 38.4, 37.9, 29.2, 27.8, 27.6, 25.5, 24.9, 24.6, 23.1, 23.04, 23.00, 22.96, 22.5, 19.6, 17.5. HRMS *m/z* $[M+H]^+$ calc for: $C_{39}H_{60}N_9O_8^+$: 782.4559;

found: 782.4572. $[M+Na]^+$ calcd for $C_{39}H_{59}N_9NaO_8^+$: 804.4379; found: 804.4390. HPLC purity: 98.7% (254 nm), t_R: 6.30 min; 100% (220 nm), t_R: 6.30 min.

MIC Determination. MIC values were determined against *M. tuberculosis* (H37Rv) and other bacteria using the standard microbroth dilution method as previously described,[28] which is based on the methods by the Clinical and Laboratory Standards Institute.[29, 30] The maximum test concentration used was 200 μg/mL. MIC values of compounds against *C. difficile* were determined as described previously.[31, 32]

Cytotoxicity Assays. Cytotoxicity was assessed *in vitro* using Vero cells (African green monkey kidney epithelial cells, ATCC CCL-81). In brief, monolayers of cells cultured in Dulbecco's Modified Eagle Medium (DMEM)/10% fetal bovine serum (FBS) were trypsinized, seeded at approximately 10% confluence in 96-well plates, and incubated overnight. Medium was replaced with DMEM/FBS containing 2-fold serial dilutions of test compounds. Detection was performed using MTT (CellTiter96, Promega) with overnight solubilization according to the manufacturer's instructions.

Membrane potential measurement. These experiments were performed as described and run in three biological replicates.[33] Essentially, flow cytometry was used to access the population of cells showing red or green fluorescence using the fluorescent probe diethyloxacarbocyanine dye $DiOC_2(3)$. While the emission of red fluorescence is dependent on the membrane potential, the emission of green fluorescence is independent of the membrane potential. *S. aureus* NRS70 was grown to exponential phase (OD_{600}nm ≈ 0.3) in Mueller-Hinton II broth, and compounds were added to 4× their MIC values. After incubation for 30 min at 37°C, cells were stained with 30 μM of $DiOC_2(3)$ for 5 min, before samples were analyzed in BD LSR II

flow cytometer. $DiOC_2(3)$ was excited using the 488-nm excitation laser. Its green fluorescence emission was detected using FITC filters while its red fluorescence emission was detected using PI-A filters; 10,000 events were collected for each sample. The effects of compounds on the membrane potential were analyzed using the software FlowJo 10.4. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma-Aldrich) was included as a positive control. This compound completely dissipates the membrane potential.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI:

Full characterization data of key precursors and spectroscopic data (^{1}H and ^{13}C NMR, HRMS, and HPLC chromatograms) (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes of Health grant (R15AI092315 and P20GM103466) and American Lebanese Syrian Associated Charities (ALSAC), St. Jude Children's Research Hospital (SJCRH). We thank Mr. Justin Reinicke for his assistance with the HRMS data.

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