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The Design, Synthesis, and Biological Evaluation of Analogues of the Serine-Threonine Protein Phosphatase 1 and 2A Selective Inhibitor Microcystin LA: Rational Modifications Imparting PP1 Selectivity

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Abstract—Based on the results from previously reported molecular modeling analyses of the interactions between the inhibitor microcystin and the serine-threonine protein phosphatases 1 and 2A, we have designed analogues of microcystin LA with structural modifications intended to impart PP1 selectivity. The synthesis of several first generation analogues followed by inhibition assays revealed that all three are PP1-selective, as predicted. Although the observed selectivities are modest, one of the designed analogues is more selective for PP1 than any known small molecule inhibitor. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Members of the structurally diverse group of natural toxins that includes okadaic acid, calyculin, microcystin LA, and tautomycin exert their cytotoxic effects by inhibiting the serine-threonine protein phosphatases PP1 and PP2A.[1,2] This activity dramatically increases the phosphorylation state of a variety of proteins within the cell, a process that can have profound effects such as the disruption of intracellular signal trafficking, the deposition of proteinacious plaques and fibrils, and unregulated cellular proliferation. Interestingly, despite the dissimilarities of the toxins' structures, these compounds are competitive inhibitors (i.e. their PP1/PP2A binding sites overlap).

As a group these toxins inhibit PP1 and PP2A quite potently and specifically relative to other known phosphatases, such as PP2B (calcineurin), PP2C, and the tyrosine phosphatases. Consequently, several members of this group have become important probes of intracellular signalling pathways. Even so, there are problems of poor selectivity between PP1 and PP2A that can result in ambiguous results when attempts are being made to define the separate roles of these phosphatases in a given process. In particular, there is no known small molecule that inhibits PP1 with good selectivity. A recently published X-ray structure of a PP1-microcystin LR complex[3] provides the opportunity to explore the binding interactions of these inhibitors with both PP1 and PP2A, and to design new selective inhibitors based on these natural products.[4]

Analogue Design

We previously reported the results of a molecular modeling study in which the structure of a PP2A-microcystin complex was calculated based on the known structure of PP1 bound microcystin, and the sequence homology of PP2A to PP1.[4a] Inspection of the two enzyme–inhibitor complexes resulted in the identification of three differential contacts that might be exploited for achieving PP1 selectivity. These contacts occur between three adjacent residues in the toxin, L-leucine, D-alanine, and *N*-methyldehydroalanine, and the β-12 and β-13 loops of the enzymes, a region that is known to be important for inhibitor selectivity based on site-directed mutagenesis studies.[3,5]

Based on a comparision of the X-ray structure with the calculated PP2A complex, a pocket occupied by the leucine isopropyl group of the inhibitor appears to be

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Key words: microcystin; inhibitor; PP1; PP2A; selective.

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more sterically demanding in PP2A than in PP1 (Fig. 1), suggesting that enlarging this group would deleteriously affect binding affinity to a greater extent in PP2A. It is of interest to note that this is one of the most variable positions in the many naturally occuring microcystins; toxic variants containing arginine, tyrosine, phenylalanine, alanine, tryptophan, and homotyrosine at this position have been isolated,[6,7] but little is known about the effect of these substitutions on selectivity. In PP1, the leucine isopropyl group is in close proximity to tyrosine 272, and is flanked by the unimposing glycine 274. In PP2A, however, a second tyrosine replaces glycine, and the leucine isopropyl group is then juxtaposed to both tyrosines (numbered 261 and 263 in PP2A). Inspection of the models suggested that groups as large as cyclohexyl might be better accommodated in PP1, leading to the choice of L-cyclohexylalanine as a substitute for leucine in the first generation non-natural microcystin.

The adjacent residue, D-alanine, has as nearest neighbors residues of opposite charge in the two enzymes (Fig. 2); in PP1 the inhibitor makes contact with glutamate 275, while in PP2A the corresponding residue is the arginine 264 side chain. The formation of mixed charge complements (i.e. salt bridges) can be a dominant contributor to binding affinity, despite the considerable desolvation penalty that is incurred upon binding in many systems.[9–11] The formation of these charge complements primarily acts to decrease the off rate of a ligand bound to its receptor, rather than increase the on rate, and the contribution to binding affinity by a single salt bridge can be as high as 3.5 kcal/mol.[10] The strategic placement of an ammonium group on the D-alanine residue of the inhibitor might thus be expected to favor inhibition of PP1 over PP2A by forming a salt bridge with glutamate 275 in PP1, while suffering electrostatic repulsion with arginine 264 in PP2A. We therefore chose to substitute the D-alanine residue found in natural microcystin LA with the corresponding amino-substituted amino acid, D-2,3-diaminoproprionic acid.

The final differential contact to be modified is one made by the the *N*-methyl group (Fig. 3). PP1 has a sizable

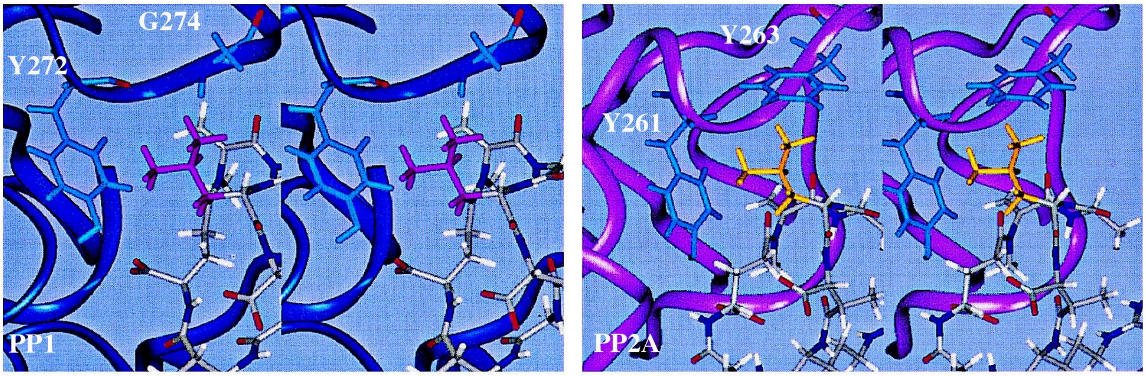


Figure 1. Stereoviews of the close contacts between the leucine isopropyl group in microcystin LR[8] (shown in magenta in the complex with PP1; shown in yellow in the complex with PP2A) and the enzymes PP1 (left, dark blue ribbon) and PP2A (right, magenta ribbon). The close contact residues in the enzymes are light blue, oxygen atoms are red, and the residue numbers are indicated in white bold. The remaining visible portion of microcystin has blue nitrogen atoms, grey carbon atoms, and white hydrogen atoms. The microcystin LR–PP1 complex is based on the known crystal structure, and the microcystin LR–PP2A complex is a theoretical structure based on modeling efforts.[4a]

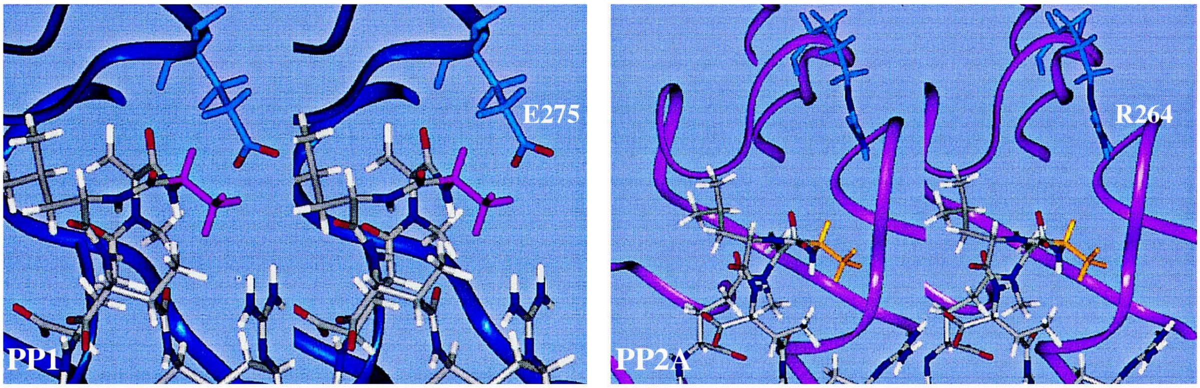


Figure 2. Stereoviews of the close contacts between the D-alanine methyl in microcystin LR[8] (shown in magenta in the complex with PP1; shown in yellow in the complex with PP2A) and the enzymes PP1 (left, dark blue ribbon) and PP2A (right, magenta ribbon). The close contact residues in the enzymes are light blue, oxygen atoms are red, and the residue numbers are indicated in white bold. The remaining visible portion of microcystin has blue nitrogen atoms, grey carbon atoms, and white hydrogen atoms. The microcystin LR–PP1 complex is based on the known crystal structure, and the microcystin LR–PP2A complex is a theoretical structure based on modeling efforts.[4a]

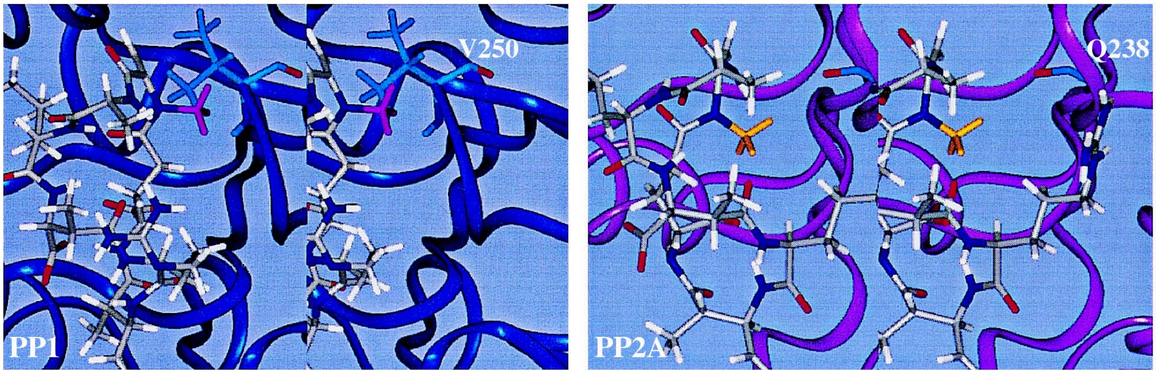


Figure 3. Stereoviews of the close contacts between the *N*-methyldehydroalanine methyl group in microcystin LR[8] (shown in magenta in the complex with PP1; shown in yellow in the complex with PP2A) and the enzymes PP1 (left, dark blue ribbon) and PP2A (right, magenta ribbon). The close contact residues in the enzymes are light blue, oxygen atoms are red, and the residue numbers are indicated in white bold. The remaining visible portion of microcystin has blue nitrogen atoms, grey carbon atoms, and white hydrogen atoms. The microcystin LR–PP1 complex is based on the known crystal structure, and the microcystin LR–PP2A complex is a theoretical structure based on modelling efforts.[4a]

hydrophobic cleft in the region directly behind the *N*-methyl group, while in PP2A the backbone carbonyl of glutamine 238 resides within 2.09 angstroms of the *N*-methyl carbon atom. The introduction of a larger *N*-methyl substituent therefore appeared to be a reasonable tactic, and the specific analogue chosen was the *N*-cyclohexyl derivative. The proposed structural modifications are summarized in Figure 4.

Synthetic Plan

The general plan for preparing analogues was to follow the published route in the synthesis of microcystin LA[12] as closely as possible, taking advantage of the fact that all of the modified residues reside in a single subunit employed in the original synthesis (Fragment **4**, Scheme 1). This fortuitous situation allows direct use of the stockpiles of Fragments **5** and **6** remaining from the natural product synthesis, and the analogues **2a** and **2c** were in principle accessible with no changes except substituting the appropriate modified amino acids.

On the other hand, incorporation of the aminoalanine residue in analogue **2b** (Scheme 1) did require a change in protecting group strategy in response to the additional amino group. To avoid possible complications of base catalyzed isomerization of the aminoalanyl group, neutral or mildly acidic conditions were desired for the final deprotection of this side chain. An obvious choice of *N*-protecting groups compatible with these requirements, the 2,2,2-trichloroethoxycarbonyl (Troc) group,[13] cannot be selectively removed in the presence of the 2,2,2-trichloroethyl (Tce) ester employed in our original microcystin LA synthesis, but this problem can be circumvented in principle simply by the use of a *t*-butyl ester (instead of Tce) to protect the carboxy terminus of the dipeptide fragment **5**. Another potential problem with the use of the Troc protecting group in the established route was the likelihood that it would not survive removal of a Cbz (*Z*) group by catalytic hydrogenation.[14] However, assembling the alkene-containing tetrapeptide fragments in a more convergent 2+2 segment coupling, rather than stepwise as per the original route (Scheme 2), circumvents this problem by allowing for removal of the *Z* group before introduction of the Troc-protected residue. This strategy also provides a common dipeptide fragment that could be used in synthesizing two of the proposed analogues.

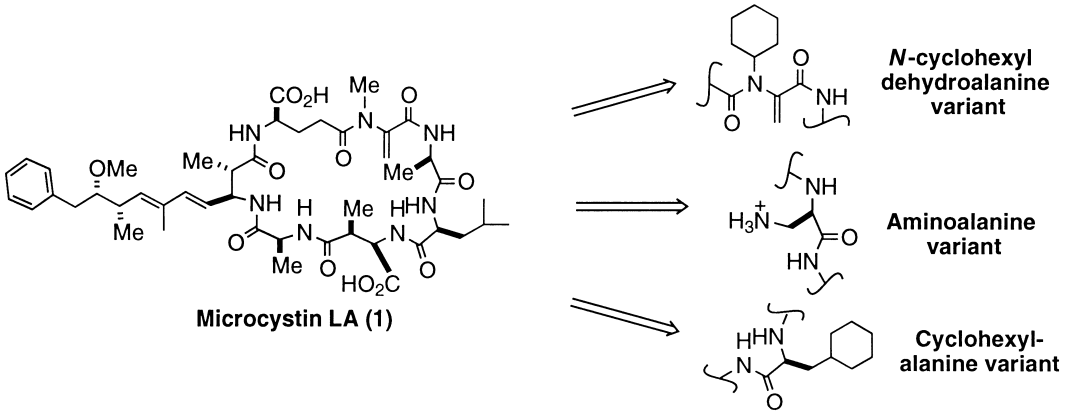
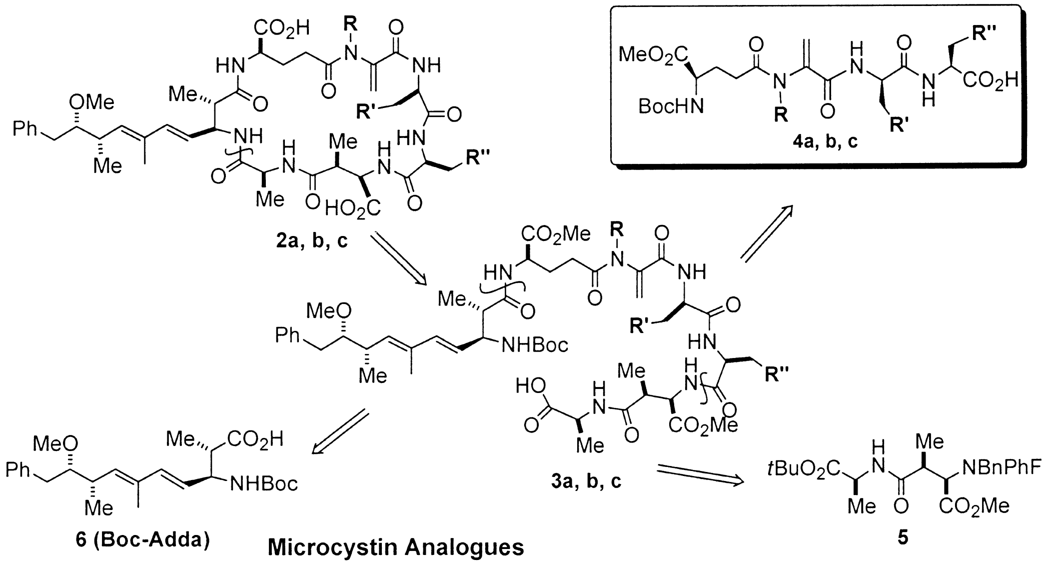


Figure 4. Summary of designed microcystin LA analogues.

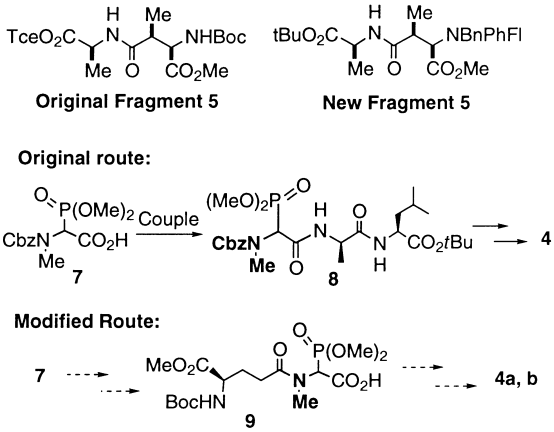


2a) Cyclohexylalanine variant: R = Me, R' = H, R'' = cyhx

2b) Aminoalanine variant: R = Me, R' = NH_3+, R'' = *i*Pro

2c) *N*-cyclohexyl variant: R = cyhx, R' = H, R'' = *i*Pro

Scheme 1. Retrosynthesis of microcystin LA analogues.



Scheme 2. Changes in the synthetic route.

