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Diastereoselective synthesis of cyclic tetrapeptide pseudoxylallemycin A illuminates the impact of base during macrolactamization†

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Therapeutic agents with unique molecular structures and new mechanisms of action are needed to confront the phenomenon of multidrug resistance among bacteria. Pseudoxylallemycins, cyclic tetrapeptide (CTP) natural products, have exhibited modest antibiotic activity, but their synthesis has proven challenging. Inherent ring strain in CTPs decreases the rate of cyclization in lieu of polymerization and racemization pathways, which has resulted in previous syntheses describing mixtures of diastereomers containing predominantly an undesired epimer. We have optimized the cyclization step of pseudoxylallemycin A to favor production of the natural diastereomer; notably, variation of the base, temperature, and solvent with peptide coupling reagent propylphosphonic anhydride (T3P) afforded exquisite selectivity for the natural product in as high as 97 : 3 DR, and our conditions can provide the natural product in up to 32% overall yield through 8 steps. Employing weaker bases than those typically used in peptide coupling reactions led to the greatest improvement in diastereoselectivity, and these studies demonstrated that the identity of the amine base has enormous impact on the rate of C-terminal epimerization when T3P is used, a variable usually considered of lesser consequence when combined with typical amide coupling reagents. Toward fully characterizing pseudoxylallemycin stereoisomers, variable temperature NMR was described as a tool to more clearly analyze CTPs that exhibit multiple conformational states. These synthetic and spectroscopic insights were applied toward synthesizing several natural product analogues, and their antibacterial activity was examined using microdilution assays.

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Introduction

Cyclic peptides as therapeutics have been gaining interest in recent decades,[1–3] as they have shown wide ranging bioactivity as antibacterial, anticancer, antifungal, antiviral, and anti-inflammatory agents.[4,5] Compared to linear peptides, they are more proteolytically resistant, providing enhanced biostability, bioavailability,[6] and membrane permeability.[7,8] The ring architecture also rigidifies the conformation, which can result in enhanced binding affinities for biological targets due to a smaller entropic cost.[9] Further, cyclic peptides can be iteratively modified both chemically, through the use of differing amino acids, and stereochemically, *via* the incorporation of L and D amino acids, toward the exploration of structure activity relationships (SAR), allowing for optimization of biological properties. Despite this promise, the synthesis of medium sized cyclic peptides (9–12-membered rings, tri- or tetrapeptides) remains a challenge due to ring strain brought about by unfavorable transannular interactions amongst amino acid side chains.[3,10,11]

Pseudoxylallemycins A–F (Fig. 1a), a class of 12-membered cyclic tetrapeptide (CTP) natural products isolated from the *Pseudoxylaria* sp. X802 fungus, were shown to have modest antibacterial activity against *Pseudomonas aeruginosa* K799/61 and *Mycobacterium vaccae* 10670.[12] As antibiotic resistant Gram-negative bacteria have been particularly challenging to target with new antibiotics,[13,14] often due to the impermeability of their outer cell membrane and various efflux factors,[15–18] we were intrigued by the growth inhibition these natural products exhibited toward *P. aeruginosa*. A subsequent biosynthetic natural product diversification study, where the fungal strain was grown in the presence of modified amino acid biosynthetic precursors, produced 21 additional pseudoxylallemycin analogues with varied substituents on the phenyl ring (Fig. 1b).[19] This investigation demonstrated that the phenyl substitution had minimal impacts on the antibacterial activity, as all compounds examined produced uniform growth

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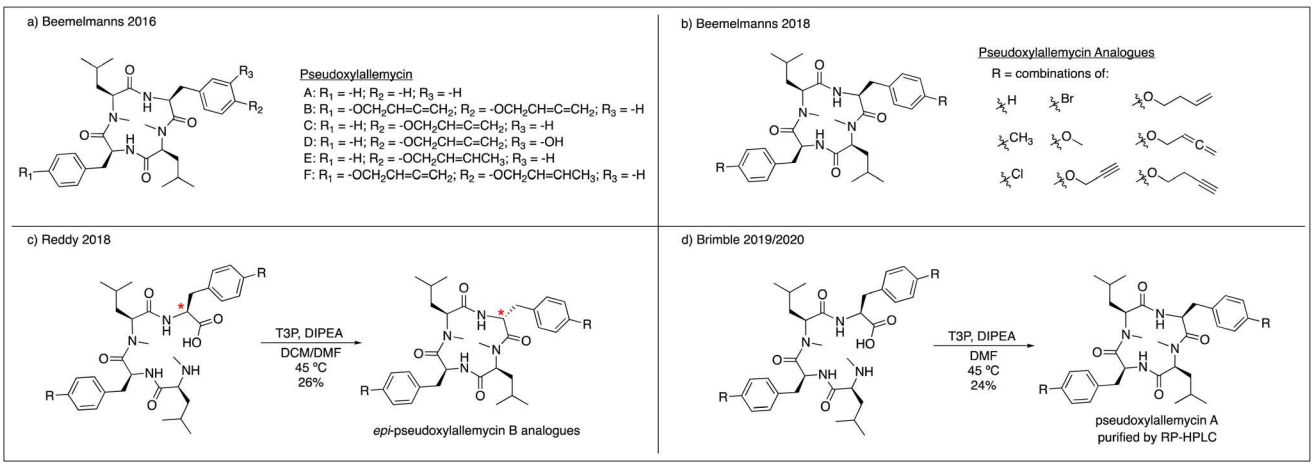


Fig. 1 Literature precedent toward our current study. (a) The structures of pseudoxylallemycin natural products. (b) SAR study using biosynthetically derived analogues. (c) Initial efforts toward pseudoxylallemycin synthesis result in complete epimerization of the C-terminal amino acid. (d) Subsequent synthetic efforts provide natural pseudoxylallemycin.

inhibition of the *P. aeruginosa* strain at 12.5 μg mL^{-1} (>20 μM), which suggested that more significant changes to the amino acid identity or stereochemistry would likely be required to materially impact antibacterial activity.

Efforts toward chemical synthesis of pseudoxylallemycin have been complicated by the common factors troubling the cyclization of all L-configuration CTP natural products,[3] including C-terminal epimerization of the linear tetrapeptide (LTP) and dimerization/polymerization pathways predominating over the desired cyclization of the LTP.[20–24] As the LTP amides commonly exist in an s-*trans* conformations, the amine and activated ester are held at a distance, preventing the proximity required for the desired cyclization reaction. Turn-inducing elements such as proline residues or *N*-methylation can favor the s-*cis* amide form to increase the rate of LTP cyclization. Numerous studies have focused on the incorporation of temporary or permanent turn-inducing elements,[25–28] but even when common turn inducing elements are present, successful cyclization is far from guaranteed, demonstrated by currently synthetically inaccessible all L-cyclo-[Pro-Tyr-Pro-Val], despite this natural product's two turn-inducing proline residues.[23,24] The first published effort toward the synthesis of pseudoxylallemycin by Reddy and coworkers described complete epimerization of the C-terminus in the CTP cyclization step, converting an all L-LTP to an LLLD-CTP in 26% yield without any trace of the desired product reported (Fig. 1c).[29] The authors postulated that this complete configurational inversion may be due to *epi*-pseudoxylallemycin being more thermodynamically stable than the natural product. Two additional reports of pseudoxylallemycin natural product syntheses from the Brimble laboratory demonstrated that a simple solvent modification to the CTP cyclization conditions (9 : 1 DCM/DMF → 100% DMF) can slow the rate of C-terminal epimerization, producing the natural product in a 35 : 65 ratio favoring the undesired epimer as the major product (Fig. 1d). Subsequent separation of the cyclization isomers using reverse phase-HPLC provided the desired natural product in 24% yield, providing access to pseudoxylallemycin A in 18% overall yield facilitated by solid-phase synthesis of the LTP precursor.[30,31] These synthetic efforts provided important insights that allowed our laboratory to initiate independent synthesis efforts toward improving the chemistry and elucidating additional SAR trends for antibacterial activity.

Herein, we discuss our scalable solution phase synthesis of the LTP precursor to pseudoxylallemycin A as well as an efficient and diastereoselective cyclization to afford the natural product. Most notably, we disclose how base identity greatly impacts diastereoselectivity of the cyclization, with weaker bases typically avoiding epimerization pathways in favour of direct cyclization to the natural product. As CTP macrolactamization efficiency is largely dependent on amino acid sequence and stereochemistry, we investigated the applicability of our optimized pseudoxylallemycin cyclization conditions to several analogues with modified stereochemistry or alanine replacement. In our efforts to fully characterize pseudoxylallemycin analogues, we described how variable temperature NMR can be a useful tool for more complete characterization efforts of CTPs, as this compound class often exists in multiple conformations, leading to NMR signal broadening at room temperature. Finally, we attempted to examine the antibacterial SAR of pseudoxylallemycin analogues with multiple strains of *P. aeruginosa*, and the data suggest that further studies will be required to ascertain the source of the bioactivity reported from the natural product extracts.

