Bioorganic & Medicinal Chemistry Letters 19 (2009) 5773–5777

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Structure–activity relationship and improved hydrolytic stability of pyrazole derivatives that are allosteric inhibitors of West Nile Virus NS2B-NS3 proteinase

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ARTICLE INFO

Article history:
Received 26 March 2009
Revised 28 July 2009
Accepted 30 July 2009
Available online 3 August 2009

Keywords:
West Nile virus
Flavivirus
Proteinase inhibitors
NS2B-NS3 proteinase
Antiviral

ABSTRACT

West Nile Virus (WNV) is a potentially deadly mosquito-borne flavivirus which has spread rapidly throughout the world. Currently there is no effective vaccine against flaviviral infections. We previously reported the identification of pyrazole ester derivatives as allosteric inhibitors of WNV NS2B-NS3 proteinase. These compounds degrade rapidly in pH 8 buffer with a half life of 1–2 h. We now report the design, synthesis and in vitro evaluation of pyrazole derivatives that are inhibitors of WNV NS2B-NS3 proteinase with greatly improved stability in the assay medium.

West Nile virus (WNV) is a mosquito-borne pathogen of the genus, *Flavivirus*.[1] This genus also contains many other human pathogens, such as Dengue virus (Den), Japanese encephalitis virus (JE), and yellow fever virus (YF). First isolated in 1937 from Uganda, WNV has since been widely distributed around the world.[2] Infections in humans are usually asymptomatic or cause a mild flu-like illness for a few days called West Nile fever. However, recent infections of WNV have been associated with much higher fatality rates particularly among the senior population.[3] Since the identification of WNV in New York in 1999 the virus has distributed itself widely throughout North America, infecting over 28,000 people (see the Center for Disease Control and Prevention site on the World Wide Web at www.cdc.gov/ncidod/dvbid/westnile/index.htm). Currently there is no effective vaccine against flaviviral infections.[2] WNV has a single-stranded RNA genome, which encodes a single polyprotein. This consists of three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. Post translational processing of the polyprotein precursor is required to produce functional viral proteins.[4] In particular, the WNV NS2B-NS3 proteinase holds promise as a potential target for therapeutic intervention with small molecule drugs.[5–9] Thus the identification of potent small molecule inhibitors of WNV would be a highly useful starting point for drug discovery and development.

We previously reported the discovery of pyrazole derivatives as allosteric inhibitors of WNV NS2B-NS3 proteinase identified by high-throughput screening (HTS) of a small molecule library through the NIH Molecular Libraries Initiative (MLI).[10] The structures and data for compounds were deposited to the PubChem database under AID 577 (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=577&loc=ea_ras) and AID 653 (http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=653&loc=ea_ras). The hit compounds exhibited potencies ranging from 0.105 to 1.353 μM (Figure 1). However, these pyrazolyl benzoic acid ester derivatives

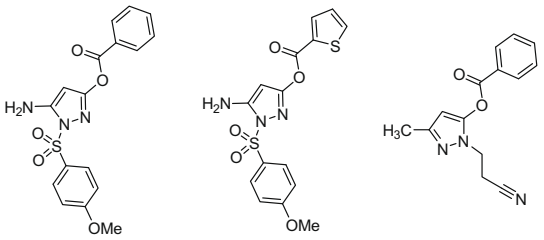

Figure 1. WNV NS2B-NS3 proteinase inhibitor hits from screening.

