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Microcystin Analogues Comprised Only of Adda and a Single Additional Amino Acid Retain Moderate Activity as PP1/PP2A Inhibitors

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Abstract—A series of greatly simplified microcystin analogues comprised only of Adda (the β-amino acid common to the microcystins, nodularins, and motuporin,) and a single additional amino acid residue was synthesized and screened for inhibition of the protein phosphatases 1 and 2A. Several of the analogues were shown to be mid-nanomolar inhibitors of the enzymes.

The protein phosphatases are a ubiquitous enzymes that regulate many crucial biological functions, including neurotransmission, learning and memory, and cell division.[1] Of the serine-threonine phosphatases, two of the most prevalent are protein phosphatase 1 and 2A (PP1 and PP2A). Understanding the roles of these two enzymes in cellular signal transduction has benefited greatly from the availability of natural toxin inhibitors such as the microcystins, tautomycin, okadaic acid, and others;[2] however, their respective pharmacological functions of PP1 and PP2A can be difficult to distinguish because of the structural similarity between the two and the resultant scarcity of selective inhibitors, particularly those that more strongly inhibit PP1.[2]

The microcystins (illustrated by microcystin-LA, **1**, Fig. 1) illustrate the point. They are a group of more than 60 cyclic heptapeptides that potently inhibit PP1 and PP2A relative to other serine-threonine phosphatases but that do not distinguish between the two ($IC_{50} = 0.3$ nM for both PP1 and PP2A).[3] One structural feature shared by the microcystins, nodularins, and motuporin—and which is essential for activity—is the unusual β-amino acid (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, **2**, generally referred to by its acronym 'Adda.'[4] This actually is one of two structural features of these toxins that are known to be essential for potent inhibition of PP1/PP2A by microcystin, the other being a free carboxylate.[5] Although Adda is necessary for inhibition, it is not alone sufficient, as the parent amino acid and its *N*-acylated derivatives **2**, **3**, and **4** are only very weakly active at best.[6]

Our group previously synthesized **1**[7] and several 'full-sized' analogues that were selective for PP1,[3] albeit modestly (up to 7:1). Due to the substantial synthetic effort required to prepare these macrocyclic 'non-natural' heptapeptides, we have subsequently investigated the possibility that much simpler derivatives, while they

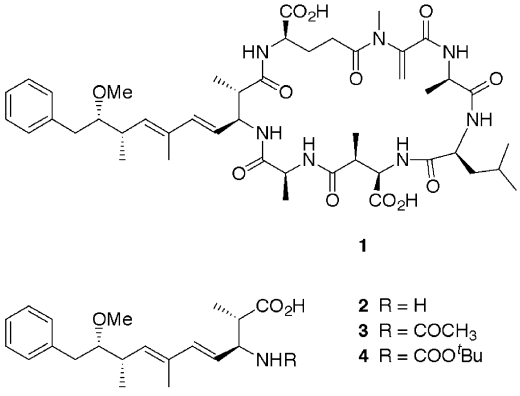

Figure 1. Microcystin (**1**), adda (**2**), *N*-Ac-adda (**3**), and *N*-Boc-adda (**4**).

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may be expected to be less potent then the natural toxins, might nonetheless have desirable properties such as enhanced selectivity. In preliminary experiments toward this goal, we have found—despite the rather un-encouraging precedent for Adda itself—that simple, acyclic analogues containing only *N*-acetylated Adda and a single additional amino acid—a surrogate for the *iso*-glutamyl residue of microcystin—retain mid-nanomolar inhibitory potency.[8] We also find that further simplification, in the Adda side chain, produces only inactive compounds.

