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Bioorganic & Medicinal Chemistry Letters 13 (2003) 2363–2367

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Polymer-Assisted Solution-Phase (PASP) Parallel Synthesis of an α-Ketothiazole Library as Tissue Factor VIIa Inhibitors

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Received 31 January 2003; revised 15 April 2003; accepted 16 April 2003

Abstract—A solution-phase synthesis of an α-ketothiazole library of the general form D-Phe-L-AA-L-Arg-α-ketothiazole is described. The five-step synthesis is accomplished using a combination of polymeric reagents and polymer-assisted solution-phase purification protocols, including reactant-sequestering resins, reagent-sequestering resins, and tagged reagents. The multi-step synthesis affords the desired α-ketothiazole products in excellent purities and yields. A variety of L-amino acid inputs were used to probe the S2 pocket of the tissue factor (TF) VIIa enzyme to influence both potency and selectivity. An X-ray crystal structure of compound **10e** bound to the TF/VIIa complex was obtained that explains the observed selectivity. The α-ketothiazoles were found to be potent, reversible-covalent inhibitors of tissue factor VIIa, with some analogues demonstrating selectivity versus thrombin.

Cardiovascular disease is the most common cause of mortality in the western world. The disease is characterized by the acute coronary syndromes unstable angina and myocardial infarction, which often lead to sudden death.[1] This inappropriate thrombus formation is initiated via the extrinsic coagulation cascade by a plaque rupture, which exposes tissue factor to the serine protease VIIa in circulating blood forming the TF/VIIa complex. This complex then further activates the serine proteases IX to IXa and X to Xa, which in turn activate prothrombin to thrombin and fibrinogen to fibrin. Upon combination with activated platelets, a fibrin clot may result in a life threatening thrombus formation.[2] There is evidence from our own laboratories as well as others that selective inhibition of the TF/VIIa complex may provide effective anticoagulation while lessening the risk of bleeding side effects when compared to other antithrombotic mechanisms such as IIb/IIIa antagonists and inhibitors of Xa and thrombin.[3]

An approach to the design of serine protease inhibitors has been the replacement of the scissile amide bond by an electron-deficient carbonyl group.[4] A series of aldehydes,[5] α-fluoroketones,[6] α-keto esters/amides,[7] and α-ketothiazoles[8] have been incorporated into peptidyl protease inhibitors. Since the report of the X-ray crystal structure of D-Phe-L-Phe-L-Arg chloromethyl ketone (DFFRCMK) bound to the active site of tissue factor VIIa,[9] comparisons of the various pockets with other closely related proteases such as thrombin and Xa have been possible. The most notable difference is the S2 pocket of VIIa. The S2 pocket of TF/VIIa is larger than that of thrombin and Xa and differs by a key residue, Asp 60, contained only in TF/VIIa. We initiated a program to identify a selective tissue factor VIIa inhibitor. At the outset of the program, we sought to utilize tripeptide transition state analogues closely related to DFFRCMK. The design was of the general form D-Phe-L-AA-L-Arg-α-ketothiazole, utilizing the DFFRCMK backbone with α-ketothiazole in place of the chloromethyl ketone to provide a covalent-reversible inhibitor. The L-amino acid allows for variation at the P2 position, which directly overlaps the S2 pocket of

