Synthesis and in vitro Evaluation of West Nile Virus Protease Inhibitors Based on the 2-{6-[2-(5-Phenyl-4H-[1,2,4]triazol-3-ylsulfanyl)acetylamino]benzothiazol-2-ylsulfanylandacetamide Scaffold

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In recent years, clinical symptoms resulting from West Nile virus (WNV) infection have worsened in severity, with an increased frequency in neuroinvasive diseases among the elderly. As there are presently no successful therapies against WNV for use in humans, continual efforts to develop new chemotherapeutics against this virus are highly desired. The viral NS2B-NS3 protease is a promising target for viral inhibition due to its importance in viral replication and its unique substrate preference. In this study, a WNV NS2B-NS3 protease inhibitor with the compound interferes with productive interactions of the NS2B cofactor with the NS3 protease and is an allosteric inhibitor of the WNV NS3 protease.

Introduction

The West Nile virus (WNV), a neurotropic Flavivirus, is a mosquito-borne, reemerging pathogen causing outbreaks on multiple continents.[1] The clinical manifestations of WNV-infected humans vary significantly, from asymptomatic to the development of severe neuroinvasive diseases such as meningitis, flaccid paralysis, and encephalitis.[2] Close follow-up with WNV-infected humans have shown that patients who manifest symptomatic infections often suffer lifetime consequences, such as long-term cognitive and neurological impairment.[3] Despite the growing public health concern associated with WNV infection, to date there are no effective and safe antiviral therapies or vaccines available for human use against WNV.

WNV is a small enveloped virus containing a 10.8 kb, single-stranded, positive sense RNA genome approximately 11 000 nucleotides in length.[4] The RNA genome encodes three structural proteins (C, capsid; M, membrane; E, envelope) which form the virion and seven non-structural proteins (NS1, NS2A, NS3, NS4A, NS4B, and NS5) which are required for viral replication.[5] The full-length NS3 protein represents a multifunctional protein in which the N-terminal portion encodes a serine protease and the C-terminal portion encodes an RNA helicase. Interaction between the NS2B cofactor and the NS3 protease domain was found to be essential for the NS3 protease to exhibit its catalytic activity. The WNV NS2B-NS3 protease preferentially cleaves peptide substrates at the C-terminus, which has a pair of basic amino acids (Arg and Lys) at the P1 and P2 positions.[6] Site-directed mutagenesis studies have shown that mutation of essential catalytic residues on the NS3 protease cleavage sites lead to a halt in viral infectivity. This dibasic P2/P1 requirement for substrate recognition indicates that the NS2B-NS3 protease could be an attractive therapeutic target for anti-WNV drug design.[7] However, the design of WNV NS2B-NS3 protease inhibitors has met with only modest success thus far. Earlier studies identified various peptidic compounds containing highly charged moieties which demonstrate good inhibitory activity against the WNV NS2B-NS3 protease in vitro.[8] However, their short physiological half-lives and high clearance rates in vivo curtail their potential for drug development. For non-peptidic inhibitors, a number of sulfonyl pyrazolyl derivatives are the most potent compounds known to date (IC_{50} = 0.37 m).[9] However, these allosteric inhibitors were unstable under physiological conditions and degraded within an hour in aqueous buffer. Attempts to improve their stability without compromising biological activities proved to be unsatisfactory.[10] Other non-peptidic inhibitors with moderate to good activities include compounds with isothiourea (IC_{50} = 18 m),[11] guanidino (K = 13 ± 1 m),[12] 8-hydroxyquinoline (K = 3.2 ± 0.3 m),[13] or isoquinoline (IC_{50} = 30 m) derivatives and 9,10-dihydro-3H,4H-1,3,9,10a-tetraaza-phenanthren-4-one (K = 4.47 ± 0.37 m) derivatives. As part of our ongoing study on the inhibition of WNV NS2B-NS3 protease, we describe herein the identification of a new scaffold with promising inhibitory activity against WNV NS2B-NS3 protease, synthesis and biological evaluation of 39 analogues of the lead compound, and the
structure–activity relationship (SAR) and cytotoxicity properties of the most potent inhibitors.

