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1, 2-DISUBSTITUTED CYCLOHEXANE DERIVED TRIPEPTIDE ALDEHYDES AS NOVEL SELECTIVE THROMBIN INHIBITORS

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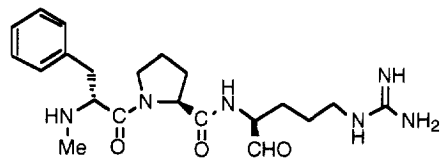
Abstract: A series of tripeptide arginine aldehydes was synthesized by replacement of proline with 1,2-disubstituted cyclohexane derivatives in the sequence of D-MePhe-Pro-Arg-H. Based on molecular modeling, further modification of the D-MePhe residue resulted in a potent and selective thrombin inhibitor.

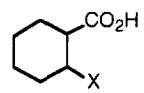
Thrombin is a serine protease which plays a key role in both haemostasis and thrombosis[1]. The biological functions of thrombin have stimulated intense searches for small, orally active direct inhibitors of this enzyme as potential anti-coagulant agents[1-5].

Our studies for the discovery of new inhibitors was focused on the peptide arginal GYKI 14766 **1**, a reaction intermediate based inhibitor which inhibits thrombin by two stage, slow and tight binding kinetics at nanomolar concentrations[6]. Recent reports from the literature show the discovery of potent thrombin inhibitors derived from modification of the D-MePhe-Pro-Arg-H sequence of GYKI 14766 either by substitution of the proline residue with other cyclic structures[7], by introduction of constrained arginine side chain modifications[8], by isosteric replacement of the C-terminal aldehyde with other groups such as borate esters[9] or the transformation of the C-terminus to various arginoyl heterocycles[10].

The aim of our work, taking **1** as a lead, was to replace the central proline residue with other structures of defined stereochemistry, which would orientate the lateral fragments for favourable interaction with the enzyme site whilst also producing analogues with less peptide characteristics compared to the lead molecule. With these modifications we hoped to produce a low molecular weight compound metabolically more stable compared to GYKI 14766 and yet maintaining a high thrombin inhibitory activity . In our studies we chose the individual stereoisomers of 1,2-cyclohexane dicarboxylic acid **2** and 2–aminocyclohexane carboxylic acid **3** as modifiers.



GYKI 14766 **1**



X = CO_2H **2**
= NH_2 **3**

Chemistry: The synthesis of both series of compounds starts from each stereoisomer of cyclohexane 1, 2-dicarboxylic acid mono-methyl ester **4**, which were prepared individually as previously described[11]. The synthesis of the first series of compounds **8**, is outlined in **scheme 1**. Carefully controlled saponification conditions were needed to obtain **5**[12]. The coupling with protected Arg-(Z)-lactam **6**, the reduction by $LiAlH_4$ and the hydrogenolysis to the final products **8**, were carried out according to previously described methods[6]

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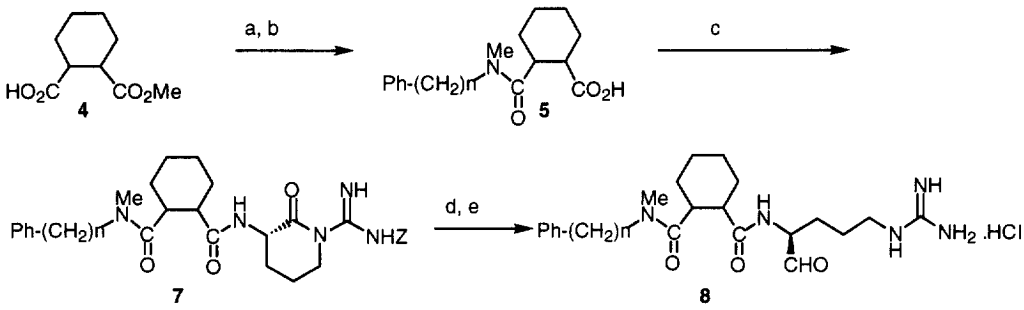
Scheme 1