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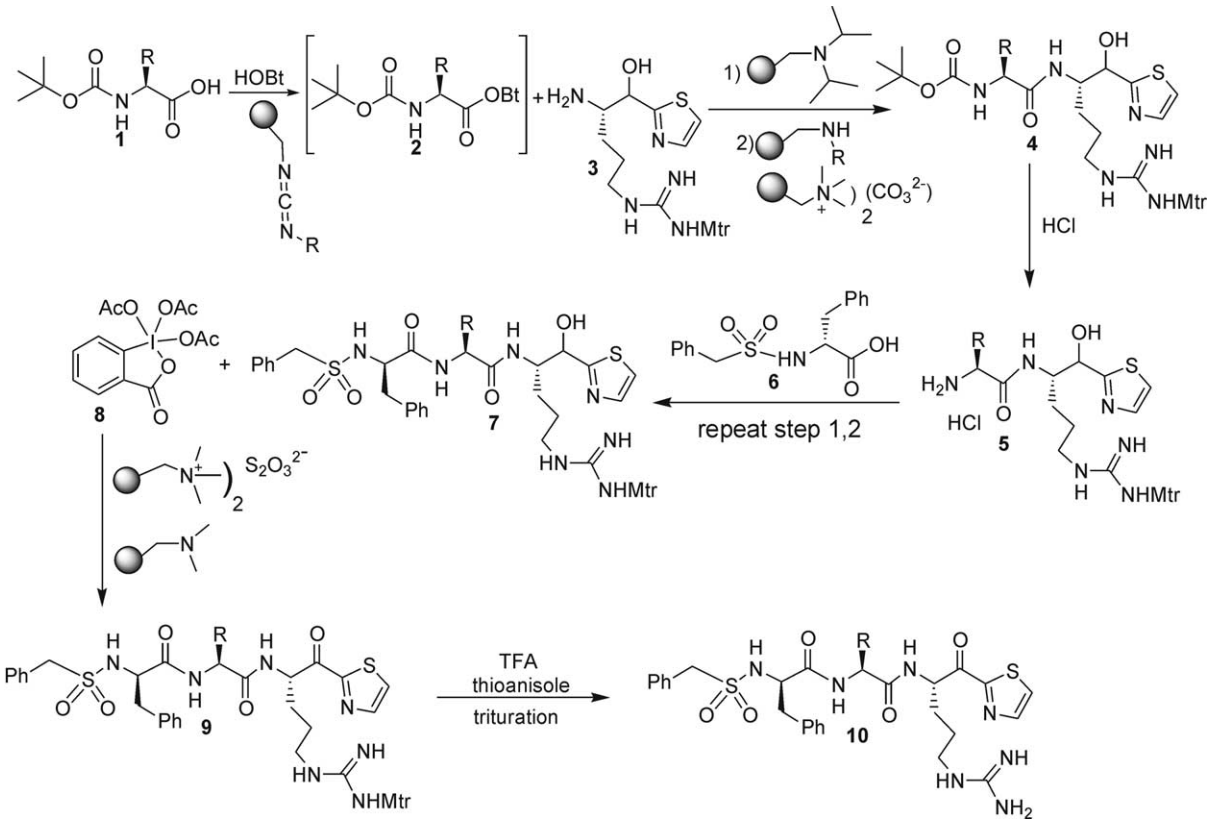
doi:10.1016/S0960-894X(03)00398-6

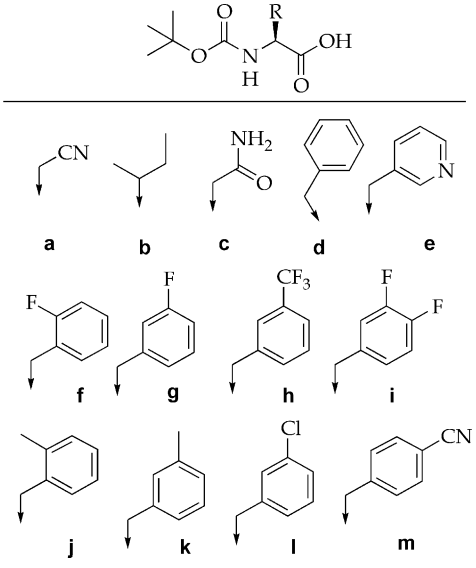


Scheme 1. PASP Synthesis of an α-ketothiazole library.

the enzyme and is expected to influence both potency and selectivity. In an effort to rapidly prepare libraries of α-ketothiazole peptidyl protease inhibitors in a parallel format, a solution-phase library synthesis was developed utilizing polymer-assisted solution-phase (PASP) technology.[10] Herein, we report a five-step PASP synthesis and biological activity of α-ketothiazoles with the structural motif D-Phe-L-AA-LArg-α-ketothiazole.

