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IDENTIFICATION OF A POTENT ANALOGUE OF NAZUMAMIDE A THROUGH ITERATION OF COMBINATORIAL TETRAPEPTIDE LIBRARIES

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Abstract: Five sets of N-acylated tetrapeptide libraries and sublibraries related to Nazumamide A have been prepared using 25 natural and unnatural amino acids. They were evaluated in antithrombin assay, in order to quantify inhibition at each step of the tetrapeptide sublibrary iteration. The studies led to the identification of 2,5-dihydroxybenzoyl-lysyl-isoleucyl-phenylalanyl-arginine as a novel inhibitor of thrombin and was found to be at least 25 times more potent than the natural tetrapeptide 2,5- dihydroxybenzoyl-arginyl-prolyl-isoleucyl-α-aminobutyric acid (NAZA).

One of the many potential targets for drug intervention in the coagulation cascade is thrombin inhibition which is of extreme interest due to its dual role in promoting coagulation as well as stimulating platelet aggregation. Much attention has recently been focussed on the inhibition of thrombin as an emerging means to specifically interrrupt the coagulation process. Although a number of potent thrombin inhibitors have been reported[1,2], they either lack a high selectivity over other trypsin like enzymes or have poor oral bioavailability with inappropriate plasma half lives. Design and synthesis of noncleavable ligands that span the site of proteolytic cleavage offers an interesting approach as they may be able to address the issue of specificity. Hirugen and hirulogs are examples of this class of compounds with a noncleavable peptide bond analogue, and even though they exhibited potent and specific inhibition, in clinical trials they showed pronounced incidence of serious adverse effects[3].

Nazumamide A[4,5] (NAZA), isolated from the marine sponge *Theonella Sp.* and comprised of 2,5-Dihydroxybenzoyl-Arg-Pro-Ile-αAbu offers another strategy for preparing noncleavable substrates. The synthetic NAZA was reported to be endowed with antithrombin activity without inhibiting trypsin even at concentrations up to 165 μM. Recently, Nienabar *et al*[6] on the basis of x-ray crystallography of complexes with thrombin reported that NAZA has weak binding properties which they attributed to: 1) the absence of a methylene residue in the side chain of Arg as it is unable to form salt bridge with Asp-198 in thrombin, 2) loss of antiparallel ß-sheet hydrogen bonds which are typical for peptide substrates and 3) the presence of α-aminobutyrate group at position 4 which destabilises the binding of NAZA with thrombin. Thus, although NAZA may not be a therapeutically suitable candidate as anticoagulant, it provides an interesting pharmacophore for the design of more potent and specific thrombin inhibitors. This prompted us to use combinatorial approach to create a large library of NAZA analogues with the view to identify congener with higher

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antithrombin activity. In recent years combinatorial methodology[7] has led to the identification of several interesting hits from the pharmacological point of view. In this paper we describe the synthesis and screening of tetrapeptide libraries based on the premix method[7].

In the first instance a tetrapeptide library was prepared on chlorotrityl resin using a pipetting robot peptide synthesizer. The peptide library, comprising the calculated total number of 390,625 tetrapeptides was generated from an equimolar mixture of 25 natural and unnatural amino acids. This was followed by acylation of the resin bound tetrapeptide library with nine different mono/dihydroxy benzoic acids (MHB/2,5-DHB) separately, resulting in nine tetrapeptide libraries represented by the general formula $O^1X^2X^3X^4X^5$ (where O^1 is defined). The peptide mixtures were cleaved from the resin using TFA/phenol/ethanedithiol/thianisol/water mixture and precipitated with the addition of diethylether. The precipitates were washed with ether and lyophilised from *t*-BuOH-H_2O (4:1). The libraries were characterised using ES-MS and the observed mass distribution for different libraries showed good correlation with the simulated mass distribution[7]. Amino acid analysis of libraries confirmed ES-MS results and the expected equimolarity with the accuracy of such analysis (+/– 10%). These libraries were then evaluated for their antithrombin activity and results have been summarised in Figure 1.

As is evident, one of the libraries having 2,5-dihydroxybenzoic acid at position O^1 exhibited highest order of inhibition of thrombin and was therefore selected for further iteration in order to define the remaining four mixture positions. This was carried out by synthesizing peptide mixtures in which one position was individually defined with each of the 25 amino acids at a time, coupled with antithrombin assay of the resulting sublibrary. Final iteration of the fifth and last mixture position resulted in the synthesis of 25 single peptides with all the positions defined. The purity and identity of all individual peptides were characterised by RP-HPLC (purities ranged from 80-95%) and ES-MS. The most active peptide ultimately found through iteration was synthesised and purified to more than 98% homogeneity by RP-HPLC value for final IC_{50} value determination.

