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Critical Effect of Peptide Cyclization on the Potency of Peptide Inhibitors against Dengue Virus NS2B-NS3 Protease

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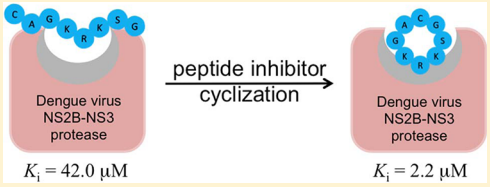
Supporting Information

ABSTRACT: Dengue virus (DENV) infection is a serious public health threat worldwide that demands effective treatment. In the search for potent virus protease inhibitors, several cone snail venoms were screened against serotype 2 DENV NS2B-NS3 protease, and one conotoxin, MrIA, was identified to have inhibitory activity. The inhibitory activity was attributed to a disulfide bond-mediated loop, from which rational optimization was made to improve the potency and stability. An eight-residue cyclic peptide inhibitor was finally obtained with high potency (inhibitory constant 2.2 μM), stability, and cell permeability. This inhibitor can thus serve as a good lead for DENV drug development. In addition, this work highlights the critical effect of peptide cyclization on the potency of oligopeptide inhibitors against DENV protease, which may advance the design of peptide inhibitors for homologous virus proteases.



INTRODUCTION

Dengue virus (DENV), a member of flavivirus family, is one of the most widespread pathogens in tropical and subtropical areas.[1] DENV infection causes severe epidemic diseases such as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS).[2,3] About 55% of the world population is at risk of DENV infection every year, and 5% of infections are lethal, which underscores the need for effective treatment of DENV infection. However, DENV has four distinct serotypes (DENV 1−4) based on antigenic properties and sequence similarity.[1] Infection by one serotype of DENV does not protect from, but actually enhances, subsequent infection by any of the other three serotypes.[4] This antibody-dependent enhancement effect (ADE) of DENV renders the vaccine study particularly difficult. Up to now, there has been no approved vaccine against DENV. Therefore, many efforts have been made to develop antiviral therapeutic agents against other targets.[5,6]

DENV NS3 protease is considered a promising antiviral target as it is responsible for the cleavage of virus protein precursors and thus is indispensable for the virus replication life cycle.[7,8] The strategy of inhibiting the viral protease has been successfully utilized in the treatment of HIV (human immunodeficiency virus) and HCV (hepatitis C virus) infections.[9] Previous studies have shown that the activity of DENV NS3 protease, the N-terminal region of the non-structural protein 3 (NS3), is dependent on a 40 amino acid region of another NS, NS2B.[10] Therefore, the fused NS2B-NS3 protease has been used for characterization and inhibitor screening.[11] Its recognition sequence has been deduced from a cleavage site sequence alignment and peptide array study.[12] So far, neither high-throughput screening of compound libraries nor modification of substrate-mimicking oligopeptides has produced any DENV NS2B-NS3 protease inhibitor suitable for clinical development.[5] On the other hand, rational design of inhibitors specific to DENV NS2B-NS3 protease is rather difficult, because the active site of this protease is quite flat. In order for an inhibitor to bind, the substrate binding site requires a substantial conformational change of the NS2B fragment, the structure of which has only become available very recently.[13,14] Thus, a novel strategy would be insightful in a DENV NS2B-NS3 protease inhibitor study.

In this work, an ideal natural peptide library, conotoxins, was used for DENV NS2B-NS3 protease inhibitor screening. Conotoxins are a mixture of peptide neurotoxins produced by cone snails to paralyze prey and for defense.[15] Each *Conus* species has about 50−200 components in its venom; there are about 700 *Conus* species in the world with little overlap in their venom reservoir. As a consequence, conotoxins have a potential capacity of 70000 peptides. Conotoxins are often disulfide-rich short peptides of 10−40 residues that exhibit astonishing

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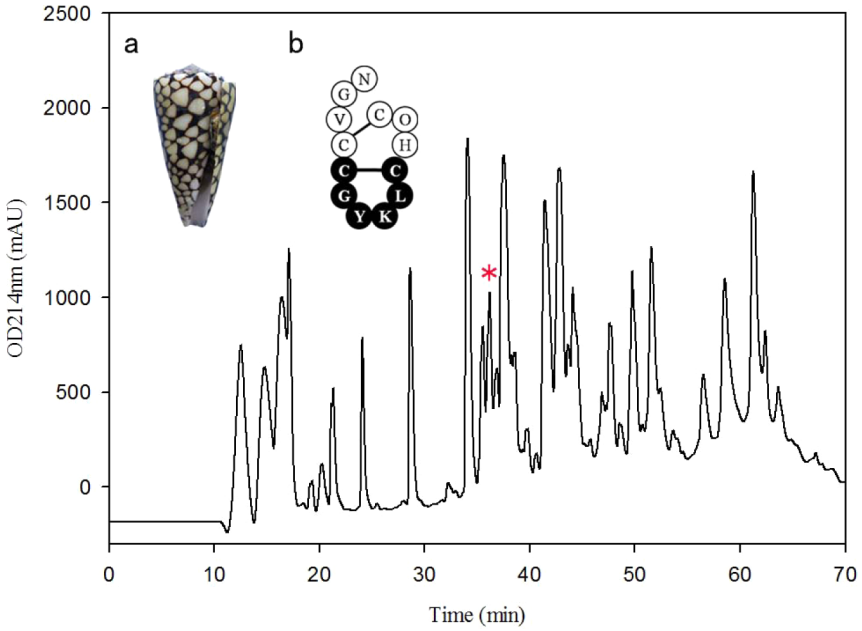

Figure 1. Purification of MrIA from the venom of *C. marmoreus*. The crude venom extract of *C. marmoreus* was separated on a ZORBAX 300SB-C18 semipreparative column with a linear gradient of 0−70% buffer B (0.1% TFA in acetronitrile) in 70 min. The flow rate was 1 mL/min. The asterisk indicates the peak corresponding to MrIA. It eluted at 36.2 min (insets: a, shell of *C. marmoreus*; b, topological structure of MrIA).

structural diversity.[16] We take advantage of this diversity to obtain a novel DENV NS2B-NS3 protease inhibitor.

