Computer-aided identification, synthesis and evaluation of substituted thienopyrimidines as novel inhibitors of HCV replication.

Marcella Bassetto\textsuperscript{a} \textsuperscript{1}, Pieter Leyssen\textsuperscript{b}, Johan Neyts\textsuperscript{b}, Mark M. Yerukhimovich\textsuperscript{c}, David N. Frick\textsuperscript{c}, Andrea Brancale\textsuperscript{a}

\textsuperscript{a}Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff, King Edward VII Avenue, Cardiff CF103NB, UK
\textsuperscript{b}Rega Institute for Medical Research, University of Leuven, Belgium
\textsuperscript{c}Department of Chemistry & Biochemistry, University of Wisconsin- Milwaukee, Milwaukee, Wisconsin 53211, United States

Abstract
A structure-based virtual screening technique was applied to the study of the HCV NS3 helicase, with the aim to find novel inhibitors of the HCV replication. A library of \textasciitilde450000 commercially available compounds was analysed \textit{in silico} and 21 structures were selected for biological evaluation in the HCV replicon assay. One hit characterised by a substituted thieno-pyrimidine scaffold was found to inhibit the viral replication with an EC\textsubscript{50} value in the sub-micromolar range and a good selectivity index. Different series of novel thieno-pyrimidine derivatives were designed and synthesised; several new structures showed antiviral activity in the low or sub-micromolar range.

Key words
Structure-based virtual screening; substituted thieno-pyrimidines; anti-HCV activity.

1. Introduction
Hepatitis C virus (HCV) is one of the first causes of chronic liver disease and it currently affects 130-150 million people worldwide [1]. The infection becomes chronic in 60-85\% of patients and leads to the development of hepatic steatosis, fibrosis, cirrhosis and hepatocellular carcinoma [2, 3]. A vaccine against this virus is still not available, while the standard of care was for long a combination of pegylated interferon (pegIFN) and ribavirin, a therapy not specific for HCV and efficient in 50\% of treated patients, with many associated side effects [4]. HCV single-stranded, positive-sense, 9.6 kb RNA genome encodes six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B), all essential for the viral replication [5]. In the last decade great progress has been made in the development of safe and highly potent inhibitors of HCV replication targeting the NS3 protease [6, 7], the NS5B polymerase [8], the NS4B protein [9] and the NS5A protein [10]. Even if all-oral treatment regimens of chronically infected patients with a combination therapy of either viral NS5B polymerase, NS3 protease or NS5A inhibitors are showing promising results, these treatments are associated with high costs [11, 12], while resistant viral strains have been reported for each clinically approved direct-acting antiviral [13-17]. These

\textsuperscript{1}Corresponding author
E-mail address: bassettom@cardiff.ac.uk (Dr. M. Bassetto)
limitations highlight the need for new therapeutics that will reduce the costs of treatment and avoid the development of resistance.

Despite the abundance of structural and mechanistic information on the HCV NS3 helicase [18], this enzyme is still underexploited, with very few inhibitors reported so far, none of which taken into clinical development [19]. The protein main function, essential for the viral replication [20, 21], is the unwinding of double-stranded RNA sequences, formed as an intermediate of the viral nucleic acid synthesis; this process is permitted by ATP hydrolysis [22, 23].

Due to the essential role of a functional NS3 helicase for HCV replication, this enzyme was chosen as target for the computer-aided identification and synthesis of viral replication inhibitors. The SPECS library of commercially available small molecules [24] was screened in silico on the enzyme known RNA binding cleft, leading to the selection of 21 compounds that were analysed in the HCV replicon assay. A first hit was found to inhibit the viral replication with an EC$_{50}$ in the sub-micromolar range and a good selectivity index. Its thieno-pyrimidine scaffold was the starting point for the design and synthesis of different series of new analogues, with which the main chemical features were explored. Several new derivatives were found to inhibit the HCV replication with EC$_{50}$ values in the low or sub-micromolar range.

2 Results and discussion

2.1 Structure-based virtual screening

The HCV NS3 helicase is formed by three domains and occupies the C-terminal portion of the NS3 protein. It presents multiple ligand-binding regions, the main ones being an ATP binding site in the cleft separating domain 1 from domain 2 and a single-stranded nucleic acid binding site at the interface of the three domains [25]. The potential interference with the known nucleic acid binding cleft was evaluated to screen the SPECS library of commercially available compounds [24]. The 3KQH crystal structure, which corresponds to the enzyme high-affinity open conformation bound to a single-stranded DNA substrate [26], was used for all the analyses performed. The area defined by Glu493, Asn556 and Phe557 was chosen as centre for the creation of a pharmacophoric filter, and the residues essential for the helicase unwinding activity (Thr269, Arg393, Thr411, Glu493 and Trp501) [27] were considered to build the pharmacophore using MOE 2014.10 (Figure 1) [28].
Among the approximately 450000 structures in the SPECS database, which were analysed with MOE 2014.10 conformational search tool prior to filtration [28], 3000 hits were found to match the pharmacophoric query. Molecular docking of these compounds in the 3KQH nucleic acid binding cleft was performed using Glide in the standard precision SP mode [29]. The output poses were re-scored with Glide XP [30], Plants ChemPLP [31] and FlexX [32] scoring functions, and the scoring results were combined using a previously reported consensus scoring procedure [33]. 21 derivatives were finally selected after visual inspection, purchased and tested in the HCV replicon assay (Table S1). Compound 1a (Figure 2) showed a promising antiviral effect against HCV replication, with an estimated EC$_{50}$ <1 µM and a good selectivity index >141 (Table 1).