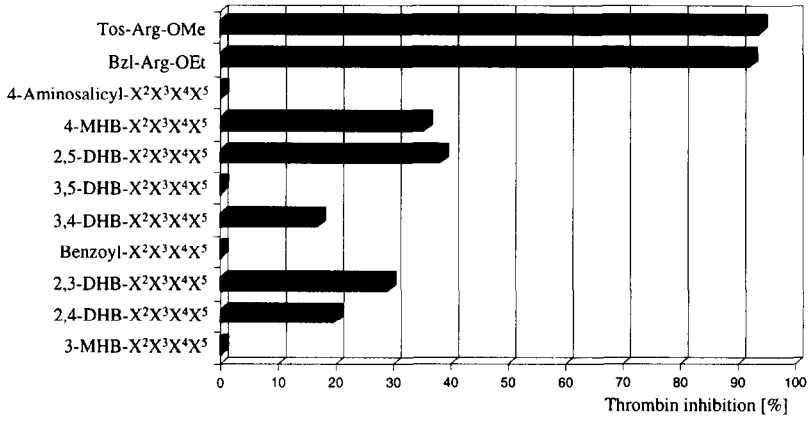

Figure 1: Inhibition of thrombin by various libraries and standards at 1.25 mM concentration.

Table 1: IC_{50} values for iterations carried out on 2,5-DHB-$X^2X^3X^4X^5$

Iteration 1	IC_{50} [μM]	Iteration 2	IC_{50} [μM]	Iteration 3	IC_{50} [μM]	Iteration 4	IC_{50} [μM]
2,5-DHB-ßA$X^3X^4X^5$	3542	2,5-DHB-KßAX^4X^5	821	2,5-DHB-KIßAX^5	406	2,5-DHB-KIFßA	29
2,5-DHB-A$X^3X^4X^5$	1749	2,5-DHB-KAX^4X^5	126	2,5-DHB-KIAX^5	183	2,5-DHB-KIFA	5.8
2,5-DHB-R$X^3X^4X^5$	1606	2,52,5-DHB-KRX^4X^5	273	2,5-DHB-KIRX^5	26	**2,5-DHB-KIFR**	**1.9**
2,5-DHB-N$X^3X^4X^5$	3386	2,5-DHB-KNX^4X^5	711	2,5-DHB-KINX^5	341	2,5-DHB-KIFN	21
2,5-DHB-D$X^3X^4X^5$	2462	2,5-DHB-KDX^4X^5	1262	2,5-DHB-KIDX^5	680	2,5-DHB-KIFD	69
2,5-DHB-Q$X^3X^4X^5$	2545	2,5-DHB-KQX^4X^5	300	2,5-DHB-KIQX^5	82	2,5-DHB-KIFQ	6.4
2,5-DHB-E$X^3X^4X^5$	3326	2,5-DHB-KEX^4X^5	54	2,5-DHB-KIEX^5	458	2,5-DHB-KIFE	200
2,5-DHB-G$X^3X^4X^5$	2989	2,5-DHB-KGX^4X^5	428	2,5-DHB-KIGX^5	446	2,5-DHB-KIFG	20
2,5-DHB-H$X^3X^4X^5$	2803	2,5-DHB-KHX^4X^5	422	2,5-DHB-KIHX^5	109	2,5-DHB-KFH	13
2,5-DHB-I$X^3X^4X^5$	2710	**2,5-DHB-KIX^4X^5**	**25**	2,5-DHB-KIIX^5	257	2,5-DHB-KIFI	42
2,5-DHB-L$X^3X^4X^5$	1887	2,5-DHB-KLX^4X^5	46	2,5-DHB-KILX^5	137	2,5-DHB-KIFL	9
2,5-DHB-KX^3X^{4}X^5	**1141**	2,5-DHB-KKX^4X^5	748	2,5-DHB-KIKX^5	68	2,5-DHB-KIFK	2.5
2,5-DHB-M$X^3X^4X^5$	2630	2,5-DHB-KMX^4X^5	49	2,5-DHB-KIMX^5	46	2,5-DHB-KIFM	7
2,5-DHB-F$X^3X^4X^5$	2206	2,5-DHB-KFX^4X^5	195	**2,5-DHB-KIFX^5**	**15**	2,5-DHB-KIFF	27
2,5-DHB-P$X^3X^4X^5$	3383	2,5-DHB-KPX^4X^5	160	2,5-DHB-KIPX^5	582	2,5-DHB-KIFP	>500
2,5-DHB-S$X^3X^4X^5$	2328	2,5-DHB-KSX^4X^5	446	2,5-DHB-KISX^5	106	2,5-DHB-KIFS	15
2,5-DHB-T$X^3X^4X^5$	3153	2,5-DHB-KTX^4X^5	546	2,5-DHB-KITX^5	394	2,5-DHB-KIFT	17
2,5-DHB-W$X^3X^4X^5$	1713	2,5-DHB-KWX^4X^5	169	2,5-DHB-KIWX^5	44	2,5-DHB-KIFW	43
2,5-DHB-Y$X^3X^4X^5$	2430	2,5-DHB-KYX^4X^5	672	2,5-DHB-KIYX^5	25	2,5-DHB-KIFY	38
2,5-DHB-V$X^3X^4X^5$	2576	2,5-DHB-KVX^4X^5	318	2,5-DHB-KIVX^5	194	2,5-DHB-KIFV	31
2,5-DHB-u$X^3X^4X^5$	2873	2,5-DHB-KpX^4X^5	1256.	2,5-DHB-KIuX^5	294	2,5-DHB-KIFu	21
2,5-DHB-g$X^3X^4X^5$	3013	2,5-DHB-KyX^4X^5	298	2,5-DHB-KIgX^5	262	2,5-DHB-KIFg	11
2,5-DHB-x$X^3X^4X^5$	2164	2,5-DHB-KzX^4X^5	378	2,5-DHB-KIxX^5	168	2,5-DHB-KIFx	13
2,5-DHB-a$X^3X^4X^5$	2956	2,5-DHB-KaX^4X^5	308	2,5-DHB-KIaX^5	593	2,5-DHB-KIFa	54
2,5-DHB-l$X^3X^4X^5$	2776	2,5-DHB-KlX^4X^5	507	2,5-DHB-KIlX^5	507	2,5-DHB-KIFl	51
-	-	-	-	Tos-Arg-OMe	89	**2,5-DHB-KIFX^5**	**14**