Results and discussion

Linear tetrapeptide construction

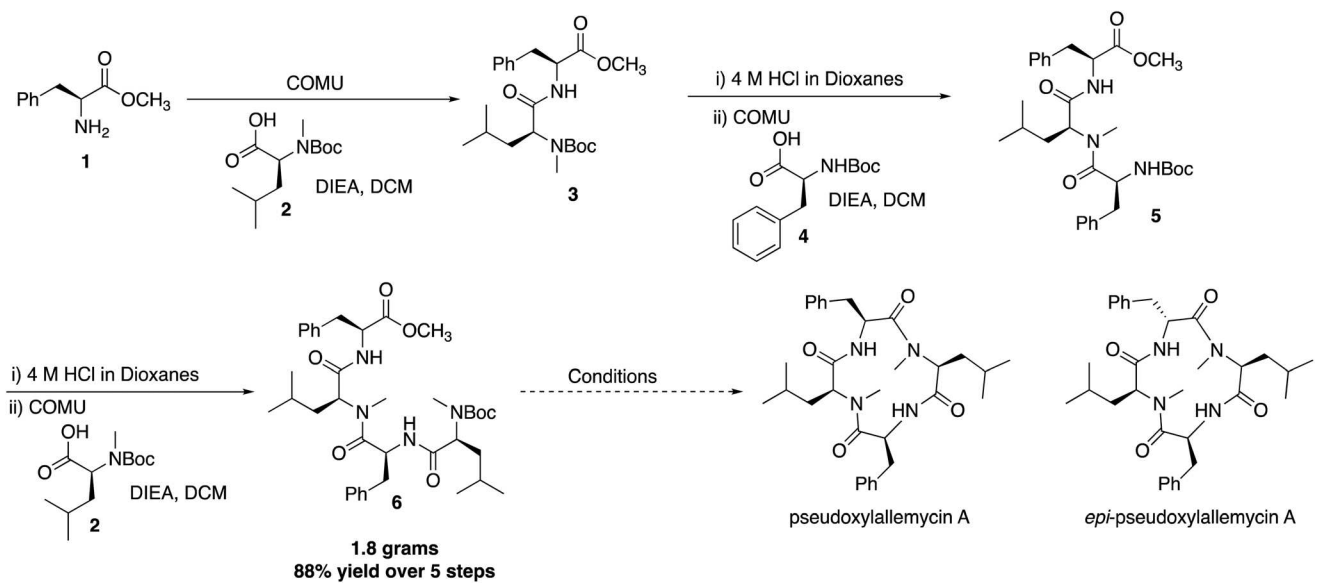
While many of the pseudoxylallemycin natural product analogues contained structurally unique allenyl modifications to the phenylalanine amino acid, precedent already demon-

strated that those structural components appeared to have a minimal impact on the antibacterial activity of these compounds.[12,19] Therefore, our efforts focused on developing a diastereoselective approach to pseudoxylallemycin A that could then be modified with different amino acids to examine how the side chains and configurations impacted the biological activity. It was clear that the cyclization step would require the greatest optimization efforts, so we desired methods that would allow for the simple and efficient production of significant quantities of the LTP precursor **6**. While solid-phase peptide synthesis provides many conveniences, it also has drawbacks such as a lack of atom economy (coupling steps requiring 4–5-fold excess of coupling reagents, amino acids, and the amine base) and a reliance on RP-HPLC after cleavage from the resin, which in many cases complicates large scale production of linear peptide precursors. We alternatively produced the LTP through solution-phase peptide coupling methods using safe and effective peptide coupling reagent COMU[32] in the individual coupling steps (Scheme 1). Briefly, phenylalanine methyl ester **1** was coupled to *N*-Boc-*N*-methyl leucine **2** to produce linear dipeptide **3**, which was found to be suitable for subsequent steps without chromatographic purification. After Boc-deprotection of **3** using 4 M HCl in dioxanes, the free amine dipeptide was coupled to *N*-Boc phenylalanine **4** to afford tripeptide **5**, which again was used directly in Boc-deprotection and peptide coupling with **2** without purification. These efforts expeditiously fashioned differentially protected LTP **6** on a gram-scale, which was purified using normal-phase flash column chromatography to provide the pure CTP precursor in 88% yield over three peptide coupling steps and multiple Boc-deprotections. With this compound in hand, we were poised to evaluate cyclization conditions toward a diastereoselective synthesis of pseudoxylallemycin A.

Linear tetrapeptide cyclization strategy

Five classes of *N*-methylated CTP natural products containing proteinogenic amino acids have been reported, including the endolides,[20,33,34] hirsutides,[35,36] onychocins,[22,37,38] pseudoxylallemycins,[12,19,29–31] and auxarthrides.[34] While uronium (*e.g.* HATU) and phosphonium (*e.g.* PyAOP) coupling reagents typically prove among the most effective in promoting LTP cyclization from precursors that lack *N*-methylation,[3] these reagents have been uniformly ineffective in promoting the diastereoselective synthesis of all-L *N*-methylated CTPs. Indeed, the only somewhat successful use of these reagents involved attempted cyclization of all-L MeHN-Leu–Phe–*N*-Me-Leu–Phe-OH to the desired all-L cyclo(NMe-Leu–Phe)$_2$ with PyAOP, which was accompanied by almost quantitative C-terminal epimerization to the cyclo(LLLD) form of the natural product. Instead, the most effective strategies for *N*-methylated CTP cyclization have involved either the formation of a pentafluorophenyl ester (Pfp) with *in situ* cyclization[35,37] or utilization of propanephosphonic acid anhydride (T3P).[20–22,29–31] Because of T3P's recent success involving the endolides, onychocins, and pseudoxylallemycins, we investigated its use toward improving the diastereoselectivity of pseudoxylallemycin synthesis toward the production of analogues to investigate the antibacterial structure activity relationships (SAR).

Efforts by the Reddy and Brimble groups demonstrated a major link between solvent identity and diastereoselectivity of cyclization, as both groups used similar amounts of T3P and diisopropylethylamine (DIPEA) in an identical solvent concentration, but 9 : 1 DCM/DMF led to complete epimerization to produce a 26% yield of *epi*-pseudoxylallemycin[29] while pure DMF led to a 35 : 65 diastereomeric ratio (DR), still favoring

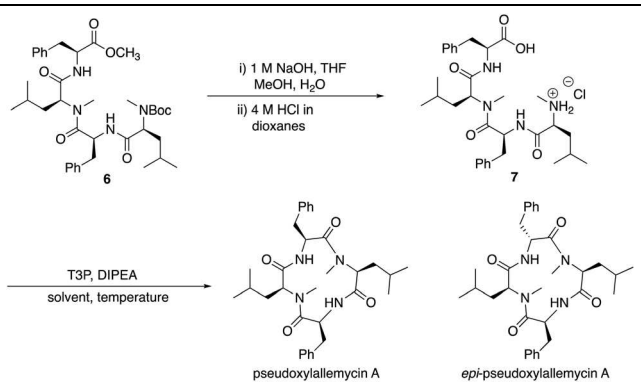


Scheme 1 Solution-phase synthesis of pseudoxylallemycin precursor LTP.

the epimer (24% isolated yield of the natural product).[30] Both groups also utilized a slightly elevated temperature for the macrolactamization reaction (45 °C), and the data indicated that 45 °C instead of room temperature appeared to shift the DR slightly toward more of the natural product.[30] Therefore, our initial optimization efforts examined how higher temperatures may improve the DR and yield. To begin, we sequentially saponified the protected LTP **6** using aqueous lithium hydroxide in THF/methanol and Boc-deprotected using 4 M HCl in dioxanes, providing the unprotected LTP **7**, which was subjected to cyclization conditions without any additional purification (Table 1). To establish a baseline yield and DR (comparable to literature conditions), we subjected this material to 3 eq. of T3P and DIPEA at a 1 mM concentration in DMF (Table 1, entry 1), and obtained a 42% isolated yield of the diastereomeric mixture over three steps (saponification, boc-deprotection, cyclization). Notably, we determined that the natural product could be isolated from its epimer using normal-phase column chromatography, something that had only been done previously using semi-preparative HPLC, which allowed our laboratory to efficiently separate the diastereomers and calculate a 28 : 72 DR, again producing more of the epimer than the desired natural product (in agreement with the literature). To determine if higher temperatures could enhance yield and DR, we elevated the reaction temperature to 80 °C in DMF, and this modestly improved DR (36 : 64) while having a minimal impact on yield (entry 2). Further increasing the reaction temperature above 100 °C caused decomposition. Since solvent identity was shown to largely impact the DR at 45 °C, we naturally investigated how changing the solvent at higher temperatures would affect the formation of natural product. To accommodate this increase in temperature, dichloroethane (DCE) was selected as an alternative high-boiling solvent to DCM, allowing us to run cyclization reactions around 80 °C. Reactions run in 1 : 1 DCE/DMF (entry 3) provided a minor increase to both DR (39 : 61) and yield (46%), while pure DCE (entry 4) boosted yield (57%) with a slight decrease in the DR (32 : 68). With the highest yield of pure natural product by mass, pure DCE at 80 °C appeared to be the most attractive solvent and temperature conditions for subsequent cyclization attempts; however, increasing temperature overall demonstrated relatively modest improvements in DR and yield.

