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SYNTHESIS OF CYCLIC PEPTIDES MODELLED ON THE MICROCYSTIN AND NODULARIN RINGS.

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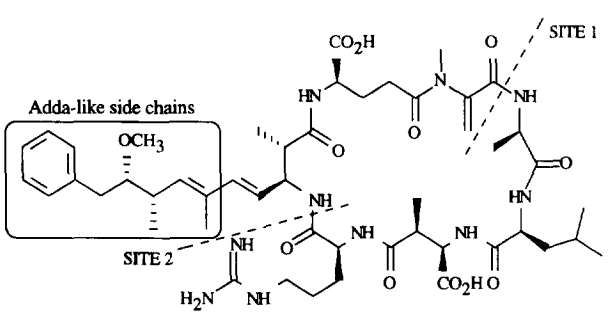
Abstract: Several precursor cyclic peptides modelled on the ring systems of microcystin-LR and nodularin were synthesized. Synthesis was best achieved using solid-phase cyclization with solution-phase methods being successful in only one instance. These precursor cyclic peptides are suitable for elaboration with Adda-like hydrophobic side chains in order to produce structure-activity information.

Microcystin-LR is a naturally occurring protein phosphatase inhibitor (Figure 1).[1] Numerous groups world-wide are currently involved in the isolation and characterization of naturally occurring microcystins.[2] Even so, there remains little structure-activity data available. Several derivatives of the microcystins have been semi-synthesized from the natural products in an attempt to further understand the mechanism by which they interact with the protein phosphatases.[2-4]

The majority of the synthetic work has concentrated on the novel Adda amino residue. Hydrogenation of the Adda diene system produced an inactive derivative.[3] Likewise, ozonolysis produced an inactive microcystin derivative. The free Adda amino acid obtained by hydrolysis of microcystin-LR and nodularin was unstable and, as a result, the toxicity of the naturally occurring free Adda has not yet been determined. The N-protected and unprotected Adda amino residues have been synthesized and in both instances there was little or no toxicity shown in mouse liver assay.[4] These results suggested that the Adda itself was not active, and that other factors such as functionality, shape or spatial alignment of other amino acids were important. Recently, the total synthesis of motuporin was reported, however, no toxicity data was available.[5]

Figure 1: Sites of ring closure in microcystin-LR.



Preliminary synthetic studies of the peptidic backbone of the microcystins were reported.[4] Linear analogues of three microcystins, M-RR, M-YM, and M-LA, were synthesized. The novel amino residue Adda was not incorporated in the synthesis of the three analogues, nor was it replaced by another hydrophobic residue. Intraperitoneal injections of 10-1000 mg/mL aliquots of the synthetic analogues in saline were not lethal

and did not reveal any other signs of toxicity in white mice. All three analogues were found to be inactive. It was concluded that the Adda residue was essential for activity.

In light of the limited structure-activity studies on the microcystins, the synthesis of both linear and cyclic peptide precursors modelled on the microcystin ring was undertaken. Two cyclization sites were examined, as shown in Figure 1. It was envisaged to subsequently add Adda and Adda-like side chains (Figure 1) to these precursors in order to provide structure-activity information. In order to design target compounds, the structures of the known microcystins were considered. Table 1 summarizes the amino residues tolerated in each position within the microcystin and nodularin cyclic cores. Using this information as a guide, a template of the microcystins was developed.

Table 1: Variation of the Amino Acid Residues Within the Microcystin and Nodularin Families.

Residue Number	Amino Acid Residues In Microcystin-LR	Variations of Microcystin-LR	Variation of Nodularin and Motuporin
1	D-Ala	D-Ser	----------
2	L-Leu	L-Ala, L-Arg, L-Hty, L-Met, L-Met(O), L-Phe, L-Trp , L-Tyr	----------
3	*erythro*-β-methyl-D-aspartic acid	D-isoaspartic acid	*erythro*-β-methyl-D-isoaspartic acid
4	L-Arg	L-Aba, L-Ala, L-Har, L-Hph, L-Leu, L-Met L-Phe, L-Tyr, L-Val	L-Arg L-Val
5	Adda	ADMAdda, DMAdda	ADMAdda, DMAdda
6	D-isoglutamic acid	NOT VARIANT	NOT VARIANT
7	*N*-Methyl dehydroalanine	L-Ala, L-Ser Dehydroalanine *N*-Methyl serine	*N*-Methyl dehydrobutyric acid