From the venom of *Conus marmoreus*, conotoxin MrIA was found to significantly inhibit NS2B-NS3 protease activity, although this toxin was originally identified as a noradrenaline transporter (NET) inhibitor.[17] A mutational study revealed that a disulfide-mediated loop is responsible for NS2B-NS3 inhibitory activity with a Lys residue at the active site. Further optimization gave a potent and stable eight-residue cyclic peptide inhibitor against DENV NS2B-NS3 protease. This work highlights the critical effect of peptide cyclization for the potency of inhibitors, which may, at least partially, explain the unsatisfactory results of previous peptide inhibitor studies and which will shed new light on virus protease inhibitor development.

RESULTS

Screening DENV NS2B-NS3 Protease Inhibitor from Conotoxins. While crude venom extractions of *Conus generalis*, *Conus lividus*, *Conus vitulinus*, *Conus marmoreus*, and *Conus caracteristicus* were tested for inhibitory activity against DENV2 NS2B-NS3 protease, only the venom of *C. marmoreus* showed obvious inhibitory activity. Then, this venom was fractionated on an high-performance liquid chromatography (HPLC) C18 column (Figure 1). The activity of each fraction was followed, which excluded all of the components except the single peak that eluted at 36.203 min. Edman degradation sequencing of this peak gave a 13-residue sequence (NGVCCGYKLCHOC, where O is hydroxyl proline), which was confirmed by mass spectrometry (data not shown). These results demonstrate that this peak is conotoxin MrIA, which was originally identified in 2000[18] and was found to inhibit NET.[17]

To confirm that the DENV2 NS2B-NS3 protease inhibitor activity is from MrIA rather than another minor component of this peak, MrIA was chemically synthesized with Cys4 and Cys13 selectively protected with acetamidomethyl (Acm). Stepwise oxidation gave correctly folded MrIA, which coeluted with the native MrIA on an analytical C18 reverse-phase HPLC column (Supporting Information, Figure S1). Indeed, the synthetic MrIA inhibits DENV NS2B-NS3 protease with a K_i of 9.0 μM (Supporting Information, Figure S2).

Active Site of MrIA for Inhibitory Activity. The solution structure of MrIA was published in 2005.[19] The connectivity of two disulfide bonds gives this toxin a double loop structure (Figure 1, inset b), with the lower loop being the active site for NET inhibitor activity.[20] To test whether this loop is also responsible for the protease inhibitory activity, the six-residue sequence (CGYKLC) of MrIA was chemically synthesized, and the disulfide bond between two Cys residues was formed with air oxidation. The resultant single loop peptide with one disulfide bond, termed MrIA-SL, displayed similar inhibitory activity against DENV2 NS2B-NS3 protease (K_i = 12.5 μM) as the full-length MrIA. Therefore, the lower loop of MrIA is indeed responsible for the inhibitory activity. It is worth pointing out that, when the disulfide bond is not formed, the linear peptide of MrIA-SL has no inhibitory activity even at a concentration of 167 μM, which strongly suggests that the conformation of the cyclic peptide is crucial for the inhibitory activity.

To narrow down the active site in this six-residue loop, residues except Cys and Gly were individually replaced with Gly. The inhibitory activities of these three point mutants, inhibitors **1**, **2**, and **3**, respectively, clearly showed that Lys is the key site for activity, while the mutation of Tyr or Leu does not affect activity (Table 1). This six-residue cyclic peptide MrIA-SL was taken as the starting template for further optimization.

Optimization of Cyclic Peptide Inhibitors. Previous studies have shown that DENV NS2B-NS3 protease favors basic residues at the P1−P3 positions.[12] To improve the binding of inhibitors to the protease, six-residue peptides **4** and **5** with two or three basic residues were synthesized, oxidized, and tested. Indeed, the K_i of inhibitors **4** and **5** are 2.5- and 3.5-fold lower than the K_i of MrIA-SL, indicating that multiple

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Table 1. Molecular Masses and Inhibitory Constants of Peptide Inhibitors against DENV2 NS2B-NS3 Protease and Trypsin

Inhibitor No.	Sequence	Mass (Da)	K_i against NS2B-NS3 (μM)	K_i against trypsin (μM)
MrIA[a]	NGVCCGYKLCHOC	1408.5	9.0 ± 0.4	/[b]
MrIA-SL	CGYKLC	683.3	12.5 ± 0.3	>100
1	CG**G**KLC	577.7	15.1 ± 1.4	/
2	CGY**G**LC	612.7	>100	/
3	CGYK**G**C	627.8	12.0 ± 1.0	/
4	CGYKRC	726.9	5.0 ± 0.1	/
5	CGKRRC	719.9	3.5 ± 0.2	/
6	CGKRKLC	805.0	2.5 ± 0.3	/
7	CGKRKSC	779.0	1.4 ± 0.1	>100
8	AGKRKSG	684.8	11.3 ± 1.4	/
9	CAGKRKSG	787.9	2.2 ± 0.2	>100
10	CAGKRKSG	805.9	42.0 ± 4.2	/

[a]In the MrIA sequence, O is hydroxyproline. [b]Not determined.

basic residues, presumably at the P1−P3 positions, improve the inhibitory activity of peptide inhibitors against DENV NS2B-NS3 protease (Table 1). The apparent difference between the K_i of inhibitors **4** and **5** (5.0 ± 0.1 and 3.5 ± 0.2 μM, respectively) indicates that triple basic residues are preferred than double basic residues by DENV NS2B-NS3 protease.

