Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 40–43

Peptide inhibitors of dengue virus NS3 protease. Part 2: SAR study of tetrapeptide aldehyde inhibitors

Zheng Yin,[a,*] Sejal J. Patel,[a] Wei-Ling Wang,[a] Wai-Ling Chan,[a] K. R. Ranga Rao,[a] Gang Wang,[a] Xinyi Ngew,[a] Viral Patel,[a] David Beer,[a] John E. Knox,[a] Ngai Ling Ma,[a] Claus Ehrhardt,[b] Siew Pheng Lim,[a] Subhash G. Vasudevan[a] and Thomas H. Keller[a]

[a]*Novartis Institute for Tropical Diseases, 10 Biopolis Road, #05-01 Chromos, 138670, Singapore*
[b]*Novartis Institute for BioMedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland*

Received 15 July 2005; revised 11 September 2005; accepted 20 September 2005
Available online 21 October 2005

Abstract—With the aim of discovering potent and selective dengue NS3 protease inhibitors, we systematically synthesized and evaluated a series of tetrapeptide aldehydes based on lead aldehyde **1** (Bz-Nle-Lys-Arg-Arg-H, K_i = 5.8 μM). In general, we observe that interactions of P_2 side chain are more important than P_1 followed by P_3 and P_4. Tripeptide and dipeptide aldehyde inhibitors also show low micromolar activity. Additionally, an effective non-basic, uncharged replacement of P_1 Arg is identified.

Dengue virus is a member of the *Flaviviridae* family which causes dengue fever and dengue hemorrhagic fever in millions of people each year in tropical and subtropical regions of the world. Currently, there is no vaccine or effective antiviral therapy for the four known serologically related virus types (dengue 1–4).[1] Dengue virus genome is a 11-kb single positive-stranded RNA that encodes three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A and NS4B, and NS5).[2] NS3 protein is essential for viral replication and maturation of virions, hence it serves as an attractive therapeutic target for the dengue virus infections.[3]

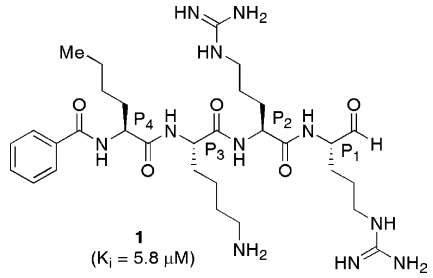
Dengue NS3 protein is a multifunctional protein composed of protease, nucleoside triphosphatase, 5′-RNA triphosphatase, and helicase activities.[3a,4] The amino terminal 618 amino acid residues contain a trypsin-like serine protease (NS3-pro) that has preferences for the dibasic residues at P_1 and P_2 (Arg and Lys) positions.[3a] Protease activity is enhanced by NS2B protein, which acts as an essential cofactor for NS3 protease.[5] Two high affinity non-prime substrates [Bz-Nle-Lys-Arg-Arg (K_m 12.42 μM) and Bz-Nle-Lys-Thr-Arg (K_m 33.9 μM)] were identified recently through substrate profiling of dengue protease using tetrapeptides.[6] Based on these peptide substrates, we synthesized and evaluated several dengue protease inhibitors using both ionic and covalent warheads (see preceding paper[1b]). We found only peptides containing electrophilic covalent warheads such as aldehydes, trifluoromethyl ketones, and boronic acids to be effective in inhibiting the enzyme activity. The structure of DEN2 NS3-pro with the mung-bean Bowman–Birk inhibitor (MbBBI) has been reported (pdb code: 1DF9), but NS3-pro (without the NS2B cofactor) can only hydrolyze the substrate Arg-*p*-nitrophenylanilide efficiently and is essentially inactive on tri- and tetrapeptide substrates.[7] As a consequence, the available structural information is only of limited use for drug discovery and in the absence of active NS3/NS2B structure, rational improvements of selectivity and inhibitory activity of lead compounds can be difficult. We chose therefore to use classical structure–activity relationship (SAR) techniques to probe the specificity of the enzyme binding pockets of NS2B/NS3-pro complex.

