

N-Sulfonylanthranilic Acid Derivatives as Allosteric Inhibitors of Dengue Viral RNA-Dependent RNA Polymerase

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Abstract: A novel class of compounds containing *N*-sulfonylanthranilic acid was found to specifically inhibit dengue viral polymerase. The structural requirements for inhibition and a preliminary structure–activity relationship are described. A UV cross-linking experiment was used to map the allosteric binding site of the compound on the viral polymerase.

The World Health Organization has estimated that the four serotypes of the dengue virus (DENV^a) cause 50–100 million human infections annually worldwide.¹ Approximately 500 000 of the infected individuals progress to dengue hemorrhagic fever, leading to 22 000 deaths.² DENV is mosquito-borne and threatens up to 2.5 billion people in more than 100 endemic countries. Although the morbidity of dengue infection is low, its socioeconomic impact is high.³ Currently there is no vaccine or effective antiviral treatment available.⁴ As a result, development of a safe, effective therapeutic agent for DENV is urgently needed.

DENV is a member of the genus *flavivirus* in the family *Flaviviridae*.⁵ The viral genome is a single-stranded RNA (~10.7 kb) of positive polarity. The viral genome encodes three structural proteins and seven nonstructural proteins. The nonstructural protein 5 (NS5) functions as a methyltransferase and an RNA-dependent RNA polymerase (RdRp) that are essential for viral replication.⁶ The RdRp is responsible for the synthesis of both minus-sense and positive-sense viral RNAs during replication. Similar to other polymerases, the structure of DENV RdRp exhibits a canonical right-hand conformation and consists of fingers, palm, and thumb domains.⁷ The active site of the enzyme is defined by a conserved GDD motif in the palm domain.

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^aAbbreviations: DENV, dengue virus; RdRp, RNA-dependent RNA polymerase; NS5, nonstructural protein 5; SPA, scintillation proximity assay; GTP, guanine triphosphate.

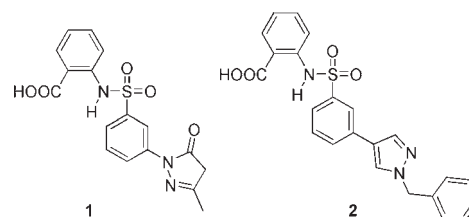
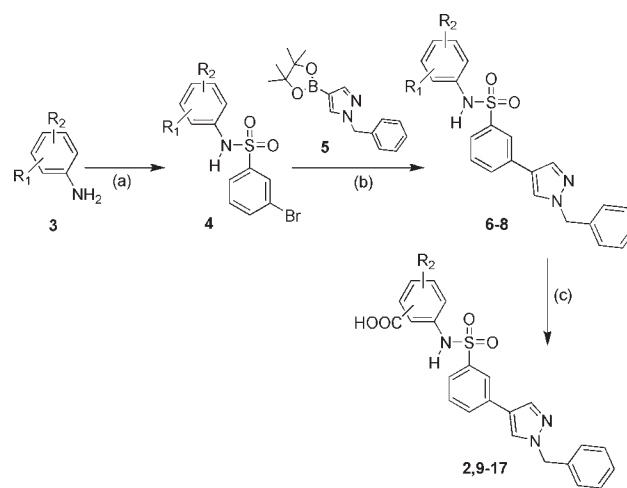


Figure 1. High throughput hit **1** and *N*-sulfonylanthranilic acid lead **2**.

Scheme 1. Synthesis of Inhibitors **2** and **6–17**^a

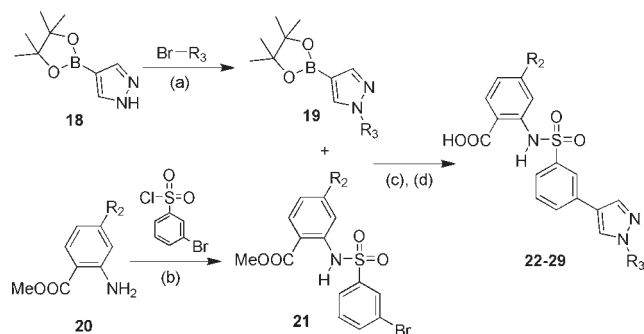


^aReagents and conditions: (a) 3-bromobenzenesulfonyl chloride, pyridine; (b) Pd(Ph₃)₄, K₂CO₃, DMF; (c) LiOH, MeOH.

