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Synthesis and disulfide bond connectivity–activity studies of a kalata B1-inspired cyclopeptide against dengue NS2B–NS3 protease

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ABSTRACT

Kalata B1 is a plant protein with remarkable thermal, chemical and enzymatic stability. Its potential applications could be centered on the possibility of using its cyclic structure and cystine knot motif as a scaffold for the design of stable pharmaceuticals. To discover potent dengue NS2B–NS3 protease inhibitors, we have prepared various kalata B1 analogues by varying the amino acid sequence. Mass spectrometric and biochemical investigations of these analogues revealed a cyclopeptide whose two fully oxidized forms are substrate-competitive inhibitors of the dengue viral NS2B–NS3 protease. Both oxidized forms showed potent inhibition with K_i of 1.39 ± 0.35 and 3.03 ± 0.75 μM, respectively.

1. Introduction

Dengue virus, an arthropod-borne human pathogen transmitted by the *Aedes aegypti* mosquitoes belongs to the virus family of flaviviridae and is the causative agent of dengue fever, dengue shock syndrome and dengue hemorrhagic fever. It is estimated that about 40% of the world population is at risk of dengue virus infection and of the one million cases which are diagnosed yearly, about 5% are fatal, making dengue fever the most important mosquito-borne viral disease affecting humans.[1] To-date, there are four known serotypes of dengue virus, known as DEN1–4. Due to the large serological distance among these serotypes, a host who has recovered from infection by one serotype cannot render himself immune to the others. Hence in endemic areas, dengue virus often circulates as a complex of four serotypes and currently there is no approved vaccine or effective antiviral therapy for these diseases.[2]

Like other flaviviruses, the dengue virus contains a single, positive-sense RNA genome of ~11 kb in size which is firstly translated into a polypeptide and subsequently processed by host cell proteases and the virus-encoded NS3 protease to generate three structural proteins (C (capsid), prM (precursor-membrane) and E (envelope)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).[3] Optimal activity of the NS3 protease is thus essential for the maturation of the virus. The NS3 protease is known to exhibit activity with some model substrates for serine proteases and its activity has been shown to be markedly enhanced when the protease is coupled to its cofactor, the non-structural protein NS2B.[4] This NS2B–NS3 protease also efficiently catalyzes the *cis* cleavage of NS2A–NS2B and NS2B–NS3, as well as the *trans* cleavage of NS3–NS4A and NS4B–NS5 sites in the polyprotein.[5] These sites have in common dibasic amino acids, Lys-Arg, Arg-Arg and Arg-Lys, and occasionally Gln-Arg followed by a short side-chain residue such as a Gly, Ser or Ala amino acid in the cleavage site. In addition, the viral NS2B–NS3 protease also cleaves internally within NS2A[6] and NS3.[7] Since NS2B–NS3 protease plays important roles in post-translational proteolytic processing of the viral polyprotein and in viral replication and maturation, it is an attractive therapeutic target for anti-dengue viral drug design. However, the design of flavivirus protease inhibitors has so far met with limited success. Only a few peptidic[3,8] and non-peptidic[9] inhibitors of the dengue serine protease with moderate activities have been reported. Yin et al. have shown that substitution of the amide group of the Bz-Nle-Lys-Arg-Arg-H tetrapeptide inhibitor (K_i = 5.8 μM) for an aldehyde greatly increased the inhibition (K_i of 1.5 μM, **A** in Fig. 1).[8c] Incorporating trifluoromethyl ketone

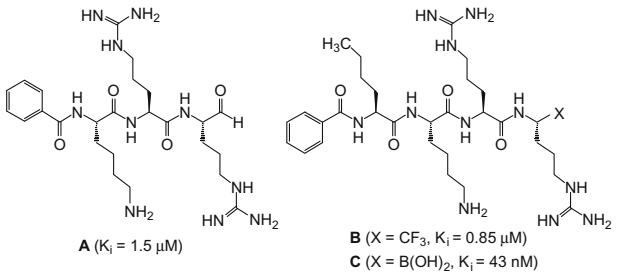

Figure 1. Some known inhibitors of dengue NS3 protease.