In our warheads study, aldehyde **1** showed reversible, competitive binding with a K_i value of 5.8 μM and was also active in full-length dengue NS3 protein (K_i = 7.0 μM).[8] Even though, aldehyde inhibitors were not the most potent inhibitors, they were chosen to examine the SAR as they are readily amenable to

Keywords: Dengue virus NS3 protease; Peptide inhibitors.
* Corresponding author. Tel.: +65 6722 2981; fax: +65 6722 2918; e-mail: zheng.yin@novartis.com

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.09.049

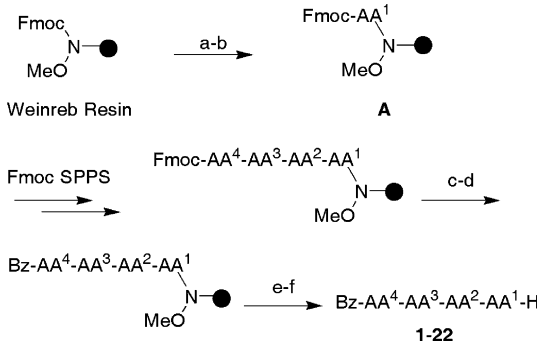
high-throughput synthesis. Aldehyde **1** served as the starting lead molecule to understand the essential pharmacophore and inhibitor–enzyme interactions. In this paper, we will communicate a systematic SAR study within the aldehyde class of inhibitors.



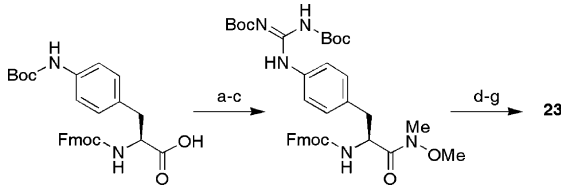
1
(K_i = 5.8 μM)

There are various methods available for the solution-phase synthesis of peptide aldehydes, but the solid-phase synthesis using Weinreb amide was chosen due to fast work-up and availability to perform parallel synthesis.[9] In this method, the aldehyde functionality is liberated in the last step after the acidic removal of side-chain protecting group. This sequence of events minimizes the condensation between the side-chain guanidine of arginine and the aldehyde to an inactive cyclic dehydro compound.[10]

Peptide aldehydes **1**–**22** were assembled by stepwise solid-phase synthesis using Fmoc-protected Weinreb resin (Scheme 1). Acid labile protecting groups (Boc, Pbf) were utilized on the side chain of individual amino acids. Deprotection of the Fmoc was achieved with 20% piperidine solution in DMF. All amino acids were activated with HATU and DIEA in DMF. All peptides were benzoylated using benzoyl chloride and DIEA after the final Fmoc removal. Aldehyde inhibitors were deprotected using 10% TMS-OTf followed by cleavage from the solid support using $LiAlH_4$. Synthesis of peptide aldehydes **24**–**30** was achieved by directly coupling previously synthesized tripeptide [Bz-Nle-Lys(Boc)-Arg(Pbf)-OH] to corresponding intermediate **A**, followed by deprotection and cleavage. Synthesis of rigid arginine mimetic tetrapeptide aldehyde **23** was achieved using standard solution-phase peptide chemistry as shown in Scheme 2.



Scheme 1. Solid-phase synthesis of aldehyde inhibitors using Weinreb resin (SPPS, solid-phase peptide synthesis). Reagents: (a) 20% piperidine, DMF; (b) Fmoc-AA^1-OH, HATU, DIEA, DMF; (c) 20% piperidine, DMF; (d) BzCl, DIEA; (e) TMS-OTf, CH_2Cl_2; (f) $LiAlH_4$.