In the strongest inhibitor yet, peptide **5**, the P1 position is next to a Cys residue in a disulfide bond, which may cause some constraint of the conformation or flexibility. To test this possibility, peptide **6**, with the same spacing residue Leu as in MrIA-SL, was studied. As compared to inhibitor **5**, the activity of inhibitor **6** is definitely increased, with K_i = 2.5 ± 0.3 μM (Table 1 and Supporting Information, Figure S2).

DENV NS2B-NS3 protease has a strong preference for Ser at the P1′ position.[12] To test whether a more appropriate P1′ residue would further improve the activity, Leu in **6** was mutated to Ser, resulting in peptide **7**. Unsurprisingly, the K_i of inhibitor **7** decreased further to 1.4 ± 0.1 μM (Table 1 and Supporting Information, Figure S2), showing the strongest inhibitory activity in this study.

Inhibitors Cyclized through Peptide Bonds. The potential utility of the DENV NS2B-NS3 protease inhibitor is to block protease activity during viral replication in host cells, where the reducing cytoplasmic environment may break the disulfide bond of the cyclic peptide inhibitors above. Therefore, peptide **8**, cyclized through a peptide bond, was synthesized, and its activity was determined. The length and sequence of peptide **7** are retained, except that two Cys residues were replaced with Ala and Gly, respectively. However, the inhibitory activity of inhibitor **8** is much weaker than peptide **7**, with K_i = 11.3 μM (Table 1 and Supporting Information, Figure S2). One possible reason could be that the ring cyclized through the peptide bond is smaller than the ring mediated through the disulfide bond of the Cys side chain thiol groups.

We then tried another cyclic peptide with one residue longer than peptide **8**, to approximate the ring size, and hence the inhibitory activity, of peptide **7**. The eight-residue cyclic peptide **9** indeed has stronger activity than the seven-residue peptide **8**. The K_i of peptide **9** is 2.2 μM (Table 1 and Supporting Information, Figure S2), similar to the K_i values of the strongest peptide inhibitors, peptides **6** and **7**.

To confirm that the potency of peptide **9** depends not only on the sequence but also on the cyclization of the peptide, the linear peptide with the same sequence, peptide **10**, was synthesized and tested. This sequence matches well the recognition sequence of DENV NS2B-NS3 protease,[12] so that peptide **10** does have inhibitory activity, but it is much weaker than the cyclic peptide **9**. The K_i of peptide **10** is 42 μM, about 20-fold higher than the K_i of peptide **9**. This result clearly demonstrates that the conformation of the cyclic peptide plays an important role in inhibiting DENV2 NS2B-NS3 protease by favoring the binding between protease and inhibitor.

Stability and Specificity of Cyclic Peptide Inhibitors. In addition to the potency of inhibitory activity, the stability of protease inhibitors is another major concern for their potential development as drugs. The stabilities of inhibitors **6**−**9**, as well as MrIA-SL, were measured by checking the remaining inhibitor activity after incubating the inhibitor with NS2B-NS3 protease for different times. A time course plot (Figure 2)

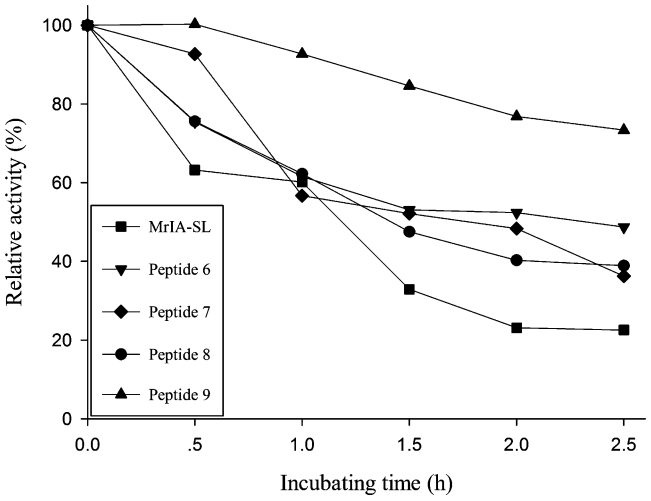

Figure 2. Stability of different peptide inhibitors against DENV2 NS2B-NS3 protease. The inhibitory activities of the inhibitors were determined every 30 min for 2.5 h.

showed that stronger inhibitors have better stability and that peptide bond cyclization further enhances the stability of peptide inhibitors. MrIA-SL is the least stable, with only 20% activity remaining after 2.5 h of incubation with NS2B-NS3 protease. Peptides **6** and **7**, both of which are stronger inhibitors than MrIA-SL, are also more stable than MrIA-SL; about half of their inhibitory activities are lost after 2.5 h incubation. Peptide **8**, cyclized through a peptide bond, has a similar stability as peptide **6** and **7**, although it has a significantly weaker inhibitory activity. The stability of peptide **9** is the best; only 25% inhibitory activity was lost after 2.5 h of incubation with protease.

