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Linearized and Truncated Microcystin Analogues as Inhibitors of Protein Phosphatases 1 and 2A

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Abstract—A series of acyclic, truncated microcystin analogues, comprised of the dienic β-amino acid (Adda) and up to four additional amino acids characteristic of the parent toxin, was synthesized and screened for activity as inhibitors of PP1 and PP2A. Despite a recent report to the contrary for a microcystin-derived tetrapeptide degradation product, none approaches the potency of microcystin itself.

The protein phosphatases 1 and 2A, (PP1 and PP2A, respectively), are crucial elements in the regulation of many cell signaling pathways.[1] The inhibition of these enzymes can disrupt numerous physiological processes, ranging from glycogen synthesis,[2] cell division,[3] to memory and learning.[4] Only recently have the respective roles of PP1 and PP2A begun to be delineated, due in large part to the discovery of selective inhibitors—generally naturally occurring toxins—for cell biology studies.[5]

Among the most potent inhibitors of PP1 and PP2A are the microcystins (e.g., microcystin-LR, **1**, Fig. 1), a large family of natural toxins characterized by the presence of an otherwise 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 (generally referred to by its acronym 'Adda'). While these cyclic heptapeptides are quite potent and selective for PP1 and PP2A over other serine-threonine phosphatases (e.g., PP2B, PP2C, etc.), they do not distinguish at all between the two ($IC_{50} = 0.3$ nM for PP1 and PP2A).[6] To initiate a search for more selective inhibitors, our group synthesized one member of this family, microcystin-LA[7] as well as a series of rationally-designed 'full-sized' variants.[6] Assays of PP1/PP2A inhibition by these analogues demonstrated that modification of the dehydroalanine, D-alanine, or L-leucine residues gives modest increases in selectivity for PP1. While these initial results were gratifying, the rather laborious synthesis of the macrocyclic heptapeptides suggested that it would be prudent to investigate whether simplified, acyclic analogues might achieve the same goal.

Recently, Jones et al. reported studies on the natural enzymatic degradation pathway of **1**,[8,9] including the

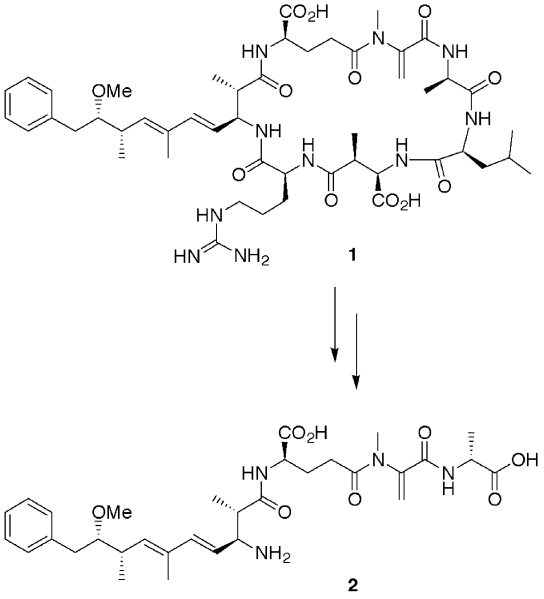

Figure 1. Enzymatic degradation of MC-LR.

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identities and inhibitory potencies of two 'linearized,' acyclic Adda-containing peptides. These experiments address the important question of whether fragments of the parent toxin might retain significant activity and thus pose a toxicological risk in their own right. The first degradation product in the pathway is the acyclic heptapeptide resulting from scission of the Adda-arginine amide bond to open the macrocyclic ring. This linearized product was reported to be less active than its macrocyclic precursor, **1**, but it appears to retain substantial potency (PP1 $IC_{50}=90$ nM). More surprisingly, another degradation product, the linearized and truncated tetrapeptide **2**, was reported to be a low nanomolar inhibitor (PP1 $IC_{50}=12$ nM). This admittedly is nearly a 100-fold less potent than the macrocyclic parent but nonetheless amazingly potent considering it lacks both the macrocyclic ring and three of the seven amino acid residues present in **1**. Before proceeding with simplified microcystin analogue design based on this promising precedent, we decided to prepare the reported tetrapeptide degradation product and several higher and lower homologues (e.g., the corresponding penta- and dipeptides) for comparison. We relate in this Letter that all of these synthetic linearized and truncated peptides, including the reportedly potent tetrapeptide degradation product, are only moderately active inhibitors of PP2A and even less potent towards PP1.

