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INHIBITION OF PROTEIN PHOSPHATASE 2A BY CYCLIC PEPTIDES MODELLED ON THE MICROCYSTIN RING

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Abstract: Several synthetic analogues of microcystin-LR and nodularin at 1 mM inhibited PP2A. The active microcystin analogues were cyclic heptapeptides envisaged to interact with the catalytic subunit of PP1 and PP2A when an Adda-type hydrophobic group was added. The cyclic core was found to have some intrinsic phosphatase inhibitory activity. Copyright © 1996 Elsevier Science Ltd

Microcystin-LR, a naturally occcurring cyclic heptapeptide, is a member of the okadaic acid class protein phosphatase inhibitors (Figure 1).[1] Investigation of the microcystins, okadaic acid, and the calyculins led to the development of an okadaic acid pharmacophore model.[2] Figure 2 highlights the four regions of microcystin-LR considered essential for activity; a conserved acidic site, a hydrophobic side chain, and two potential hydrogen bonding sites (I and II). The novel Adda residue has been extensively investigated[3] and the synthesis of three linear microcystin derivatives was recently reported[4], however, little information is currently available on the cyclic core itself. In the previous paper, we reported the synthesis of several linear and cyclic microcystin-LR analogues.[5] The inhibition of protein phosphatase 2A activity of these analogues was examined and is now reported.

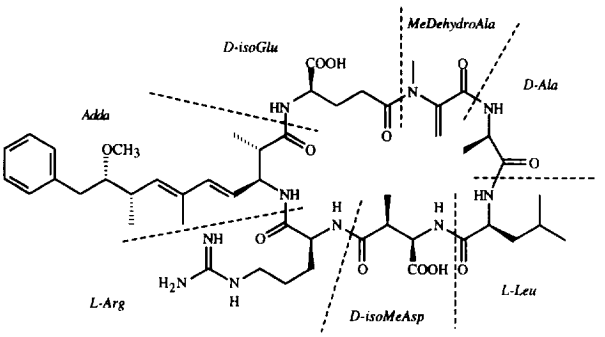


Figure 1: Microcystin-LR

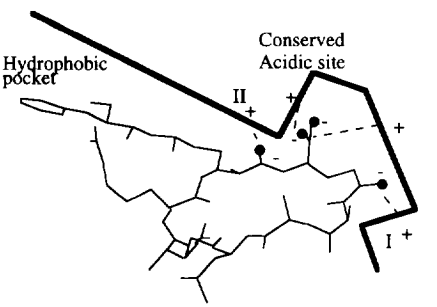


Figure 2: The conserved acid binding domain of microcystin-LR, showing the potential hydrogen bonding areas I (Glu carbonyl O) and II (Adda carbonyl O), the conserved acid and the hydrophobic side chain.

Microcystin-LR interacts with PP1 and PP2A with approximately the same affinity.[6] Partially purified PP2A was used in this investigation.[7] Microcystin-LR inhibited the dephosphorylation of [^{32}P]phosporylase *a* by protein phosphatase 2A with an IC_{50} value of 0.2 nM.[8]

Table 1: Inhibition of PP2A by Synthetic Peptides Modelled on Microcystin-LR and Nodularin.

Microcystin/Nodularin Analogues		Inhibition of PP2A at 1mM	IC_{50} (mM)
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Asp**-D-isoGlu-L-Ala-CO_2H	**1**	21%	
H_2N-D-Ala-L-Leu-D-Asp-L-**Ala**-**L-Asp**-D-Glu-L-Ala-CO_2H	**2**	11%	
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Ala-CO_2H	**3**	12%	
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Ala**-D-isoGlu-L-Ala-CO_2H	**4**	22%	
H_2N-D-Ala-L-Leu-β-Ala-L-Ala-**L-Asp**-D-isoGlu-L-Ala-CO_2H	**5**	12%	
H_2N-D-Ala-L-Leu-β-Ala-L-Ala-**L-Asp**-D-Glu-L-Ala-CO_2H	**6**	14%	
cyclo(-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Ala-)	**7**	3%	
H_2N-β-**Ala**-D-Glu-L-Ala-D-Ala-L-Leu-D-Asp-L-Ala-CO_2H	**8**	16%	
H_2N-β-**Ala**-D-isoGlu-L-Ala-D-Ala-L-Leu-D-isoAsp-L-Ala-CO_2H	**9**	15%	
H_2N-**L-Cys**-D-Glu-L-Ala-D-Ala-L-Leu-D-Asp-L-Ala-CO_2H	**10**	22%	
H_2N-**L-Cys**-D-isoGlu-L-Ala-D-Ala-L-Leu-D-isoAsp-L-Ala-CO_2H	**11**	19%	
H_2N-β-**Ala**-D-Glu-L-Ala-D-Asp-L-Ala-CO_2H	**12**	30%	1-10
H_2N-β-**Ala**-D-isoGlu-L-Ala-D-isoAsp-L-Ala-CO_2H	**13**	19%	
H_2N-D-Ala-L-Leu-D-Asp-L-Ala-**L-Cys**-D-Glu-L-Asp-$CONH_2$	**14**	17%	
cyclo(-D-Ala-L-Leu-D-Asp-L-Ala-**L-Cys**-D-Glu-L-Asp-)	**15**	86%	0.5
H_2N-D-Ala-L-Leu-D-Asp-L-Ala-β-**Ala**-D-Glu-L-Asp-$CONH_2$	**16**	1%	
cyclo(-D-Ala-L-Leu-D-Asp-L-Ala-β-**Ala**-D-Glu-L-Asp-)	**17**	21%	
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Cys**-D-isoGlu-L-Asp-$CONH_2$	**18**	18%	
cyclo(-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Cys**-D-isoGlu-L-Asp-)	**19**	45%	1-10
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Asp-$CONH_2$	**20**	11%	
cyclo(-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Asp-)	**21**	42%	1-10