Results and Discussion

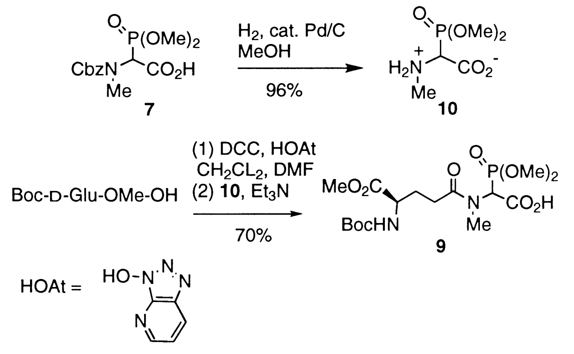
Synthesis of microcystin LA analogues

According to this modified route, the phosphonate-containing dipeptide **9** was to be employed as a common precursor to the *N*-methyl analogues **2a** and **2b**. Catalytic hydrogenolysis of the Cbz-phosphosarcosine free acid **7**[12] gave the free amino acid **10** in near quantitative yield (Scheme 3). Formation of the HOAt active ester[15] of Boc-D-Glu-OMe-OH[12] proceeded smoothly, and the crude material was coupled with the zwitterion **10** to give the requisite dipeptide **9** in 70% yield.

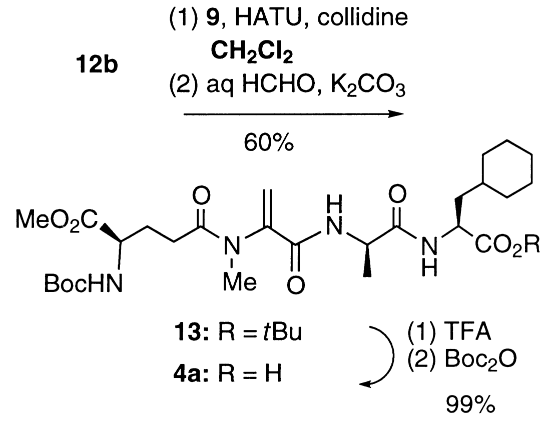
Having established a route to **9**, assembly of the tetrapeptide fragments could proceed. Toward this end, L-cyclohexylalanine ester **11** was coupled with Boc-D-alanine (EDCI/HOAt), followed by selective Boc acidolysis to give **12b** (Scheme 4). Initial attempts to couple **12b** and **9** were conducted using DMF as the solvent, and to reduce the problems associated with NMR analysis of the phosphonate-containing tetrapeptide (diastereomers, rotamers, and phosphorous–proton coupling), the crude material from the coupling reaction was subjected to the next step (Horner–Emmons olefination to give **13**) without purification beyond an aqueous workup. However, the low yield (<25%) we observed over this sequence prompted a more thorough investigation.

After conducting the sequence in stepwise fashion, we observed that the olefination step was very efficient (>90%), and that the low yields arose during the coupling step. Coupling *N*-alkylated α-amino acids in the C-terminal direction can give high yields, but is often accompanied by significant racemization.[16,17] This well-known racemization process occurs via formation of the cyclic oxazolonium ion, and reversible deprotonation of the greatly acidified α-proton scrambles the stereocenter (Fig. 5). In light of the poor coupling yield in our case, we suspect that the presence of the phosphonate moiety acidifies the α-proton to such a degree that deprotonation of the cyclic oxazolonium ion is essentially irreversible under the basic coupling conditions, and that the pseudoaromatic species formed is unreactive towards nucleophiles.

The use of less polar solvents is known to reduce the problems of racemization in coupling reactions,[18] therefore CH_2Cl_2 was substituted for DMF, leading to an improved 60% yield in the coupling of **9** with **12b** (Scheme 5).[19] Deprotection of **13** with TFA, followed by re-protection of the amine with Boc_2O, gave the desired free acid **4a** in near quantitative yield.



Scheme 3. Synthesis of the phosphonate dipeptide **9**.

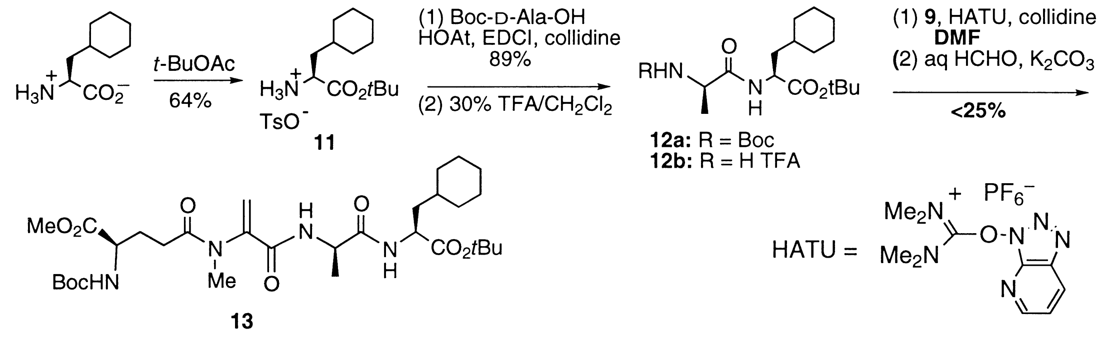


Scheme 5. Completion of fragment **4a**.

Having proven the viability of the modified route to the dehydrotetrapeptides, the extension of this route to the preparation of fragment **4b** could proceed. We next required a convenient route to the appropriately protected 2,3-diaminopropionic acid (aminoalanine) residue, but none of the existing methods proved to be satisfactory for our purposes.[20,21] Of the various methods for preparing related derivatives, the report that serine lactone can be opened at the β-position with ammonia in THF[21j] was the most attractive. The lactone **14** was readily prepared following the procedure of Vederas et al. (Scheme 6),[21j,22] but to our disappointment, attempts to open the lactone with ammonia in THF gave exclusively the primary amide resulting from opening at the carbonyl position. Fortunately, subsequent experiments showed that sodium azide opens lactone **14** cleanly at the β-position to give the azidoacid **15** in excellent yield. Reduction of the azide by catalytic hydrogenation, followed by amine protection, gave the Troc-Boc-aminoalanine **17** in 80% overall yield from lactone **14**.

In order to complete fragment **4b**, **17** was coupled with L-Leu-O*t*Bu (HATU) to give the corresponding dipeptide **18** in 77% yield (Scheme 7). Much to our surprise, based on our previous experiences with related couplings, when the dipeptide **18** was carried through the sequence of coupling with **9**, followed by Horner–Emmons olefination, a 1:1 mixture of tetrapeptides was obtained. The compounds were separated by chromatography, and the higher R_f material corresponded with the structure of the desired dehydrotetrapeptide **19** by ^{1}H NMR and FABMS. The lower R_f material, however, was identified as the formaldehyde adduct with the Troc protected amine (**20**).[23] In further experiments, we found that reducing the equivalents of formaldehyde from 10 to 1 resulted in clean conversion to the desired olefin. Double deprotection of the resulting tetrapeptide **19** (TFA), followed by amine re-protection gave the tetrapeptide free acid **4b** in 56% (from the dipeptide **18**). As no formaldehyde adduct was seen with the Boc group in any of the other substrates, this result highlights the greater acidity of Troc groups relative to Boc groups, which has been of use in Mitsunobu reactions involving protected amines.[24]

With the tetrapeptide fragments **4a** and **4b** completed, our next objective was to prepare the *N*-cyclohexyl



Scheme 4. Low yielding coupling.

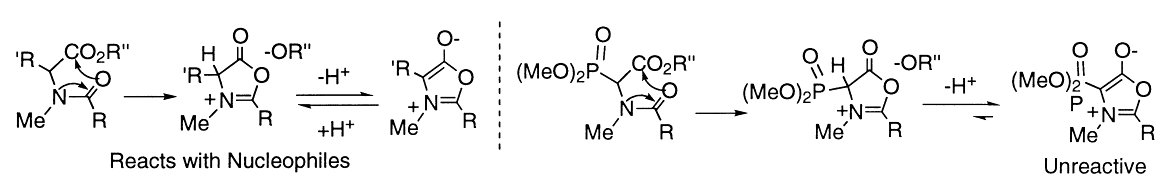
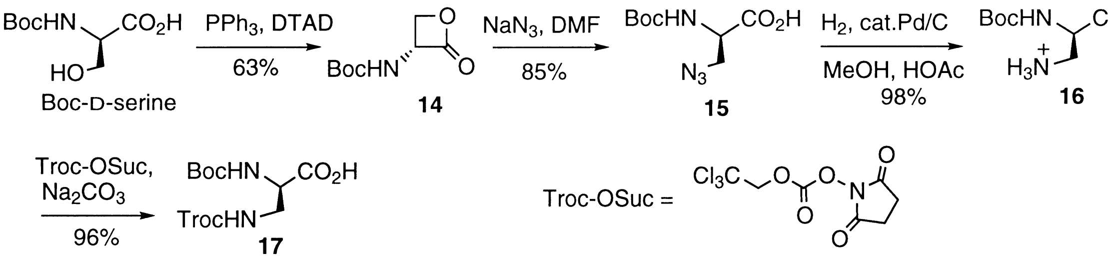
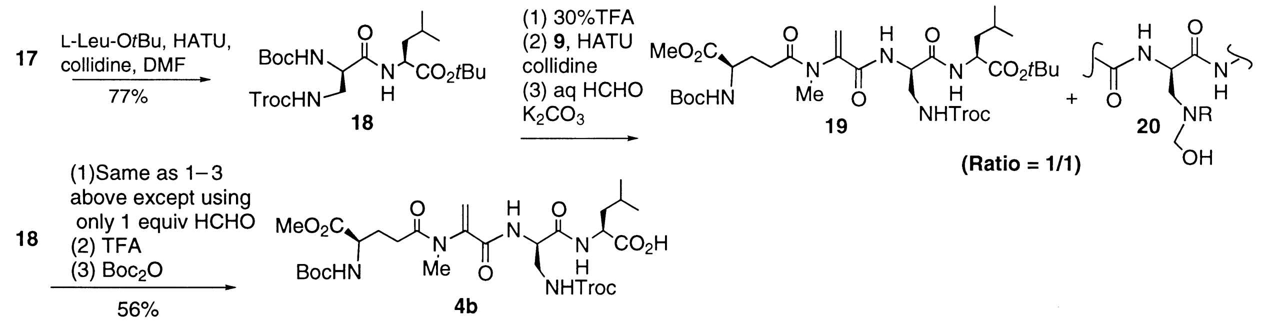


Figure 5. Oxazolonium formation and deprotonation.



Scheme 6. Synthesis of the differentially protected aminoalanine amino acid.



Scheme 7. Synthesis of fragment **4b**.

tetrapeptide **4c**. As mentioned above, we were initially interested in synthesizing the *N*-cyclohexyldehydroalanine-containing analogue **2c**. To obtain **4c** following the previous synthetic route, an acid-catalyzed condensation of Cbz-cyclohexylamine and the hemiacetal of methylglyoxylate was required. In the case of Cbz-methylamine, the condensation is an equilibrium process that slightly favors the starting materials, but it provides a 30% yield of the desired hemiaminal.[12] Not surprisingly, the corresponding equilibrium for the Cbz-cyclohexylamine lies far to the left, and only trace amounts of product were suggested by TLC (Fig. 6).

At that juncture, we reconsidered the importance of the dehydroamino acid for binding and activity. It is known that the dehydroalanine residue in microcystin reversibly forms a covalent bond with the cysteine 273 thiol in the binding site.[5c] While this interaction no doubt stabilizes the enzyme–ligand complex, it does not appear to greatly influence the apparent binding affinities. Indeed, a C273A mutant of PP1 (alanine replacement at the cysteine residue in question) demonstrated no significant change in IC_{50} values from the wild type for several inhibitors of the okadaic acid class, including microcystin.[5c] In addition, several reports state that reduction of the dehydro-residue present in microcystin produces two isomers that are as potent inhibitors as the natural material.[25] The Michael adducts of microcystin with aminoethanethiol[26] and ethanedithiol[27] are nearly as potent as the natural material in inhibiting PP1 and PP2A. Finally, a naturally occurring microcystin toxin, (Mser[7])-microcystin LR, contains an *N*-methyl serine residue in place of the dehydroalanine residue and still retains potent toxicity.[28] In light of these reports, we reasoned that substituting *N*-methylglycine (sarcosine) for the dehydroalanine residue should not interfere significantly with binding.[25d,e] Consequently, the decision was made to replace the *N*-cyclohexyldehydroalanine originally designed with *N*-cyclohexylglycine (*N*-cyhxGly).

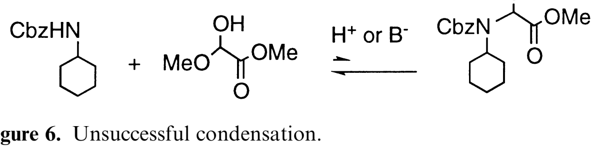
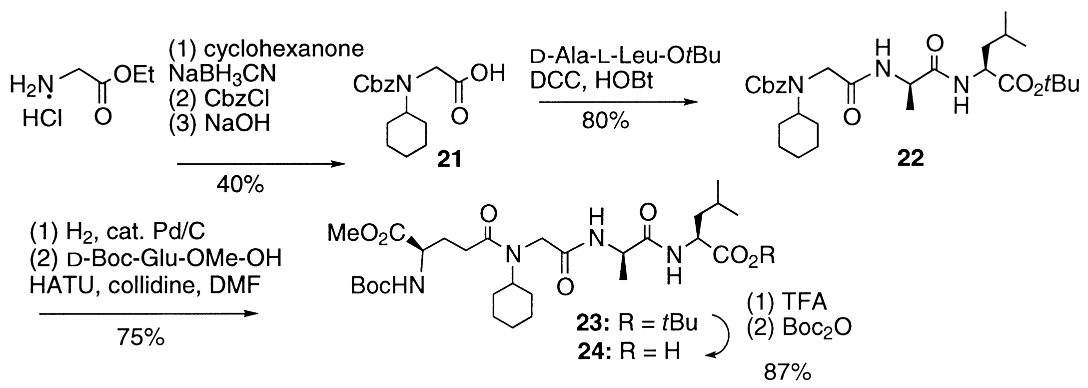


Figure 6. Unsuccessful condensation.

A concern arising from the implementation of this substitution, however, was that it might deleteriously influence the macrocyclization,[17,29–34] but this change did not appear to affect the cyclization efficiency (vide infra). The decision to replace the *N*-MdhA residue with *N*-cyhxGly involved changing two structural variables at once, so the *N*-methylglycine (*N*-meGly)-containing analogue itself was also prepared to serve as a control.

The requisite tetrapeptide fragments were easily prepared from the corresponding *N*-alkylated glycines. Reductive amination of Gly-OEt hydrochloride with cyclohexanone ($NaCNBH_3$), followed by amine protection (Cbz-Cl) and ethyl ester hydrolysis gave the *N*-cyhxGly residue **21** in 40% unoptimized yield (Scheme 8). Coupling of **21** with D-Ala-L-Leu-O*t*Bu[12] (DCC/HOBt) gave tripeptide **22** in excellent yield. N-terminal coupling to *N*-alkylated amino acids is known to be problematic, typically resulting in low yields and significant epimerization (when coupling to α-amino acids).[18] New methods and reagents, particularly Carpino's HATU reagent,[35] have lead to dramatic improvements in many cases,[17] but we were still concerned about how efficiently the bulky *N*-cyclohexyl-containing tripeptide would couple in the N-terminal

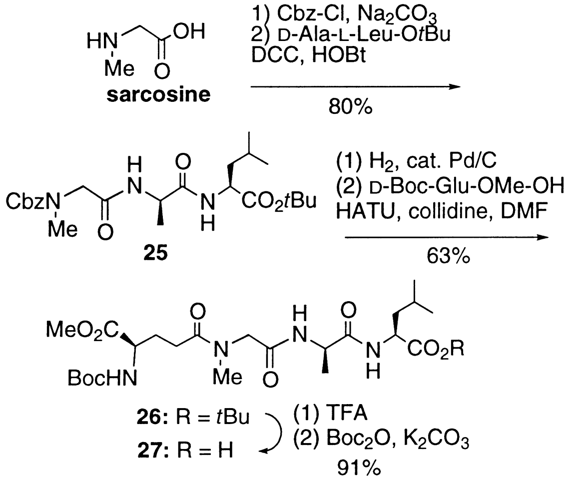


Scheme 8. Synthesis of the *N*-cyhxGly tetrapeptide **24**.

direction. Our concerns were allayed when we found that following hydrogenolysis of the Cbz-group in **22**, the *N*-cyhxGly-D-Ala-L-Leu-O*t*Bu peptide coupled with Boc-D-Glu-OMe-OH to give **23** in 75% unoptimized yield using HATU. That this coupling proceeded cleanly attests to the power of the HATU reagent in effecting these difficult transformations. Finally, treatment of the tetrapeptide **23** with TFA, followed by amine re-protection (Boc_2O) gave the free acid **24** in excellent yield.

Preparation of the corresponding *N*-meGly containing tetrapeptide proceeded along similar lines. Coupling of Cbz-N-MeGlycine (Cbz-sarcosine) with D-Ala-L-Leu-O*t*Bu (DCC/HOBt) gave the tripeptide **25** (Scheme 9). Hydrogenolysis, followed by HATU-mediated coupling with Boc-D-Glu-Ome-OH, gave the tetrapeptide **26**. Double deprotection, followed by amine re-protection gave the desired free acid **27**, thus completing the last of the requisite tetrapeptide fragments.

