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High-Throughput Synthesis and Optimization of Thrombin Inhibitors Via Urazole α-Addition and Michael Addition

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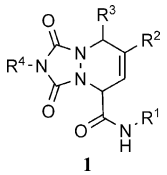
Abstract—A novel α-addition of propiolates to urazoles followed by Michael addition of a variety of nucleophiles has been developed for rapid production and optimization of peptidomimetic drug leads. This technology has produced a number of highly potent and selective inhibitors of the serine protease, thrombin.

The serine protease thrombin has been the subject of intense pharmaceutical research for several years. This research has produced significant advances in the development of highly active and selective inhibitors of thrombin.[1] Despite these advances, no orally active direct thrombin inhibitor is currently widely available for the treatment of thrombotic diseases. We have been developing modular synthetic approaches in the context of small-molecule mimetics of peptide secondary structure for use as drug lead discovery and optimization tools.[2] As part of a program to develop peptidomimetic inhibitors for serine proteases generally, we have developed new methods for the rapid generation and optimization of lead compounds using solid phase organic synthesis. This technology has been deployed for the rapid synthesis and optimization of active site thrombin inhibitors.



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Recently, we disclosed bicyclic β-strand mimetics to be useful as scaffolds for synthesis of protease inhibitors based on the triazolopyridazine structure (**1**).[3] We have been examining the reactivity of this system in order to expand the scope of chemistry that can be utilized to rapidly produce and optimize diverse drug leads. Herein we report the utilization of new triazolopyridazine chemistry that extends the scope of this chemical class for the synthesis of new libraries of bioactive compounds and application of this chemistry for rapid generation and optimization of thrombin inhibitor compounds.

The synthesis of the β-strand mimetic template (triazolopyridazine template) relies on a highly efficient hetero Diels–Alder reaction between a diene and the dienophile, 1,2,4-triazolinedione, obtained by an in situ oxidation of the corresponding urazole.[4] This oxidation occurs under very mild conditions resulting in reliable synthetic methods that are well suited to solid or solution phase organic synthesis. A wide variety of substitution on the diene and urazole is tolerated making these methods ideal for library production. However, the 4-substituted urazoles, used as diversity elements in the library synthesis, impose restrictions on the library design, as few urazoles are readily available from commercial sources and others require custom synthesis.[5] Therefore, development of solid phase reactions, suitable for functionalization of unsubstituted triazolopyridazine **2** (Scheme 1) has been an emphasis of our research efforts.

A recent publication[6] detailed the reaction of phthalimide with propiolates in toluene at high temperature (90 °C to reflux) mediated by triphenyl phosphine and acid buffer leading to the formation of acrylates via

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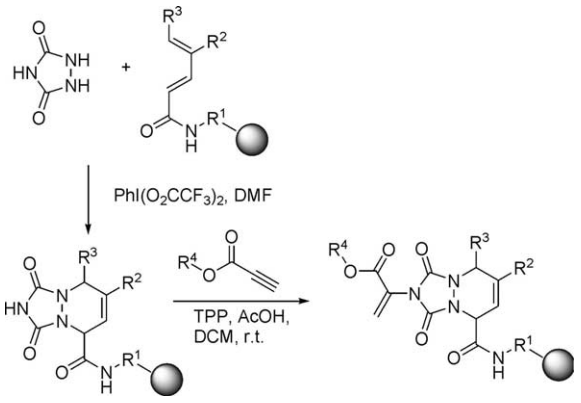
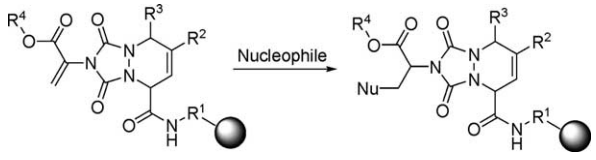


Scheme 1. Triazolopyridizine addition to acrylates.

α-addition of phthalimide to propiolate esters. We found that triazolopyridazine reacts similarly to phthalimide to give analogous acrylate products when reacted under the reported conditions (Scheme 1).