Results and Discussion

Hit identification and analogue synthesis

In the preliminary WNV NS2B-NS3 protease inhibition assay, a collection of 110 compounds from our in-house compound library with a history of antimicrobial, antiviral, and anticancer activities were screened at 100 \( \mu \text{M} \) to identify potential inhibitors. Eight compounds were found to exhibit detectable inhibition ability toward WNV NS2B-NS3 protease. These compounds were further refined by cellular cytotoxicity assays. This resulted in the identification of compound 1a as a lead compound. To optimize the potency of this compound, a library of structurally diverse analogues of compound 1a was synthesized and evaluated for inhibitory activity.

To the best of our knowledge, the synthesis of compound 1a has not been reported previously. To develop a synthetic strategy for expedient preparation of compound 1a, we carried out a retrosynthetic analysis of the compound (Figure 1). Disconnection of compound 1a at the amide and C=S bonds resulted in four fragments: triazole 2a, amino benzothiazole (or benzimidazole)-2-thione 3, amine 4, and bromoacetyl bromide.

Compound 2a was prepared from the respective benzoic acid 5 (Scheme 1A) by first treating it with either methanol and concentrated \( \text{H}_2\text{SO}_4 \) under microwave irradiation for 15 min, or oxalyl chloride and DMF at room temperature for 2.5 h \(^{(17)}\) to provide the respective methyl ester 6a or acyl chloride 6b in good yields. Li and co-workers \(^{(18)}\) have previously reported that reacting compound 6a \((R^1 = \text{H})\) with hydrazine hydrate in ethanol at reflux overnight gave benzohydrazide 7 in 83% yield. To shorten the reaction time, we explored combining compound 6a with neat hydrazine hydrate at reflux. The reaction was complete within 20 min, and benzohydrazide 7 precipitated from the cold reaction mixture in good yield. Conversion of 7 to compound 8 was adapted from an earlier reported procedure in which the reaction was performed on silica gel under microwave irradiation. \(^{(19)}\) However, we found that treating compound 7 with alkylisothiocyanate under microwave irradiation at 130°C in the absence of silica gel gave compound 8 in higher yields (76–86%). It is worth noting that when \( R^1 \) was not an \( \text{NH}_2 \) moiety, compound 8 could be obtained directly from acyl chloride 6b by reacting it with thiosemicarbazide at room temperature. \(^{(20)}\) With compound 8 in our hands, we proceeded with the cyclization reaction in sodium hydroxide solution under microwave irradiation at 150°C. The reaction was completed in 3 min (versus 1 h under conventional reflux) to provide 1,2,4-triazole-5(4H)-thione 2a in good yield. We also synthesized compound 2a12 from 2a6 by reducing the nitro group with iron and concentrated hydrochloric acid (Scheme 1B).

Compound 3 was prepared by first treating 2-aminothiophenol or o-phenylenediamine 9 with \( \text{CS}_2 \) in the presence of KOH under microwave irradiation to provide intermediate 10 (Scheme 2). Nitration of compound 3, followed by reduction of the nitro group with iron powder and concentrated hydrochloric acid, afforded compound 3 in moderately good yield.

Compounds 2, 3, and various commercially available amines or anilines 4 were coupled as shown in Scheme 3. Compound 4 was treated with bromoacetyl bromide to yield compound 12, which was then reacted with the thiolate anion, generated

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**Figure 1. Retrosynthesis of compound 1a.**

**Scheme 1. Synthesis of compounds A) 2a and B) 2a12. Reagents and conditions: a) MeOH, conc. H\(_2\)SO\(_4\) (cat), 130°C, MW, 15 min; b) (COCl\(_2\)), DMF (cat), CH\(_2\)Cl\(_2\), RT, 2.5 h; c) thiosemicarbazide, THF, 0°C — RT, 1.5 h; d) NH\(_2\)NH\(_2\)·H\(_2\)O, reflux, 20 min; e) R\(_2\)NCS, THF, 130°C, MW, 8 min; f) NaOH (2\( \mu \)m), 150°C, MW, 3 min; g) Fe, conc. HCl, EtOH (aq, 80%), reflux, 45 min.**
in situ from the reaction of compound 3 with potassium hydroxide, to provide compound 13. Subsequent coupling of the amino group on 13 with another molecule of bromoacetyl bromide gave intermediate 14, which precipitated readily from the reaction mixture. Finally, regioselective coupling of compound 2a with compound 14 under basic conditions gave compound 1a in moderately good yield.

