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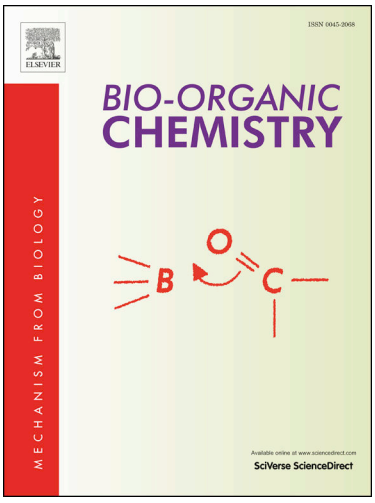

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Synthesis and evaluation of novel *S*-benzyl- and *S*-alkylphthalimide- oxadiazole - benzenesulfonamide hybrids as inhibitors of dengue virus protease

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Abstract

Direct acting antiviral drugs (DAADs) are becoming therapeutics of choice for the treatment of viral infections. Successful development of anti HIV and HCV drugs by targeting the viral proteases has provided impetus for discovering newer DAADs. Dengue virus (DENV) protease, which is composed of two nonstructural proteins, NS2B and NS3pro, can be likewise exploited for discovering new anti-dengue therapeutics. In this study, we have linked together two pharmaceutically interesting motifs, namely 1,3,4-oxadiazole and benzenesulfonamide in two alternative series to develop novel *S*-benzylated and *S*-alkylphthalimidated hybrids. For the first series of hybrids, 4-aminobenzoic acid (**1**) was reacted with substituted benzenesulfonyl chlorides *via* its amino group, whereas the carboxylic acid side was elaborated to sulfonamido-1,3,4-oxadiazole-2-thiols (**6a/b**) in three steps. At this stage, the intermediates **6a/b** were bifurcated to either *S*-alkylphthalimidated (**8a-j**) or *S*-benzylated (**9a-c**) hybrids by reacting with corresponding halides. For the alternative series of hybrids, the carboxylic acid group of probenecid (**10**) was similarly elaborated to sulfonamido-1,3,4-oxadiazole-2-thiols (**13**), and diverged to *S*-alkylphthalimidated (**14a-f**) and *S*-benzylated hybrids (**15a-e**). Bioactivity assays demonstrated that **8g** and **8h** are the most potent inhibitors among the synthesized analogs, exhibiting the IC_{50} values of $13.9\mu M$ and $15.1\mu M$, respectively. Computational assessment predicted the binding of the inhibitors at an allosteric site developed in the open conformation of DENV2 NS2B/NS3pro. Taken together these findings point out that the synthesized hybrid inhibitors possess a great potential for further antiviral drug development.

1. Introduction

Recent decades have witnessed a sudden rise in flaviviral infections in tropical and subtropical regions, with a potential of penetrating into remote areas due to international travel and trade. Particularly, the infections caused by dengue virus (DENV) and Zika virus (ZIKV) have raised major public health concerns [1-4]. Epidemiological data suggest that the DENV infections affect approximately 400 million people each year globally [1]. Clinical symptoms of DENV infection range from a mild self-limited fibril illness to the fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [5]. In the recent epidemic outbreaks, four serotypes of DENV (DENV1-4) have been detected along with the emergence of a fifth one in Southeast Asia [6].

To date, no specific drug is available for the treatment of DENV infection. Although, Sanofi-Pasteur has developed a tetravalent dengue vaccine (CYD-TDV, *Dengvaxia*), which is licensed for clinical use in several endemic countries [7, 8], yet its effectiveness in general population has been questioned [9, 10]. Therefore, development of direct-acting antiviral drugs (DAADs) drugs for the treatment of DENV infection is highly demanded. In this regard, various viral factors have been proposed as drug targets [11, 12]; amongst them the complex NS2B/NS3pro is becoming a prominant target for discovering anti-dengue DAA therapeutics[13, 14].

Previous efforts for discovering peptidic, peptidomimetic, and small molecule based inhibitors of DENV NS2B/NS3pro and closely related flaviviruses have been reviewed recently [13, 14, 15]. Despite the identification of several potent inhibitors of the protease [16], unfavorable pharmacokinetics and pharmacodynamic characteristics no inhibitor of DENV NS2B/NS3pro has hitherto entered the clinical trial. This predicament demands a continuous effort toward the screening of small organic molecules for discovering newer hits for the development of potent anti-dengue drug. Carrying on our efforts toward discovering novel inhibitors of DENV NS2B/NS3pro, we describe here results of newly synthesized oxadiazole-sulfonamide hybrids.

1,3,4-Oxadiazoles are privileged structural scaffolds with diverse synthetic utility and broad spectrum of biological activities including anti-cancer [17], anti-mitotic [18], anti-inflammatory [19-23], antimicrobial [24-28], enzyme inhibitory [29-35] and others [36, 37]. Whereas, sulfonamides are another useful class of compounds having antibacterial, antimicrobial and

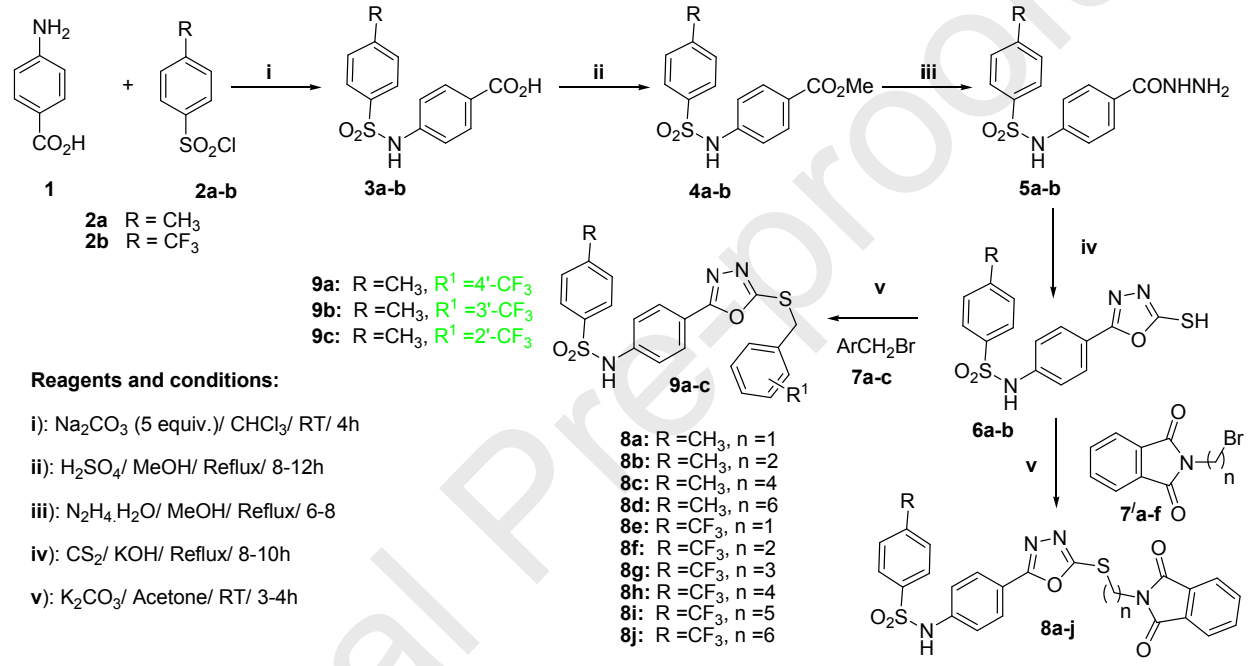
anticancer activities [38]. Presence of arylsulfonamide moiety at the P′-position has been found to enhance the inhibitory potential peptidomimetic inhibitors of hepatitis C virus [39]. Additionally, the replacement of the cleavage site peptide bond with a sulfonamide bond in peptide-based inhibitors of protease results in enhanced stability of the inhibitors against proteolytic degradation [40]. Recently, phthalimide containing sulfonamides have been reported as dengue virus protease inhibitors [41, 42, 43], along with other interesting biological activities [44-46]. Impressed by the biological profile of these structural scaffolds, we envisioned to combine these pharmacophores to synthesize novel *S*-benzyl-oxadiazole-benzene sulfonamides and *S*-alkylphthalimide-oxadiazole benzenesufon-amides. This article reports on the synthesis, bioassay screening against dengue protease (serotype-2), DENV2 NS2B/NS3pro, molecular docking analyses of these hybrids.

2. Results and discussion

2.1. Chemistry

The syntheses of the target *S*-alkylphthalimide-oxadiazole benzenesulfonamides (**8a-j; 14a-f**) and *S*-benzyl-oxadiazole benzenesufonamides (**9a-c;15a-e)** were achieved by following a multistep synthetic approach as outlined in Scheme 1 and 2. For the synthesis of *N*-(4-(5-((1,3-dioxoisoindolin-2-yl)alkylthio)-1,3,4-oxadiazol-2-yl)phenyl)-4-methyl benzenesulfonamide (**8a-j)** and *N*-(4-(5-(trifluoromethyl substituted)benzylthio)-1,3,4-oxadiazol-2-yl)phenyl)-4-methyl benzene-sulfona-mide **(9a-c)**, a common starting material 4-amino benzoic acid (**1**) was used. The reaction of 4-amino benzoic acid (**1**) with 4-methylbenzene-1-sulfonyl chloride (**2a**) or 4-(trifluoromethyl)benzene-1-sulfonyl chloride (**2b**) in the presence of sodium carbonate provided 4-(tosylamino) benzoic acid (**3a**) or 4-(4-(trifluoromethyl)phenylsulfonamido) benzoic acid (**3b**) in 82% and 86% yield, respectively [47]. The sulfonamide acids **3a**, and **3b** were esterified in methanol in the presence of sulfuric acid to obtain methyl 4-(4-methylphenyl-sulfonamido) benzoate (**4a**), and methyl 4-(4-(trifluoromethyl)phenylsulfonamido) benzoate (**4b**) in 83% and 80% yield, respectively. The hydrazinolysis of **4a** and **4b** with 80% hydrazine hydrate in methanol under reflux afforded 4- (4-methylphenyl-sulfonamido) benzohydrazide (**5a**) and 4-(4-(trifluoromethyl)phenylsulfonamido) benzohydrazide (**5b**) in 78% and 71% yield, respectively [48]. The hydrazide **5a** and **5b** were then treated with carbon disulfide and potassium hydroxide under reflux in methanol to afford 4-Methyl-N-(4-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-

yl)phenyl) benzenesulfonamide (**6a**) and N-(4-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl)-4-(trifluoromethyl) benzenesulfonamide (**6b**) in 77% and 70% yield, respectively [49-51]. Finally, the reaction of **6a** and **6b** with phthalimido-alkyl bromides **7́a-f** or substituted benzyl bromides **7a-e** in the presence of potassium carbonate in acetone at room temperature resulted in the synthesis of the desired structures **8a-j** and **9a-c** in decent overall yields [52] (Scheme 1).