Design and Synthesis

The simplest analogue of the series to be synthesized was the dipeptide **4**, which consists of Adda and an isoglutamyl residue capped with a dimethyl amide. Previous studies had shown that Adda alone is not sufficient for inhibition,[10] so this dipeptide was chosen as the simplest member of the linearized and truncated series to be investigated. The synthesis was performed by coupling **3**, synthesized by a route disclosed recently by Rinehart,[11] with a suitably protected glutamate, followed by standard protecting group manipulations to yield **4** (Fig. 2).

The degradation tetrapeptide, **2**, was synthesized using the methodology developed previously for the synthesis of the parent toxin **1**.[7] The dipeptide **5** (Fig. 3), was deprotected by hydrogenolysis, followed by coupling of the resultant free amine **6** to selectively protected glutamate and in situ olefination to give the iso-glutamyl tripeptide **7**. Next, **7** was coupled to **3** using HATU to yield the protected tetrapeptide **8**, which upon saponification of the methyl esters and deprotection of the amine afforded the degradation tetrapeptide **2**.[12] Unlike the saponification of the methyl esters in our synthesis of microcystin-LA, where significant epimerization was observed, there was only a trace of the unwanted diastereomer formed in this acyclic case.

The degradation peptide **2** differs from the parent toxin not only in lacking the macrocyclic ring and two amino acid residues, but also in having a basic amino group at the N-terminus of Adda rather than an amide group. In order to address the issue of whether reducing the basicity of this nitrogen might have an effect on potency, we prepared the corresponding *N*-acetylated tetrapeptide **9**, which mimics the amide group found at the corresponding position in the parent toxin. This analogue was synthesized (Fig. 4) from the intermediate **8**, as shown.

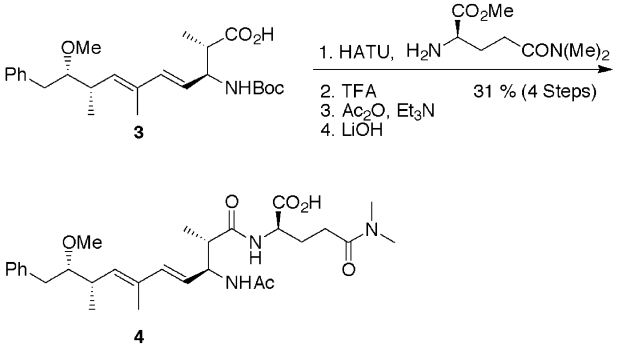

Figure 2. Synthesis of dipeptide **4**.

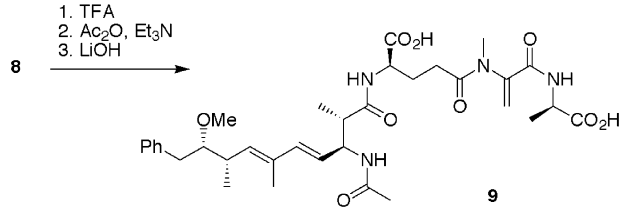

Figure 4. Synthesis of acylated tetrapeptide **9**.

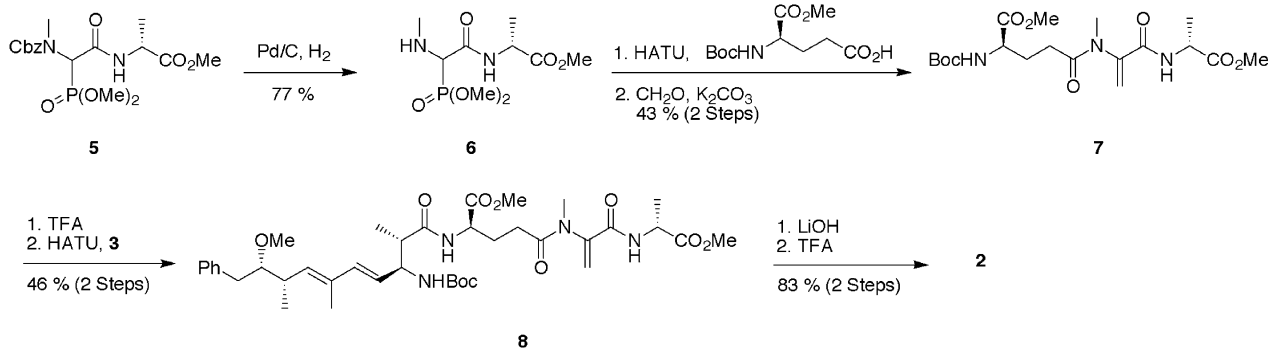

Figure 3. Synthesis of the degradation tetrapeptide.