Structure–activity relationship (SAR) studies

A representative set of 39 analogues of compound 1a was synthesized and tested in a WNV assay at 50 μM concentration to identify potential inhibitors. Inhibitors exhibiting more than 50% inhibition were further evaluated for their effects at different concentrations, and the IC50 value of each compound was calculated using GraphPad Prism software (Table 1). The fluorogenic peptide Pyr-RTKR-AMC was used as a substrate for the WNV NS2B-NS3 protease, and the kH value was determined to be 3.5 ± 0.4 μM using the protocol from the SensoLyte 440 West Nile Virus protease assay kit.

In our preliminary SAR studies, we examined the effects of substituents on the inhibitory activity of the compound by varying the X, R1, R2, and R5 groups of compound 1a (Table 1, 1a2–1a10). However, these changes did not provide any significant improvement in activity. Hence, we proceeded to screen the unsubstituted compound for inhibitory activity by changing both R1 and R2 on compound 1a1 and 1a2, and the IC50 value of each compound was calculated. Further modifications by varying the X and R5 groups on compound 1a1 resulted in a loss of inhibitory activity. Next, we proceeded to explore the effect of the methylene linker. Analogues of 1a11 were synthesized with various ani-

### Table 1. IC50 values of compound 1a[^a]

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[^a] Concentration required to inhibit WNV NS2B-NS3 protease activity by 50%; data represent the mean ±SE of triplicate experiments. [^b] NA: no activity. [^c] Phenyl group containing R1 was replaced by a cyclohexyl ring. [^d] Phenyl group containing R2 was replaced by an n-propyl group. [^e] Phenyl group containing R5 was replaced by a pyridine-4-yl ring. [^f] Phenyl group containing R4 and R5 was replaced by a cyclohexyl ring.
line derivatives (4; Table 1, 1a19–1a29), and screening results showed that the presence of an R2 alkyl substituent (Table 1, 1a23–1a25) significantly enhanced a compound’s inhibitory activity. The best inhibition was observed for compound 1a24, which contains an ethyl group at the para position (R2) of the phenyl ring. Further derivatizing compound 1a24 by varying the substituents at X, R1, and R2 (Table 1, 1a30–1a39) did not result in further improvement of inhibitory activity, confirming the importance of the 5-phenyl-1,2,4-triazole and benzothiazole fragments for the compound’s inhibitory activity. In addition, replacing the phenyl ring with a cyclohexyl moiety (Table 1, 1a40) provided a compound with modest inhibition against WNV NS2B-NS3 protease.

The stabilities of compounds 1a22, 1a23, 1a24, 1a25, and 1a37 in the assay buffer (pH 8) were determined by measuring the amount of compound remaining over time using analytical HPLC and the Shimadzu LC Solution software. Compounds 1a22, 1a23, 1a24, 1a25, and 1a37 showed excellent stability in pH 8 buffer at 37 °C.

Next, we evaluated the cytotoxicities of compounds 1a22, 1a23, 1a24, 1a25, and 1a37 against baby hamster kidney fibroblast (BHK21) cells after various incubation times. Test compounds were dissolved in dimethyl sulfoxide (DMSO), which was also included alone as a control. The amount of DMSO used in the assay was controlled at 0.2% of the total volume to ensure that DMSO did not result in cytotoxicity. The results showed (Figure 2) that the three strongest candidates identified from our earlier SAR studies, compounds 1a24, 1a25, and 1a37, were also less cytotoxic to BHK21 cells than compounds 1a22 and 1a23, with compound 1a24 having the lowest toxicity of the three compounds. The inhibition mode of 1a24, the most potent compound, was determined from a Dixon plot (Figure 3) and revealed an uncompetitive inhibition mode with a Ki value of 2.8 ± 0.1 μM (where Ki = IC50/(1 + K0/S)).