The predicted binding mode found for this structure suggests a good spatial occupation of the target site of the HCV NS3 helicase, with the cyclohexyl portion of the molecule in close proximity to Trp501, the pyrimidine ring filling the space defined by Glu493 and Asn556, and the opportunity of hydrogen-bond formation between the 2'-hydroxyphenyl group and Arg393 lateral chain. Interestingly, 1a shows significant structural similarities with a series of previously published HCV NS3 inhibitors, QU663.
(Figure 3) and its derivatives [34]. Furthermore, the predicted binding mode suggested for these structures involves the same sub-pocket of the enzyme nucleic acid binding cleft chosen as target in this study.

![QU663](image)

**Figure 3:** Chemical structure of potent HCV NS3 helicase inhibitor QU663.

After the structure of 1a was re-synthesised and its potential to inhibit the HCV replication was confirmed (Table 1), starting from its scaffold different series of novel thieno-pyrimidine derivatives were designed, synthesised and evaluated.

### 2.2 Chemistry
The structure of 1a is characterized by a central thieno-pyrimidinic nucleus, condensed with a tetrahydrobenzene system at positions 4 and 5 of the thiophene ring, and functionalised with a 2,5-dihydroxyphenyl-ethylidene moiety via a hydrazone linker at position 4 of the pyrimidine ring. Several new derivatives were designed in order to explore the role of the aromatic substituents, the hydrazine linker and the cyclic aliphatic moiety on the thiophene portion.

A first series of differently substituted thieno[2,3-d]pyrimidin-4-yl-hydrazone was synthesised according to an optimised five-step synthetic pathway, developed from previously reported procedures (Scheme 1) [35-38].
Scheme 1: Reagents and conditions: (i) Et₃N, EtOH, reflux, 24 h (5a-e) or Et₃N, DMF, 45 °C, 1 h (5f); (ii) 10% Pd/C, PhMe, reflux, 5 d; (iii) formamide, reflux, 6 h (6a-g from 5a-g) or MeCN, HCl, 1,4-dioxane, r.t., 4 h, 100°C, 16 h (6h from 5a); (iv) N-Chlorosuccinimide, AcOH, 95 °C, 1.5 h; (v) POCl₃, reflux, 6 h; (vi) hydrazine monohydrate, MeOH, reflux, 8 h; (vii) substituted acetonaphone or benzaldehyde, EtOH, reflux, 24 h.

The general strategy started with the preparation of 2-amino-thienophene-3-carboxylic acid ethyl esters 5a-f through a Gewald reaction, which involves the condensation of a ketone or aldehyde with an α-cyanoester in the presence of elemental sulphur and a base. By treating compounds 5a-g with an excess of formamide, cyclic pyrimidinones 6a-g were obtained in good yield after crystallisation. Pyrimidinones 6a-i were chlorinated using phosphorus oxychloride to give intermediate 4-chloro-pyrimidines 7a-d, f-i.

Aromatic nucleophilic displacement of the chloride leaving group with aqueous hydrazine was then carried out to form aromatic hydrazine derivatives 8a-d, f-i, which were finally used for the formation of Schiff bases with differently substituted aromatic ketones and aldehydes, giving the final compounds 1, 9-24. With the purpose to better understand the role of the tetrahydrobenzene substituent on the thiophene ring of 1a, along with its replacement with different aliphatic functionalities, the aromatisation of this group was planned and carried out. Saturated intermediate 5a was converted into its oxidised...
analogue 5g by treatment with 10% Pd/C in toluene for 5 days [39]. As a means to explore the role of the aromatic proton in the pyrimidine ring, its replacement with a methyl group was designed and achieved by condensing aminoester intermediate 5a with acetonitrile instead of formamide. The reaction was performed in a saturated HCl solution in dioxane, under pressure in a sealed tube [40]. The mixture was sonicated in an ultrasonic bath for 4 h in order to solubilise the starting material, then heated at 100 °C for 16 h to obtain pyrimidinone 6h with a methyl group replacing the aromatic proton, after precipitation by the addition of water. With the aim to insert a chlorine substituent at position 6 of the thiophene ring, pyrimidinone 6e was treated with N-chlorosuccinimide in glacial acetic acid for 1.5 h under reflux conditions, giving the desired chlorinated product 6i, which was isolated after precipitation from water in good yield [41].

Synthetic efforts were also directed to the replacement of the hydrazone linker in the original active scaffold (Scheme 2). In particular, three different chemical functionalities were explored, by the substitution of this bond with a hydrazide, sulfonylhydrazide and amino group, respectively.