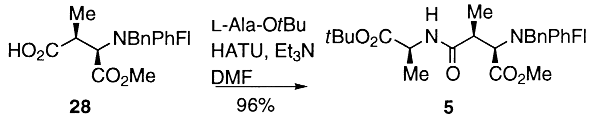
The final subunit required was the protected β-methylaspartate-containing dipeptide **5**. In developing the synthetic route to the natural product itself, we found that the *N*-BnPhFl-protected aspartate derivative **28** coupled efficiently with amino acids,[12] but that protecting group strategy did not find its way into the published route. However, it was exhumed for our current purposes, and coupling of the aspartate derivative **28** with L-Ala-O*t*Bu (HATU) gave the dipeptide **5** in near quantitative yield (Scheme 10).



Scheme 9. Synthesis of the *N*-meGly tetrapeptide **27**.

With this dipeptide in hand, we were ready to investigate the 4+2 segment couplings. In considering these sometimes difficult coupling reactions, we were mindful of some unpleasant possibilities associated with our specific protection format; aspartate (and to a lesser extent glutamate) residues in peptide chains are prone to base- and acid-catalyzed imide formation.[36–39] Protection of aspartate side chains as bulky *t*-butyl[40] or β-phenacyl[37] esters is therefore recommended in order to avoid base- and acid-catalyzed imide formation, respectively. However, even these measures do not always provide complete protection.[37,41]

Our strategy, however, builds upon the previously reported regio- and diastereoselective aspartate alkylation,[42] and more importantly, a delicate final saponification of the methyl esters.[12] Rather than re-work the entire sequence employing *t*-butyl esters, which would also have required other changes in the protecting group scheme, we decided to face the challenges put forth by incorporating the aspartate residue with methyl ester side chain protection. In the first attempt at coupling **4a** with the dipeptide free amine resulting from selective nitrogen de-protection of **5**, the free amine was used in excess, and the only coupled product observed was the hexapeptide imide, as demonstrated by FABMS and ^1H NMR (loss of one methyl ester singlet). We suspected that the imide formation was due to the presence of excess free amine component, so we carried out a second coupling using the amine TFA salt as the component in excess. In this case, using the mild base collidine in excess to effect deprotonation, we observed efficient coupling with only a trace of imide formation evident by TLC analysis. In light of this result, we recommend that base sensitive couplings of this type be conducted in a similar fasion. These conditions buffer the reaction



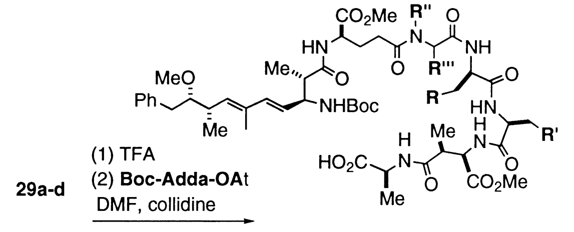
Scheme 10. Completion of fragment **5**.

at a lower pH, thus reducing base catalyzed side-reactions. Other amine salts, such as HCl and TsOH salts, also give excellent results (vide infra).[43]

Once the problem of imide formation had been solved, the four tetrapeptides were carried through the segment coupling process (Scheme 11). The standard method was selective deprotection of the dipeptide **5** to produce the amine TFA salt. This component was then coupled with the appropriate tetrapeptide (HATU/collidine) to give the hexapeptides **29a–d** in good purified yields, with little or no imide formation observed.

With the completion of the hexapeptide fragments, the stage was set for coupling to the Adda[12] fragment **6**. The plan was to couple the doubly deprotected (N- and carboxy- termini) hexapeptides to a pre-activated Adda, a strategy that would provide products that were ready to enter the macrocyclization sequence with no further deprotections required. The HOAt active ester of the Adda fragment **6** was therefore prepared (DCC/HOAt), the crude active ester was combined with the doubly deprotected hexapeptide amine salt (resulting from treatment of the hexapeptides **29a–d** with TFA), and the coupling was promoted by the addition of collidine (Scheme 12). Following this general protocol, the various heptapeptides (**3a–b**, **30a–b**) were produced in 55 to 68% overall yield after purification.[44]

The macrocyclizations were carried out, using the general procedure employed for the synthesis of the natural product,[12] as follows: the heptapeptide free acids (**3a–b**, **30a–b**) were converted into the respective pentafluorophenyl esters (Scheme 13), followed by amine deprotection (TFA) and addition to a rapidly stirred two-phase chloroform/aqueous phosphate buffer mixture. As anticipated, the cyclohexylalanine variant **31a** and the *N*-Troc-aminoalanine variant **31b** were obtained in yields comparable to that obtained for the parent microcystin LA. Interestingly, the *N*-cyhxGly dervative cyclized equally efficiently to give **31c**, but the *N*-meGly variant cyclized to give **31d** in significantly lower yield. It may be that the higher cyclization yields observed for the *N*-cyhxGly analogue relative to the corresponding *N*-methyl derivative simply reflect a greater tendency for the larger *N*-cyclohexyl substituent to induce a favorable turn structure in the cyclization precursor.[33,45]



3a (57%): R = H, R' = cyhx, R'' = Me, R''' = CH_2

3b (58%): R = NHTroc, R' = *i*Pro, R'' = Me, R''' = CH_2

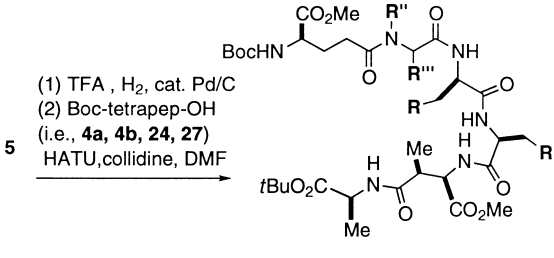
30a (55%): R = H, R' = *i*Pro, R'' = cyhx, R''' = H

30b (68%): R = H, R' = *i*Pro, R'' = Me, R''' = H

Scheme 12. Coupling with the Adda-active ester to form the cyclization precursors.

The completion of the cyhxAla variant **2a**, the *N*-cyhxGly variant **32a**, and the *N*-meGly variant **32b**, were carried out by treatment of the corresponding diesters with LiOH, giving a mixture of three isomers in each case (Scheme 14).[46] The by-products, which we believe to be the result of isomerization prior to saponification, were separated from the desired material and each other by chromatography. The desired isomer was identified in each case by re-esterification using TMS-diazomethane, followed by comparison with authentic samples of the di-esters.[47] Several alternative methods of saponification were attempted, including the use of potassium trimethylsilanoate,[48] KOH in methanol,[49] $CeCl_3$ and $CoCl_2$ in aqueous THF,[50] horse serum butyrylcholinesterase,[51] and lipases from *Candida cylindracea* and *Pseudomonas* species,[52] but either no improvement or no reaction was observed in these cases.

The reductive Troc removal to complete the aminoalanine variant **2b** proved troublesome. Our initial efforts



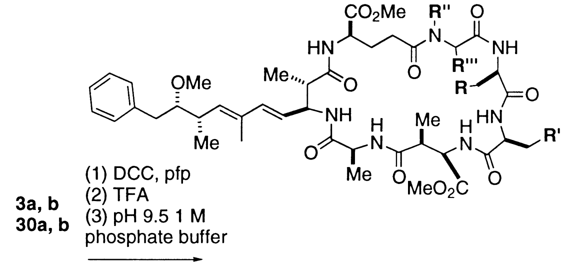
29a (70%): R = H, R' = cyhx, R'' = Me, R''' = CH_2

29b (76%): R = NHTroc, R' = *i*Pro, R'' = Me, R''' = CH_2

29c (72%): R = H, R' = *i*Pro, R'' = cyhx, R''' = H

29d (57%): R = H, R' = *i*Pro, R'' = Me, R''' = H

Scheme 11. 4+2 segment couplings.



31a (43%): R = H, R' = cyhx, R'' = Me, R''' = CH_2

31b (47%): R = NHTroc, R' = *i*Pro, R'' = Me, R''' = CH_2

31c (43%): R = H, R' = *i*Pro, R'' = cyhx, R''' = H

31d (26%): R = H, R' = *i*Pro, R'' = Me, R''' = H

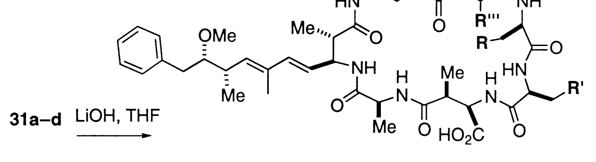
Scheme 13. Macrocyclizations.

using Zn in either acetic acid[13] or an aqueous buffer[53] resulted in decomposition, while using the milder reducing agent, cadmium,[14] gave no reaction. Using Zn–Cu couple[54] in acetic acid resulted in removal of the Troc group, but the product precipitated out with the metal salts as an intractable mixture.[55] This obstacle was overcome by the use of a more acidic medium, thereby maintaining the carboxylic acids in a protonated state and preventing their complexation with zinc.[56] Thus, treatment of the Troc-protected diacid **32c** with Zn–Cu couple in MeOH/0.1 M pH 3.0 phosphate buffer gave the final analogue **2b** in excellent yield (Scheme 14).

Inhibition assays

The activity of the analogues as PP1 and PP2A inhibitors was determined as IC$_{50}$ values using purified PP1 and PP2A catalytic subunits via the established radiolabeled phosphorylase-*a* assay.[57] Synthetic microcystin LA, **1**, was tested first and found to be comparable in activity to the values reported for the natural product,[58] giving sub-nanomolar IC$_{50}$ values and showing no selectivity as an inhibitor of PP1 and PP2A (Table 1). The derivative lacking only the methylidene group of the parent compound (i.e. the *N*-methylglycine (sarcosine) variant **32b**) was only slightly less potent an inhibitor, but slightly selective for PP2A. However, as predicted by the model, replacing the *N*-methyl substituent with the larger *N*-cylcohexyl group (**32a**) resulted in a relative fourfold increase in selectivity in favor of PP1, from 1:2 to 2:1. A somewhat higher selectivity of 3:1 was found for the aminoalanine analogue **2b**, again, consistent with predictions (although the IC$_{50}$ values are an order of magnitude or more higher). Finally, the cyclohexylalanyl derivative **2a** has an IC$_{50}$ less than twofold higher for PP1, but more than tenfold higher for PP2A, resulting in a selectivity of 7:1 in favor of PP1, again, as predicted by the modeling.

While these selectivities are admitedly modest, they are all consistent with predictions and provide some reassurance that the models upon which the analogue design is based have some validity. It is also worth noting that at 7:1 the cyclohexylalanine derivative **2a** is more PP1-selective than any previously known small molecule inhibitor (tautomycin previously held the record at 5:1).[1a] With this encouragement, the design and synthesis of second generation inhibitors are in progress.



2a (41%): R = H, R' = cyhx, R'' = Me, R''' = CH$_2$

32a (40%): R = H, R' = *i*Pro, R'' = cyhx, R''' = H

32b (44%): R = H, R' = *i*Pro, R'' = Me, R''' = H

Zn/Cu pH 3.0

32c (31%): R = NHTroc, R' = *i*Pro, R'' = Me, R''' = CH$_2$

2b (82%): R = NH$_3$+, R' = *i*Pro, R'' = Me, R''' = CH$_2$

Scheme 14. Completion of the microcystin LA analogues.

Table 1. Comparison of the IC$_{50}$ values of synthetic microcystin LA[12] and the microcystin analogues towards the purified catalytic subunits of PP1 and PP2A

Inhibitor	IC$_{50}$(nm) PP1-C	IC$_{50}$(nm) PP2A-C	Selectivity PP1:PP2A
1 (synthetic)[12]	0.3	0.3	1
32b (*N*-meGly)	1.1	0.58	1:2
32a (*N*-cyhxGly)	0.8	1.5	2:1
2b (aminoala)	3	9	3:1
2a (cyhxAla)	0.52	3.4	7:1

Experimental

General

^{1}H and ^{13}C NMR spectra were obtained on an Omega 500 (500 MHz), a General Electric GN-500 (500 MHz), a Bruker 500 (500 MHz), or a Bruker 400 (400 MHz) spectrometer. For spectra measured in organic solvents, data are reported in ppm from internal tetramethylsilane for ^{1}H NMR and in ppm from the solvent for ^{13}C NMR. For ^{1}H spectra taken in D$_2$O, data are reported in ppm relative to HDO (4.80 ppm) unless otherwise stated. Data are reported as follows: chemical shift, multiplicity (app = apparent, par obsc = partially obscured, ovrlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, abq = ab quartet), coupling constant, and integration. Infrared (IR) spectra were taken with a Perkin–Elmer model 1600 series FTIR spectrophotometer. Melting points (mp) were obtained from a Laboratory Devices Mel-Temp melting-point apparatus and are reported uncorrected. Thin-layer chromatography (TLC) was performed on 0.25 mm Merck precoated silica gel plates (60 F-254), and silica gel chromatography was performed using ICN 200–400 mesh silica gel. Reversed phase preparative HPLC was conducted with a 25×100 mm Waters prep Nova-Pak HR C$_{18}$ cartridge (WAT038510). Reversed-phase analytical HPLC was conducted with a 4.6×250 mm Rainin Microsorb-MV C$_{18}$ column (86-200-C5). Solution pH measurements were made with Whatman type CF pH 0–14 indicator paper strips. Inert atmosphere operations were conducted under nitrogen passed through a Drierite drying tube in oven or flame-dried glassware. Anhydrous tetrahydrofuran (THF) was distilled first from calcium hydride, and then from potassium; anhydrous ether was distilled from potassium. Triethylamine and methylene chloride were purified by distillation from calcium hydride. Diisopropylethylamine (DIEA) and DMF were dried over sieves. HATU was purchased from PerSeptive Biosystems Inc. D-Boc-Glu(OH)-OMe was prepared by a one-pot procedure[12] not described in the text, full experimental details will be published elsewhere. All other reagents were used as purchased from Aldrich, Sigma-Aldrich, or Acros unless otherwise stated.

Chemistry

α-Dimethylphosphonylsarcosine (10). Compound **7** (3.67 g, 11.07 mmol) was hydrogenated in 80 mL of methanol over 370 mg of 10% palladium on carbon for 8.5 h at 1 atm, then filtered, and concentrated in vacuo to give 2.07 g (95%) of a white solid: mp 107–109 °C (dec); IR (KBr pellet) 3441 br, 2964, 1654, 1245, 1041 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 2.86 (s, 3H), 3.92 (d, *J* = 11 Hz, 3H), 3.93 (d, *J* = 11.5 Hz, 3H), 4.30 (d, *J* = 19.5 Hz, 1H); ^{13}C NMR (125 MHz, D_2O with sodium 3-trimethylsilylproprionate-2,2,3,3-d_4) δ 36.5 (d, *J* = 7.4 Hz, *N*-Me), 57.7 (ovrlp d, *J* = 7.5, 7.6 Hz, OMe groups), 62.1 (d, *J* = 141.9 Hz, α-carbon), 168.9; HRMS *m/e* calcd for $C_5H_{13}NO_5P+$ $(M+H)^+$: 198.0532. Found: 198.0530.

Boc-D-*iso*-Glu(OMe)-α-dimethylphosphonylsarcosine (9). To an oven-dried flask containing 556 mg (2.13 mmol) of Boc-D-Glu-OMe-OH and 319 mg (2.34 mmol) of HOAt was added 6 mL of CH_2Cl_2, followed by 1 mL of DMF. The solution was cooled to 0 °C and 461 mg (2.24 mmol) of DCC was added. The mixture was stirred for 2 h, warmed to rt, and stirred for an additional 9 h. The mixture was then filtered through Celite into a second oven-dried flask, and the filtrate was concentrated in vacuo to remove the CH_2Cl_2. To the resulting solution was added 400 mg (2.03 mmol) of amino acid **12**, followed by 9 mL DMF. The solution was cooled to 0 °C, and 431 mg (4.26 mmol) of triethylamine was added over 5 min. The yellow solution was stirred for 2.5 h, warmed to rt, stirred for an additional 13 h, and stored at −16 °C overnight. The solution was partitioned between ether and water, and the phases were separated. The aqueous phase was washed once with ether, acidified to pH 1 with 1 M $NaHSO_4$, saturated with solid NaCl, and extracted three times with EtOAc. The combined EtOAc phases were washed twice with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography ($CHCl_3$ to elute less polar material, then 1/5/94: HOAc/IPA/$CHCl_3$ to elute HOAt, then 1/25/74: HOAc/IPA/$CHCl_3$ to elute product, 200 mL silica gel) gave 623 mg (70%) of the diastereomeric phosphonates as a yellow solid: R_f 0.14 (1/10/89: HOAc/IPA/$CHCl_3$); IR (thin film) 3368 br, 2952, 1736, 1708, 1643, 1171, 1045 cm^{-1}; FABMS *m/e* calcd for $C_{16}H_{30}N_2O_{10}P+$ $(M+H)^+$: 441.1639. Found: 441.1636. Attempts to separate the diastereomers for analytical purposes by chromatography and crystallization (EtOAc/hexanes) failed to produce any pure samples. However, ^{1}H NMR analysis of the first fraction collected containing product from the column showed mostly a single diastereomer, thus spectral data is given for the higher R_f material: ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 1.38 (s, 9H), 1.85 (m, 1H), 1.96 (m, 1H), 2.45 (par obsc m, 2H), 3.08 (s, 3H), 3.63 (s, 3H), 3.66 (d, *J* = 11.0 Hz, 3H), 3.72 (d, *J* = 11.0 Hz, 3H), 4.04 (m, 1H), 5.71 (d, *J* = 25.5 Hz, 1H), 6.85 (br, 1H).

