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O-BENZYL HYDROXYPROLINE AS A BIOISOSTERE FOR PHE-PRO: NOVEL DIPEPTIDE THROMBIN INHIBITORS

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Abstract: A series of analogs were prepared based on the known thrombin inhibitor PPACK, in which the D-Phe-Pro dipeptide has been replaced by trans-4-O-benzyl hydroxyproline. One of these analogs is a more potent inhibitor of thrombin, and is more selective, than PPACK itself.

Introduction:

Thrombin, the final enzyme of the coagulation cascade, plays a central role in the processes of thrombus formation.[1] This serine protease is responsible for the conversion of fibrinogen to fibrin monomers and promotes its own formation via the activation of factors V and VIII.[2] Thrombin is also a potent agonist of platelet activation.[3] Inefficient control of thrombin activity is a major contributor to thrombotic events.[4] With such a pivotal role in the processes of thrombogenesis, thrombin has long been a target for intervention in the treatment of thrombotic disorders.[5]

The prototypical small peptide thrombin inhibitor is D-Phenylalanine-Proline-Arginine chloromethylketone (PPACK).[6] This molecule has been shown to be an effective antithrombotic agent in vivo.[7] The use of PPACK as a drug is hampered by the presence of the chemically reactive chloromethylketone, as well as a series of undesirable properties, including poor selectivity, short half-life, and low oral bioavailability.[8] In order to begin addressing some of these problems an attempt was made to simplify the structure of PPACK and in the process reduce the peptide nature of the molecule. During these studies the chloromethylketone was left intact in order to facilitate comparisons to a well established potent inhibitor of thrombin.

Molecular models indicate that a benzyloxy group introduced at the 4-position of the proline ring should be able to occupy the same region in space as the D-phenylalanine side chain in PPACK. Consequently, a series of functionalized hydroxyproline-arginine dipeptide chloromethylketones were prepared. The difference in the activity between the *cis* -and *trans* -4-benzyloxy Hyp derivatives was found to mirror the differences observed in tripeptides containing D-Phe and L-Phe.[9] One of the compounds prepared was found to be a more potent inhibitor of thrombin than PPACK itself, with an improved selectivity profile.

Results and Discussion:

IC_{50} values for the inhibition of thrombin were obtained following the method of Seghatchian.[10]

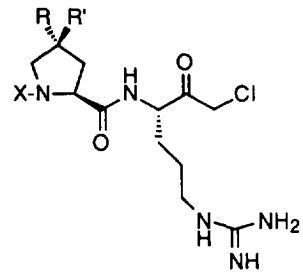
The first analogs that were prepared involved appending a benzyloxy group directly onto the PPACK structure by substituting 4-O-benzyl hydroxyproline for proline. Introduction of a *trans* -4-benzyloxy group onto the proline residue of PPACK results in a twofold loss of potency for the inhibition of thrombin. A *cis* -4-benzyloxy substituent reduces potency almost fivefold. These relatively small decreases in potency may be an indication that in these two analogs the phenylalanine side chain occupies the same region in the enzyme as it does in PPACK and the additional phenyl ring from the benzyl group is collapsed upon the Phe sidechain in the same hydrophobic pocket. This packing together of two hydrophobes has been observed in the crystal structure of thrombin with a number of other inhibitors.[11] With **2** in hand as a lead, D-phenylalanine was replaced with a variety of acyl groups in an attempt to begin to simplify the structure of the tripeptide.

While removal of the terminal amino group from the D-Phe residue resulted in a tenfold loss in potency, replacement of the entire amino acid by an acetyl group led to compound **5**, which was actually a better inhibitor of thrombin than PPACK. Thus the lead compound was reduced from a tripeptide to a more potent dipeptide analog. Eliminating the carbonyl group of **5** to give the N-ethyl analog **6** resulted in an almost 10,000-fold loss in potency. Both the propionyl- and methanesulfonyl-containing analogs were also less potent than **5**. These results demonstrate both the importance of the carbonyl group attached to the proline nitrogen (**5** vs **6**) and the strict size requirement for the acyl group at this position (**5** vs **7** vs **4**).