We next sought to explore other factors that could impact epimerization during macrolactamization. C-terminal epimerization is a well-documented challenge in peptide chemistry, and it is broadly understood that the major pathway leading to the loss of stereochemical integrity involves oxazolone formation at the C-terminus followed by aromatization of the ring, which occurs by α-proton abstraction in the oxazolone.[39–41] Formation of the oxazolone can be suppressed in many cases by combining traditional peptide coupling reagents with hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt) additives, which form a less reactive active ester species, decreasing the rate of oxazolone formation and epimerization of the α-stereocenter.[41] Indeed, some of the most highly utilized peptide coupling reagents (*e.g.* HBTU, HATU, PyBOP, and PyAOP) come with the HOBt or HOAt as a pre-formed adduct with the electrophilic component that forms the initial activated ester. In contrast, the bases included in peptide coupling reactions are typically considered to have less impact on C-terminal racemization, demonstrated experimentally by DIPEA, 2,2,6,6-tetramethylpiperidine (TMP), and *N*-methylmorpholine (NMM) commonly providing similar racemization rates when included alongside various peptide coupling reagents (HATU, HBTU, COMU, DCC-HOAt, DCC-HOBt,

Table 1 Impact of temperature and solvent on macrolactamization



Entry[a]	Temp (°C)	Solvent (1 mM)	% Total CTP Yield[a]	% pseudoxylallemycin A Yield[b]	Natural/*epi* ratio[c]
1	45	DMF	41.8	11.7	28 : 72
2	80	DMF	40.9	14.7	36 : 64
3	80	1 : 1 DCE/DMF	46.4	18.1	39 : 61
4	80	DCE	56.7	18.1	32 : 68

[a] Cyclization reactions performed using 50 mg of LTP **7** diluted to 1 mM, 3 eq. of T3P and 3 eq. of DIPEA. [b] Isolated yields. [c] Determined by mass after separation using flash chromatography.

etc.).[32,42,43] However, impact of the base while using T3P for couplings has been examined much less than traditional carbodiimide/benzotriazole combinations.

We endeavored to explore whether base identity could improve diastereoselectivity and yield of cyclizations by expanding beyond DIPEA. To improve the throughput of our optimization efforts, we miniaturized the reactions and examined yield and diastereoselectivity of the reactions at 80 °C using HPLC absorbance of the product signals against a standard curve (Fig. S1 and S2†). In optimizing this approach, entries 2–4 of Table 1 were repeated on smaller scale (Table 2, entries 1–3), and the yield trend remained (DCE was highest) while DR was observed to be lower on the smaller scale (more epimerization). TMP provided no major gains to DR with a modest gain in yield (entries 4–6), but NMM produced a notable increase in DR when used in DCE as a solvent (NMM: 37/63, TMP: 27/73, DIPEA: 17/83). We began with these bases because they are employed commonly in peptide coupling reactions, and it was noteworthy that NMM is the weakest of the three bases (NMM conj. acid pK_a = 7.38 *versus* around 10–11 for DIPEA and TMP). In considering that the base could be promoting the racemization of the C-terminus, we postulated that bases weaker than NMM may further decrease the rate of this process without impacting the rate of ring closure. To explore this, collidine (conj. acid pK_a = 7.43), pyridine (conj. acid pK_a = 5.2), and 2,6-di-*tert*-butylpyridine (D*t*BP conj. acid pK_a = 3.58) were included in our optimization efforts (Table 2, entries 10–15). The cyclization using collidine in DCE performed similarly to that using NMM, as the two are almost equally basic (although with major steric differences), but with a slight increase to DR and yield of the natural product (entry 10). Gratifyingly, when weaker base pyridine was used, a significant jump in diastereoselectivity was observed (59 : 41 DR), marking the first time that the cyclization favored the natural product over its epimer (entry 12). Weakest base D*t*BP continued to demonstrate this trend, which, remarkably, produced almost exclusively natural product (92 : 8 DR) albeit at diminished yield (entry 14). Racemization suppressing agents HOBt and HOAt were included alongside pyridine to examine whether the DR and yield improvements could be further amplified (entries 16 and 17), but these additives in combination with T3P were ineffective.

Table 2 Impact of base and solvent on macrolactamization

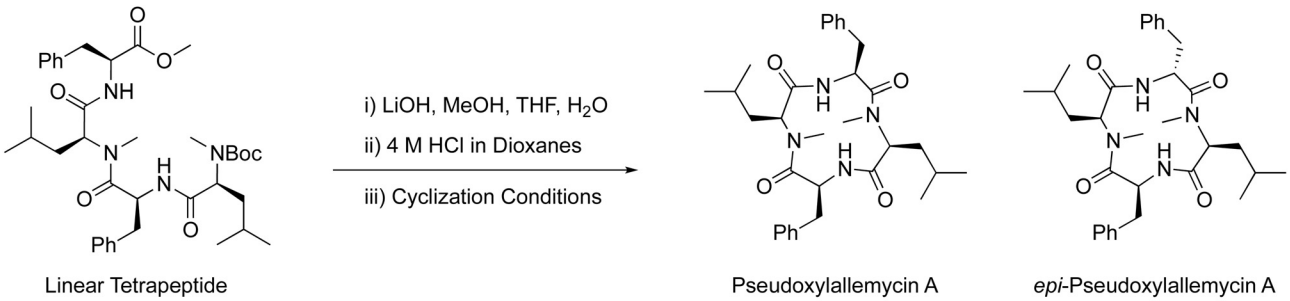
Entry[a]	Base/additive	Solvent	% yield[b]	Natural/*epi* ratio[c]
1	DIPEA	DCE/DMF (1 : 1)	45	25 : 75
2	DIPEA	DCE	67	17 : 83
3	DIPEA	DMF	53	31 : 69
4	TMP	DCE/DMF (1 : 1)	43	19 : 81
5	TMP	DCE	78	27 : 73
6	TMP	DMF	46	28 : 72
7	NMM	DCE/DMF (1 : 1)	30	24 : 76
8	NMM	DCE	77	37 : 63
9	NMM	DMF	42	32 : 68
10	Collidine	DCE	73	41 : 59
11	Collidine	DMF	11	66 : 34
12	Pyridine	DCE	58	59 : 41
13	Pyridine	DMF	1	61 : 39
14	D*t*BP	DCE	26	92 : 8
15	D*t*BP	DMF	7	91 : 9
16	Pyridine/HOBt	DCE	10	0 : 100
17	Pyridine/HOAt	DCE	0	n/a

[a] For all entries, LTP was diluted to 1 mM and heated to 80 °C followed by addition of 3 eq. coupling reagent then 3 eq. base (also 3 eq. of HOBt or HOAt for entries 16 and 17). Aliquots of reaction mixture were obtained at 48 hours. Those containing DMF were concentrated then dissolved in the same volume of DCE. All aliquots were diluted with MeOH at a 2 : 1 ratio before HPLC injection. [b] % yield is sum of natural product and epimer yields interpolated from a standard curve of integration units as a function of concentration (Fig. S1†) based on peak area measured at 221 nm on a Shimadzu Prominence HPLC using a 0–100% gradient of solvent B over 12 minutes at 1 mL min^{-1} on a Phenomenex Luna C18 3 μM 50 mm × 4.60 mm column at room temperature. Solvent A = 100% H_2O + 0.1% formic acid. Solvent B = 100% MeCN + 0.1% formic acid. [c] Ratio of diastereomers based on % area of natural product and epimer peaks.

Encouraged by these results and knowing that our former isolated yields showed modest differences from our HPLC analyzed reaction scale, we increased the scale of these reactions approximately 10-fold, isolated the natural product and epimer *via* flash chromatography, and saw further improvement to DR for all three bases on larger scale (as occurred in the initial scale-ups from Table 1). Overall, we observed that collidine maximized yield of the natural product and epimer (36% natural product, 30% epimer), pyridine provided high natural product yield (32%) while maintaining good diastereocontrol (89 : 11 DR), and D*t*BP maximized diastereocontrol (97 : 3 DR) at the cost of yield (19%) (Fig. 2). These results demonstrated that diastereoselectivity of the cyclization of pseudoxylallemycins is highly dependent on base strength, with weaker bases minimizing epimerization in favor of natural product formation. More generally, it indicated that when T3P is employed as a coupling reagent, significant changes in yield and C-terminal stereochemical integrity can be associated with the identity of the base used in the reaction, a variable that is often less important when more typical peptide coupling reagents are used. Overall, our synthesis of pseudoxylallemycin A improved upon preceding syntheses in terms of yield (as high as 32% over eight steps *versus* 18%), diastereoselectivity (as high as 97 : 3 DR, *versus* 35 : 65 DR), and efficiency (gram-scale synthesis of LTP **6** and purification by flash chromatography *versus* RP-HPLC).[30]

Variable temperature (VT) NMR improves characterization of CTPs

Linear and cyclic peptides often exist as multiple rotational conformers due to the ability of the amides to exist in *cis*- and *trans*-conformations that are distinguishable on an NMR timescale. This causes individual protons to often be split into many separate NMR resonances, and sometimes the signals broaden to an undetectable level. For example, apicidin, a



	Conditions	Yield of Natural Product	Yield of Epimer
High Yield, High Diastereocontrol	T3P, pyridine, DCE 80°C, 48 h	32%	4%
Maximum Yield	T3P, collidine, DCE 80°C, 48 h	36%	30%
Maximum Diastereocontrol	T3P, di-*t*Bu-pyridine, DCE 80°C, 48 h	19%	<1%