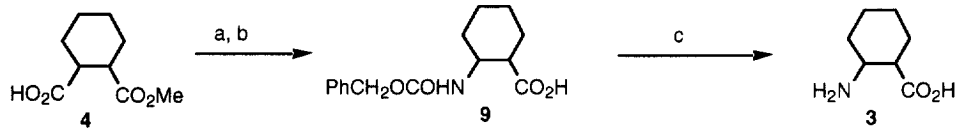


a) (i) $(COCl)_2$, cat.DMF, (ii) $Ph(CH_2)nNHMe$, NEt_3; b) 0.7M NaOH, H_2O/DMSO 1/4; c) DCC, HOBt, NEt_3, N^G-Z-Arginine-lactam-**6**; d) $LiAlH_4$, THF, -60°C; e) H_2, Pd/C, HCl, MeOH

In the synthesis of the second series of compounds, the stereospecific Curtius rearrangement was the key method to provide each enantiomer of 2-aminocyclohexane carboxylic acid **3**, starting from a given stereoisomer of **4** as outlined in **scheme 2**. Chiral HPLC and NMR analysis of the individual stereoisomers of intermediate **9** indicated that no epimerisation or racemisation of the substrates occurred during the ester hydrolysis.

Each stereoisomer of the acid **3** was then used to prepare target compounds **13** as depicted in **scheme 3**.

Scheme 2

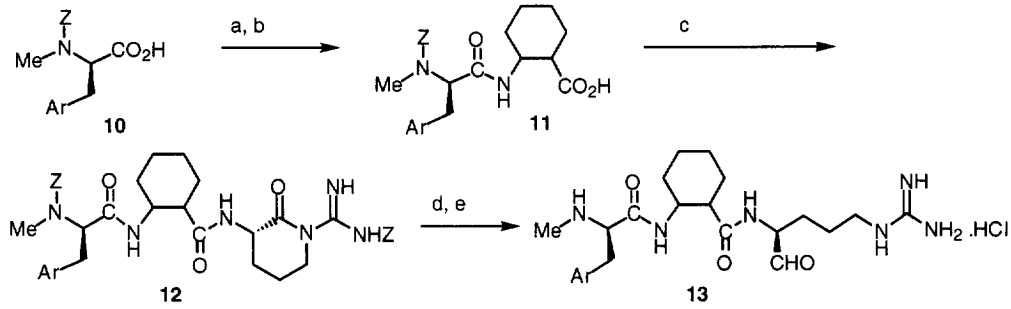


a) DPPA, toluene, $PhCH_2OH$, NEt_3, Δ; b) 2M NaOH, MeOH; c) H_2, Pd/C, MeOH

Results and Discussion: The compounds in both series were evaluated for their activity and the values are reported as a percentage of inhibition of the thrombin enzyme at a concentration of 100 μM. The IC_{50} values of the most active compounds and GYKI 14766 were then evaluated, **Table 1**. The compounds **8a-8d** and **13a-13b** containing the cyclohexane with *trans* stereochemistry were found to have poor thrombin inhibitory activity for both configurations 1S, 2S and 1R, 2R. However changing the stereochemistry of the cyclohexane ring from *trans* to *cis*, brought about noticeably improved inhibitory activity for both series. The compound **8e** containing the racemic *cis* cyclohexane dicarboxylate unit (measured initially as racemates due to the stereochemical instability of **5**)[12] had modest thrombin inhibition activity (36%) at 100 μM, while elongation by one methylene group of the phenyl alkyl side chain resulted in **8f**, with an IC_{50} = 84 μM. The pure enantiomer **8g** showed an IC_{50} = 12 μM, indicating that activity results predominantly from the 1R, 2S configuration in the cyclohexane ring. A similar relationship between stereochemistry of the central cyclohexane ring and activity was observed in the compounds bearing the 2-aminocyclohexane carboxylate unit where **13c** with the *cis* 1R, 2S configuration gave an IC_{50} value of 4μM for thrombin inhibition. Changing to the opposite configuration in **13d** (*cis* 1S, 2R) again resulted in a loss of activity.