A summary description of the library synthesis is depicted in Scheme 1. Each of the steps in the synthesis underwent independent validation in order to optimize conditions such that each transformation could be performed in a high-yielding, parallel format. The synthesis was accomplished using a combination of polymeric reagents and polymer-assisted solution-phase purification protocols, including reactant-sequestering resins, reagent-sequestering resins, and tagged reagents. Compound **3** was selected as the starting scaffold in its reduced form as the alcohol to avoid side reactions during the ensuing steps. As a result, the synthesis was devised such that oxidation of the alcohol to the ketone was conducted near the end. The synthesis is general and allows for variation of either amino acid. The initial library was designed with D-Phe as the terminal amino acid capped as the benzylsulfonamide. The benzylsulfonamide moiety, expected to occupy the S3 pocket, was selected based on the structure activity relationship (SAR) from the thrombin literature and the similarity of the S3 pockets of factor VIIa and thrombin. Considering the larger size of the S2 pocket of TF/VIIa compared to thrombin and Xa, a variety of L-amino acids were used to probe the S2 pocket with the hope of providing potency and selectivity.

The first step of the synthesis in Scheme 1 involved an amide coupling using the starting scaffold **3** and a variety of BOC protected L-amino acids **1**, which are shown in Scheme 2. The polymer-bound carbodiimide resin was used as the coupling agent with hydroxybenzotriazole. Upon completion of the reaction, a



Scheme 2. Amino acid inputs.

mixed-resin bed of the polyamine and carbonate resins were added to sequester the remaining reactants. Simple filtration and evaporation of the solvents left highly purified products **4**.

Deprotection of the BOC group was accomplished by treatment with 4*N* hydrochloric acid in dioxane to afford the amine hydrochloride salts **5**. The next step of the synthesis involved an amide coupling using the amine intermediate **5** and *N*-(benzylsulfonyl)-D-phenylalanine **6**. The same reaction conditions were applied from step 1 except that the sequestration process involved using only the polyamine resin. The carbonate resin was omitted in this step since the strongly basic carbonate resin sequesters the desired products **7** due to the acidic proton on the sulfonamide. The oxidation of the alcohol to the ketone utilized a PASP purification protocol.[11] The alcohol **7** was oxidized with an excess of the periodinane reagent **8**. Upon completion of the reaction, the thiosulfate resin was added to the product mixture, which reduced the excess Dess–Martin reagent **8** and the I^{III} by-product species to the sequesterable 2-iodobenzoic acid. In addition, the thiosulfate resin sequestered the majority of the 2-iodobenzoic acid. Amberlyst A-21 was added to sequester the remaining acid. Simple filtration and rinsing with dichloromethane yielded a filtrate, whereupon evaporation of the solvents left purified products **9** from each parallel reaction. The last step of the synthesis involved deprotection of the 2,3,6-trimethyl-4-methoxybenzenesulfonyl (Mtr) protecting group using thioanisole in TFA to afford the unprotected guanidine. Upon completion of the reaction, the solvent was evaporated and the residue was triturated to afford desired product **10** from each reaction chamber. The HPLC purities for the desired α-ketothiazoles **10** ranged from 70 to 99% with an average purity level of 88%. The overall yields ranged from 12 to 37% based on mass recovery.[12]

The compounds **10a**–**m** were screened for potency on TF/VIIa and for other enzymes affecting coagulation, such as Xa and Thrombin (IIa), to determine specificity, Table 1. Each enzyme assay consisted of the specific enzyme and chromogenic substrate for that enzyme. Enzyme activity was determined by monitoring the increase in absorbance at 405 nm caused by the release of *p*-nitroaniline when the substrate is cleaved by the protease. Inhibition of the enzyme reduces the change in absorbance with the data reported as IC_{50} values. All of the α-ketothiazoles **10** prepared were active against TF/VIIa with IC_{50} values ranging from 0.042 to 1.45 μM. The most potent compound of the library was **10d** with the L-amino acid as phenylalanine. The TF/VIIa activity was fairly flat across the set of compounds with the substituted phenylalanine derivatives exhibiting consistent activity with IC_{50}'s below 1 μM. A key requirement for the program was to demonstrate that selectivity of TF/VIIa versus thrombin could be achieved. Several compounds, **10e**, **f**, **h**, **m** have selectivity ratios (VIIa/IIa) of greater than 100, with **10e** having a selectivity factor of 500. The data in Table 1 confirmed that a potent TF/VIIa inhibitor with selectivity versus thrombin could indeed be developed.