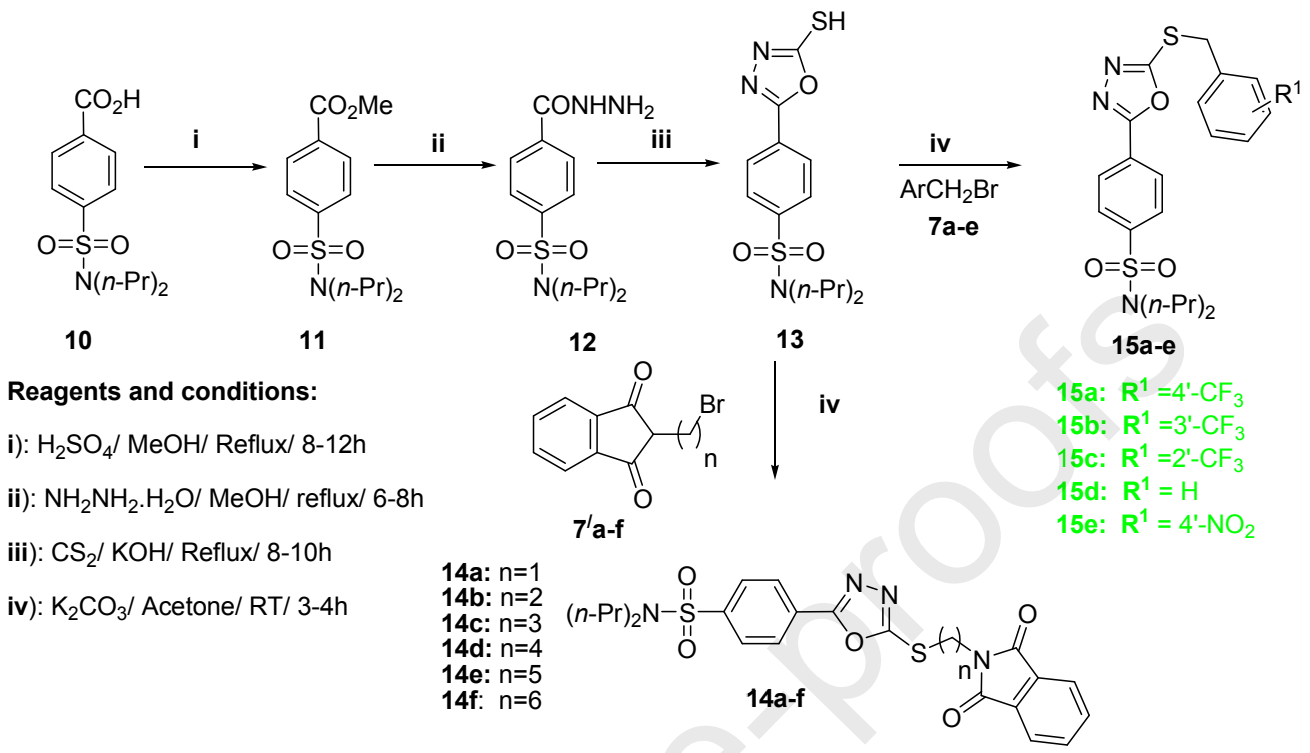
Scheme 1. Synthetic route of **8a-j** and **9a-c** from 4-aminobenzoic acid (**1**).

The target compounds and intermediates were purified by recrystallization and the final products were characterized by ^{1}H & ^{13}C NMR spectroscopy, FT-IR, and CHNS analyses. In the ^{1}HNMR, NH proton of **8a-j** and **9a-c** displayed a characteristic broad singlet at δ 11.13-10.71 ppm. The twelve aromatic protons of **8a-j** and **9a-c** were located in the range of δ 8.05-7.06 ppm. The singlet corresponding to CH_3 group on the phenyl ring of **8a-d** and **9a-c** appeared between δ 2.32-2.31 ppm. The singlet for two protons of *S*-CH_2 group in compounds **8a** and **8e** was found at δ 5.28 and 5.31 ppm, respectively whereas, the singlet for *S*-CH_2 protons of **9a-c** showed up in the range of δ 4.68-4.62 ppm. Similarly, in compounds **8b-d** and **8f-j** a triplet in the range of δ 3.56-3.24 ppm corresponded to two protons of *N*-CH_2. All the remaining methylene groups

present between the *S*-CH_2 and *N*-CH_2 group of **8c-d** and **8g-j** were found as multiplets in the range of δ 2.21-1.29 ppm. In ^{13}C NMR, the two carbonyl signals of phthalimido group were located between δ 168-164 ppm. The two oxadiazole carbons were present between δ 160-165 ppm and δ 140-145 ppm. All the aromatic carbons of **8a-j** and **9a-c** were obtained in the range of δ 141-118 ppm. All the aliphatic carbons were observed below δ 50 ppm.

For the synthesis of 4-(5-((1,3-dioxoisoindolin-2-yl)alkylthio)-1,3,4-oxadiazol-2-yl)-*N,N*-dipropyl benzenesulfonamide (**14a-f**) and 4-(5-(substituted)benzylthio)-1,3,4-oxadiazol-2-yl)-*N,N*-dipropyl benzenesulfonamide (**15a-e**), we followed the similar synthetic route; however, by using a different starting material, i.e. probenecid (**10**) containing an *n*-propylsulfonamide moiety. Thus, esterification of **10** was followed by hydrazinolysis of the resulting ester **11** with 80% hydrazine hydrate in methanol under reflux to afford 4-(dipropylsulfamoyl)benzo-hydrazide (**12**) in 76% yield. The cyclization of **12** was accomplished with CS_2/KOH/MeOH system under refluxing conditions to afford *N,N*-dipropyl-4-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl) benzenesulfonamide **(13)** in 73% yield. The synthetic route was again bifurcated at this stage in the two series **14a-f** and **15a-e** by reaction of **13** with phthalimido-alkyl bromides **7́ a-f** and substituted benzyl bromides **7a-e** in the presence of potassium carbonate in acetone at room temperature, in fairly good yields (Scheme 2).

The ^{1}HNMR of compounds **14a-f** displayed the characteristic peaks for two isopropyl groups and two aromatic rings. In addition, the singlet for *S*-CH_2 group of **14a** was found at δ 5.42 ppm. The triplets for the *S*-CH_2 and *N*-CH_2 group of **14b-f** were present in the range of δ-3.64-3.31 ppm and δ 4.23-3.69 ppm, respectively. The multiplet for the CH_2 group present between *S*-CH_2 and *N*-CH_2 group of **14c** was found at δ 2.34-2.27 ppm. The four protons of two methylene groups of compound **14d** present between *S*-CH_2 and *N*-CH_2 group were obtained as a multiplet at δ 1.97-1.87 ppm. The other protons of remaining methylene groups of 14e and 14f were found between δ 1.93-1.56 ppm. The ^{13}C NMR spectrum of **14a-c** revealed characteristic peaks for two carbonyl carbons between δ 166-164 ppm, two oxadiazole carbons at around δ 162 and 143 ppm, aromatic carbons between δ 138- 120 ppm and aliphatic carbons below δ 50 ppm.



Scheme 2. Synthetic route of **14a-f** and **15a-e** from probenecid **(10).**

In addition to the characteristic signals of the *n*-propyl group in ^{1}H NMR spectra, compounds **15a-e** showed peaks for aromatic protons between δ 8.23-7.62 ppm and singlets for two benzylic protons between δ 4.76-4.58 ppm. Similarly, ^{13}C NMR for compounds **15a-e** showed peaks for carbonyl groups, aromatic carbons, oxadiazole carbons and *n*-propyl carbons. The benzylic carbons were found between δ 39-33 ppm.

2.2 DENV2 protease activity

All the synthesized compounds were tested for DENV2 protease inhibitory activity by using the recombinant Gly_4-Ser-Gly_4 linked NS2B/NS3pro [53] in the presence of fluorogenic Bz-Nle-Lys-Arg-Arg-AMC substrate. The assay measures the cleavage of the fluorescent tag, 7-amino-4-methylcoumarine (AMC) from the substrate as a function of time. A dose of 50μM of each of the sulfonamide-oxadiazol hybrids was incubated with 100nM protease and reduction in the fluorescence was deemed as the inhibition of the protease. A separate experiment was run to measure background fluorescence originating from the synthetic compounds or quenching of AMC by the compounds to rule out the false positive inhibition (data not shown). None of the

tested compounds showed any significant background fluorescence or quenching effect under the conditions used for these experiments. Moreover, the enzymatic reactions of potent inhibitors were repeated by replacing the zwitterionic detergent with non-ionic detergent to rule out the possibility of false positive hit due to aggregation of compounds.

The hybrids containing *S*-alkylphthalimide moiety, i.e. **8** and **14** demonstrated overall a better inhibition than the *S*-benzylated analogs, as shown in Fig. 1 (A & C versus B & D). Presence of trifluoromethyl (CF_3) moiety on the benzene ring of sulfonamide moiety in **8e-j** seems to improve the inhibition of the protease as compared to the methyl moiety in **8a-d** (Fig 1, A); however, the inhibitory activity is strictly depended on the length of the alkyl chain flanking between the phthalimide and oxadiazole moieties. The analogs containing shorter alkyl chain (n=1, 2) in **8** and **14** showed <50% inhibition at 50μM concentration. The most potent inhibition

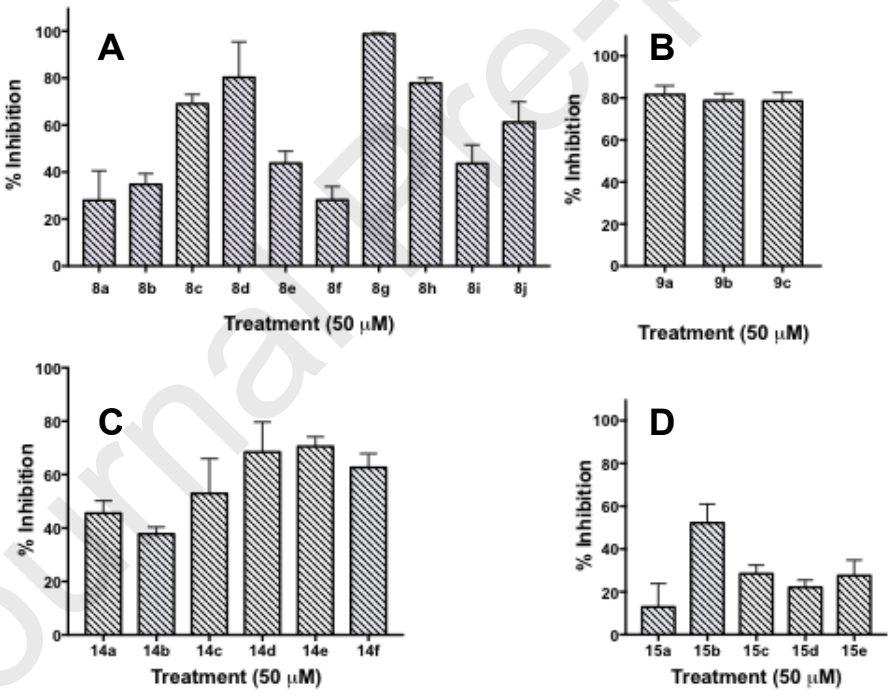


Fig. 1. Inhibition of DENV2 NS2B/NS3pro by *S*-alkylphthalimide- (**A,C**) and *S*-benzyl-sulfonamide-oxadiazole hybrids (**B, D**). A dose of 50 μM of the hybrids was incubated in the presence of 100 nM recombinant DENV2 NS2B/NS3pro, 5μM tetrapeptide-AMC substrate in a Tris buffer at pH 9.5. Time dependent release of fluorescent AMC tag was measured for 30 min and compared with the control, which was run in the absence of the hybrid under the same assay conditions. The results are reported as percentage of inhibition.

of the DENV2 NS2B/NS3pro (>90%) was demonstrated by **8g**, with a propyl group (n =3) between them.