The inhibitory activity against trypsin, a typical protease recognizing basic residues, was also measured for MrIA, peptides **7** and **9**. None of them exhibits any inhibition at concentrations as high as 100 μM. Their strong specificity

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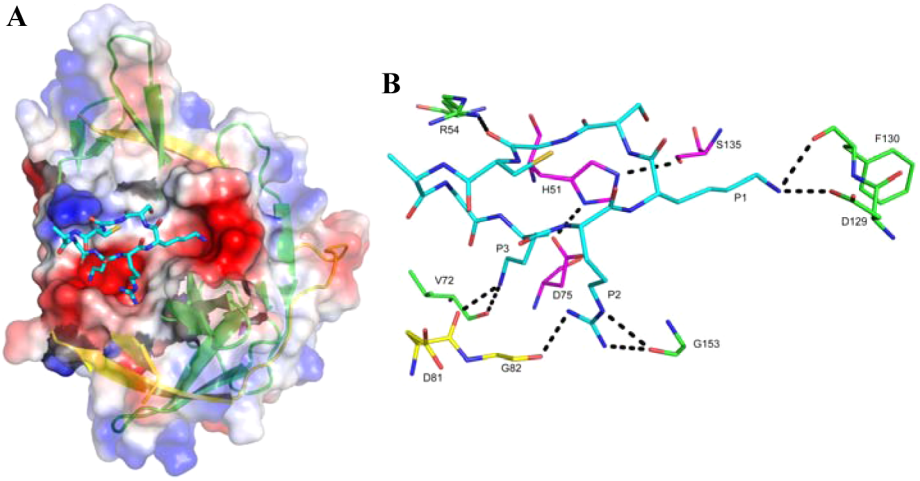

Figure 3. Modeled interaction of cyclic peptide **9** with DENV2 NS2B-NS3 protease. (A) Surface representation of the generated "closed" form of DENV2 NS2B-NS3 protease. The negative electrostatic potential is colored red, and the positive is colored blue. The NS2B cartoon is shown in yellow, and the NS3 cartoon is in green. Cyclic peptide **9** is showed as cyan sticks. (B) Detailed hydrogen bond interactions between cyclic peptide **9** and DENV2 NS2B-NS3 protease. Residues are colored as in panel A except that the catalytic triad is in magenta; potential hydrogen bonds are shown as black dashes.

toward DENV NS2B-NS3 protease is another advantage for clinical utilization.

Consistent with the lack of inhibitory activity against trypsin, peptide **9** is a poor substrate of trypsin. While peptide **10** was completed digested in 30 min, about half of peptide **9** remained intact after trypsin digestion for 4 h (Supporting Information, Figure S3). This demonstrates that the stability of the cyclic peptide **9** is significantly enhanced in comparison with the linear peptide **10**. Furthermore, peptide **9** is totally resistant to the digestion of cathepsin S, another physiologically important protease (Supporting Information, Figure S4). The stability of peptide **9** against these different proteases suggests that this inhibitor may have a long half-life in physiological condition.

Putative Binding Interactions between Cyclic Peptide 9 and NS2B-NS3 Protease. To investigate the possible interaction between the cyclic peptide **9** and DENV NS2B-NS3 protease, a model of peptide **9** was built and docked into the catalytic site of DENV2 NS2B-NS3 protease, which was modeled using the crystal structure of DENV3 NS2B-NS3 protease as a template.[14] The fitted low energy conformation shows that cyclic peptide **9** is anchored in the catalytic pocket of NS2B-NS3 protease tightly. The side chains of Lys and Arg of peptide **9** are nicely inserted into the negatively charged cleft of the binding pocket (Figure 3A), forming an extensive hydrogen-bonding network (Figure 3B). The P1 Lys side chain forms a hydrogen bond with the carbonyl of F130 of NS3 and a salt bridge with D129. The P2 Arg side chain interacts with the main chain of G82 of NS2B, and the P3 Lys side chain forms a strong hydrogen bond interaction with the carbonyl of D81 of NS2B. These interactions secure the β-hairpin of NS2B to peptide **9**, thus making the closed conformation stable. Additionally, the P2 and P3 side chains form hydrogen bonds with the main chain of G153 and V72 of NS3. R54 of NS3 interacts with the backbone of peptide **9**. Taken together, the active site of the NS2B-NS3 protease is fully occupied by cyclic peptide **9**, which may explain the strong inhibitory activity of this peptide against the NS2B-NS3 protease.

In another binding conformation with slightly lower energy, peptide **9** adopts a reversed orientation, which does not fit the canonical protease-inhibitor binding mode (Supporting Information, Figure S5). However, the binding pattern and hydrogen network are rather similar in these two conformations. More experimental work would be needed to deduce the real binding orientation of this cyclic peptide; however, competitively occupying the active site of NS2B-NS3 protease is most probably the inhibition mechanism of this cyclic peptide.

Cell Permeability. To check the cell permeability of peptide **9**, this cyclic peptide, as well as glutathione (GSH) and a cell-permeable 13-residue Tat peptide,[21] was conjugated with tetramethylrhodamine-5-maleimide (TMR) and then used to incubate BHK-21 and Vero cell lines. As a negative control, TMR-labeled GSH was not taken up by BHK-21 cells even at 20 μM. In clear contrast, the Tat peptide and inhibitor **9** efficiently permeated into BHK-21 cells when used at 2 μM (Figure 4). Higher concentrations of peptide **9** did not further increase the cell permeability, but the fluorescence signal in cells was much lower when a low concentration (0.2 μM) peptide **9** was used for incubation (Supporting Information, Figure S6). Peptide **9** shows similarly good cell permeability on Vero cells (Supporting Information, Figure S7), suggesting that permeability is an intrinsic property of this peptide.

DISCUSSION AND CONCLUSION

The serious situation of DENV infection worldwide demands effective treatment, but the specific ADE problem for DENV vaccine development makes the situation even more urgent. To discover potent and promising virus protease inhibitors, we screened several conotoxin venoms and found one component with considerable inhibitory activity. Through rational optimization, we finally obtained a potent and stable cyclic peptide inhibitor of DENV2 NS2B-NS3 protease.