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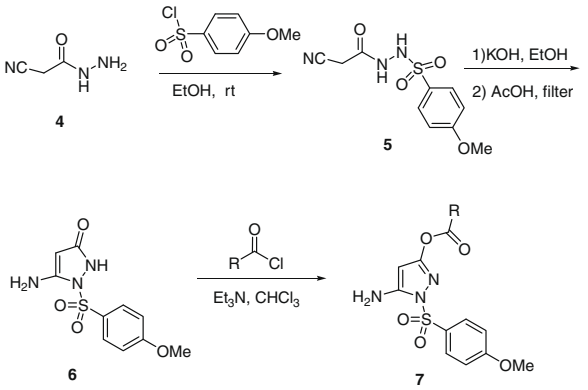
doi:10.1016/j.bmcl.2009.07.150

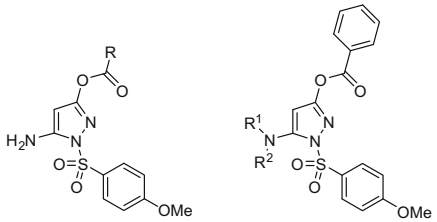


Scheme 1. Synthetic route to prepare pyrazole esters.

(**1–3**) were rapidly hydrolyzed in an aqueous buffer (pH 8) to the corresponding pyrazol-3-ol in approximately 1–2 h. Clearly these compounds, while potent, were of limited use as tools to investigate the biochemistry and enzymatic kinetics of WNV NS2B-NS3 proteinase in vitro because of their instability. We hypothesized that it might be possible to design and synthesize analogues of the hits which retain potency as inhibitors of WNV NS2B-NS3 proteinase and, in addition, exhibit improved hydrolytic stability in the assay medium. Herein, we report the synthesis and structure–activity relationship (SAR) of pyrazole WNV NS2B-NS3 proteinase inhibitors with substantially increased stability towards hydrolysis in an aqueous medium.

The initial phase of studies to address this problem focused on the development of modified benzoate ester analogues of the lead structures. Our approach to the design of ester derivatives with the potential for improved stability in buffer was to introduce substituents in the *ortho* positions of the aryl ring in an attempt to

Table 1
Summary of in vitro data from first focused library.



7 **7q** $R^1 = CH_3$, $R^2 = H$ **7r** $R^1 = R^2 = CH_3$

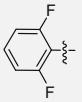
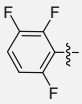
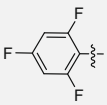
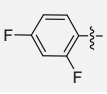
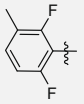
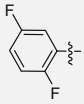
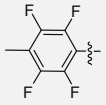
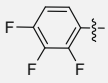
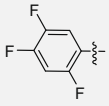
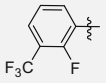
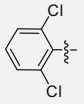
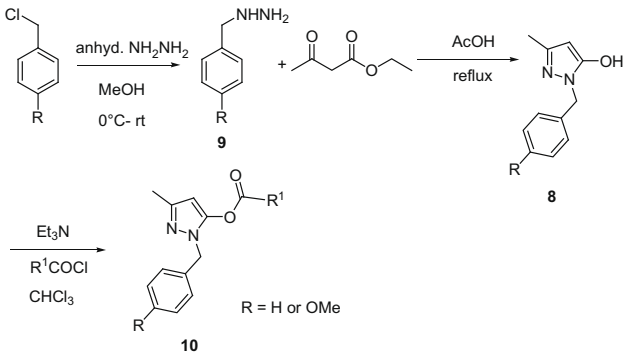
Compound	R	IC_{50} (μM)
1		0.105
2		0.11
7a		0.31
7b		0.97
7c		1.22
7d		1.83
7e		1.96

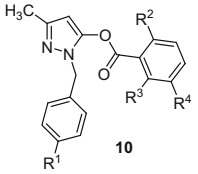
Table 1 (*continued*)

Compound	R	IC_{50} (μM)
7f		3.55
7g		4.28
7h		4.90
7i		5.00
7j		10.69
7k		11.92
7l		26.18
7m		45.49
7n		>100
7o		>100
7p		>100



Scheme 2. Synthetic route to prepare pyrazole esters **10**.