3-Cyclohexyl-L-alanine *tert*-butylester, *p*-toluene sulfonate (11). To a flask containing 70 mL *t*BuOAc was added 1.11 g (5.84 mmol) of L-cyclohexylalanine hydrate and 0.65 mL (6.4 mmol) of 70% aqueous perchloric acid. The mixture clarified within 45 min, and was stirred for 96 h. The solution was then cooled to 0 °C, and extracted twice with water. The organic phase was diluted with hexanes, and extracted twice more with water. The combined aqueous phases were basified to pH > 10 by adding solid K_2CO_3, and were then extracted twice with ether. The combined ether phases were dried over anhydrous K_2CO_3, and filtered through cotton into a solution of 1.12 g (5.90 mmol) *p*-toluenesulfonic acid in ether. The precipitate was isolated by filtration to give 1.55 g (64%) of **13** as a white solid: mp 135–136.5 °C (dec with gas evolution, re-solidified to a solid with mp 184–185 °C); IR (KBr pellet) 3448, 2925, 1747, 1125 cm^{-1}; ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.98 (m, 2H), 1.18–1.36 (m, 3H), 1.46 (par obsc m, 1H), 1.52 (s, 9H), 1.57–1.83 (m, 7H), 2.37 (s, 3H), 3.91 (app t, *J* = 7.2 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 2H), 7.71 (d, *J* = 8.0 Hz, 2H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 21.3, 27.0, 27.1, 27.3, 28.1, 33.9, 35.0, 39.1, 52.3, 85.0, 127.0, 129.8, 141.7, 143.6, 170.2; FABMS *m/e* calcd for C_{13} $H_{26}NO_2$ $+(M-TsO^-)+$: 228.1964. Found: 228.1972.

Boc-D-Ala-3-cyclohexyl-L-Ala-O*t*Bu (12a). To an oven dried flask was added 105 mg (0.55 mmol) of Boc-D-alanine-OH, 255 mg (0.61 mmol) of **11**, and 83 mg (0.61 mmol) of HOAt. The mixture was dissolved in 2.7 mL of DMF, and the solution was cooled to 0 °C in an ice bath. 138 mg (1.10 mmol) of 2,4,6-collidine was added, followed by 138 mg (0.72 mmol) of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI). The resulting yellow solution was stirred at 0 °C for 2 h, warmed to rt, and stirred for an additional 11 h. The solution was then partitioned between water and ether, and the phases were separated. The aqueous phase was extracted twice with ether. The combined organic phases were washed once each with water, 1 M $NaHSO_4$, water, and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated to an oil. Chromatography (20 EtOAc/80 hexanes, 10 mL silica gel) gave 198 mg (89%) of the dipeptide as a white foam: R_f 0.59 (30 EtOAc/70 hexanes); IR (thin film) 3310 br, 2918, 1719, 1655, 1164 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 70 °C) δ 0.84 (m, 1H), 0.93 (m, 1H), 1.14 (br, 2H), 1.18 (obsc, 1H), 1.19 (d, *J* = 7.1 Hz, 3H), 1.31 (m, 1H), 1.38 (s, 9H), 1.40 (s, 9H), 1.51 (m, 2H), 1.59 (br, 1H), 1.64 (m, 4H), 3.99 (app pentet, *J* = 7.3 Hz, 1H), 4.18 (ddd, *J* = 5.7, 8.3, 8.8 Hz, 1H), 6.47 (br, 1H), 7.63 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6, 70 °C) δ 18.2, 25.4, 25.5, 25.8, 27.5, 28.0, 31.7, 32.8, 33.6, 38.5, 49.8, 50.3, 78.0, 80.3, 154.6, 171.3, 172.4; HRMS *m/e* calcd for $C_{21}H_{39}N_2O_5^+$ $(M+H)^+$: 399.2861. Found: 399.2860.

Boc-D-*iso*-Glu(OMe)-MeΔAla-D-Ala-3-cyclohexyl-L-Ala-OtBu (13). To 103 mg (0.26 mmol) of **12a** in an oven dried flask was added 0.9 mL of CH_2Cl_2. The solution was cooled to 0 °C, and 0.4 mL of TFA was added. After 2.25 h, the solution was diluted with hexanes and concentrated in vacuo. The residue was re-concentrated twice from hexanes to remove residual TFA. To the resulting oil, **12b**, was added 103 mg (0.23 mmol) of **9**, and 98 mg (0.26 mmol) of HATU. The mixture was suspended in 1.6 mL of CH_2Cl_2, cooled to 0 °C, and 110 mg (0.94 mmol) of collidine was added over 5 min. The yellow mixture was stirred for 2 h at 0 °C, warmed to rt, and stirred for an additional 12 h. The mixture was partitioned between water and ether, and the phases were separated. The aqueous phase was extracted twice with ether. The combined organic phases were washed once with 50% sat. $NaHCO_3$, diluted with 0.25 volumes of hexane, washed twice with 1 M $NaHSO_4$, and once each with water and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo to give a tan foam. The crude tetrapeptide phosphonate was dissolved in 2.83 mL of 70 THF/30 water, and 130 mg (0.94 mmol) of K_2CO_3 was added to the resulting solution. 0.019 mL (0.23 mmol) of 37% aq HCHO was added, and the solution was stirred for 8.5 h. The solution was partitioned between water and ether, and the phases were separated. The aqueous phase was extracted once with ether. The combined organic phases were washed once each with 0.2 M $NaHSO_4$ and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo to give a yellow residue. Chromatography (90 EtOAc/10 hexanes, 14 mL silica gel) gave 88 mg (60%) of the tetrapeptide **13** as a white foam: R_f 0.54 (EtOAc); IR (thin film) 3318, 2931, 1734, 1719, 1660, 1155 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.82–0.96 (m, 2H), 1.15 (m, 3H), 1.29 (d, J = 7.1 Hz, 3H), 1.36 (s, 9H), 1.39 (s, 9H), 1.46–1.59 (m, 4H), 1.64 (m, 4H), 1.81 (m, 1H), 1.95 (m, 1H), 2.31 (m, 2H), 3.00 (obsc s, 3H), 3.61 (s, 3H), 3.98 (ddd, J = 5.6, 8.0, 8.3 Hz, 1H), 4.20 (ddd, J = 5.7, 8.2, 8.9 Hz, 1H), 4.39 (dq, J = 7.2, 7.3 Hz, 1H), 5.51 (s, 1H), 5.96 (s, 1H), 6.72 (br, 1H), 7.69 (d, J = 7.2 Hz, 1H), 7.82 (d, J = 7.4 Hz, 1H); FABMS m/e calcd for $C_{31}H_{53}N_4O_9+$ $(M+H)^+$: 625.3815. Found: 625.3811.

Boc-D-*iso*-Glu(OMe)-MeΔAla-D-Ala-3-cyclohexyl-L-Ala-OH (4a). To 92 mg (0.15 mmol) of the tetrapeptide **13** was added 1 mL TFA. After 50 min, the solution was concentrated in vacuo, and the residue was re-concentrated three times from hexanes to remove residual TFA. The residue was dissolved in 0.6 mL of water, and 78 mg (0.74 mmol) of Na_2CO_3 in 0.3 mL of water was added. The solution was cooled to 0 °C, and 37 mg (0.17 mmol) of di-*tert*-butyldicarbonate in 0.6 mL dioxane was added. The mixture was stirred at 0 °C for 2 h, followed by warming to rt and stirring for an additional 6.5 h. The mixture was partitioned between ether and water, and the phases were separated. The aqueous phase was washed once with ether. The combined ether phases were back-extracted twice with water. The combined aqueous phases were acidified with 1 M NaHSO4, and extracted twice with EtOAc. The combined EtOAc phases were washed once each with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to a residue. Chromatography (1/5/94: HOAc/IPA/$CHCl_3$ to 1/10/89: HOAc/IPA/$CHCl_3$ to elute product, 12 mL silica gel) gave 84 mg (99%) of the desired tetrapeptide **4a** as a white foam: R_f 0.42 (1/10/89: HOAc/IPA/$CHCl_3$); IR (thin film) 3309 br, 2921, 1738, 1717, 1652 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.82–0.96 (m, 2 H), 1.11–1.21 (m, 3H), 1.29 (d, J = 7.1 Hz, 3H), 1.33 (obsc m, 1H), 1.37 (s, 9H), 1.49–1.68 (m, 7H), 1.82 (m, 1H), 1.96 (m, 1H), 2.31 (m, 2H), 3.00 (par obsc s, 3H), 3.61 (s, 3H), 3.98 (ddd, J = 5.6, 8.1, 8.4 Hz, 1H), 4.29 (ddd, J = 5.2, 8.3, 9.2 Hz, 1H), 4.40 (dq, J = 7.1, 7.3 Hz, 1H), 5.51 (s, 1H), 5.97 (s, 1H), 6.69 (br, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H); FABMS m/e calcd for $C_{27}H_{45}N_4O_9+$ $(M+H)^+$: 569.3186. Found: 569.3185.

2-(*R*)-(*tert*-Butoxycarbonyl)-amino-3-azidoproprionic acid (15). To an oven dried flask was added 500 mg (2.67 mmol) of Boc-D-serine lactone (**14**),[22] and 15 mL of DMF. The solution was cooled to 0 °C, and 261 mg (4.01 mmol) of NaN_3 was added. The mixture was stirred for 45 min, warmed to rt, and stirred for two additional h. The solution was partitioned between ether and water, and the phases were separated. The ether phase was back-extracted twice with water. The combined aqueous phases were acidified to pH 3 with sat. citric acid, and were extracted twice with EtOAc. The combined EtOAc phases were washed twice with water, once with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to an oil. Chromatography (1/30/69: HOAc/EtOAc/hexanes, 50 mL silica gel) gave 523 mg (85%) of the azide as a colorless oil: R_f 0.30 (1/59/40: HOAc/EtOAc/hexanes); IR (thin film) 3329 br, 2980, 2106 str, 1714 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 70 °C) δ 1.39 (s, 9H), 3.57 (m, 2H), 4.13 (ddd, J = 6.3, 6.3, 7.4 Hz, 1H), 6.89 (br, 1H); ^{13}C NMR (125 MHz, DMSO-d_6, 70 °C) δ 27.8, 50.8, 53.3, 78.2, 154.8, 170.6; HRMS m/e calcd for $C_8H_{18}N_5O_4+$ $(M+NH_4)^+$: 248.1359. Found: 248.1356.

***N*^{2}-*tert*-Butoxycarbonyl-(*R*)-2,3-diaminoproprionic acid (16).** Compound **15** (385 mg, 1.67 mmol) was hydrogenated in 15 mL of methanol and 1 mL HOAc over 89 mg of 10% palladium on carbon for 45 min at 1 atm. The mixture was filtered, and the filtrate was concentrated in vacuo to an oil. The oil was re-concentrated twice from toluene, to remove residual methanol, to give 342 mg (99%) of a white solid that would typically be used without further purification. To obtain analytically pure material, the solids were recrystallized from MeOH to give 245 mg (72%) of the amino acid as a white solid obtained in three crops: mp 196–198 °C (dec, Lit. 198–200 °C dec)[21f]; IR (KBr pellet) 3342, 2968 br, 1688 cm^{-1}; ^{1}H NMR (500 MHz, MeOH-d_4) δ 1.37 (s, 9H), 3.03 (dd, J = 6.2, 12.4 Hz, 1H), 3.08 (dd, J = 6.8, 12.4 Hz, 1H), 3.96 (m, 1H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 28.7, 43.1, 53.9, 80.9, 158.0, 174.8; HRMS m/e calcd for $C_8H_{17}N_2O_4{}^+$ $(M+H)^+$: 205.1189. Found: 205.1186.

***N*^{2}-*tert*-Butoxycarbonyl-*N*^{3}-2,2,2-trichloroethoxycarbonyl-(*R*)-diaminoproprionic acid (17).** To a flask was added 230 mg (1.13 mmol) of **16** and 4 mL of water. 358 mg (3.38 mmol) of Na_2CO_3 was added, and the resuting solution was cooled to 0 °C. To this 360 mg (1.24 mmol) of succinimidyl 2,2,2-trichloroethycarbonate (Troc-OSuc, available from Aldrich) in 3 mL dioxane was added. After 2 h, the mixture was warmed to rt, and was stirred for an additional 12 h. The solution was partitioned between ether and water, and the phases were separated. The aqueous phase was washed once with ether. The combined ether phases were back-extracted twice with water, and were discarded. The combined aqueous phases were acidified to pH 3 with sat. citric acid, and were extracted twice with ether. The combined ether phases were washed twice with water, once with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give 413 mg (96%) of the di-protected amino alanine derivative as a white solid that was used without further purification: mp 134.5–136 °C (dec); R_f 0.20 (1/45/54: HOAc/EtOAc/hexanes); IR (thin film) 3330 br, 2974, 1715, 1688 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 70 °C) δ 1.38 (s, 9H), 3.37 (m, 2H), 4.08 (m, 1H), 4.77 (s, 2H), 6.67 (br, 1H), 7.50 (br, 1H); ^{13}C NMR (125 MHz, DMSO-d_6, 60 °C) δ 27.8, 41.6, 53.2, 73.4, 78.1, 96.0, 154.3, 155.0, 171.6; HRMS *m/e* calcd for $C_{11}H_{18}Cl_3N_2O_6+$ $(M+H)^+$: 379.0232 (Cl = 34.9689). Found: 379.0226.

***N*²-Boc-*N*³-Troc-(*R*)-Aminoala-L-Leu-O*t*Bu (18).** The title compound was prepared following the same procedure as that used to prepare **12a**. The materials used were 150 mg (0.40 mmol) of **17**, 97 mg (0.44 mmol) of the HCl salt of L-Leu-O-*t*Bu, and the amounts of the other reagents and solvents were adjusted accordingly. Chromatography (20 EtOAc/80 hexanes, 10 mL silica gel) of the residue gave 168 mg (77%) of the dipeptide as a white solid: R_f 0.37 (25 EtOAc/75 hexanes); IR (thin film) 3328, 3190, 2963, 1734, 1719, 1688, 1654 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 70 °C) δ 0.83 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.38 (s, 9H), 1.39 (s, 9H), 1.50 (m, 2H), 1.63 (m, 1H), 3.31 (m, 2H), 4.13 (m, 2H), 4.74 (d, J = 12.3 Hz, 1H), 4.77 (d, J = 12.3 Hz, 1H), 6.50 (br, 1H), 7.41 (br, 1H), 7.81 (d, J = 7.5 Hz, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 21.3, 22.8, 24.1, 27.6, 27.9, 28.1, 42.5, 51.1, 54.3, 73.4, 78.3, 80.4, 96.1, 154.6, 155.0, 169.9, 171.5; FABMS *m/e* calcd for $C_{21}H_{37}Cl_3N_3O_7+$ $(M+H)^+$: 548.1697 (Cl = 34.9689). Found: 548.1686.

Boc-D-*iso*-Glu(OMe)-MeΔAla-*N*²-Boc-*N*³-Troc-(*R*)-Aminoala-L-Leu-O*t*Bu (19). An oven-dried flask was charged with 153 mg (0.28 mmol) of **18**, and this was treated with TFA as in the preparation of **13**. The resulting oil was combined with 112 mg (0.25 mmol) of **9**, and 106 mg (0.28 mmol) of HATU. The amounts of solvents and additional reagents were adjusted according to the procedure to prepare **13**. After the prescribed time, the mixture was partitioned between ether and water, and the phases were separated. The aqueous phase was extracted twice with ether. The combined organic phases were washed once each with 50% sat. $NaHCO_3$, 1 M $NaHSO_4$, water, and brine. The organic phase was then dried over $MgSO_4$, filtered and concentrated in vacuo to give a yellow foam.

The olefination was carried out according to the procedure to prepare **13**, with the exception that only 0.021 mL (0.25 mmol) of 37% aq fomaldehyde was used. Chromatography (65 EtOAc/35 hexanes, 16 mL silica gel) of the residue gave 119 mg (60%) of the desired tetrapeptide as a white foam: R_f 0.55 (75 EtOAc/25 hexanes); IR (thin film) 3311, 2955, 1725, 1657 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.84 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.37 (s, 9H), 1.40 (s, 9H), 1.52 (app t, J = 7.2 Hz, 2H), 1.64 (m, 1H), 1.83 (m, 1H), 1.97 (m, 1H), 2.34 (m, 2H), 3.02 (s, 3H), 3.45 (app t, J = 6.1 Hz, 2H), 3.62 (s, 3H), 3.98 (ddd, J = 5.6, 8.1, 8.1 Hz, 1H), 4.17 (ddd, J = 7.4, 7.4, 7.5 Hz, 1H), 4.52 (ddd, J = 6.4, 6.4, 8.0 Hz, 1H), 4.74 (s, 2H), 5.51 (s, 1H), 5.95 (s, 1H), 6.66 (br, 1H), 7.28 (br, 1H), 7.74 (d, J = 7.4 Hz, 1H), 8.0 (d, J = 8.0 Hz, 1H); FABMS *m/e* calcd for $C_{31}H_{51}Cl_3N_5O_{11}+$ $(M+H)^+$: 774.2654 (Cl = 34.9689). Found: 774.2647.

