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Peptide inhibitors of dengue virus NS3 protease. Part 1: Warhead

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Abstract—Substrate-based tetrapeptide inhibitors with various warheads were designed, synthesized, and evaluated against the Dengue virus NS3 protease. Effective inhibition was achieved by peptide inhibitors with electrophilic warheads such as aldehyde, trifluoromethyl ketone, and boronic acid. A boronic acid has the highest affinity, exhibiting a K_i of 43 nM.

The mosquito-borne dengue virus is endemic to most tropical and sub-tropical regions throughout the world, making dengue fever the most important mosquito-borne viral disease affecting humans. Its global distribution is comparable to that of malaria, and an estimated 2.5 billion people live in areas at risk for epidemic transmission.[1] The four serotypes of dengue cause a self-limiting viral fever which can lead to life-threatening conditions such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Due to the public health threat posed by dengue virus infection and the lack of vaccines or antiviral therapies, there is an urgent need to develop new chemotherapeutic approaches to counter this emerging infectious disease.

The dengue virus genome contains a trypsin-like protease with a classical serine protease catalytic triad (His51, Asp75, and Ser135) which constitutes part of the nonstructural protein 3 (NS3). The enzymatic activity of NS3 protease is enhanced by interactions with the NS2B protein, which acts as an essential cofactor.[2] NS3 protease is vital for the post-translational proteolytic processing of the polyprotein precursor and is essential for viral replication and maturation of infectious dengue virons.[3] Therefore, dengue NS3 protease is an attractive therapeutic target for dengue virus infections.

Classical inhibitors of serine proteases are ineffective or only effective at high concentration against the dengue NS3 protease, except for aprotinin, which is a large protein likely to prevent to the substrate from accessing the protease active site by enveloping the enzyme.[4a] Additionally, only a few peptidic and non-peptidic inhibitors of the dengue serine protease with moderate activities have been reported (Fig. 1).[4,5] In view of the general lack of information about dengue protease inhibitors, it was desirable to identify peptides with good inhibitory activities that can be used as prototype enzyme inhibitors for future drug discovery efforts.

Unlike trypsin, dengue protease has a marked cleavage site preference for dibasic residues. Two high affinity non-prime side substrates: Bz-Nle-Lys-Arg-Arg ($\mathbf{S}^1$) (K_m 12.42 μM) and Bz-Nle-Lys-Thr-Arg ($\mathbf{S}^2$) (K_m 33.9 μM), have been recently identified through substrate profiling of dengue protease using positional scanning tetrapeptides.[6] We sought to capitalize on the substrate information of NS3 protease to develop potent small molecule inhibitors of the dengue serine protease. In order to rapidly map the active site, we decided, as a first step, to examine several well-established serine

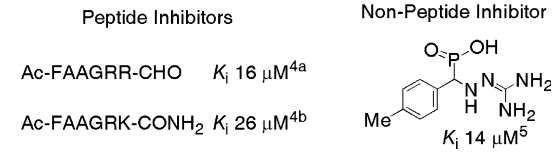

Figure 1. Inhibitors of dengue virus NS3 protease.

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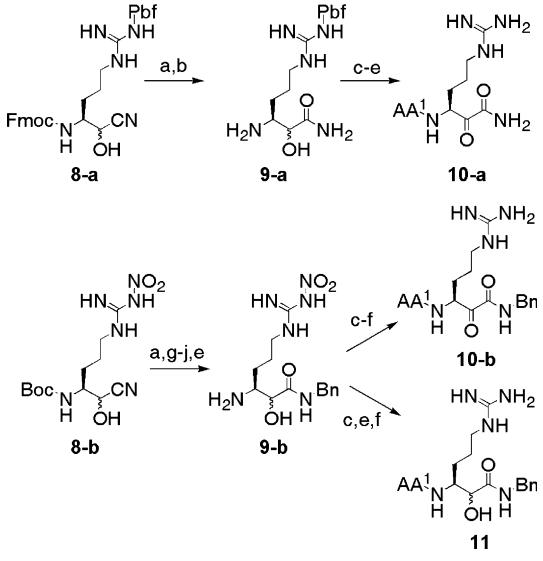
protease warheads. In this paper, we describe the design, synthesis, and evaluation of dengue protease inhibitors based on the tetrapeptide substrates. The importance of the warheads and their contributions in the binding to the NS3 protease will also be discussed.