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or boronic acid warheads into the tetrapeptide further reduced the K_i values to 0.85 μM and 43 nM, respectively (**B** and **C** in Fig. 1).[8b] However the low bioavailability and high toxicity of these substrate inhibitors with warheads could limit its potential as effective chemotherapeutics.[8e] Cyclic peptides are appealing targets due to the conformational restriction imposed by cyclization.[10] In general, backbone cyclic peptides have several advantages over their non-cyclic counterparts. They are resistant to attack by exopeptidases, making them less vulnerable to degradation and can have an increased thermal stability.[11] In addition for cyclic peptides, the unfavorable entropic losses upon binding to the target proteins are significantly reduced, resulting in a thermodynamically more efficient binding interaction.[12] These biological advantages of backbone cyclized peptides may lead to their use as scaffolds for the design of stable pharmaceuticals.[13]

There have been an increasing number of reports over the last few years on naturally occurring and synthetic cyclic proteins. Of particular interest among the naturally occurring examples are a series of plant-derived proteins referred to as the cyclotides.[14] These include kalata B1, the circulins, cyclopsychotride and several peptides from the Viola species.[15] These peptides are small disulfide-rich compounds that have the unusual feature of a cyclic backbone. They usually comprise 29–31 amino acids, including six highly conserved Cys residues that form a cystine knot.[16] In this structural motif, an embedded ring formed by two disulfide bonds and their connecting backbone segments is penetrated by a third disulfide bond. The combination of a cystine knot embedded in a cyclic backbone produces a unique protein fold that is topologically complex and has exceptional chemical and biological stability.[16b] This unique structural and diverse biological activities found in cyclotides[17] inspired us to design cyclopeptides as inhibitors of dengue virus protease. In this paper, we have designed a range of polypeptides based on the kalata B1 molecular framework but with modifications such as (i) incorporating the viral protease substrate recognition sequences, (ii) substituting a disulfide bond with a salt bridge or (iii) introducing both (i) and (ii) into the peptide sequence (Table 1). These polypeptides were evaluated for their inhibitory activities against the DEN2 NS2B–NS3 protease which comprised of a 83-residue hydrophilic region of NS2B fused in-frame to the N-terminal 181 amino acids of NS3. Acyclic kalata B1 and kalata B1 did not demonstrate any inhibition activity probably because they do not contain the dengue virus protease substrate sequences. Changing the last amino acid at the C-terminal from N to R (polypeptide 1) resulted in a slight increase in the inhibition activity. When the dengue virus protease substrate sequences RRG and RRS were incorporated (polypeptides 2 and 3), a progressive improvement in the inhibitory activity was observed. Polypeptide 4, which contained three viral protease substrate recognition sequences and a salt bridge due to the substitutions of two cysteines (Cys9 and Cys21) with Glu and Arg, respectively, demonstrated the best inhibition amongst the linear polypeptides. When polypeptide 4 was cyclized (designated as cyclopeptide **1**) the inhibition further improved and inhibitory activity at low micromolar level was observed. This could be attributed to the more effective binding interaction between cyclopeptide **1** and the target protease. Interestingly, the oxidation of **1** gave a mixture of 3 isomers arising from the different disulfide bond connectivities and each isomer exhibited different activity against the NS2B–NS3 protease. We herein report our findings in the design, characterization of the disulfide bond connectivity and inhibitory studies of **1**.

Table 1
Peptides designed as potential inhibitors to DEN2 NS2B–NS3 protease[a]

Peptide	Sequence
Acyclic Kalata B1	*5 9 14 19 21 26* GLPVCGETCVGGTCNTPGCTCSWPVCTRN
Kalata B1	┌GLPVCGETCVGGTCNTPGCTCSWPVCTRN┐
Polypeptide 1	GLPVCGETCVGGTCNTPGCTCSWPVCTR**R**
Polypeptide 2	TPGCTCS**RRS**CG**RRG**LPVCGETCVGGTCN
Polypeptide 3	GLPVC**RRS**CKRGCNTPGCTCS**RRS**CGR**R**
Polypeptide 4	LPVCGSEES**RRG**CNTPGC**RRS**WPVCT**RRG**
Cyclopeptide **1**	┌**G**LPVCGSEES**RRG**CNTPGC**RRS**WPVCT**RR**┐

[a] Amino acids shown in bold represent the dengue virus protease substrate sequences or part of it.

2. Results and discussion

2.1. Design and oxidative refolding of cyclopeptide 1

In the design of the cyclopeptide **1**, we have incorporated the 3 dengue virus protease substrate sequences, RRG(11-13), RRS(20-22) and RRG(27-1),[4] with the aim of conferring specificity for the binding between the peptide molecule and the DEN2 NS2B–NS3 protease. We have also changed Cys9 to Glu and Cys21 to Arg to create a salt bridge which will increase the solubility of **1**. Hence cyclopeptide **1** possesses only four cysteines instead of the six found in kalata B1 and structurally there will be three possible isomers (Fig. 2) arising from the different disulfide bond connectivity.[14,15a]

