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Synthesis of Functionalised Cyclic Pentapeptide Analogues of the Serine-Threonine Protein Phosphatase Inhibitor Nodularin.

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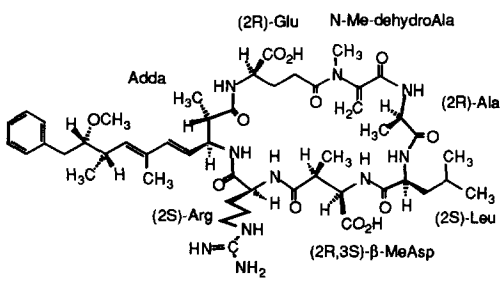
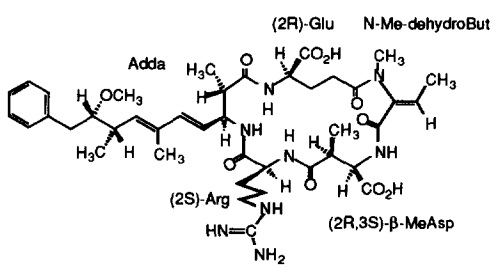
Abstract. A generic synthesis of cyclic peptidic analogues of nodularin incorporating suitable functionality for synthetic elaboration is described, providing access to new protein phosphatase inhibitors.

It is now widely accepted that the reversible phosphorylation of proteins on serine, threonine and tyrosine residues, as catalysed by protein kinases and phosphatases, is the principal mechanism by which eukaryotic cells respond to external stimuli.[1,2] The natural cyclic isopeptides microcystin (**1**) and nodularin (**2**), previously established as potent hepatotoxins,[3] are now known to inhibit the catalytic subunit of mammalian serine-threonine protein phosphatases PP1 and PP2A with subnanomolar inhibition constants.[4] There has been heightened



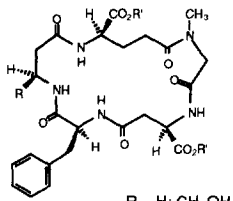
Microcystin-LR **1**



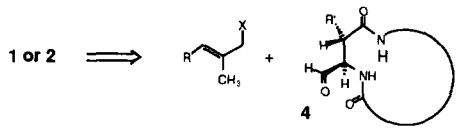
Nodularin **2**

awareness of the extremely toxic properties of microcystins (cyanoginosins) as reservoir water contaminated with cyanobacteria (blue-green algae) has caused death.[3] Liver cells possess a special ability to take-up microcystins which explains their susceptibility to such toxins. The powerful tumour promotor okadaic acid, responsible for diarrhetic shellfish poisoning, and calyculin A, tautomycin and cantharidin are also inhibitors of cat-PP1 and cat-PP2A.[5] These enzymes are highly homologous (~50% identical)[2] and there has been much interest in understanding how such diverse structures might interact with the enzymes. The only structural information for Ser-Thr PP'ases is derived from a very recent X-ray crystal structure of a cat-PP1-microcystin complex.[6] Our interests were to synthesise new analogues of microcystin and nodularin to probe the active-site binding interactions. Specific inhibitors for either PP1 or PP2A are not presently available but are required to delineate the individual physiological roles of the enzymes. Here we describe a short efficient synthesis of rationally designed stripped-down nodularin macrocycles (**3**, R = H and R = CH_2OH), and some of their properties.

Both microcystins and nodularins contain an Adda residue, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, or structural variants. The stereochemistry of the double bond at C-6 is not crucial for biological activity[7], thus, structurally diverse lipophilic groups might be introduced in place of the Adda side-chain. The first disconnection was, therefore, introduced between C-4 and C-5 to give an allyl halide and the macrocyclic peptidic aldehyde (**4**, R' = CH_3), Scheme 1, and attention was turned to the construction of suitable macrocycles.