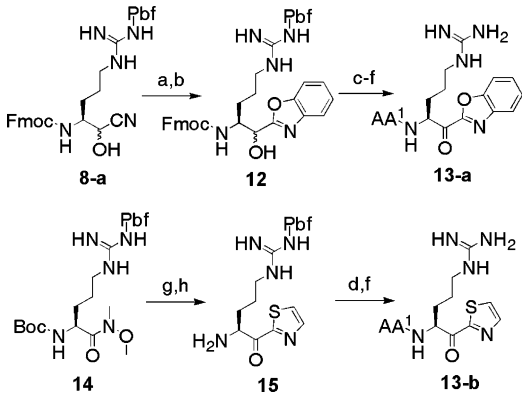
Peptides including compounds **1**, **2**, Bz-Nle-Lys(Boc)-Arg(Pbf)-OH and Bz-Nle-Lys(Boc)-Thr(*t*-Bu)-OH, were either synthesized according to standard solution-phase peptide chemistry[7] or acquired from commercial sources. The synthesis of compounds **3** and **4** was accomplished by standard solid-phase peptide chemistry using rink amide resin as solid support.[8] Trifluoroacylsulfonamide **5** was obtained from tetrapeptide **1** and trifluoromethylsulfonamide using EDC. Tetrapeptide aldehydes **6** and **7** were prepared according to previously reported methods.[9]

Various substrate-based tetrapeptide inhibitors possessing α-ketoamides were synthesized as shown in Scheme 1. Protected Arg-OH was converted to corresponding 'reduced' warheads using previously reported methodologies from Weinreb amides[9b,10] and resulting intermediates were used for subsequent coupling reactions. Tetrapeptide α-ketoamides **10-a** and **10-b** were prepared by Dess–Martin periodinane oxidation[11] of the corresponding alcohols followed by removal of protecting groups. Tetrapeptide α-hydroxyamide **11** was also synthesized by coupling **9-b** with tripeptide followed by simple removal of protecting group.

Peptide α-ketobenzoxazole **13-a** and α-ketothiazole **13-b** were prepared as shown in Scheme 2. Treatment of cyanohydrin **8-a** with anhydrous HCl and ethanol in a Pinner reaction afforded the intermediate imino ether hydrochloride, which was then cyclized with *o*-aminophenol to afford α-hydroxybenzoxazole **12**.[12] α-Ketobenzoxazole **13-a** was achieved by oxidation of the corresponding alcohol. Meanwhile, α-ketothiazole **13-b** was prepared through Arg α-ketothiazole, which was constructed through reaction of arginine Weinreb amide **14** with excess 2-lithiothiazole at low temperature.[13]



Scheme 1. Reagents: (a) HCl, CH_2Cl_2; (b) piperidine, DMF; (c) Bz-Nle-Lys(Boc)-Arg(Pbf)-OH, EDC, HOBt, DIEA, DMF; (d) Dess–Martin periodinane, CH_2Cl_2; (e) 95% TFA, CH_2Cl_2; (f) H_2, Pd/C, AcOH/MeOH; (g) HCl, MeOH; (h) $(Boc)_2O$, $NaHCO_3$, THF; (i) LiOH, THF/H_2O; (j) Benzylamine, EDC, HOBt, DIEA, DMF. [AA^1 = Bz-Nle-Lys-Arg.]



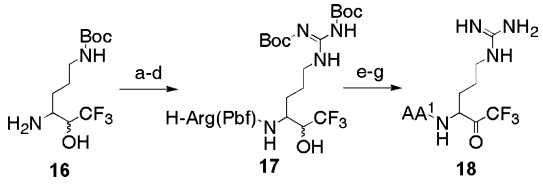
Scheme 2. Reagents: (a) AcCl, EtOH; (b) 2-aminophenol, CH_2Cl_2; (c) piperidine, DMF; (d) Bz-Nle-Lys(Boc)-Arg(Pbf)-OH, EDC, HOBt, DIEA, DMF; (e) Dess–Martin periodinane, CH_2Cl_2; (f) 95% TFA, CH_2Cl_2; (g) thiazole, BuLi, THF, −78 °C; (h) 20% TFA, CH_2Cl_2. [AA^1 = Bz-Nle-Lys-Arg.]

Scheme 3 outlines the synthesis of substrate-based tetrapeptide trifluoromethyl ketone **18**. The fluorinated building block **16** was prepared in a few steps starting with a modified Dakin–West reaction.[14] The fluorinated alcohol **17** was assembled through guanylation using bis-Boc-pyrazole-1-carboxamidine.[15] Subsequently, the tetrapeptide trifluoromethyl ketone **18** was achieved through Dess–Martin periodinane oxidation of tetrapeptide fluorinated alcohol followed by removal of protecting groups.