Both D-Ala (residue 1) and L-Leu (residue 2) can be substituted without affecting activity and both residues are absent in nodularin, suggesting that they are not essential for activity. D-Ala and L-Leu were chosen for the template (Table 2). The *erythro*-β-methyl-D-aspartic acid residue can tolerate the loss of the methyl group with little effect on the biological activity, therefore D-isoaspartic acid was chosen for the template. L-Arg can be replaced with a number of L-amino residues with little or no change in phosphatase activity and L-Ala was included in the template. Molecular modelling studies suggest that other hydrophobic groups such as the side chains of okadaic acid (terminal spiroketal) and calyculin A (nitrile side chain) occupy the same hydrophobic pocket as Adda.[6] In order to probe this region of the molecule, several precursor peptides, containing either β-Ala, L-Ala, L-Cys or L-Asp at the Adda position, were synthesized. Side chains of varying lengths, chemical composition and three dimensional shape may later be incorporated. The D-isoGlu residue is non-variant and was included in the template. Mdha can be replaced by L-Ala, Dha, Mser or L-Ser in the microcystins and by *N*-methyl dehydrobutyric acid in the nodularins. L-Ala was included in the template.

The D-isoGlu and *erythro*-β-methyl-D-isoAsp/D-isoAsp residues are conserved in all microcystins, nodularins, and motuporin. A second template (Table 2) was designed, incorporating these two acidic residues via their α-carboxylic acids to establish the optimum ring size and shape for activity.

Table 2: Template

Residue Number	1	2	3	4	5	6	7
Microcystin-LR	D-Ala	L-Leu	*erythro*-β-methyl-D-isoaspartic acid	L-Arg	Adda	D-isoGlu	*N*-Methyl dehydroalanine
Template 1	D-Ala	L-Leu	D-isoaspartic acid	L-Ala	Various	D-isoGlu	L-Ala
Template 2	D-Ala	L-Leu	D-Asp (or β-Ala)	L-Ala	Various	D-Glu	L-Ala

Linear peptides were prepared using an in situ neutralization protocol for Boc-protection chemistry.[7] The crude peptide mixtures were obtained using standard HF cleavage protocols, precipitated with ice-cold ether, re-dissolved with 20% acetic acid and lyophilized. The crude synthetic peptides were analyzed by HPLC, MS, and NMR. For investigation of site 1 cyclization, four heptapeptides (**1-4**) were synthesized (Table 3). In order to lower the number of cyclization sites the D-isoAsp/D-Asp (residue 3) was replaced with β-Ala in two additional heptapeptides **5** and **6** (Table 3).

Table 3: Site 1 Cyclization
(Residue Number 5, corresponding to Adda is highlighted)

Peptide	
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Asp**-D-isoGlu-L-Ala-CO_2H	**1**
H_2N-D-Ala-L-Leu-D-Asp-L-Ala-**L-Asp**-D-Glu-L-Ala-CO_2H	**2**
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala- **β-Ala**-D-isoGlu-L-Ala-CO_2H	**3**
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Ala**-D-isoGlu-L-Ala-CO_2H	**4**
H_2N-D-Ala-L-Leu- β-Ala-L-Ala-**L-Asp**-D-isoGlu-L-Ala-CO_2H	**5**
H_2N-D-Ala-L-Leu- β-Ala-L-Ala-**L-Asp**-D-Glu-L-Ala-CO_2H	**6**