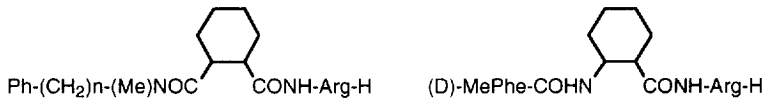
This dependence on the stereochemistry of attachment of the lateral fragments to the central cyclohexane ring in both series led us to seek further improvements in the biological activity by preparing side chain

Scheme 3



a) DCC, THF, 3,4,6-trichlorophenol(TCP) ; b) 2-aminocyclohexane carboxylic acid-**3**, pyridine; c)iBuOCOCl, N-Methyl morpholine, N^G-Arginine[Z]lactam-**6**; d) $LiAlH_4$, THF, -60°C; e) H_2, Pd/C, HCl, MeOH

Table 1. Thrombin Inhibitory Activity[a] of the Cyclohexane 1,2-Dicarboxylate Series, (**8a-g**) and the 2-Aminocyclohexane Carboxylate series (**13a-d**).



8a-g **13a-d**

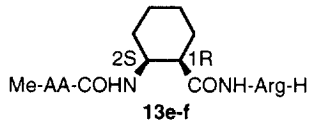
Cpd.	Stereochemistry	n	% Inhibition at 100 μM	IC_{50} (μM)
8a	*trans* 1S, 2S	1	27	n.d.
8b	*trans* 1S, 2S	2	15	n.d.
8c	*trans* 1R, 2R	1	8	n.d.
8d	*trans*1R, 2R	2	14	n.d.
8e	(±)*cis*	1	36[b]	n.d.
8f	(±)*cis*	2	100	84[b]
8g	*cis* 1R, 2S	2	100	12
13a	*trans* 1R, 2R	-	6	n.d.
13b	*trans* 1S, 2S	-	1	n.d.
13c	*cis* 1R, 2S	-	100	4
13d	*cis* 1S, 2R	-	39	n.d.
GYKI 14766				0.046

a) The activity was determined using the synthetic substrate Chromozym TH (Boehringer Mannheim, FRG), according to the experimental procedure detailed by the Supplier. b) Due to the stereochemical instability of the *cis* Cyclohexane-dicarboxylate unit in the synthetic intermediate **5**, some of the compounds in this series containing this moiety were studied for enzyme inhibition activity as their racemates. c) GKYI 14766 was synthesized according to Bajusz et al.[6]

modified analogues while maintaining a fixed configuration as cis 1R,2S. Based on indications from molecular modeling we chose to replace the side chain phenyl group with large hydrophobic groups in order to improve the interaction with the S_3 pocket of the active site of thrombin. As our two leads **8g** and **13c** were active at concentrations well below 100 μM, the subsequent enzyme inhibition studies were carried out at a concentration of 10 μM, **Table 2.** We found that in the case of modifications to **8g**, changing from phenyl to cyclohexyl, **8h**, and to 1-naphthyl, **8i**, resulted in a heavy decrease or practically complete loss of inhibition at the concentration used. In contrast changing the phenylalanine residue of **13c** to the larger β-(2-naphthyl)alanine gave **13e** with an IC_{50} of 22nM, twice as active as **1**. Replacing the N-terminal amino acid with D-phenylglycine, **13f**, caused activity to be lost again.

Table 2. Thrombin Inhibitory Activity of the Side Chain Substituted Compounds.

8h-i



13e-f

Cpd.	Side Chain R or AA	% Inhibition at 10 μM	IC_{50} (nM)
8h	c-C_6H_{11}	10	n.d.
8i	1-Naphthyl	0	n.d.
13e	(D)β-(2-Naphthyl)Ala	100	22
13f	(D)Phg	0	n.d.
GYKI 14766			46

The selectivity of thrombin inhibition compared to activity against three other serine proteases Trypsin, Plasmin and tPA was studied for the most active compounds in our series, **Table 3.** The compounds **8g** and **13c** gave superior selectivity for thrombin inhibition vs. trypsin compared to the lead **1**, but much poorer selectivity against plasmin inhibition. The highest selectivity was showed against t-PA inhibition. On the other hand, the compound **13e**, the most active one in our series, showed markedly superior selectivity for thrombin inhibition vs. all the other serine proteases compared to the lead compound **1**.

Table 3. Selectivity for Thrombin Inhibition vs. Other Serine Proteases [a].

Cpd.	trypsin / thrombin[b]	plasmin / thrombin[c]	t-PA / thrombin[c]
8g	57	67	308
13c	74	38	1000
13e	3409	4545	>5000
GYKI 14766	2	413	n.d.

a) Selectivity is defined as the ratio of IC_{50}'s. b) Trypsin inhibition was determined according to the ref.17. c) Plasmin and t-PA inhibitions were determined using the synthetic substrates Chromozym PL and Chromozym t-PA, respectively, according to the experimental procedure detailed by the Supplier (Boehringer Mannheim, FRG).