Scheme 2. Solution-phase synthesis of aldehyde inhibitor **23**. Reagents: (a) HNMe(OMe), EDC, HOBt, DIEA, DMF; (b) 10% TFA, CH_2Cl_2; (c) bis-Boc-pyrazole-1-carboxamidine, DMAP, THF; (d) 20% piperidine, DMF; (e) Bz-Nle-Lys(Boc)-Arg(Pbf)-OH, EDC, HOBt, DIEA, DMF; (f) $LiAlH_4$; (g) 10% TMS-OTf, CH_2Cl_2.

All tetrapeptide aldehyde inhibitors were evaluated in an enzyme inhibition assay against truncated dengue 2 NS3 enzyme fused via a flexible linker to a 47 amino acid region of NS2B.[11]

The inhibition data from the substitution of each side chain residue in tetrapeptide aldehyde **1** with alanine, phenylalanine, lysine, proline, and D- and *N*-Me amino acid scans are summarized in Table 1. The alanine scan suggested that the side chain of P_2(Arg) made the strongest contributions to enzyme binding. While substitution of P_2(Arg) led to a peptide without measurable inhibition (**3**), replacement of P_1(Arg) with alanine resulted in a weak inhibitor (**2**). Results from phenylalanine scan were analogous to those observed during alanine scan. Replacement of P_2(Arg) with phenylalanine had the most detrimental effect (**7**). Variations at P_3 were tolerated without significant reduction in potency (**4** and **8**). Substitutions at P_4 with either alanine or phenylalanine resulted in equipotent inhibitors (**5** and **9**), implying minimal contribution of P_4 in the enzyme binding.

Table 1. Results from different scans (alanine, phenylalanine, lysine, and proline, D- and *N*-Me amino acids) and varying the inhibitor length

Compound	Aldehyde inhibitors	K_i[a] (μM)
1	**Bz-Nle-Lys-Arg-Arg-H**	**5.8**
2	Bz-Nle-Lys-Arg-**Ala**-H	193.0
3	Bz-Nle-Lys-**Ala**-Arg-H	>500
4	Bz-Nle-**Ala**-Arg-Arg-H	22.1
5	Bz-**Ala**-Lys-Arg-Arg-H	5.3
6	Bz-Nle-Lys-Arg-**Phe**-H	15.9
7	Bz-Nle-Lys-**Phe**-Arg-H	40.7
8	Bz-Nle-**Phe**-Arg-Arg-H	15.8
9	Bz-**Phe**-Lys-Arg-Arg-H	6.8
10	Bz-Nle-Lys-Arg-**Lys**-H	20.5
11	Bz-Nle-Lys-**Lys**-Arg-H	41.3
12	Bz-Nle-Lys-**Pro**-Arg-H	109.0
13	Bz-Nle-**Pro**-Arg-Arg-H	61.4
14	Bz-Nle-Lys-***N*-Me**-Arg-Arg-H	47.4
15	Bz-Nle-***N*-Me**-Lys-Arg-Arg-H	113.3
16	Bz-***N*-Me**-Nle-Lys-Arg-Arg-H	43.7
17	Bz-Nle-Lys-Arg-D-**Arg**-H	51.0
18	Bz-Nle-Lys-D-**Arg**-Arg-H	115.0
19	Bz-Nle-D-**Lys**-Arg-Arg-H	28.6
20	Bz-D-**Nle**-Lys-Arg-Arg-H	9.4
21	Bz-Lys-Arg-Arg-H	1.5
22	Bz-Arg-Arg-H	12.0
23	Bz-Nle-Lys-Arg-(***p*-guanidinyl**)**Phe**-H	2.8

[a] Each K_i value is the mean of at least two independent experiments.