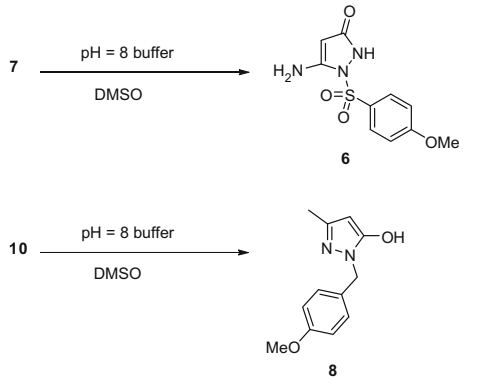
Table 2
Summary of in vitro data for pyrazole esters **10**.



Compounds	R^1	R^2	R^3	R^4	IC_{50} (μM)
10a	OMe	H	H	H	4.03
10b	H	F	F	H	8.04
10c	OMe	F	F	H	8.71
10d	OMe	F	F	CH_3	9.26
10e	H	F	F	CH_3	9.43

prevent hydrolysis of the ester. Compounds **1** and **2** were used as starting points because they were the most potent in that series.

Table 3
Time-dependant degradation of pyrazole series **7** and **10**.[a]



Compounds	$t_{1/2}$ (min)[b]
7a	90
7b	90
7d	255
7e	450
7g	150
7n	300
10a	900
10c	90

[a] The compounds were dissolved in <10% DMSO, pH 8 buffer (1 mM) and Chlorpromazine (15 mM).
[b] Degradation of compound w.r.t. standard.

We therefore designed and synthesized a focused library of pyrazole ester analogues. The synthetic chemistry used for the preparation of the pyrazole ester analogues of **1** and **2** is outlined in Scheme 1.[11]

The reaction of commercially available cyanoacetohydrazide **4** with 4-methoxyphenylsulfonyl chloride in ethanol yielded the sulfonamide **5** which precipitated from solution. Cyclization of sulfonamide **5** to pyrazolone **6** was achieved in an ethanolic solution of KOH, followed by neutralization with AcOH. Lastly, pyrazolone **6** was treated with an arylcarbonyl chloride to obtain the pyrazole