Design and Synthesis

In order to determine the minimum structural requirements for inhibition by simplified analogues at our target levels (sub-micromolar IC_{50}), we envisioned simply linking the two structural elements known to be required for activity in **1**, namely Adda and a carboxylic acid, via a peptide bond. The resultant dipeptides, derived from *N*-acetyl-Adda (**3**) and D-alanine, glycine, and L-alanine (**5**–**7**, respectively in Fig. 2),[4] retain only the Adda group and a remnant of the adjacent D-isoglutamyl residue (or its L-epimer) that have been, in effect, 'excised' from the parent macrocyclic heptapeptide. In practice, the three were prepared by coupling alanine or glycine to *N*-Ac-Adda with HATU, then sapponifying the esters.[6]

Further simplification of these dipeptides, specifically in the Adda side chain, would be highly desirable because obtaining quantities of Adda itself requires a substantial multi-step synthetic effort. Although we were mindful that double bond isomerization, hydrogenation, or ozonolysis of the Adda side chain renders **1** inactive,[9] some structural variation is known to be tolerated. For example, in microcystin itself a free alcohol or acetate may substitute for the methyl ether with little effect on activity; thus, modifications were limited to this part of the side chain[4] of our simplified analogues, leaving the diene substitution pattern and stereochemistry intact.

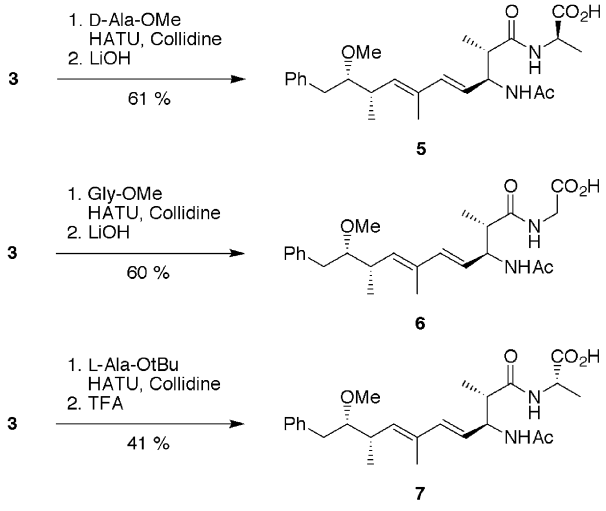


Figure 2. Syntheses of Adda coupled to D-alanine (**5**), glycine (**6**), and L-alanine (**7**).

With these considerations in mind, three Adda surrogates were designed. The first, **9** (Fig. 3), lacks both the methoxy and methyl substituent of the parent structure. Since neither of these substituents is thought to contact the enzymes, their absence might have minimal effect on inhibition, unless they play an important conformational role. This Adda analogue was to be incorporated into a dipeptide (**11**) with D-alanine to give a simplified dipeptide for direct comparison with **5**[10] (Fig. 3). The other two simplified analogues, **13** and **15** (Fig. 4), are intended to constrain the side chain to a conformation similar to that observed in the PP1-bound state of **1**, in which the methyl and methoxy groups are approximately coplanar.[11] All three of the simplified Adda analogues were synthesized[12] as previously described for Adda itself but employing the modified aldehydes **8**, **12**, and **14**. The route employed was patterned after on developed by Rinehart et al., which is not convergent but is high yielding and convenient. This linear approach provides the diene after two sequential Wittig olefinations, and sets the two stereocenters via a diastereoselective aldol reaction. Each of these simplified Adda analogues was then converted into the corresponding dipeptides **11**, **13**, and **15**, respectively.

Another set of analogues was designed to test combinations of Adda itself with alternative, slightly more complex *iso*-glutamyl surrogates. These derivatives were

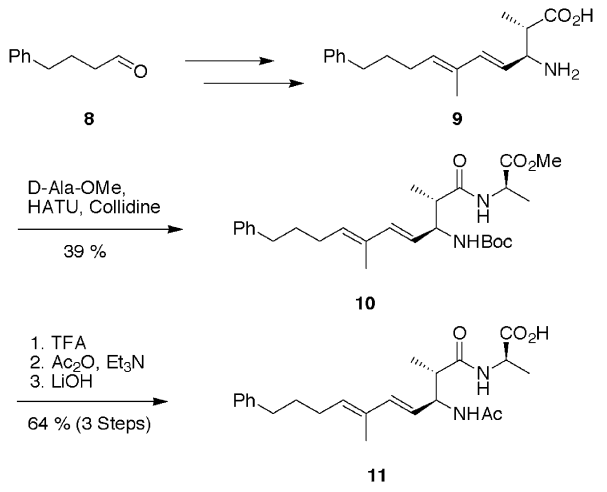


Figure 3. Synthesis of simplified Adda analogues **11**.