The peptide **3** (5 mg), dissolved in DMF, was stirred continuously with the activating agents, HBTU [*O*-(benzotriazol-1-yl)1,1:3,3-tetramethyluronium hexafluorophosphate] and DIEA [diisopropylethylamine]. After 30 mins the parent peptide, *m/z* 660Da (ion spray MS), was absent and a new peak at *m/z* 642Da detected. Partially pure cyclo(-D-Ala-L-Leu-D-isoAsp-L-Ala-β-Ala-D-isoGlu-L-Ala-) (**7**) (R_t 21.12 mins, 0.39 mg, yield = 7.8%) was obtained by HPLC. The molecular ion, *m/z* 642Da, was subjected to tandem mass spectrometry (MS/MS). Table 4 lists the b-series fragmentation generated from the MS/MS of **3** and **7**. A loss of 18Da could also result from formation of either the Glu succinimide or Asp succinimide products. Formation of the succinimides was ruled out by comparison of the expected daughter ions with those actually observed (Table 4).

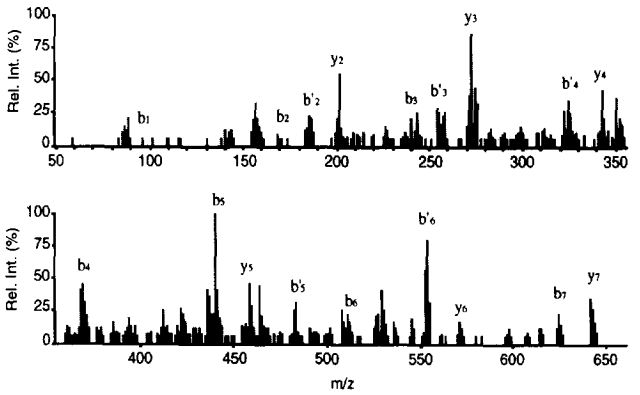
Table 4: Observed and Expected Masses for Daughter Ions of the b-Series Generated by CID of the Molecular Ion of **3**, **7**, and Related By-products. (a. Observed Masses, b. Expected Masses).

Compound	b1	b2	b3	b4	b5	b6	b7
3 [a]	72.0	185.1	300.2	371.1	442.1	571.3	642.0
3 Glu suc [b]	72.0	185.1	300.2	371.1	442.1	---	624.1
3 Asp suc [b]	72.0	185.1	---	353.1	424.1	553.3	624.1
7 [a]	98.0	169.1	240.1	369.0	439.8	511.0	624.1

The two sequences Ala^1-Leu^2-Asp^3-Ala^4-Ala^5 and Leu^2-Ala^1-Ala^7-Glu^6-Ala^5-Ala^4 were identified from the MS/MS spectrum of **7** (Figure 2). The second sequence confirms the cyclic nature of the peptide indicating bond formation between the two terminal residues Ala^1 and Ala^7. The MS/MS data not only identified the two sequences confirming the cyclic nature of **7**, but also ruled out the two other possible cyclic products, cyclo(A-L-D-A-A-G)-A and cyclo(A-L-D)-A-A-G-A.

Assignment of the cyclic product was based on comparison with the NMR data obtained for the linear parent.[8] Six peaks were observed in the amide bond region of the ^1H NMR of the linear parent, **3**. The proton signals in this particular region could not be easily distinguished, however, integration of this region indicated the presence of seven hydrogens. Coupling was observed between the α proton of the D-Ala residue, 4.14 ppm, and its NH proton, 7.94 ppm, in the DQF-COSY spectrum. This coupling indicated the D-Ala residue was no longer a N-terminal residue and that cyclization was successful. The NOESY spectrum showed through space interactions between the D-Ala^1 residue and the L-Ala^7 residue, confirming the cyclic nature of the peptide.

Figure 2: MSMS Spectrum of **7**.



The two activating agents, BOP [(benzotriazolyloxy) tris (dimethylamino) phosphonium hexafluorophosphate] and HATU [*O*-(7-azabenzotriazol-1-yl)-1,1:3,3-tetramethyluronium hexafluorophosphate] did not improve the yield of cyclization of **3**.