WNV NS2B-NS3 protease and the dengue virus DENV2 NS2B-NS3 protease are different serocomplex subgroups with ~70% amino acid sequence identity. Therefore, to determine selectivity, compound 1a24 was also analyzed for in vitro inhibitory activity using recombinant DENV2 NS2B-NS3 protease. Compound 1a24 showed only slight inhibition of DENV2 protease activity (IC50 = 42.9 ± 1.9 μM), indicating that this compound is ~13-fold more potent against WNV NS2B-NS3 protease than DENV2 NS2B-NS3 protease.

Docking analysis

A docking study was performed to rationalize the inhibition and kinetics result as well as to identify the possible binding sites for compounds on the WNV NS2B-NS3 protease. The crystal structure of the WNV NS2B-NS3 protease in complex with peptide Bz-Nle-Lys-Arg-Arg-H (PDB: 2FP7) has been previously reported. To identify the potential binding site of compound 1a24 on the NS2B-NS3 protease, in silico docking studies were performed with Molecular Operating Environment (MOE) using these published crystal structure coordinates. As compound 1a24 is an uncompetitive inhibitor of the WNV NS2B-NS3 protease, we focused our analysis on regions beyond the substrate binding sites. Earlier studies showed that integration of residues 78–87 of NS2B into the protease–cofactor complex affected the formation of the catalytic active sites. Deletion or mutagenesis of these residues produced an inert enzyme. Therefore, the region on the NS3 protease where key interactions with the NS2B cofactor (residue 78–87) occur is important for NS2B-NS3 protease activity, and particular attention was paid to this region during our docking studies.

The best docked pose for compound 1a24 and the WNV NS2B-NS3 protease is shown in Figure 4. The docking analysis predicted a strong π-cation-type interaction between the guanidine group of Arg78 of the NS2B cofactor and the phenyl ring directly attached to the triazole. Arg78 was also observed...
to form a hydrogen bond with the triazole nitrogen. As the interaction of Arg78 with a ligand interferes with the productive interaction of the NS2B cofactor with the catalytic active site in the NS3 protease, these bonding interactions between compound 1a24 and Arg78 are important factors contributing to the compound’s inhibitory potency. At the same time, the triazole nitrogen atom and the oxygen atom on the amide group directly linked to the benzothiazole of 1a24 were observed to interact with Lys73 of NS3 via hydrogen bonding. These results support our experimental inhibition assay data that show compound 1a24 as an allosteric inhibitor of the WNV NS2B-NS3 protease.

Conclusions

In this study, we have identified a lead compound (1a1) with moderate inhibitory activity against the WNV NS2B-NS3 protease. Refinement of this initial hit by synthesizing a focused library of 1a1 derivatives for SAR studies provided compound 1a24, which inhibited the interaction of the NS2B cofactor and the NS3 protease at low micromolar concentration (IC50 = 3.4 ± 0.2 μM) and in an uncompetitive manner. Compound 1a24 is stable under physiological conditions and is non-cytotoxic to BHK21 cells. Compared with the other physiologically stable, non-peptidic inhibitors which were reported earlier, compound 1a24 is also a stronger inhibitor of the WNV NS2B-NS3 protease. Further studies are presently ongoing to develop compound 1a24 as a potential antiviral therapeutic.

Experimental Section

General: All chemical reagents and anhydrous solvents were obtained from Sigma–Aldrich, Merck, Lancaster, or Fluka and were used without further purification. Flash chromatography was performed with silica (Merck, 70–230 mesh), while TLC was carried out on pre-coated plates (Merck silica gel 60, F254) and visualized with UV light. Final compounds were purified on a Gilson preparative HPLC system with a Waters C18 column (250 × 10.0 mm, 5 μm).