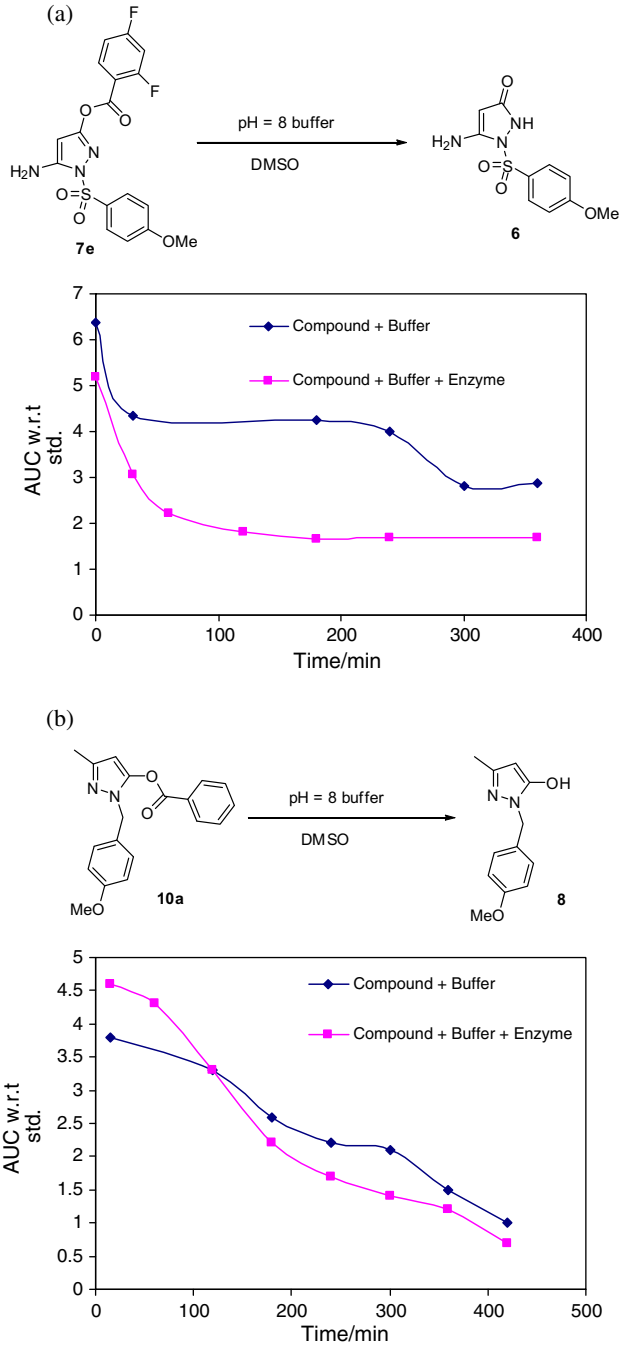


Figure 2. Stability of compounds **7e** and **10a** in the presence and absence of WNV NS2B-NS3 proteinase. (a) Stability studies on compound **7e**[a]. (b) Stability studies on compound **10a**[a]. [a]10 mM Tris–HCl buffer, pH 8.0, containing 20% (v/v) glycerol and 0.005% Brij 35. The substrate and enzyme concentrations were 25 μM and 10 nM, respectively.

esters **7**. The analogues in this library were designed to probe both the steric and electronic requirements of the benzoic acid ester moiety and the in vitro data for selected examples are shown in Table 1. Although the introduction of *ortho* difluoro substituents on the aryl ring was tolerated (e.g., **7a**, **7b**) with a small loss of potency, more bulky substituents such as dichloro (**7n**) led to a complete loss of activity. Similarly, the presence of electron donating (**7o**) or withdrawing (**7p**) substituents in the *ortho* position gave inactive analogues, although the *ortho* methyl derivative (**7f**) retained its micromolar inhibitory activity. In general the addition of substituents led to a reduction in potency for both the benzoate and the thiophenecarboxylate (**7c**, **7i**) analogues (Table 1). To test the effect of N-alkylation of the pyrazole 5-amino group on inhibitory activity we synthesized the monomethylamino (**7q**) and dimethylamino (**7r**) derivatives which were prepared by methylation of compound **1** using $(CH_3)_2SO_4$. Unfortunately this led to loss of inhibitory activity (IC_{50} >100 μM). It should be noted that the products of ester hydrolysis **6** and **8** are inactive (IC_{50} >100 μM) as inhibitors of WNV NS2B-NS3 proteinase.[10]

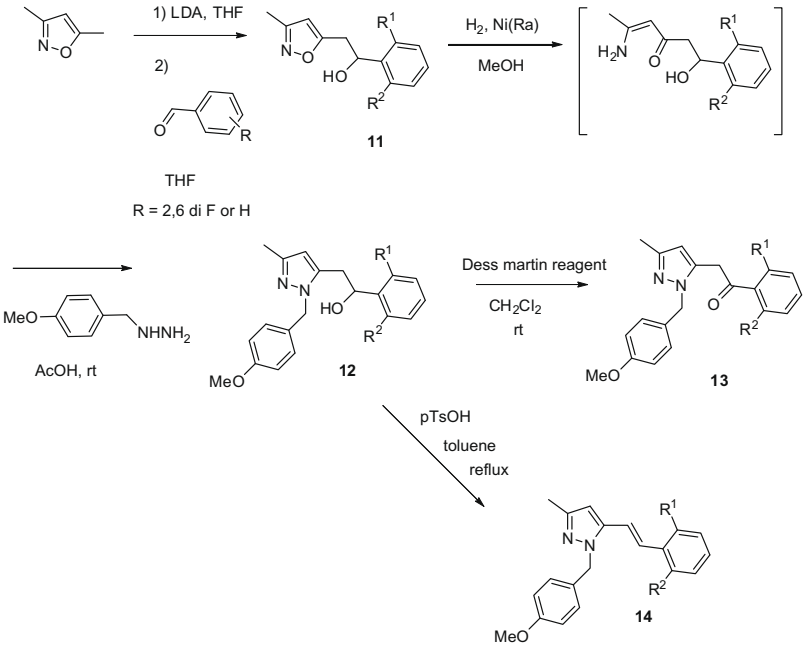
We also investigated the structural requirements important for potency and stability of 3-substituted pyrazolyl esters related to compound **3**. Pyrazole derivative **8** was selected as the key synthon for the preparation of ester analogues. The synthetic chemistry used to prepare the second series of pyrazole ester derivatives is outlined in Scheme 2. The reaction of commercially available chloromethyl methoxybenzene with anhydrous hydrazine in MeOH provided the benzyl hydrazine derivative **9**, which reacted with ethylacetoacetate in AcOH under reflux to obtain pyrazole **8** in excellent yield. Lastly, the treatment of **8** with the appropriate arylcarbonyl chloride in the presence of Et_3N in $CHCl_3$ furnished the corresponding pyrazole esters **10**.[12]