We used modeling to correlate the biological results in order to explain the influence of structural changes and to infer from this the three dimensional geometrical requirements for binding of these inhibitors to thrombin. Since the X-ray coordinates of GYKI 14766 complexed to human α-thrombin are not available, we chose PPACK (D-Phe-Pro-Arg-CH_2Cl)[13] as a reference and a model of the active site was constructed from its crystal structure bound to thrombin[14]. Conformational analysis was performed with the Discover Molecular Mechanics program, and use of the Consistent Valence Force Field method (CVFF)[15-16], on compounds **8a-g** and **13a-d** by varying in sequence their most significant torsion angles. The obtained low energy conformations (those within 8 kcal/mole of the global energy minimum) were overlaid to PPACK (complexed with human α thrombin) and the conformers (one for each compound) having maximum overlap of important groups such as the carbonyl groups near or attached to the cyclohexane ring, the cyclohexane ring (with the proline ring of PPACK) and the guanidinium group, were selected. The chosen conformation of compounds **8a-g** and **13a-d** were docked onto the model of the active site of human α-thrombin containing PPACK in such a manner that the atoms of **8a-g** and **13a-d** were superimposed with the topologically equivalent ones of PPACK and finally PPACK was removed.

All the intermolecular interactions stabilizing the complex have been analyzed in detail. The compounds in both series containing the *trans*-cyclohexane residue did not satisfy the similarity criteria at all. We therefore decided to consider only models concerning the compounds **8 f, g** and **13 c, d** with a *cis*-stereochemistry of the cyclohexane ring; these models were therefore minimized using Discover [15].

The S_1-S_3 sites in **8f, g**-thrombin complexes and **13c, d**-thrombin complexes are occupied in an anti-parallel fashion by the side chain of Arg, the cyclohexane carboxylic acid and D-Phe groups respectively. Asp189 is positioned at the bottom of the S_1 pocket to make a salt bridge with the arginine residue of the inhibitor. The cyclohexane ring is positioned in a hydrophobic pocket formed by residues His 57, Ser214, Leu99, Tyr60A and Trp60 D with the latter two residues forming a lid to the pocket. The interaction energy of our compounds with these residues is mainly of the Van der Waals type.

The S_2 site is ideally suited to the binding of medium size non-polar residues. In this region there is a second hydrophobic pocket (S_3) lined by the residues Ile174, Trp215 and segment 97-99, which is occupied by the phenyl ring of our compounds and is particularly suited to aromatic residues. The interaction energy is mainly of the Van der Waals type and Trp215 is the residue with the strongest contribution term.

Figure 1. Docking of **13e** onto the model of the active site of thrombin.

A *cis*- 1R, 2S configuration seems to be favoured in the complex and the spatial disposition of the inhibitor inside the active site is such that the interaction of both cyclohexane and phenyl groups, with the residues of the hydrophobic pockets in which they are positioned, is maximized.

Intermolecular interactions of **13c** with the S_2, S_3 pockets are stronger than those of compound **8g**. The S_3 pocket in which the phenyl ring is inserted is big enough to contain a larger aromatic group. Replacement of the phenyl ring with a naphthyl ring, giving **13e**, improved the intermolecular interaction energy and contributed to a greater stability of the inhibitor-enzyme complex (see Figure 1).

In summary, replacement of the proline residue in GYKI 14766, **1**, with a 1, 2 disubstituted cyclohexane moiety gave rise to potent and selective thrombin inhibitors. In the modeling studies the stereochemistry of the central cyclohexane unit was shown to be an important criterion for superimposition onto PPACK. This was confirmed by the profile of inhibition activity of our series of compounds. Of the two types of mimics used, the 2-aminocyclohexane carboxylic acid unit gave overall the best results for activity. Furthermore modeling studies indicated the importance of an enlargement of the N-terminal side chain to increase hydrophobic interactions with the S_3 pocket of the active site. This further modification led to compound **13e** which is not only the more active compared to **1**, but also a much more selective thrombin inhibitor.