Fig. 2 Three step reaction yields and diastereoselectivities vary according to base. Modification to the base in macrolactamization provided condition that maximized yield at the expense of diastereocontrol (collidine), maximized diastereocontrol at the expense of yield (D*t*BP), and provided high diastereocontrol with moderately high yield (pyridine).

thoroughly studied CTP natural product investigated for use as a histone deacetylase inhibitor,[44–46] exists as a mixture of three detectable conformers in approximately an 80 : 15 : 5 ratio.[47] ^{1}H and ^{13}C NMR characterization of pseudoxylallemycin suggests that it exists almost exclusively as a single conformation, as the signals and their relative integrations match the expected number. Pseudoxylallemycin has C_2-symmetry, meaning the number of unique resonances it produces is half that of *epi*-pseudoxylallemycin. While our synthesized *epi*-pseudoxylallemycin fully matched literature characterization, we noted that the NMR spectra lacked clarity in a variety of ways, likely due to the molecule existing in multiple conformational states. For example, the ^{1}H NMR showed only three of the four expected a-proton signals and the methyl signals associated with leucine's isobutyl side chains appeared as broad multiplets instead of the expected resolved doublets. In the case of the ^{13}C NMR previously published, many of the carbon signals are indistinguishable from noise due to signal broadening, leading to far fewer discernable signals than expected. Indeed, while *epi*-pseudoxylallemycin is expected to have 28 unique ^{13}C NMR signals, the literature precedent was only able to peak-pick 16 carbons.[30] As the spectral ambiguity from signal broadening in both the ^{1}H and ^{13}C NMR spectra were expected to be derived from slow conformational interconversions, we postulated that acquiring the spectra at an increased temperature would result in an increased rate of equilibrium between the various conformational isomers, and this may cause signal averaging, making individual signals more pronounced.[48] To examine this, ^{1}H NMR was acquired at room temperature, 45 °C, and 85 °C, and immense peak sharpening was achieved at higher temperature (Fig. 3a). Of note, the spectrum at room temperature showed only three a-protons while at 85 °C four were clearly visible (3.75–4.75 ppm, red box). Further, the broad multiplets associated with the isobutyl methyl groups sharpened to two resolved doublets and additional overlapping doublets slightly farther downfield (0.75–1.00 ppm, blue box). Regarding ^{13}C NMR, a relatively concentrated NMR sample of *epi*-pseudoxylallemycin was prepared (~27 mg mL^{-1}), and a ^{13}C NMR was run for 10 240 scans at 101 MHz examining whether the sample concentration and large number of scans may provide more resolution than the 16 signals associated with the literature spectrum. These conditions provided a spectrum with 19 signals, still short of the 28 unique carbon atoms present in *epi*-pseudoxylallemycin (Fig. 3b). To examine the impact of increased temperature, the same sample was examined at 85 °C for the same number of scans on the same instrument, which resulted in significant sharpening. Gratifyingly, all 28 expected carbon signals were observed. Noteworthy areas with impressive resolution gains included the amide carbonyls, where only one observable carbonyl signal at room temperature gave way to four clear signals at 85 °C (Fig. 3b, 169.5–172.5 ppm, red box). Further, only three of the six carbon signals associated with the methyl and methine carbons of leucine's side chains were visible at room temperature, and they gained full resolution at 85 °C (Fig. 3b, 22–24 ppm, blue box). While greater resolution of the signals could have been gained on a higher field instrument, our data

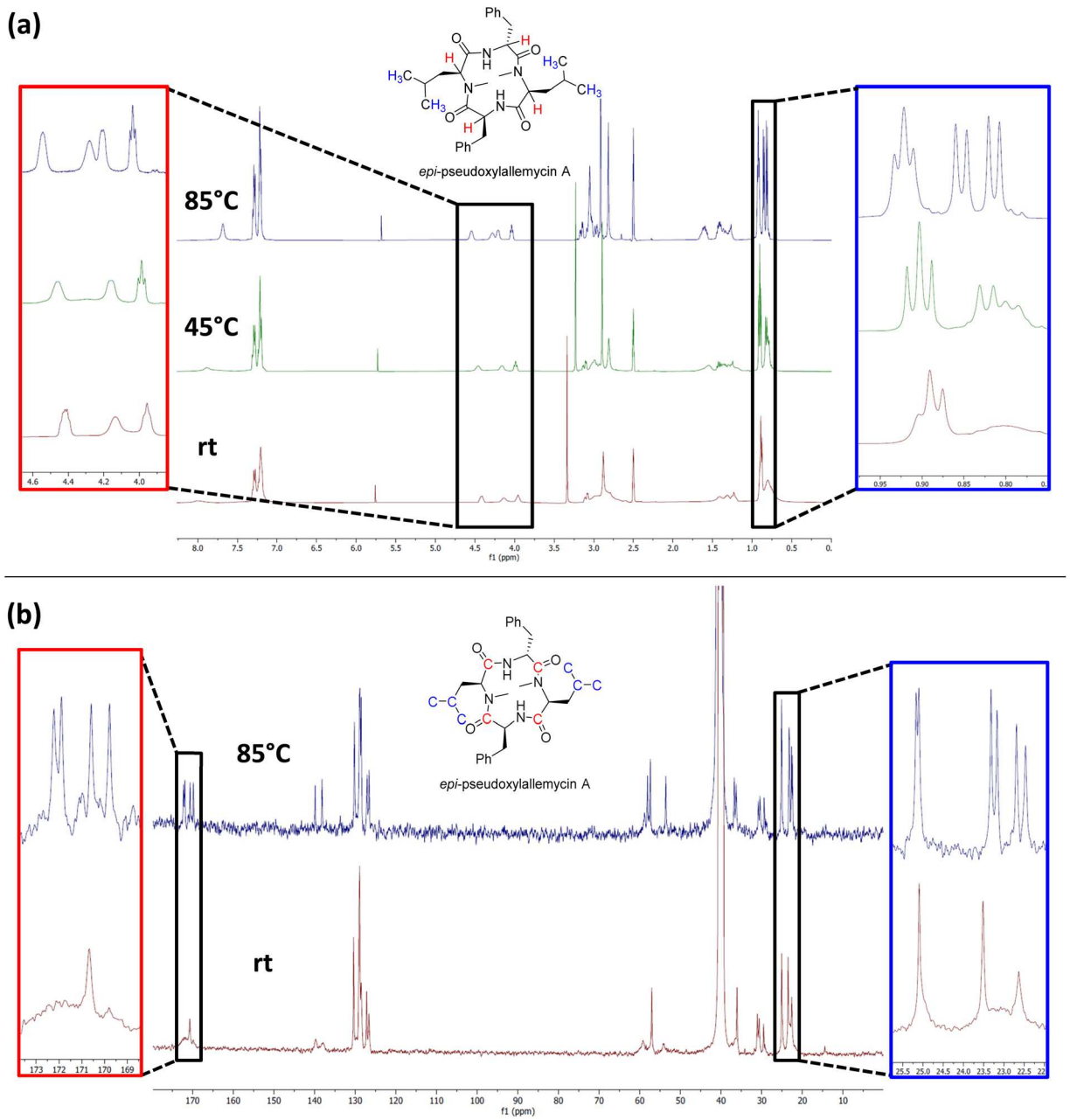


Fig. 3 Variable temperature NMR resolves *epi*-pseudoxylallemycin signals. (a) ^{1}H NMR acquired at room temperature, 45 °C, and 85 °C provides spectra with enhanced resolution in various regions. (b) ^{13}C NMR at room temperature *versus* 85 °C increases the number of signals from 19 to 28.

make it clear that VT-NMR can vastly improve signal resolution when those tools are inaccessible, aiding in CTP characterization efforts.

Synthesis of pseudoxylallemycin analogues

It is widely understood that a molecule's stereochemistry can have immense impacts on the biological activity.[49–51] More specifically, the stereochemical identity of individual amino acid components of cyclic peptides can play a significant role in their biological properties.[2,3] After developing efficient syntheses of pseudoxylallemycin and *epi*-pseudoxylallemycin and determining more efficient ways of characterizing CTPs in general, we endeavored to synthesize stereoisomers of pseudoxylallemycin A (all-L cyclo(*N*-Me-Leu–Phe)$_2$), which we could screen for antibacterial activity alongside the natural product and its epimer, attempting to understand how the stereochemistry of pseudoxylallemycin A impacts its antibacterial activity. We focused on exploring the symmetrical stereoisomers of the natural product, including its enantiomer (all-D cyclo(*N*-Me-Leu–Phe)$_2$) and both diastereomers alternating L and D amino acids (cyclo(L-*N*-Me-Leu–D-Phe)$_2$ and (cyclo(D-*N*-Me-Leu–L-Phe)$_2$). Further, synthesis of stereochemically dis-

tinct pseudoxylallemycin A analogues would allow us to examine whether our method of utilizing weak bases to minimize C-terminal racemization extends to other stereoisomers. The LTPs of these diastereomers were synthesized using the same methods as LTP **6** (synthesis and characterization details available in experimental and ESI†). The all-D LTP enantiomer of pseudoxylallemycin was subjected to cyclization with collidine, as that base resulted in the highest yield. As expected, this produced a mixture of diastereomers (64 : 36 all-D/epimer), and a 43% yield of the desired all-D diastereomer was isolated using flash chromatography (Table 3, entry 1). The LDLD and DLDL LTPs, however, completely epimerized under these same conditions. While a single set of conditions for analogue synthesis would be desirable, it is broadly understood that amino acid identity and configuration has enormous impacts on the efficiency of macrolactamization.[3,21,22,30] As phosphonium coupling reagent PyAOP led to total or nearly total epimerization of the all-L LTP, ostensibly cyclizing the LLLD-LTP precursor in good yield, we considered that it may be poised to promote a racemization-free macrolactamization of mixed stereochemistry LTPs, such as the LDLD- or DLDL-LTP precursors. Gratifyingly, cyclization of LDLD- and DLDL-LTPs using PyAOP and DIPEA led exclusively to formation of the desired diastereomer in relatively good yields (53% and 52%, entries 2 and 3). This remarkable difference in cyclization efficiency among the different diastereomers subjected to identical conditions further illustrates the sequence-based temperamentality of cyclizing LTPs, where one is required to match specific LTPs with unique coupling reagent combinations to promote the desired macrolactamization. In this case, T3P matched with an ideal base is most effective at suppressing racemization of all-D or all-L LTPs while PyAOP is far superior when cyclizing mixed stereochemistry LTPs.