In order to develop conditions more suitable for solid phase organic synthesis (SPOS) using resin bound urazole substrates, we surveyed a number of solvents at reflux temperature, finding DCM, chloroform and DCE most suitable for the reaction. DMF, a solvent widely used for SPOS did not provide any of the expected product. The acetic acid/sodium acetate buffer, reported for the phthalimide addition, suffers from poor solubility in these reaction media. Further optimization showed that soluble mixtures of AcOH/DIEA or AcOH/Pyridine could replace this buffer. The reaction proceeds equally well with acid/base ratios from 2:1 to 1:2, allowing for work with acid sensitive resins. Finally, the outcome of the reaction at lower temperatures was investigated. For the propiolate esters, the reaction can be performed at room temperature without a significant decrease in chemical yield or purity. Reaction of propiolic amides however, required higher temperature (60–70 °C) for complete conversion of the starting material within a reasonable time period (24 h).



Scheme 2. Michael addition to unsaturated esters.

Table 1. General reaction conditions for Michael addition to **3**

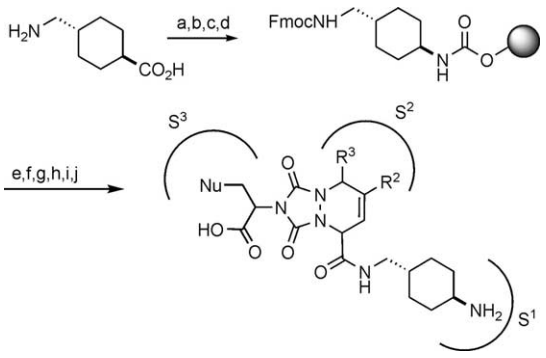
Entry	Nucleophile[a]	Time/temp	Yield[b]	Purity[c]
1	RSH/ArSH	2 h/rt	>85%	>90%
2	R_2NH	3 h/rt	>90%	>90%
3	Phenol	48 h/rt	No reaction	NA
4	Aniline	48 h/rt	No reaction	NA
5	$P(OMe)_3$	48–72 h/40 °C	>80%	>90%
6	EWG_2CH_2/DBU	16–24 h/rt	>85%	>90%

[a]R groups defined in text, EWG = .
[b]Based on isolated product.
[c]Determined by HPLC/MS.

A variety of propiolic acid esters was used to provide **3** (R4 = methyl, ethyl, isopropyl, cyclohexyl and *t*-butyl) in excellent yield.[7] The α,β-unsaturated ester intermediate **3** proved to be a valuable Michael-type acceptor for a variety of nucleophiles (Scheme 2). Primary and secondary amines as well as alkyl and aryl thiols smoothly underwent conjugate addition at rt to afford the corresponding β-substituted propionates in very good yield.[8,9] Table 1 shows the range of nucleophiles and general reaction conditions which were studied. As entries 3 and 4 show, phenols and anilines failed to add even after two days. Alkyl phosphite addition required 2–3 days at 40 °C to go to completion while that of stabilized anions required the use of DBU and periods of 16–24 h. Meldrum's acid and methyl nitroacetate added smoothly to **3** in the presence of DBU. Even under forcing conditions, however, *t*-butyl malonate failed to react. Every product was formed as a mixture of two diastereomers in approximately equal amounts indicating that the existing chiral centers are too remote to induce stereoselectivity in the addition.

More than 600 compounds have been synthesized in this fashion and screened in a number of protease assays. Several compounds active as inhibitors of thrombin were discovered and the α-addition chemistry was used to generate compounds with optimized activity. Addition of thiol nucleophiles to the Michael acceptor followed by acidic (TFA/CH_2Cl_2, 3:1) cleavage from the resin gave the inhibitors, which were assayed as mixtures of diastereomers.

The *trans*-4-aminomethylcyclohexylamine moiety has been shown to be effective at binding in the S^1 pocket of the thrombin active site and to impart high selectivity for thrombin over trypsin and other serine proteases.[8] Resin bound *trans*-4-aminomethylcyclohexylamine was synthesized by a slight modification of the reported route (Scheme 3). *trans*-4-Aminomethylcyclexane carboxylic acid was protected using FmocOSu and the acid was converted to the isocyanate via a Curtius rearrangement. The isocyanate was reacted with Wang resin under acid catalysis. Deprotection of the amine function and coupling of pentadienoic acid, followed by Diels–