Table 1. IC_{50} Values of α-ketothiazoles **10**

Compd **10**	IC_{50} (μM)			
	VIIa	IIa	Xa	VIIa/IIa
a	1.45	100	2.4	69
b	0.30	19.3	0.38	64
c	1.03	60	2.1	58
d	0.042	4.0	0.027	95
e	0.20	100	0.29	500
f	0.09	>30	0.21	>333
g	0.20	15	0.25	75
h	0.80	84.9	1.0	106
i	0.16	11.2	0.21	70
j	0.24	21.7	0.33	90
k	0.17	12.0	0.16	71
l	0.23	14.6	0.27	63
m	0.34	50	0.90	147

The X-ray crystal structure of compound **10e** bound in the active site of TF/VIIa complex is shown in Figure 1. The ketothiazole inhibitor **10e** is bound well in the active site of TF/VIIa, forming several hydrogen bonding interactions with the protein atoms. The arginine side chain at P1 forms four strong hydrogen bonds at the bottom of the S1 pocket with the side chains of Asp 189 and Ser 190 as well as to the backbone carbonyl oxygen of Gly 219. The active site serine, Ser 195, forms

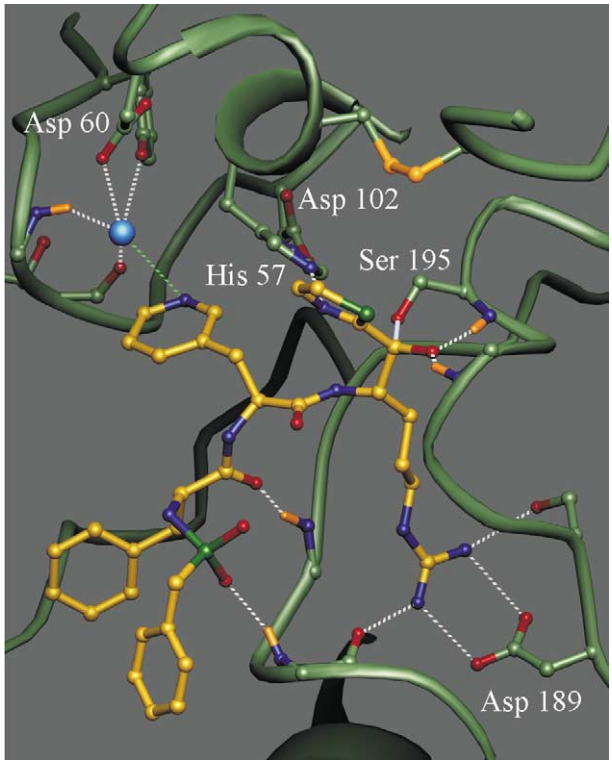


Figure 1. Crystal structure of ketothiazole inhibitor **10e** bound in the active site of TF/VIIa complex. Some of the key side chains of factor VIIa are displayed (C: green, N: dark blue, O: red, S: yellow and H: orange). The carbon atoms of the inhibitor are shown in gold color. The hydrogen bonds formed by the inhibitor and a bound water molecule in the S2 site are shown in dotted white lines. The close interaction between the bound solvent and the pyridyl nitrogen of the inhibitor is shown in dotted green line. The active site serine, Ser 195, forms a covalent bond (thin solid line) with the activated carbon of the inhibitor.

