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THE COMBINATORIAL SYNTHESIS OF A 30,752-COMPOUND LIBRARY: DISCOVERY OF SAR AROUND THE ENDOTHELIN ANTAGONIST, FR-139,317

Nicholas K. Terrett,* Dejan Bojanic,‡ David Brown, Peter J. Bungay,‡ Mark Gardner, David W. Gordon, Carolyn J. Mayers‡ and John Steele

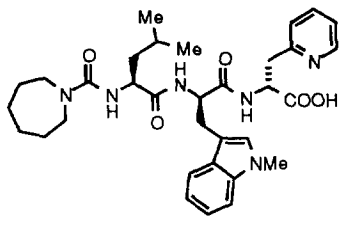
Departments of Discovery Chemistry and Discovery Biology,‡ Pfizer Central Research, Sandwich, Kent CT13 9NJ, United Kingdom

Abstract: A combinatorial library of 30,752 compounds has been synthesised from a set of 32 natural and unnatural amino acids. The library was designed to include the known endothelin antagonist, FR-139,317, as a positive control, and this and a number of close analogues were shown to be the most potent compounds. Thus, combinatorial libraries may be used both to discover leads and rapidly explore SAR.

Introduction: In the search for ever more rapid ways of discovering new biologically active molecules, the advent of combinatorial synthesis has presented a method for significantly accelerating the process of drug discovery. The resin-based mix-and-split strategy for combinatorial synthesis was first described by Furka et al[1] and has been explored since by several groups.[2] In this communication we describe the use of combinatorial synthesis to prepare a library of 30,752 trimeric compounds, and we show that this technique permits the identification of the known endothelin antagonist, FR-139,317, and defines structure-activity relationships around this lead.

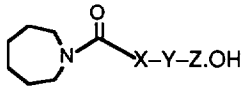
The endothelins[3] are a family of peptides with potent and sustained vasoconstrictor activity. Raised plasma levels have been observed in conditions related to systemic vasoconstriction, such as hypertension, heart failure and angina, and endothelin is thought to play a key role in acute and chronic renal failure. Consequently, there has been a concerted search for antagonists of the endothelin receptor subtypes. A number of antagonists have been described in the literature, and one of these, with selectivity for the ET_A receptor subtype, is the modified tripeptide, FR-139,317 from Fujisawa[4] (Figure 1).

Figure 1. FR-139,317



The Library Synthesis: A compound library of trimeric structures (Figure 2) was prepared from a set of 32 monomeric amino acids containing both L- and D- natural amino acids and a number of unnatural amino acids (see Table).[5] The monomers were chosen to include a range of chain lengths between the amine and the carboxylic acid, and a variety of functionality on the side-chains. As this library was to be tested against the endothelin receptor, specific monomers were selected to ensure that FR-139,317 was synthesised as a positive control within the library, and that close analogues containing portions of this structure would also occur in the library. Thus, the three amino acids that constitute this antagonist, *L-Leu*, *D-N-Me-Trp* and *D-2-Pyr-Ala*, were included within the monomer set, and all trimers were capped on the N-terminus to give the homopiperidine urea derivative. It was observed that D-2-Pyr-Ala would only couple in very low yield under the reaction conditions used for library synthesis, and thus this amino acid was omitted from the X and Y positions. The library synthesis followed a standard procedure using Wang polystyrene resin and Fmoc-protected monomers to allow the formation of C-terminal carboxylic acids following trifluoroacetic acid (TFA)-catalysed cleavage from the resin beads.[6] The analysis of complex compound mixtures is clearly still a challenge. However, we included trial synthetic and analytical experiments to show us that the chemistry employed was successful.[7]

Figure 2. The general structure of the library components

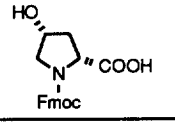
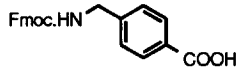
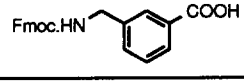
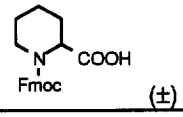
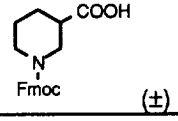


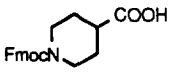
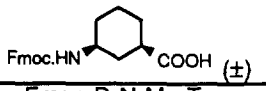
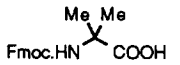
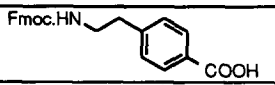
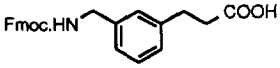
Z = set of 32 monomers