The olefin of the *N*-methyl-dehydroalanine residue is another element of interest that may or may not play a significant role in controlling the activity in this series of linearized and truncated analogues. It has been shown for the parent microcystins that neither borohydride reduction of this residue to the corresponding *N*-methyl-alanine diastereomers,[13] nor replacement with sarcosine (*N*-methyl-glycine) has any effect on PP1/PP2A inhibition.[6] This insensitivity towards removal of the olefin group is particularly interesting in view of the fact that microcystin-LR is known to undergo conjugate addition to this residue, upon binding to PP1, by a free cysteine in the active site. Nonetheless, the olefin still may be an important structural feature for the linearized analogues, particularly in view of additional potential conformational effects not present in the macrocyclic parent toxin. Thus, two tetrapeptides substituting sarcosine for dehydroalanine, **11** and **12**, were synthesized from the dipeptide **10** (Fig. 5) using chemistry similar to that employed in the preparation of **2** and **9**, as shown.

Because our previous studies[6] had shown that modification of the leucine residue of microcystin-LA can increase selectivity towards PP1, it was of interest to prepare the next higher linearized homologue, the C-terminal residue of which is that corresponding to leucine. Two pentapeptide analogues were thus synthesized (**14** and **15**, Fig. 6) with and without the olefin as discussed above, from the dipeptide **13** employing the phosphosarcosine and sarcosine routes described for other members of the series.

Biological Assays

All of the analogues were screened in a *p*NPP assay[14,15] against PP1 and PP2A to determine IC_{50} values (Table 1).

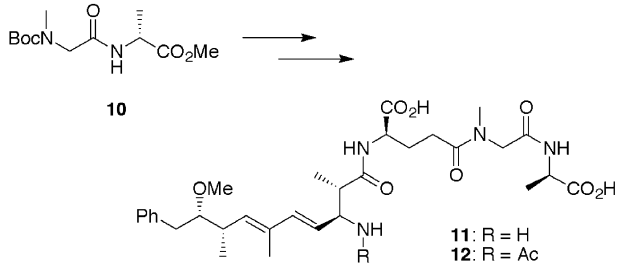

Figure 5. Sarcosine analogues **11** and **12**.

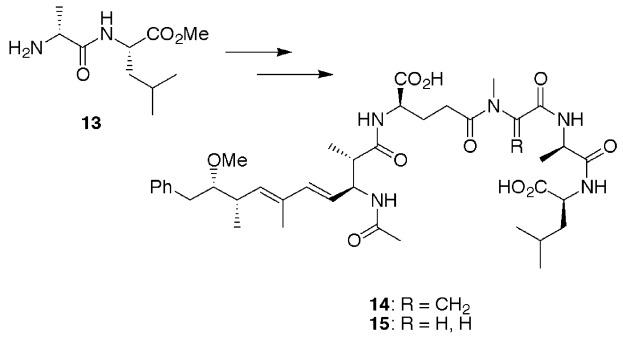

Figure 6. Pentapeptide analogues **14** and **15**.

Additionally, two other compounds were screened as controls: microcystin-LR (**1**), which gave IC_{50}'s in good agreement with literature values,[6] and the methyl ester of **4**, which served as a negative control.

Discussion

The impetus for preparing this series of linearized analogues was the surprisingly potent activity of the degradation tetrapeptide **2**, which was reported to have an IC_{50} of 12 nM (PP1). Unfortunately, we were unable to confirm this result with our synthetic tetrapeptide, measuring instead a value more than three orders of magnitude higher: $IC_{50} = 45$ μM for PP1 (slightly better for PP2A, $IC_{50} = 25$ μM). A part—but probably not all—of this discrepancy may be ascribed to the use of a different assay (*p*NPP) than in the original report (phosphorylase *a*), but the results obtained in the two assays should differ only by an order of magnitude or so. For example, in the assays reported here, microcystin has an IC_{50} of 3 nM, only a factor of 10 higher than the 0.3 nM value we have determined previously in phosphorylase *a* assays. This discrepancy may be due to a trace contaminant in the isolated degradation sample that was responsible for most of the observed activity. This type of contamination is always a concern when testing by-products isolated from mixtures containing very active constituents. In this particular case, the presence of only ~1% of the parent toxin (or some other equally active inhibitor) could have resulted in the reported IC_{50} of 12 nM reported for the degradation product.

