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Incrementally increasing the length of a peptide backbone: effect on macrocyclisation efficiency†

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Md. Iqbal Ahmed, Jason B. Harper and Luke Hunter*

Three novel analogues of the cyclic pentapeptide sansalvamide A have been synthesised in high yield. A leucine residue in the lead compound is replaced with either a glycine, β-alanine or GABA residue, and the corresponding linear precursor peptides are found to cyclise with dramatically improved efficiency. This correlates with an increase in the effective molarity (EM) of the cyclisation reactions.

Peptide based drug design is an important branch of contemporary medicinal chemistry research.[1] Of particular interest are small peptides that can modulate protein–protein interactions, fundamental process that are involved in a myriad of diseases including cancer, metabolic disorders, and diseases associated with hormone dysfunction.[2] Cyclic peptides often exhibit superior biological activity relative to their linear counterparts, due to their conformational rigidity and resistance to proteolytic degradation.[3] However, a limiting factor in the development of cyclic peptide drugs is that their synthesis is often very inefficient. During the macrocyclisation process, there are energetic and entropic costs associated with preorganising the linear precursor peptide into a conformation that is amenable to cyclisation. This can lead to the appearance of sideproducts arising from polymerisation, cyclooligomerisation and C-terminal epimerisation processes, which erode the yield of the desired cyclic peptide.[3] The difficulty is greatest in the case of peptides containing seven or fewer amino acids, as these have the least conformational mobility.[3,4]

Several strategies have been developed to improve the efficiency of peptide macrocyclisation,[3] for example employing highly dilute reaction conditions,[5] incorporating turn-inducing components such as pseudoproline residues into the linear precursor,[6] exploiting a two-step ring contraction strategy to access small cyclic peptides,[7] and harnessing the templating effect of a suitably-sized metal ion during cyclisation.[8] However, despite these considerable advances no individual method is suitable for every peptide, and so there is an ongoing need to develop new strategies for improving the efficiency of peptide cyclisation.

A potential new strategy is inspired by the recently reported total synthesis of unguisin A (**1**, Fig. 1).[9] This natural product is a cyclic heptapeptide derived from the marine fungus *Emericella unguis*,[10] and it uniquely contains the nonproteinogenic amino acid γ-aminobutyric acid (GABA) within the ring. During the total synthesis of the peptide **1**,[9] cyclisation of the corresponding linear precursor peptide was found to be exceptionally rapid and efficient (<1 min reaction time, 81% isolated yield after preparative HPLC), and the ease of cyclisation was attributed, in part, to the flexibility imparted by the GABA residue that was positioned in the centre of the linear precursor peptide. It was hypothesised that this result could form the basis of a more general strategy for assisting the cyclisation of

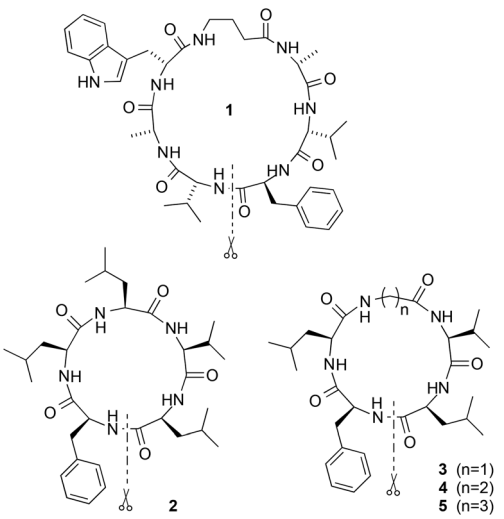

Fig. 1 Structures of unguisin A (**1**),[9,10] sansalvamide A amide (**2**),[11,12] and the new targets of this work (**3**–**5**).

School of Chemistry, UNSW Australia, Sydney, NSW 2052, Australia.
E-mail: l.hunter@unsw.edu.au

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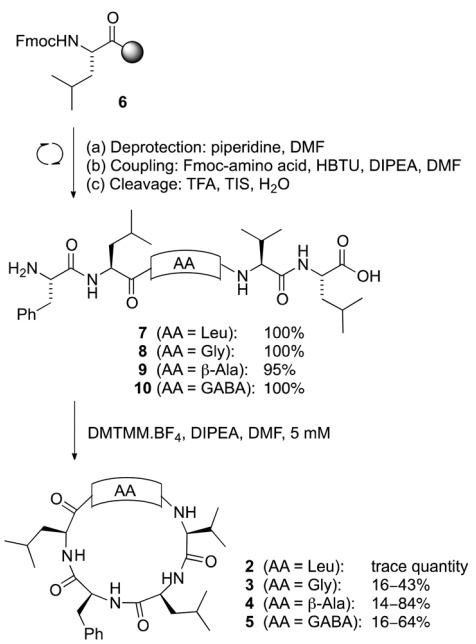
analogues of "difficult" peptides: the concept would be to incrementally increase the backbone length of one constituent amino acid, and investigate whether the resulting increase in flexibility could improve the cyclisation efficiency.