In this work, we find that peptide cyclization is crucial for the potency of peptide inhibitors against DENV2 NS2B-NS3 protease. This issue has been overlooked previously, so that mainly linear peptide inhibitors were screened and studied. However, their activities were moderate without chemical modifications.[22,23] Only recently, a modeling study proposed that cyclopeptides may have better binding affinity and inhibitory activity against DENV NS2B-NS3 protease, but no

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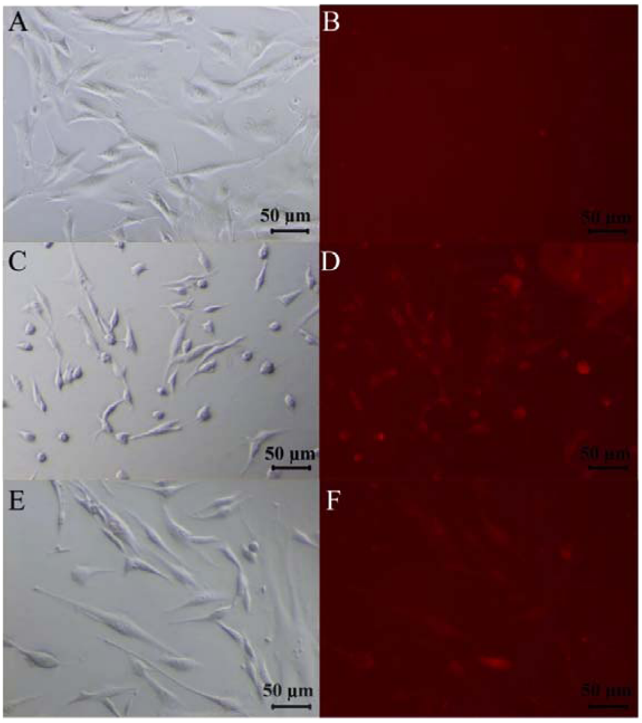

Figure 4. Cell permeability of inhibitor **9** in BHK-21 cells. One × 10^4 cells were incubated with 0.5 mL of peptide solution for 30 min at 37 °C before observation. (A and B) Cells incubated with 20 μM TMR-labeled GSH; (C and D) cells incubated with 2 μM TMR-labeled Tat-(48−60) peptide; (E and F) cells incubated with 2 μM TMR-labeled cyclic inhibitor **9**. (A, C, and E) Cells observed in bright field; (B, D, and F) cells observed with fluorescence channel.

experimental result was provided.[24] Here, the basis of peptide cyclization was obtained from studying a naturally occurring conotoxin, MrIA. This work demonstrates that conotoxins can serve as a rich natural library of structurally diversified peptides.

Peptide cyclization probably confers a more defined conformation to the peptide inhibitor, which significantly facilitates the binding of the inhibitor to the protease. Similarly, many natural proteinaceous inhibitors have their active sites located at a convex binding loop complementary to the active site of corresponding proteases.[25] Because of the significant structural conservation between proteases of different flaviviruses,[14] this cyclization strategy can also be applied to designing peptide inhibitors for proteases of other flaviviruses, such as West Nile virus and Japanese encephalitis virus. In fact, cyclic peptide inhibitors have been prepared and shown very good activities inhibiting HIV and HCV proteases.[26] For HCV NS3/4A protease, several potent cyclic inhibitors have already been in clinical trials.[27−29]

The cyclic peptide inhibitor **9** that we obtained has satisfactory cell permeability, which is an additional advantage for its potential drug development. Cell permeability is probably attributed by the continuous basic residues in the sequence, as poly-Arg peptides have been found to be able to enter cell membranes autonomously.[21] Taken together with its specificity against DENV protease (Table 1) and the stability against trypsin and cathepsin S (Supporting Information, Figures S3 and S4), this cyclic peptide inhibitor can serve as a good lead for DENV drug development.

In conclusion, starting from identifying a conotoxin with inhibitory activity against DENV protease, we obtained a potent cyclic peptide inhibitor with an inhibitory constant of 2.2 μM. The high potency, stability, specificity, and cell permeability of this inhibitor make it an ideal lead for a DENV protease inhibition drug. This work confirms the substrate recognition sequence of DENV NS2B-NS3 protease and, more importantly, establishes the crucial effect of peptide cyclization for DENV NS2B-NS3 protease inhibitors, shedding new light on the design of peptide inhibitors against various viral proteases.

EXPERIMENTAL SECTION

Materials. Specimens of cone snails were collected from Sanya near the South China Sea, and the crude venom was extracted as previously described.[30] The serotype 2 DENV NS2B-NS3 protease was expressed with plasmid kindly provided by Dr. Siew Phengsuozeg Lim, Novartis Institute for Tropical Diseases, Singapore.[12] Bovine trypsin was from Sigma (St. Louis, MO). Human cathepsin S was purchased from Sino Biological Inc. (Beijing, China). Trifluoroacetic acid (TFA) and acetonitrile for HPLC were from Merck (Darmstadt, Germany). Other solvents and reagents were of analytical grade.

Purification and Characterization of Natural Conotoxin. The lyophilized crude venom of cone snails was dissolved in buffer A (0.1% TFA in water) and separated on an HPLC Zorbax 300SB-C18 semipreparative column (250 mm × 9.4 mm, Agilent Technologies) with acetonitrile gradient elution. All major elution peaks were manually collected and lyophilized. Further purification of the peptide was performed on a PepMap C18 analytical column (250 mm × 4.6 mm, 5 μm, 300 Å, PerSeptive Biosystems).