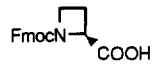
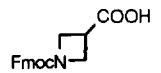
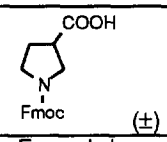
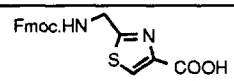
X and Y = 31 monomers
(set Z without D-2-Pyr-Ala)

Table. Monomers used in compound library synthesis

Monomer number	Structure
1	
2	
3	
4	
5	
6	Fmoc-D-Lys
7	Fmoc-L-Lys
8	Fmoc-D-Ser
9	Fmoc-L-Ser
10	Fmoc-D-Asp
11	Fmoc-L-Asp
12	Fmoc-D-Asn
13	Fmoc-L-Asn

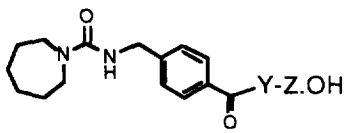
Monomer number	Structure
14	
15	
16	
17	 (±)
18	 (±)

Monomer number	Structure
19	
20	
21	Fmoc-D-N-Me-Trp
22	
23	
24	Fmoc-L-Val
25	

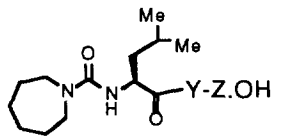
Monomer number	Structure
26	Fmoc-D-Phe
27	
28	
29	
30	Fmoc-L-Leu
31	
32	Fmoc-D-2-Pyr-Ala

Library Screening: After the synthesis of the library, the 30,952 compounds were arranged in 31 mixtures of 992 (i.e. 31 x 32) compounds each. The position X was fixed in each mixture as one specific monomer, and the other positions, Y and Z, were random mixtures of 31 and 32 amino acids respectively. These mixtures and both a positive control (an authentic sample of FR-139,317) and a negative control (reaction well CM, containing only the residue from evaporation of the TFA-based cleavage mixture) were tested[8] against dog spleen ET_A receptors and IC_{50} values obtained (see Table of first round results). Only three mixtures had significant activity when compared with background (see Figure 3).

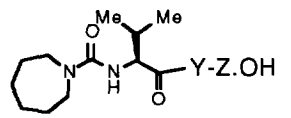
Figure 3. Structures of the three most active mixtures following first round screening
IC_{50} values based on total mixture concentration



IC_{50} = 11 μM
(11 nM per component)



IC_{50} = 35 μM
(35 nM per component)

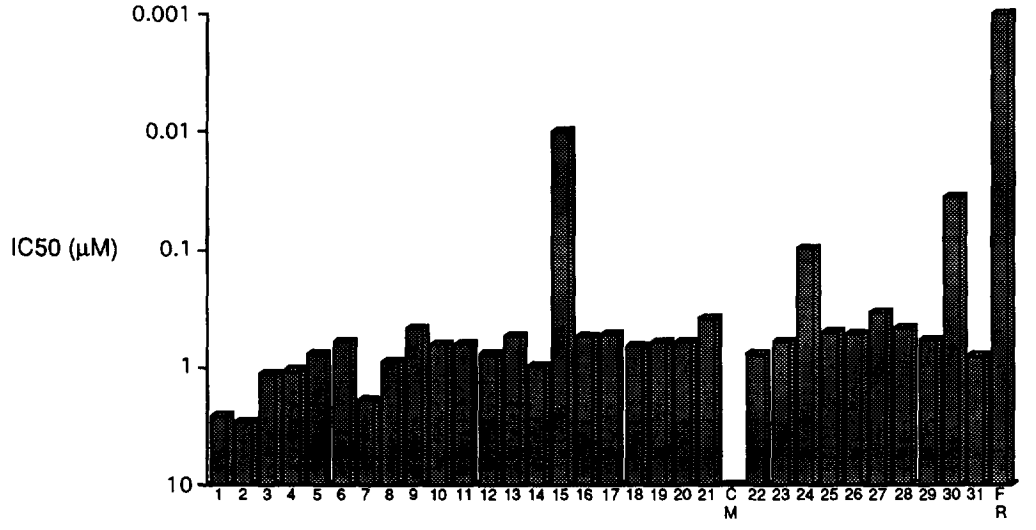


IC_{50} = 97 μM
(98 nM per component)

The significance of the IC_{50} value of a mixture of 992 compounds deserves further discussion. The most potent mixture had an IC_{50} value of 11 μM, and this may be caused by the presence of one compound with 11 nM affinity amongst 991 inactive compounds. Alternatively, the mixture may contain 992 weakly active compounds with average IC_{50} activity of 11 μM. Most probably, the mixtures contain compounds with a range of activities. Furthermore, it is not a safe assumption that all compounds have been produced in quantitative yield and in equal

amounts. It is probable that overall yields for the synthesis of some of these compounds may be significantly below 100%, and this may explain the discrepancy between the IC_{50} of pure FR-139,317 and the IC_{50} values of mixtures that are expected to contain this compound.