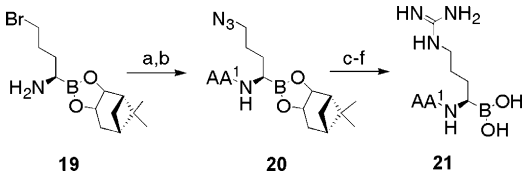
Finally, a peptide boronic acid inhibitor was synthesized through known intermediate **19** as shown in Scheme 4.[16]

All tetrapeptide inhibitors were evaluated in an enzyme inhibition assay against the viral NS3 protease Den 2 CF40·NS3pro, a truncated dengue 2 NS3 enzyme fused via a flexible linker to a 47 amino acid region of NS2B.[17] The activity of the inhibitors is summarized in Table 1.

A non-covalent, charged warhead, such as a carboxylic acid, theoretically could provide binding efficiency via electrostatic interactions. A product based peptide acid was reported as a competitive inhibitor of the hepatitis



Scheme 3. Reagents: (a) Fmoc-Arg(Pbf)-OH, EDC, HOBt, DIEA, DMF; (b) 20% TFA, CH_2Cl_2; (c) bis-Boc-pyrazole-1-carboxamidine, DMAP, THF; (d) piperidine, DMF; (e) Bz-Nle-Lys(Boc)-OH, EDC, HOBt, DIEA, DMF; (f) Dess–Martin periodinane, CH_2Cl_2; (g) 95% TFA, CH_2Cl_2. [AA^1 = Bz-Nle-Lys-Arg.]



Scheme 4. Reagents: (a) Bz-Nle-Lys(Boc)-Arg(Pbf)-OH, IBCF, THF; (b) NaN_3, DMF; (c) H_2, $Pd(OH)_2/C$, MeOH; (d) bis-Boc-pyrazole-1-carboxamidine, DMAP, MeOH; (e) TFA, CH_2Cl_2; (f) $PhB(OH)_2$, diethyl ether/water. [AA^1 = Bz-Nle-Lys-Arg.]

Table 1. Inhibition of dengue virus NS3 protease by tetrapeptide analogs containing different warheads

Compound		K_i (μM)[a]
1	Bz-Nle-Lys-Thr-Arg-OH	>500
2	Bz-Nle-Lys-Arg-Arg-OH	>500
3	Bz-Nle-Lys-Thr-Arg-NH_2	>500
4	Bz-Nle-Lys-Arg-Arg-NH_2	127.5
5	Bz-Nle-Lys-Arg-Arg-$NHSO_2CF_3$	>500
6	Bz-Nle-Lys-Thr-Arg-H	>500
7	Bz-Nle-Lys-Arg-Arg-H	5.8
11	Bz-Nle-Lys-Arg-Arg(OH)-CONH-Bn	178
13-a	Bz-Nle-Lys-Arg-Arg-Benzoxazole	82.9
13-b	Bz-Nle-Lys-Arg-Arg-Thiazole	42.8
18	Bz-Nle-Lys-Arg-Arg-CF_3	0.85
21	Bz-Nle-Lys-Arg-Arg-$B(OH)_2$	0.043

[a] Each K_i is the average of at least two independent determinations.

C virus NS3-4A serine protease with a K_i of 0.6 μM.[18] In our hands, carboxylic acids **1** and **2** failed to show activity against dengue serine protease. Furthermore, peptide analog **5** containing trifluoroacetylsulfonamide, which is a known isostere for carboxylic acids, was not active as well.[19] Simple amides are known to be relatively inert toward certain serine proteases and can serve as substrate-like enzyme inhibitors.[20] As a consequence, substrate-based tetrapeptide amides **3** and **4** were explored. Amide **4** corresponding to the best substrate $\mathbf{S}^1$ showed activity at high micromolar concentration. In general, peptides without well-established serine protease electrophilic warheads did not show useful inhibition of dengue serine protease.

Peptide analogs containing electrophilic warheads are, in general, good inhibitors of serine proteases.[21] Therefore, the effect of different electrophilic warheads on the inhibition of dengue protease was studied. We initially chose to study the substrate-based peptide aldehydes due to the ease of synthesis, which resulted in the identification of low micromolar inhibitors (e.g., aldehyde **7**, K_i 5.8 μM). Not surprisingly, this aldehyde (**7**) was found to be a competitive inhibitor of dengue NS3 protease as shown by Lineweaver–Burk plot (Fig. 2). However, the aldehdye analog (**6**), corresponding to the second best substrate $\mathbf{S}^2$,[6] did not show any activity against dengue protease.