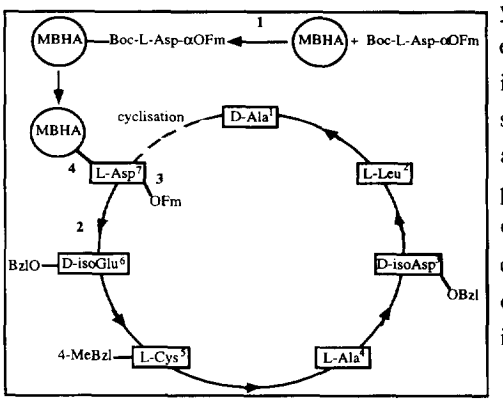
Solution cyclization of the linear precursors **1**, **2**, **4**, and **5** was unsuccessful. A peak corresponding to the cyclized product of **6** was detected, however, it was present in trace amounts only and could not be characterized by MS/MS. The yield could not be improved even though the concentration of the peptide was increased and the reaction mixture allowed to stir for 24 h.

Cyclization at site 2 was investigated with four heptapeptides (**8**-**11**) and two pentapeptides based on nodularin (**12**, **13**) (Table 5). Solution cyclization was unsuccessful for all six peptides.

Table 5: Site 2 Cyclization
(Residue Number 5, corresponding to Adda is highlighted)

Peptide	
H_2N--**β-Ala**-D-Glu-L-Ala-D-Ala-L-Leu-D-Asp-L-Ala-CO_2H	**8**
H_2N--**β-Ala**-D-isoGlu-L-Ala-D-Ala-L-Leu-D-isoAsp-L-Ala-CO_2H	**9**
H_2N--**L-Cys**-D-Glu-L-Ala-D-Ala-L-Leu-D-Asp-L-Ala-CO_2H	**10**
H_2N--**L-Cys**-D-isoGlu-L-Ala-D-Ala-L-Leu-D-isoAsp-L-Ala-CO_2H	**11**
H_2N--**β-Ala**-D-Glu-L-Ala-D-Asp-L-Ala-CO_2H	**12**
H_2N--**β-Ala**-D-isoGlu-L-Ala-D-isoAsp-L-Ala-CO_2H	**13**

Figure 3: Schematic of Solid-phase Cyclization.



Solution cyclization of linear peptides tends to be a low yielding step, usually requiring high-dilution conditions and extensive purification. Solid-phase peptide cyclization was investigated (Figure 3). The synthetic approach involved (1) side chain anchoring of an initial partially protected amino acid residue to a resin support, (2) stepwise solid-phase peptide synthesis using an in situ neutralization protocol, (3) orthogonal deprotection to selectively liberate a free Cα-carboxyl group for subsequent cyclization, and (4) final deprotection and cleavage to release the free cyclic peptide into solution.

The four linear precursors, **14**, **16**, **18**, and **20** were cyclized at site 1 on resin (Table 6). These linear peptides were bound to the MBHA resin via the γ-carboxyl group of Boc-L-Asp-α-OFm. The α-carboxyl group was selectively deprotected and subsequent cyclization was achieved using HBTU and DIEA. The four cyclic peptides and their linear precursors were cleaved from the resin with HF and analysed by HPLC, MS, MS/MS, and NMR. The MS of the crude cyclic peptides showed the major product in each case was 18Da less than the

parent peptide. MS/MS data indicated the compounds were cyclic in nature. Assignment of the cyclic products was simplified by the fact that there was only one possible cyclization product.

Table 6: Solid-phase Site 1 Cyclization
(Residue Number 5, corresponding to Adda is highlighted)

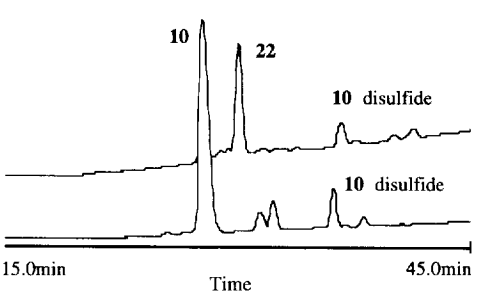
Linear		Cyclo(- -)	
H_2N-D-Ala-L-Leu-D-Asp-L-Ala-**L-Cys**-D-Glu-L-Asp-$CONH_2$	**14**	(-D-Ala-L-Leu-D-Asp-L-Ala-**L-Cys**-D-Glu-L-Asp-)	**15**
H_2N-D-Ala-L-Leu-D-Asp-L-Ala- β-**Ala**-D-Glu-L-Asp-$CONH_2$	**16**	(-D-Ala-L-Leu-D-Asp-L-Ala-β-**Ala**-D-Glu-L-Asp-)	**17**
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Cys**-D-isoGlu-L-Asp-$CONH_2$	**18**	(-D-Ala-L-Leu-D-isoAsp-L-Ala-**L-Cys**-D-isoGlu-L-Asp-)	**19**
H_2N-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Asp-$CONH_2$	**20**	(-D-Ala-L-Leu-D-isoAsp-L-Ala-β-**Ala**-D-isoGlu-L-Asp-)	**21**