We also investigated the replacement of charged and basic P_1, P_2 arginine with the basic-amino acid lysine. Lysine substitution at P_1 showed a 4-fold decrease in activity, while the same change at P_2 showed a 8-fold decrease (**10** and **11**). Hence, besides P_1, interactions of charged arginine at P_2 position seemed crucial in enzyme binding. These results are surprising since the crystal structure of a complex of NS3-pro and Bowman–Birk inhibitor suggests that the most extensive interactions are in the P_1 sites.[7a] However, they are in agreement with our recently published substrate studies, which showed that the P_2 side chain was a very important determinant for substrate binding (substrate Bz-nKRR-ACMC showed a significantly higher K_m than Bz-nKTR-ACMC) and is consistent with the prediction that side chains lining the S_2 pocket, especially Q35, may hydrogen bond with the guanidine group of arginine.[6]

Loss of activity during the exchange of P_2 and P_3 residues with proline indicated that turn geometry was not favorable for inhibitor binding (**12** and **13**). Introduction of *N*-Me amino acids caused a major loss of potency (**14**–**16**), indicating once again the importance of backbonding hydrogen bonds in enzyme binding. Switching of the L-amino acids at positions 1–3 with isomeric D-amino acids was not well tolerated (**17**–**19**). However, replacement of L-Nle at P_4 with D-Nle resulted in only a slight decrease in potency (**20**).

Encouraged by repeated observations that diverse substitutions were permitted at P_4 position (Phe, Ala, and D-Nle), we examined the effect of inhibitor length on the activity. Truncating the aldehyde **1** to tripeptide aldehyde **21** afforded a slightly improved activity (K_i = 1.5 μM). These results match our substrate studies in which a suboptimal substitution at P_4 maintained K_m but displayed 7-fold decrease in k_{cat} [substrates Bz-nKRR-ACMC (k_{cat} = 1.39 s^{-1}) and Bz-TKRR-ACMC (k_{cat} = 0.20 s^{-1})].[6] Moreover, they are in agreement with the recently published data that pointed out marked destabilization of the enzyme–inhibitor interactions in the presence of a small chain residue such as Ala or Ser at P_4.[12] Of even greater significance is that dipeptide aldehyde **22** also exhibited a comparatively higher inhibitory potency (K_i = 12 μM). This finding is in agreement with a strong preference for dibasic residues at the P_1 and P_2 positions.

During our studies of different warheads, we observed that some of the inhibitors containing arginine at P_1 position were unstable and could easily cyclize to inactive derivatives (see, α-ketoamide data in the preceding paper[16]). Fairlie and co-workers have reasoned that aldehyde inhibitors containing arginine residue at P_1 show only modest activity against flavivirus NS3 proteases, because they are in equilibrium with their hydrate and cyclic forms, with only about 5% of the active aldehyde functionality exposed for the interaction with the active site serine hydroxyl group.[13] However, in our hands rigid arginine-mimetic derivative at P_1 [**23** (*p*-guanidinyl)Phe] only slightly improved inhibitory activity (K_i = 2.8 μM). This experiment implies that aldehyde inhibitors with arginine at P_1 are in a fast equilibrium with their hydrate and cyclic forms.

Table 2. Effect of modifications in the P_1 position of the tetrapeptide aldehydes (Bz-Nle-Lys-Arg-P_1-H)

Compound	P_1	K_i[a] (μM)
6	**Phe**	**15.9**
24	Phg	33.0
25	homoPhe	>500
26	(*p*-Cl)Phe	138.0
27	(*p*-CN)Phe	18.6
28	(*p*-Me)Phe	6.0
29	(*p*-Ph)Phe	11.6
30	Trp	7.5

[a] Each K_i value is the mean of at least two independent experiments.

We also investigated whether the basic, charged P_1 Arg residue can be replaced with neutral, uncharged side chains. During our phenylalanine scan, we had observed that removal of P_1 Arg with Phe afforded modest activity (aldehyde **6**, K_i = 15.9 μM). A bifurcated, redundant recognition mode for P_1 arginine is observed in the published structure of DEN2 NS3-pro:MbBBI (1DF9). In one mode, P_1 Arg makes electrostatic interactions with Y150 and S163, while in the other mode, it makes two H-bonding interactions with D129.[7] Even though, the significance of this discovery is unclear in the absence of NS2B cofactor, we hypothesized that π–π interactions may exist between Y150 in the S_1 pocket and phenylalanine at P_1. We wanted to capitalize on this information and decided to examine other aromatic amino acid derivatives at P_1 position (Table 2).

