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DISPLAY OF FIXED RESIDUES ON VARIABLE CYCLIC SCAFFOLDS: A NOVEL APPROACH TO PEPTIDE COMBINATORIAL LIBRARY SYNTHESIS

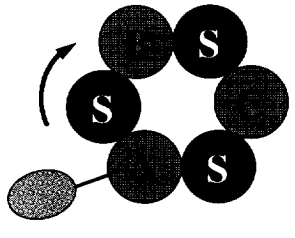
Ian Sanders*, Mark C. Allen, Ian M. Matthews
NOVARTIS Horsham Research Centre, Wimblehurst Road, Horsham, West Sussex RH12 4AB, UK.
Malcolm M. Campbell
School of Chemistry, University of Bath, Bath, BA2 7AY, U.K.

Abstract: A synthetic peptide library is described which displays three nominated residues in varied spacing around cyclic peptides. Diversity of ring size was achieved by the use of binary combinatorial synthesis, and the inclusion of hydrophilic spacer molecules. The order of residue occurrence and chirality was fully varied while duplication was avoided using a modified split-and-mix protocol. © 1997 Elsevier Science Ltd.

Combinatorial libraries have become a significant tool in the pursuit and optimisation of new drug candidates. Synthetic peptide libraries particularly have been widely applied to probe the binding requirements of proteins and receptors.[1] More recently, methods of imposing conformational constraint upon such libraries have been sought to improve the potency of active members and to facilitate the subsequent design of small molecule mimics.[2] Peptide libraries have been cyclised,[3] incorporated into proteins of known topography[4] or residues displayed upon rigid scaffolds[5] which may themselves be cyclic peptides.[6] Examples include variable residues attached to the side chains of a constant cyclic lysine scaffold,[7] or additional diversity achieved by first introducing variable spacer molecules onto the side chains.[8] Originally peptide libraries comprised random combinations of all natural amino acids. Latterly, the membership of libraries has been focused toward the target in question by the selection of a synthetic template,[9] inclusion of known significant residues[10] or by pre-selection using sub-library kits.[11]

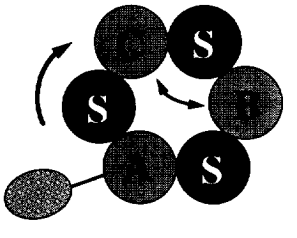
We present for the first time a library whose central element of diversity is a variable template, whilst the displayed residues remain constant.

Three sequentially disparate residues of Tissue Factor, thought to be significant in the binding of Tissue Factor to Factor VIIa,[12] were chosen for display in the library. The three residues were incorporated into cyclic peptides. Diversity was achieved by varying the order of inclusion, the mix of chirality and the distances between each of the chosen residues within the cyclic scaffold (**Figure 1**).



A,B,C = L-or D-Lysine, Isoleucine, Aspartic acid, in any order without duplication.
S = One of five spacer molecules, or no coupling "skip codon"

Fig. 1 Library Membership

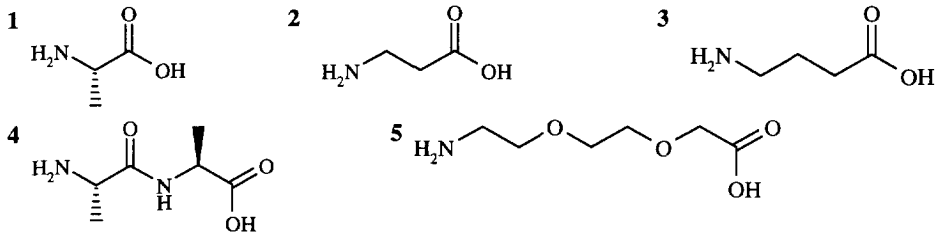


All order variants are generated from the exchange of two residues, **B** and **C** from the structure in fig.1.

Fig. 3 Strategy for diversity of residue occurrence without duplication

ian.sanders@pharma.novartis.com Fax (0)1403 323307

The variable residue spacing was achieved by random inclusion of different spacer molecules, which also bestowed diverse ring size on the peptides. Additional diversity was conferred by the complete deletion of a spacer molecule (skip codon) from the synthetic sequence. The latter technique has been applied to linear peptide libraries by Furka et al[13] as Binary Combinatorial Synthesis. The molecules occupy the spatial equivalent of one to three α-linked amino acids. The size range was selected by inspection of the published crystal structure of Tissue Factor "binding patch".[14] Spacer molecules (**1-5**) were chosen for hydrophilicity and modest side chain functionality. Amino ethoxy acids[15] and similar[16] have been employed successfully as hydrophilic spacer molecules in peptidomimetics.



The Split and Mix protocol[17] was employed for the library synthesis (**Figure 2**). Within the scheme, all possible order variants of the chosen residues were generated by allowing variation at only two residue positions (**Figure 3**). The system minimises the number of components in each sub library by eliminating repeated residues. The principle was discussed by Furka et al.[11] in their use of an Occurrence Library. The possibility of duplicate sequences arising from a head-to-tail cyclic library is also avoided.