Cyclopeptide **1**, in its fully reduced form, showed a single peak which corresponded to 3201 Da (monoisotopic masses were used and this value was calculated through the deconvolution of the multiple charged ions) in the reversed-phase LC–MS. However the LC analysis profile (Fig. 3a) of the reaction mixture after oxidation with 20% DMSO[18] or 1.5% H_2O_2 displayed two distinct peaks and a minor peak at a slightly shorter retention time. LC–MS studies suggested that these three fractions showed identical molecular mass of 3197 Da. The molecular entities embedded in these peaks may be attributed to the different isomers arising from different disulfide connectivity. The differences in the HPLC retention time may also indicate that there were differences in the biophysical activities among these isomers. Since the LC results show only two distinct peaks, it is possible that the third isomer may be difficult to form because of structural conformation restrictions. The percentage of each isomer as calculated from the LC profile is <1% of isomer **1A**, 35% of isomer **1B** and 64% of isomer **1C**, respectively. Isomer **1A** was formed in too small a quantity to identify its disulfide bond connectivity and perform biological tests.

2.2. Determination of disulfide bond connectivity in 1B and 1C

To determine the disulfide bond connectivity in isomers **1B** and **1C**, two mapping strategies were used to study the disulfide connectivity. In the first strategy (Scheme 1), isomers **1B** and **1C** were treated with TCEP[19] under acidic conditions (pH 3.0) to avoid reshuffling of the native disulfide bonds. The resulting partially reduced species were then cyanylated with CDAP[20] and the mixture obtained was separated by RP-HPLC and the HPLC fractions

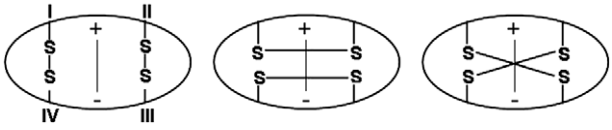


Figure 2. Possible isomers of cyclopeptide **1**.

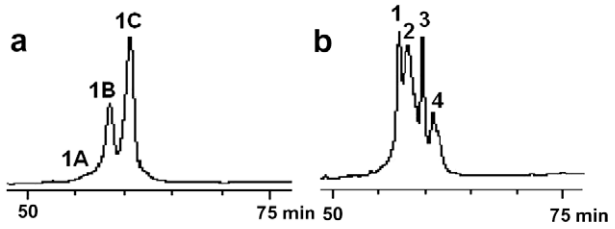
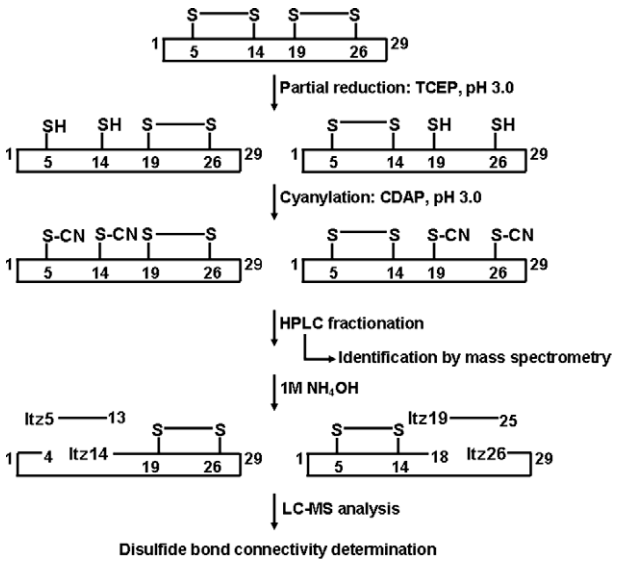


Figure 3. (a) HPLC profile of cyclopeptide **1** after subjecting to oxidation; (b) LC–MS fractions of isomer **1B** after partial reduction and cyanylation.



Scheme 1. Schematic representation of strategy 1 used for disulfide bond connectivity determination of **1B**.

obtained were analyzed by LC–MS. When this strategy was applied to isomer **1B**, four peaks with molecular mass of 3249, 3197, 3301 and 3249 Da, respectively, were observed (Fig. 3b). Since peaks 1 and 4 have molecular mass 3249 Da, this may be attributed to isomer **1B** which has been partially reduced and cyanylated (CN molecular mass: 26 Da). Peak 2 showed no change in the molecular mass (3197 Da) indicating that the partial reduction did not proceed to completion and isomer **1B** was still present in the reaction mixture. Peak 3 (molecular mass 3301 Da) has a molecular mass that is 104 Da higher than 3197. This peak could be attributed to the fully reduced and cyanylated **1B**.