Boc-D-*iso*-Glu(OMe)-MeΔAla-*N*²-Boc-*N*³-Troc-(*R*)-Aminoala-L-Leu-OH (4b). The title compound was prepared following the same procedure as that used to prepare **4a**. 104 mg (0.13 mmol) of **19** was used, and all other reagents and solvents were used in the appropriate amounts. The residue was filtered through silica (1/5/94: HOAc/IPA/$CHCl_3$ to 1/10/89: HOAc/IPA/$CHCl_3$ to elute product, 16 mL silica gel) to give 89 mg (93%) of the desired acid as a white solid: R_f 0.47 (1 HOAc/10 IPA/89 $CHCl_3$); IR (thin film) 3318 br, 2953, 1736, 1716, 1655 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.85 (d, J = 6.5 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.37 (s, 9H), 1.55 (m, 2H), 1.64 (m, 1H), 1.82 (m, 1H), 1.97 (m, 1H), 2.33 (m, 2H), 3.01 (s, 3H), 3.45 (m, 2H), 3.61 (s, 3H), 3.99 (ddd, J = 5.5, 8.1, 8.2 Hz, 1H), 4.26 (ddd, J = 6.6, 7.9, 7.9 Hz, 1H), 4.54 (ddd, J = 6.2, 6.2, 8.2 Hz, 1H), 4.74 (s, 2H), 5.52 (s, 1H), 5.96 (s, 1H), 6.67 (br, 1H), 7.27 (br, 1H), 7.74 (d, J = 7.9 Hz, 1H), 7.88 (d, J = 8.3 Hz, 1H); FABMS *m/e* calcd for $C_{27}H_{43}Cl_3N_5O_{11}+$ $(M+H)^+$: 718.2038 (Cl = 34.9689). Found: 718.2045.

N-(cyclohexyl)-Glycine Ethylester, Hydrochloride

Glycine ethyl ester hydrochloride (2.00 g, 14.33 mmol) and sodium cyanoborohydride (0.90 g, 14.33 mmol) were combined in a flask, followed by the addition of 30 ml of methanol. Cyclohexanone was filtered through basic alumina, and 1.41 g (14.33 mmol) of this was added to the methanolic slurry. The mixture was stirred for 12 h. At this point, 1.5 mL of conc. HCl was added, and after stirring for an additional 3 h, the mixture was concentrated to a white solid in vacuo. The solids were dissolved in the minimum volume of water, this solution was basified to pH > 10 by adding solid K_2CO_3, and was then extracted twice with ether. The combined organic phases were washed once with 10% aq K_2CO_3, once with brine, dried over K_2CO_3, filtered, and concentrated in vacuo to an oil. The resulting oil was dissolved in 150 mL absolute ethanol, 1.5 mL conc. HCl was added, and the solution was concentrated in vacuo to approximately 10 mL. The product was precipitated by the addition of ether, and was isolated by filtration to give 1.39 g (44%) of the amine HCl salt as a white solid: mp 178.5–180 °C; IR (KBr pellet) 2937, 2722, 1747, 1234 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 1.23 (m, 1H), 1.33 (t, J = 7.2 Hz, 3H), 1.32–1.46 (par obsc m, 4H), 1.71 (m, 1H), 1.89 (m, 2H), 2.11 (m, 2H), 3.23 (dddd, J = 3.7, 3.8, 11.2, 11.3 Hz, 1H), 4.05 (s, 2H), 4.34 (q, J = 7.2 Hz, 2H); ^{13}C NMR (125 MHz, D_2O with sodium 2-trimethylsilylproprionate-2,2,3,3-d_4) δ 16.0, 26.8, 27.3, 31.7, 47.5, 60.4, 66.2, 170.7; HRMS *m/e* calcd for $C_{10}H_{19}NO_2$+ (M−HCl)$^+$: 185.1417. Found: 185.1414.

Cbz-*N*-(cyclohexyl)-Glycine Ethylester

To an oven dried flask containing 1.50 g (6.76 mmol) of the HCl salt of *N*-(cyhx)-Gly-OEt was added 40 mL of CH_2Cl_2, and the flask was fitted with a reflux condensor. 1.37 g (13.56 mmol) of triethylamine was added, the mixture was heated to reflux, and 1.73 g (10.14 mmol) of benzylchloroformate was added to the warm mixture. After 7 h, approximately 10 mg (0.08 mmol) of DMAP was added. 1 h later, significant conversion was noted by TLC, and 100 mg (0.81 mmol) of DMAP and an additional 1.67 g (9.80 mmol) of benzylchloro-formate were added. After an additional 2 h, the reaction was allowed to cool to rt and stirred overnight. The mixture was extracted once with 1 M HCl, and the aqueous phase was back-extracted once with CH_2Cl_2. The combined organic phases were washed once each with water and brine, and filtered through cotton. The filtrate was concentrated in vacuo to an oil. Chromatography (10 EtOAc/90 hexanes, 170 mL silica gel) gave 2.10 g (98%) of the title compound as a clear oil: R_f 0.32 (10 EtOAc/90 hexanes); IR (thin film) 2930, 1748, 1699 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 130 °C) δ 1.09 (m, 1H), 1.15 (t, J = 7.1 Hz, 3H), 1.26 (m, 2H), 1.38 (m, 2H), 1.56 (app d, J = 12.7 Hz, 1H), 1.72 (m, 4H), 3.80 (m, 1H), 3.91 (s, 2H), 4.06 (q, J = 7.1 Hz, 2H), 5.07 (s, 2H), 7.26–7.32 (m, 5H); ^{13}C NMR (125 MHz, DMSO-d_6, 130 °C) δ 13.1, 24.2, 24.8, 29.6, 44.4, 55.5, 59.5, 65.7, 126.5, 126.8, 127.4, 136.3, 154.4, 169.1; HRMS *m/e* calcd for $C_{18}H_{25}NO_4$+ (M)$^+$: 319.1785. Found: 319.1780.

Cbz-*N*-(cyclohexyl)-Glycine-OH (21). To 1.00 g (3.13 mmol) of Cbz-*N*-cyhxGly-OEt in 15 mL of THF was added 9.4 mL (9.4 mmol) of 1.0 M LiOH. After 26 h, the solution was partitioned between ether and water, and the phases were separated. The aqueous phase was acidified to pH 1 with 1 M $NaHSO_4$, and was extracted once with ether. The ether phase was washed once each with water and brine, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to give 800 mg (88%) of the title compound as a colorless oil that solidified over time to a white solid. Analytically pure material was obtained by recrystallization from EtOAc/hexanes: mp 110.5–112.5 °C; R_f 0.63 (1/49/50: HOAc/EtOAc/hexanes); IR (thin film) 3077 br, 2929, 1703 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 130 °C) δ 1.08 (ddddd, J = 3.4, 3.4, 12.8, 12.8, 12.8 Hz, 1H), 1.26 (m, 2H), 1.39 (m, 2H), 1.57 (app d, J = 13.1 Hz, 1H), 1.72 (m, 4H), 3.77 (dddd, J = 3.4, 3.4, 11.9, 11.9 Hz, 1H), 3.85 (s, 2H), 5.07 (s, 2H), 7.24–7.34 (m, 5H); ^{13}C NMR (125 MHz, DMSO-d_6, 130 °C) δ 24.3, 24.8, 29.7, 44.2, 55.5, 65.7, 126.5, 126.8, 127.5, 136.5, 154.6, 170.3; HRMS *m/e* calcd for $C_{16}H_{22}NO_4$+ (M + H)$^+$: 292.1549. Found: 292.1552.

Cbz-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-O*t*Bu (22). Cbz-D-Ala-L-Leu-O*t*Bu (121 mg, 0.31 mmol) was hydrogenated in 1 mL of methanol over 24 mg of 10% palladium on carbon for 3 h at 1 atm, filtered, and concentrated in vacuo to an oil. The oil was re-concentrated twice from hexanes to remove residual methanol. 82 mg (0.28 mM) of **21** in 1 mL CH_2Cl_2 was added, and the solution was concentrated in vacuo to give a white foam. To this were added 42 mg (0.31 mmol) of HOBt and 1.2 mL DMF, the resulting solution was cooled to 0 °C, and 69 mg (0.36 mmol) of EDCI was added. The mixture was stirred for 2 h, followed by warming to rt and stirring for an additional 11 h. The solution was partitioned between water and ether, and the phases were separated. The aqueous phase was extracted twice with ether. The combined organic phases were washed once each with 50% sat. $NaHCO_3$, water, 1 M $NaHSO_4$, water, and brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated under vacuum to give 135 mg (91%) of the desired tripeptide as a white foam: R_f 0.42 (50 EtOAc/50 hexanes); IR (thin film) 3297, 3071, 2930, 1731, 1694, 1651 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.84 (d, J = 6.6 Hz, 3H), 0.89 (d, J = 6.6 Hz, 3H), 1.06 (m, 1H), 1.19 (d, J = 7.0 Hz, 3H), 1.23–1.36 (m, 4H), 1.39 (s, 9H), 1.47-1.58 (m, 3H), 1.63 (m, 1H), 1.74 (m, 4H), 3.78 (par obsc m, 1H), 3.79 (d, J = 16.9 Hz, 1H), 3.84 (d, J = 17.0 Hz, 1H), 4.15 (ddd, J = 6.5, 8.0, 8.0 Hz, 1H), 4.33 (dq, J = 7.1, 7.1 Hz, 1H), 5.06 (s, 2H), 7.25–7.36 (m, 5H), 7.56 (d, J = 7.5 Hz, 1H), 7.71 (d, J = 7.4 Hz, 1H); HRMS *m/e* calcd for $C_{29}H_{46}N_3O_6$+ (M + H)$^+$: 532.3389. Found: 532.3404.

Boc-D-*iso*-Glu(OMe)-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-O*t*Bu (23). Compound **22** (115 mg, 0.22 mmol) was hydrogenated in 1 mL of methanol over 21 mg of 10% palladium on carbon for 4.2 h at 1 atm, then filtered, and concentrated. The residue was re-concentrated twice from hexanes to remove residual methanol, and the remaining oil was dissolved in 1 mL DMF. Boc-D-Glu-OMe (65 mg, 0.25 mmol) and HATU (99 mg, 0.25 mmol) were added, and after cooling to 0 °C, 79 mg (0.65 mmol) of collidine was added. The yellow solution was stirred for 2 h, followed by warming to rt and stirring for an additional 10 h. The reaction was worked up as in the procedure to prepare **22**, to give a colorless residue. Chromatography (50 EtOAc/50 hexanes to 70 EtOAc/30 hexanes to elute product, 21 mL silica gel) gave 104 mg (75%) of the tetrapeptide as a white foam: R_f 0.13 (50 EtOAc/50 hexanes); IR (thin film) 3311, 2935, 1730, 1715, 1652 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 110 °C) δ 0.85 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 6.5 Hz, 3H), 1.08 (m, 1H), 1.23 (d, J = 7.0 Hz, 3H), 1.31 (par obsc m, 4H), 1.38 (s, 9H), 1.40 (s, 9H), 1.52–1.74 (m, 8H), 1.86 (m, 1H), 1.98 (m, 1H), 2.35 (br, 2H), 3.62 (s, 3H), 3.84 (m, 2H), 4.02 (m, 1H), 4.16 (app q, J = 6.7 Hz, 1H), 4.32 (m, 1H), 6.59 (br, 1H), 7.55 (br, 2H); FABMS *m/e* calcd for $C_{32}H_{57}N_4O_9$+ (M + H)$^+$: 641.4128. Found: 641.4126.

Boc-D-*iso*-Glu(OMe)-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-OH (24). The title compound was prepared following the same procedure as that used to prepare **4a**, with only two minor exceptions: the TFA treatment was for 1.5 h, and ether was used instead of EtOAc in the workup. 90 mg (0.14 mmol) of **23** was used, and the amounts of the solvents and additional reagents were adjusted accordingly. Filtration of the residue through silica (1 HOAc/5 IPA/94 $CHCl_3$) gave 71 mg (87%) of the desired acid as a white solid: R_f 0.50 (1 HOAc/10 IPA/89 $CHCl_3$); IR (thin film) 3300 br, 2935, 1720, 1694, 1652 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 130 °C) δ 0.86 (d, *J* = 6.4 Hz, 3H), 0.89 (d, *J* = 6.4 Hz, 3H), 1.08 (m, 1H), 1.23 (d, *J* = 7.0 Hz, 3H), 1.38 (obsc m, 4H), 1.38 (s, 9H), 1.55–1.74 (m, 8H), 1.87 (m, 1H), 2.00 (m, 1H), 2.37 (m, 2H), 3.63 (s, 3H), 3.85 (m, 2H), 4.04 (br, 1H), 4.27–4.33 (m, 2H), 6.45 (br, 1H), 7.46 (br, 2H); FABMS *m/e* calcd for $C_{28}H_{49}N_4O_9+$ $(M+H)^+$: 585.3501. Found: 585.3494.

Cbz-*N*-(Me)-Glycine. 2.00 g (22.4 mmol) of sarcosine and 7.12 g (67.2 mmol) of Na_2CO_3 in a flask were dissolved in 60 mL of water, and the solution was cooled to 0 °C. 4.21 g (24.6 mmol) of benzylchloroformate in 20 mL of dioxane was added over 5 min. The mixture was stirred for 1.5 h, followed by stirring at rt for an additional 11 h. The cloudy mixture was partitioned between ether and water, and the phases were separated. The aqueous phase was acidified with 1M $NaHSO_4$, and was extracted twice with EtOAc. The combined EtOAc phases were washed once with water, once with brine, dried over anhydrous $MgSO_4$, filtered, and the filtrate was concentrated in vacuo to give 4.98 g (>99%) of *Z*-sarcosine as a clear oil: R_f 0.50 (1 HOAc/10 IPA/89 $CHCl_3$); IR (thin film) 3458, 3067 br, 2940, 1698 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 2.92 (s, 3H), 3.94 (s, 2H), 5.08 (s, 2H), 7.28–7.33 (m, 5H); ^{13}C NMR (125 MHz, DMSO-d_6, 90 °C) δ 34.9, 49.9, 66.0, 126.9, 127.3, 127.9, 136.6, 155.5, 170.2; HRMS *m/e* calcd for $C_{11}H_{13}NO_4$ $(M)^+$: 223.0845. Found: 223.0843.

Cbz-*N*-(Me)-Gly-D-Ala-L-Leu-O*t*Bu (25). The title compound was prepared by following the procedure used to prepare **22**, with the exceptions that 128 mg (0.57 mmol) of *Z*-sarcosine was used as the acid component, and the amounts of the required solvents and reagents were adjusted accordingly. Chromatography (60 EtOAc/40 hexanes, 40 mL silica gel) of the resulting oil gave 211 mg (80%) of the desired tripeptide as a colorless oil: R_f 0.20 (50 EtOAc/50 hexanes); IR (thin film) 3315, 2952, 1731, 1710, 1651 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.84 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 1.21 (d, *J* = 7.1 Hz, 3H), 1.39 (s, 9H), 1.51 (m, 2H), 1.62 (m, 1H), 2.88 (s, 3H), 3.86 (d, *J* = 16.6 Hz, 1H), 3.92 (d, *J* = 16.6 Hz, 1H), 4.17 (ddd, *J* = 6.4, 8.1, 8.1 Hz, 1H), 4.36 (dq, *J* = 7.2, 7.2 Hz, 1H), 5.05 (d, *J* = 13.1 Hz, 1H), 5.07 (d, *J* = 13.1 Hz, 1H), 7.26–7.33 (m, 5H), 7.69 (app d, *J* = 6.4 Hz, 2H); HRMS *m/e* calcd for $C_{24}H_{38}N_3O_6+$ $(M+H)^+$: 464.2762. Found: 464.2756.

Boc-D-*iso*-Glu(OMe)-*N*-(Me)-Gly-D-Ala-L-Leu-O*t*Bu (26). The title compound was prepared following the same procedure as that used to prepare **23**. 165 mg (0.36 mmol) of **25** was used, and the amounts of the additional solvents and reagents were adjusted accordingly. Chromatography (EtOAc, 25 mL silica gel) of the residue gave 128 mg (63%) of a white solid: R_f 0.40 (EtOAc); IR (thin film) 3300, 2966, 1725, 1652 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 110 °C) δ 0.85 (d, *J* = 6.7 Hz, 3H), 0.89 (d, *J* = 6.6 Hz, 3H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.38 (s, 9H), 1.40 (s, 9H), 1.53 (m, 2H), 1.64 (m, 1H), 1.85 (m, 1H), 1.96 (m, 1H), 2.35 (br, 2H), 2.88 (obsc s, 3H), 3.63 (s, 3H), 3.91 (d, *J* = 16.4 Hz, 1H), 3.97 (d, *J* = 16.4 Hz, 1H), 4.03 (ddd, *J* = 5.4, 8.1, 8.1 Hz, 1H), 4.17 (ddd, *J* = 6.4, 8.0, 8.0 Hz, 1H), 4.34 (dq, *J* = 6.7, 6.8 Hz, 1H), 6.63 (br, 1H), 7.59 (br, 2H); FABMS *m/e* calcd for $C_{27}H_{49}N_4O_9+$ $(M+H)^+$: 573.3501. Found: 573.3488.

Boc-D-*iso*-Glu(OMe)-*N*-(Me)-Gly-D-Ala-L-Leu-OH (27). The title compound was prepared following the same procedure as that used to prepare **4a**, using 110 mg (0.19 mmol) of **26**, with one exception: the TFA treatment was for 1.5 h. Flash chromatography (1 HOAc/15 IPA/84 $CHCl_3$, 12 mL silica gel) of the residue gave 90 mg (91%) of the desired acid as a white solid: R_f 0.17 (1 HOAc/10 IPA/89 $CHCl_3$); IR (thin film) 3310 br, 2955, 1730, 1662 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 120 °C) δ 0.86 (d, *J* = 6.5 Hz, 3H), 0.89 (d, *J* = 6.5 Hz, 3H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.38 (s, 9H), 1.56 (m, 2H), 1.65 (m, 1H), 1.85 (m, 1H), 1.96 (m, 1H), 2.34 (m, 2H), 3.62 (s, 3H), 3.91 (d, *J* = 16.8 Hz, 1H), 3.97 (d, *J* = 16.5 Hz, 1H), 4.03 (m, 1H), 4.26 (m, 1H), 4.35 (m, 1H), 6.57 (br, 1H), 7.52 (br, 2H); FABMS *m/e* calcd for $C_{23}H_{40}N_4NaO_9+$ $(M+Na)^+$: 539.2695. Found: 539.2694.