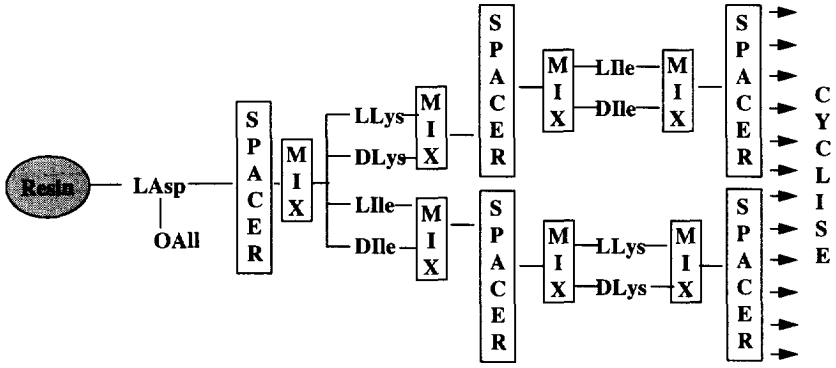


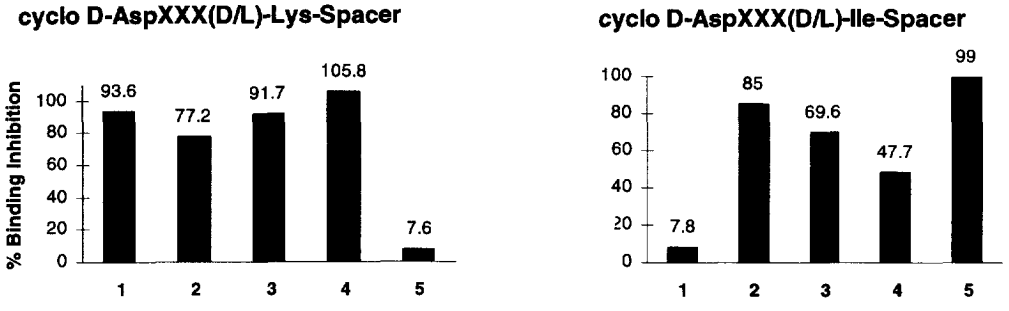
Fig. 2 Synthetic scheme

The fortuitous inclusion of aspartic acid among the three residues of interest allowed the synthesis to be achieved by anchoring the side chain of aspartic acid α-allyl-ester to the resin followed by library synthesis and on-resin cyclisation in the reported manner.[18] Good generality is reported on cyclisation of a library of aspartic acid side-chain anchored peptides.[19] A subset of the intended library synthesised singly using the chemistry described achieved 90% cyclic monomeric products by LC-MS. The synthesis was performed in glass scinter funnels using 8g Wang resin. FMOC-DAsp-OAll was synthesised according to published methods[20] for the

L-amino acid. Resin loading was achieved at 0.2 mmol/g by the method of Sieber.[21] FMOC amino acids were activated by HBTU/HOBT/DIEA(1:1:2). Resin (100mg) from each synthetic step was preserved for future deconvolution and analysis. After synthesis, the allyl-ester protection was removed with Pd(0),[22] before cyclisation with HBTU for 5 hours.

Synthetic progress was monitored by electrospray mass spectrometry and reverse phase HPLC after cleavage of the small resin samples removed after each synthetic step.[23] Initially, the relatively low number of compounds per sub library permitted observation of the discrete calculated mass ions and expected number of HPLC peaks. Later in the synthesis, coupled LC-MS was employed to separate the components and successively identify the expected ions. Impurities attributed to incomplete and linear sequences were observed at less than 15% base peak abundance. Ten sub libraries with 144 members each were produced. A separate set of sub libraries was synthesised beginning with D-Asp-OAll. Inhibitory activity was measured by ELISA assay.[24] Example results from the first round of screening are shown below. **Figure 4.**

Fig. 4



In conclusion, we have synthesised a peptide library where three fixed residues of interest are presented at many different distances and orientations from one another by merit of a highly diverse scaffold. The scaffold is conformationally constrained by ring formation. A modification of the split and mix procedure eliminates unwanted sequences containing repeated residues or duplicate sequences, maximising differences between sub libraries. The protocol generates library members widely variant in ring size, whilst retaining a constant number of synthetic steps. The general principle may also be applicable to the optimisation of pharmacophore display within biologically active peptides.

Acknowledgement

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24. Immulon-2 plate coated with r-FVIIa (200µl, 50nM, 0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.5, 12hrs, 4°C). Remaining active plate blocked with 1mg/ml BSA in base buffer,1 hr Candidate library added (10µl, 50% aq.DMSO) followed by r-TF (100µl, 5nM, base buffer, 50nM $CaCl_2$), giving180µM total library peptide, incubation 90min. Primary antibody anti-TF monoclonal(100ul, 0.25µg/ml, wash buffer) incubated 90 min. Secondary antibody antimouse IgG conjugated to alkaline phosphatase incubated in blocking solution for 90 min. Substrate (p-nitro-phenylphosphate, 150µl, 0.5mg/ml) added and incubated until colour developed. All steps washed with base buffer + 0.05% Tween 20, 5mM $CaCl_2$. Base buffer = 0.1M Tris, 0.15M NaCl, 5mM $CaCl_2$, pH 8.0. OD readings converted to % inhibition using no-compound control as 100%, subtracting no-VIIa controls. Values quoted are averages of duplicate experiments, with no more than 10% variation between duplicates.

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