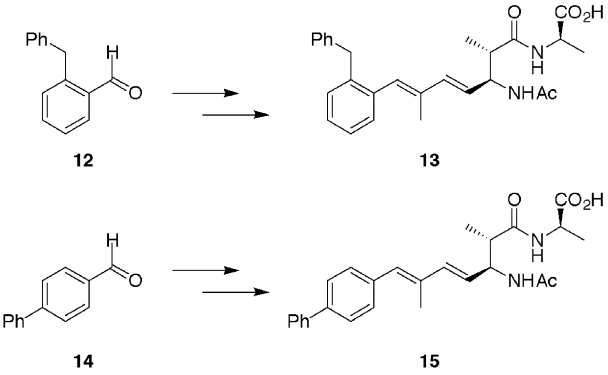
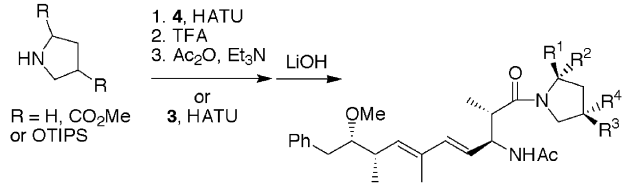


Figure 4. Simplified Adda analogues **13** and **15**.

designed to test the effect of incorporating a scaffold for (later) attachment of substituents that might alter PP1/PP2A selectivity. Proline was chosen as a test scaffold, bearing in mind that modified prolyl amide bond conformational preferences unfavorable to binding could derail this plan.

To test whether the scaffold backbone alone is tolerated, the dipeptides **17** and **21** were synthesized (Table 1) by coupling **3** to both enantiomers of proline (D- and L-proline, **17**[13] and **20** respectively). The pyrrolidine-based analogue **16**, a control that was not expected to show any activity because it lacks the carboxylate group that plays the essential role of phosphate mimic, was also prepared in a similar manner. As a preliminary test of functionalized scaffolds, several 4-substituted proline dipeptides were prepared, including the hydroxyproline derivative **18** and the proline dicarboxylates, **19**, **20**, and **22**. Additionally, several 5-substituted proline dicarboxylates were chosen because inspection of the microcystin-PP1 X-ray structure[11] suggested that a second carboxylate in this position on the pyrrolidine ring could possibly interact with Arg96 and/or Tyr134 in the active site of PP1. We prepared this series of dipeptides from the corresponding amines (**23**, **24**, and **29**), which were in turn synthesized from pyroglutamatic acid by modifying previously published routes,[14,15] to yield the 2,5-diacid analogues **27** and **28** (Fig. 5), with the corresponding acyclic diacid **31**, prepared analogously, as a control (Fig. 6).

Table 1. Syntheses of 4-substituted proline analogues



Compd	R^1	R^2	R^3	R^4
16	H	H	H	H
17	CO_2H	H	H	H
18	CO_2H	H	OTIPS	H
19	CO_2H	H	CO_2H	H
20	CO_2H	H	H	CO_2H
21	H	CO_2H	H	H
22	H	CO_2H	CO_2H	H

Biological Assays

All of the target analogues were screened either in a phosphorylase-*a* assay[16] (Table 2) or a *p*NPP assay assay[17] (Table 3) to determine IC_{50} values against PP1 and PP2A. Two additional compounds, **32** and **33**, the methyl ester precursors to **17** and **21**, respectively, were tested as negative controls. While the IC_{50} values determined in a given assay under standard conditions are directly comparable, 'cross-assay' comparisons are not. However, the values obtained for **5**, which was screened in both assays, show that the *p*NPP assay under these conditions gives IC_{50} values that are a factor of 25–50 higher than those determined by the phosphorylase-*a* procedure. This difference is also consistent with the 3.0 nM IC_{50} determined for microcystin-LR in the *p*NPP assay (Table 3), which is 10- to 30-fold higher than the 0.1–0.3 nM literature values in the phosphorylase-*a* assay.[2]