For the present work, the cyclic pentapeptide sansalvamide A amide (**2**, Fig. 1) was selected as a test case to investigate this hypothesis. Peptide **2** is a heat shock protein 90 inhibitor, and has previously been shown to exhibit potent *in vitro* activity against multiple cancer cell lines including colon, pancreatic, breast and prostate.[11] However, the medicinal development of this lead molecule is somewhat hampered by difficulties associated with peptide cyclisation: although a large number of analogues of **2** have been synthesised they are often obtained in very low (<1%) yield.[11] Previous structure–activity relationship studies have shown that alteration of one leucine residue of **2** is possible without eroding the biological activity.[12] Therefore, the new targets of the present work are peptides **3–5** (Fig. 1), in which a leucine residue of the parent compound **2** is replaced with glycine, β-alanine and GABA respectively. If the new analogues turn out to be more synthetically accessible this could benefit the medicinal development of the lead compound **2**, but more importantly this could also represent a general approach for synthesising analogues of other "difficult" cyclic peptides.

The requisite linear precursor peptides (**7–10**, Scheme 1) were readily assembled by solid phase peptide synthesis, employing Wang resin as the solid support and HTBU/DIPEA as the coupling reagents.[13] With the linear peptides **7–10** in hand, attention was next turned to their macrocyclisation. For these reactions, identical conditions were employed to those reported for the total synthesis of unguisin A (**1**),[9] *i.e.* the coupling reagents DMTMM·BF_4/DIPEA were used,[13] and the reactions were performed at 5 mM peptide concentration. Under these conditions none of the lead compound (**2**) was isolated (Scheme 1).[14] Gratifyingly however, a dramatic improvement was observed for each of the new analogues **3–5**, with these targets being isolated in up to 43%, 84% and 64% yields respectively after HPLC purification (Scheme 1).

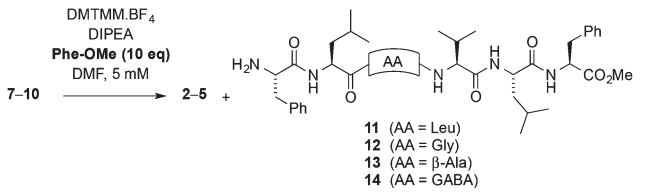


Scheme 1 Synthesis of cyclic peptides **2–5**.

It was of interest to rank these new peptide cyclisations (**8** → **3**, **9** → **4** and **10** → **5**) in terms of their synthetic efficiency. However, simply comparing the isolated yields of **3–5** was an unsatisfactory measure since these results were highly variable across different experimental repetitions (Scheme 1), presumably due to mechanical losses of material during HPLC purifications. Therefore, the rates of reaction were investigated as an alternative way to rank the cyclisation efficiencies. Time-course LCMS analyses were attempted, but it was found that all three cyclisations (**8** → **3**, **9** → **4** and **10** → **5**) were surprisingly rapid; all appeared to reach completion within 1 min, and so it was not possible to rank the cyclisations by reaction rate either. Finally, the effective molarity (EM) of each linear peptide was measured as a novel way of comparing the peptide cyclisation efficiencies.

Effective molarity is defined as the ratio between the rate constants of an intramolecular reaction and the corresponding intermolecular process.[15] The EM concept is commonly associated with descriptions of enzyme-catalysed reactions,[16] where high intramolecular reaction rates involving enzyme–substrate conjugates correspond to high EM values. However, to our knowledge the EM concept has not previously been employed to measure and/or explain peptide macrocyclisation efficiency. To determine EM values of the linear peptides **7–10**, a series of inter-/intramolecular competition experiments were performed (Table 1). These competition experiments involved repeating the peptide cyclisation reactions in the presence of excess phenylalanine methyl ester. In this way, two competing products are formed: the cyclic pentapeptides **2–5** resulting from the intramolecular process; and the linear hexapeptides **11–14** resulting from an intermolecular process. By quantifying the