All experiments were performed using Shimadzu LC Solution software. 1H and 13C NMR spectra were recorded using CDCl3, [D6]DMSO as solvents and tetramethylsilane (TMS) as an internal reference on a Bruker AMX500 or a Bruker ACFC300 Fourier transform spectrometer. The following abbreviations were used to explain multiplicities: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), q (quartet), and m (multiplet). The number of protons (n) for a given resonance is indicated as nh. Mass spectrometry was performed on a Finnigan/MAT LCQ mass spectrometer using electron spray ionization (ESI).

General procedure for the synthesis of 6a (L = OMe): Concentrated H2SO4 (0.39 mmol) was added to a solution of the corresponding benzoic acid 5 (3.9 mmol) in MeOH (15 mL). The reaction mixture was microwave irradiated at 130 °C (with the heating program starting at 150 W) for 15 min. Thereafter, the reaction mixture was concentrated, and saturated NaHCO3 was added slowly at room temperature until no more bubbling occurred. The reaction mixture was then extracted with EtOAc (20 × 3 mL), and the combined organic layer was dried over Na2SO4, concentrated, and purified via short column chromatography using EtOAc/hexane (2:3) as the eluent. The resulting product was dried overnight in a vacuum oven at 50 °C to provide compound 6a in 86–95% yield.

General procedure for the synthesis of 6b (L = Cl): Oxalyl chloride (27.5 mmol) and DMF (2 drops) were added to a solution of the corresponding benzoic acid 5 (9.0 mmol) in CH2Cl2 (30 mL) at 0 °C. The mixture was allowed to stir at room temperature for 2.5 h, then the reaction mixture was concentrated, and the crude product was used directly in the next reaction.

General procedure for the synthesis of 7: Hydrazine hydrate (5 mL, 100 mmol) was added to the corresponding methyl benzoate 6a (3 mmol). The reaction mixture was stirred and heated at reflux for 20 min. Thereafter, the reaction mixture was cooled to room temperature and then to −15 °C for 10 min. The resulting precipitate was filtered and washed with ice-cold water (3 mL), then the crude product was dried overnight in a vacuum oven at 50 °C and used in the next reaction. The crude yield of 7 was 82–90%.

General procedure for the synthesis of 8 (R= Me or Et): The corresponding benzohydrazide 7 (3.6 mmol) was added to a solution of alkyl isothiocyanate (3.96 mmol) in THF (12 mL). The mixture...
was irradiated under microwave at 130 °C (with the heating pro-
gram starting at 150 W) for 8 min. Thereafter, the reaction mixture 
was concentrated and extracted with EtOAc/H2O. The combined 
organic layer was dried over Na2SO4, concentrated, and purified by 
column chromatography using EtOAc/hexane (starting from 3:7 to 
3:2) as the eluent. The resulting precipitate was filtered and dissolved in a vacuum oven at 50 °C to provide compound 8 in 76–86 % yield.

**General procedure for the synthesis of 8** (R² = H): Commercially available acid chloride (9.0 mmol) or crude product 6b (L = Cl, 9.0 mmol) was added slowly to a mixture of thiosemicarbazide (19.6 mmol) in THF (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. The reaction mixture was then con-
centrated, and the residue was triturated with water. The product that precipitated from the reaction mixture was filtered, washed with water, and dried overnight under vacuum at 50 °C to yield product 8 in 60–87 % yield. This product was used directly in the next reaction.

**General procedure for the synthesis of 2a:** NaOH (2 m, 15 mL) was added to compound 8 (3.4 mmol), and the reaction mixture was irradiated under microwave at 150 °C (with the heating pro-
gram starting at 150 W) for 3 min. Thereafter, the solution was neutral-
ized with acetic acid and extracted with EtOAc (3 × 15 mL). The combined organic layers were dried over Na2SO4, concentrated, and purified by flash column chromatography using EtOAc/hexane (from 1:5 to 1:2) as the eluent. The product was dried overnight under vacuum at 50 °C to provide compound 2a in 71–94 % yield.