The *n*-propyl sulfonamide-oxadiazol-phthalimide hybrids (**14a-f)** (Fig. 1, C) also showed the alkyl-chain dependent inhibitory activity. The analogs **14c-f** containing the alkyl chain with n=3-6 demonstrated similar inhibitions (~ 60 to 70%) of the protease, indicating similar mode of binding with the protease as exhibited by **8i-j**.

Interestingly, the introduction of trifluoromethyl (CF_3) moiety in the *S*-benzyl group in **9a-c** (Fig. 1, B) enhanced the inhibition up to ~80%, regardless the position of CF_3 on the aromatic ring. The inhibitory activity of the *S*-benzylated analogs was reduced significantly, even in the presence of the CF_3 moiety in the *n*-propylsulfonamide hybrids **15a-e** (Fig. 1, D).

The hybrids showing close to or better than 70% DENV2 NS2B/NS3pro inhibition were further tested in the dose response experiments. A 3-fold serial dilution of the compounds, ranging from

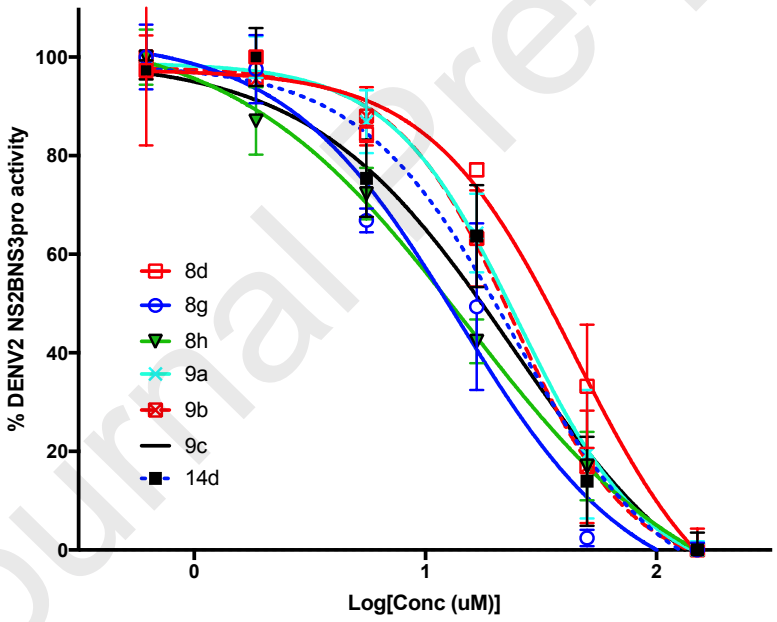


Fig. 2. Dose response of selected hybrids for the inhibition of DENV2 NS2B/NS3 protease activity. Three-fold serial dilution of selected hybrids were screen between 150μM and 620nM concentration in DENV2 NS2B/NS3pro assay in triplicate.

150μM to 620nM was screened in triplicate. The kinetic data were fit into the dose response model embedded in Graphpad Prism software (version 7.0). Half-maximal inhibitory dose of all selected hybrids was observed within a range of approximately 14μM to 45μM concentration. As

shown in Table 1 and Fig 2, **8g** and **8h** demonstrated promising inhibitory profiles with IC_{50} values of 13.9 ± 1.4μM and 15.1 ± 1.3μM, respectively. When experiments were conducted in the presence of nonionic detergent Brij35, both compounds **8g** and **8h** showed IC_{50} values of 14.1±1.5μM and 16.3±1.1μM, respectively.

Table 1.

Activity and predicted binding free energy of the selected *S*-alkylphthalimide-oxadiazole-sulfonamide inhibitor against DENV2 NS2B/NS3pro.

Compound	**Inhibition (%)[a]**	**IC_{50} (μM)**	**Docking score (kcal/mol)**
8d	71.7 ± 2.9	44.3 ± 1.5	-6.9
8g*	99.9 ± 1.3	13.9 ± 1.4 (14.1±1.5)	-9.0
8h*	77.6 ± 2.1	15.1 ± 1.3 (15.7±1.1)	-8.8
9a	83.9 ± 1.3	25.2 ± 1.2	-8.6
9b	80.4 ± 1.7	23.9 ± 1.2	-8.6
9c	80.8 ± 1.0	24.0 ± 1.6	-8.6
14d	74.8 ± 1.0	23.9 ± 1.3	-6.8
Aprotinin (control, 5 μM)	~99.8 ± 0.8	0.026 ± 0.003	-11.0

[a]Percentage of inhibition was measured in triplicate by using a single dose (50 μM) of the test compound.

*The IC_{50} value for the compound **8g** and **8h** was within 5% error when experiments were conducted in the presence of nonionic detergent Brij35 as shown in the parenthesis.

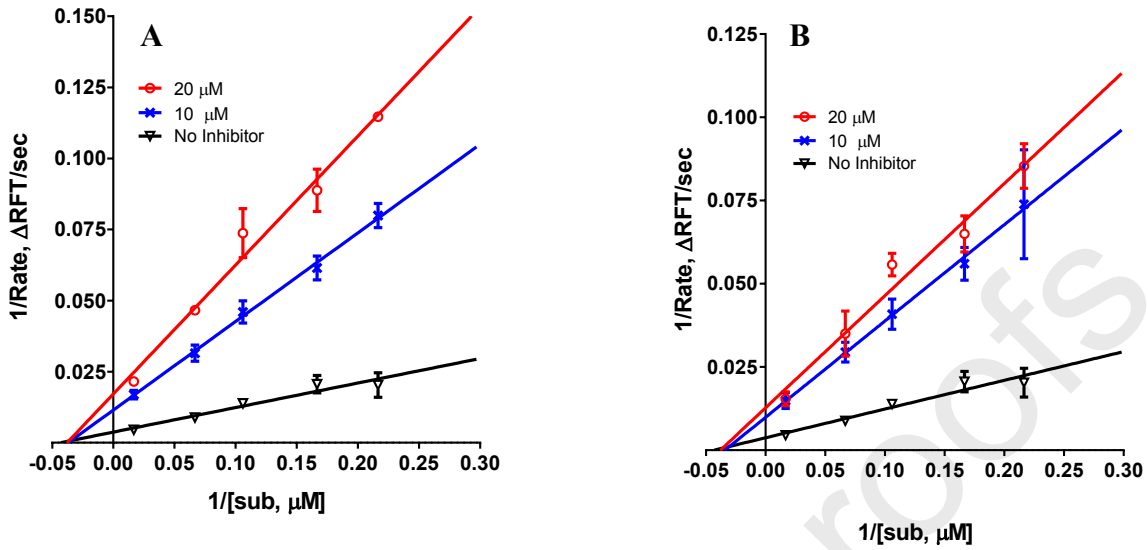


Fig 3. Mode of inhibition. Lineweaver-Burk plots showing non-competitive inhibition of DENV2 NS2B/NS3pro in the presence **of 8g (A)** and **8h (B).** Initial rates of enzymatic reactions were measured at varying concentration of the substrate (4.6-60 μM) in the absence (▽) and in the presence of 10 μM (x) and 20 μM (o) of inhibitors. Each data point is a mean of three replicates and error bars show the standard deviation of the replicates.

Mode of inhibition of the protease by **8g** and **8h** was determined by measuring the protease activity at varying concentrations (4.6-60μM) of the tetrapeptide substrate in the presence of different concentrations of the inhibitors (0, 10, 20 μM). The data obtained were fitted to the Lineweaver-Burk model and the linear plots corresponding to the different concentrations of **8g** and **8h** were found to be passing through different *y*-intercepts, but intercepting at the same point on the negative *x*-axis. This indicated further that no change in the value of K_M of the protease (31.3 ± 4.5 μM) occurred in the presence of different amounts of the inhibitors; however the V_{max} was reduced in response to the higher concentration of the inhibitor. Therefore, from the kinetics analysis, we concluded a non-competitive type inhibition by these inhibitors, with *Ki* values of 12.3 ± 3.8 μM and 13.8 ± 5.1 μM, for **8g** and **8h**, respectively (Fig 3).

To get an insight into atomic level details of binding of the synthesized inhibitors to DENV2 NS2B/NS3pro, we performed molecular docking assessments by using AutoDock Vina [54]. For this purpose, we used the recently reported co-crystal structure of DENV2 NS2B/NS3 protease (PDB ID: 6MO0) containing a bound inhibitor [55]. The docking files for the receptor (protease) and the selected inhibitors were prepared in AutoDock Tool. As shown in the Fig 4 and Table 1,

all of the tested compounds were found to bind with favorable binding energies (between -9.0 to -6.8 kcal/mol) at an allosteric allosteric cleft between two β-sheets in the open conformation of the protease. The polar sulfonamide and oxadiazol moieties of the docked structures were found to be pointing away from the cavity in the solvent exposed side. In all of the docked models, the N-atoms of the Trp-83 and Asn-167 exhibited H-bonding with the O-atoms of the carbonyl of the phthalimide moiety. However, another dipole-dipole interaction is observed between the hydroxyl group of Thr-118 and the trifluoromethyl group of **8g.** This interaction seems to be absent in **8h** and **8d**, most probably due to longer alkyl chains (n=4 in **8h**, and n=6 in **8d**). A number of residues in the cleft shown hydrophobic interactions with the non-polar parts of the inhibitors. The docking data demonstrated the synthesized inhibitors are allosteric in nature and have potential to bind at the open conformation of DENV2 protease. Similar computational binding interactions were also observed when other crystal structures of DENV2 NS2B/NS3pro (e.g., 2FOM) were used in the docking analyses (data not shown). To the best of our knowledge, all of the reported crystal structures from the DENV2 NS2B/NS3pro have an under-developed substrate binding site in the so-called open conformation. Therefore, the estimated binding interactions could be biased toward the allosteric site. To minimize this bias, we also conducted the docking analyses by using the crystal structure of DENV3 NS2B/NS3pro (3U1I), having a fully developed substrate binding site (closed conformation). With the exception of a loop comprising of Gly151 to Glu171 residues and located near the allosteric site, both the structures depicted by 3U1I and 6MO0 align very well. The loop Gly151-Glu171 seems highly dynamic in the open conformation of the protease and thus did not show significant electron density in the crystal structure 6MO0; however, it is very well resolved in the closed conformation (crystal structure 3U1I). Docking analyses of **8g** and **8h** by using the closed conformation of 3U1I showed that both of the inhibitors bind at the same allosteric site with slightly weaker binding energies (~ -8.0 kcal/mol), probably due to the closeness of the G151-Glu171 loop. Computational assessment, therefore, indicated that the inhibitors **8g** and **8h** have higher affinity towards the open conformation of the protease for binding at the allosteric site, and corroborated their non-competitive behavior in the enzyme inhibition assays. Interestingly, Yao *et al.* have very recently published an article reporting the inhibition and X-ray crystallographic data of allosteric inhibitors that also bind at the same cleft [55]. This finding demonstrated that our

synthesized hybrid inhibitors can be further developed into novel therapeutics for the treatment of DENV2 infections.