3 R = H; CH_2OH
R' = CH_3, Li^+



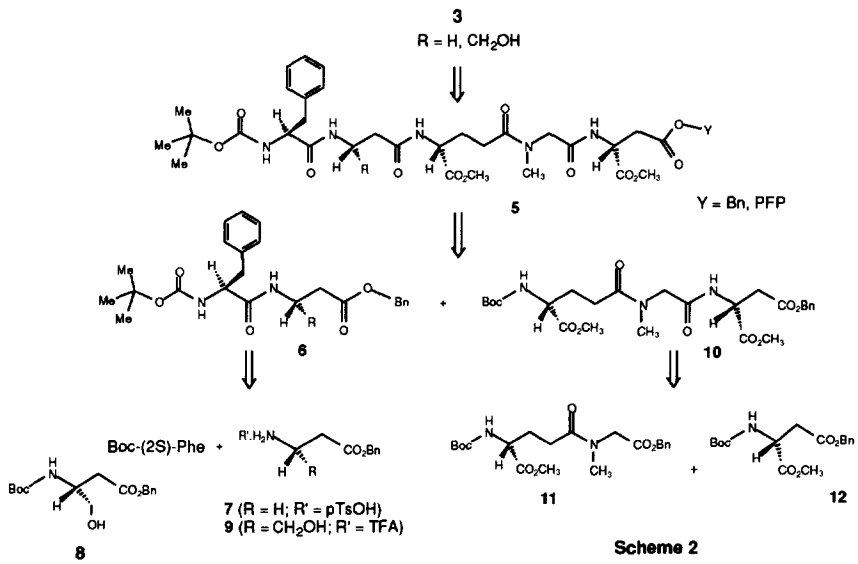
Scheme 1

Over 50 structurally diverse variants of these macrocycles (**1**) and (**2**) are known and all tested examples show biological activity. For example, microcystin-LR contains the variable amino acids (2S)-Leu and (2S)-Arg whereas for nodularin (**2**), the Arg residue can be replaced by (2S)-Val to give motuporin.[8] In defining a target for the synthesis of the macrocycle it was reasoned, on the basis of computer aided design, that the Arg residue in (**1**) or (**2**) could be replaced by Phe and that a methyl group from each of the two β-amino acids could be removed without significant detriment to activity. To test the importance of N-methyldehydroalanine in conferring biological activity by serving as a Michael acceptor, microcystin-LR (**1**) was reduced with sodium borohydride in wet methanol to give a mixture of dihydromicrocystin diastereomers (*ca.* ~5:1), each of which were potent inhibitors of cat-PP2A.[9] Although it is now known that Cys-273 of cat-PP1 does form a Michael adduct with the N-methyl dehydroalanine residue of microcystin,[6] other workers have reached similar conclusions to our own by showing that the replacement of Cys-273 by alanine does not significantly compromise the inhibitory properties of microcystin.[10] Hence, covalent bond formation is a secondary event and must occur reversibly. The results with the dihydromicrocystins also indicated that the stereochemistry, and indeed, hybridisation state at C-2 of the N-methylated residue is not important in conferring activity. Rationalising this information in the design of a target for synthesis allows the N-methyldehydrobutyrine residue in nodularin to be replaced by sarcosine to give macrocycle (**3**, R = CH_2OH). This was disconnected as shown in Scheme 3.

The γ-glutamyl sarcosine diester (**11**) was prepared by treating N-Boc (2R)-glutamic acid γ-benzyl ester with ethereal diazomethane followed by catalytic hydrogenolysis of the benzyl ester. Treatment of the activated γ-carboxy group with sarcosine benzyl ester tosylate salt in THF/DMF gave amide (**11**) in 90% overall yield. This was subjected to hydrogenolysis to uncover the sarcosine carboxy group which was activated and then treated with the (2R)-aspartate α-methyl β-benzyl diester [derived from the deprotection of (**12**) with dry HCl] to give the common intermediate tripeptide triester (**10**) in 92% yield. The N-Boc protection was removed from (**10**) using hydrogen chloride gas in ethyl acetate in 89% yield. Note that compound (**12**) was itself prepared from the α-free acid and diazomethane.

The synthesis of the peptidic macrocycle was first optimised using an unsubstituted β-alanine benzyl ester, compound (**7**). Reaction with mixed anhydride activated N-Boc (2S)-phenylalanine gave the dipeptide benzyl ester (**6**, R = H) in 85% yield. Removal of the benzyl ester protection followed by activation and treatment with the N-terminal deprotected tripeptide triester derived from (**10**) gave the pentapeptide triester (**5**, R = H, Y = Bn) in 81% yield. The C-terminal benzyl ester of (**5**, R = H, Y = Bn) was removed through catalytic hydrogenolysis and was replaced by a pentafluorophenyl ester group (PFP group), using pentafluorophenol and EDCI in 69%

yield after chromatographic purification. Deprotection of the N-Boc pentapeptide PFP ester (**5**, R = H, Y = PFP) with TFA in dichloromethane gave the amine TFA salt which was thoroughly dried under high vacuum and then treated with DIPEA in dichloromethane. After several days at 20 °C, the required macrocycle (**3**, R = H) formed and was isolated in 45% yield. The compound and all of its intermediates were fully characterised[11] and NMR spectra showed the existence of three stable populated conformations. A small sample was quantitatively converted to the dilithium salt (**3**, R = H, R' = Li^+) with lithium hydroxide in aqueous methanol. Note in addition to those reported here, several other cyclisation conditions were tried and alternative disconnections were tested (*e.g.*, macrolactamisation between the (2R)-aspartyl amino group and sarcosine), all with no success. However, the strategy described here has successfully given other nodularin and microcystin macrocycles which will be reported in due course.