Table of First Round Results

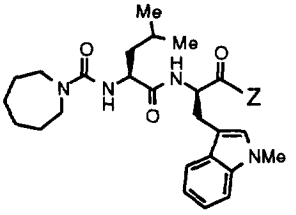


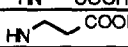
Results from the first round of synthesis and testing. The numbers refer to the monomer in the X position. Also included in the reaction block is a negative control (CM = cleavage mixture only) and a positive control (FR = an authentic sample of FR-139,317). IC_{50} values are displayed as the individual compound concentrations within mixtures to allow direct comparison with FR-139,317.

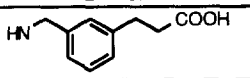
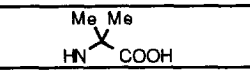
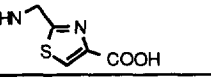
Whilst it was not surprising to observe[9] the potency of the mixtures containing L-Leu or the closely related amino acid L-Val, we were surprised to observe that the mixture with X = *p*-(aminomethyl)benzoic acid was the most potent. Both the *p*-(aminomethyl)benzoic acid and the L-Leu mixtures were followed-up by further synthesis. Surprisingly, none of the second round mixtures (position Y fixed) containing X = *p*-(aminomethyl)benzoic acid had significant activity.[10] The mixture that contained X = L-Leu was resynthesised as 31 mixtures of 32 compounds each. In each of these second round mixtures, X was kept constant as L-Leu, the position Z was a random mixture of the 32 amino acids, and position Y was one specific amino acid in each mixture. Screening of the 31 mixtures revealed that the mixture with X = L-Leu and Y = N-Me-D-Trp was the *only* active mixture (IC_{50} = 285 nM total compound concentration; equivalent to 8.9 nM per component; all other mixtures had IC_{50} > 100 μM).

In the third round of synthesis, 32 individual compounds were synthesised with X = L-Leu and Y = D-N-Me-Trp. A number of endothelin antagonists were identified and their structures confirmed by ms (see Figure 4).

Figure 4. Activity of Individual Compounds.



Z =	IC_{50} (nM)
	220
	62
	140
	>3500
	>3500

Z =	IC_{50} (nM)
D-Phe	2.5
D-2-Pyr-Ala (FR-139,317)	5.3
D-Ser	8
D-N-Me-Trp	8
D-Asp	295
D-Asn	>3500
	420
	730
L-Leu	1320
	1100

All other compounds synthesised in the final round had IC_{50} values greater than 3.5μM.

The results on these single compounds reveal some SAR around the FR-139,317 structure.[11] Clearly chain length of the Z residue is important with maximal activity observed with the β-alanine and γ-aminobutyric acid derivatives and less affinity from the glycine derivative. Amongst the α-amino acids, those with D-configuration and aromatic side-chains are preferred, although the more polar D-serine appears to confer good receptor affinity. Unsurprisingly, FR-139,317 was one of the most potent compounds discovered from this library.

Summary: In conclusion, this work has demonstrated the value of combinatorial library synthesis for the rapid assembly of drug-like molecules. We have shown that a known lead, incorporated as a positive control, can be detected, and that exploration of SAR is feasible by combinatorial methods. With the development of new synthetic reactions on solid-phase, the possibility will emerge of using combinatorial libraries for the discovery of novel leads, or for preparing targeted libraries to optimise known biologically active molecules .

Acknowledgements: We acknowledge the valuable technical assistance of Michelle Freer, Michelle Hunter, Debbie Malloy and Hannah Vuong, and the Department of Physical Sciences, Pfizer Central Research.

References and Notes

1. Furka. A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Abstr. 14th Int. Congr. Biochem., Prague, Czechoslovakia,* **1988**, *5*, 47. Furka. A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Peptide Protein Res.*, **1991**, *37*, 487.

