Pergamon

Tetrahedron Letters, Vol. 38, No. 18, pp. 3305-3308, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00592-3

Design of a novel class of bifunctional thrombin inhibitors, synthesised by the first application of peptide boronates in solid phase chemistry

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Abstract: Borologs containing peptide boronates are new bifunctional biologically active molecules which bind to and inhibit thrombin. These compounds are designed based on the C-terminal sequence of hirudin. The inhibitors consists of four parts, i) an active site inhibitor, D-Phe Pro-Boro(aa)-OPin. ii) an anion binding exosite association moiety, Hirudin, iii) a spacer to link these components and iv) a novel 'flexor' non-peptide unit to allow correct orientation. The bivalent nature of the inhibitor [-D-PheProBoroBpgOPin]$CO(CH_2)_3COGly_2$Hir enhanced binding up to 10 fold greater than the corresponding native peptide Z-D-PheProBoroBpgOPin or the mixture of non covalently linked units, and resulted in a potent and selective inhibitor of thrombin having a Ki of 0.6nM. For the synthesis of these compounds suitably protected aminoboronate derivatives were shown to be compatible with FMOC solid phase chemistry. © 1997 Elsevier Science Ltd.

Thrombin is a multifunctional enzyme of the blood coagulation cascade. The most important biological functions of this enzyme are: its interaction with fibrinogen and the activation of both procoagulant (e.g. factor XIII), and, when bound to thrombomodulin, fibrinolytic (e.g. protein C) factors, and diverse cellular activities as on platelets and endothelial cells.

The most potent thrombin inhibitor known is hirudin, a family of isoproteins isolated from the glandular secretion of the leech Hirudo medicinalis[1]. This single chain protein containing 65 residues has a high affinity for α-thrombin ($K_D=10^{-12}$ to 10^{-15} M) and does not inhibit other haemocoagulant enzymes[2]. It consists of an active site blocking moiety, (Hirudin $^{1-48}$), a fibrinogen-recognition exo-site binding moiety, (Hirudin $^{55-65}$), and a linker, (Hirudin $^{49-54}$), connecting these inhibitor moieties.

Hirulog[3] is a bifunctional peptide based upon the structure of hirudin. It combines a fragment of the C-terminus of hirudin (interacting with the anion binding exosite of thrombin) with various low molecular weight inhibitor structural units joined by a spacer peptide sequence.

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H-D-Phe-Pro-Arg-Pro was used as an N-terminus fragment[3] (which interacts with the catalytic site of thrombin) in Hirulog-8, i.e. H-D-Phe-Pro-Arg-Pro$(Gly)_4Hir^{53-64}$, giving a Ki value of 2.5 nM.

Hirulog-8 was elaborated[4] to compound P79, Ac-D-Phe-Pro-Arg$\psi(COCH_2)CH_2CH_2COHir^{49-65}$, by incorporation of a ketomethylene isosteric replacement which improved the potency to Ki= 0.33nM.

The active site blocking moiety was also replaced by a small non substrate-like active site inhibitor of thrombin[5], e.g., dansyl-Arg-(D-pipecolic acid), to improve the affinity (Ki) of the inhibitor 10-100-fold (down to $1.7X\ 10^{-11}$ M) compared to that of similar compounds having D-Phe-Pro-Arg as their substrate type inhibitor moiety ($Ki=10^{-9}$-10^{-10} M).

The proposed design of NAPAP$(Gly)_4Hir^{53-65}$ was reported with expectation of enhanced potency and specificity for thrombin[6].

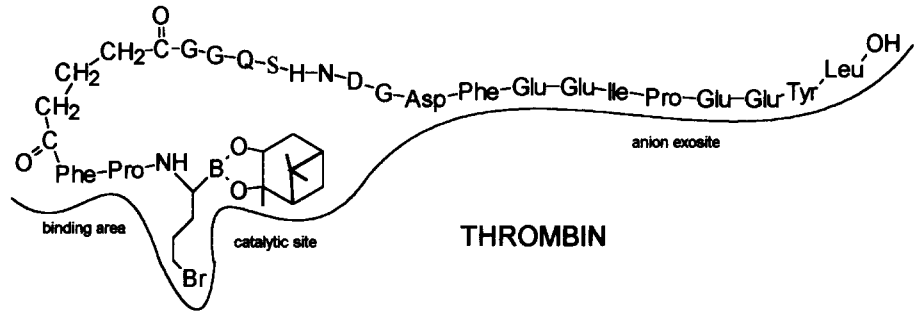

Figure: Schematic drawing of the hirulog [-D-PheProBoroBpgOPin]$CO(CH_2)_3$COGGHir$^{49-64}$ bound to thrombin.

More generally in the field of drug discovery, developments in the areas of rapid screening for biological activity , have lead to interest in combinatorial libraries, based for instance on peptidic units, which utilise solid phase chemistry techniques to produce sets of homologous compounds. Incorporation of specific synthetic units, of known affinity for target enzymes, such as the peptidophosphonates of Campbell et al.[7], has been used to ehance biological activity of the libraries.