The partially reduced and cyanylated product embedded in peak 1 was selected and cleaved with 1.0 M ammonium hydroxide. The resulting mixture was analyzed by LC–MS which indicated (Table 2) that the amide bonds between Gly18-Cys19 and Val25-Cys26 have been cleaved. This means that the sulfhydryl groups of Cys19 and Cys26 were cyanylated and isomer **1B** possesses I–II and III–IV disulfide bond connectivities. When the same disulfide connectivity determination procedure was applied to isomer **1C**, the LC–MS data indicated that this isomer possessed I–IV and II–III disulfide bond connectivities.

To further verify the disulfide bond connectivity, we applied strategy 2 for the partial reduction of the disulfide bonds in isomer **1C** (Scheme 2). In this strategy, the partial reduction of the disulfide bonds was achieved using TCEP[19] and the reaction mixture was then separated by HPLC. The partially reduced peptide obtained was alkylated with *N*-ethylmaleimide (NEM) then fully reduced with dithiothreitol and alkylated with iodoacetamide to give the fully alkylated peptide which was isolated, lyophilized and cleaved with trypsin. The resulting cleavage mixture was analyzed by LC–MS (Table 3) which indicated that the sulfhydryl groups of Cys5 and Cys26 were alkylated with NEM whilst those of Cys14 and Cys19 residues were alkylated by iodoacetamide. Thus this confirms that isomer **1C** possesses I–IV and II–III disulfide bond connectivities.

2.3. Inhibition of dengue NS2B–NS3 protease

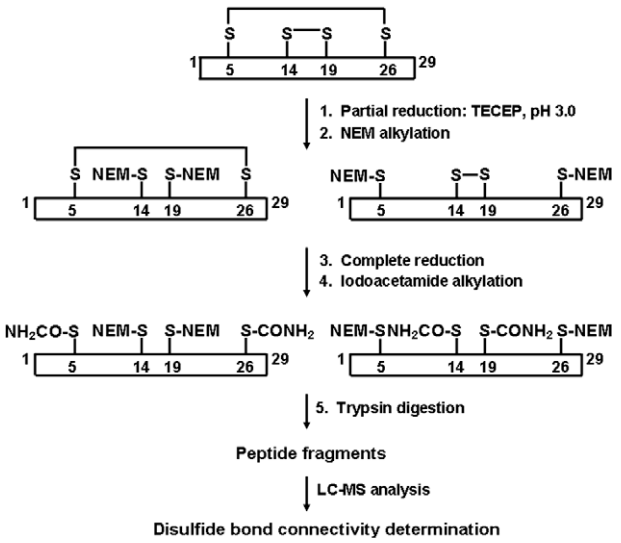
A fluorogenic dengue NS2B–NS3 protease substrate, Dabcyl-KGRRSSKL-Edans, was designed based on the sequence of the KGRR↓SSKL cleavage site.[22] The K_m of the DEN2 NS2B–NS3 protease on Dabcyl-KGRRSSKL-Edans was determined to be 9.94 ± 3.52 μM.

Kalata B1, its acyclic form and its designed derivatives were subsequently evaluated as potential inhibitors to the DEN2 NS2B–NS3 protease. Although kalata B1 and acyclic kalata B1 showed no inhibitory activity, cyclopeptide **1** showed the best inhibition in the preliminary screening of our designed peptides. However HPLC analysis showed that under the conditions used for screening (pH 9), cyclopeptide **1** was easily converted to the oxidized form and thus a mixture of both the reduced and oxidized forms co-existed in the assay solution. It was therefore necessary to isolate the individual forms to determine their efficacy against the DEN2 NS2B–NS3 protease. Thus after fractionation and purification by HPLC, the inhibitory activities of isomers **1B** and **1C** were measured against DEN2 NS2B–NS3 protease. Interestingly, each disulfide connectivity isomer displayed different inhibitory activity against the dengue viral protease (Table 4). Thus, it appears that

Table 2
Fragments after NH_4OH cleavage for assignment of disulfide bond connectivity

Observed fragments[a]	MW_{Calc}	Observed ions	MW_{Exp}[b]
Isomer **1B**			
Itz-CTRRGLPVCGSEE GPTNCGRRS	2357.04	590.34^{4+}, 786.49^{3+}	2357.26
Itz-CRRSWPV	927.45	310.23^{3+}, 464.72^{2+}	927.57
One nick	3266.52	545.46^{6+}, 654.41^{5+}, 817.78^{4+}, 1090.12^{3+}	3267.15
Isomer **1C**			
Itz-CRRSWPVCTRRS GRRSEESGCVPLG	2858.37	953.86^{3+}, 715.64^{4+}, 572.72^{5+}	2858.57[c]
Itz-CGPTN	515.18	516.24^{+}	515.23
No cleavage	3248.48	1083.91^{3+}, 813.22^{4+}, 650.74^{5+}	3248.79

[a] Itz at the N-terminal represents iminothiazolidine derivative, C-terminal is an amide.[21]
[b] MW_{Exp} is calculated through deconvolution of the multiply charged ions.
[c] A disulfide bond was broken during the cleavage by NH_4OH.