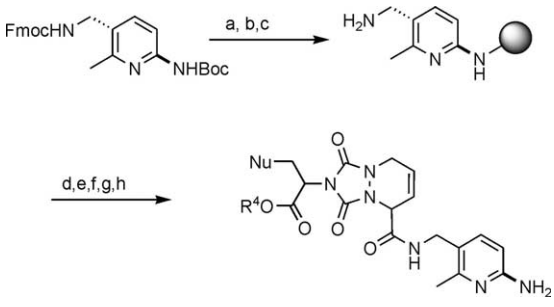
Scheme 3. Reaction conditions. (a) FmocOSu, *i*-Pr_2NEt, DMF; (b) oxalyl chloride, cat. DMF, CH_2Cl_2; (c) NaN_3, cat. *n*-Bu_4NN_3, toluene/water then toluene 90 °C; (d) Wang resin, cat. HCl, CH_2Cl_2; (e) piperidine/DMF; (f) dienoic acid, PyBOP, DMF; (g) urazole, $PhI(O_2CCF_3)_2$, DMF, rt; (h) R^4O_2CCCH, TPP, AcOH/Pyridine (1:2), DCM, rt; (i) NuH, *i*-Pr_2NEt, DMF, rt; (j) TFA/CH_2Cl_2, rt.

Alder cycloaddition with urazole gave the peptidomimetic core. α-Addition of propiolate esters followed by nucleophillic addition and cleavage from the resin gave the compounds tested as thrombin inhibitors. The putative binding mode of these inhibitors with thrombin is indicated in Scheme 3 with binding of the basic group in the S^1 subsite and hydrophobic group in the S^3 subsite of thrombin.[1,2]

The less basic 2-amino-6-methylpyridine moiety has a greater potential for oral absorption while maintaining sufficient basicity to bind in the S^1 pocket of thrombin.[9] Synthesis of differentially protected 2-amino-5-aminomethyl-6-methylpyridine has been previously reported. For the present purpose, the Boc group was cleaved and the product was attached to 2-chorotrityl resin (Scheme 4). Following Fmoc cleavage, the resin was used for the synthesis of thrombin inhibitors as described for the cyclohexylamine compounds.

For optimization studies, small sets of compounds with the strand mimetic and these two moieties were synthesized and screened against human thrombin at a fixed concentration (400 nM)[10] in order to determine the effects of various groups around the mimetic core. Data from screening of the original collection suggested that substituents in the R^2 and R^3 positions were deleterious to thrombin binding, therefore, the optimization set was unsubstituted at these positions. The compounds were synthesized as acids (from concurrent *t*-butyl ester and resin cleavage) and methyl esters and the nucleophile was varied in order to determine the breadth of substitution that could be incorporated into the S^3 binding portion of the mimetic. Table 2 summarizes the screening results from this collection of compounds.

The S^1 aminocyclohexyl compounds were consistently more active than the less basic aminopyridine, as expected. P^3 SAR for these compounds follows the expected trend with aromatic and alkyl groups giving the highest inhibition potency. Compounds **5**, **6** and **8** with halogenated aromatic groups were particularly good thrombin inhibitors. Compounds with bulkier aromatic groups and aliphatic groups were less effective. Compounds **19–21** which contained an amine function in or near the S^3 binding portion were much less effective. Interestingly, the methyl ester analogues were all



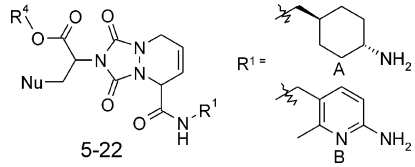
Scheme 4. Reaction conditions. (a) TFA/CH_2Cl_2; (b) 2-chorotrityl chloride resin, *i*-Pr_2NEt, CH_2Cl_2; (c) 20% piperidine/DMF; (d) dienoic acid, PyBOP, DMF; (e) urazole, $PhI(O_2CCF_3)_2$, DMF; (f) R^4O_2CCCH, TPP, AcOH/Pyridine (1:2), CH_2Cl_2, rt; (g) NuH, *i*-Pr_2NEt, DMF, rt; (h) TFA/CH_2Cl_2, rt.

greater than 10-fold more active than the acid counterparts, suggesting that the carboxylate group is involved in binding with the protein and a negative charge is detrimental.