References and Notes

1. Kimball, S. D. *Current Pharmaceutical Design*, **1995**, *1*, 441.
2. Tapparelli, C.; Metternich, R.; Ehrhardt, C.; Cook, N. S. *TIPS*, **1993**, *14*, 366.
3. Bajusz, S.; Barabas, E.; Tolnay, P.; Szell, E.; Bagdy, D. *Int. J. Peptide Protein Res.*, **1978**, *12*, 217.
4. Schuman, R. T.; Rothenberger, R. B.; Campbell, C. S.; Smith, G. F.; Gifford-Moore D. S.; Gesellchen, P. D. *J. Med. Chem.*, **1993**, *36*, 314.
5. Deadman, J. J.; Elgendy, S.; Goodwin, C. A.; Green, D.; Baban, J. A.; Patel, G.; Skordalakes, E.; Chino, N.; Claeson, G.; Kakkar, V. V.; Scully, M. F. *J. Med. Chem.*, **1995**, *38*, 1511.
6. Bajusz, S.; Szell, E.; Bagdy, D.; Barabas, E.; Horvath, G.; Horvath, G.; Dioszegi, M.; Zittler, Z.; Szabo, G.; Juhasz, A.; Tomori, E.; Szilagyi, G. *J. Med. Chem.*, **1990**, *33*, 1729.
7. Tamura, S. Y.; Goldman, E. A.; Brunck, T. K.; Ripka, W. C.; Semple, J. E. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 331.
8. (a) Levy, O. E.; Semple, J. E.; Lim, M.L.; Reiner, J.; Rote, W.E.; Dempsey, E.; Richard, B.M.; Zhang, E.; Tulinsky, A.; Ripka, W.C.; Nutt, R.F., *J. Med. Chem.*, **1996**, *39*, 4527. b) Lyle, T.A.; Chen, Z.; Appleby, S.D.; Freidinger, R.M.; Gardell S.J.; Lewis, S.D.; Li, Y.; Lyle E.A.; Lynch Jr., J.J.; Mulichak, A.M.; Assunta Ng S.; Naylor-Olsen, A.M., Sanders, W.M. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 67.
9. Dominguez C.; Carini D.J.; Weber P.C.; Knabb, R.M.; Alexander, R.S.; Kettner, C.A.; Wexler, R.R. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 79.
10. (a) Akiyama, Y.; Tsutsumi, S.; Hatsushiba, E.; Ohuchi, S.; Okonogi, T.; *Bioorg. Med. Cem. Lett.*, **1997**, *7*, 533. (b) Tamura, S.Y.; Shamblin, B.M.; Brunck, T.K.; Ripka, W.C. *ibid*, 1359.
11. Turbanti, L.; Cerbai, G.; Di Bugno, C.; Giorgi, R.; Garzelli, G.; Criscuoli, M.; Renzetti, A. R.; Subissi, A.; Bramanti, G. *J. Med. Chem.*, **1993**, *36*, 699.
12. We found in our initial syntheses that these substrates undergo both extensive *cis/trans* epimerization and racemisation during the saponification when the amide group in the precursor was not alkylated It was also found that even the isolated pure *cis*-isomers of the acid amide **5** had to be converted immediately, as it racemised completely on conserving at low temperature for a prolonged period.
13. Ketter, C.; Shaw, E. *Throm. Res.*, **1979**, *14*, 969.
14. The atomic co-ordinates of the complex of human α-thrombin with PPACK were taken from the Brookhaven Protein Data Bank (data base designation 1 dwe), Bernstein, F.C.; Koetzle, T.F.; Williams G.J.B.; Meyer, E.F.; Brice, M.D.; Rodgers, J.R.; Kennard, O.; Shimanouchi, T., Tasumi, M., *J. Mol. Biol.*, **1977**, *112*, 535.
15. Discover™, Biosym Technologies Inc., San Diego, CA 92121-2777; the selected force field was CVFF, a dielectric constant dependent on distance of $\varepsilon = 4$ was used.
16. Hagler, A.T.; Lifson, S. *J. Am. Chem. Soc.*, **1974**, *96*, 5319.
17. Bergmeyer H.U., In *Methods in Enzymatic Analysis*, Academic Press inc., 1974 Edition, p. 815.