Subsequently, α-ketoamides were synthesized to determine their efficacy against dengue protease and they showed no effect up to 500 μM concentration, whereas α-hydroxyamides showed inhibitory activity at high

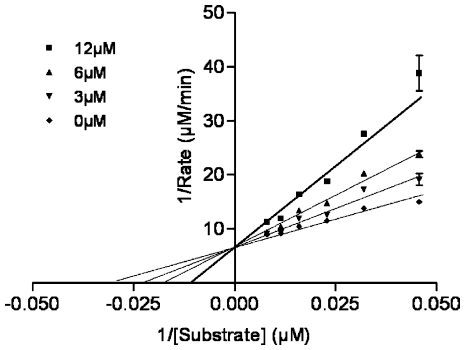


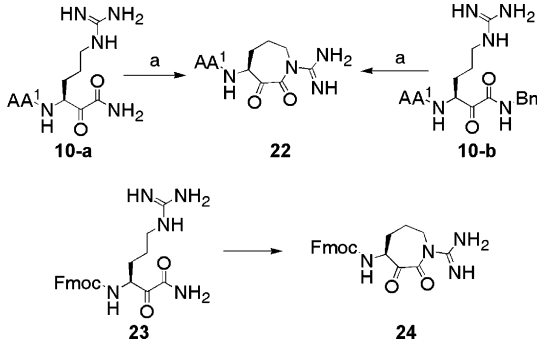
Figure 2. Lineweaver–Burk plot of aldehyde **7**.

micromolar concentration. The reason for this unusual result was found to be the quick degradation of α-ketoamides in the enzyme assay buffer medium (pH 8.5) during incubation to a seven-membered ring lactam (**22**), which presumably, is inactive because of the loss of the key interaction between P_1(Arg) side-chain and the enzyme. To provide additional structural evidence for the formation of lactam, a model study was performed as shown in Scheme 5 and the structure of lactam **24** was confirmed by 2D NMR.

Inhibitors incorporating the α-keto heterocycle moiety like **13-a** and **13-b** proved to be less active than aldehyde **7**. These heterocyclic groups gave potent inhibitors with the related serine proteases elastase, chymase, and thrombin.[22] We surmise that their relatively poor activity compared to aldehyde **7** may be due to either the difference of carbonyl reactivities or some steric restriction in the active site of the enzyme, which prevented optimal binding.

Potent inhibitors were identified by incorporating trifluoromethyl ketone and boronic acid onto the substrate peptide. Tetrapeptide boronic acid **21** proved to be the most potent inhibitor of dengue virus NS3 protease with a K_i of 43 nM. The affinity of trifluromethyl ketone **18** was intermediate between those of peptide aldehyde **7** and peptide boronic acid **21**.

In the present investigation, we have examined the major requirements for nanomolar inhibition of the dengue NS3 protease. Starting with the best known



Scheme 5. Degradation of α-ketoamides. Reagents and conditions: (a) Enzyme assay buffer medium, pH 8.5.

substrate peptide sequence, we have determined that, in contrast to HCV NS3 protease, cleavage products and their analogs do not appreciably inhibit the Dengue NS3 protease. Peptide inhibitors with electrophilic warheads, such as aldehyde, trifluoromethyl ketone, and boronic acid, were needed to see effective inhibition of the enzyme activity. Among the examined warheads, tetrapeptide boronic acid has the highest affinity, exhibiting a K_i of 43 nM. We believe that these small molecule inhibitors offer valuable insights into the development of chemotherapeutics for the treatment of dengue infection in humans.

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17. Enzyme inhibition assay: Inhibitors were assayed in a 96-well plate format using 50 mM Tris, pH 8.5, 1 mM CHAPS in a final volume of 50 μL. Typically enzyme conjugate Den 2 CF40 · NS3pro (50 nM) was pre-incubated with various concentrations of test compounds at 37 °C for 30 min. The reaction was then initiated by the addition of substrate Bz-nKRR-AMC at 20 μM. Reaction progress was monitored continuously by following the increase in fluorescence (excitation 385 nm, emission 465 nm) on a TECAN Safire plate reader. K_i values were derived for inhibitors by fitting the calculated initial velocity's to a nonlinear regression curve fit using GraphPad prism software and then applying the Cheng and Prusoff relationships (1973) for competitive inhibitor.
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