Scheme 2. Schematic representation of strategy 2 used for disulfide bond connectivity determination of **1C**.

Table 3
Fragments after trypsin cleavage for assignment of disulfide bond connectivity

Observed fragments[a]	MW_{Calc}	Observed ions	MW_{Exp}[b]
GLPVC($CONH_2$)GSEESR	1189.54	596.02^{2+}	1190.04
GLPVC($CONH_2$)GSEESR(R)	1345.65	449.53^{3+}, 673.92^{2+}	1345.63
RGC(NEM)NTPGC(NEM)R (R)RT(CONH2)CVPWSR	2413.17	403.21^{6+}, 805.45^{3+}	2413.31
SWPVC($CONH_2$)TR	904.43	302.53^{3+}, 453.51^{2+}, 905.53^{+}	904.53

[a] Trypsin prefers to cleave the site after arginine, therefore, fragments may have one, two or more arginine residues according to the cyclopeptide **1** sequence. R residue in the bracket may exist in the C- or N-terminal.
[b] MW_{Exp} is calculated through deconvolution of the multiply charged ions.

Table 4
Inhibition of dengue NS2B–NS3 protease by isomers **1B** and **1C**

Peptide	IC_{50} (μM)	K_i (μM)
Isomer **1B**	4.3	1.39 ± 0.35
Isomer **1C**	9.3	3.03 ± 0.75

the nature of the disulfide bond connectivity is essential for useful inhibition of the dengue viral protease. Since the most potent dengue viral inhibitor (without the electrophilic warheads) known to-date has activity in the low micromolar concentration (**A** in Fig. 1),[8c] the above results also demonstrate that highly potent inhibitors of DEN2 NS2–NS3 protease have been designed and synthesized based on molecular frameworks embedded in naturally occurring cyclotides.

3. Conclusion

In summary, various analogues of the kalata B1 cyclotide were evaluated for their inhibitory activities against DEN2 NS2B–NS3 protease. A cyclopeptide which exists in two distinct oxidized forms, isomers **1B** and **1C** showed potent inhibition with K_i of 1.39 ± 0.35 and 3.03 ± 0.75 μM, respectively. The disulfide bond connectivity of isomers **1B** and **1C** were mapped by two different mapping methods which showed that isomer **1B** has I–II and III–IV disulfide connectivity and isomer **1C** has I–IV and II–III disulfide connectivity. The inhibitors reported here represent the first NS2B–NS3 protease inhibitor designed from a natural cyclotide. Since the NS2B–NS3 protease carries out various functions which are essential for viral replication, its inhibition is of significance pharmacologically.

4. Experimental

4.1. Chemicals and synthetic peptides

All oligonucleotides used for the gene synthesis were purchased from Sigma-Proligo (Singapore). *Escherichia coli* strain DH5α (Stratagene, La Jolla, CA) was used as the host for all cloning experiments while protein expression studies were performed using *E. coli* strain BL21(DE3) (Novagen, Madison, WI) as host. Linear synthetic peptides were purchased from Pepscan Systems (The Netherlands) or Sigma–Aldrich. Cyclopeptide **1** was purchased from Pepscan Systems while kalata B1 was obtained from GL Biochem (Shanghai). Chemicals such as dimethyl sulfoxide (DMSO), dithiothreitol (DTT), tris(2-chloroethyl)phosphate (TCEP), *N*-ethylmaleimide (NEM), iodoacetamide, 1-cyano-4-dimethyl-aminopyridinium tetrafluoroborate (CDAP) and the designed substrate for the dengue viral protease activity assay, Dabcyl-KGRRSSKL-Edans, were purchased from Sigma–Aldrich.

4.2. Oxidative refolding of cyclopeptide 1 and purification of the individual isomers

Cyclopeptide **1** was dissolved in 50 mM ammonium bicarbonate buffer (pH 8.0) to a concentration of 0.5 mg/mL. The peptide solution was then treated with 20% DMSO[17] or 1.5% H_2O_2 solution. After incubating the reaction mixture at 25 °C for overnight (for DMSO oxidation) or at 0 °C for 20 min (for H_2O_2 oxidation), the reaction mixture was directly analyzed or fractionated using RP-HPLC carried out on a Shimadzu LC-10Avp series apparatus with a UV detector and a VP-ODS C_{18} column (250 mm × 4.6 mm, 5 μm and 300 Å). The solvents used for the gradient elution were 0.05% trifluoroacetic acid (TFA) in water, henceforth known as solvent A, and 0.05% TFA in 85% acetonitrile in water, henceforth known as solvent B. The flow rate was 0.5 mL/min with solvent B increasing from 0% to 50% in 90 min. The chromatograms were recorded at 220 nm. The collected fractions were either used directly for MS analysis or lyophilized for activity studies.