Table 1 Competition experiments were performed to calculate the effective molarity (EM) of linear peptides **7–10**



Starting material	Ratio of cyclic pentapeptide : linear hexapeptide[a]	EM[b] (mM)
7	12 : 88	7 ± 1
8	21 : 79	13 ± 3
9	27 : 73	18 ± 2
10	26 : 74	18 ± 3

[a] Determined by analytical HPLC. [b] Uncertainties are reported as half-the-range from at least three experiments.

relative amounts of these intra- and intermolecular reaction products, the effective molarity of the cyclisation reactions of species **7**–**10** can be calculated.[17]

The observed EM values of the cyclisations of **7**–**10** are illuminating. The cyclisation of **7** has the lowest EM value (Table 1, entry 1), which was to be expected since this reaction was already known to be low-yielding (Scheme 1).[18] The corresponding EM value for linear peptide **8** is higher (Table 1, entry 2), and this correlates with the higher yield in the cyclisation of **8** relative to **7** (Scheme 1). Upon proceeding to peptide **9**, which contains an additional methylene group relative to peptide **8**, a further increase in EM is observed (Table 1, entry 3). Finally, the cyclisation of linear peptide **10** was found to have an EM value that was essentially identical to that of **9** (Table 1, entry 4), demonstrating that the incorporation of yet another methylene group has no further effect on the cyclisation efficiency.

Overall, the EM values of **7**–**10** (Table 1) confirm that the incorporation of a glycine or backbone-homologated residue can improve peptide cyclisation efficiency. Also, comparing the EM values constitutes a more precise method of ranking these peptides' propensity for cyclisation than other measurements attempted in this work (*i.e.*, reaction rate and product yield). It is notable that the changes in EM are quite small in magnitude; this implies that while adding extra rotatable bonds does reduce the enthalpic penalty of cyclisation, this is somewhat offset by an increased entropic penalty. The maximum benefit is achieved with β-alanine (*i.e.*, **9** → **4**).

Having identified three synthetically accessible analogues of the lead compound **2**, it became of interest to investigate whether these new compounds maintained useful levels of biological activity. Accordingly, the cytotoxicities of **3**–**5** were measured against the HCT-116 human colon cancer cell line using the CCK assay method (Table 2). Analogue **3** was found to suffer a reduction in activity relative to parent **2** (only 10% inhibition by **3** at 100 μM, *cf.* 35% inhibition by **2** at 50 μM), which was disappointing in light of previous structure–activity data suggesting that the variable amino acid was not critical for activity.[12] However, it was gratifying to observe that some activity was recovered in analogues **4** and **5** (Table 2). Compound **5** now appears to be an interesting candidate for further development; one possible avenue of future work may be to incorporate conformationally-biased GABA analogues[19] in order to optimise the biological activity while retaining synthetic efficiency.

Table 2 Cytotoxicity of **2**–**5** towards human colon cancer cells. The positive and negative controls were 17-AAG and DMSO respectively

Compound	Concentration (μM)	% inhibition of HCT-116 cell growth
2	50[a]	35[a]
3	100[b]	10 ± 1[c]
4	100[b]	15 ± 1[c]
5	100[b]	19 ± 1[c]

[a] Data taken from ref. 11. [b] Higher concentrations were also investigated in an attempt to match the level of inhibition shown by **2**, but this led to solubility problems. [c] Uncertainty is reported as the standard error of at least four experiments.

In summary, three novel analogues of the medicinally relevant cyclic pentapeptide **2** have been synthesised in high yield. Replacing a leucine residue in the lead compound with glycine leads to a dramatic improvement in macrocyclisation efficiency (analogue **3**), and this is attributed to the increased flexibility of the linear precursor peptide **8**. Progressing to analogues containing the backbone-homologated residues β-alanine (**4**) and GABA (**5**) gives a further increase in flexibility and cyclisation efficiency. These results should facilitate the medicinal development of **2** towards anticancer therapeutic applications, but more broadly this work may also represent a novel strategy for assisting the synthesis of analogues of "difficult" cyclic peptides.