A number of these compounds were seleceted for further investigation and production of analogues. Thrombin K_i's were measured and the selectivity of the inhibitors vs various serine proteases were determined.[11] Indeed, several of the compounds were tested for trypsin inhibition and had K_i's higher than 5 μM and did not inhibit other serine proteases at concentrations up to 40 μM. As can be seen from Table 3, the SAR for these inhibitors is similar to that observed for other thrombin inhibitors in which an aromatic group is preferred in the P^3 pocket.

The effect of the carboxylate group was of interest and a number of ester and amide analogues were synthesized in order to investigate the effect of steric and electronic variations in this moeity. However, the products of the conjugate addition reaction are stable to acidic cleavage conditions but not to basic conditions. Attempts to hydrolyze the methyl esters under basic conditions were unsuccessful giving predominantly acrylates resulting from β-elimination. Thus, synthesis of amide or ester analogues by an ester hydrolysis/coupling route is problematic. Additionally, the aminopyridine moiety was linked to 2-chlorotrityl resin and this linkage was found to be unstable to the high temperatures required for α-addition of propiolate amides. To circumvent these problems, we prepared the desired propiolate amides

Table 2. Thrombin inhibition activity of selected compounds



Compd	Nu	% Inhibition @ 400 nM[a]			
		R^1 = A		R^1 = B	
	R^4	CH_3	H	CH_3	H
5a,b,c,d	4-Br-PhS	NA	95	83	NA
6a,b,c,d	4-Cl-PhS	100	92	82	32
7a,b,c,d	4-CH_3O-PhS	100	84	80	29
8a,b,c,d	3,4-diCl-PhS	100	88	78	22
9a,b,c,d	4-F-PhS	98	75	50	34
10a,b,c,d	PhS	98	69	32	15
11a,b,c,d	2,4-Di-CH_3-PhS	94	48	NA	6
12a,b,c,d	2-NaphthylS	92	13	6	7
13a,b,c,d	CyclohexylS	91	53	28	11
14a,b,c,d	$(CH_3)_2CHCH_2S$	87	32	21	NA
15a,b,c,d	1-NaphthylS	88	24	15	3
16a,b,c,d	4-$(CH_3)_3C$-PhS	64	10	10	3
17a,b,c,d	$CH_3CH_2CH_2S$	64	NA	8	NA
18a,b,c,d	$HOCH_2CH(OH)CH_2S$	62	9	NA	0
19a,b,c,d	$PhCH_2NH$	33	7	8	6
20a,b,c,d	CyclohexylNH	25	NA	NA	7
21a,b,c,d	$(CH_3)_2CHCH_2NH$	11	16	NA	NA
22a,b,c,d	4-AcNH-PhS	0	NA	34	5

[a]Inhibition was measured on the crude compound versus 100% activity for the negative control.

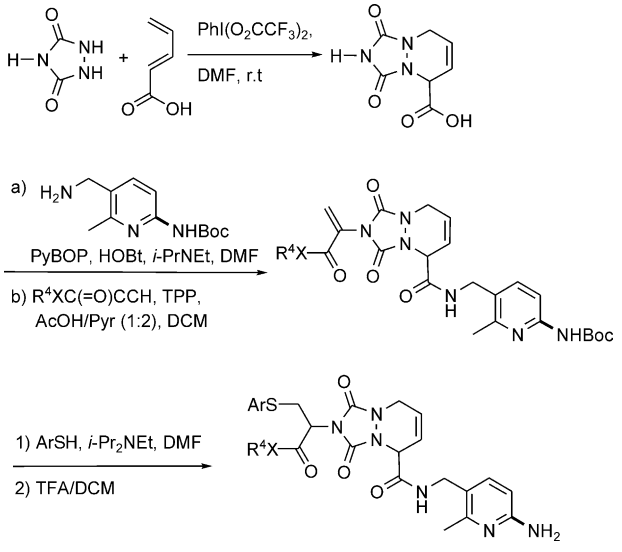
Table 3. Thrombin activity of selected acid and methyl ester analogues