4.3. Mass spectrophotometric analysis of disulfide bond connectivity in cyclopeptide 1

Two strategies were used to determine the disulfide bond connectivity. For strategy 1, partial reduction was carried out by mixing 3 μL of 20 mM TCEP in 200 mM citrate buffer (pH 3.0) with 30 μL cyclopeptide **1** (1 mg/mL in 200 mM citrate buffer, pH 3.0 containing 6 M guanidine·HCl). The reaction mixture was then incubated at room temperature for 30 min to achieve partial reduction of the disulfide bonds. After which, cyanylation was carried out by treating the peptide mixture with 10 μL of 100 mM CDAP in 0.2 M citrate buffer, pH 3.0. The partially reduced and cyanylated peptides were separated and collected by RP-HPLC using the same elution system as described above. The HPLC fractions obtained were lyophilized and 10 μL of 6 M guanidine·HCl was added to dissolve the peptide followed by 25 μL of 1 M aqueous NH_4OH. The reaction mixture was allowed to stand at room temperature for 1 h. After which, the reaction mixture was applied to LC–MS (Shimadzu LC–MS 2010EV) for mass analysis. The molecular mass measured for each peptide fragment was compared with the theoretical value. The possible cleavage sites were deduced from the molecular mass which in turn provides information about

the disulfide bond connectivity in the peptide. For strategy 2, partial reduction was achieved by mixing 10 μL of 20 mM TCEP in 200 mM citrate buffer (pH 3.0) with 30 μL cyclopeptide **1** (1 mg/mL in 200 mM citrate buffer, pH 3.0 containing 6 M guanidine·HCl). The reaction mixture was incubated at room temperature for 15 min and then the reaction was stopped by immediately injecting the reaction mixture into the HPLC. The flow rate was 0.5 mL/min with solvent B increasing from 0% to 50% in 50 min. This condition was also used in the purification step after the NEM alkylation. For NEM alkylation, the volume of the partially reduced peptide collected was treated with the same volume of 200 mM NEM in 200 mM citrate buffer, pH 3.0. The reaction mixture was incubated for 2 h at room temperature and then directly injected into the RP-HPLC. The manually collected HPLC fractions were analyzed by mass spectrometry, dried by lyophilization and then cleaved with trypsin (0.1 μg in 20 μg of 100 mM NH_4HCO_3, pH 8.0, 37 °C, 5 min). After the reaction, the reaction mixture was injected into the LC–MS for mass spectrometry analysis.

4.4. Inhibitory activity assay against dengue virus type 2 NS2B–NS3 protease

Recombinant protease TRX-NS2B–NS3 was expressed in *E. coli* according to an earlier described procedure.[23] Briefly, the pET32 vector generated for the expression of the TRX-NS2B–NS3 protein was transformed into competent BL21(DE3) cells. The bacterial cultures were grown and induced with isopropyl-D-thiogalactopyranoside (IPTG) under conditions described by Cui et al.[23b] The cells were harvested by centrifugation (5000*g* for 10 min) at 5 h post-induction and lysed with BugBuster HT protein extraction reagent (Novagen). The soluble protein fractions were collected by centrifugation at 8000*g* for 20 min at 4 °C and purified in a single chromatographic step using nickel as the metal ion in immobilized metal affinity chromatography.[23a] The recombinant protease was eluted in an elution buffer (1 M imidazole, 50 mM NaCl, 20 mM Tris–HCl, pH 7.9) and dialyzed against 5 mM Tris–Cl, pH 8.0, using 12 kDa molecular weight cutoff tubing. The recombinant proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and the concentration of TRX-NS2B–NS3 recovered was estimated by BCA assay (Pierce, Rockford, IL, USA). The cleavage of TRX-NS2B–NS3 releasing DEN2 NS2B–NS3 protease was performed using enterokinase (Novagen) at 37 °C for 2 h. The cleavage reaction mixture was directly used for the protease assay.