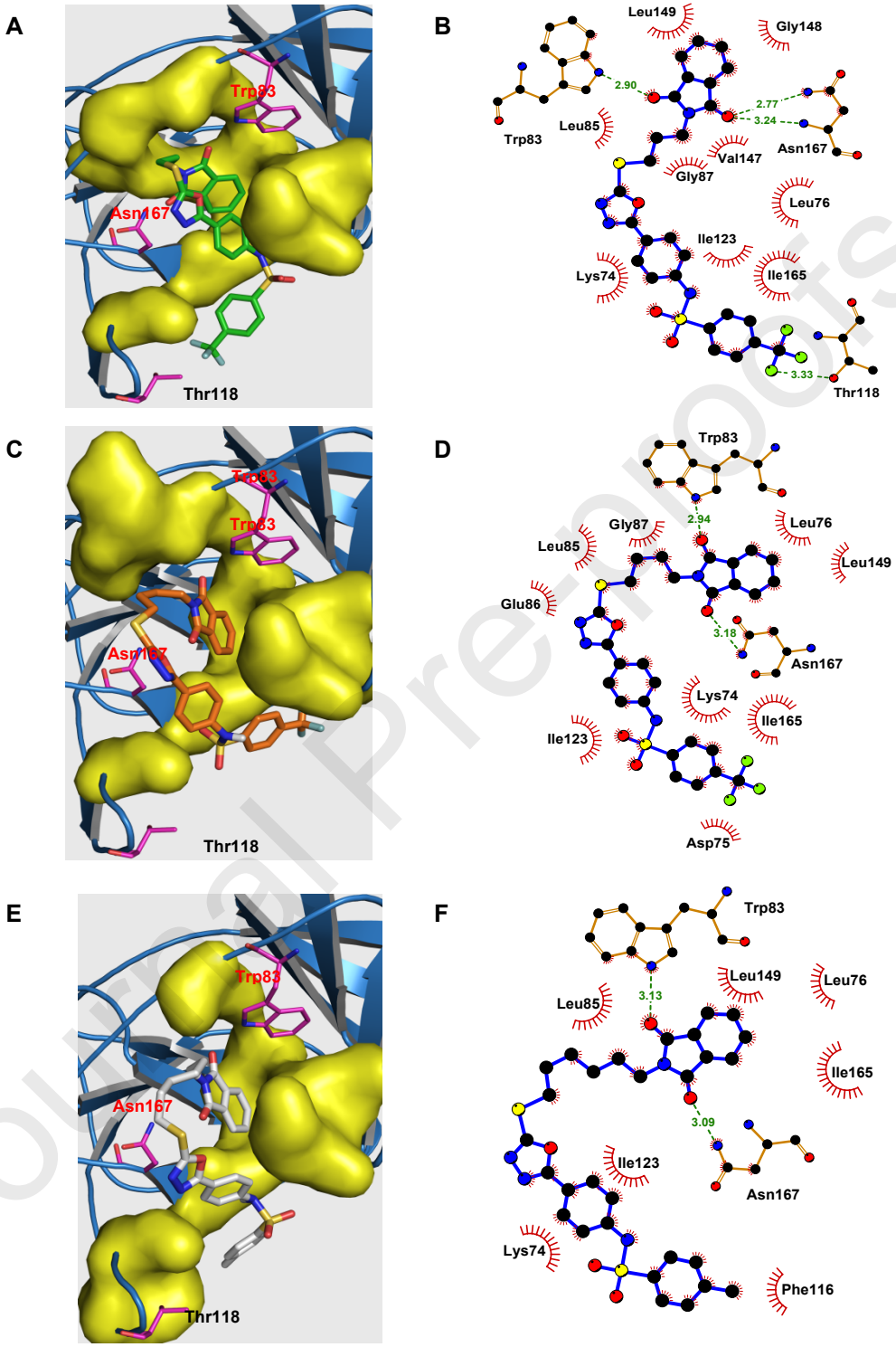


Figure 4. Molecular docking interaction of 8g, 8h, and 8d with DENV2 NS2B/NS3pro. **A,C,E**) The protease is shown as blue cartoon, the residues showing hydrophobic interactions are shown as yellow surface, and those showing H-bonding as magenta colored sticks with the docked **8g (A), 8h (C)**, and **8d (E)** as stick models. Interactions of the individual residues with **8g, 8h**, and **8d** are depicted in LigPlot and shown in **B, D,** and **F**, respectively.

3. Experimental section

3.1. Chemistry

3.1.1. General

All the laboratory grade chemicals and reagents were obtained commercially and used without further purification. Analytical grade solvents were used without drying/ distillation. Melting points were recorded in open capillaries using a Gallenkamp melting point apparatus (MP-D) and are uncorrected. All the reactions were monitored using thin layer chromatography, which was accomplished on Merck pre-coated plates (silica gel 60 F254, 0.25 mm) and visualized using fluorescence quenching under UV light (254 nm). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-300 spectrometer (300 MHz). IR spectra were recorded on Shimadzu Fourier Transform Infra-Red spectrophotometer model using ATR (Attenuated Total Reflectance). Mass spectra were recorded on a Fisons VG Autospec X double-focusing mass spectrometer.

*3.1.2. General procedure for the synthesis of 4- (4-alkylphenylsulfonamido)benzoic acid **(3a-b)***

A 250-mL single necked round bottom flask was charged with 4-aminobenzoic acid **2** (30mmol) dissolved in 40 mL of aqueous sodium carbonate (60 mmol). A solution of respective sulfonyl chloride **1a-b** (31mmol) in 25 mL of chloroform was added slowly and the mixture was stirred at room temperature for 4 hours. Aqueous layer was acidified with 2N HCl to precipitate pure **3a** and **3b**.

3a: Colorless solid; yield: 82%; R_f 0.51 (*n*-hexane:ethylacetate, 6:4); mp: 232-234°C (lit. m.p. 230-232); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 12.85 (bs, 1H, OH), 10.78 (s, 1H, NH), 7.79 (d, J = 8.7 Hz, 2H, Ar-H), 7.70 (d, J = 8.4 Hz, 2H, Ar-H), 7.35 (d, J = 8.1 Hz, 2H, Ar-H), 7.19 (d, 2H, J = 8.7 Hz, Ar-H), 2.32 (s, 3H, Ar-CH_3); IR (cm^{-1}): 3560-3400 (OH), 3320 (N-H), 3025 (C-H, aromatic), 2823 (C-H, aliphatic), 1738 (C=O), 1348, 1158 (O=S=O).

3b: Colorless solid; yield: 86%; R_f 0.34 (*n*-hexane: ethylacetate, 6:4); m.p.: 298-300°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 12.79 (bs, 1H, OH), 11.06 (bs, 1H, NH), 8.04 (d, J = 9 Hz 2H, Ar-H), 7.96 (d, J = 9H, 2H, Ar-H), 7.83 (d, J = 9 Hz, 2H, Ar-H), 7.21 (d, J = 9 Hz, 2H, Ar-H); ^{13}C NMR (75MHz, DMSO-d_6): δ ppm 167.12, 143.59, 141.83, 133.47, 133.04, 131.31, 128.14, 127.26, 127.23, 127.18, 127.12, 126.62, 125.56, 121.95, 119.09. IR (cm^{-1}): 3400 (OH), 3263 (N-H), 3020 (C-H, aromatic), 2826 (C-H, aliphatic), 1673 (C=O), 1323, 1163 (O=S=O).

3.1.3. General procedure for the synthesis of carboxylate esters **(4a-b & 11)**

The carboxylic acid **3a**, **3b** and **10** (0.02 mol) was dissolved in 30 mL of methanol and concentrated sulfuric acid (0.5 mL) and stirred at reflux temperature for 8-10 hours. The reaction mixture was concentrated at reduced pressure. The reaction mixture was neutralized with saturated aqueous sodium bicarbonate solution (150 mL) and extracted with ethyl acetate (3 x 50 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated to obtain pure products **4a**, **4b** and **11**.

4a: Colorless solid; yield: 83%; R_f 0.83 (*n*-hexane: ethylacetate, 6:4), m.p.: 204-206°C (lit. mp 202-204); ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.81 (s, 1H, NH), 7.81 (d, J = 8.7 Hz, 2H, Ar-H), 7.70 (d, J = 8.1 Hz, 2H, Ar-H), 7.35 (d, J = 8.1 Hz, 2H, Ar-H), 7.20 (d, J = 9 Hz, 2H, Ar-H), 3.77 (s, 3H, OCH_3), 2.32 (s, 3H, Ar-CH_3); IR (cm^{-1}): 3300 (NH), 3059, 3025 (C-H, aromatic), 2874 (C-H, aliphatic), 1691 (C=O), 1338 (asym), 1158 (sym) (O=S=O).

4b: Colorless solid; yield: 80%; R_f: 0.90 (*n*-hexane: ethylacetate, 6:4); m.p.: 162-163°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 11.10 (bs, 1H, NH), 8.02 (d, J = 9 Hz, 2H, Ar-H), 7.96 (d, J = 9 Hz,2H, Ar-H), 7.86-7.83 (m, 2H, Ar-H), 7.26-7.22 (m, 2H, Ar-H), 3.77 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 166.02, 143.52, 142.22, 133.51, 133.08, 131.21, 128.14, 127.27, 127.24, 127.19, 127.13, 125.55, 125.38, 119.09, 52.43; IR (cm^{-1}): 3400 (NH), 3066, (C-H, aromatic), 2950 (C-H, aliphatic), 1692 (C=O), 1349 (asym), 1162 (sym) (O=S=O).

11: Colorless solid; yield: 84%; R_f 0.77 (*n*-hexane: ethylacetate, 9:1); m.p.: 65-66°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.18-8.15 (m, 2H, Ar-H), 7.90-7.87 (m, 2H, Ar-H), 3.97 (s, 3H, OCH_3), 3.14-3.09 (m, 4H, $N(CH_2)_2$), 1.62-1.49 (m, 4H, $N(CH_2\underline{CH_2}CH_3)_2$), 0.88 (t, J = 15 Hz, 6H, $(CH_2\underline{CH_3})_2$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 165.76, 144.27, 133.41, 130.22, 127.00, 52.61, 49.89, 21.91, 11.15; IR (cm^{-1}): 3100, 3051 (C-H, aromatic), 2935, 2873 (C-H, aliphatic), 1726 (C=O), 1341 (asym), 1156 (sym) (O=S=O).

3.1.4. Synthesis of Carboxylic Acid Hydrazides **(5a**, **5b & 12)**

To the solution of carboxylate esters **4a**, **4b** and **11** (0.02 mol) in 30 mL of methanol, hydrazine hydrate (80%, 0.06 m0l) was added. The reaction mixture was subjected to reflux for 6-8 hours. The mixture was brought to the room temperature and cold water was added. The precipitated solid was filtered, dried and recrystallized from methanol to obtain pure compound.

5a: Colorless solid; yield: 78%, R_f: 0.43 (Chloroform: acetone, 9:1); m.p.: 270-272°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.58 (bs, 1H, NH), 9.60 (s, 1H, NH), 7,68 (dd, J = 8.4, 3.9 Hz, 4H, Ar-H), 7.34 (d, J = 8.4 Hz 2H, Ar-H), 7.12 (d, J = 8.7 Hz, 2H, Ar-H), 4.43 (bs, 2H, NH_2), 2.32 (s, 3H, Ar-CH_3); IR (cm^{-1}): 3340, 3290 (NH_2), 3220, 3100 (N-H), 3048, 2940 (C-H, aromatic.), 2874 (C-H, aliphatic), 1650 (C=O), 1333 (asym.), 1153 (sym.) O=S=O.