The purified active conotoxin fraction was dissolved in 100 mM Tris-HCl, pH 8.7, and 1 mM EDTA, then reduced with a 100-fold excess of dithiothreitol (DTT) at 42 °C for 0.5 h. Then, the reduced peptide was alkylated with a 250-fold excess of iodacetamide at room temperature. The N-terminal amino acid sequence of the S-alkylated peptide was analyzed with an Edman degradation sequencer (ABI 491A). The molecular masses of all native and synthetic peptides were analyzed by a Q-trap mass spectrometry (Applied Biosystems, Foster City, CA).

Peptide Synthesis and Refolding. All linear peptides were synthesized using the standard Fmoc chemistry by GL Biochem (Shanghai). For the refolding of full-length MrIA, a two-step selective oxidation was used.[31] The two nonprotected cysteines were oxidized by 1 mM oxidized glutathione (GSSG)/GSH to form the first disulfide bond in 0.1 M Tris-HCl, pH 8.7, and 2 mM EDTA at 4 °C for 2 h. The Acm-protecting groups of two other cysteine residues were removed by iodine, which accompanies the formation of the second disulfide bond. Other synthetic linear peptides with two cysteine residues were oxidized with GSSG/GSH at room temperature for 2 h. The folded peptides were purified on a Zorbax C-18 analytical column with gradient elution. Disulfide formation was confirmed with mass spectrometry.

Cyclic peptide **8** was synthesized by using standard Fmoc chemistry and cyclized in solution by GL Biochem (Shanghai). For cyclic peptide **9**, di-Fmoc-3,4-diaminobenzoic acid was first attached to rink-amide MBHA resin (Novabiochem), and then, the linear peptide was synthesized with Fmoc chemistry except that the last Cys residue was protected with Boc and Trt. The linear peptide, after being cleaved from resin, was cyclized in 100 mM ammonium acetate and 20 mM DTT.

Determination of Inhibitory Constant K_i. The inhibitory activities of the synthetic peptides against DENV2 NS2B-NS3 protease were measured at 37 °C in reaction buffer (50 mM Tris-HCl, pH 9.0, 10 mM NaCl, 20% glycerol, 1 mM CHAPS, and 0.04% NaN_3) using Benzoyl-Nle-Lys-Arg-Arg-AMC as a substrate.[12] The enzyme was first incubated with different amounts of inhibitor at 37 °C for 3 min to allow equilibrium to be reached between enzyme and inhibitor, and the substrate was then added. The residual enzyme activity was measured with a Hitachi F-2500 fluorescence spectrophotometer. The excitation and emission wavelengths were 356 and 438 nm,

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respectively. The inhibition constant (K_i) values were determined with Dixon plot ($1/v$ against concentration of inhibitor),[32] using two different concentrations of substrate (33 and 66 μM). Data were averaged from triplicate measurements.

The trypsin inhibitory activity was measured in 0.05 M Tris-HCl, pH 7.8, and 20 mM $CaCl_2$ containing 1 μg/mL trypsin, and 100 μM *N*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) was used as substrate. All assays were carried out at 25 °C. The residual trypsin activity was measured at 410 nm with a Hitachi U-2800 spectrophotometer. The value of K_i was measured with the Dixon plot method as described above. Substrate concentrations were 50 and 100 μM.

To measure their stability, the peptide inhibitors were first incubated with NS2B-NS3 protease at 37 °C for 0−2.5 h before substrate was added to measure the enzyme activity. The half maximal inhibitory concentration (IC_{50}) of each inhibitor was used in this stability measurement (MrIA-SL, 16.7 μM; inhibitor **6**, 6.7 μM; inhibitor **7**, 3.3 μM; inhibitor **8**, 16.7 μM; and inhibitor **9**, 6.7 μM).

The stabilities of peptides **9** and **10** against trypsin and cathepsin S were checked by analyzing the peptides on an HPLC C18 analytical column after protease digestion. Trypsin digestion was performed in 0.05 M Tris-HCl, pH 7.8, and 20 mM $CaCl_2$ at 37 °C, and cathepsin S digestion was in 50 mM NaAc, pH 4.5, 25 mM NaCl, and 5 mM DTT at room temperature.

Computational Docking. All computational work was done using Discovery Studio 3.0 package (Accelrys Inc.). The cyclic peptide **9** was built using the "Build and Edit Protein" module. The closed conformation of DENV2 NS2B-NS3 protease was modeled by MODELER using the crystal structure of DENV3 NS2B-NS3 protease complexed with Bz-nKRR-H (PDB ID: 3U1I)[14] as a template. The sequence identity between DENV2 and DENV3 NS2B-NS3 protease is 61.4%. The validity of the closed model of DENV2 NS2B-NS3 protease was examined by VERIFY-3D. Then, CDOCKER was used to perform the docking of the cyclic peptide **9** to the catalytic site of NS2B-NS3 protease. Forty random conformations of peptide **9** were generated using high temperature molecular dynamics, and the random conformations were docked and refined by grid-based simulated annealing to generate the candidate poses. The full potential minimization step was applied to those docking poses. These poses were ranked based on the total docking energy.

Fluorescence Labeling of Peptides. GSH, Tat-(48−60) peptide with a C-terminal Cys residue (GRKKRRQRRRPPQC)[21] and inhibitor **9** were dissolved in 50 mM Tris-HCl, pH 7.5 buffer, reacted for 10 min in the dark at 4 °C with equimolar TMR dissolved in dimethyl sulfoxide (DMSO). Fluorescence-labeled peptides were purified by HPLC (purity >98%) and then confirmed by mass spectrometry. These modified peptides were lyophilized in the dark, quantified by optical absorbance at 541 nm, and stored at −20 °C in the dark until further use.