Beyond the impact of amino acid stereochemistry on pseudoxylallemycin's structure activity relationships, the side chain identity can have immense effects on the biological activity. Toward exploring the side chain SAR, we performed a brief alanine scan of pseudoxylallemycin A. One aspect of the pseudoxylallemycin cyclization that simplified our study was that the natural product had half as many signals as the epimer due to its symmetry, allowing easy confirmation of CTP stereochemistry. If we substituted a single amino acid from pseudoxylallemycin with an alanine, this would result in a loss of symmetry. Without symmetry, it would remove our ability to distinguish stereoisomers using NMR spectra, greatly complicating our effort. To ease this challenge, we opted to perform a symmetrical alanine scan, synthesizing one analogue replacing both *N*-methyl leucine residues (FAFA) and a second where both phenylalanine residues were replaced (ALAL). Therefore, LTP analogues *N*-Me-Ala–Phe–*N*-Me-Ala-Phe and *N*-Me-Leu–Ala–*N*-Me-Leu-Ala were synthesized using the same methods as LTP **6**. Cyclization of FAFA was initially attempted using T3P with collidine (highest yielding conditions for pseudoxylallemycin), and a 50/50 ratio of the all-L and epimeric material was observed. Flash chromatography was attempted to separate the isomers, but solubility challenges complicated these efforts. Specifically, the product streaked off the column slowly, and the pure stereoisomers were unable to be isolated. A second effort was made utilizing DIPEA as the base (Table 3, entry 4), and this provided a 40/60 diastereomeric ratio favoring the epimer. Purification of this mixture had similar solubility complications, but a 5% yield of the epimeric material was able to be isolated and characterized. For synthesis of ALAL, T3P with collidine provided an 11% yield of the desired product (entry 5), and no racemization of the C-terminus was observed. These efforts again demonstrated that relatively minor changes (Leu → Ala or Phe → Ala) can significantly influence the efficiency of cyclization while also impacting properties such as solubility or chromatographic retention of the different stereoisomers.

Pseudoxylallemycin and analogues thereof lack antibacterial activity

With pseudoxylallemycin A, a collection of stereoisomers, and two alanine analogs in hand, we began analysis of the antibiotic properties of these CTPs against a common laboratory

Table 3 Macrolactamzation of pseudoxylallemycin analogs

Entry[a]	Residues	Configuration	Coupling reagent	Base	% yield[d]	Natural/*epi* ratio[e]
1		DDDD	T3P[b]	Collidine	43	64 : 36
2		LDLD	PyAOP[c]	DIPEA	52	100 : 0
3		DLDL	PyAOP[c]	DIPEA	53	100 : 0
4		LLLL	T3P[b]	DIPEA	5 (*epi*)	40 : 60
5		LLLL	T3P[b]	Collidine	11	nd[f]

[a] For all entries, the LTP was diluted to 1 mM in DCE and heated to a given temperature, followed by addition of 3 eq. coupling reagent then 3 eq. base. Reactions were stirred for 48 hours, then the reaction mixture was diluted with MeOH at a 2 : 1 ratio before HPLC injection. [b] Reactions with T3P run at 80 °C. [c] Reactions with PyAOP run at 45 °C. [d] Isolated yield. [e] Ratio of diastereomers based on % area of natural product and epimer peaks measured at 221 nm on a Shimadzu Prominence HPLC using a 0–100% gradient of solvent B over 12 minutes at 1 mL min^{-1} on a Phenomenex Luna C18 3 μM 50 mm × 4.60 mm column at room temperature. Solvent A = 100% H_2O + 0.1% formic acid. Solvent B = 100% MeCN + 0.1% formic acid. [f] ALAL lacked significant UV absorbance, and product ratio was unable to be determined using HPLC. No epimer was isolated following flash chromatography.

strain of *P. aeruginosa* (ATCC 27853) and *P. aeruginosa* clinical isolates associated with cystic fibrosis (NR-51337) and septicemia (NR-51335). Surprisingly, neither pseudoxylallemycin nor any of its analogues inhibited the growth of these strains at concentrations as high as 256 μg mL^{-1}. The initial antibacterial activity of the pseudoxylallemycins was described in a *P. aeruginosa* strain called K799/Z61,[12,19] which has increased membrane permeability due to a deletion of various efflux pumps[52–58] among its other mutations. To examine whether the lack of observed antibacterial activity in wild type *P. aeruginosa* strains was due to the efflux pumps being unimpaired, we decided to co-dose pseudoxylallemycin A and its analogues with broad-spectrum efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN), as it has been shown to synergize with known antibiotics, potentiating their activity in *P. aeruginosa*.[59–61] Despite this coadministration, all pseudoxylallemycin stereoisomers failed to inhibit the growth of these wild type strains, indicating that active efflux was not the sole issue causing the compounds to lack antibacterial activity. Next, we acquired the *P. aeruginosa* K799 and K799/Z61 strains, to compare the activity of our synthetically prepared pseudoxylallemycins in an identical strain to that which was used with the natural product extracts. Again, pseudoxylallemycin A and its stereoisomers failed to inhibit the growth of K799 or the more susceptible K799/Z61 mutated strain. While our compounds were inactive in all *P. aeruginosa* strains, we screened them additionally against clinical isolates of other ESKAPE pathogens, including the *E. faecium* HF 50104 and E980, *S. aureus* USA 300 and ATCC 12600, *K. pneumoniae* 160_1080, UHKPC32 and BIDMC1, *A. baumannii* WC-487, 3-137 and Naval-18, and *E. cloacae* complex BEI01 strains, as well as other commonly probed pathogens, *E. faecalis* B3286, *S. epidermidis* NRS6 and NRS7, *B. cereus* G9241, and *E. coli* NR-8 and CFT673; again, the compounds were uniformly inactive for growth inhibition. While unexpected, all former anti-*pseudomonal* activity was reported for the natural product extracts, and the *P. aeruginosa* K799/Z61 growth inhibition occurred at a relatively high concentration (12.5 μg mL^{-1}). Two plausible explanations for the different biological observations associated with the extract both include secondary minor chemical entities (<5%) that either (1) act independently as antibacterial agents or (2) synergize with pseudoxylallemycins in an unexpected way. Despite the lack of antibacterial activity, we look forward to supplying pseudoxylallemycin A and its stereoisomers to other laboratories or high-throughput screening facilities to potentially identify other applications of these compounds.

Conclusions

Previously reported syntheses of pseudoxylallemycins were plagued with unwanted racemization in the final cyclization step, leading to mostly the natural product's unwanted epimer. We have optimized the synthesis of pseudoxylallemycin A, providing the natural product in as high as 32% overall yield through 8 steps with high cyclization diastereoselectivity. The increased yield and diastereoselectivity was accomplished primarily by combining T3P with bases of varying strength during the macrolactamization, and conditions are available that maximize yield or diastereocontrol. In the process of characterizing the natural product epimer, we discovered that variable temperature NMR could be used to more thoroughly characterize cyclic tetrapeptides that exist in numerous conformational states, and we believe this method can be used more generally for improved characterization of CTP natural products and analogues thereof. Our synthetic and spectroscopic insights were leveraged to synthesize several stereoisomers and alanine substituted derivatives of the natural product for antibacterial SAR studies. Macrolactamization to prepare these analogues provided insight into how stereochemistry and amino acid identity play a large role in the likelihood of successful cyclization. Unexpectedly, 96-well plate antibacterial microdilution assays revealed that pseudoxylallemycin A and all analogues lacked any antibacterial activity, even at concentrations as high as 256 μg mL^{-1}. Future studies will aim to further examine the properties of pseudoxylallemycins and other CTPs toward the goal of discovering bioactive cyclic peptides.