5b: Colorless solid; yield: 71%; R_f: 0.58 (Chloroform: methanol, 9:1); m.p: 236-238°C. ^{1}HNMR (300 MHz, DMSO-d_6): δ ppm: 10.59 (bs, 1H, NH), 9.63 (bs, 1H, NH), 8.02-7.95 (m, 4H, Ar-H), 7.70 (d, J = 9 Hz, 2H, Ar-H), 7.15 (d, J = 9 Hz, 2H, Ar-H), 4.45(bs, 2H, NH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 165.64, 143.69, 140.18, 133.39, 132.96, 129.39, 128.76, 128.12, 127.14, 119.36; IR (cm^{-1}): 3320, 3304 (NH_2), 3130 (N-H), 3050, (C-H, aromatic.), 2936, 2877 (C-H, aliphatic), 1666 (C=O), 1322 (asym.), 1159 (sym.) O=S=O.

12: Colorless solid; yield: 76%; R_f: 6.50 (*n*-hexane: ethyl acetate 6:4); m.p.: 116-118°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.00 (s, 1H, NH), 7.98 (d, J = 9 Hz, 2H, Ar-H), 7.86 (d, J = 9 Hz, 2H, Ar-H), 4.59 (s, 2H, NH_2), 3.04 (m, J = 15 Hz, 4H, $N(CH_2)_2$), 1.52-1.42 (m, 4H, $(CH_2\underline{CH_2}CH_3)_2$), 0.80 (6H, t, J = 15 Hz $(CH_2\underline{CH_3})_2$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 164.94, 142.06, 137.31, 128.42, 127.29, 50.09, 22.09, 11.42. IR (cm^{-1}): 3297, 3211 (NH2), 3086 (N-H), 3036 (C-H aromatic.), 2964, 2873 (C-H, aliphatic), 1658 (C=O), 1328 (asym.), 1155 (sym.) O=S=O.

3.1.5. Synthesis of 5-aryl-1,3,4-oxadiazole-2-thiols/thiones **(6a-b & 13)**

Hydrazide **5a**, **5b & 12** (0.02 mol) were dissolved in methanol and a 20-mL of methanolic solution of potassium hydroxide (0.03 mole) was added. Carbon disulfide (0.06 mol) was added dropwise after 10 mints. The yellowish reaction mixture was subjected to reflux for 12-14 hours. The mixture was cooled to room temperature and concentrated than placed in ice cold water. Crude solid product precipitated out on treatment with dilute HCl up to pH 2. The precipitate was filtered and washed with warm water and recrystallized from methanol to afford pure oxadiazoles **6a**, **6b** and **13**.

6a: Colorless solid; yield: 77%; R_f: 0.45 (Chloroform: methanol, 9:1); m.p.: 221-223°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 14.68 (s, 1H, NH), 10.83 (s, 1H, NH), 7.73 (m, 4H, Ar-H), 7.36 (d, J = 6 Hz, 2H, Ar-H), 7.27 (d, J = 9 Hz, 2H, Ar-H), 2.32 (s, 3H, Ar-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 177.65, 160.65, 144.21, 141.89, 136.79, 130.35, 127.93, 119.40,

117.75, 21.43; IR (cm^{-1}): 3232 (NH), 3061 (C-H, aromatic), 2945 (C-H, aliphatic), 1615, 1511 (C=N), 1320 (C-S), 1175 (O=S=O) [42-44].

6b: Colorless solid; yield: 70%; R_f: 2.25 (*n*-hexane:ethylacetate 6:4); m.p.: 259-260°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 14.70 (s, 1H, NH), 11.28 (s, 1H, NH), 8.00 (dd, J = 18, 9 Hz, 4H, Ar-H), 7.77 (d, J = 9 Hz, 2H, Ar-H), 7.30 (d, J = 9 Hz, 2H, Ar-H); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 177.69, 160.53, 143.51, 141.16, 133.30, 128.15, 128.08, 127.29, 127.25, 119.99, 118.42; IR (cm^{-1}): 3198 (NH), 3071 (C-H, aromatic), 1612, 1516 (C=N), 1322 (C-S), 1165 (O=S=O).

13: Colorless solid; yield: 73%; R_f: 0.47 (n-hexane:ethylacetate, 6:4); m.p.: 177-180°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 14.92 (s, 1H, NH), 8.06 (2H, d, J = 9Hz, Ar-H), 7.97 (2H, d, J = 9Hz, Ar-H), 3.06 (t, 4H, J = 15Hz, $N(CH_2)_2$), 1.46 (quin, 4H, J = 15 Hz, $CH_2\underline{CH_2}CH_3$), 0.80 (t, 6H, J = 15 Hz, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6,): δ ppm 178.12, 159.79, 142.78, 128.24, 127.49, 126.44; IR (cm^{-1}) 3068 (C-H, aromatic), 2968 (C-H, aliphatic), 1612, 1592 (C=N), 1340 (C-S), 1158 (O=S=O).

3.1.6. General method for the synthesis of S-alkyl 1,3,4- oxadiazole **(8a-j) & (9a-c)**

S-alkylation of the products was achieved by following a modified method [38]. The oxadiazoles (**6a**, **6b**, **13**) (0.02, mol) and K_2CO_3 (0.022, mol) were stirred in 10 mL of acetone for 20 mints followed by the addition of alkyl bromides **7a-c** and **7d-m** (0.022, mol). The reaction mixture was stirred for 4-6 hours at room temperature. The reaction mixture was concentrated under vacuum and crude solid gained was recrystallized from methanol to get pure products.

8a: Colorless solid, 55% yield, R_f: 0.55 (chloroform: acetone, 9:1), m.p. 224-226°C. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.71 (s, 1H, NH), 7.91-7.84 (m, 4H, Ar-H), 7.64 (dd, 4H, J = 18, 9Hz, Ar-H), 7.28 (d, 2H, J = 9 Hz, Ar-H), 7.06 (d, 2H, J = 9Hz, Ar-H), 5.28 (s, 2H, $S\underline{CH_2}N$), 2.31 (s, 3H, Ar-$\underline{CH_3}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 167.02, 166.74, 160.01, 144.00, 142.03, 140.01, 135.44, 131.76, 129.72, 127.89, 126.94, 124.06, 119.95, 118.11, 43.00, 21.37; IR (cm^{-1}) 3260 (N-H), 3025 (C-H, aromatic), 2948 (C-H, aliphatic), 1716 (C=O), 1602 (C=N), 1308, 1176 (O=S=O); Anal. Calcd for $C_{24}H_{18}N_4O_5S_2$: C, 56.91; H, 3.58; N, 11.06; O, 15.79; S, 12.66 Found: C, 56.98; H, 3.61; N, 11.11; O, 15.79; S, 12.72.

8b: Colorless solid; yield; 70 %, R_f: 0.33 (Chloroform: acetone, 9:1); m.p.; 214-216°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.81 (1H, s, NH), 7.78-7.71 (m, 8H, Ar-H), 7.37 (d, 2H, J = 8.1 Hz, Ar-H), 7.26 (d, 2H, J = 9 Hz, Ar-H), 4.02 (t, 2H, J = 6Hz, N-CH_2), 3.56 (t, 2H, J = 6Hz, S-CH_2), 2.32 (s, 3H, Ar-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.00, 165.00, 163.21, 144.13, 141.72, 136.81, 134.84, 131.83, 130.35, 128.12, 127.21, 123.50, 119.44, 118.36, 37.34, 31.12, 21.40; IR (cm^{-1}): 3268 (N-H), 3027 (C-H, aromatic), 2948 (C-H, aliphatic), 1714 (C=O), 1609, 1579 (C=N), 1331, 1152 (O=S=O); Anal. Calcd for $C_{25}H_{20}N_4O_5S_2$: C, 57.68; H, 3.87; N, 10.76; O, 15.37; S, 12.32 Found: C, 57.70; H, 3.88; N, 10.82; O, 15.38; S, 12.34.

8c: Colorless solid; yield: 78%; R_f: 0.28 (Chloroform: acetone, 9:1), m.p.: 220-223 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.78 (s, 1H, NH), 7.83-7.70 (m, 8H, Ar-H), 7.35 (d, 2H, J = Hz, Ar-H), 7.26 (d, 2H, J = 9 Hz, Ar-H), 3.59 (t, 2H, J = 12 Hz, NCH_2), 3.28 (t, 2H, J = 12 Hz, S-CH_2), 2.31 (s, 3H, Ar-CH_3), 1.75-173 (m, 4H, $CH_2\underline{CH_2}\underline{CH_2}CH_2$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.41, 165.13, 163.66, 144.14, 141.67, 136.85, 134.76, 132.01, 130.32, 128.13, 127.20, 123.41, 119.52, 118.40,37.27, 3212, 27.24, 28.18, 21.42; IR (cm^{-1}): 3240 (N-H), 3027 (C-H, aromatic), 2942 (C-H, aliphatic), 1700 (C=O), 1611, 1595 (C=N), 1338, 1192 (O=S=O); Anal. Calcd for $C_{27}H_{24}N_4O_5S_2$. C, 59.11; H, 4.41; N, 10.21; O, 14.58; S,11.69 Found: C, 59.30; H, 4.54; N, 10.31; O, 14.62; S, 11.71.

8d: Colorless solid; yield: 75%; R_f: 0.37 (Chloroform: acetone, 9:1); m.p.; 162-164 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.79 (s, 1H), 7.85-7.78 (m, 6H, Ar-H), 7.70 (d, 2H, J = 9 Hz, Ar-H), 7.34 (d, 2H, J = 9 Hz, Ar-H), 7.25 (d, 2H, J = 9 Hz, Ar-H), 3.54 (t, 2H, J = 15 Hz, NCH_2), 3.24 (t, 2H, J = 15 Hz, SCH_2), 2.31 (s 3H, Ar-CH_3), 1.73-1.66 (m, 2H, $NCH_2\underline{CH_2}$), 1.60-1.53 (m, 2H, $SCH_2\underline{CH_2}$), 1.43-1.29 (m 4H, $CH_2\underline{CH_2}\underline{CH_2}CH_2$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.40, 165.16, 163.75, 143.95, 142.19, 137.13, 134.79, 132.06, 130.26, 128.10, 127.17, 123.43, 119.58, 118.10, 37.71 32.43, 29.23, 28.21, 27.80, 26.09, 21.41; IR (cm^{-1}) 3211 (N-H), 3025 (C-H, aromatic), 2929 (C-H, aliphatic), 1705 (C=O), 1611, 1568 (C=N), 1331, 1157 (O=S=O); Anal. Calcd for $C_{29}H_{28}N_4O_5S_2$ C, 60.40; H, 4.89; N, 9.72; O, 13.87; S, 11.12 Found: C, 60.45; H, 4.94; N, 9.78; O, 13.82; S, 11.18.

8e: Colorless solid; yield: 65 %; R_f: 0.34 (Chloroform: acetone, 9:1); m.p.: 201-203°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 11.136 (s, 1H, NH), 8.06 (d, 2H, J = 8.4 Hz, Ar-H), 8.7 (d, 2H, J = 9 Hz, Ar-H), 7.89-7.83 (m, 6H, Ar-H), 7.30 (d, 2H, J = 9 Hz, Ar-H), 5.31 (s, 2H, SCH_2N); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 166.78, 166.15, 161.20, 143.51, 141.21,

135.44, 133.56, 131.72, 128.47, 128.18, 127.28, 127.24, 125.55, 124.04, 120.04, 118.90, 40.69; IR (cm^{-1}) 3241 (N-H), 3064 (C-H, aromatic), 2923 (C-H, aliphatic), 1711 (C=O), 1614, 1503 (C=N), 1350, 1165 (O=S=O); Anal. Calcd for $C_{24}H_{15}F_3N_4O_5S_2$ C, 51.43; H, 2.70; F, 10.17; N, 10.00; O, 14.27; S, 11.44 Found: C, 51.54; H, 2.78; F, 10.82; N, 10.21; O, 14.32; S, 11.51.