D-*erythro*-*N*-(9-Phenylfluoren-9-yl)-*N*-benzyl-β-Me-*iso*-Asp(OMe)-L-Ala-O*t*Bu (5). To 1.00 g (2.03 mmol) of the *N*-BnPhFl-B-methyl aspartate **28** in an oven-dried flask was added 0.701 g (3.86 mmol) of the HCl salt of L-Ala-O*t*Bu, and 852 mg (2.24 mmol) of HATU. The mixture was dissolved in 10 mL of DMF, cooled to 0 °C, and 786 mg (6.10 mmol) of diisopropylethylamine was added over 5 min. The yellow solution was stirred at 0 °C for 3 h, warmed to rt, and stirred for an additional 12 h. The solution was partitioned between ether and 10% aq $NaHCO_3$, and the phases were separated. The aqueous was extracted once with ether. The combined ether phases were washed once each with water, 50% sat citric acid, water, water, and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to give 1.20 g (96%) of a white solid that was pure by NMR analysis: R_f 0.39 (25 EtOAc/75 hexanes); IR (thin film) 3145, 3056, 2971, 1729, 1677, 1153 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.50 (d, *J* = 6.8 Hz, 3H), 1.28 (d, *J* = 7.2 Hz, 3H), 1.64 (s, 9H), 2.12 (dq, *J* = 6.8, 10.9 Hz, 1H), 2.91 (s, 3H), 3.70 (d, *J* = 10.9 Hz, 1H), 4.25 (dq, *J* = 7.2, 7.2 Hz, 1H), 4.38 (d, *J* = 13.3 Hz, 1H), 4.64 (d, *J* = 13.5 Hz, 1H), 4.93 (d, *J* = 6.9 Hz, 1H), 7.17–7.37 (m, 10H), 7.42 (t, *J* = 7.4 Hz, 1H), 7.58 (d, *J* = 6.7 Hz, 1H), 7.60 (d, *J* = 6.9 Hz, 1H), 7.63 (d, *J* = 7.7 Hz, 1H), 7.75 (d, *J* = 7.5 Hz, 1H), 7.88 (par obsc m, 2H), 7.91 (d, *J* = 7.3 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.6, 18.0, 28.2, 41.8, 49.0, 50.5, 50.9, 63.0, 80.6, 81.2, 119.0, 120.0, 126.6, 127.3, 127.3, 127.3, 127.5, 127.6, 127.7, 128.0, 128.2, 128.4, 130.8, 138.8, 142.2, 142.6, 144.6, 144.9, 147.3, 171.2, 172.6, 173.2; FABMS *m/e* calcd for $C_{39}H_{43}N_2O_5+$ $(M+H)^+$: 619.3172. Found: 619.3169.

Boc-D-*iso*-Glu(OMe)-MeΔAla-D-Ala-3-cyclohexyl-L-Ala-D-β-Me-*iso*-Asp(OMe)-L-Ala-O*t*Bu (29a). **5** (154 mg, 0.25 mmol) was hydrogenated in 1.92 mL of MeOH with 28 mg (0.25 mmol) of TFA over 30 mg of 10% palladium on carbon for 13 h at 1 atm. The mixture was filtered through Celite, concentrated in vacuo, and re-concentrated three times from hexanes to remove residual MeOH. To the residue were added 129 mg (0.23 mmol) of **4a**, 95 mg (0.25 mmol) of HATU, and 1.5 mL of DMF. The solution was cooled to 0 °C, and 110 mg (0.91 mmol) of collidine was added over 5 min. The solution was stirred for 2 h, warmed to rt, and stirred for an additional 16 h. The solution was partitioned between ether and water, and the phases were separated. The aqueous phase was back-extracted twice with ether. The combined ether phases were washed once each with 50% sat. $NaHCO_3$, water, 1 M $NaHSO_4$, water, brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to a yellow foam. Chromatography (EtOAc, 32 mL silica gel) gave 133 mg (70%) of the hexapeptide as a white solid: R_f 0.23 (EtOAc); IR (thin film) 3311, 2924, 1736, 1652, 1516 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.83–0.94 (m, 3H), 1.05 (d, J = 7.2 Hz, 3H), 1.13 (m, 3H), 1.22 (d, J = 7.2 Hz, 3H), 1.30 (d, J = 7.1 Hz, 3H), 1.37 (s, 9H), 1.40 (s, 9H), 1.46–1.66 (m, 7H), 1.82 (m, 1H), 1.96 (m, 1H), 2.31 (m, 2H), 2.95 (par obsc m, 1H), 3.00 (par obsc s, 3H), 3.59 (s, 3H), 3.61 (s, 3H), 3.98 (ddd, J = 5.5, 5.5, 8.1 Hz, 1H), 4.10 (dq, J = 7.2, 7.3 Hz, 1H), 4.30 (ddd, J = 5.1, 8.1, 9.7 Hz, 1H), 4.38 (dq, J = 7.2, 7.3 Hz, 1H), 4.48 (dd, J = 5.5, 8.6 Hz, 1H), 5.51 (s, 1H), 5.97 (s, 1H), 6.70 (br, 1H), 7.68 (d, J = 8.5 Hz, 1H), 7.77 (d, J = 7.9 Hz, 1H), 7.80 (d, J = 7.9 Hz, 1H), 7.90 (d, J = 6.9 Hz, 1H); FABMS *m/e* calcd for $C_{40}H_{67}N_6O_{13}+$ $(M+H)^+$: 839.4769. Found: 839.4754.

Boc-D-*iso*-Glu(OMe)-MeΔAla-N^2-Boc-N^3-Troc-(*R*)-Aminoala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-O*t*Bu (29b). The title compound was prepared following the same procedure as that used to prepare **29a**. The materials used were 66 mg (0.11 mmol) of **5**, 71 mg (0.10 mmol) of **4b**, and all other reagents and solvents were used in the appropriate amounts. Chromatography (80 EtOAc/20 hexanes, 25 mL silica gel) of the residue gave 74 mg (76%) of the hexapeptide as a white solid: R_f 0.43 (EtOAc); IR (thin film) 3320, 2955, 1735, 1652 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.83 (d, J = 6.5 Hz, 3H), 0.86 (d, J = 6.5 Hz, 3H), 1.06 (d, J = 7.2 Hz, 3H), 1.23 (d, J = 7.2 Hz, 3H), 1.38 (s, 9H), 1.41 (s, 9H), 1.53 (m, 2H), 1.61 (m 1H), 1.83 (m, 1H), 1.97 (m, 1H), 2.36 (m, 2H), 3.01 (par obsc s, 3H), 3.46 (m, 2H), 3.60 (s, 3H), 3.62 (s, 3H), 3.99 (m, 1H), 4.13 (dq, J = 7.2, 7.2 Hz, 1H), 4.26 (ddd, J = 6.6, 7.9, 7.9 Hz, 1H), 4.50 (dd, J = 5.5, 7.3 Hz, 1H), 4.51 (par obsc m, 1H), 4.75 (s, 2H), 5.52 (s, 1H), 5.96 (s, 1H), 6.71 (br, 1H), 7.36 (br, 1H), 7.74 (d, J = 8.9 Hz, 1H), 7.86 (m, 2H), 7.95 (d, J = 7.4 Hz, 1H); FABMS *m/e* calcd for $C_{40}H_{65}Cl_3N_7O_{15}+$ $(M+H)^+$: 988.3608 (Cl = 34.9689). Found: 988.3610.

Boc-D-*iso*-Glu(OMe)-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-O*t*Bu (29c). The title compound was prepared following the same procedure as that used to prepare **29a**. The amounts of materials used were 73 mg (0.12 mmol) of **5**, 63 mg (0.11 mmol) of **24**, and amounts of the required solvents and additional reagents were adjusted accordingly. Chromatography (95 EtOAc/5 hexanes, 18 mL silica gel) of the residue gave 66 mg (72%) of the hexapeptide as a white solid: R_f 0.39 (EtOAc); IR (thin film) 3308, 2930, 1737, 1651 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 130 °C) δ 0.84 (d, J = 6.4 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H), 1.07 (d, J = 7.2 Hz, 3H), 1.07 (obsc m, 1H), 1.24 (d, J = 7.3 Hz, 3H), 1.25 (d, J = 7.1 Hz, 3H), 1.31 (par obsc m, 4H), 1.38 (s, 9H), 1.41 (s, 9H), 1.49–1.75 (m, 8H), 1.88 (m, 1H), 2.00 (m, 1H), 2.37 (m, 2H), 2.95 (dq, J = 5.5, 7.2 Hz, 1H), 3.60 (s, 3H), 3.63 (s, 3H), 3.83 (d, J = 16.7 Hz, 1H), 3.88 (d, J = 16.8 Hz, 1H), 4.04 (ddd, J = 5.6, 7.9, 7.9 Hz, 1H), 4.14 (dq, J = 7.2, 7.2 Hz, 1H), 4.27 (ddd, J = 5.3, 8.1, 8.7 Hz, 1H), 4.32 (par obsc dq, J = 7.2, 7.2 Hz, 1H), 4.49 (dd, J = 5.5, 8.6 Hz, 1H), 6.41 (br, 1H), 7.37 (br, 1H), 5.54 (m, 2H), 7.64 (d, J = 7.0 Hz, 1H); FABMS *m/e* calcd for $C_{41}H_{71}N_6O_{13}+$ $(M+H)^+$: 855.5082. Found: 855.5091.

Boc-D-*iso*-Glu(OMe)-*N*-(Me)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-O*t*Bu (29d). The title compound was prepared following the same procedure as that used to prepare **29a**. 27 mg (0.04 mmol) of **5** and 20 mg (0.04 mmol) of **27** were used, and the amounts of solvents and additional reagents were adjusted accordingly. Chromatography (10 IPA/90 $CHCl_3$, 6 mL silica gel) of the residue gave 17 mg (57%) of the hexapeptide as a white solid: R_f 0.19 (10 IPA/90 $CHCl_3$); IR (thin film) 3299, 2961, 1740, 1650 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 110 °C) δ 0.83 (d, J = 6.6 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H), 1.05 (d, J = 7.1 Hz, 3H), 1.23 (d, J = 7.0 Hz, 3H), 1.25 (d, J = 7.0 Hz, 3H), 1.38 (s, 9H), 1.41 (s, 9H), 1.48–1.66 (m, 3H), 1.86 (m, 1H), 1.98 (m, 1H), 2.36 (m, 2H), 2.90 (br.s, 3H), 2.94 (par obsc dq, J = 5.5, 7.1 Hz, 1H), 3.60 (s, 3H), 3.63 (s, 3H), 3.90 (d, J = 16.4 Hz, 1H), 3.98 (d, J = 16.3 Hz, 1H), 4.04 (m, 1H), 4.13 (dq, J = 7.1, 7.1 Hz, 1H), 4.26 (m, 1H), 4.34 (m, 1H), 4.49 (dd, J = 5.4, 8.6 Hz, 1H), 6.47 (br, 1H), 7.49 (br, 1H), 7.57 (m, 2H), 7.67 (br, 1H); FABMS *m/e* calcd for $C_{36}H_{63}N_6O_{13}+$ $(M+H)^+$: 787.4456. Found: 787.4447.

Boc-Adda-D-*iso*-Glu(OMe)-MeΔAla-D-Ala-3-cyclohexyl-L-Ala-D-β-Me-*iso*-Asp(OMe)-L-Ala-OH (3a). To 23 mg (0.05 mmol) of Boc-Adda-OH was added 9 mg (0.06 mmol) of HOAt, and the mixture was concentrated once from toluene. The residue was dissolved in 0.75 mL of 20 DMF/80 CH_2Cl_2, and the solution was cooled to 0 °C. 12 mg (0.06 mmol) of DCC was added, and the mixture was stirred at 0 °C for 2 h, followed by warming to rt and stirring for an additional 7 h. In a separate flask, 47 mg (0.06 mmol) of **29a** was treated with 0.5 mL of freshly distilled TFA for 1.5 h, followed by concentration and re-concentration once from toluene and twice from hexanes to remove residual TFA. The resulting foam was dissolved in 0.4 mL of DMF. The active ester solution prepared above was cooled to 0 °C, and the hexapeptide solution was added to this via cannula. To the mixture was added 25 mg (0.21 mmol) of collidine, and the mixture was stirred for 2 h, followed by warming to rt and stirring for an additional 12 h. The mixture was partitioned between EtOAc and water, and the phases were separated. The aqueous phase was extracted once with EtOAc. The combined EtOAc phases were washed once each with 1 M $NaHSO_4$ and water, and these aqueous phases were discarded. The organic phase was diluted with one volume of 50 ether/50 hexanes, and extracted once each with 10% $NaHCO_3$ and water. The combined aqueous phases were washed once with 50 ether/50 hexanes, acidified to pH 1 with 1 M $NaHSO_4$, and extracted three times with ether. The combined ether phases were washed once each with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography (1/5/94: HOAc/IPA/$CHCl_3$, 10 mL silica gel) of the residue gave 33 mg (57%) of the heptapeptide as a white solid: R_f 0.16 (1/5/94: HOAc/IPA/$CHCl_3$); IR (thin film) 3311 br, 2921, 1741, 1717, 1650 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.84–0.94 (m, 3H), 0.97 (d, $J = 6.8$ Hz, 3H), 1.02 (d, $J = 7.0$ Hz, 3H), 1.06 (d, $J = 7.2$ Hz, 3H), 1.15 (m, 3H), 1.26 (d, $J = 7.0$ Hz, 3H), 1.31 (d, $J = 7.1$ Hz, 3H), 1.39 (s, 9H), 1.50 (m, 1H), 1.57 (s, 3H), 1.61–1.65 (m, 6H), 1.85 (m, 1H), 1.98 (m, 1H), 2.27–2.35 (m, 2H), 2.57 (m, 2H), 2.67 (dd, $J = 7.3$, 14.1 Hz, 1H), 2.75 (dd, $J = 4.9$, 14.1 Hz, 1H), 3.18 (par obsc s, 3H), 3.26 (ddd, $J = 5.7$, 6.9, 6.9 Hz, 1H), 3.59 (s, 3H), 3.60 (s, 3H), 4.08 (m, 1H), 4.18–4.26 (m, 2H), 4.30 (m, 1H), 4.40 (dq, $J = 7.0$, 7.0 Hz, 1H), 4.49 (dd, $J = 5.8$, 8.0 Hz, 1H), 5.38 (d, $J = 10.4$ Hz, 1H), 5.46 (par obsc dd, 1H), 5.47 (s, 1H), 5.96 (s, 1H), 6.07 (d, $J = 16.0$ Hz, 1H), 6.21 (d, $J = 7.6$ Hz, 1H), 7.15–7.26 (m, 5H), 7.72 (d, $J = 8.2$ Hz, 1H), 7.77 (d, $J = 7.3$ Hz, 1H), 7.82 (d, $J = 7.1$ Hz, 1H), 7.87 (d, $J = 6.9$ Hz, 1H), 7.90 (d, $J = 6.7$ Hz, 1H); FABMS *m/e* calcd for $C_{56}H_{86}N_7O_{15}+$ $(M+H)^+$: 1096.6. Found: 1096.7. FABMS *m/e* calcd for $C_{56}H_{85}NaN_7O_{15}+$ $(M+Na)^+$: 1118.6. Found: 1118.6.