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Notes and references

1 (*a*) S. H. Joo, *Biomol. Ther.*, 2012, **20**, 19–26; (*b*) D. Raucher, S. Moktan, I. Massodi and G. L. Bidwell III, *Expert Opin. Drug Delivery*, 2009, **6**, 1049–1064; (*c*) P. Vlieghe, V. Lisowski, J. Martinez and M. Khrestchatisky, *Drug Discovery Today*, 2010, **15**, 40–56.
2 B. Groner, *Peptides as Drugs: Discovery and Development*, Wiley-VCH, Weinheim, 2009.
3 C. J. White and A. K. Yudin, *Nat. Chem.*, 2011, **3**, 509–524.
4 (*a*) A. Ehrlich, H.-U. Heyne, R. Winter, M. Beyermann, H. Haber, L. A. Carpino and M. Bienert, *J. Org. Chem.*, 1996, **61**, 8831–8838; (*b*) R. Hili, V. Rai and A. K. Yudin, *J. Am. Chem. Soc.*, 2010, **132**, 2889–2891.
5 M. Malesevic, U. Strijowski, D. Bächle and N. Sewald, *J. Biotechnol.*, 2004, **112**, 73–77.
6 D. Skropeta, K. A. Jolliffe and P. Turner, *J. Org. Chem.*, 2004, **69**, 8804–8809.
7 W. D. F. Meutermans, S. W. Golding, G. T. Bourne, L. P. Miranda, M. J. Dooley, P. F. Alewood and M. L. Smythe, *J. Am. Chem. Soc.*, 1999, **121**, 9790–9796.
8 M. Liu, Y.-C. Tang, K.-Q. Fan, X. Jiang, L.-H. Lai and Y. H. Ye, *J. Pept. Res.*, 2005, **65**, 55–64.
9 L. Hunter and J. H. Chung, *J. Org. Chem.*, 2011, **76**, 5502–5505.
10 J. Malmstrøm, *J. Nat. Prod.*, 1999, **62**, 787–789.
11 R. P. Sellers, L. D. Alexander, V. A. Johnson, C.-C. Lin, J. Savage, R. Corral, J. Moss, T. S. Slugocki, E. K. Singh, M. R. Davis, S. Ravula, J. E. Spicer, J. L. Oelrich, A. Thornquist, C.-M. Pan and S. R. McAlpine, *Bioorg. Med. Chem.*, 2010, **18**, 6822–6856.

12 M. R. Davis, E. K. Singh, H. Wahyudi, L. D. Alexander, J. B. Kunicki, L. A. Nazarova, K. A. Fairweather, A. M. Giltrap, K. A. Jolliffe and S. R. McAlpine, *Tetrahedron*, 2012, **68**, 1029–1051.

13 W. C. Chan and P. D. White, *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press Inc., New York, 2000. HBTU = *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; DIPEA = *N*,*N*-diisopropylethylamine; DMTMM·BF_4 = 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium tetrafluoroborate.

14 It is possible to obtain **2** in moderate yield under different reaction conditions, see: (*a*) W. Gu, S. Liu and R. B. Silverman, *Org. Lett.*, 2002, **4**, 4171–4174; (*b*) A. I. Fernández-Llamazares, J. García, V. Soto-Cerrato, R. Pérez-Tomás, J. Spengler and F. Albericio, *Chem. Commun.*, 2013, **49**, 6430–6432. However, we were primarily interested in comparing **2** with the other targets (**1**, **3**–**5**) which is why the quoted conditions were employed.

15 (*a*) A. J. Kirby, *Adv. Phys. Org. Chem.*, 1980, **17**, 183–278; (*b*) M. A. Winnik, *Chem. Rev.*, 1981, **81**, 491–524; (*c*) R. Cacciapaglia, S. Di Stefano and L. Mandolini, *Acc. Chem. Res.*, 2004, **37**, 113–122.

16 V. M. Krishnamurthy, V. Semetey, P. J. Bracher, N. Shen and G. M. Whitesides, *J. Am. Chem. Soc.*, 2007, **129**, 1312–1320.

17 See ESI.†

18 It is noteworthy that the EM of the cyclisation of peptide **7** (*i.e.*, 7 mM) is close to the concentration at which the reaction was actually performed (*i.e.*, 5 mM), which may explain the low yield of **2**. We therefore repeated the reaction at a lower peptide concentration of 0.5 mM, but unfortunately still did not obtain an isolable quantity of **2**.

19 (*a*) L. Hunter, K. A. Jolliffe, M. J. T. Jordan, P. Jensen and R. B. Macquart, *Chem. – Eur. J.*, 2011, **17**, 2340–2343; (*b*) L. Hunter, S. Butler and S. B. Ludbrook, *Org. Biomol. Chem.*, 2012, **10**, 8911–8918.