8f: Colorless solid; yield: 74%; R_f: 0.23 (Chloroform: acetone, 9:1) m.p.; 176-179°C; ^1H NMR (300 MHz, DMSO-d_6): δ ppm 11.09 (s,1H, NH), 8.05 (d, 2H, J = 9 Hz, Ar-H), 7.99 (d, 2H, J = 9 Hz, Ar-H), 7.81-7.70 (m, 6H, Ar-H), 7.30 (d, 2H, J = 9Hz, Ar-H), 4.02 (t, 2H, J = 12Hz, NCH_2), 3.56 (t, 2H, J = 12 Hz, SCH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.05, 164.96, 163.40, 153.52, 140.95, 134.82, 133.52, 133.09, 131.84, 128.26, 128.19, 127.29, 127.24, 123.51, 120.06, 118.91, 37.31, 31.12; IR (cm^{-1}) 3391, 3218 (N-H), 2927 (C-H, aliphatic), 1713 (C=O), 1614, 1503 (C=N), 1366, 1166 (O=S=O); Anal. Calcd for $C_{25}H_{17}F_3N_4O_5S_2$ C, 52.26; H, 2.98; F, 9.92; N, 9.75; O, 13.92; S, 11.16 Found: C, 52.31; H, 3.10; F, 9.98; N, 9.81; O, 13.96; S, 11.74.

8g: Colorless solid; yield: 75%; R_f: 0.25 (Chloroform: acetone, 9:1); m.p.170-173 °C; ^1H NMR (300 MHz, DMSO-d_6): δ ppm 11.08 (s, 1H, NH), 8.03 (d, 2H, J = 9 Hz, Ar-H), 7.97 (d, 2H, J = 9 Hz, Ar-H), 7.86-7.79 (m, 6H, Ar-H), 7.29 (d, 2H, J = 9 Hz, Ar-H), 3.71 (t, 2H, J = 12 Hz, NCH_2), 3.30 (t, 2H, J = 12 Hz, SCH_2), 2.13-2.06 (m, 2H, $SCH_2\underline{CH_2}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.50, 165.05, 163.79, 143.54, 140.91, 134.75, 133.50, 133.07, 132.18, 128.28, 128.16, 127.26, 127.21, 125.56, 123.43, 121.94, 120.11, 119.08, 36.58, 30.09, 28.67; IR (cm^{-1}): 3200, 3400 (N-H), 2930 (C-H, aliphatic), 1713 (C=O), 1608, 1503 (C=N), 1321, 1159 (O=S=O); Anal. Calcd for $C_{26}H_{19}F_3N_4O_5S_2$: C, 53.06; H, 3.25; F, 9.68; N, 9.52; O, 13.59; S, 10.90. Found C, 53.12; H, 3.32; F, 9.82; N, 9.62; O, 13.64; S, 10.95.

8h: Colorless solid; yield: 76%; R_f: 0.26 (Chloroform: acetone, 9:1); m.p.; 210-213 °C; ^1H NMR (300 MHz, DMSO-d_6): δ ppm 11.08 (s, 1H, NH), 8.04 (d, 2H, J = 8.4 Hz, Ar-H), 7.99 (d, 2H, J = 9 Hz, Ar-H), 7.83-7.75 (m, 6H, Ar-H), 7.30 (d, 2H, J = 9 Hz, Ar-H), 3.59 (t, 2H, J = 12 Hz, NCH_2), 3.28 (t, 2H, J = 12 Hz, SCH_2), 1.75-1.73 (m, 4H, $SCH_2\underline{CH_2}\underline{CH_2}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.43,165.03, 163.81, 143.54, 140.93, 134.77, 133.49, 132.02, 128.28, 128.17, 127.27, 123.42, 120.10, 119.07, 37.26, 32.12, 27.23, 26.79; IR (cm^{-1}): 3218 (N-H), 2942 (C-H, aliphatic), 1699 (C=O), 1612, 1503 (C=N), 1343, 1165 (O=S=O); Anal. Calcd for $C_{27}H_{21}F_3N_4O_5S_2$. C, 53.81; H, 3.51; F, 9.46; N, 9.30; O, 13.28; S, 10.64 Found C, 53.88; H, 3.57; F, 9.51; N, 9.35; O, 13.32; S, 10.73.

8i: Off-white solid; yield: 77%; R_f: 0.31 (Chloroform: acetone, 9:1); m.p.; 180-181 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 11.08 (s 1H, NH), 8.03 (d, 2H, J = 9 Hz, Ar-H), 7.97 (d, 2H, J = 9 Hz, Ar-H), 7.86-7.78 (m, 6H, Ar-H), 7.30 (d, 2H, J = 9 Hz, Ar-H), 3.56 (t, 2H, J = 15 Hz, NCH_2), 3.24 (t, 2H, J = 15 Hz, SCH_2), 1.76 (quin, 2H, J = 15, 9 Hz, $NCH_2\underline{CH_2}$), 1.62 (2H, quin, J = 15, 9 Hz, $SCH_2\underline{CH_2}$), 1.39 (quin, 2H, , J = 15, 9 Hz, $SCH_2CH_2\underline{CH_2}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.40, 165.00, 163.90, 143.53, 140.88, 134.79, 133.06, 132.03, 128.29, 128.16, 127.26, 127.21, 123.43, 120.12, 119.14, 37.61, 32.32, 28.94, 27.81, 25.52; IR (cm^{-1}): 3400, 3200 (N-H), 3137 (C-H, aromatic), 2945 (C-H, aliphatic), 1714 (C=O), 1606, 1569 (C=N), 1353, 1159 (O=S=O); Anal. Calcd for $C_{28}H_{23}F_3N_4O_5S_2$: C, 54.54; H, 3.76; F, 9.24; N, 9.09; O, 12.97; S, 10.40 Found C, 54.58; H, 3.82; F, 9.34; N, 9.13; O, 13.01; S, 10.68.

8j: Colorless solid; yield: 78%; R_f: 0.38 (Chloroform: acetone, 9:1); m.p.; 168-171°C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 11.07 (s, 1H, NH), 8.02 (d, 2H, J = 8.4 Hz, Ar-H), 7.96 (d, 2H, J = 9 Hz, Ar-H), 7.85-7.79 (m, 6H, Ar-H), 7.29 (d, 2H, J = 9 Hz, Ar-H), 3.54 (t, 2H, J = 15 Hz, NCH_2), 3.24 (t, 2H, J = 15 Hz, S-CH_2), 1.71 (quin, 2H, J = 15, 6 Hz, $NCH_2\underline{CH_2}$), 1.57 (quin, 2H, J = 15, 6 Hz, $SCH_2\underline{CH_2}$), 1.46-1.29 (m, 4H, $CH_2\underline{CH_2}\underline{CH_2}CH_2$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 168.40, 165.00, 163.96, 143.57, 140.94, 134.79, 132.06, 128.28, 128.15, 127.21, 125.56, 123.43, 120.13, 119.09, 37.71, 32.43, 29.22, 28.21, 27.80 26.08; IR (cm^{-1}): 3238 (N-H), 2941 (C-H, Aliph.), 1711 (C=O), 1611, 1504 (C=N), 1335, 1186 (O=S=O); Anal. Calcd for $C_{29}H_{25}F_3N_4O_5S_2$: C, 55.23; H, 4.00; F, 9.04; N, 8.88; O, 12.68; S, 10.17. Found C, 55.29; H, 4.10; F, 9.08; N, 8.91; O, 12.71; S, 10.21.

9a: Off-white solid; yield: 71%; R_f: 0.50 (Chloroform: acetone, 9:1); m.p. 164-165 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.81 (s, 1H, NH), 7.78 (d, 2H, J = 9 Hz, Ar-H), 7.70 (d, 6H, J = 9 Hz, Ar-H), 7.35 (d, 2H, J = 9 Hz, Ar-H), 7.27 (d, 2H, J = 9 Hz, Ar-H), 4.62 (s, 2H, SCH_2), 2.32 (s, 3H, Ar-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 165.44, 162.96, 144.17, 142.35, 141.75, 136.79, 130.30, 128.83, 128.41, 128.20, 127.20, 125.90, 125.85, 119.46, 118.25, 35.58, 21.41; IR (cm^{-1}) 3175 (N-H), 2950 (C-H, aliphatic), 1609 (C=N), 1347 (C-S). 1322, 1159 (O=S=O); Anal. Calcd for $C_{23}H_{18}F_3N_3O_3S_2$: C, 54.64; H, 3.59; F, 11.27; N, 8.31; O, 9.49; S, 12.69 Found C, 54.68; H, 3.62; F, 11.81; N, 8.38; O, 9.53; S, 12.74.

9b: Colorless solid; yield: 74%; R_f: 0.41 (Chloroform: acetone, 9:1); m.p.: 160-161 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.80 (s, 1H, NH), 7.85 (s, 1H, Ar-H), 7.78 (d, 3H, J = 9 Hz, Ar-H), 7.70 (d, 2H, J = 6 Hz, Ar-H), 7.63 (d, 1H, J = 6 Hz, Ar-H), 7.57 (t, 1H, J = 15 Hz,

Ar-H), 7.35 (d, 2H, *J* = 9 Hz, Ar-H), 7.26 (d, 2H, *J* = 9 Hz, Ar-H), 4.62 (s, 2H, SCH_2), 2.32 (s, 3H, Ar-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 165.43, 162.99, 144.17, 141.73, 139.10, 136.77, 133.65, 130.31, 130.11, 129.78, 129.36, 128.18, 127.20, 126.31, 126.16, 126.11, 124.89, 124.84, 122.69, 119.47, 118.28, 35.54, 21.40; IR (cm^{-1}): 3166 (N-H), 2926 (C-H, aliphatic), 1609 (C=N), 1327 (C-S). 1302, 1155 (O=S=O); Anal. Calcd for $C_{23}H_{18}F_3N_3O_3S_2$: C, 54.64; H, 3.59; F, 11.27; N, 8.31; O, 9.49; S, 12.69 Found C, 54.68; H, 3.62; F, 11.81; N, 8.38; O, 9.53; S, 12.74.

9c: Colorless solid; yield: 73%; R_f: 0.56 (Chloroform: acetone, 9:1); m.p.: 143-144°C; ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 10.82 (s, 1H, NH), 7.81-7.29 (m, 12, Ar-H), 4.68 (s, 2H, SCH_2), 2.31 (s, 3H, Ar-CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 165.63, 162.60, 144.18, 141.81, 136.78, 134.80, 133.53, 132.53, 130.33, 129.21, 128.26, 127.21, 126.79, 126.51, 119.46, 118.24, 33.59, 21.41; IR (cm^{-1}): 3205 (N-H), 2940 (C-H, aliphatic), 1611 (C=N), 1345 (C-S). 1303, 1151 (O=S=O); Anal. Calcd for $C_{23}H_{18}F_3N_3O_3S_2$: C, 54.64; H, 3.59; F, 11.27; N, 8.31; O, 9.49; S, 12.69 Found C, 54.68; H, 3.62; F, 11.81; N, 8.38; O, 9.53; S, 12.74.