2. For early publications that describe the use of combinatorial mix-and-split synthesis see: Houghten, R.A.; Pinilla, C.; Blondelle, S.E.; Appel, J.R.; Dooley, C.T.; Cuervo, J.H. *Nature*, **1991**, *354*, 84. Owens, R.A.; Gesellchen, P.D.; Houchins, B.J.; DiMarchi, R.D. *Biochem. and Biophys. Res. Comm.*, **1991**, *181*, 402. Hortin, G.L.; Staatz, W.D.; Santoro, S.A. *Biochem. International*, **1992**, *26*, 731.

3. Doherty, A.M. *J. Med. Chem.*, **1992**, *35*, 1493.

4. Hemmi, K.; Fukami, N.; Hashimoto, M.; Tanaka, H.; Kayakiri, N. *World Patent* WO93/10144, **1991.** Sogabe, K.; Nirei, H.; Shoubo, M.; Nomoto, A.; Ao, S.; Notsu, Y.; Ono, T. *J. Pharmacol. Exp. Ther.* **1993**, *264*, 1040.

5. As four of the amino acids were used as racemates, the library in fact consists of 44,100 compounds (35x35x36), although not in equimolar amounts.

6. Each monomer (45μmoles) was loaded onto Wang cross-linked polystyrene resin beads, and the total quantity of resin intimately mixed before distributing as a DMF slurry to 31 reaction wells of either an Abimed AMS 422 or Advanced Chemtech 396 multiple peptide synthesiser. The Fmoc protecting groups were removed with 50% piperidine/DMF (successive incubations over 21 minutes of 500μl, 250μl and 250μl), washed (DMF, 5 x 0.75ml) and then each well individually coupled with an Fmoc-protected amino acid (225μmoles), HOBt (225μmoles), TBTU (285μmoles), and Hunig's base (440μmoles) in DMF (1.3 ml) over 1h. The resins were washed with DMF (6 x 1ml), and thoroughly dried prior to combining and mixing. The resins were again distributed to 31 reaction wells and treated as above for deprotection and the second coupling. Each well was treated with homopiperidine carbonyl chloride (50μmoles) and Hunig's base (50μmoles) in DMF (100μl) for 18h, and then drained and washed with DMF (5 x 0.2ml). The mixtures were cleaved from resin by treating with TFA/ethanedithiol/water (95:1:4)(0.5ml) for 2h. The tubes were filtered, and the filtrate evaporated and taken up in DMSO (0.6ml) for screening.

7. To check that the proposed library chemistry was feasible, we firstly successfully synthesised and characterised the Fujisawa compound as an individual compound on solid-phase using the same conditions as employed in the library synthesis. During the library synthesis itself we prepared cap-Leu-Leu-Gly.OH in a separate reaction well as a check on the operations of the multiple peptide synthesiser. Fmoc-protected dimer mixtures were analysed by LC-MS, and in the case of Fmoc-Leu-X.OH (the intermediates in the Fujisawa series) the majority of expected components were identified.

8. Competition binding experiments were performed in an ET_A receptor assay using Bolton-Hunter labelled [^{125}I]ET_1 binding to dog spleen membranes which have previously been shown to contain endothelin receptors characterised as ET_A. Incubations containing [^{125}I]ET_1, dog spleen membranes and test compounds were carried out at room temperature for 2h with shaking before filtration on a Brandel cell harvester. Non-specific binding was determined using an excess of unlabelled ET_1. Radioactivity remaining on filter disks after washing off unbound radioactivity was determined with a gamma counter. Data handling was carried out using programs to calculated percentage specific binding for each sample and IC_{50} values after applying curve fitting routines.

9. The mixtures were screened without knowledge of their identity. IC_{50} values quoted for mixtures followed-up are n=3. Final round individual compounds were synthesised twice, and IC_{50} values are a mean of up to four determinations. The IC_{50} values have not been adjusted for variation in chemical yields, and as yields will inevitably vary from compound to compound, the SAR derived needs to be treated cautiously. This is a restriction imposed by high speed synthetic methods where there is minimal purification of the product compounds, and is an issue we are actively seeking to resolve in our current library work.

10. The analogue of FR-139,317 that contains *p*-(aminomethyl)benzoic acid in the place of L-Leu was synthesised independently and found to have an IC_{50} for the ET_A receptor of 1.1μM. The activity of the *p*-(aminomethyl)benzoic acid mixture is somewhat of a mystery to us. One explanation could be that there are a large number of weakly active components in this mixture that additively give rise to high receptor affinity.

11. Much of the SAR correlates well with previously disclosed SAR from Fujisawa (see European Patent, EP 457,195, 1991) and Banyu (for example see European Patent, EP-460,679, 1991).

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