The structures of all the linear and cyclic peptides were verified by analysis of their ^{1}H NMR, DQF-COSY and NOESY spectra. The cyclic peptides were assigned by comparison with their linear precursors. Seven peaks were observed in the amide bond region of the ^{1}H NMR of the cyclic peptide **21** compared with only six for the linear peptide **20**. These seven peaks verified the presence of seven amide bonds, as expected for a cyclic product. The αH of the D-Ala residue coupled to a methyl proton signal at 1.15 ppm and an amide proton signal at 7.94 ppm in the DQF-COSY spectrum indicating that it was no longer an N-terminus residue. The NOESY spectrum of **21** showed through space interactions between the Asp($CONH_2$) and D-Ala residues and between the Asp($CONH_2$) and L-Leu residues. Through space interaction of Asp($CONH_2$) to D-Ala confirmed the cyclic nature of this peptide. The other cyclic peptides were characterized following the same procedure.

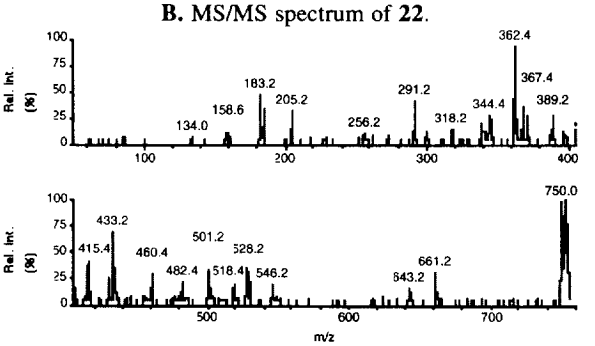
In order to directly compare the two cyclization methods, solution cyclization of peptide **14**, which was successfully cyclized on resin, was attempted. The solution cyclization was unsuccessful.

Preliminary experiments were also conducted incorporating a model side chain at the L-Cys residue. Bromoacetic acid was added to a solution of **10** dissolved in 0.1 M $NaHCO_3$, and the mixture stirred for 1 h. Aliquots of a control experiment and the reaction mixture were injected into the ion spray mass spectrometer. Starting material, *m/z* 692Da, was detected in the control. The ligated compound, H_2N-L-Cys(CH_2CO_2H)-D-Glu-L-Ala-D-Ala-L-Leu-D-Asp-L-Ala-CO_2H (**22**), was detected at *m/z* 750Da, in the reaction mixture. HPLC analysis of the control showed the presence of the starting material, as well as the disulfide. HPLC analysis of the reaction mixture showed the presence of a major product, **22**, and the disulfide (Figure 4A). MS/MS analysis of **22** confirmed the extension of the side chain at the L-Cys residue (Figure 4B). Unfortunately there was not enough of the ligated product available to confirm the structure by NMR.

Figure 4: A. HPLC of **10** and **22.**



B. MS/MS spectrum of **22.**



This study showed that both solution-phase and solid-phase cyclization of linear peptides was possible at site 1, but that solid-phase cyclization was more successful. At site 2 solution-phase cyclization was unsuccessful. It was also shown that alkylation of the precursor peptides was possible. Protein phosphatase activity of these precursor peptides is reported in the following paper.

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8. The ^{1}H and 2D NMR spectra of all synthetic peptides were obtained in DMSO-d_6 using a Varian 600Mhz NMR instrument.

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