14a: Off-white solid; yield: 81%; R_f: 0.46 (Chloroform: acetone, 9:1); m.p.: 154-155 °C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.20 (d, 2H, *J* = 9 Hz, Ar-H), 7.95 (d, 2H, *J* = 9 Hz, Ar-H), 7.91-7.87 (m, 2H, Ar-H), 7.82-7.77 (m, 2H, Ar-H), 5.42 (s, 2H, SCH_2N), 3.17-3.11 (m, 4H, $N\underline{CH_2}$), 1.69-1.54 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.90 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 166.45, 165.51, 162.41, 143.29, 134.75, 131.70, 127.75, 127.49, 126.77, 123.97, 49.97, 39.79, 21.99, 11.20; IR (cm^{-1}): 3025 (C-H, aromatic), 2960 (C-H, aliphatic), 1723 (C=O), 1606, 1568 (C=N), 1327, 1154 (O=S=O); Anal. Calcd for $C_{23}H_{24}N_4O_5S_2$: C, 55.18; H, 4.83; N, 11.19; O, 15.98; S, 12.81 Found C, 55.90; H, 4.88; N, 11.24; O, 16.01; S, 12.84.

14b: Colorless solid; yield: 78%; R_f: 0.49 (Chloroform: acetone, 9:1); m.p.: 131-132 °C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.15 (dd, 2H, *J* = 6.9, 5.4 Hz, Ar-H), 7.95-7.92 (m, 2H, Ar-H), 7.88-7.82 (m, 2H, Ar-H), 7.76-7.72 (m, 2H, Ar-H), 4.23 (t, 2H, *J* = 12 Hz, NCH_2), 3.64 (t, 2H, *J* = 15 Hz, SCH_2), 3.15-3.10 (m, 4H, $N\underline{CH_2}CH_2$), 1.63-1.50 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.88 (t, 6H, *J* = 5 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 168.01, 164.67, 164.25, 143.05, 134.27, 131.79, 127.69, 127.24, 126.84,123.52, 49.89, 36.62, 30.90, 21.92, 11.18; IR (cm^{-1}): 3050 (C-H, aromatic), 2968 (C-H, aliphatic), 1703 (C=O), 1614, 1549 (C=N),

1337, 1154 (O=S=O); Anal. Calcd for $C_{24}H_{26}N_4O_5S_2$: C, 56.01; H, 5.09; N, 10.89; O, 15.54; S, 12.46 Found C, 56.05; H, 5.13; N, 10.92; O, 15.62; S, 12.51.

14c: Colorless solid; yield: 79%; R_f: 0.45 (Chloroform: acetone, 9:1); m.p.: 157-158°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.13 (d, 2H, *J* = 9 Hz, Ar-H), 7.93 (d, 2H, *J* = 9 Hz, ar-H), 7.89-7.84 (m, 2H, Ar-H), 7.78-7.74 (m, 2H, Ar-H), 3.91 (t, 2H, *J* = 12 Hz, NCH_2), 3.37 (t, 2H, *J* = 15 Hz, SCH_2), 3.13 (t, 4H, *J* = 15 Hz, $N\underline{CH_2}CH_2$), 2.34-3.27 (m, 2H, $NCH_2\underline{CH_2}$), 1.63-1.50 (4H, m), 0.89 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 168.41, 165.18, 164.55, 143.00, 134.19, 131.94, 127.71, 127.17, 126.91, 123.42, 49.88, 36.49, 29.98, 28.90, 21.92, 11.19; IR (cm^{-1}): 3025 (C-H, aromatic), 2937 (C-H, aliphatic), 1708 (C=O), 1620, 1545 (C=N), 1337, 1151 (O=S=O); Anal. Calcd for $C_{25}H_{28}N_4O_5S_2$: C, 56.80; H, 5.34; N, 10.60; O, 15.13; S, 12.13 Found C, 56.84; H, 5.38; N, 10.66; O, 15.18; S, 12.16.

14d: Off-white solid; yield: 83%; R_f: 0.51 (Chloroform: acetone, 9:1); m.p.; 117-119 °C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.15-8.11 (m, 2H, Ar-H), 7.95-7.91 (m, 2H, Ar-H), 7.86-7.82 (m, 2H, Ar-H), 7.76-7.71 (m, 2H, Ar-H), 3.76 (t, 2H, *J* = 12 Hz, NCH_2), 3.38 (t, 2H, *J* = 12 Hz, SCH_2), 3.15-3.10 (m, 4H, $N\underline{CH_2}CH_2$), 1.97-1.87 (m, 4H, $NCH_2\underline{CH_2}\underline{CH_2}CH_2S$), 1.63-1.50 (m, 4H, $NCH_2\underline{CH_2}CH_3$),0.88 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*, δ ppm)168.38, 165.34, 164.47, 142.99, 134.05, 132.01, 127.70, 127.15, 126.94, 123.30, 49.88, 37.14, 31.99, 27.52, 26.52, 21.92, 11.18; IR (cm^{-1}): 3088 (C-H, aromatic), 2970 (C-H, aliphatic), 1703 (C=O), 1617, 1543 (C=N), 1332, 1160 (O=S=O); Anal. Calcd for $C_{26}H_{30}N_4O_5S_2$: C, 57.54; H, 5.57; N, 10.32; O, 14.74; S, 11.82 Found C, 57.63; H, 5.65; N, 10.38; O, 14.79; S, 11.88.

14e: Colorless solid; yield: 80%; R_f: 0.53 (Chloroform: acetone, 9:1); m.p.: 106-109°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.15-8.12 (m, 2H, Ar-H), 7.93 (dd, 2H, *J* = 6, 3 Hz, Ar-H), 7.87.7.82 (m, 2H, Ar-H), 7.76-7.71 (m, 2H, Ar-H), 3.72 (t, 2H, *J* = 15 Hz, NCH_2), 3.32 (t, 2H, *J* = 15 Hz, SCH_2), 3.15-3.10 (m, 4H, $N\underline{CH_2}CH_2CH_3$), 1.93 (2H, quin, *J* = 15, 9 Hz, $NCH_2\underline{CH_2}CH_2CH_2CH_2S$), 1.76 (2H, quin, *J* = 15, 9 Hz, $NCH_2CH_2CH_2\underline{CH_2}CH_2S$), 1.63-1.50 (m, 6H, $NCH_2CH_2\underline{CH_2}CH_2CH_2S$ and $NCH_2\underline{CH_2}CH_3$), 0.88 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 168.45, 165.55, 164.41, 142.93, 134.00, 132.06, 127.70, 127.15, 126.98, 123.25 ,49.85, 37.62, 32.36, 28.75, 28.06, 25.81, 21.92, 11.19; IR (cm^{-1}): 3088 (C-H, aromatic), 2947 (C-H, aliphatic), 1712 (C=O), 1617, 1550 (C=N), 1336, 1177 (O=S=O). Anal. Calcd for $C_{27}H_{32}N_4O_5S_2$: C, 58.25; H, 5.79; N, 10.06; O, 14.37; S, 11.52 Found C, 58.31; H, 5.84; N, 10.72; O, 14.42; S, 11.58.

14f: Colorless solid; yield: 82%; R_f: 0.55 (Chloroform: acetone, 9:1); m.p.; 124-126°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.13 (dd, 2H, *J* = 6, 3 Hz, Ar-H), 7.94-7.92 (m, 2H, Ar-H), 7.83 (dd, 2H, *J* = 6, 3 Hz, Ar-H), 7.71 (dd, 2H, *J* = 6, 3 Hz, Ar-H), 3.69 (t, 2H, *J* = 15 Hz, NCH_2), 3.31 (t, 2H, *J* = 15 Hz, SCH_2), 3.15-3.1 (m, 4H, $N\underline{CH_2}CH_2CH_3$), 1.86-1.84 (m, 2H, $NCH_2\underline{CH_2}$), 1.73-1.68 (m, 2H, $\underline{CH_2}CH_2S$), 1.60-1.52 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 1.43-1.41 (m, 4H, $NCH_2CH_2\underline{CH_2}\underline{CH_2}CH_2CH_2S$), 0.88 (t, 6H, J = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 168.46, 165.64, 164.38, 142.93, 133.94, 132.09, 127.69, 127.13, 126.99, 123.21, 49.88, 37.77, 32.50, 29.03, 28.40, 28.09, 26.25, 21.92, 11.18; IR (cm^{-1}): 3070 (C-H, aromatic), 2931 (C-H, aliphatic), 1704 (C=O), 1614, 1553 (C=N), 1340, 1153 (O=S=O). Anal. Calcd for $C_{28}H_{34}N_4O_5S_2$: C, 58.93; H, 6.00; N, 9.82; O, 14.02; S, 11.24 Found C, 58.98; H, 6.07; N, 9.89; O, 14.12; S, 11.31.

15a: Colorless solid; yield: 78%; R_f: 0.68 (Chloroform: acetone, 9:1); m.p.: 120-121°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.1 (d, 2H, *J* = 12 Hz, Ar-H), 7.93 (d, 2H, *J* = 9 Hz, Ar-H), 7.62 (s, 4H, Ar-H), 4.58 (s, 2H, SCH_2), 3.15-3.10 (m, 4H, $N\underline{CH_2}CH_2CH_3$), 1.62-1.5 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.88 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 164.80, 164.41, 143.22, 139.77, 130.58, 130.14, 129.57, 127.73, 127.16, 126.72, 125.79, 125.74, 122.10, 49.87, 35.97, 21.90, 11.16; IR (cm^{-1}): 3100 (C-H, aromatic), 2969 (C-H, aliphatic), 1617 (C=N), 1372 (C-S). 1325, 1153 (O=S=O); Anal. Calcd for $C_{22}H_{24}F_3N_3O_3S_2$: C, 52.89; H, 4.84; F, 11.41; N, 8.41; O, 9.61; S, 12.84 Found C, 52.91; H, 4.88; F, 11.47; N, 8.46; O, 9.67; S, 12.88.

15b: Colorless solid; yield: 80%; R_f: 0.68 (Chloroform: acetone, 9:1); m.p.: 93-94°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.11 (d, 2H, *J* = 9 Hz, Ar-H), 7.93 (d, 2H, *J* = 9 Hz, Ar-H), 7.74 (d, 1H, J = 6 Hz, Ar-H), 7.70 (s, 1H, Ar-H), 7.58 (d, 1H, *J* = 9 Hz, Ar-H), 7.49 (t, 1H, *J* = 18 Hz, Ar-H), 4.59 (s, 2H, SCH_2), 3.13 (t, 4H, *J* = 15 Hz, $N\underline{CH_2}CH_2CH_3$), 1.62-1.50 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.88 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 164.78, 164.41, 143.20, 136.69, 132.64, 131.42, 130.99, 129.36, 127.73, 127.18, 126.74, 125.91, 125.86, 125.08, 125.03, 122.01, 49.86, 36.08, 21.90, 11.16; IR (cm^{-1}): 2970 (C-H, aliphatic), 1613 (C=N), 1370 (C-S). 1328, 1153 (O=S=O); Anal. Calcd for $C_{22}H_{24}F_3N_3O_3S_2$: C, 52.89; H, 4.84; F, 11.41; N, 8.41; O, 9.61; S, 12.84 Found C, 52.91; H, 4.88; F, 11.47; N, 8.46; O, 9.67; S, 12.88.