With an acetyl group in place at the proline nitrogen, the difference in activity between the *cis* - and *trans* -4-benzyloxy derivatives is much more significant than was observed for compounds **2** and **3**. The N-acetyl-*cis* -4-benzyloxy analog (**9**) is more than 5000-times less potent than its trans counterpart **5**. This great difference in potency is to be expected if the *trans* -4-benzyloxy group is now filling the same role that the D-phenylalanine residue does in PPACK. Similar differences are noted between tripeptides containing a D-Phe or an L-Phe residue at P3.[9] In addition, deletion of the benzyloxy substituent, as in **10**, gives a relatively inactive compound. This is further evidence that the *trans* -4-benzyloxy group plays a major role in recognition of these inhibitors by thrombin.

Simple modifications to the benzyloxy group in **5** all lead to decreases in potency. The best tolerated change involves saturation of the phenyl ring to give the cyclohexylmethyloxy analog **12**. This compound is fivefold less potent than its benzyloxy counterpart. The least tolerated change is the replacement of the oxygen with a methylene group to give the all carbon analog **11**, which is 40-fold less potent. Other changes that involve lengthening the benzyl group or increasing/decreasing its steric bulk give analogs with potencies between those of **11** and **12**.

Table 1. Hydroxyproline Based Thrombin Inhibitors.



	X	R	R'	thrombin IC_{50} (nM)	IC_{50} Factor Xa / IC_{50} thrombin	IC_{50} plasmin / IC_{50} thrombin
1	D-Phe	H	H	1.5	83	166
2	D-Phe	OCH_2Ph	H	3.0	366	266
3	D-Phe	H	OCH_2Ph	7.2		
4	$Ph(CH_2)_2CO$	OCH_2Ph	H	31.7		
5	CH_3CO	OCH_2Ph	H	0.9	1088	244
6	CH_3CH_2	OCH_2Ph	H	8600		
7	CH_3CH_2CO	OCH_2Ph	H	13.0	115	31
8	CH_3SO_2	OCH_2Ph	H	24.0		
9	CH_3CO	H	OCH_2Ph	4600		
10	CH_3CO	H	H	1350		
11	CH_3CO	CH_2CH_2Ph	H	40.0		
12	CH_3CO	$OCH_2C_6H_{11}$	H	4.9	120	183
13	CH_3CO	$O(CH_2)_3CH_3$	H	21.6		
14	CH_3CO	$O(CH_2)_3Ph$	H	14.0		
15	CH_3CO	$O(CH_2)_2CHPh_2$	H	10.0		

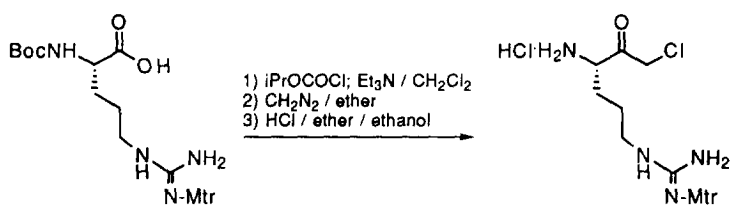
This then demonstrates that the P3-P2 dipeptide of a typical tripeptide thrombin inhibitor may by replaced by a single, appropriately substituted residue. In this case *trans* -4-O-benzyl hydroxyproline serves as an isostere for D-Phe-Pro.

Several of the analogs in Table 1 were tested for their inhibition of two other related serine proteases, Factor Xa and plasmin. Compound **5**, the most potent inhibitor of thrombin, was more than an order of magnitude more selective towards Factor Xa than PPACK (**1**). Selectivity vs plasmin was increased much more modestly.

In addition, one compound (**11**) was tested for antithrombotic efficacy in vivo using a model of $FeCl_3$ induced thrombosis in the rat carotid artery.[12] The compound was administered as an intravenous bolus (3mg/kg) plus a constant infusion (0.3 mg/kg/min) beginning five minutes before the application of $FeCl_3$ and continuing until formation of an occlusive thrombus or for a maximum of 65 minutes. Vessel patency was maintained for the entire experiment in the four rats given compound **11** versus an average time to occlusion of 18 ± 1 minute for four rats given saline. Thrombus mass was also reduced from 5.5 ± 0.2 mg in the saline group to 2.9 ± 0.2 mg in the group given **11**. Activated partial thromboplastin time and prothrombin time were not different between groups.