Boc-Adda-D-*iso*-Glu(OMe)-MeΔAla-N^2-Boc-N^3-Troc-(*R*)-Aminoala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-OH (3b). The Boc-Adda activation was carried out as in procedure to prepare **3a**. The materials used were 22 mg (0.05 mmol) of Boc-Adda-OH, 8 mg (0.06 mmol) of HOAt, 12 mg (0.06 mmol) of DCC, and 0.6 mL of CH_2Cl_2 as the solvent. In a separate flask, 50 mg (0.05 mmol) of **29b** was treated with 0.5 mL of freshly distilled TFA for 1.5 h, followed by concentration and re-concentration three times from hexanes to remove residual TFA. The resulting foam was dissolved in 0.5 mL of CH_2Cl_2. The active ester solution prepared above was cooled to 0 °C, and the hexapeptide solution was added to this via cannula. To the mixture was added 25 mg (0.213 mmol) of collidine. The mixture was stirred for 4 h, and 0.5 mL of DMF was added to dissolve a precipitate that hindered stirring. After this period, the mixture was warmed to rt, and was stirred for an additional 12 h. The mixture was partitioned between EtOAc and water, and the phases were separated. The aqueous phase was extracted once with EtOAc. The combined EtOAc phases were washed once each 50% sat. citric acid, water, and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography (1/5/94: HOAc/IPA/$CHCl_3$, 12 mL silica gel) of the residue gave 37 mg (58%) of the heptapeptide as a white solid: R_f 0.37 (1/5/94: HOAc/IPA/$CHCl_3$); IR (thin film) 3311 br, 2945, 1741, 1647 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.82 (d, $J = 6.5$ Hz, 3H), 0.85 (d, $J = 6.6$ Hz, 3H), 0.96 (d, $J = 6.8$ Hz, 3H), 1.02 (d, $J = 7.0$ Hz, 3H), 1.05 (d, $J = 7.1$ Hz, 3H), 1.25 (d, $J = 7.2$ Hz, 3H), 1.37 (s, 9H), 1.52 (m, 2H), 1.56 (d, $J = 1.1$ Hz, 3H), 1.61 (par obsc m, 1H), 1.85 (m, 1H), 1.98 (m, 1H), 2.33 (m, 2H), 2.57 (m, 3H), 2.66 (dd, $J = 7.1$, 14.0 Hz, 1H), 2.73 (dd, $J = 4.9$, 14.0 Hz, 1H), 2.99 (par obsc s, 3H), 3.17 (s, 3H), 3.24 (ddd, $J = 5.2$, 5.4, 7.2 Hz, 1H), 3.46 (m, 2H), 3.59 (s, 3H), 3.59 (s, 3H), 4.07 (m, 1H), 4.19–4.29 (m, 3H), 4.46–4.55 (m, 2H), 4.74 (s, 2H), 5.37 (d, $J = 10.0$ Hz, 1H), 5.45 (obsc, 1H), 5.45 (dd, $J = 6.4$, 15.6 Hz, 1H), 5.93 (br. s, 1H), 6.07 (d, $J = 15.6$ Hz, 1H), 6.20 (d, $J = 7.5$ Hz, 1H), 7.13–7.26 (m, 5H), 7.31 (br, 1H), 7.75 (d, $J = 8.6$ Hz, 1H), 7.86 (m, 3H), 7.93 (d, $J = 7.4$ Hz, 1H); FABMS *m/e* calcd for $C_{56}H_{84}Cl_3N_8O_{17}+$ $(M+H)^+ = 1245$ (Cl = 34.9689). Found: 1245. FABMS *m/e* calcd for $C_{56}H_{83}Cl_3NaN_8O_{17}+$ $(M+Na)^+$: 1267 (Cl = 34.9689). Found: 1267.

Boc-Adda-D-*iso*-Glu(OMe)-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-OH (30a). The Boc-Adda activation was carried out as in the procedure to prepare **3a**. The materials used were 20 mg (0.05 mmol) of Boc-Adda-OH, 7 mg (0.05 mmol) of HOAt, 10 mg (0.05 mmol) of DCC, and 0.6 mL of 20 DMF/80 CH_2Cl_2 as the solvent. In a separate flask, 43 mg (0.05 mmol) of **29c** was treated with TFA as required. The resulting foam was dissolved in 0.5 mL of DMF, combined with the active ester solution at 0 °C, and 18 mg (0.14 mmol) of collidine was added. The mixture was stirred for 2 h, warmed to rt, and stirred for an additional 12 h. The mixture was partitioned between ether and water, and the phases were separated. The aqueous phase was extracted once with EtOAc. The combined organic phases were washed once each with 1 M $NaHSO_4$, water, and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Chromatography (1 HOAc/7 IPA/92 $CHCl_3$, 12 mL silica gel) of the residue gave 28 mg (55%) of the heptapeptide as a white solid: R_f 0.28 (1 HOAc/10 IPA/89 $CHCl_3$); IR (thin film) 3295, 2931, 1736, 1648 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6, 90 °C) δ 0.82 (d, $J = 6.5$ Hz, 3H), 0.86 (d, $J = 6.5$ Hz, 3H), 0.96 (d, $J = 6.7$ Hz, 3H), 1.02 (d, $J = 6.9$ Hz, 3H), 1.04 (d, $J = 7.1$ Hz, 3H), 1.05 (obsc m, 1H), 1.25 (d, $J = 7.1$ Hz, 3H), 1.27 (d, $J = 7.2$ Hz, 3H), 1.27–1.38 (par obsc m, 4H), 1.38 (s, 9H), 1.47–1.57 (par obsc m, 2H), 1.57 (d, $J = 1.2$ Hz, 3H), 1.59–1.74 (m, 6H), 1.88 (m, 1H), 2.01 (m, 1H), 2.33 (m, 2H), 2.58 (m 3H), 2.66 (par obsc dd, $J = 7.2$, 14.2 Hz, 1H), 2.74 (par obsc dd, $J = 4.9$, 14.2 Hz, 1H), 2.96 (par obsc dd, $J = 5.5$, 7.1 Hz, 1H), 3.18 (s, 3H), 3.25 (ddd, $J = 5.1$, 5.4, 7.2 Hz, 1H), 3.60 (s, 3H), 3.61 (s, 3H), 3.85 (m, 2H), 4.08 (m, 1H), 4.17–4.30 (m, 4H), 4.47 (dd, $J = 5.6$, 8.6 Hz, 1H), 5.37 (d, $J = 9.6$ Hz, 1H), 5.48 (dd, $J = 6.6$, 15.6 Hz, 1H), 6.08 (obsc, 1H), 6.08 (d, $J = 15.6$ Hz, 1H), 7.14–7.24 (m, 5H), 7.45 (br, 1H), 7.60 (m, 2H), 7.73 (m, 2H); FABMS *m/e* calcd for $C_{57}H_{90}N_7O_{15}+$ $(M+H)^+$: 1112. Found: 1112.

Boc-Adda-D-*iso*-Glu(OMe)-*N*-(Me)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala-OH (30b). The Boc-Adda activation was carried out as in the procedure to prepare

3a using 20 mg (0.05 mmol) of Boc-Adda-OH. In a separate flask, 28 mg (0.04 mmol) of **29d** was treated with TFA as required. The resulting foam was combined with the active ester solution at 0 °C, and 13 mg (0.11 mmol) of collidine was added. After stirring for the appropriate time, the mixture was worked up, with the exception to the general procedure that the initial aqueous phase was extracted twice with EtOAc. Chromatography (1/15/84: HOAc/IPA/$CHCl_3$, 5 mL silica gel) of the residue gave 25 mg (68%) of the heptapeptide as a white solid: R_f 0.23 (1/20/79: HOAc/IPA/$CHCl_3$); IR (thin film) 3300 br, 2955, 1741, 1720, 1657 cm^{-1}; FABMS *m/e* calcd for $C_{52}H_{81}NaN_7O_{15}+$ $(M+Na)^+$: 1066.6. Found: 1066.6. FABMS *m/e* calcd for $C_{52}H_{81}LiN_7O_{15}+$ $(M+Li)^+$: 1050.6. Found: 1050.6. Attempts to analyze this material by ^1H NMR failed because it decomposed rapidly to imide (loss of one methyl ester resonance) at the elevated temperatures necessary to reduce the rotamer induced peak broadening and doubling. In contrast to this, spectral information was easily obtained after the macrocyclization event.

Cyclo-[Adda-D-*iso*-Glu(OMe)-MeΔAla-D-Ala-3-cyclohexyl-L-Ala-D-β-Me-*iso*-Asp(OMe)-L-Ala] (31a). To 45 mg (0.041 mmol) of **3a** in an oven dried flask was added 15 mg (0.08 mmol) of pentafluorophenol in 1.01 mL freshly distilled EtOAc. The solution was cooled to 0 °C, and 9.7 mg (0.05 mmol) of DCC was added. The mixture was stirred for 1.5 h, warmed to rt, and stirred for an additional 6.5 h. The solvent was removed in vacuo, and the residue was reconcentrated once from hexanes to remove residual EtOAc. The residue was treated with 2 mL of freshly distilled TFA for 20 min, concentrated in vacuo, and re-concentrated three times from hexanes. The residue was then dissolved in 20 mL of $CHCl_3$, transferred to a dropping funnel, and added dropwise to a rapidly stirring biphasic mixture of 35 mL of $CHCl_3$ and 35 mL of 1 M pH 9.5 phosphate buffer over 10 min. After 30 additional min, the phases were separated, and the aqueous phase was extracted twice with EtOAc. The combined EtOAc phases were washed once with water, combined with the $CHCl_3$ phase, and washed once with brine. The organic phase was dried over $MgSO_4$, filtered, and concentrated in vacuo to give a yellow residue. The residue was flashed chromatographed ($CHCl_3$ to elute nonpolar material, then 10 IPA/90 $CHCl_3$ to elute product, 6 mL silica gel) to give a white solid. Further purification by preparative reversed phase HPLC (10 THF/42 ACN/48 10 mM HOAc, retention time of product = 23 min) gave 17 mg (43%) of the desired macrocycle as a white solid: R_f 0.20 (10 IPA/90 $CHCl_3$); IR (thin film) 3373, 3278, 2923, 1740, 1672, 1646 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.85–0.92 (m, 3H), 1.02 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.8 Hz, 3H), 1.18 (d, J = 7.1 Hz, 3H), 1.24 (par obsc m, 3H), 1.33 (d, J = 7.3 Hz, 3H), 1.37 (d, J = 7.2 Hz, 3H), 1.45 (m, 1H), 1.61 (s, 3H), 1.67 (par obsc m, 6H), 2.27 (m, 1H), 2.33 (m, 1H), 2.53 (m, 3H), 2.59 (ddq, J = 6.6, 6.6, 9.7 Hz, 1H), 2.68 (dd, J = 7.3, 13.9 Hz, 1H), 2.68 (obsc m, 1H), 2.79 (dd, J = 4.7, 13.9 Hz, 1H), 3.20 (ddd, J = 4.8, 6.1, 7.3 Hz, 1H), 3.24 (s, 3H), 3.27 (s, 3H), 3.70 (s, 3H), 3.87 (s, 3H), 4.32 (m, 1H), 4.38 (dq, J = 7.4, 7.8 Hz, 1H), 4.54 (m, 1H), 4.61 (dd, J = 2.8, 8.3 Hz, 1H), 4.68 (m, 1H), 4.86 (m, 1H), 5.31 (s, 1H), 5.37 (dd, J = 7.9, 15.6 Hz, 1H), 5.41 (d, J = 9.9 Hz, 1H), 5.99 (s, 1H), 6.14 (d, J = 8.0 Hz, 1H), 6.23 (d, J = 15.6 Hz, 1H), 6.58 (d, J = 8.8 Hz, 1H), 6.79 (d, J = 8.9 Hz, 1H), 6.96 (d, J = 9.2 Hz, 1H), 7.17–7.27 (m, 5H), 7.61 (d, J = 6.7 Hz, 1H), 7.92 (d, J = 8.2 Hz, 1H); FABMS *m/e* calcd for $C_{51}H_{75}NaN_7O_{12}+$ $(M+Na)^+$: 1000.5375. Found: 1000.5379.

Cyclo-[Adda-D-*iso*-Glu(OMe)-MeΔAla-N^2-Boc-N^3-Troc-(*R*)-Aminoala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala] (31b). The activation, Boc cleavage, and macrocyclization were carried out as in the preparation of **31a**. 52 mg (0.04 mmol) of **3b** was used, and all other reagents and solvents were used in the appropriate amounts. After 1 h, the mixture was worked up. The residue was filtered through silica ($CHCl_3$ to elute nonpolar material, then 5 IPA/95 $CHCl_3$ to elute product, 6 mL silica gel) to give a white solid. Further purification by preparative reversed phase HPLC (12 THF/46 ACN/42 10 mM HOAc, retention time of product = 19.4 min) gave 22 mg (47%) of the desired macrocycle as a white solid: R_f 0.17 (5 IPA/95 $CHCl_3$); IR (thin film) 3373, 3289, 2955, 1735, 1667, 1646 cm^{-1}; ^1H NMR (500 MHz, $CDCL_3$) δ 0.87 (d, J = 6.5 Hz, 3H), 0.91 (d, J = 6.4 Hz, 3H), 1.02 (d, J = 6.7 Hz, 3H), 1.13 (d, J = 6.7 Hz, 3H), 1.20 (d, J = 6.7 Hz, 3H), 1.37 (d, J = 7.1 Hz, 3H), 1.61 (par obsc s, 3H), 2.25 (m, 2H), 2.56 (m, 3H), 2.68 (dd, J = 7.3, 13.9 Hz, 1H), 2.71 (obsc m, 1H), 2.80 (dd, J = 4.5, 13.9 Hz, 1H), 3.20 (ddd, J = 5.5, 6.6, 6.8 Hz, 1H), 3.24 (s, 3H), 3.25 (s, 3H), 3.55 (m, 1H), 3.69 (par obsc m, 1H), 3.69 (apps, 6H), 3.82 (par obsc m, 1H), 4.28 (m, 1H), 4.51 (par obsc m, 2H), 4.55 (d, J = 12.1 Hz, 1H), 4.65 (m, 2H), 4.75 (d, J = 11.9 Hz, 1H), 4.88 (m, 1H), 5.27 (s, 1H), 5.38 (dd, J = 8.3, 15.6 Hz, 1H), 5.42 (d, J = 10.0 Hz, 1H), 5.63 (br, 1H), 5.91 (s, 1H), 6.18 (d, J = 8.2 Hz, 1H), 6.22 (d, J = 15.6 Hz, 1H), 6.61 (d, J = 8.8 Hz, 1H), 6.71 (d, J = 8.0 Hz, 1H), 7.17–7.29 (par obsc m, 6 H), 7.73 (d, J = 6.4 Hz, 1H), 8.10 (d, J = 7.3 Hz, 1H); FABMS *m/e* calcd for $C_{51}H_{74}Cl_3N_8O_{14}$ $(M+H)^+$: 1127 (Cl = 34.9689). Found: 1127. FABMS *m/e* calcd for $C_{51}H_{73}Cl_3NaN_8O_{14}$ $(N+Na)^+$: 1149 (Cl = 34.9689). Found: 1149.

Cyclo-[Adda-D-*iso*-Glu(OMe)-*N*-(cyclohexyl)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala] (31c). The activation, Boc cleavage, and macrocyclization were carried out as in the procedure to prepare **31a**. 23 mg (0.02 mmol) **30a** was used, and the amounts of solvents and additional reagents were adjusted accordingly. The residue was flashed chromatographed ($CHCl_3$ to elute nonpolar material, then 5 IPA/95 $CHCl_3$ to elute product, 3 mL silica gel) to give a white solid. Further purification by preparative reversed phase HPLC (11 THF/45 ACN/44 10 mM HOAc, retention time of product = 19.5 min) gave 9 mg (43%) of the desired macrocycle as a white solid: R_f 0.42 (10 IPA/90 $CHCl_3$); IR (thin film) 3383, 3289, 2934, 1735, 1672, 1646 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 1.02 (d, J = 6.7 Hz, 3H), 1.10 (par obsc m, 1H), 1.13 (d, J = 6.8 Hz, 3H), 1.17 (d, J = 7.2 Hz, 3H), 1.30 (d, J = 7.3 Hz, 3H), 1.37 (d, J = 7.2 Hz, 3H), 1.42 (m, 2H), 1.45 (m, 2H), 1.61 (d, J = 0.9 Hz, 3H), 1.64–1.88 (m, 10 H), 2.25 (m, 1H), 2.50 (m, 2H), 2.60 (ddq, $J=6.3$, 6.6, 9.8 Hz, 1H), 2.65 (par obsc m, 1H), 2.68 (dd, $J=7.5$, 14.1 Hz, 1H), 2.80 (dd, $J=4.7$, 14.0 Hz, 1H), 3.20 (ddd, $J=4.7$, 6.3, 7.2 Hz, 1H), 3.24 (s, 3H), 3.60 (m, 1H), 3.71 (s, 3H), 3.80 (d, $J=16.7$ Hz, 1H), 3.87 (s, 3H), 3.95 (d, $J=16.7$ Hz, 1H), 4.29 (ddd, $J=6.1$, 6.2, 9.3 Hz, 1H), 4.47 (dq, $J=7.3$, 8.6 Hz, 1H), 4.53 (ddd, $J=8.3$, 8.6, 9.2 Hz, 1H), 4.63 (dd, $J=2.7$, 8.4 Hz, 1H), 4.64 (par obsc m, 1H), 4.82 (dq, $J=7.3$, 9.6 Hz, 1H), 5.34 (dd, $J=8.4$, 15.4 Hz, 1H), 5.41 (d, $J=9.8$ Hz, 1H), 6.11 (d, $J=8.6$ Hz, 1H), 6.25 (d, $J=15.4$ Hz, 1H), 6.61 (d, $J=8.6$ Hz, 1H), 6.75 (d, $J=8.4$ Hz, 1H), 6.90 (d, $J=9.8$ Hz, 1H), 7.18–7.28 (m, 5H), 7.43 (d, $J=6.1$ Hz, 1H), 8.14 (d, $J=8.6$ Hz, 1H); FABMS *m*/*e* calcd for $C_{52}H_{80}N_7O_{12}+$ $(M+H)^+$: 994. Found: 994. FABMS *m*/*e* calcd for $C_{52}H_{79}KN_7O_{12}+$ $(M+K)^+$: 1032. Found: 1032.