Surprisingly, Phg at P_1 reduced the activity by 2-fold but homoPhe gave completely inactive inhibitor (**24** and **25**). We suspect that an extra methylene unit of homoPhe causes a disruption of the stabilizing electrostatic interactions in the S_1 pocket. Electron-withdrawing aromatic substitution on phenylalanine, such as *p*-chloro (**26**) and *p*-CN (**27**), proved less desirable. Nevertheless, installation of (*p*-methyl)Phe (**28**) at P_1 afforded equipotent inhibitor to the lead aldehyde **1**. In addition, (*p*-phenyl)Phe (**29**) and Trp (**30**) also exhibited low micromolar

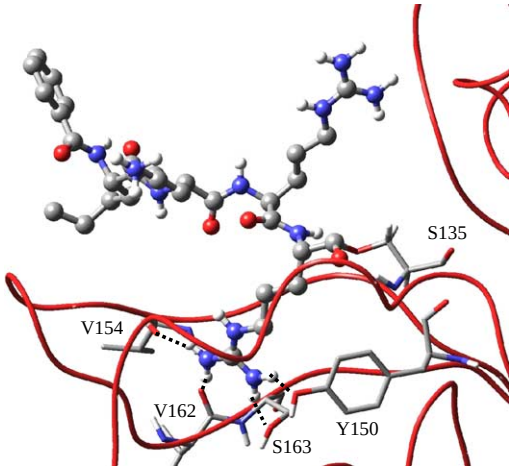


Figure 1. Putative interactions of aldehyde **1** with NS3-pro, with hydrogen bond interactions between the P_1 Arg and residues in the S_1 pocket highlighted by dotted line. For simplicity, the hydrogen-bonding interactions between P_2 Arg and Q35/H51 are not shown here.

activity. Thus, we were able to identify at least two good replacements of P_1 arginine residue with uncharged and neutral aromatic amino acid derivatives.

Furthermore, in our preliminary modeling study[14] of aldehyde **1**, we observed the retention of the key hydrogen bonds formed by MbBBI with Y150 and S163 (Fig. 1), and additional interactions of P_1 Arg with V154 and V162. When P_1 Arg was replaced by Phe (inhibitor **6**), the hydrogen bonding interactions with Y150 and S163 were lost; however, our model suggests that there are possible π–π interactions between P_1 phenylalanine and Y150.[15] This stabilizing force might be the reason for the comparable activity of aldehydes **1** and **6**.

In conclusion, we have described a systematic SAR study based on substrate-based aldehyde inhibitor **1**. During various scans, we observed that P_2 Arg residue was more important for enzyme interactions than P_1 Arg, and tri- and dipeptide aldehyde inhibitors afforded low micromolar activity. Furthermore, successful non-basic and uncharged replacements of P_1 arginine were identified. Even though we were unable to enhance the potency of aldehyde inhibitors, this systematic study has significantly enhanced the pharmacophore knowledge of dengue NS3 protease inhibitors and future investigations are in progress toward more potent inhibitors of dengue protease.

Acknowledgment

The authors gratefully acknowledge Jeasie Tan for analytical support.