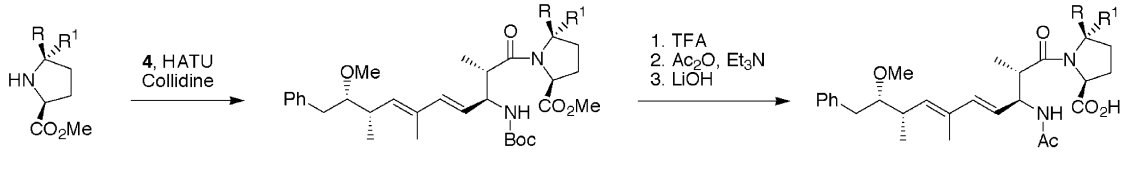
Discussion

Not surprisingly, the dipeptides **5**–**7** inhibited both PP1 and PP2A with a substantial loss in activity compared to the extremely potent microcystin-LA (**1**, $IC_{50} = 0.3$ nM for both PP1 and PP2A in the phosphorylase-*a* assay[3]). However, it is rather remarkable that **5**, which lacks five of the seven amino acids present in the parent microcystin (and obviously also the macrocyclic ring) is still a 160 nM inhibitor of PP2A in the phosphorylase-*a* assay (7 μM in the *p*NPP assay). This, the most potent of these three analogues, is the (*R*)-alanyl derivative and therefore directly analogous to the natural (*R*)-*iso*-glutamyl residue found in **1**. Interestingly, of the three it is also the most microcystin-like in its PP1/PP2A selectivity, at 1:6 in favor of PP2A, compared to 1:1 for microcystin itself. The glycine-based analogue **6** was both less active and more selective for PP2A (1:11), and

Table 2. Inhibitory activity (IC_{50}'s) against PP1 and PP2A for analogues

Analogue	IC_{50} (μM) PP1[a]	IC_{50} (μM) PP2A[a]	Ratio PP1: PP2A
1	0.0003	0.0003	1:1
5	1.0	**0.16**	1:6
6	5.6	0.5	1:11
7	170	7.8	1:22

[a]Values determined using the phosphorylase-*a* assay.



23 R = CO_2Me, R^1 = H
24 R = H, R^1 = CO_2Me

25 R = CO_2Me, R^1 = H; 62 %
26 R = H, R^1 = CO_2Me; 63 %

27 R = CO_2H, R^1 = H; 29 %
28 R = H, R^1 = CO_2H; 28 %

Figure 5. Syntheses of 5-substituted proline dicarboxylates.

Table 3. Inhibitory activity (IC_{50}'s) against PP1 and PP2A for analogues

Analogue	IC_{50} (μM) PP1[a]	IC_{50} (μM) PP2A[a]	Ratio PP1: PP2A
MC-LR	0.003	0.003	1:1
5	25	**7.0**	1:4
11	>100	>100	—
13	>100	>100	—
15	>100	>100	—
16	>100	>100	—
17	55	25	1:2
18	>100	63	>1:2
19	>100	>100	—
20	>100	>100	—
21	>100	>100	—
22	>100	>100	—
27	>50	10	>1:5
28	>50	39	>1:1.3
31	>100	>100	—
32	>100	>100	—
33	>100	>100	—

[a]Values determined using the *p*NPP assay.

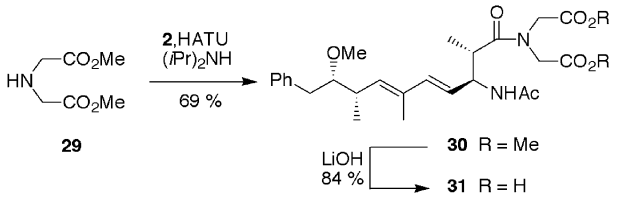


Figure 6. Synthesis of acyclic diacid **31**.

the (*S*)-alanyl analogue **7** was the least potent of the three but with redoubled selectivity for PP2A (1:22). Taken as a whole, these results suggest an as-yet-unexplained role of the *iso*-glutamyl residue of microcystin in modulating PP1/PP2A selectivity. They also provide a benchmark dipeptide, **5**, against which to compare activities of the other analogues.