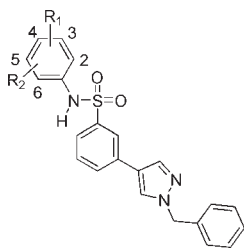
The DENV RdRp is a preferred antiviral target because of the established druggability of this class of viral enzymes,⁸ its essentiality for viral replication, its conservation among all four serotypes, and the lack of counterparts in mammalian cells.⁹ To date, no non-nucleoside inhibitor of the DENV RdRp has been reported.

In our search for DENV RdRp inhibitors, we developed a scintillation proximity assay (SPA), using a DENV-2 full-length recombinant NS5, polyG as a primer, and biotinylated-oligo C as a template.¹⁰ The assay measured the incorporation of radioactive ³H-GTP into the polyC/biotinylated-oligo G complex that was captured on the streptavidin-coated scintillation beads. High throughput screening of the Novartis corporate compound archive led to the identification of 2-[3-(3-methyl-5-oxo-4,5-dihydropyrazol-1-yl)benzenesulfonylamino]benzoic acid (**1**) with an IC₅₀ of 7.2 μM. Derivatization of **1** rapidly produced *N*-sulfonylanthranilic acid (**2**), which had a IC₅₀ of 0.7 μM (Figure 1). In this communication, we report the synthesis and biological activity of this new series of *N*-sulfonylanthranilic acids.

Scheme 1 depicts the general procedure for the synthesis of **2** and derivatives with a fixed benzylpyrazole fragment. 3-Bromobenzenesulfonyl chloride was reacted with appropriate anilines **3** to form sulfonamides **4**, and subsequent coupling with commercially available benzyl pyrazole boronic ester (**5**) through a palladium catalyzed Suzuki reaction¹¹ gave the desired *N*-sulfonylanthranilic acid analogues.

Scheme 2. Synthesis of Inhibitors **22–29**^a

^aReagents and conditions: (a) CsCO₃, CH₃CN; (b) pyridine; (c) Pd(Ph₃)₄, K₂CO₃, DMF; (d) LiOH, MeOH.

Table 1. Substitution Effect on the Phenyl Ring of *N*-Sulfonylanthranilic Acids

compd	R ₁	R ₂	IC ₅₀ (μM)
2	2-COOH	H	0.7
6	H	H	> 20
7	2-COOMe	H	> 20
8	2-CONH ₂	H	> 20
9	3-COOH	H	> 20
10	4-COOH	H	> 20
11	2-COOH	6-Cl	18.02
12	2-COOH	5-Cl	0.77
13	2-COOH	4-Cl	2.56
14	2-COOH	3-Cl	> 20
15	2-COOH	5-F	0.37
16	2-COOH	5-methoxy	9.99
17	2-COOH	4,5-dimethoxy	> 20

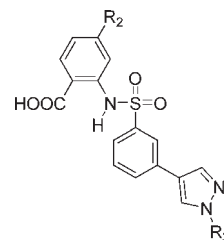
The carboxylic acid targets were produced by hydrolysis of the corresponding methyl esters.

Compounds **22–29** were synthesized as shown in Scheme 2. Modifications of the pyrazole were achieved by direct alkylation of commercially available pyrazole boronic ester (**18**) in the presence of cesium carbonate. Suzuki coupling reaction of the modified pyrazole boronic esters **19** with 3-bromobenzenesulfonamides **21** followed by hydrolysis furnished the desired *N*-sulfonylanthranilic acids.

Efforts to understand the structure–activity relationships of the lead molecule started with the carboxylic acid moiety.

As shown in Table 1, removal (**6**) or replacement of carboxylic acid with functional groups such as ester and amide (**7** and **8**) resulted in lower potency compounds, indicating that the carboxylic acid is essential for the activity. Moreover, compounds having the carboxylic acid functional group in meta- and para-position (**9** and **10**) were inactive against RdRp, demonstrating the importance of the position of the carboxylic acid (Table 1).

The effect of substituents on the phenyl ring adjacent to the carboxylic acid was explored subsequently (Table 1). A chlorine atom was well tolerated in the 5-position (**12**),

Table 2. Replacement of Benzyl on Pyrazole

compd	R ₂	R ₃	IC ₅₀ (μM)
2	H	benzyl	0.7
22	H	H	> 20
23	H	Me	> 20
24	H	isobutyl	> 20
25	H	isopentyl	6.77
26	H	ethylmorpholine	> 20
27	H	methyl-2-naphthalene	0.37
28	F	methyl-2-naphthalene	0.26

while substitution at the 4- (**13**), 6- (**11**), and 3- (**14**) position led to progressively inferior inhibition. On the basis of this initial structure–activity information, further work focused on the 5-position of the phenyl ring. Replacing the chlorine with the stronger electron-withdrawing fluorine (**15**) improved the activity slightly, while an electron-donating methoxy group (**16**) reduced the activity significantly. These results suggest that electron-withdrawing groups may be generally preferred at the 5-position of the phenyl ring. The results suggest that the electron withdrawing group increases the acidity of the carboxylic acid which may be critical for the interaction with the polymerase enzyme. In agreement with this hypothesis, significant loss of potency was observed when a second methoxy group was introduced at the 4-position (**17**) (Table 1).