Experimental details

General synthesis considerations

All starting materials, reagents, and solvents were purchased from commercial suppliers and were used without purification. Reaction progress was monitored by thin-layer chromatography (TLC) performed on TLC Silica gel 60 F254 from Supelco. Visualization of TLC plates was accomplished *via* UV light (254 nm) and/or the use of iodine and potassium permanganate staining. Flash chromatography was performed using normal phase Silica RediSep Silver Rf flash columns on a CombiFlash Rf automated flash chromatography system. ^{1}H and ^{13}C NMR spectra were obtained on a 400 MHz or 500 MHz JEOL spectrometer. Chemical shifts are reported in ppm relative to residual solvent peaks as an internal standard (δ 7.26 and δ 77.16 for $CDCl_3$ or δ 2.50 and δ 39.52 DMSO-*d*6). Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, dd = doublet of doublets, td = triplet of doublets, m = multiplet), coupling constant (Hz), integration. Data for ^{13}C NMR are reported in chemical shift (δ ppm). High resolution mass spectra (HRMS) were recorded on an AB Sciex 5600 TripleTOF using electrospray ionization in positive mode. Analytical high performance liquid chromatography (HPLC) was performed on a Shimadzu Prominence analytical HPLC with UV detection at 221 nm and 254 nm along with full spectrum UV detection using a Phenomenex Luna C18 3 mM 50 mm × 4.60 mm column. Optical rotations were obtained on a PerkinElmer Model 341 polarimeter.

Typical procedure for synthesis of linear peptides

Methods A and B described below were cycled until the peptide chain was poised for cyclization.

General method A: representative COMU amide coupling (dipeptide 3)

A round bottomed flask equipped with a magnetic stir bar was charged with L-phenylalanine methyl ester **1** (0.646 g, 3.00 mmol), L-*N*-Boc-*N*-methyl leucine **2** (0.736 g, 3.00 mmol), and DIPEA (1.57 mL, 9 mmol) dissolved in dimethylformamide (30 mL). The mixture was cooled to 0 °C, and COMU was added, all at once. The reaction was stirred at 0 °C, and the ice bath was allowed to warm to ambient temperature over the following two hours before leaving the reaction mixture to stir overnight at room temperature. The reaction was diluted with diethyl ether (100 mL), and the mixture was washed with sodium bicarbonate (2 × 50 mL), 1 M HCl (2 × 50 mL), and brine (50 mL). The organic was dried with sodium sulphate, filtered, and concentrated under reduced pressure, yielding a yellow oil in quantitative yield, which was used in subsequent amide coupling steps without further purification.

General method B: representative Boc-deprotection method (dipeptide 3 deprotection)

Dipeptide **3** (1.219 g, 3.00 mmol) was transferred to a round bottom flask equipped with a magnetic stir bar. To this flask was added 4 M HCl in dioxanes (15.0 mL, 60.0 mmol). The reaction was stirred at rt for 2 h. The reaction mixture was concentrated under reduced pressure and further dried under high vacuum. The deprotected dipeptide was afforded as a white solid in quantitative yield, used in subsequent amide coupling steps without further purification.

Typical procedure for synthesis of cyclic peptides

Once the linear tetrapeptide was constructed, the C-terminus was saponified according to method C below, the N-terminus was Boc-deprotected according to method B above, and cyclization is described for the individual examples.

General method C: saponification of the methyl ester (described from LTP 6)

LTP **6** (1.763 g, 2.59 mmol) was transferred to a round bottomed flask equipped with a stir bar, and was dissolved in a mixture of THF (12.3 mL) and methanol (8.1 mL). The mixture was stirred at 0 °C, and 0.5 M LiOH (13 mL, 6.47 mmol) was slowly added. The reaction was allowed to stir and gradually warm to room temperature over two hours and was shown to be complete by TLC. At this point, it was transferred to a separatory funnel and partitioned between diethyl ether (75 mL) and 1 M HCl (75 mL). The mixture was extracted with diethyl ether (3 × 75 mL), and the combined organic was dried with sodium sulphate, filtered, and concentrated under reduced pressure. The saponified tetrapeptide was afforded as a light yellow oil (1.717 g, 99%), and it was subsequently used in Boc-deprotection and cyclization without further purification.

Preparation of linear peptides

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-leucyl-L-phenylalanyl-*N*-methyl-L-leucyl-L-phenylalaninate (6, all-L MeHN-Leu–Phe–*N*-Me-Leu–Phe-OH). It was prepared using general methods A and B and was purified by column chromatography (eluting with 20/80 EtOAc/hexanes to 50/50 EtOAc/hexanes) to afford an off-white solid (1.8 g, 88%, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.33–6.97 (m, 10H), 5.12–4.80 (m, 2H), 4.81–4.66 (m, 1H), 4.65–4.41 (m, 1H), 3.79–3.60 (m, 3H), 3.22–3.02 (m, 1H), 3.02–2.77 (m, 2H), 2.76–2.63 (m, 1H), 2.62–2.55 (b, 2H), 2.46 (s, 3H), 2.37 (s, 1H), 1.69–1.57 (m, 1H), 1.56–1.47 (m, 3H) 1.47–1.43 (m, 9H), 1.43–1.27 (m, 4H), 0.90–0.77 (m, 12H). ^{13}C NMR (126 MHz, room temperature, $CDCl_3$) δ 172.39, 171.92, 171.87, 171.05, 170.72, 170.16, 168.82, 136.08, 129.38, 129.22, 129.12, 129.00, 128.73, 128.63, 127.27, 126.91, 80.45, 58.53, 55.90, 54.87, 53.99, 53.15, 52.53, 52.26, 50.19, 50.05, 38.54, 37.82, 37.55, 37.22, 36.48, 36.21, 30.49, 29.79, 29.59, 28.93, 28.45, 28.43, 24.74, 24.70, 23.38, 23.10, 22.45, 22.00, 21.46. HRMS (ESI$^+$) *m*/*z* calculated for $C_{38}H_{56}N_4O_7$ $[M + H]^+$ = 681.4227, found: 681.4256. $[\alpha]_D^{25}$ −70.4 (*c* = 0.05, MeOH).

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-D-leucyl-D-phenylalanyl-*N*-methyl-D-leucyl-D-phenylalaninate (all-D MeHN-Leu–Phe–*N*-Me-Leu–Phe-OH). It was prepared using general methods A and B and was purified by column chromatography (eluting with 20/80 EtOAc/hexanes to 50/50 EtOAc/hexanes) after each coupling to afford an off-white solid (1.067 g, 33% yield, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.36–6.97 (m, 10H), 5.15–4.82 (m, 2H), 4.82–4.67 (m, 1H), 4.66–4.43 (m, 1H), 3.80–3.59 (m, 3H), 3.21–3.07 (m, 1H), 2.98–2.78 (m, 2H), 2.77–2.65 (m, 1H), 2.64–2.53 (b, 2H), 2.53–2.42 (m, 3H), 2.39 (s, 1H), 1.70–1.59 (m, 1H), 1.58–1.48 (m, 3H) 1.48–1.45 (m, 9H), 1.43–1.20 (m, 4H), 0.90–0.78 (m, 12H). ^{13}C NMR (126 MHz, room temperature, $CDCl_3$) δ 172.35, 171.83, 171.44, 171.03, 170.68, 170.15, 168.76, 136.07, 129.35, 129.19, 129.09, 129.00, 128.68, 128.59, 127.21, 126.87, 80.37, 58.48, 55.85, 54.84, 53.94, 53.12, 52.46, 52.20, 50.20, 50.03, 38.50, 37.75, 37.50, 37.16, 36.46, 36.23, 30.48, 29.72, 29.54, 28.89, 28.41, 28.39, 24.70, 24.67, 23.36, 23.08, 22.40, 21.97, 21.44. HRMS (ESI$^+$) *m*/*z* calculated for $C_{38}H_{56}N_4O_7$ $[M + H]^+$ = 681.4227, found: 681.4227. $[\alpha]_D^{25}$ + 63.4 (*c* = 0.05, MeOH).

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-D-leucyl-L-phenylalanyl-*N*-methyl-D-leucyl-L-phenylalaninate (D-*N*-Me-Leu—L-Phe—D-*N*-Me-Leu—L-Phe). It was prepared using general methods A and B and was purified by column chromatography (eluting with a gradient of 20/80 EtOAc/hexanes to 50/50 EtOAc/hexanes) to afford an off-white solid (1.421 g, 40% yield, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.28–7.10 (m, 10H), 5.16–5.01 (m, 1H), 5.00–4.80 (m, 1H), 4.80–4.56 (m, 2H), 3.62 (s, 3H), 3.06–2.92 (m, 4H), 2.75–2.71 (m, 2H), 2.69–2.61 (m, 4H), 1.71–1.59 (m, 3H), 1.59–1.49 (m, 2H), 1.48–1.43 (m, 11H), 1.42–1.34 (m, 1H), 0.90–0.73 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.58, 172.45, 172.32, 171.53, 170.64, 170.37, 157.01, 136.94, 135.48, 129.34, 129.10,

128.82, 128.52, 127.43, 126.89, 80.71, 57.64, 56.02, 54.94, 53.71, 53.44, 52.20, 52.09, 51.35, 51.16, 38.37, 38.04, 37.51, 36.97, 36.65, 36.25, 35.88, 30.82, 30.10, 29.53, 28.50, 28.45, 28.40, 24.74, 24.51, 24.33, 23.26, 22.40, 21.81, 21.33. Extra signals are present due to conformational isomerism. HRMS (ESI^+) m/z calculated for $C_{38}H_{56}N_4O_7$ $[M + H]^+$ = 681.4227, found: 681.4218. $[\alpha]_D^{25}$ + 70.5 (c = 0.10, MeOH).