**Synthesis of 3-(4-aminophenyl)-1H-1,2,4-triazole-5(4H)-thione (2a12):** Concentrated HCl (1 mmol) was added to a mixture of iron powder (10 mmol) in 80 % aqueous EtOH (5 mL), and the reaction mixture was stirred in a warm (40 °C) water bath. Thereafter, 3-(4-nitrophenyl)-1H-1,2,4-triazole-5(4H)-thione 2a6 (R² = NO2, 2 mmol) was added portionwise, and the resulting reaction mixture was stirred and heated at reflux for 45 min. The EtOH was then re-
moved by rotary evaporator, and the residual reaction mixture was adjusted to pH 13 with NaOH. The reaction mixture was then fil-
tered by suction filtration, and the filtrate was adjusted to pH 5 with glacial acetic acid. The resulting precipitate was filtered and purified by flash column chromatography using EtOAc/hexane (from 1:1 to 2:1) as eluent. The resulting product was dried over-
night under vacuum at 50 °C to yield compound 2a12 in 61% yield.

**General procedure for the synthesis of 10:** Carbon disulfide (5.5 mmol) was added to a mixture of KOH (5.5 mmol) in 80 % aqueous EtOH (7.5 mL), and the reaction mixture was stirred at room temperature for 15 min. Corresponding aniline 9 (5 mmol) was added, and the reaction mixture was irradiated under micro-
wave at 130 °C (with the heating program starting at 150 W) for 15 min and then cooled to room temperature. The reaction mix-
ture was then diluted with water (10 mL), adjusted to pH 5 with glacial acetic acid, and cooled to 0 °C for 1 h to complete the crys-
tallization. The shiny precipitate that formed was filtered and dried overnight under vacuum at 50 °C to yield compound 10 in 85–94 % yield.

**General procedure for the synthesis of 11:** The corresponding azole derivative 10 (3.0 mmol) was slowly dissolved in concentrat-
ed H2SO4 (1.4 mL, 25.7 mmol), cooling from 0 °C to −5 °C. A mix-
ture of fuming HNO3 (0.4 mL, 9.6 mmol) and concentrated H2SO4 
(0.3 mL, 5.6 mmol) was added dropwise, and the reaction mixture 
was stirred for 1 h at 0 °C to −5 °C before pouring into ice. The re-
sulting precipitate was filtered and dissolved in a minimum volume of 25 % aqueous ammonia solution. The resulting solution was heated at 50 °C and filtered under suction. The filtrate was cooled to 0 °C and adjusted to pH 2 with 6 M HCl. The precipitate that formed was filtered and purified by flash column chromatog-
raphy using EtOAc/hexane (from 1:2 to 1:1) as eluent. The product was dried overnight under vacuum at 50 °C to obtain compound 11 in 48–65 % yield.

**General procedure for the synthesis of 3:** The synthesis for com-
 pound 3 is similar to that used for the synthesis of 2a12. However, the resulting precipitate was purified by flash column chromatog-
raphy using EtOAc/hexane (from 2:3 to 2:1) as eluent. The product was dried overnight under vacuum at 50 °C to obtain compound 3 in 67–74 % yield.

**General procedure for the synthesis of 12:** The corresponding amine or aniline 4 (4.4 mmol) was added dropwise to a mixture of bromoacetyl bromide (5.3 mmol) in CH3CN (50 mL) at 0 °C. After addition, the mixture was stirred at room temperature for 1 h. CH3CN was removed by rotary evaporation, and the residue was extracted with an EtOAc/H2O solvent system. The combined organic layer was concentrated and purified by flash column chromatog-
raphy using EtOAc/hexane (from 1:10 to 3:10) as eluent. The product was dried overnight under vacuum at 50 °C to obtain compound 12 in 68–87 % yield.

**General procedure for the synthesis of 13:** Compound 3 (0.3 mmol) was dissolved in a mixture of KOH (0.6 mmol) in 60 % aqueous EtOH (20 mL). The corresponding intermediate 12 (0.3 mmol) was added, and the reaction mixture was stirred for 1 h at room temperature. After completion of the reaction (monitored by TLC), EtOH was removed by rotary evaporation, and the residual mixture was neutralized with dilute acetic acid followed by extrac-
tion with EtOAc (3 × 15 mL). The combined organic layers were concentrated and purified using a short silica column with EtOAc/ hexane (from 3:10 to 1:4) as eluent. The resulting semi-pure prod-
uct was dried under high vacuum at room temperature and used for the next step of the reaction.