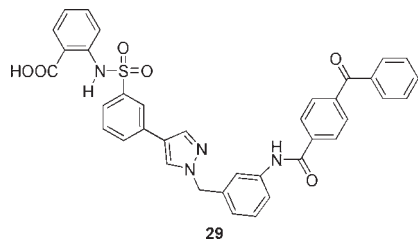
With 2-[3-(1*H*-pyrazol-4-yl)benzenesulfonylamino]benzoic acid as a fixed fragment, replacement of the benzyl group on the pyrazole ring was investigated to identify the minimum scaffold capable of inhibiting the dengue NS5 polymerase (Table 2). Removal (**22**) or replacement of the benzyl group with small hydrophobic substituents such as methyl (**23**) on pyrazole was detrimental to the activity. Interestingly, while the isobutyl derivative (**24**) was not able to inhibit the dengue polymerase, the compound with an isopentyl replacement (**25**) (one carbon longer aliphatic chain) showed activity at low micromolar range. Moreover, no activity at the concentration tested was observed when a hydrophilic morpholine (**26**) was introduced.

Therefore, the structural feature that gives rise to potency is likely to be a hydrophobic terminal group linked to the headgroup by a defined chain length. This could be demonstrated by the potency increase of the methyl-2-naphthalene replacement (**27**) that extends the hydrophobic substituent. A further (albeit small) improvement in potency was made through a combination of structural fragments showing the best activity. Compound **28** with fluoro substitution on the phenyl ring of carboxylic acid and methyl-2-naphthalene on pyrazole displayed an IC₅₀ of 0.26 μM against the dengue polymerase (Table 2).

Inhibitors **2**, **15**, and **28** were assessed for selectivity against several other polymerases, including human DNA polymerase α and β and hepatitis C RdRp. The inhibitors did not inhibit these polymerases, exhibiting IC₅₀ of > 20 μM

Table 3. Selectivity Panel

compd	IC ₅₀ (μM)			
	dengue RdRp	HCV RdRp	human DNA Pol α	human DNA Pol β
2	0.7	> 20	> 20	> 20
15	0.37	> 20	> 20	> 20
28	0.26	> 20	> 20	> 20

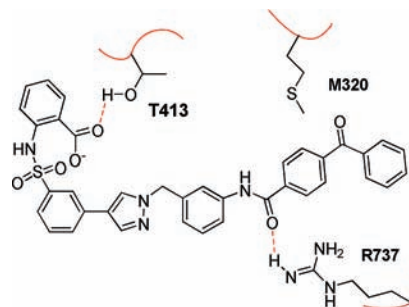
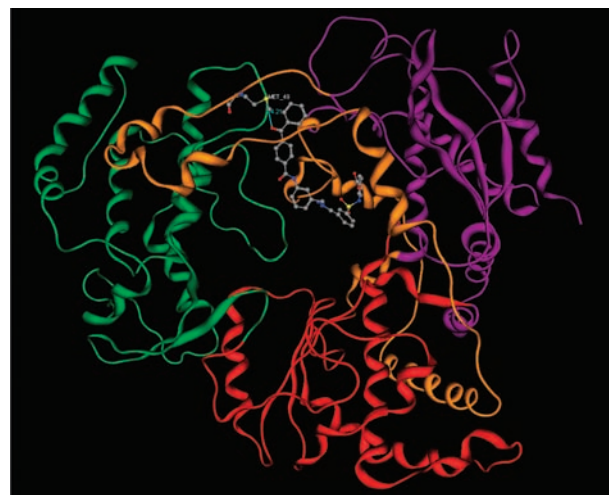
**Figure 2.** Structure of inhibitor **29**.

(Table 3). These results demonstrate that these novel inhibitors specifically suppress DENV RdRp.