Cyclo-[Adda-D-*iso*-Glu(OMe)-*N*-(Me)-Gly-D-Ala-L-Leu-D-β-Me-*iso*-Asp(OMe)-L-Ala] (31d). The activation, Boc cleavage, and macrocyclization were carried out as in the procedure to prepare **31a**, using 22 mg (0.02 mmol) of **30b**, and the amounts of solvents and additional reagents were adjusted accordingly. After 1.5 h, the mixture was worked up. The residue was flashed chromatographed ($CHCl_3$ to elute nonpolar material, then 20 IPA/80 $CHCl_3$ to elute product; 4 mL silica gel) to give a white solid. Further purification by preparative reversed phase HPLC (52 ACN/48 10 mM HOAc), retention time of product = 13.1 min) gave 5 mg (26%) of the desired macrocycle as a white solid: R_f 0.20 (20 IPA/80 $CHCl_3$); IR (thin film) 3292, 2945, 1740, 1658 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.90 (d, $J=6.1$ Hz, 3H), 0.93 (d, $J=6.2$ Hz, 3H), 1.00 (d, $J=6.7$ Hz, 3H), 1.16 (d, $J=6.9$ Hz, 3H), 1.21 (d, $J=7.1$ Hz, 3H), 1.25 (par obsc m, 1H), 1.36 (d, $J=7.1$ Hz, 3H), 1.39 (d, $J=7.0$ Hz, 3H), 1.71 (m, 3H), 2.33–2.48 (m, 3H), 2.59 (m, 2H), 2.67 (dd, $J=7.4$, 14.0 Hz, 1H), 2.77 (dd, $J=4.7$, 14.0 Hz, 1H), 2.81 (dq, $J=3.3$, 7.0 Hz, 1H), 3.09 (s, 3H), 3.18 (ddd, $J=4.9$, 6.0, 7.2 Hz, 1H), 3.23 (s, 3H), 3.75 (apps, 6H), 4.10 (s, 2H), 4.37 (ddd, $J=4.2$, 6.9, 11.1 Hz, 1H), 4.42 (dq, $J=7.2$, 7.3 Hz, 1H), 4.50 (m, 1H), 4.57 (dd, $J=3.3$, 8.4 Hz, 1H), 4.65 (ddd, $J=3.2$, 7.2, 10.6 Hz, 1H), 4.71 (dq, $J=7.5$, 7.7 Hz, 1H), 5.39 (d, $J=9.7$ Hz, 1H), 5.45 (dd, $J=6.8$, 15.6 Hz, 1H), 6.19 (obsc, 1H), 6.20 (d, $J=15.6$ Hz, 1H), 6.60 (d, $J=8.0$ Hz, 1H), 7.16–7.28 (m, 7H), 7.31 (br, 1H), 7.65 (d, $J=8.0$ Hz, 1H); FABMS *m*/*e* calcd for $C_{47}H_{72}N_7O_{12}+$ $(M+H)^+$: 926.5242. Found: 926.5249.

Microcystin, cyclohexylalanine variant (2a). To 10 mg (0.01 mmol) of the dimethyl ester **3a** in a vial was added 2 mL of THF. The solution was cooled to 0 °C, and 36 drops of water was added, followed by 10 drops of 1 M LiOH. After 1 h, the solution was partitioned between 1 M $NaHSO_4$ and EtOAc, and the phases were separated. The aqueous was extracted twice with EtOAc. The combined EtOAc phases were washed once with brine, dried over Na_2SO_4, micro-filtered, and concentrated to a white solid. The solid was subjected to preparative reversed phase HPLC (70 MeOH/30 (0.2%) aq TFA). Two major peaks were observed, and the material corresponding to each peak was isolated. The faster running peak (retention time = 14 min) was the desired product (4 mg, 41%), isolated as a white solid: R_f 0.37 (DOC-A: 0.9 H_2O/13 HOAc/17.1 MeOH/69 CH_2Cl_2); IR (thin film) 3354, 3281 br, 2926, 1721, 1659 cm^{-1}; ^1H NMR (500 MHz, pH 7.5, 50 mM potassium phosphate in D_2O) δ 0.90 (m, 1H), 1.00 (m, 1H), 1.05 (app d, $J=6.6$ Hz, 6H), 1.08 (d, $J=6.9$ Hz, 3H), 1.15–1.27 (m, 4H), 1.31 (d, $J=7.3$ Hz, 3H), 1.42 (d, $J=7.4$ Hz, 3H), 1.63–1.74 (m, 6H), 1.75 (s, 3H), 1.95 (m, 2H), 2.10 (m, 1H), 2.61 (ddd, $J=3.9$, 12.5, 16.4 Hz, 1H), 2.69–2.85 (m, 4H), 2.97 (dd, $J=4.0$, 14.2 Hz, 1H), 3.05 (dq, $J=6.9$, 10.5 Hz, 1H), 3.19 (dq, $J=4.6$, 7.0 Hz, 1H), 3.30 (s, 3H), 3.43 (s, 3H), 3.52 (m, 1H), 3.95 (dd, $J=6.9$, 8.2 Hz, 1H), 4.38 (m, 2H), 4.43 (app d, $J=4.3$ Hz, 1H), 4.49 (m, 2H), 5.58 (par obsc d, $J=10.9$ Hz, 1H), 5.58 (s, 1H), 5.62 (par obsc dd, $J=8.6$, 15.7 Hz, 1H), 5.97 (s, 1H), 6.35 (d, $J=15.6$ Hz, 1H), 7.29–7.40 (m, 5H); FABMS *m*/*e* calcd for $C_{49}H_{71}NaN_7O_{12}+$ $(M+Na)^+$: 972. Found: 972. FABMS *m*/*e* calcd for $C_{49}H_{71}LiN_7O_{12}+$ $(M+Li)^+$: 968. Found: 968.

The slower running peak (retention time = 20.6 min) was two compounds by TLC; R_f 0.37 for the faster running material, and 0.21 for the slower running material (DOC-A). The two materials were separated by preparative TLC (DOC-A, silica gel 60) to give 2 mg each of white solids that were isomeric with the desired product by FABMS, but that were both different than the desired product by ^1H NMR.

To identify which of the three isomers obtained corresponded with the starting di-ester, a small amount of each of these three materials was re-esterified using the following general proceedure: Approximately 0.2 mg of the purified hydrolysis product was dissolved in 0.2 mL EtOAc, and three drops of MeOH were added. The solution was cooled to 0 °C, and two drops of 2 M trimethylsilyldiazomethane (TMS-diazomethane) were added. After 10 min, the reaction was quenched with 1 drop of HOAc, and the solution was concentrated in vacuo to a residue. The residue was filtered through silica (10 IPA/90 $CHCl_3$), and the filtrate was concentrated to a solid. The solid was analyzed by ^1H NMR, and compared to authentic di-ester by TLC cospotting assays. Only the material that had eluted first from the reversed phase HPLC purification resulted in esterified material that was identical to the original di-ester.

Microcystin, *N*-(cyclohexyl)-glycine variant (32a). The title compound was prepared using the same procedure as that used to prepare **2a**. 8 mg (0.008 mmol) of **31c** was used, and the amounts of solvents and additional reagents were adjusted accordingly. After 40 min, the reaction was worked up. The resulting solid was subjected to preparative reversed phase HPLC (75 MeOH/25 (0.2%) aq TFA). Two major peaks were observed, and the material corresponding to each peak was isolated. The faster running peak (retention time = 17.6 min) was the desired product (3.5 mg, 44%), isolated as a white solid: R_f 0.44 (DOC-A); IR (thin film) 3354, 3302 br, 2926, 1721, 1655 cm^{-1}; ^1H NMR (500 MHz, pH 7.5, 50 mM potassium phosphate in D_2O) δ 0.89 (d, $J=$

6.0 Hz, 3H), 0.97 (d, J = 6.1 Hz, 3H), 1.04 (d, J = 6.5 Hz, 3H), 1.05 (d, J = 6.3 Hz, 3H), 1.09 (d, J = 6.8 Hz, 3H), 1.10 (par obsc m, 1H), 1.31 (d, J = 7.2 Hz, 3H), 1.38 (d, J = 7.4 Hz, 3H), 1.41–1.71 (m, 6H), 1.74 (s, 3H), 1.79–2.13 (m, 8H), 2.57 (m, 1H), 2.70–2.84 (m, 3H), 2.98 (dd, J = 3.9, 14.3 Hz, 1H), 3.03 (dq, J = 7.0, 10.7 Hz, 1H), 3.15 (dq, J = 4.2, 7.1 Hz, 1H), 3.30 (s, 3H), 3.51 (m, 1H), 3.88 (m, 1H), 3.96 (d, J = 17.4 Hz, 1H), 4.00 (par obsc m, 1H), 4.05 (d, J = 17.3 Hz, 1H), 4.31 (m, 1H), 4.40 (m, 2H), 4.49 (app t, J = 9.7 Hz, 1H), 4.54 (app q, J = 7.4 Hz, 1H), 5.60 (m, 2H), 6.36 (d, J = 15.6 Hz, 1H), 7.29–7.40 (m, 5H); FABMS *m/e* calcd for $C_{50}H_{75}NaN_7O_{12}$ + $(M + Na)^+$: 988. Found: 988.

The slower running peak (retention time = 21.5 min) was two compounds by TLC; R_f 0.51 for the faster running material, and 0.33 for the slower running material (DOC-A). The two materials were separated by preparative TLC (DOC-A, silica gel 60) to give 2 mg each of white solids that were isomeric with the desired product by FABMS, but that were both different than the desired product by ^{1}H NMR.[59]

Microcystin, *N*-methylglycine variant (32b). The title compound was prepared using the same procedure as that used to prepare **2a**, using 5 mg (0.005 mmol) of **31d**, 1 mL THF, 20 drops of water, and 5 drops of 1 M LiOH. After 50 min, the reaction was worked up. The resulting solid was subjected to preparative reversed phase HPLC (71 MeOH/29 (0.2%) aq TFA). Two major peaks were observed, and the material corresponding to each peak was isolated. The faster running peak (retention time = 14 min) was the desired product (2 mg, 40%), isolated as a white solid: R_f 0.24 (DOC-A); IR (thin film) 3286 br, 2920, 1651 cm^{-1}; ^{1}H NMR (500 MHz, pH 7.5, 50 mM potassium phosphate in D_2O) δ 0.86 (d, J = 5.7 Hz, 3H), 0.94 (d, J = 5.8 Hz, 3H), 1.03 (app d, J = 7.0 Hz, 6H), 1.07 (d, J = 6.9 Hz, 3H), 1.28 (d, J = 6.9 Hz, 3H), 1.40 (d, J = 7.3 Hz, 3H), 1.68 (m, 3H), 1.72 (s, 3H), 1.95 (par obsc m, 1H), 2.04 (m, 1H), 2.52 (m, 1H), 2.68–2.79 (m, 3H), 2.90–2.98 (m, 2H), 3.13 (dq, J = 4.8, 7.2 Hz, 1H), 3.23 (s, 3H), 3.28 (s, 3H), 3.50 (m, 1H), 3.90 (d, J = 17.0 Hz, 1H), 3.95 (m, 1H), 4.15 (d, J = 17.0 Hz, 1H), 4.32 (m, 2H), 4.41–4.51 (m, 3H), 5.55 (d, J = 9.8 Hz, 1H), 5.60 (dd, J = 8.5, 15.6 Hz, 1H), 6.34 (d, J = 15.6 Hz, 1H), 7.28–7.38 (m, 5H); FABMS *m/e* calcd for $C_{45}H_{67}NaN_7O_{12}$ + $(M + Na)^+$: 920. Found: 920.

As seen before, the slower running peak (retention time = 17.2 min) was two compounds by TLC; R_f 0.24 for the faster running material, and 0.11 for the slower (DOC-A). The materials were separated by preparative TLC (DOC-A, silica gel 60) to give 1 mg each of white solids that were isomeric with the desired product by FABMS, but that were both different than the desired product by ^{1}H NMR.[59]

Microcystin, *N*-Troc-Aminoalanine variant (32c). The title compound was prepared using the same procedure as that used to prepare **2a**. The materials used were 13 mg (0.01 mmol) of **31b**, 2.6 mL THF, 42 drops of water, and 13 drops of 1 M LiOH. After 40 min, the reaction was worked up. The resulting solid was subjected to preparative reversed phase HPLC (74 MeOH/26 (0.2%) aq TFA). Two major peaks were observed, and the material corresponding to each peak was isolated. The faster running peak (retention time = 23 min) was the desired product (3.5 mg, 31%), isolated as a white solid: R_f 0.40 (DOC-A); IR (thin film) 3312 br, 2947, 1721, 1643 cm^{-1}; ^{1}H NMR (500 MHz, pH 7.5, 50 mM potassium phosphate in D_2O) δ 0.89 (d, J = 5.8 Hz, 3H), 0.94 (d, J = 5.9 Hz, 3H), 1.05 (d, J = 6.4 Hz, 3H), 1.06 (d, J = 5.9 Hz, 3H), 1.09 (d, J = 6.7 Hz, 3H), 1.32 (d, J = 6.7 Hz, 3H), 1.71 (par obsc m, 2H), 1.74 (s, 3H), 1.92 (m, 2H), 2.11 (m, 1H), 2.58 (m, 1H), 2.66–2.79 (m, 4 H), 2.96 (dd, J = 4.0, 14.0 Hz, 1H), 3.05 (dq, J = 6.9, 10.0 Hz, 1H), 3.17 (dq, J = 4.2, 6.7 Hz, 1H), 3.29 (s, 3H), 3.37 (s, 3H), 3.43–3.53 (m, 2H), 3.78 (dd, J = 3.8, 14.6 Hz, 1H), 4.09 (dd, J = 6.7, 8.2 Hz, 1H), 4.28 (m, 1H), 4.39 (d, J = 3.6 Hz, 1H), 4.50 (m, 2H), 5.58 (par obsc m, 2H), 5.60 (s, 1H), 5.94 (s, 1H), 6.35 (d, J = 15.4 Hz, 1H), 7.29–7.40 (m, 5H); FABMS *m/e* calcd for $C_{49}H_{69}Cl_3NaN_8O_{14}$ + $(M + Na)^+$: 1121 (Cl = 34.9689). Found: 1121. FABMS *m/e* calcd for $C_{49}H_{70}Cl_3N_8O_{14}$ + $(M + H)^+$: 1099 (Cl = 34.9689). Found: 1099.

The slower running peak (retention time = 26.2 min) was two compounds by TLC; R_f 0.37 for the faster running material, and 0.21 for the slower running material (DOC-A). The two materials were separated by preparative TLC (DOC-A, silica gel 60) to give 2 mg each of white solids that were isomeric with the desired product by FABMS, but that were both different than the desired product by ^{1}H NMR.[59]

Microcystin, aminoalanine variant (2b). To 0.25 mg (0.23 mmol) of **32c** in 0.15 mL MeOH were added 0.2 mL of 0.1 M pH 3.0 phosphate buffer and 12 mg Zn/Cu couple.[58] The mixture was stirred vigorously for 22.5 h, followed by filtration through Celite using 50 MeOH/50 0.1M pH 3.0 phosphate buffer to rinse. The filtrate was purified by analytical reversed phase HPLC (70 MeOH/30 pH 3.0 50 mM phosphate buffer to begin elution, followed by ramping to 80 MeOH/20 pH 3.0 50 mM phosphate buffer over 10 min at 15 min elution time; retention time of the product was 32.6 min). The fractions containing product were concentrated in vacuo to remove the MeOH, followed by increasing the pH to 7.0 by the dropwise addition of 1 M pH 7.0 phosophate buffer. The solution was then concentrated in vacuo to give a solid residue. The residue was slurried in methanol, and filtered through Celite to remove most of the salts. The filtrate was concentrated to half volume, and was filtered through a second pad of Celite. The filtrate was concentrated in vacuo to give a white solid, 0.18 mg (82%) of the title compound was obtained based on UV absorption: ^{1}H NMR (500 MHz, MeOH-d_4) δ 0.88 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 6.7 Hz, 3H), 0.93 (d, J = 6.4 Hz, 3H), 1.01 (d, J = 6.8 Hz, 3H), 1.07 (d, J = 6.8 Hz, 3H), 1.14 (d, J = 6.2 Hz, 3H), 1.59 (par obsc m, 3H), 1.62 (s, 3H), 1.78 (m, 1H), 1.93 (m, 1H), 2.58 (m, 2H), 2.69 (m, 2H), 2.81 (m, 1H), 3.03 (m, 3H), 3.23 (par obsc s, 3H), 3.53 (m, 2H), 4.21 (m, 1H), 4.38 (m, 2H), 4.52 (m, 1H), 4.58 (m, 2H), 5.45 (m, 3H), 5.91 (s, 1H), 6.25 (d, $J = 15.8$ Hz, 1H), 7.16–7.24 (m, 5H); FABMS *m/e* calcd for $C_{46}H_{69}$ $N_8O_{12}+$ $(M+2H-Na_2HPO_4)^+$: 925. Found: 925.

Biological evaluation

Inhibition assays. The catalytic subunits of PP1 and PP2A were purified from rabbit skeletal muscle as described.[57] ^{32}[P]-phosphorylase-α ($1-3\times10^6$ cpm/nmol) was prepared from phosphorylase-*b* as described.[57] PP1 and PP2A activities were assayed using ^{32}[P]-phosphorylase-α as substrate essentially as described.[57] Assays (final volume 30 μL) contained 50 mM Tris–HCl, 0.15 mM EGTA, 15 mM 2-mercaptoethanol, 0.01% (w/w) Brij 35, 0.3 mg/mL BSA, 5 mM caffeine, 10 μM ^{32}[P]-phosphorylase-α, inhibitors, and PP1 or PP2A. Inhibitors were preincubated with PP1 or PP2A for 10 min at 30 °C, and assays were initiated by addition of ^{32}[P]-phosphorylase.

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23. The ^{1}H NMR spectra of the aldehyde adduct at rt in DMSO-*d*6 appeared very similar to the desired product, with the following exceptions: an additional multiplet integrating to two protons centered at 4.65 ppm, a broad doublet of triplets integrating to one proton centered at 5.80 ppm, and the lack of the lower field carbamate N–H found at 7.35 ppm in the desired material. Shaking with D_2O resulted in the disappearance of the two broad triplets at 5.80 ppm, and heating to 90 °C resulted in sharpening the multiplet centered at 4.65 ppm to a pair of doublets that coupled each other with $J = 12.5$ Hz.

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