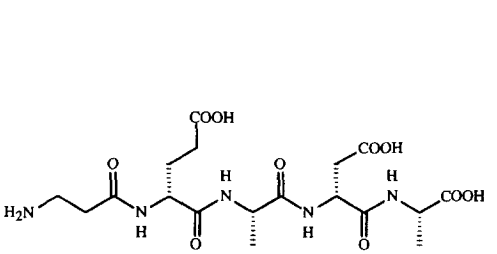
The synthetic analogues of microcystin-LR/nodularin, listed in Table 1, were screened at 1mM for the inhibition of PP2A. The assays were conducted using [^{32}P]phosphorylase *a*, partially purified PP2A (ABC type) and compound at 1 mM, following the procedure used for microcystin-LR.[8] Each experiment was run in duplicate. The results showed that the activity of most of these analogues was extremely low compared with microcystin-LR (Table 1). This was most likely a direct result of the absence of the hydrophobic side chain, Adda. Compounds **12**, **15**, **19**, and **21** showed 30% or greater inhibition of PP2A at 1 mM. These compounds were further tested to determine their IC_{50} values. The assay was conducted using [^{32}P]phosphorylase *a* as the substrate, partially purified PP2A (ABC type) isolated from mouse brain and compound (3 concentrations ranging from 0.01 to 1 mM), in duplicate. The IC_{50} value for **15** was determined to be 0.5 mM. Compounds **12**, **19**, and **21** exhibited IC_{50} values of approximately 1-10 mM. Accurate determination of the IC_{50} values was not possible due to lack of solubility.

Unexpectedly, the results showed that these compounds which are Adda deficient precursors modelled on the microcystins, had some inhibitory activity against PP2A. This supports the concept that not only the Adda is essential, but that the cyclic system plays an important role in the interaction of the microcystins with the catalytic subunit of PP2A.

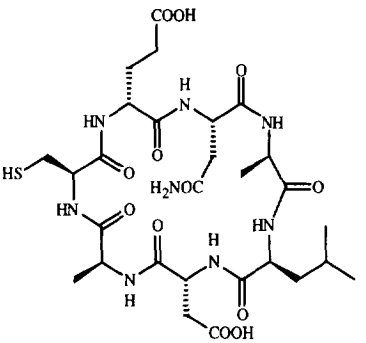
The Adda residue was substituted with either β-Ala or L-Cys. The β-Ala series of cyclic compounds, **17** (21% inhibition) and **21** (42% inhibition), were less potent than the respective L-Cys series, **15** (86% inhibition) and **19** (45% inhibition). The β-Ala residue is unable to extend into the hydrophobic pocket whereas the L-Cys side chain, although only two bonds, may still be able to interact with the catalytic subunit. This does, however,

provide some evidence that further addition of a hydrophobic, Adda-like side chain to the L-Cys will increase inhibition of PP2A.

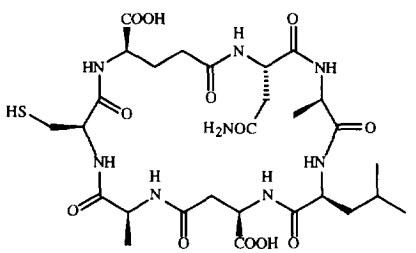
Figure 3: Synthetic cyclic peptides **15**, **19**, and **21** modelled on microcystin and the synthetic linear peptide **12** modelled on nodularin.