Compd	Nu	Thrombin K_i (nM)			
		$R^1 = A$		$R^1 = B$	
	R^4	CH_3	H	CH_3	H
5a,b,c,d	4-Br-PhS	0.023	2.6	83	NA
6a,b,cd	4-Cl-PhS	0.32	5.7	82	32
7a,b,c,d	4-CH_3O-PhS	0.75	4.7	80	29
8a,b,c,d	3,4-diCl-PhS	0.24	13	78	22
10a,b,c,d	PhS	2.9	36	32	15
13a,b,c,d	CyclohexylS	91	53	28	11

and esters then carried out the remainder of the synthesis in solution using the acid **23** (Scheme 5). Esters (ethyl, isopropyl, and cyclohexyl) uniformly underwent α-addition under mild conditions as described for the methyl and *t*-butyl esters. However, treatment of **1** with propiolate amides (pyrrolidyl, piperidyl and morpholyl) under the typical reaction conditions did not produce any of the desired product. Heating the reaction mixture to 70 °C for several days to effect α-addition to propiolate amides afforded the product in moderate yields (30–50% yield).

Table 4 summarizes results for determination of thrombin inhibition with these analogues. The steric bulk of the ester has little effect on thrombin inhibition suggesting that the ester group may be oriented toward bulk solvent. The amide analogues were slightly less active than the esters. The primary amide analogue of compound **5b** was synthesized ($R^4X = NH_2$) and the thrombin K_i was determined to be 0.19 nM versus 2.6 nM for **5b**. The effect of disubstitution on the amide is more pronounced as can be seen from the data for pyrolidinyl and morpholinyl amides for which the thrombin inhibition activity is >100-fold less than the primary amide.

In summary, the α-addition of propiolates to urazoles for the production of unique β-strand mimetics has been developed. Utilization of the intermediate acrylates for



Scheme 5. Solution phase synthesis of thrombin inhibitors.

Table 4. Thrombin activity of selected esters and amides

R^4X	Thrombin K_i (nM)		
	Ar		
	4-Br-Ph	4-Cl-Ph	3,4-di-Cl-Ph
EtO	0.27	NA	28
I-PrO	0.087	1.5	53
c-HexO	1.5	0.057	87
	30	24	18
	39	7.7	NA

the Michael addition of thiols and amines has provided a valuable method for the rapid generation and optimization of lead compounds. The scope of this reaction sequence has been investigated and it has been found that propiolate esters readily undergo α-addition of the urazole while amides are more resistant to addition but undergo addition under more vigorous conditions. A variety of nucleophiles may be added to the resulting unsaturated esters and amides to produce novel protease inhibitors in a high-throughput fashion. The ease of synthesis and general high purity of the products results in a valuable method for the production of biologically relevant compounds that have potential as therapeutic agents or leads for the development of such. We have used information gathered from these studies to optimize the drug qualities of a series of thrombin inhibitors. We have also employed this strategy for the synthesis of inhibitors of a number of proteases. These will be reported in future publications.

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7. General procedure: PAL, Wang and Chlorotrityl resins (Polystyrene, 1% DVB crosslinked core) were used. General procedure 1: Resin 2 suspended in solvent (DCM, $CHCl_3$ or DCE; the reaction does not work in DMF) at rt was treated with AcOH (5 equiv), base (3–6 equiv), propiolate ester (5–10 equiv) and TPP (2 equiv) and shaken at rt for 10–18 h. Then the resin was washed with DMF and DCM. The products

were cleaved off resin by treatment with TFA/H_2O (95:5) for 40 min. General procedure 2: Resin 3 was treated with 2.5% solution of nucleophile in solvent and base (0.2–0.6 equiv)at the specified temperature and for the designated time (see Table 1). After the specified time the resin was washed with DMF and DCM. The products were cleaved from the resin by treatment with TFA/H_2O (95:5) for 40 min and dried under vacuum to give the inhibitors **5**–**22**.

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10. Inhibition was determined for thrombin and trypsin by florometric measurement of cleavage of the substrate tosyl-Gly-Pro-Arg-AMC at 405nM. Screening assays were performed by adjustment of the final concentration of the inhibitor to (400 nM for thrombin and 5 μM for trypsin) and measurment of the absorption of cleaved substrate versus that of a negative control.

11. K_i determinations were performed as described in ref 10 using eight concentrations of inhibitor and curve fitting using Prism software.