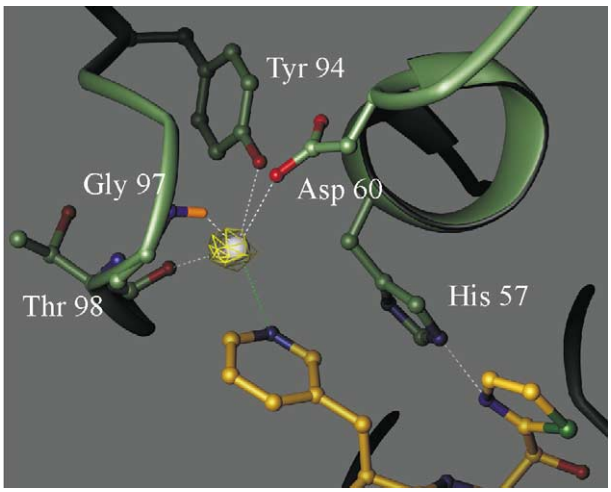


Figure 2. Expanded view of the bound solvent molecule in the S2 pocket. Shown in yellow is the |Fo|-|Fc| electron density contoured at 3.0 σ. The atoms are colored as in Figure 1. The hydrogen bonds formed by the solvent are shown in dotted white line while the close interaction between the water molecule and the pyridyl nitrogen is shown in dotted green line.

a covalent bond to the activated carbon of the inhibitor as expected. This results in the formation of a transition state analogue. The resulting hydroxyl group binds in the oxyanion hole, forming two hydrogen bonds with the amide nitrogen of Gly 193 and Ser 195. The thiazole ring stacks parallel to the side chain of active site histidine, His 57, with a hydrogen bond between the nitrogen of the thiazole and one of the nitrogens of the histidine side chain. The inhibitor forms two other hydrogen bonds with peptide nitrogens of Gly 216 and Gly 219.

The side chain pyridyl ring traps a water molecule at the S2 site of the enzyme active site, Figure 2. The bound solvent molecule forms hydrogen bonding interactions with the side chains of Asp 60 and Tyr 94. In addition, two other hydrogen bonds are formed by the solvent molecule with the backbone atoms of Gly 97 and Thr 98. Moreover, the pyridyl nitrogen of the inhibitor is 3.0 Å away from the water molecule although the geometry is not appropriate for a direct hydrogen bond. The pyridyl ring of the inhibitor is almost orthogonal to the plane of the His 57 side chain. The S2 pocket of factor VIIa is relatively open and has a negative potential due to the presence of Asp 60. The pyridyl group of the inhibitor takes advantages of these structural features to form strong interactions in the S2 pocket of VIIa. Among the coagulation proteases, only factor VIIa has a negatively charged residue at position 60. In contrast, factor Xa has a tyrosine at position 60. Moreover, the S2 pocket of factor Xa is occluded by the side chain of Tyr 99. Thrombin has a large insertion in the S2 pocket with a number of aromatic amino acid residues. These structural differences would account for the enhanced selectivity of compound **10e** for TF/VIIa versus thrombin.

In conclusion, a series of α-ketothiazoles with the general structure $BzSO_2$-D-Phe-L-AA-L-Arg-α-ketothiazole was synthesized using combinations of polymer-assisted solution-phase purification protocols. The compounds were screened for potency on TF/VIIa and for other enzymes affecting coagulation to determine specificity, Table 1. All of the compounds exhibited activity on tissue factor VIIa with IC_{50}'s ranging from 0.042 to 1.45 μM. Several compounds had excellent selectivity for VIIa versus thrombin, such as compound **10e** with a selectivity ratio (IIa/VIIa) of 500. This demonstrated that selectivity for TF/VIIa versus thrombin was possible. The structural information from the X-ray crystal structure of compound **10e** from this study, combined with the crystal structure of other related serine proteases such as thrombin and Xa, facilitated the development of a noncovalent-reversible non-peptidic tissue factor VIIa inhibitor which will be the topic of a future publication.

Acknowledgements

The authors thank Dr. Huey Shieh for some of the early crystallographic refinements of the TF/VIIa structure. Diffraction data for the TF/VIIa complex with the ketothiazole inhibitor were collected at beamline 17-ID in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. IMCA-CAT facilities are supported by the corporate members of the IMCA and through a contract with Illinois Institute of Technology (IIT), executed through the IIT's Center for Synchrotron Radiation Research and Instrumentation. Use of the Advanced Photon Source was supported by the US Department of Energy, Basic Energy Sciences, Office of Science, under Contract No. W-31-109-Eng-38.

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