Dengue NS2B–NS3 protease inhibition assay was performed in 96-well black plates in a final volume of 100 μL. Fluorogenic substrate Dabcyl-KGRRSSKL-Edans was used for the K_m determination. The substrate, in eight different concentrations [S] (ranging from 1 to 80 μM), was incubated at 37 °C with 0.2 μM protease dilute in a buffer containing 50 mM Tris–HCl buffer, 0.1% Triton X-100, pH 9.0, containing 30% (v/v) glycerol. The increase in fluorescence intensity was monitored continuously using an Infinite F200 microplate reader (Tecan, Switzerland) at an excitation wavelength of 340 nm and emission wavelength of 480 nm. The initial velocity was determined from the linear portion of the progress curve and K_m was calculated from the Michaelis–Menten equation, where $v = V_{max}[S]/([S] + K_m)$. Triplicate measurements were taken for each data point. The data are reported as mean ± SE. For the IC_{50} calculation, the respective polypeptide inhibitor in eight concentrations, each in triplicate, ranging from 0.6 to 67 μM, and protease were mixed with the assay buffer (50 mM Tris–HCl buffer, 0.1% Triton X-100, pH 9.0, containing 30% (v/v) glycerol) to give a final protease concentration of 0.2 μM. The enzyme with the polypeptide inhibitor was preincubated at 37 °C for 15 min. After which, catalysis was initiated by adding substrate Dabcyl-KGRRSSKL-Edans to a final concentration of 20 μM. The increase in fluorescence intensity was monitored continuously using an Infinite F200 microplate reader at an excitation wavelength of 340 nm and emission wavelength of 480 nm. Fluorescence values obtained in the absence of an inhibitor were taken as 100% and designated as the control value. The fluorescence values obtained in the presence of the respective polypeptide inhibitors were plotted as the percentage of inhibition of the control using Microsoft Excel. IC_{50} values were determined using sigmoidal dose-response by GraphPad Prism software (San Diego, USA). K_i values were calculated from IC_{50} values according to the equation: $K_i = IC_{50}/(1 + [S]/K_m)$. Triplicate measurements were taken and the data were reported as mean ± SE.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.026.

References and notes

1. (a) http://www.nathnac.org/pro/factsheets/dengue.htm#Risk.; (b) Wilder-Smith, A.; Schwartz, E. *N. Engl. J. Med.* **2005**, *353*, 924; (c) http://www.who.int/csr/disease/dengue/en/.
2. (a) Rigau-Perez, J. G.; Clark, G. G.; Gubler, D. J.; Reiter, P.; Sanders, E. J.; Vorndam, A. V. *Lancet* **1998**, *352*, 971; (b) Monath, T. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 2395.
3. Leung, D.; Schroder, K.; White, H.; Fang, N. X.; Stoermer, M. J.; Abbenante, G.; Martin, J. L.; Young, P. R.; Fairlier, D. P. *J. Biol. Chem.* **2001**, *276*, 45762.
4. Yusof, R.; Clum, S.; Wetzel, M.; Murthy, H. M. K.; Padmanabhan, R. *J. Biol. Chem.* **2000**, *275*, 9963.
5. (a) Clum, S.; Ebner, K. E.; Padmanabhan, R. *J. Biol. Chem.* **1997**, *272*, 30715; (b) Cahour, A.; Falgout, B.; Lai, C. J. *J. Virol.* **1992**, *66*, 1535.
6. Nestorowicz, A.; Chambers, T. J.; Rice, C. M. *Virology* **1994**, *199*, 114.
7. (a) Falgout, B.; Pethel, M.; Zhang, Y. M.; Lai, C. J. *J. Virol.* **1991**, *65*, 2467; (b) Teo, K. F.; Wright, P. J. *J. Gen. Virol.* **1997**, *78*, 337.
8. (a) Chanprapaph, S.; Saparpakorn, P.; Sangma, C.; Niyomrattanakit, P.; Hannongbua, S.; Angsuthanasombat, C.; Katzenmeier, G. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 1237; (b) Yin, Z.; Patel, S. J.; Wang, W.-L.; Wang, G.; Chan, W.-L.; Rao, K. R. 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; (c) Yin, Z.; Patel, S. J.; Wang, W.-L.; Chan, W.-L.; Rao, K. R. R.; Wang, G.; Ngew, X.; Patel, V.; Beer, D.; Knox, J. E.; Ma, N. L.; Ehrhardt, C.; Lim, S. P.; Vasudevan, S. G.; Keller, T. H. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 40; (d) Knox, J. E.; Ma, N. L.; Yin, Z.; Patel, S. J.; Wang, W.-L.; Chan, W.-L.; Rao, K. R. R.; Wang, G.; Ngew, X.; Patel, V.; Beer, D.; Lim, S. P.; Vasudevan, S. G.; Keller, T. H. *J. Med. Chem.* **2006**, *49*, 6585; (e) Chappell, K. J.; Stoermer, M. J.; Fairlie, D. P.; Young, R. *Curr. Med. Chem.* **2008**, *15*, 2771.
9. (a) Ganesh, V. K.; Muller, N.; Judge, K.; Luan, C.-H.; Padmanabhan, R.; Murthy, K. H. M. *Biol. Med. Chem.* **2005**, *13*, 257; (b) Kiat, T. S.; Pippen, R.; Yusof, R.; Ibrahim, H.; Khalid, N.; Rahman, N. A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3337; (c) Mueller, N. H.; Pattabiraman, N.; Ansarah-Sobrinho, C.; Viswanathan, P.; Pierson, T. C.; Padmanabhan, R. *Antimicrob. Agents Chemother.* **2008**, *52*, 3385; (d) Johnston, P. A.; Phillips, J.; Shun, T. Y.; Shinde, S.; Lazo, J. S.; Huryn, D. M.; Myers, M. C.; Ratnikov, B. I.; Smith, J. W.; Su, Y.; Dahl, R.; Cosford, N. D. P.; Shiryaev, S. A.; Strongin, A. Y. *Assay Drug Dev. Technol.* **2007**, *5*, 737.
10. (a) Spatola, A. F.; Crozet, Y.; deWit, D.; Yanagisawa, M. *J. Med. Chem.* **1996**, *39*, 3842; (b) Giebel, L. B.; Cass, R.; Milligan, D. L.; Young, D.; Arze, R.; Johnson, C. *Biochemistry* **1995**, *34*, 15430.
11. Iwai, H.; Pluckthun, A. *FEBS Lett.* **1999**, *459*, 166.
12. Trabi, M.; Craik, D. J. *Trends Biochem. Sci.* **2002**, *27*, 132.
13. Craik, D. J.; Simonsen, S.; Daly, N. L. *Curr. Opin. Drug Discov. Devel.* **2002**, *5*, 251.
14. Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. *J. Mol. Biol.* **1999**, *294*, 1327.
15. (a) Saether, O.; Craik, D. J.; Campbell, I. D.; Sletten, K.; Juul, J.; Norman, D. G. *Biochemistry* **1995**, *34*, 4147; (b) Gustafson, K. R.; Sowder, R. C., II; Henderson, L. E.; Parsons, I. C.; Kashman, Y.; Cardellina, J. H., II; McMahon, J. B.; Buckheit, R. W., Jr.; Pannell, L. K.; Boyd, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 9337; (c) Witherup, K. M.; Bogusky, M. J.; Anderson, P. S.; Ramjit, H.; Ransom, R. W.; Wood, T.; Sardana, M. *J. Nat. Prod.* **1994**, *57*, 1619; (d) Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. *J. Nat. Prod.* **1998**, *61*, 77.