**General procedure for the synthesis of 14:** Bromoacetetyl bromide (5.3 mmol) was added dropwise to the corresponding intermediate 13 (4.4 mmol) in CH3CN (30 mL) at 0 °C. After addition, the reaction mixture was stirred at room temperature for 1 h. The product that precipitated from the reaction mixture was filtered, washed with CH3CN (3 × 15 mL), and dried overnight under vacuum at 50 °C to provide compound 14 in 73–86 % yield.

**General procedure for the synthesis of 1a:** The corresponding tri-
zole compound 2a (0.22 mmol) was dissolved in a mixture of 
KOH (0.66 mmol) in 60 % aqueous EtOH (16 mL). The correspond-
ing intermediate 14 (0.22 mmol) was added, and the reaction mix-
ture was stirred for 2 h at room temperature. The EtOH was then re-
moved by rotary evaporation, and the residual mixture was neutral-
ized with dilute acetic acid, followed by extraction with EtOAc/ H2O. The combined organic layer was concentrated and purified by reverse phase HPLC using solvent A (0.1 % TFA in water) and solvent B (0.1 % TFA in CH3CN) as the mobile phase. The mobile phase was a gradient, with solvent B increasing from 15 % to 95 % over 30 min at a flow rate of 8.0 mL min⁻¹. Detection was conduct-
ed at 254 nm. Fractions containing the pure product were com-
bined, concentrated, and dried overnight under vacuum at 50 °C to give compound 1a in 56–83 % yield.

**Enzyme inhibition evaluation**

All of the compounds used in biological testing were at least 95 % pure. Compounds were tested against the WNV NS2B-NS3 pro-

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**ChemMedChem 2013, 8, 994 – 1001**
substrate. According to the kit protocol, the protease assay kit uses the fluorogenic peptide Pyr-RTKR-AMC as substrate. Anaspec (catalogue numbers 72079 and 72081, respectively). This kit was used to measure the activity of 16.7 nM WNV protease using the SensoLite 440 WNV protease assay kit and the CHEM MED protease inhibition assay. The enzyme kinetics were initiated by adding substrate Pyr-RTKR-AMC to obtain a final concentration of 50 μM to filter out potential inhibitors. Compounds that showed more than 50% inhibition were further investigated to determine their IC_{50} values.

**Docking**

All docking studies were performed using the Molecular Operating Environment (MOE 2009.10) software system designed by the Chemical Computing Group. The program operated under the Windows Vista operating system installed on an Intel Core2 Duo PC with a 2.4 MHz processor and 4.0 GB RAM. The docked compound was built using the MOE builder and the energy was minimized using molecular mechanics (MM) with the MMFF94x force field. The active site of the WNV protease was prepared using the crystal structure of the WNV NS2B-NS3 protease in complex with peptide Bz-Nle-Lys-Arg-Arg-H (PDB: 2FP7). The structure was stripped of all water molecules and bound ligand, followed by the addition of valence hydrogen atoms to the protein. By keeping in mind the amino acid residues in NS2B which are responsible for the activation of the catalytic triad, the active site in NS2B-NS3 was identified using the Alpha Site Finder tool. The Triangle Matcher placement method was used to generate docking poses, and each conformation was scored based on the London ΔG scoring function that estimates the free energy of binding (kcal mol^{-1}). Refinement and the geometry of resulting complex was evaluated using the Discovery Studio 3.1 client.

**Supporting Information**

Structures of derivatives used in the synthesis of 1a; Inhibition curves used to determine IC_{50} values for compounds 1a23, 1a24, 1a25, and 1a37. H and 13C NMR data for all compounds synthesized; and 1H and 13C NMR spectra for compounds 1a23, 1a24, 1a25, and 1a37 can be found in the Supporting Information.

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