Cell Permeability. The BHK-21 (C-13) and Vero cells were cultured in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin−streptomycin, and 2 mM glutamine (HyClone). After exponentially growing cells were treated with 0.25% trypsin and 0.03% EDTA solution (HyClone), 1×10^4 cells were seeded per well in a 24-well culture plate (Corning Inc.) and cultured overnight. Then, the cells were washed once with DPBS (pH 7.4) (HyClone) and preincubated in 0.5 mL of serum free medium (SFM) at 37 °C for 30 min. After that, the cell monolayers were incubated at 37 °C with peptide dissolved in SFM for 30 min. Cells were then washed three times with DPBS (pH 7.4) at room temperature and used for direct observation. The distribution of the fluorescence was analyzed with a 20× objective on a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with a Photometrics Cool-SNAP HQ charge-coupled device camera.

■ ASSOCIATED CONTENT

Supporting Information

K_i determination plots of MrIA and peptides **6**−**9**, the stability test of peptide **9** against trypsin and cathepsin S, and an alternative modeled conformation of peptide **9** bound to DENV2 NS2B-NS3 protease. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

TFA, trifluoroacetic acid; NS, nonstructural protein; Acm, acetamidomethyl; DENV, dengue virus; GSH, glutathione; GSSG, oxidized glutathione; BApNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; DTT, dithiothreitol; MS, mass spectrometry; HIV, human immunodeficiency virus; HCV, hepatitis C virus; NET, noradrenaline transporter; TMR, tetramethylrhodamine-5-maleimide; ADE, antibody-dependent enhancement effect

■ REFERENCES

(1) Guzman, M. G.; Halstead, S. B.; Artsob, H.; Buchy, P.; Farrar, J.; Gubler, D. J.; Hunsperger, E.; Kroeger, A.; Margolis, H. S.; Martinez, E.; Nathan, M. B.; Pelegrino, J. L.; Simmons, C.; Yoksan, S.; Peeling, R. W. Dengue: A continuing global threat. *Nat. Rev. Microbiol.* **2010**, *8*, S7−16.

(2) Rigau-Perez, J. G.; Clark, G. G.; Gubler, D. J.; Reiter, P.; Sanders, E. J.; Vorndam, A. V. Dengue and dengue haemorrhagic fever. *Lancet* **1998**, *352*, 971−977.

(3) Murgue, B. Severe dengue: Questioning the paradigm. *Microbes Infect.* **2010**, *12*, 113−118.

(4) Goncalvez, A. P.; Engle, R. E.; St Claire, M.; Purcell, R. H.; Lai, C. J. Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9422−9427.

(5) Noble, C. G.; Chen, Y. L.; Dong, H.; Gu, F.; Lim, S. P.; Schul, W.; Wang, Q. Y.; Shi, P. Y. Strategies for development of Dengue virus inhibitors. *Antiviral Res.* **2010**, *85*, 450−462.

(6) Chaturvedi, U. C.; Shrivastava, R.; Nagar, R. Dengue vaccines: Problems and prospects. *Indian J. Med. Res.* **2005**, *121*, 639−652.

(7) Melino, S.; Paci, M. Progress for dengue virus diseases. Towards the NS2B-NS3pro inhibition for a therapeutic-based approach. *FEBS J.* **2007**, *274*, 2986−3002.

(8) Lescar, J.; Luo, D.; Xu, T.; Sampath, A.; Lim, S. P.; Canard, B.; Vasudevan, S. G. Towards the design of antiviral inhibitors against flaviviruses: The case for the multifunctional NS3 protein from Dengue virus as a target. *Antiviral Res.* **2008**, *80*, 94−101.

(9) Steuber, H.; Hilgenfeld, R. Recent advances in targeting viral proteases for the discovery of novel antivirals. *Curr. Top. Med. Chem.* **2010**, *10*, 323−345.

(10) Falgout, B.; Miller, R. H.; Lai, C. J. Deletion analysis of dengue virus type 4 nonstructural protein NS2B: Identification of a domain required for NS2B-NS3 protease activity. *J. Virol.* **1993**, *67*, 2034−2042.