References and notes

1. (a) Jacobs, M. G.; Young, P. R. *Curr. Opin. Infect. Dis.* **1998**, *11*, 319; (b) Gubler, D. *J. Clin. Microbiol. Rev.* **1998**, *11*, 480; (c) Monath, T. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2395.
2. Chambers, T. J.; Hahn, C. S.; Galler, R.; Rice, C. M. *Annu. Rev. Microbiol.* **1990**, *44*, 649, *Abbreviations used:* C, Capsid; prM, precursor membrane; E, envelope; NS, nonstructural.
3. (a) Falgout, B.; Pethel, M.; Zhang, Y. M.; Lai, C.-J. *J. Virol.* **1991**, *65*, 2467; (b) Zhang, L.; Mohan, P. M.; Padmanabhan, R. *J. Virol.* **1992**, *66*, 7549; (c) Ramachandran, M.; Sasaguri, Y.; Nakano, R.; Padmanabhan, R. *Methods Enzymol.* **1996**, *275*, 168.
4. (a) Wengler, G.; Wengler, G. *Virology* **1991**, *184*, 707; (b) Li, H. T.; Clum, S.; You, S. H.; Ebner, K. E.; Padmanabhan, R. *J. Virol.* **1999**, *73*, 3108.
5. (a) Clum, S.; Ebner, K. E.; Padmanabhan, R. *J. Biol. Chem.* **1997**, *272*, 30715; (b) Falgout, B.; Miller, R. H.; Lai, C.-J. *J. Virol.* **1993**, *67*, 2034.
6. Li, J.; Lim, S. P.; Beer, D.; Patel, V.; Wen, D. Y.; Tumanut, C.; Tully, D.; Williams, J. A.; Jiricek, J.; Priestle, J. P.; Harris, J. L.; Vasudevan, S. G. *J. Biol. Chem.* **2005**, *280*, 28766.
7. (a) Krishna Murthy, H. M.; Judge, K.; DeLucas, L.; Padmanabhan, R. *J. Mol. Biol.* **2000**, *301*, 759; (b) Ganesh, V. K.; Muller, N.; Judge, K.; Luan, C.-H.; Padmanabhan, R.; Murthy, K. H. M. *Biol. Med. Chem. Lett.* **2005**, *13*, 257.
8. Detailed kinetic profile of aldehyde **1** will be communicated in a separate publication.
9. Fehrentz, J.-A.; Paris, M.; Heitz, A.; Velek, J.; Liu, C.-F.; Winternitz, F.; Martinez, J. *Tetrahedron Lett.* **1995**, *36*, 7871.
10. Schultz, R. M.; Varma-Nelsons, P.; Ortiz, R.; Kozlowskin, K. A.; Orawski, A. T.; Pagast, P.; Frankfater, A. *J. Biol. Chem.* **1989**, *264*, 1497.
11. 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 DEN2 CF40-gly-NS3-pro185 (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 (Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099) for competitive inhibitor. All K_i values are reported as averages of at least two experiments.
12. Chanprapaph, S.; Saparpakorn, P.; Sangma, C.; Niyomrattanakit, P.; Hannongbua, S.; Angsuthanasombat, C.; Katzenmeier, G. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 1237.
13. Nall, T. A.; Chappell, K. J.; Stoermer, M. J.; Fang, N.-X.; Tyndall, J. D. A.; Young, P. R.; Fairlie, D. P. *J. Biol. Chem.* **2004**, *279*, 48535.
14. Modeling study of aldehydes **1** and **6** was performed by mutating the P_1–P_4 residues of the MbBBI of the Lys-head in 1DF9 (using SYBYL7), followed by minimization of the tetra-peptide (with the C_α of the P_1 residue covalently bound to side-chain oxygen on S135) and all protein residues within 10 Å of the aldehyde, using the CHARMm force field: parameter and charge assignment was done with the in-house program Witnotp (A. Widmer).
15. Weak π–π interactions of 4.5 Å were observed between the P_1 Phe and Y150 in the S_1 pocket.
16. Yin, Z.; Patel, S. J.; Wang, W-L.; Wang, G.; Chan, W-L.; Ranga Rao, K. R.; Alam, J.; Jeyaraj, D. A.; Ngew, X.; Patel, V.; Beer, D.; Lim, S. P.; Vasudevan, S. G.; Keller, T. H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 36.