15c: Colorless solid; yield: 81%; R_f: 0.89 (Chloroform: acetone, 9:1); m.p.: 99-102°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.12 (dd, 2H, *J* = 6.9, 1.5 Hz, Ar-H), 7.94 (dd, 2H, *J* = 6.9, 1.5 Hz, Ar-H), 7.82 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.70 (d, 1H, *J* = 7.8 Hz, Ar-H), 7.55 (t, 1H, *J* = 15 Hz, Ar-H), 7.45 (t, 1H, *J* = 15 Hz, Ar-H), 4.76 (s, 2H, SCH_2), 3.16-3.11 (m, 4H, $N\underline{CH_2}CH_2CH_3$), 1.65-1.50 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.88 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 165.02, 164.79, 143.17, 134.19, 132.46, 132.06, 128.93,128.50, 127.73, 127.18, 126.79, 126.53, 126.45, 126.01, 122.38, 49.88, 33.25, 21.92, 11.17; IR (cm^{-1}): 3170 (C-H, aromatic), 2969 (C-H, aliphatic), 1615 (C=N), 1370 (C-S); 1344, 1156 (O=S=O); Anal. Calcd for $C_{22}H_{24}F_3N_3O_3S_2$: C, 52.89; H, 4.84; F, 11.41; N, 8.41; O, 9.61; S, 12.84 Found C, 52.91; H, 4.88; F, 11.47; N, 8.46; O, 9.67; S, 12.88.

15d: Colorless solid; yield: 73%; R_f: 0.82 (Chloroform: acetone, 9:1); m.p.: 88-90 °C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.15 (d, 2H, *J* = 9 Hz, Ar-H), 7.99 (d, 2H, *J* = 9 Hz, AR-H), 7.49 (d, 2H, *J* = 6 Hz, Ar-H), 7.38-7.29 (m, 3H, Ar-H), 4.61 (s, 2 H, SCH_2), 3.07 (t, 4H, *J* = 15 Hz, $N\underline{CH_2}CH_2CH_3$), 1.54-1.41 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.81 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 164.75, 164.69, 142.69, 136.95, 129.55, 129.08 128.29, 127.80, 126.88, 50.05, 36.35, 22.05, 11.43; IR (cm^{-1}): 3100 (C-H, aromatic), 2964 (C-H, aliphatic), 1613 (C=N), 1373 (C-S). 1335, 1152 (O=S=O); Anal. Calcd for $C_{21}H_{25}N_3O_3S_2$: C, 58.44; H, 5.84; N, 9.74; O, 11.12; S, 14.86 Found C, 52.91; C, 58.48; H, 5.89; N, 9.77; O, 11.18; S, 14.90.

15e: Colorless solid; yield: 78%; R_f: 0.66 (Chloroform: acetone, 9:1); m.p.: 107-108°C; ^{1}H NMR (300 MHz, chloroform-*d*): δ ppm 8.23-8.20 (m, 2H, Ar-H), 8.13 (d, 2H, *J* = 9 Hz, Ar-H), 7.99 (d, 2H, *J* = 9 Hz, Ar-H), 7.78 (d, 2H, *J* = 9Hz, Ar-H), 4.73 (s, 2H, SCH_2), 3.06 (t, 4H, *J* = 5 Hz, $N\underline{CH_2}CH_2CH_3$), 1.51-1.41 (m, 4H, $NCH_2\underline{CH_2}CH_3$), 0.80 (t, 6H, *J* = 15 Hz, $NCH_2CH_2\underline{CH_3}$); ^{13}C NMR (75 MHz, chloroform-*d*): δ ppm 164.86, 164.35, 147.38, 145.38, 142.73, 130.85, 128.28, 127.83, 126.83, 124.14, 50.03,35.38, 22.03, 11.42; IR (cm^{-1}): 3170 (C-H, aromatic), 2971 (C-H, aliphatic), 1605 (C=N), 1373 (C-S). 1338, 1150 (O=S=O); Anal. Calcd for $C_{21}H_{24}N_4O_5S_2$: C, 52.93; H, 5.08; N, 11.76; O, 16.79; S, 13.46 Found C, 52.98; H, 5.14; N, 11.81; O, 16.80; S, 13.53.

3. 2. DENV-2 NS2BNS3pro assay

Recombinant DENV2 NS2B/NS3pro was prepared and characterized as described elsewhere [56]. Protease assays were performed at 37 ^{o}C in opaque 96 well plates containing a total volume of 100 µL/well of the reaction mixture. The reaction mixtures were prepared in Tris buffer (50 μM, pH 9.0) containing 10 mM NaCl, 25% glycerol, 1 mM CHAPS, and 50 mM tetrapeptide substrate, Bz-NLe-Lys-Arg-Arg-AMC. Reactions were initiated by the addition of 100nM DENV2 NS2B/NS3pro and the cleavage of the fluorophore tag, AMC was measured after every minute for a total 30 min incubation time by using a spectrofluorometer (Molecular Devices). The excitation and emission wavelengths were set at 380 and 460 nm, respectively. To avoid false-positive hits, the assays were repeated under the same conditions after replacing the CHAPS (zwitterionic detergent) by 0.005% Brij 35 (nonionic detergent). Background fluorescence of the mixture, prepared in the absence of enzyme, was measured and subtracted from those of the reaction mixtures to obtain net change in fluorescence per unit time.

3.2.1. Inhibition of DENV2 NS2B/NS3pro by the synthetic hybrids

The reaction mixture 100µL were prepared as described above in the presence of 50 μM solution (DMSO) of the test compound. Total concentration of DMSO was not more than 2.5%. Reactions were initiated by the addition of 100nM recombinant DENV2 NS2B/NS3pro and cleavage of fluorophore AMC was measured after every minute for a total 30 min. The rate of change of fluorescence (ΔRFU/sec) in the presence of the test compound (v_{exp})was compared with that in the absence of the compound (v_{con}) to calculate percent inhibition according to the following equation.

$$Inhibition = 100 - \left[\left(\frac{v_{exp}}{v_{con}}\right) \times 100\right]$$

For the determination of IC_{50} values, a three-fold serial dilutions from 150μM to 620nM of the test compounds were prepared in the reaction mixture the enzyme activity was observed as described above. The residual activity was plotted against the concentration of the test compound in Graphpad Prism (version 7) by using the non-linear regressions. For the determination of the mode of inhibition, the enzymatic assays were performed under the same conditions as described above, by varying concentration (4.6-60 μM) of the substrate in the presence (10 μM and 20 μM) and absence of the selected inhibitors. The enzyme kinetics were observed for 30 min, and the

data were linearly fit into the Lineweaver-Burk model by using Graphpad Prism software (version 7)

3.3. Molecular Docking

To investigate the binding mode of the inhibitors with the DENV2 NS2B/NS3 protease, docking studies were performed by using the reported co-crystal of DENV2 NS2/NS3pro containing an allosteric inhibitor (PDB ID: 6MO0) and DENV3 NS2B/NS3pro containing a covalently bound inhibitor at the substrate binding site (3U1I). All Het-atoms and water molecules were removed, and polar hydrogen atoms were added by using Autodock Tool. The structures of the hybrid molecules were drawn in Avogadro software and optimized by energy minimization by using MMFF94 force field. Finally, the pdbqt files were prepared for both receptor and the ligand and run on Autodock vina software. The top models (with the most negative energy) were selected for the investigation of the binding interactions by using PyMol and Ligplot.

4 Conclusion

In summary, two different series of S-benzylated and S-alklyphthalimidated hybrid structures of 1,3,4-oxadiazole and sulfonamide were synthesized in very efficient way and their potential against DENV2 NS2B/NS3pro was tested. Initial investigations have identified **8g** and **8h** are potential inhibitors of the dengue protease, demonstrating IC_{50} values as 13.9μM and 15.1μM, respectively. Computational assessment indicated that these hybrids bind in an allosteric cleft of the conformatinally open structure of the protease. Additionally, the phthalimide-moeity of the hybrid structure exhibit important hydrogen bonding interactions with Trp-83 and Asn-167 and several hydrophobic interactions with the residues in the cavity. Further work is needed to refine the binding interactions and medicinal properties before these compounds can be further developed into antiviral therapeutics.

Emails of Authors and Contributions

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All authors declare no conflict of interest.

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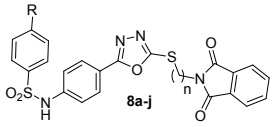
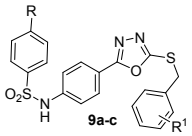
Graphical Abstract



8a: R =CH_3, n =1; **8b**: R =CH_3,n =2; **8c**: R =CH_3, n =4
8d: R =CH_3, n =6; **8e**: R =CF_3, n =1; **8f**: R =CF_3, n =2
8g: R =CF_3, n =3; **8h**: R =CF_3, n =4; **8i**: R =CF_3, n =5
8j: R =CF_3, n =6



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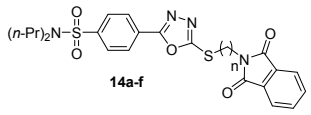
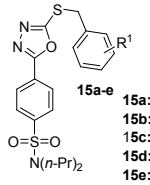
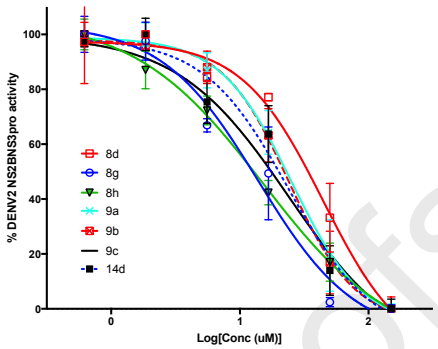
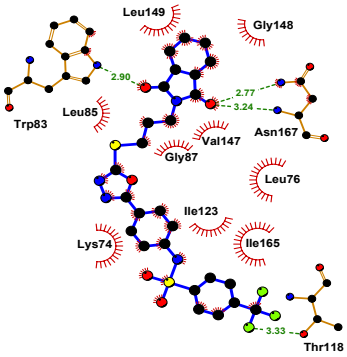


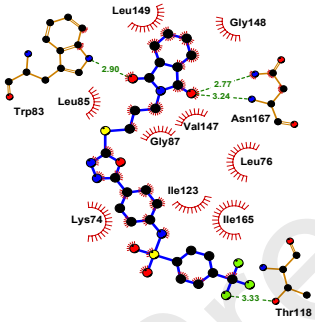
14a: n=1; **14b**: n=2; **14c**: n=3;
14d: n=4; **14e**: n=5; **14f**: n=6

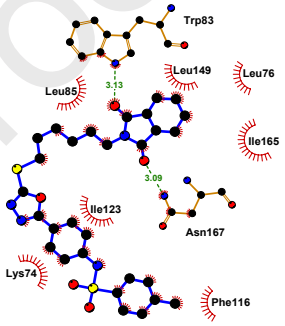


15a: R^1 =4'-CF_3
15b: R^1 =3'-CF_3
15c: R^1 =2'-CF_3
15d: R^1 =H
15e: R^1 =4'-NO_2









Highlights

A series of novel *S*-benzyl- and *S*-alkylphthalimide- oxadiazole-benzenesulfonamide hybrids was synthesized.

Bioactivity assays demonstrated that **8g** and **8h** are the most potent non-competitive inhibitors of NS2B/NS3pro among the synthesized analogs.

Computational assessment predicted the binding of the inhibitors at an allosteric site developed in the open conformation of DENV2 NS2B/NS3pro.