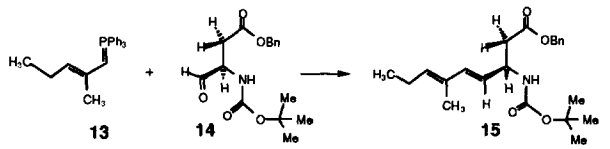


Scheme 2

In order to prepare the hydroxymethyl analogue (**3**, R = CH_2OH), N-Boc (2R)-aspartic acid β-benzyl ester [the synthetic precursor to (**12**), *vide supra*] was converted to the mixed carbonic anhydride and this was reduced with sodium borohydride in THF to give the alcohol (**8**) in 89% yield. The N-Boc protection was removed using TFA in dichloromethane to give the amine salt (**9**) which was coupled with carboxy group activated N-Boc (2S)-phenylalanine, as described previously, to afford the required dipeptide alcohol (**6**, R = CH_2OH) in 95% yield. This compound was taken through to pentapeptide (**5**, R = CH_2OH, Y = OBn) without incident (in 83% yield) and the benzyl ester was converted to the pentafluorophenyl ester (**5**, R = CH_2OH, Y = PFP). Deprotection of the N-Boc group using TFA, as described previously, followed by treatment with DIPEA gave the required peptidic macrocycle (**3**, R = CH_2OH) in 25% yield, which displayed the expected properties.[11]

Modelling studies using the crystal coordinates for the cat-PP1-microcystin complex[6] suggest that nodularin should exist in a conformation which closely matches that for microcystin in the region spanning the toxin's Glu, Adda and MeAsp residues such that the Asp carboxy group can interact with the guanidino moiety of Arg-96 on the enzyme. This conformation places the Adda diene group in a hydrophobic cleft. In support of SAR studies, the peptidic macrocycle (**3**, R = CH_2OH) will allow a range of non-natural lipophilic groups to be introduced into the Adda side chain position including those derived from esters (**3**, R = CH_2OCOR'), amides (**3**, R = CONR'R'') and olefins. The strategy for the diene was tested in a model reaction through treatment of the allylic phosphonium ylide (**13**) with the aldehyde (**14**) [prepared through periodinane oxidation in 90% yield from the

alcohol (**8**)] Scheme 3. The diene (**15**) was obtained in moderate unoptimised yield (36%) and this strategy will be used to incorporate the same diene moiety into macrocycle (**3**, R = CH_2OH) after oxidation to the aldehyde.



Scheme 3

Note that during the course of our studies a synthesis of motuporin was reported.[12] The strategy described here differs and is suitable for the introduction of structural diversity into the Adda position.

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Abbreviations.
AHB, 3-amino-4-hydroxybutyric acid; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

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11. All compounds were *fully* characterised and gave the expected spectral and analytic data. Mass data for compound (**3**; R = H; R' = CH_3). *m/z* (Found: $[M + H]^+$, 562.2513. $C_{26}H_{36}N_5O_9$ requires 562.2506); *m/z* (CI) 562 (6%, $[M + H]^+$), 530 (76, $[M + H - CH_3OH]^+$) and 59 (100, $CH_3CO_2^+$) confirmed that cyclisation had occurred. Two conformations (rotomers) were evident upon the immediate dissolution of the crystalline material in d_6-DMSO which slowly converted to two new rotomeric forms. ^1H- and ^{13}C-NMR spectra were *fully* assigned using COSY, TOCSY and HSQC techniques, full details of which will be reported in due course. The compound was sparingly soluble in C^2HCl_3 and existed largely in one of three conformations which could be distinguished by ^1H- and ^{13}C-NMR spectroscopy. Compound (**3**; R = CH_2OH; R' = CH_3) displayed the expected differences in mass and NMR spectra to the parent macrocycle; *m/z* (Found: $[M - CH_3]^+$, 576.2306. $C_{26}H_{34}N_5O_{10}$ requires 576.2291); *m/z* (CI) 591 (2%, M^+), 576 (6, $[M - CH_3]^+$) 542 (22, $[M + H - CH_3OH - H_2O]^+$) and 85 (100, $C_2H_2CO_2CH_3^+$); δ_H (500 MHz; C^2HCl_3) 1.84 (1H, m, 3-H_A, Glu), 2.35 (1H, m, 3-H_B, Glu), 2.46 (1H, m, 2-H_A, AHB), 2.47 (1H, m, 4-H_A, Glu), 2.57 (1H, m, 4-H_B, Glu), 2.69 (1H, m, 3-H_A, Asp), 2.74 (1H, m, 2-H_B, AHB), 2.77 (1H, m, 3-H_B, Asp), 3.04 (1H, m, 3-H_A, Phe), 3.09 (3H, s, N-Me), 3.23 (1H, m, 3-H_B, Phe), 3.74 (3H, s, OMe), 3.77 (3H, s, OMe), 4.21 (1H, m, 3-H, AHB), 4.49 (1H, m, 4-H_B, AHB), 4.54 (1H, m, 2-H, Glu), 4.64 (1H, m, 2-H, Phe), 4.79 (1H, m, 4-H_A, AHB), 5.68 (1H, d, *J* 7.9, NH, Glu), 6.67 (1H, d, *J* 7.3, NH, Phe), 7.17-7.33 (5H, m, aromatic), 7.37 (1H, d, *J* 6.5, NH, Asp) and 7.47 (1H, d, *J* 5.8, NH, AHB), full details will appear elsewhere.
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