Our ongoing research with thrombin has demonstrated that the bulky active site blocking moiety, (Hirudin $^{1-48}$), can effectively be replaced with a boron containing tripeptide[8], such as one based on Z-D-Phe-Pro-BoroBpgOPin. Since the compatibility of peptide boronate esters to Fmoc solid phase chemistry conditions has not previously been reported, we undertook screening studies with a variety of esters (manuscript in preparation) and found pinanediol to be stable to TFA exposure over a two hours period generally required for resin cleavage and peptide deprotection. Glutaric acid was then used as a flexible, non-peptide, unit, or 'flexor', to link the N-terminus of the linker, e.g. the dipeptide GG which is contiguos with the exosite I binding sequence, e.g. $Hir^{49-64,}$ to the N-terminus of Phe-Pro-BoroBpgOPin as shown in the figure. Selection of the flexor from a wide variety of organic diacid synthons was possible and in combination with variation of the linker (manuscript in

preparation) lead to a potent antithrombotic agent. The borolog, as synthesized, had an improved inhibition of thrombin of at least 10 fold, (see theTable below), compared to an equimolar mixture of the constitutive units.

Table: Ki values for the inhibition of thrombin.

ENTRY	PEPTIDES	Ki(μM)
1	$HOOC(CH_2)_3COGGHir^{49-64}$	12.7
2	$GGHir^{49-64}$ + Z-D-Phe-Pro-BoroBpgOPin	0.02
3	Z-D-Phe-Pro-BoroBpgOPin	0.008
4	[-D-Phe-Pro-BoroBpgOPin]$OC(CH_2)_3COGGHir^{49-64}$	0.0006

GlyGlyHir $^{49-64}$ which has the amino acid formula: H-Gly-Gly-Gln-Ser-His-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-OH, was prepared by solid phase peptide chemistry on a Milligen 9050 PepSynthesizer using an Fmoc-polystyrene continuous flow method and proprietary 9050 Plus on column monitoring software. Pre-derivatised solid support, Fmoc-Leu-PEG-PS (1.6g, 0.22meq/g) was used throughout. Fmoc groups were removed using 20% piperidine in DMF. Fmoc-amino acids (4 equiv.) as their pentafluorophenyl esters with side chain protection where appropriate (e.g. . Fmoc-L-Tyr(tBu)OPfp, Fmoc-L-Glu(tBu)OPfp, Fmoc-L-Asp(tBu)OPfp, Fmoc-L-Asn(Trt)OPfp and Fmoc-His(Boc)OPfp), were coupled sequentially. Once the required peptide sequence was complete, the N-terminal Fmoc group was removed using 20% piperdine in DMF. A positive ninhydrin test indicated that the Fmoc group had been removed. The peptide-conjugated resin was subsequently decanted on a filter and washed 'off-line' with dichloromethane, methanol and dichloromethane before being dried in-*vacuo* for a few hours.

To introduce the flexor unit, the peptide was suspended in DMF(5ml) and treated with glutaric anhydride (300mg) and 4-methyl-morpholine (200mg) in a round bottomed flask(25ml). The reaction mixture was swirled overnight. The resin was washed with DMF, DCM and MeOH, and then dried in vacuo overnight.

H-D-Phe-Pro-BoroBpgOPin was prepared by adding a 40%solution of HBr in acetic acid (20ml) to Z-D-Phe-Pro-BoroBpgOPin (2g) in a round bottomed flask (100ml) fitted with a septum and flushed with nitrogen. The flask was swirled to effect complete dissolution of the protected tripeptide. When the gas evolution ceased after approximately 30 minutes, anhydrous ether (200ml) was added and the reaction mixture was stored in a refrigerator for 4 hours. The reaction mixture was filtered, the

residue was dissolved in EtOH(1ml) and dry ether was added to precipitate the product (800mg) as a white solid (M+H), 516; Tlc (CHCl3/MeOH/acetic acid, 95/5/3), Rf=0.05.

To synthesize [-D-PheProBoroBpgOPin]$CO(CH_2)_3COGly_2Hir^{49-64}$, the dry resin $HOCO(CH_2)_3$ $COGly_2Hir^{49-64}$ was suspended in DMF (10ml), before TBTU (129mg, 0.4mmol) and H-D-PheProBoroBpgOPin (230mg, 0.4mmol) were added to the reaction mixture. After 5 minutes stirring, triethyl amine (40mg, 0.4mmol) was added and the flask left stirring overnight.

The fully protected peptide resin was washed with dichloromethane, methanol and dichloromethane and then dried under vacuum. Cleavage from the resin with simultaneous deprotection of side chain protecting groups was achieved by treating the resin with 100% TFA for two hours. TFA was removed and the free peptide with a C-terminal carboxylic acid was generated by precipitation with cold dry ether. The crude peptide was collected by filtration and washed with further portions of ether.

Purification of the crude peptide was carried out by reversed-phase HPLC using a Vydac C-18 preparative column (TP silica, 10μm, 25mmX300mm). The column was eluted with a 30-90% linear gradient of solvent A(0.1%TFA in water) and solvent B(0.1%TFA in acetonitrile). The column eluants were monitored at 230nM, and fractions were collected appropriately. The purity of the products were determined by analytical RP-HPLC and mass spectrometry.

In summary, we have demonstrated for the first time the compatibility of peptide boronate esters with Fmoc solid phase chemistry. Finally a new four component design has lead to a novel series of inhibitors deriving tight binding by utilisation of both active site and exosite I binding domains of thrombin. These features introduce the possibility of facile introduction of peptides with affinity for exosites of the target protease to peptide boronates, already identified as potent enzyme inhibitors.

Other areas of future interest which are to be focused on, are the variations in the hydrophobic binding site, the P1 side chain, the length of the spacer and the C-terminal residues of the hirulogs.

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(*Received in UK* 10 *February* 1997; *revised* 24 *March* 1997; *accepted* 26 *March* 1997)