The in vitro data for the pyrazole ester analogues based on compound **3** are shown in Table 2. The potency of the second set of analogues ranged from 4.03 to 9.43 μM in the in vitro assay[13], with compound **10a** being the most potent.

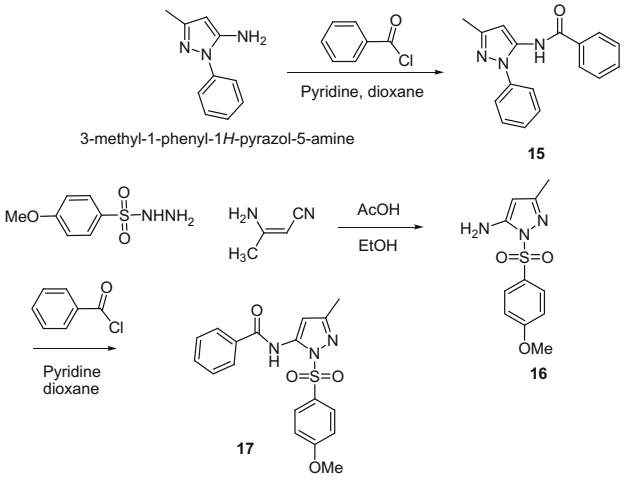
The stability of the most potent compounds in each series in pH 8 buffer was determined by analyzing the amount of compound remaining with time using LC/MS detection. To ensure an accurate quantitation of degradants chlorpromazine was used as an internal standard. The corresponding half lives are reported in Table 3. We were gratified to observe a significant improvement in aqueous stability for compounds **7e** ($t_{½}$ = 450 min) and **10a** ($t_{½}$ = 900 min) compared with the initial hits[10] (Table 3). To help address whether compounds appearing to be inactive under the assay conditions were in fact being rapidly hydrolyzed we also tested the stability of the inactive 2,6-dichloro derivative **7n** (IC_{50} >100 μM). Interestingly, as shown in Table 3, this compound exhibited significantly improved stability ($t_{½}$ = 300 min) compared with the initial hit (**1**).

Based on the data for the stability experiments in a pH 8 buffer, we selected **7e** and **10a** for additional stability studies in the in vitro assay buffer with or without the enzyme WNV NS2B-NS3 proteinase (Fig. 2). The goal in this study was to mimic the assay conditions used for the in vitro experiments and to gain insight into any differences in stability for the two chemical series. As shown in Figure 2, the results were quite different for each compound. Thus, while **7e** was relatively stable in the assay buffer, it degraded rapidly in the presence of the enzyme (Fig. 2a). On the other hand, the stability of **10a** was approximately the same in the presence or absence of the enzyme (Fig. 2b). Taken together, the stability data suggest that the relative stability of the benzoate ester derivatives may be related to electronic rather than steric effects of substituents. These results are in agreement with Charton's studies on the hydrolysis of *ortho*-substituted benzoate esters.[14,15]