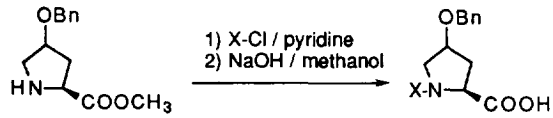
Chemistry:

Arginine chloromethylketone was prepared following the method of Kettner,[13] with several modifications. The arginine sidechain was protected as its methoxytrimethylphenylsulfonyl (Mtr) derivative and the required diazoketone was formed using isopropyl chloroformate followed by diazomethane. The chloromethylketone was then prepared with HCl in ether. Ethanol was required as a cosolvent to completely solubilize the diazoketone. The chloromethylketone was directly coupled to the remainder of the molecule. This method was found to be higher yielding than forming the chloromethyl ketone directly on the di- or tripeptides by the published route. These modifications increased the yield to 46%, from the reported 19%.



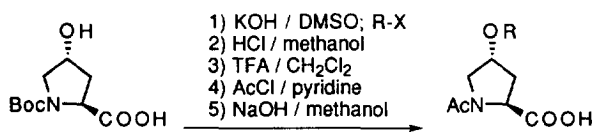
For **4-9**, *cis* - or *trans* -4-benzyloxyhydroxyproline methyl ester was first acylated or alkylated with the appropriate acid chloride or alkyl halide. Hydrolysis of the methyl ester then

gave the material that was coupled to arginine chloromethylketone. Yields for this two step conversion were between 80 and 90%.



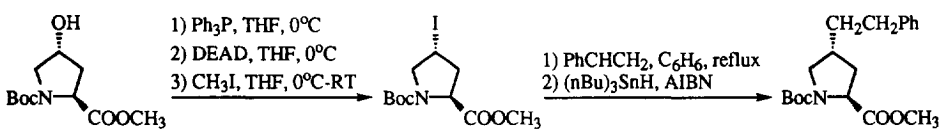
X=$Ph(CH_2)_2CO$ (**4**); CH_3CO (**5** and **9**); CH_3CH_2 (**6**); CH_3CH_2CO (**7**); CH_3SO_2 (**8**)

For **12-15**, Boc-*trans* -4-hydroxyproline was first O-alkylated with the appropriate alkyl halide using KOH in DMSO. Yields for this reaction were highly variable, ranging from 30 to 80% depending on the alkyl halide used. KOH in DMSO were found to be the optimal conditions for this reaction. Conversion to the methyl ester, deprotection, acylation and hydrolysis (>90% for 4 steps) subsequently gave the functionalized hydroxyproline that was coupled to arginine chloromethylketone.



R=$C_6H_{11}CH_2$ (**12**); $CH_3(CH_2)_3$ (**13**); $Ph(CH_2)_3$ (**14**); $Ph_2CH(CH_2)_2$ (**15**)

For compound **11**, 4-(2-phenylethyl)proline was prepared by first preparing the requisite iodide from N-Boc-4-hydroxyproline methyl ester,[14] and then trapping the corresponding free radical[15] with styrene to give the desired product in 68% yield.

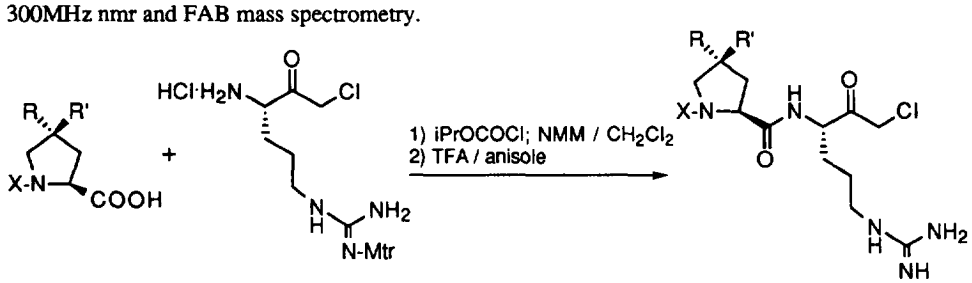


(+ *cis* iodide)

For compounds **1**, **2**, **3**, and **10**, D-Phe-Pro-OH, D-Phe-*trans* -4-benzyl Hyp-OH, D-Phe-*cis* -4-benzyl Hyp-OH, and Ac-Pro-OH, respectively, were coupled to arginine chloromethylketone.

All couplings to give the final products were performed with isopropyl chloroformate in CH_2Cl_2, with yields typically ranging from 70 and 90%. Removal of the Mtr protecting group

was accomplished with TFA in anisole (100%). Final products were purified by reverse phase HPLC using an acetonitrile/water gradient buffered with 0.1% TFA and characterized by 300MHz nmr and FAB mass spectrometry.



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