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-leucyl-D-phenylalanyl-*N*-methyl-L-leucyl-D-phenylalaninate (L-*N*-Me-Leu—D-Phe—L-*N*-Me-Leu—D-Phe). It was prepared using general methods A and B and was purified by column chromatography (eluting with a gradient of 20/80 EtOAc/hexanes to 50/50 EtOAc/hexanes) to afford an off-white solid (0.215 g, 5.3% yield, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.30–7.06 (m, 10H), 5.13–4.98 (m, 1H), 4.98–4.77 (m, 1H), 4.7–4.52 (m, 2H), 3.60 (s, 3H), 3.06–2.88 (m, 4H), 2.76–2.68 (m, 2H), 2.68–2.56 (m, 4H), 1.75–1.59 (m, 3H), 1.52–1.45 (m, 2H), 1.45–1.34 (m, 11H), 1.33–1.24 (m, 1H), 0.89–0.70 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.58, 172.45, 172.32, 172.03, 170.64, 170.38, 157.01, 136.94, 135.48, 129.34, 129.10, 128.82, 128.52, 127.43, 126.89, 80.70, 57.64, 56.02, 54.94, 53.71, 52.20, 51.35, 51.16, 38.37, 38.05, 37.50, 36.96, 36.66, 36.24, 35.88, 30.82, 30.10, 29.65, 28.50, 28.45, 28.40, 24.74, 24.51, 24.33, 23.27, 22.40, 21.81, 21.34. Extra signals are present due to conformational isomerism. HRMS (ESI^+) m/z calculated for $C_{38}H_{56}N_4O_7$ $[M + H]^+$ = 681.4227, found: 681.4222. $[\alpha]_D^{25}$ −68.3 (c = 0.10, MeOH).

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-alanyl-L-phenylalanyl-*N*-methyl-L-alanyl-L-phenylalaninate (all-L *N*-Me-Ala—Phe—*N*-Me-Ala-Phe). It was prepared using general methods A and B and was purified by column chromatography (eluting with a gradient of 50/50 EtOAc/hexanes to 75/25 EtOAc/hexanes) to afford a yellow solid (0.939 g, 42% yield, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.33–6.99 (m, 10H), 5.13–4.94 (m, 1H), 4.90–4.71 (m, 1H), 4.71–4.36 (m, 2H), 3.75–3.67 (m, 3H), 3.21–3.11 (m, 1H), 3.10–2.96 (m, 1H), 2.96–2.83 (m, 2H), 2.77–2.69 (m, 1H), 2.69–2.65 (m, 2H), 2.59 (s, 1H), 2.50 (s, 1H), 2.25 (s, 2H), 1.52–1.45 (m, 9H), 1.32–1.15 (m, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.37, 172.20, 172.17, 171.89, 170.97, 170.38, 169.78, 136.95, 136.07, 129.40, 129.36, 129.16, 129.14, 129.09, 128.74, 128.62, 127.62, 127.28, 127.17, 126.86, 55.68, 54.14, 53.16, 52.59, 52.55, 52.30, 50.78, 50.15, 38.80, 38.76, 37.75, 37.30, 30.56, 29.62, 28.47, 28.39, 13.40, 13.35. Extra signals are present due to conformational isomerism. HRMS (ESI^+) m/z calculated for $C_{31}H_{43}N_4O_7$ $[M + H]^+$ = 597.3283, found: 597.3283. $[\alpha]_D^{25}$ −59.7 (c = 0.10, MeOH).

Preparation of methyl *N*-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-leucyl-L-alanyl-*N*-methyl-L-leucyl-L-alaninate (all-L *N*-Me-Leu—Ala—*N*-Me-Leu-Ala). It was prepared using general methods A and B and was purified by column chromatography (eluting with a gradient of 50/50 EtOAc/hexanes to 100% EtOAc) to afford an off-white solid (0.784 g, 37% yield, 5 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 5.13–4.98 (t, 1H), 4.88–4.72 (m, 1H), 4.71–4.30 (m, 2H), 3.74–3.60 (m, 3H), 2.93–2.91 (m, 2H), 2.74–2.68 (m, 4H), 1.72–1.51 (m, 4H), 1.46–1.40 (m, 12H), 1.36–1.17 (m, 8H), 0.91–0.82 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.29, 173.22, 173.02, 172.70, 172.14, 170.89, 170.46, 170.18, 169.90, 169.31, 156.59, 155.50, 80.88, 80.36, 58.32, 57.48, 56.23, 56.21, 54.68, 52.51, 52.23, 48.58, 47.96, 45.51, 37.59, 36.72, 36.21, 36.03, 30.52, 30.01, 29.71, 29.02, 28.38, 24.84, 24.54, 23.31, 23.08, 23.00, 22.70, 21.96, 21.80, 21.46, 18.33, 18.15, 17.83, 17.08. Extra signals are present due to conformational isomerism. HRMS (ESI^+) m/z calculated for $C_{26}H_{49}$ N_4O_7 $[M + H]^+$ = 529.3596, found: 529.3604. $[\alpha]_D^{25}$ −95.2 (c = 0.05, MeOH).

Preparation of pseudoxylallemycin A. It was prepared by deprotecting the LTP using general methods B and C. The deprotected LTP (0.056 g, 0.094 mmol) was transferred to a round bottom flask and dissolved in DCE (93.5 mL, 1.0 mM). The mixture was heated to 80 °C, then collidine (0.037 mL, 0.28 mmol) was added followed by 50% T3P in DMF (0.083 mL, 0.28 mmol), and the reaction was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (eluting with a gradient of 40/60 EtOAc/hexanes to 100% EtOAc) to afford a white solid (0.018 g, 36% yield, 3 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.29–7.14 (m, 10H), 4.03 (s, 2H), 3.25 (dd, J = 13.9, 6.3 Hz, 2H), 3.08–3.02 (m, 2H), 2.83 (s, 6H), 1.80–1.62 (m, 2H), 1.62–1.41 (m, 3H), 1.41–1.26 (m, 3H), 0.88 (d, J = 6.4 Hz, 6H), 0.81 (d, J = 6.2 Hz, 6H). N–H signals are absent from spectrum. ^{13}C NMR (101 MHz, DMSO-$d6$) δ 172.19, 170.01, 138.51, 130.13, 128.32, 126.58, 58.79, 51.36, 38.20, 37.07, 30.65, 24.85, 23.64, 21.35. HRMS (ESI^+) m/z calculated for $C_{32}H_{44}N_4O_4$ $[M + H]^+$ = 549.3435, found: 549.3422. $[\alpha]_D^{25}$ −137.4 (c = 0.049, MeOH).

Preparation of *epi*-pseudoxylallemycin A. It was prepared by the same procedures as pseudoxylallemycin A to afford an off-white solid (0.017 g, 33% yield, 3 steps). ^{1}H NMR (400 MHz, 85 °C, DMSO-d_6) δ 7.81–7.55 (s, br, 2H), 7.36–7.09 (m, 10H), 4.62–4.47 (m, 1H), 4.38–4.24 (m, 1H), 4.24–4.14 (m, 1H), 4.08–3.99 (m, 1H), 3.19–3.11 (m, 1H), 3.11–3.03 (m, 4H), 3.03–2.92 (m, 2H), 2.92–2.88 (m, 3H), 1.71–1.54 (m, 2H), 1.50–1.18 (m, 4H), 0.92 (t, J = 5.1 Hz, 6H), 0.85 (d, J = 6.5, 3H), 0.81 (d, J = 6.5, 3H). ^{13}C NMR (101 MHz, DMSO-D_6) δ 172.23, 171.89, 170.59, 169.78, 139.84, 138.15, 130.19, 128.84, 128.81, 128.52, 127.08, 126.55, 58.83, 58.11, 57.41, 53.61, 39.44, 36.76, 36.29, 30.85, 30.43, 29.43, 25.17, 25.10, 23.32, 23.17, 22.69, 22.47. HRMS (ESI^+) m/z calculated for $C_{32}H_{44}N_4O_4$ $[M + H]^+$ = 549.3435, found: 549.3424. $[\alpha]_D^{25}$ −129.6 (c = 0.05, MeOH).