Although at this juncture we had made significant progress in enhancing the stability of the pyrazole ester derivatives, we next investigated pyrazole analogues containing non-hydrolyzable ester isosteres. In particular, we wanted to determine whether it was possible to replace the ester moiety with alcohol, ketone, alkene or amide functional groups in the pyrazole derivatives while retaining potency as inhibitors of WNV NS2B-NS3 proteinase. The pyrazole analogues containing ester isosteres were prepared according to the procedures outlined in Schemes 3 and 4. Thus, commercially available 3,5-dimethylisoxazole was reacted with LDA followed by reaction with the corresponding substituted benzaldehyde to yield compound **11** (Scheme 3). The isoxazole derivative **11** was converted to pyrazole **12**[16] using Raney Ni and **12** was then oxidized using the Dess–Martin reagent to produce the ketone derivative **13**. Acid catalyzed dehydration to afford the alkene



Scheme 3. Synthetic route to prepare ester isosteres.



Scheme 4. Synthetic routes to prepare pyrazole amide analogues.

derivative **14** was accomplished using pTsOH in hot toluene (Scheme 3).

Treatment of 3-methyl-1-phenyl-1*H*-pyrazol-5-amine with benzoyl chloride in the presence of pyridine and dioxane furnished the amide derivative **15**. *N*-(1-(4-Methoxyphenylsulfonyl)-3-methyl-1*H*-pyrazol-5-yl)benzamide (**17**) was prepared by the condensation of 3-aminocrotononitrile with 4-methoxybenzenesulfonohydrazide to afford pyrazole **16** followed by reaction of benzoyl chloride (Scheme 4). The in vitro data for some of the target compounds are shown in Table 4. All of the alcohol derivatives were inactive up to the highest concentration tested (100 μM) while the two ketone derivatives exhibited IC_{50} values in the high micromolar range. Encouragingly, however, the alkene derivative **14** was more potent with IC_{50} = 13.8 μM, while the amide derivatives **15** (IC_{50} = 16.0 μM) and **17** (IC_{50} = 9.2 μM) showed activity in a comparable range in the in vitro enzyme assay. Compounds **14**, **15** and **17** were also tested for stability in pH 8 buffer (Table 5). Interestingly, while the alkene (**14**) and amide **15** were highly stable, the amide **17** possessed a relatively short half-life of 1.25 h.

In conclusion, we have described the design and synthesis of 3-substituted pyrazole ester derivatives which are active as allosteric inhibitors of West Nile Virus NS2B-NS3 proteinase. Two compounds, **7a** and **10a**, while less potent than the original hits (1.96 and 4.03 μM IC_{50}, respectively) are significantly more stable in pH 8 buffer. In addition, we have designed, synthesized and evaluated the in vitro activity of a series of pyrazole derivatives containing ester isosteres. Of these analogues, the alkene **14** (IC_{50} = 13.8 μM) and amide **15** (IC_{50} = 16.0 μM) derivatives are highly stable inhibitors of WNV NS2B-NS3 proteinase. These compounds, which interact with an allosteric site on the enzyme,[10] are promising leads for additional optimization studies and may find utility in in vitro studies to elucidate the biochemistry and enzyme kinetics of WNV NS2B-NS3 proteinase.

Table 4
Summary of in vitro data for ester isosteres.

Compounds	R^1	R^2	IC_{50} (μM)
12a	H	H	>100
12b	F	F	>100
13a	H	H	>100
13b	F	F	23.9
13c	F	H	38.8
14	F	F	13.8

Table 5
Time-dependant degradation of pyrazoles.[a]

Compounds	$t_{1/2}$ (h)[b]
14	13
15	>96
17	1.25

[a] The compounds were dissolved in <10% DMSO, pH 8 buffer (1 mM) and Chlorpromazine (15 mM).
[b] Degradation of compound w.r.t standard.