N-Sulfonylanthranilic acid derivatives seem to inhibit the dengue RdRp during elongation; they can bind to the RdRp alone or to the RNA-RdRp complex, suggesting that compound binding does not interfere with template binding and vice versa (unpublished results). In an effort to map the site of association between the compound and the polymerase, a low micromolar inhibitor (**29**) (Figure 2) (RdRp IC₅₀ = 1.5 μM) tethered with the photoalkylating group¹² benzophenone was synthesized. Under ultraviolet light irradiation, the compound reacted with the protein and irreversibly inhibited the RdRp activity in our assay. On the basis of MALDI MS, a peptide spanning from amino acid position 313 to 326 of DENV-2 NS5 polymerase (QTGSASSMVNGVVR) was covalently linked to **29** ([M + H]⁺ *m/z* 2048.8), resulting in a mass increment of 656.17 (data not shown). Since unmodified N-terminal b₅ and C-terminal y₆ ions were observed in the corresponding MS–MS spectrum, the modification is located within the sequence SSM of the above amino acid sequence.

On the basis of the known reactivity of the *n*,*π** triplet state of benzophenone¹³ and the presence of methionine in the identified peptide sequence (e.g., *m/z* 1376.7, –CH₂CH₂S⁺), we assumed that amino acid M – 320 is most likely the cross-linking site.

A computational study was performed to rationalize the photoaffinity labeling result and to identify the possible compound binding site. In the X-ray crystallographic structure of the DENV RdRp (PDB code 2J7U),⁷ residues 311–317, 408–415, and 454–466 were not observed. As a consequence, the three-dimensional atomic coordinates for these amino acid sequences were obtained by constructing a homology structure for the DENV RdRp using MOE.¹⁴ The structure 2J7U was used as the primary template, and the Cartesian coordinates for the backbone atoms of the missing amino acids were obtained from the crystal structure of the West Nile virus RdRp (PDB code 2HCN).¹⁵ The rmsd for the backbone atoms between the X-ray structure 2J7U and the homology structure was 1.03 Å. Inhibitor **29** (with the attached benzophenone moiety) was docked into the polymerase binding pocket of the homology model using GOLD.¹⁶ The docked pose that best explained the overall SAR presented above was chosen as a reasonable initial orientation. This modeled complex of the docked ligand and

**Figure 3.** Ligand interaction map for inhibitor **29** in the binding pocket. The red dotted lines show the hydrogen bonding interactions between **29** and T413, R737. The backbone of the specific residues are shown as red lines.**Figure 4.** Proposed allosteric binding site of *N*-sulfonylanthranilic acid derivative **29** in the dengue polymerase domain.

protein structure was subjected to a molecular dynamics study in which only the loops of the protein and the ligand were allowed to move. As the photo-cross-linking experiments were done at 277 K, a NVT ensemble was used with an initial heating phase of 10 ps. The AMBER99¹⁷ force field in MOE was used for the dynamics simulation with a time step of 1 fs with sampling at every 0.5 ps over a 1 ns equilibration period. A further simulation phase of 0.5 ns was calculated using the same time parameters as before. The distance from the carbonyl O atom in the benzophenone moiety to the S in M320 in the protein was measured for each of the saved structures. It was found that this distance varied between 4.0 and 5.0 Å for 82% of the complex structures during the dynamics, and for 18% of the time of observation, this distance fluctuated between 3.0 and 4.0 Å. The average distance between the O and S atoms during the dynamics was 4.21 Å (Figure 3). The side chain of R737 formed a strong hydrogen bonding donor interaction with the carbonyl bond of the carboxamide linker moiety of **29** at a distance of 2.03 Å. Another such interaction, at a distance of 2.4 Å, was observed between the carboxylate/acid group and the hydroxyl group in the side chain of T413. The hydrogen bond between carboxylate/acid and the hydroxyl group of T413 is likely strengthened by electron withdrawing effect of the additional group at the 5-position of phenyl ring, which is consistent with initial structure–activity relationship study. These two interactions were maintained during the course of the dynamics

simulations, indicating an importance in the stabilization of the compound in the allosteric binding pocket. On the basis of these results, we hypothesize that the reported compounds bind to the dengue RdRp at an allosteric pocket that is located between the finger and the thumb regions as shown in Figure 4.

We have identified *N*-sulfonylanthranilic acid derivatives that can selectively inhibit dengue viral polymerase. Our results suggest that these compounds may bind to a novel allosteric site located between the finger and the thumb regions in dengue RdRp.

While the reported compounds are not potent enough for development, they have revealed an allosteric binding site that may be explored for future dengue NS5 polymerase inhibitors.

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Supporting Information Available: Structures, experimental methods, and characterization data for compounds **2–29**, UV cross-linking experiment, computational experimental methods, and 3D structure (PDB data file). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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