The analogues **11**, **13**, and **15** were designed as (further) simplified versions of **5**, with modifications in the terminus of the Adda side chain intended to simplify the synthesis as discussed above. However, unlike **5**, all three compounds were inactive. Although disappointing, this result further reinforces the established notion that activity is very sensitive to minor changes in the structure of Adda.

The prolyl analogue **17**, designed to test the effect of an alternative, pyrrolidine-based surrogate for the *iso*-glutamyl, proved to be only 3-fold less active than **5**. Evidence that both **5** and **17** bind in an analogous manner is that neither the pyrrolidine heterocycle alone (**16**), nor the esterified precursor (**32**) were active, suggesting the carboxylate still forms a key contact. As with D-alanyl analogue **5** and epimeric L-alanyl analogue **7**, the L-prolyl analogues (**21** and **22**) were significantly less active than D-prolyl **17**, with one notable exception (see below).

Further functionalization of the prolyl heterocycle, at the 4-position (**18**–**20**), resulted in somewhat decreased potencies. Incorporation of a silyl ether (OTIPS, **18**) was the best tolerated of the substituents, resulting in only a 2-fold loss in activity relative to **17**. Incorporation of either a *cis* (**19**) or *trans* (**20**) carboxylate resulted in at least a 4-fold reduction in potency.

In direct contrast to the deleterious effects of carboxyl-substituents at the 4-position, a 5-carboxyl group enhances activity. The *cis*-2,5-dicarboxylate **27** was almost 3-fold more active for PP2A than its simple D-prolyl parent **17** and in fact was comparable in activity to the most potent PP2A inhibitor tested, the D-alanyl dipeptide **5**. The pyrrolidine heterocycle apparently plays a role in this enhanced potency—perhaps appropriately preorganizing the two carboxyl groups of **27**—since its the acyclic cousin, **31**, was not active.

Among the Adda dipeptide examples discussed thus far, a D-amino acid is essential for maximal activity. It was therefore initially surprising that the L-prolyl dicarboxylate **28** is only 4-fold less potent than **27**, in contrast to the other L-α-analogues that were invariably much less potent than their D-diastereomers (e.g., IC_{50} for PP2A of **7**>>**5** and **21**>>**17**) This specific 5-carboxyl substituent clearly 'rescues' the activity of this L-carboxyproline analogue, perhaps by forming the aforementioned postulated additional contacts (with Arg96 and/or Tyr134) in the enzyme active site that compensates for the sub-optimal L-α-carboxy stereochemistry. This conjecture is also consistent with the increase in potency (discussed above) observed for **27**, which also has an (*S*)-5-carboxyl substituent on the proline ring.

We conclude that greatly simplifying the macrocyclic heptapeptide structure of microcystin is possible without unacceptable sacrifices in activity. While the active dipeptides reported in this Letter do not approach the sub-nanomolar potency of the parent toxins, they retain remarkable levels of activity considering that the macrocyclic ring and more than two-thirds of the amino acid residues of microcystin are absent. The *iso*-glutamyl surrogates tested demonstrate that additional steric bulk in the phosphate mimic is tolerated, and that a (*S*)-5-carboxyprolyl substituent enhances activity in several analogues, consistent with the proposed hypothesis of interactions between this substituent and several active site residues. Finally, all of the active dipeptides inhibit PP2A with varying degrees of selectivity (PP1/PP2A IC_{50} ratios ranging from 1:1.3 to 1:22), unlike the parent toxin, which is non-selective. This consistent trend suggests PP1/PP2A selectivity is in part a function of the specific structure of the phosphate mimic of the analogue. Further studies are underway to clarify this issue and to prepare second generation dipeptides with enhanced potency and selectivity.

Acknowledgements

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