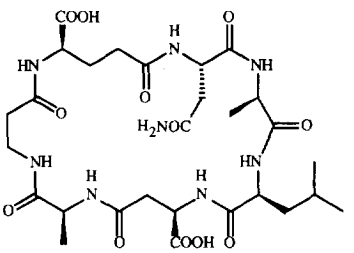
12



15



19



21

The structures of the synthetic peptides **15**, **19**, and **21** are shown in Figure 3. The most active compound **15** does not contain either the D-isoAsp or the D-isoGlu residues of the parent compound. The size of the ring system was therefore different than that of microcystin-LR but similar to nodularin. Compound **19** was structurally similar to microcystin-LR, containing the D-isoAsp and D-isoGlu residues and exhibited lower potency with only 45% inhibition of PP2A activity compared with 86% for **15.** Conversion from the α-linked acidic residues to the β and γ-linked acidic residues not only affects the ring size but also the potency of these cyclic compounds. These results support the hypothesis that the size of the cyclic core does not determine inhibitory potency and indicate that the presence of iso-linked amino residues is not essential for inhibition of PP2A.[2] The conserved acidic group may still be able to interact with the catalytic subunit whether the acids are incorporated into the peptide backbone by either their α, β or γ acids. The extension of the glutamic carboxylic acid from the cyclic core appeared to increase inhibition of PP2A in this series of compounds.

The cyclic peptide **21** contains an aspartamate side chain in place of the microcystin methyldehydroalanine residue while **7** contains an alanine side chain. The presence of the primary amide resulted in an increase in inhibition from 3% for **7** to 42% for **21**. The primary amide appears to be involved in binding to the catalytic site of PP2A.

The two linear peptides **12** and **13** were modelled on nodularin. Nodularin contains a slightly different peptide backbone than microcystin-LR, cyclo(Adda-D-isoGlu-MBut-D-MeisoAsp-L-Arg).[1, 9] The IC_{50} of **12** was determined to be between 1-10mM, but due to poor solubility an accurate result was not possible.

Comparison of the inhibitory activity of compounds **12** and **13** showed that conversion from the α-linked to the β and γ-linked acidic residues resulted in a decrease in potency from 30% to 19% inhibition. This supports the results observed for the cyclic compounds **15** and **19**.

The crystal structure of mammalian PP1 complexed with microcystin-LR[10] confirms the major aspects of the conserved acid binding domain pharmacophore model, a full analysis will be reported elsewhere. The glutamic acid carboxyl group and the Adda carbonyl group bind to the metal-binding site via metal-liganded water. In addition, the Masp carboxyl group hydrogen bonds to Arg 96 and Tyr 134. Compounds **12**, **15**, **19**, and **21** retain these structural features and would be expected to occupy the same binding site as microcystin-LR but at lower affinity. It was interesting to observe that the cyclic template has intrinsic, but low, PP2A inhibitory activity. Further work to add Adda-like hydrophobic side chains to the cyclic precursors is in progress.

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7. PP2A enzyme was prepared following the method reported in Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Nishiwaki-Matsushima, R.; Sugimura, T. *FEBS Lett.* **1991**, *279*, 115. PP2A enzyme (heterotrimeric complex ABC type: A (67kDa), B (58kDa), and C (41kDa) was isolated from mouse brain and partially purified by DEAE-cellulose column chromatography using 50 mM Tris-HCl buffer (pH 7.4). Partially purified PP2A was eluted with buffer containing 0.2 M NaCl. The enzyme activity was measured in 50 mM Tris-HCl buffer (pH 7.0) containing 100 mM EDTA, 5 mM caffeine, 0.1% 2-mercaptoethanol, 0.6 mg/mL BSA, and [^{32}P]phosphorylase *a*.
8. The inhibition of PP2A activity by microcystin-LR was determined by incubation of [^{32}P]phosphorylase *a* (the substrate), PP2A and microcystin-LR (6 concentrations ranging from 0.001 to 100 nM) for 10 min at 30 °C in 100 μL volume, in duplicate. PP2A activity was determined by the liberation of [^{32}P]phosphate into the supernatant after precipitation of protein with 100 mL of ice-cold 50% trichloroacetic acid. After centrifugation an aliquot (150 μL) of supernatant was counted in Amersham Aquasol scintillant. Data was expressed as percent inhibition with respect to a control (absence of competing compound) incubation.
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