Preparation of (3*R*,6*R*,9*R*,12*R*)-3,9-dibenzyl-6,12-diisobutyl-1,7-dimethyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone (all-D cyclo(*N*-Me-Leu–Phe)$_2$). It was prepared by deprotecting the LTP using methods B and C. The deprotected LTP (0.085 g, 0.14 mmol) was transferred to a round bottom flask and dissolved in DCE (140 mL, 1.0 mM). The mixture was heated to 80 °C, then collidine (0.056 mL, 0.42 mmol) was added followed by 50% T3P in DMF (0.13 mL, 0.42 mmol), and the reac-

tion was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (eluting with a gradient of 40/60 EtOAc/hexanes to 100% EtOAc) to afford a white solid (0.033 g, 43% yield, 3 steps). ^{1}H NMR (400 MHz, 85 °C, DMSO-d_6) δ 7.88 (d, J = 8.9 Hz, 2H), 7.23–7.07 (m, 10H), 4.75 (s, 2H), 3.87 (dd, J = 10.6, 3.8 Hz, 2H), 3.17 (dd, J = 13.9, 6.2 Hz, 2H), 2.73 (dd, J = 13.8, 8.1 Hz, 2H), 2.61 (s, 6H), 1.61–1.50 (m, 2H), 1.43–1.34 (m, 2H), 1.32–1.20 (m, 2H), 0.81 (d, J = 6.7 Hz, 6H), 0.75 (d, J = 6.5 Hz, 6H). ^{13}C NMR (126 MHz, room temperature, $CDCl_3$) δ 171.96, 171.04, 137.39, 129.75, 128.37, 126.75, 59.33, 52.19, 37.44, 30.50, 29.79, 24.91, 23.19, 21.05. HRMS (ESI$^+$) m/z calculated for $C_{32}H_{44}N_4O_4$ $[M + H]^+$ = 549.3441, found: 549.3431. $[\alpha]_D^{25}$ 173.4 (c = 0.05, MeOH).

Preparation of (3*S*,6*R*,9*S*,12*R*)-3,9-dibenzyl-6,12-diisobutyl-1,7-dimethyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone (cyclo(D-*N*-Me-Leu—L-Phe)$_2$). It was prepared by deprotecting the LTP using methods B and C. The deprotected LTP (0.075 g, 0.13 mmol) and PyAOP (0.196 g, 0.375 mmol) were transferred to a round bottom flask and dissolved in DCE (125 mL, 1.0 mM). The mixture was heated to 45 °C, then DIPEA (0.053 mL, 0.38 mmol) was added, and the reaction was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (eluting with a gradient of 15/85 EtOAc/hexanes to 40/60 EtOAc/hexanes) to afford a white solid (0.036 g, 52% yield, 3 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.28–7.11 (m, 10H), 6.70 (d, J = 10.2 Hz, 2H), 5.20 (td, J = 5.7, 5.0 Hz, 2H), 5.03z (dd, J = 7.0, 9.0 Hz, 2H), 3.22 (dd, J = 9.8, 13.0 Hz, 2H), 2.92 (dd, J = 6.2, 12.7 Hz, 2H), 2.78 (s, 6H) 1.58–1.42 (m, 4H), 1.22–1.12 (m, 2H), 0.81 (d, J = 6.5, 6H), 0.73 (d, J = 6.5, 6H). ^{13}C NMR (101 MHz, room temperature, $CDCl_3$) δ 175.72, 170.90, 136.74, 129.10, 128.60, 126.85, 53.31, 50.27, 37.40, 34.01, 29.84, 24.90, 22.99, 21.81. HRMS (ESI$^+$) m/z calculated for $C_{32}H_{44}N_4O_4$ $[M + H]^+$ = 549.3441, found: 549.3427. $[\alpha]_D^{25}$ 38.0 (c = 0.05, MeOH).

Preparation of (3*R*,6*S*,9*R*,12*S*)-3,9-dibenzyl-6,12-diisobutyl-1,7-dimethyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone (cyclo(L-*N*-Me-Leu—D-Phe)$_2$). It was prepared by deprotecting the LTP using methods B and C. The deprotected LTP (0.057 g, 0.094 mmol) and PyAOP (0.147 g, 0.28 mmol) were transferred to a round bottom flask and dissolved in DCE (94 mL, 1.0 mM). The mixture was heated to 45 °C, then DIPEA (0.040 mL, 0.28 mmol) was added, and the reaction was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (eluting with a gradient of 15/85 EtOAc/hexanes to 40/60 EtOAc/hexanes) to afford a white solid (0.027 g, 53% yield, 3 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.28–7.14 (m, 10H), 6.69 (d, J = 10.2 Hz, 2H), 5.20 (td, J = 5.8, 4.8 Hz, 2H), 5.04 (dd, J = 7.4, 9.2 Hz, 2H), 3.22 (dd, J = 9.9, 13.1 Hz, 2H), 2.91 (dd, J = 5.8, 13.3 Hz, 2H), 2.78 (s, 6H) 1.56–1.42 (m, 4H), 1.21–1.10f (m, 2H), 0.81 (d, J = 6.5, 6H), 0.72 (d, J = 6.5, 6H). ^{13}C NMR (101 MHz, room temperature, $CDCl_3$) δ 175.72, 170.90, 136.74, 129.10, 128.60, 126.85, 53.31, 50.27, 37.40, 34.01, 29.84, 24.90, 22.99, 21.81. HRMS (ESI$^+$) m/z calculated for $C_{32}H_{44}N_4O_4$ $[M + H]^+$ = 549.3441, found: 549.3429. $[\alpha]_D^{25}$ −37.4 (c = 0.05, MeOH).

Preparation of (3*S*,6*S*,9*R*,12*S*)-3,9-dibenzyl-1,6,7,12-tetramethyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone (cyclo(L-*N*-Me-Ala—L-Phe—L-*N*-Me-Ala—D-Phe)). It was prepared by deprotecting the linear peptide using methods B and C. The deprotected LTP (0.084 g, 0.16 mmol) was transferred to a round bottom flask and dissolved in DCE (162 mL, 1.0 mM). The mixture was heated to 80 °C, then DIPEA (0.085 mL, 0.49 mmol) was added followed by 50% T3P in DMF (0.14 mL, 0.49 mmol), and the reaction was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (eluting with a gradient of 50/50 EtOAc/hexanes to 100% EtOAc) to afford a white solid (0.004 g, 5% yield, 3 steps). ^{1}H NMR (400 MHz, 85 °C, DMSO-d_6) δ 8.03 (s, 1H), 7.54 (s, 1H), 7.33–7.13 (m, 10H), 4.58 (s, 1H), 4.45 (s, 1H), 4.39 (s, 1H), 4.15 (q, J = 7.1 Hz, 1H), 3.15 (dd, J = 13.7, 5.6 Hz, 1H), 3.09–3.02 (s, 3H), 3.02–2.93 (m, 3H), 2.74 (s, 3H), 1.30–1.26 (m, 6H). ^{13}C NMR (101 MHz, 1 : 1 $CDCl_3$/MeOD) δ 174.01, 173.30, 171.70, 171.62, 170.10, 137.24, 135.90, 129.80, 128.45, 127.86, 126.97, 126.34, 59.20, 56.22, 53.93, 51.29, 39.83, 37.61, 35.14, 31.68, 30.27, 30.19, 29.42, 22.42, 15.77, 13.69. Both $CDCl_3$ and MeOD were required to solubilize solid for NMR analysis; $CDCl_3$ solvent peak set as the internal standard. HRMS (ESI$^+$) m/z calculated for $C_{26}H_{32}N_4O_4$ $[M + H]^+$ = 465.2502, found: 465.2509. $[\alpha]_D^{25}$ −117.6 (c = 0.033, 1 : 2 MeOH/DCM).

Preparation of (3*S*,6*S*,9*S*,12*S*)-6,12-diisobutyl-1,3,7,9-tetramethyl-1,4,7,10-tetraazacyclododecane-2,5,8,11-tetraone (all-L cyclo(*N*-Me-Leu—Ala)$_2$). It was prepared by deprotecting the linear peptide using methods B and C. The deprotected LTP (0.080 g, 0.18 mmol) was transferred to a round bottom flask and dissolved in DCE (178 mL, 1.0 mM). The mixture was heated to 80 °C, then collidine (0.071 mL, 0.531 mmol) was added followed by 50% T3P in DMF (0.156 mL, 0.531 mmol), and the reaction was allowed to stir for 48 h. The reaction mixture was transferred to a separatory funnel and the organic phase was washed with saturated $NaHCO_3$ (2 × 100 mL), 1 M HCl (2 × 100 mL), and brine (100 mL). The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure. The crude material was purified by column chrom-

atography (eluting with a gradient of 60/40 EtOAc/hexanes to 90/10 EtOAc/hexanes) to afford a white solid (0.008 g, 11% yield, 3 steps). ^{1}H NMR (400 MHz, room temperature, $CDCl_3$) δ 7.22 (d, J = 9.4 Hz, 2H), 4.95 (t, J = 7.4 Hz, 2H), 4.24 (dt, J = 11.1, 3.7 Hz, 2H) 2.80 (s, 6H), 2.05–1.98 (m, 2H), 1.71 (ddd, J = 14.7, 11.2, 3.5 Hz, 2H) 1.52–1.43 (m, 2H), 1.31 (d, J = 6.6 Hz, 6H), 0.95 (dd, J = 6.6, 3.5 Hz, 12H). ^{13}C NMR (126 MHz, room temperature, $CDCl_3$) δ 173.44, 170.46, 59.47, 46.13, 37.35, 30.51, 24.91, 23.40, 21.24, 18.03. HRMS (ESI^+) m/z calculated for $C_{20}H_{36}N_4O_4$ $[M + H]^+$ = 397.2815, found: 397.2818. $[\alpha]_D^{25}$ −126.0 (c = 0.05, 1 : 1 MeOH/DCM).

Author contributions

MCO conceived of and managed the project. MCO and VMF wrote and reviewed the manuscript while RCR and JZ edited and approved the manuscript. All authors performed experiments and analysed data.

Conflicts of interest

There are no conflicts to declare.

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