Acknowledgments

This work was supported by NIH grants U01 AI078048 and U54 HG005033.

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12. *Synthesis of* **10a**: a solution of (4-methoxybenzyl)hydrazine (2.0 g, 0.013 mol) and ethyl acetoacetate (1.8 mL, 0.014 mol) in glacial acetic acid (54.0 mL) was stirred and heated at 100 °C over night. The solvent was evaporated and the product purified using automated medium pressure silica gel chromatography (ISCO) eluting with 20–80% EtOAc/CH_2Cl_2 to obtain 1-(4-methoxybenzyl)-3-methyl-1*H*-pyrazol-5-ol (**8**) (1.28 g, 46%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.12 (d, J = 8.70 Hz, 2H), 6.86 (d, J = 8.40 Hz, 2H), 5.15 (s, 1H), 4.86 (s, 2H), 3.71 (s, 3H), 2.00 (s, 3H). A mixture of **8** (53.4 mg, 0.24 mmol), Et_3N (0.2 mL, 1.43 mmol) and benzoyl chloride (0.03 mL, 0.29 mmol) were dissolved in $CHCl_3$ (2 mL) and allowed to react at room temperature for 5 min. The crude reaction mixture was dissolved in water (10 mL) and extracted with CH_2Cl_2 (20 mL). The organic layer was separated and the solvent was removed in vacuo. The crude residue was purified using preparative HPLC to obtain the title compound (65.3 mg, 84%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.09 (d, J = 7.80 Hz, 2H), 7.80–7.75 (m, 1H), 7.64–7.59 (m, 2H), 7.14 (d, J = 8.40 Hz, 2H), 6.85 (d, J = 8.40 Hz, 2H), 6.07 (s, 1H), 5.14 (s, 2H), 3.69 (s, 3H), 2.16 (s, 3H) ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.6, 158.6, 146.1, 143.9, 134.6, 130.0, 129.1, 128.9, 128.8, 127.4, 113.9, 94.0, 55.0, 50.2, 14.1. LRMS (ESI): 323.00 $(M+1)^+$.
13. *WNV NS2B-NS3 proteinase activity assay with Pyr-RTKR-AMC fluorogenic substrate*: the assay for WNV NS2B-NS3 protease activity was performed in 10 mM Tris–HCl buffer, pH 8.0, containing 20% (v/v) glycerol and 0.005% Brij 35. The substrates and enzyme concentrations were 25 μM and 10 nM, respectively. The total assay volume was 0.1 mL. Initial reaction velocities were monitored continuously at λ_{ex} (excitation wavelength) of 360 nm and λ_{em} (emission wavelength) of 465 nm on a Spectramax Gemini EM fluorescence spectrophotometer (Molecular Devices). All assays were performed in triplicate in wells of a 96-well plate. The K_m and k_{cat} values were derived from a double-reciprocal plot of $1/V_0$ against $1/[S]$, using the Lineweaver–Burk transformation: $1/V_0 = K_m/V_{max} \times 1/[S] + 1/V_{max}$, where V_0 is the initial velocity of substrate hydrolysis, $[S]$ is the substrate concentration, V_{max} is the maximum rate of hydrolysis, and K_m is the Michaelis–Menten constant. The concentration of the catalytically active proteinase was measured using the fluorescent assay by titration against a standard aprotinin solution of known concentration. The concentration of active NS2B-NS3 was close to 100% when compared with the total protein in the sample. For the determination of the IC_{50} value of the inhibitors, NS2B-NS3 proteinase was pre-incubated for 60 min at 18 °C with increasing concentrations of the inhibitors. Following addition of the Pyr-RTKR-AMC substrate (25 μM), the rate of substrate hydrolysis was monitored, and IC_{50} values were determined using routine kinetics software.
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