16. (a) Isaacs, N. W. *Curr. Opin. Struct. Biol.* **1995**, *5*, 391; (b) Colgrave, M. L.; Craik, D. J. *Biochemistry* **2004**, *43*, 5965.
17. (a) Hernandez, J. F.; Gagnon, J.; Chiche, L.; Nguyen, T. M.; Andrieu, J. P.; Heitz, A.; Trinh, H. T.; Pham, T. T. C.; Nguyen, D. L. *Biochemistry* **2000**, *39*, 5722; (b) Luckett, S.; Garcia, R. S.; Barker, J. J.; Konarev, A. V.; Shewry, P. R.; Clarke, A. R.; Brady, R. L. *J. Mol. Biol.* **1999**, *290*, 525.
18. Tam, J. P.; Wu, C. R.; Liu, W.; Zhang, J. W. *J. Am. Chem. Soc.* **1991**, *113*, 6657.
19. Goeransson, U.; Craik, D. J. *J. Biol. Chem.* **2003**, *278*, 48188.
20. Wu, J.; Watson, J. T. *Protein Sci.* **1997**, *6*, 391.
21. Wu, J.; Watson, J. T. *Anal. Biochem.* **1998**, *258*, 268.
22. Cui, T.; Puah, C. M.; Liew, O. W.; Lee, S. H. PCT Int. Appl. 2007, WO2007149052.
23. (a) Liew, O. W.; Chong, J. P. C.; Yandle, T. G.; Brennan, S. O. *Protein Expr. Purif.* **2005**, *41*, 332; (b) Cui, T.; Gao, Y.; Liew, O. W.; Puah, C. M.; Gutte, B. *J. Biotechnol.* **2007**, *130*, 378; (c) Zuo, Z.; Liew, O. W.; Chen, G.; Chong, P. C. J.; Lee, S. H.; Chen, K.; Jiang, H.; Puah, C. M.; Zhu, W. *J. Virol.* **2009**, *83*, 1060.