All natural amino acids have been denoted by single letter code. For other amino acids abbreviations used are: p = D-proline; z = thioproline; y =4-hydroxyproline; u = α Abu; g = γAbu; x = ε aminohexanoic acid; a = D-alanine; l = D-leucine, βA = β-alanine

The peptide mixtures were assayed for their antithrombin activity by competitive enzyme assay using the fluorogenic substrate Tos-Gly-Pro-Arg-AMC. Serial dilutions of the libraries from mM to nM range, dissolved in 50 mM Tris buffer at pH 8.3 containing 1% DMSO were used to determine IC_{50} values. Based on the lowest IC_{50} value the most effective sublibrary was then selected for further iteration (Table 1).

The first sublibrary tested consisted of 25 pools of sublibraries each containing a mixture of 25^3 different tetrapeptides. Out of these the best sublibrary for the inhibition of thrombin was found to be 2,5-DHB-Lys-$X^3X^4X^5$ (IC_{50} = 1141 μM). Lys was therefore selected for the second position, however, iteration of 2,5-DHB-Arg-$X^3X^4X^5$ was also pursued for the purpose of comparison. Thus, in the next step two sets of sublibraries: 2,5-DHB-Lys-$O^3X^4X^5$ and 2,5-DHB-Arg-$O^3X^4X^5$ were synthesised and evaluated for their antithrombin activity. From these sublibraries, best inhibition was observed for Ile (IC_{50} = 25 μM), Leu (IC_{50} = 46 μM), Met (IC_{50} = 49 μM) and Glu (IC_{50} = 54 μM) sublibraries with Lys at position 2. In contrast 2,5-DHB-Arg-Pro-X^4X^5 (IC_{50} = 72 μM, data not shown) corresponding to the natural tetrapeptide NAZA exhibited higher IC_{50} value. Therefore iteration for this sublibrary was dropped and deconvolution for 2,5-DHB-Lys-Ile-X^4X^5 was continued to define the fourth position. Out of the resulting 25 pools of sublibraries (each containing mixture of 25^1 compounds) with known amino acid at position 4, 2,5-DHB-Lys-Ile-Phe-X^5 and to a lesser extent 2,5-DHB-Lys-Ile-Tyr-X^5 and 2,5-DHB-Lys-Ile-Arg-X^5 were found to be most effective.

The fifth and final mixture position for 2,5-DHB-Lys-Ile-Phe-X^5 was then defined to complete the iterative process. After assaying for their thrombin inhibition, it was found that basic amino acids such as Arg and Lys were the most acceptable ones at this position. The most effective peptide inhibitor was found to be 2,5-DHB-Lys-Ile-Phe-Arg (IC_{50} = 1.9 μM) which was at least 25 times more potent than the natural peptide NAZA (IC_{50} = 53 μM) in our assays.

Thus, although the validity of combinatorial approach to identify inhibitors has been confirmed, we did not observe drastic gains in the potency from 2nd iteration to 4th iteration as described with other combinatorial libraries. Further modifications are nevertheless necessary to optimise our lead structure in order to enhance its inhibitory capacity.

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References

1. Das, J.; Kimball S.D.; *Bioorg. Med. Chem.* 1995, **3**, 999.
2. Ripka, W.C., Vlasuk, G.P., *Annual Reports in Med. Chem.* 1997, 32, 71.
3. Catella-Lawson F; *Coron. Artery Dis.* 1997, **8**, 105.
4. Fusetani, N.; Nakao, Y.; Matsunaga, S; *Tetrahedron Lett.* 1991, **32**, 7073.
5. Hayashi, K.; Hamada, Y.; Shioiri, T.; *Tetrahedron Lett.* 1992, **33**, 5075.
6. Nienabar, V., L.; Amparo, E., C.; *J. Am. Chem. Soc.* 1996, **118**, 6807.
7. In combinatorial peptide and nonpeptide libraries; Jung; G., Ed.; VCH; Germany, 1996.