Despite the relatively weak activity of **2**, it still serves as a benchmark for comparison with other linearized analogues that may serve as lead compounds in the search for structurally-simplified selective inhibitors. The tetrapeptide **9** more closely mimics the parent compound **1**, with an amide terminus instead of the basic amine of **2**, but this acetylated derivative shows a 3-fold decrease in PP1 inhibition, with no difference in PP2A inhibition. Further comparison of **2** with the corresponding sarcosine analogues **11** (free amine) and **12** (acetylated amine) reveals a further 5- to 10-fold erosion of potency towards both PP1 and PP2A. This reduced activity is in contrast to the parent toxin, which does not lose potency when the *N*-methyl-dehydroalanine is replaced

Table 1. Inhibitory activity (IC_{50}'s) against PP1 and PP2A for analogues

Analogue	IC_{50} (μM) PP1	IC_{50} (μM) PP2A	Ratio PP1:PP2A
1	0.003	0.003	1:1
2	45	25	1:2
4	>100	20	>1:5
9	140	25	1:6
11	280	270	1:1
12	400	135	1:3
14	75	16	1:5
15	230	17	1:14
Methyl ester of **4**	>100	>100	—

with sarcosine, as discussed above. These results suggest that for the acyclic tetrapeptides the olefin is important for binding, perhaps because it biases acyclic conformational preferences in favor of a pseudo-cyclic arrangement favored for binding. Alternatively, the aforementioned conjugate addition to the *N*-methyldehydroalanine double bond that does not affect measured potencies in the parent toxins may come into play for the acyclic analogues, slowing the off-rates for **2** and **9** relative to **11** and **12** and resulting in lower IC_{50} values for the former pair.

The next higher homologues, the pentapeptides **14** and **15**, incorporate an additional leucine residue at the C-terminus and behave similarly to the tetrapeptides in most respects, but with one interesting difference. Comparison of **9** and **14** (both with dehydroalanine) shows a slight, 2-fold improvement in PP1 and PP2A inhibition for the pentapeptide over the tetrapeptide. For **12** and **15** (with sarcosine), there was also a 2-fold improvement in PP1 inhibition, but an 8-fold increase in PP2A potency for the pentapeptide. The net effect on selectivity is that for **15**, but not **14**, there is a significant increase in PP2A selectivity in going from the tetrapeptide to the corresponding pentapeptide. Both pentapeptides (**14** and **15**) were slightly more potent than the corresponding tetrapeptides (**9** and **12**, respectively), and they exhibited a similar decrease in PP1 potency for the sarcosine-derived derivatives compared to the corresponding dehydro-peptides. Interestingly, however, the absence of the olefin in the pentapeptides does not have the same deleterious effect for PP2A; **14** and **15** have essentially identical IC_{50} values of 16 and 17 nM.

As the tetrapeptide was only slightly less potent than the pentapeptide, we were not surprised to observe that further simplification of the structure to the dipeptide **4** did not abolish activity. Indeed, for PP2A, the IC_{50} was essentially the same as the tetrapeptide **9** and the pentapeptide **14**, while the potency towards PP1 was reduced (only a lower limit could be determined due to limited solubility of the compound).

The Adda-containing analogues described herein, ranging from di- to penta-peptides with the sequence found in microcystins, exhibit remarkably similar activities. Their inhibitory potencies towards PP1 and PP2A have been found to be only moderate-to-mediocre, contradicting a literature report of potent inhibition by the acyclic tetrapeptide microcystin degradation product. What is not known is why there is no significant change in activity between the di-, tetra-, and pentapeptides. The three variable residues could bind in a similar manner to microcystin, with the beneficial binding contacts from each additional residue offset by the entropic cost of restraining the conformation for binding. Alternatively, the residues may not make any contacts, and extend away from the enzyme. Further experiments are being conducted in our laboratory to address this issue.

Despite their reduced potencies, some of the simplified analogues retain sufficient activity to serve as lead compounds for the development of new, selective inhibitors. The clear lesson is that linearized and truncated analogues of microcystin do not benefit—at least in terms of potency - from additional residues beyond those present in the Adda-containing dipeptide. In fact, we have found that further truncation of the *iso*-glutamyl-residue in this dipeptide is well tolerated, resulting in even simpler analogues that retain inhibitory activity against PP1 and PP2A. Initial results on these further simplifications are described in an accompanying Letter.

Acknowledgements

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