(11) Leung, D.; Schroder, K.; White, H.; Fang, N. X.; Stoermer, M. J.; Abbenante, G.; Martin, J. L.; Young, P. R.; Fairlie, D. P. Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. *J. Biol. Chem.* **2001**, *276*, 45762−45771.
(12) Li, J.; Lim, S. P.; Beer, D.; Patel, V.; Wen, D.; Tumanut, C.; Tully, D. C.; Williams, J. A.; Jiricek, J.; Priestle, J. P.; Harris, J. L.; Vasudevan, S. G. Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries. *J. Biol. Chem.* **2005**, *280*, 28766−28774.
(13) de la Cruz, L.; Nguyen, T. H.; Ozawa, K.; Shin, J.; Graham, B.; Huber, T.; Otting, G. Binding of low molecular weight inhibitors promotes large conformational changes in the dengue virus NS2B-NS3 protease: Fold analysis by pseudocontact shifts. *J. Am. Chem. Soc.* **2011**, *133*, 19205−19215.
(14) Noble, C. G.; Seh, C. C.; Chao, A. T.; Shi, P. Y. Ligand-bound structures of the dengue virus protease reveal the active conformation. *J. Virol.* **2012**, *86*, 438−446.
(15) Terlau, H.; Olivera, B. M. Conus venoms: A rich source of novel ion channel-targeted peptides. *Physiol. Rev.* **2004**, *84*, 41−68.
(16) Halai, R.; Craik, D. J. Conotoxins: Natural product drug leads. *Nat. Prod. Rep.* **2009**, *26*, 526−536.
(17) Sharpe, I. A.; Gehrmann, J.; Loughnan, M. L.; Thomas, L.; Adams, D. A.; Atkins, A.; Palant, E.; Craik, D. J.; Adams, D. J.; Alewood, P. F.; Lewis, R. J. Two new classes of conopeptides inhibit the alpha1-adrenoceptor and noradrenaline transporter. *Nat. Neurosci.* **2001**, *4*, 902−907.
(18) McIntosh, J. M.; Corpuz, G. O.; Layer, R. T.; Garrett, J. E.; Wagstaff, J. D.; Bulaj, G.; Vyazovkina, A.; Yoshikami, D.; Cruz, L. J.; Olivera, B. M. Isolation and characterization of a novel conus peptide with apparent antinociceptive activity. *J. Biol. Chem.* **2000**, *275*, 32391−32397.
(19) Nilsson, K. P.; Lovelace, E. S.; Caesar, C. E.; Tynngard, N.; Alewood, P. F.; Johansson, H. M.; Sharpe, I. A.; Lewis, R. J.; Daly, N. L.; Craik, D. J. Solution structure of chi-conopeptide MrIA, a modulator of the human norepinephrine transporter. *Biopolymers* **2005**, *80*, 815−823.
(20) Sharpe, I. A.; Palant, E.; Schroeder, C. I.; Kaye, D. M.; Adams, D. J.; Alewood, P. F.; Lewis, R. J. Inhibition of the norepinephrine transporter by the venom peptide chi-MrIA. Site of action, Na+ dependence, and structure-activity relationship. *J. Biol. Chem.* **2003**, *278*, 40317−40323.
(21) Vives, E.; Brodin, P.; Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **1997**, *272*, 16010−16017.
(22) Yin, Z.; Patel, S. J.; Wang, W. L.; Wang, G.; Chan, W. L.; Rao, K. R.; Alam, J.; Jeyaraj, D. A.; Ngew, X.; Patel, V.; Beer, D.; Lim, S. P.; Vasudevan, S. G.; Keller, T. H. Peptide inhibitors of Dengue virus NS3 protease. Part 1: Warhead. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 36−39.
(23) Schuller, A.; Yin, Z.; Brian Chia, C. S.; Doan, D. N.; Kim, H. K.; Shang, L.; Loh, T. P.; Hill, J.; Vasudevan, S. G. Tripeptide inhibitors of dengue and West Nile virus NS2B-NS3 protease. *Antiviral Res.* **2011**, *92*, 96−101.
(24) Tambunan, U. S.; Alamudi, S. Designing cyclic peptide inhibitor of dengue virus NS3-NS2B protease by using molecular docking approach. *Bioinformation* **2010**, *5*, 250−254.
(25) Otlewski, J.; Jelen, F.; Zakrzewska, M.; Oleksy, A. The many faces of protease-protein inhibitor interaction. *EMBO J.* **2005**, *24*, 1303−1310.
(26) Tyndall, J. D.; Fairlie, D. P. Macrocycles mimic the extended peptide conformation recognized by aspartic, serine, cysteine and metallo proteases. *Curr. Med. Chem.* **2001**, *8*, 893−907.
(27) Faucher, A. M.; Bailey, M. D.; Beaulieu, P. L.; Brochu, C.; Duceppe, J. S.; Ferland, J. M.; Ghiro, E.; Gorys, V.; Halmos, T.; Kawai, S. H.; Poirier, M.; Simoneau, B.; Tsantrizos, Y. S.; Llinas-Brunet, M. Synthesis of BILN 2061, an HCV NS3 protease inhibitor with proven antiviral effect in humans. *Org. Lett.* **2004**, *6*, 2901−2904.
(28) Liverton, N. J.; Carroll, S. S.; Dimuzio, J.; Fandozzi, C.; Graham, D. J.; Hazuda, D.; Holloway, M. K.; Ludmerer, S. W.; McCauley, J. A.; McIntyre, C. J.; Olsen, D. B.; Rudd, M. T.; Stahlhut, M.; Vacca, J. P. MK-7009, a potent and selective inhibitor of hepatitis C virus NS3/4A protease. *Antimicrob. Agents Chemother.* **2010**, *54*, 305−311.
(29) Harper, S.; McCauley, J. A.; Rudd, M. T.; Ferrara, M.; DiFilippo, M.; Crescenzi, B.; Koch, U.; Petrocchi, A.; Holloway, M. K.; Butcher, J. W.; Romano, J. J.; Bush, K. J.; Gilbert, K. F.; McIntyre, C. J.; Nguyen, K. T.; Nizi, E.; Carroll, S. S.; Ludmerer, S. W.; Burlein, C.; DiMuzio, J. M.; Graham, D. J.; McHale, C. M.; Stahlhut, M. W.; Olsen, D. B.; Monteagudo, E.; Cianetti, S.; Giuliano, C.; Pucci, V.; Trainor, N.; Fandozzi, C. M.; Rowley, M.; Coleman, P. J.; Vacca, J. P.; Summa, V.; Liverton, N. J. Discovery of MK-5172, a Macrocyclic Hepatitis C Virus NS3/4a Protease Inhibitor. *ACS Med. Chem. Lett.* **2012**, *3*, 332−336.
(30) Zhang, L.; Shao, X.; Chi, C.; Wang, C. Two short D-Phe-containing cysteine-free conopeptides from *Conus marmoreus*. *Peptides* **2010**, *31*, 177−179.
(31) Ye, M.; Hong, J.; Zhou, M.; Huang, L.; Shao, X.; Yang, Y.; Sigworth, F. J.; Chi, C.; Lin, D.; Wang, C. A novel conotoxin, qc16a, with a unique cysteine framework and folding. *Peptides* **2011**, *32*, 1159−1165.
(32) Dixon, M. The determination of enzyme inhibitor constants. *Biochem. J.* **1953**, *55*, 170−171.