Scheme 2: Reagents and conditions: (i) 2-Pyridine-sulfonyl chloride, Pyridine, 0 °C, 1 h, r.t., 24 h; (ii) Aromatic carboxylic acid, TBTU, DiPEA, THF, r.t., 5 h; (iii) Aryl-amine, NaHCO₃, iPrOH, reflux, 96 h.

Compounds 25-36, with a hydrazide linker that confers higher stability in aqueous systems while maintaining the same length of the hydrazone substituent, were obtained through a coupling reaction between hydrazine intermediates 8a-i and aromatic carboxylic acids, using TBTU as coupling agent, stirring the reaction mixture at r.t. in THF in the presence of DIPEA. Compound 37, with a sulfonyl hydrazide linker replacing the original hydrazone group, was obtained by reacting intermediate 8a with 2-pyrindinesulfonyl chloride, stirring the two starting materials in pyridine at 0 °C for 1 h and then at r.t. for 24 h.

Finally, a small series of three amino compounds, 38-40, was obtained by aromatic nucleophilic displacement of the chloride leaving group in intermediate 7a with different aromatic amines, refluxing
the reaction mixture in iPr-OH for 96 h in the presence of NaHCO₃. In this last series of structures, the original three-atom linker is shortened and the substituted phenyl ring is replaced with a condensed bicyclic aromatic moiety. This significant modification on the original structure was designed after a series of conformational analysis studies performed on the structure of 11a, as discussed in paragraph 2.3. All newly

2.3 Conformational analysis

In order to understand the role of the 2′-hydroxy group of the hit scaffold, which seems to play a key role for the antiviral activity of the new compounds (Table 1), the Conformational Search tool in MOE2014.10 was used to analyse the conformational space of 11a in vacuum [28]. An internal hydrogen bond between the 2′-hydroxy group and the nitrogen of the hydrazone linker is predicted to stabilise the lowest energy conformation found (Figure 4).

![Figure 4: Lowest energy conformation found for 11a.](image-url)

In order to freeze this interaction, the replacement of the hydrazone bond with an aromatic amino group was envisaged. This new linker was functionalised with heteroaromatic bicyclic rings, such as 2-benzimidazole (38) and 8-quinoline (39), along with 1-naphthalene (40).

2.4 Biological activity

2.4.1 HCV replicon and cytostatic assay

All the newly synthesised thieno-pyrimidines were evaluated for their potential effect against HCV replication in the Huh5-2 replicon system (Table 1) [42]. The HCV protease inhibitor Telaprevir (VX-950) was included as positive control.

<table>
<thead>
<tr>
<th>Table 1: Antiviral effect of the test compounds on hepatitis C virus replication in the Huh5-2 replicon system.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar</td>
<td>X</td>
</tr>
<tr>
<td>R₁</td>
<td>HN</td>
</tr>
<tr>
<td>R₂</td>
<td>S</td>
</tr>
<tr>
<td>R₃</td>
<td>N</td>
</tr>
<tr>
<td>Comp.</td>
<td>R₁</td>
</tr>
<tr>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>1a</td>
<td>Me</td>
</tr>
<tr>
<td>1b</td>
<td>Me</td>
</tr>
<tr>
<td>1c</td>
<td>Me</td>
</tr>
<tr>
<td>1d</td>
<td>Me</td>
</tr>
<tr>
<td>1e</td>
<td>Me</td>
</tr>
<tr>
<td>1f</td>
<td>Me</td>
</tr>
<tr>
<td>1g</td>
<td>Me</td>
</tr>
<tr>
<td>1h</td>
<td>Me</td>
</tr>
<tr>
<td>1i</td>
<td>Me</td>
</tr>
<tr>
<td>1j</td>
<td>Me</td>
</tr>
<tr>
<td>1k</td>
<td>Me</td>
</tr>
<tr>
<td>1l</td>
<td>Me</td>
</tr>
<tr>
<td>1m</td>
<td>Me</td>
</tr>
<tr>
<td>1n</td>
<td>Me</td>
</tr>
<tr>
<td>1o</td>
<td>Me</td>
</tr>
<tr>
<td>1p</td>
<td>Me</td>
</tr>
<tr>
<td>1q</td>
<td>Me</td>
</tr>
<tr>
<td>1r</td>
<td>Me</td>
</tr>
<tr>
<td>1s</td>
<td>Me</td>
</tr>
<tr>
<td>1t</td>
<td>Me</td>
</tr>
<tr>
<td>1u</td>
<td>Me</td>
</tr>
<tr>
<td>1v</td>
<td>Me</td>
</tr>
<tr>
<td>1w</td>
<td>Me</td>
</tr>
<tr>
<td>1x</td>
<td>Me</td>
</tr>
<tr>
<td>1y</td>
<td>Me</td>
</tr>
<tr>
<td>1z</td>
<td>Me</td>
</tr>
<tr>
<td>2a</td>
<td>Me</td>
</tr>
<tr>
<td>2b</td>
<td>Me</td>
</tr>
<tr>
<td>2c</td>
<td>Me</td>
</tr>
<tr>
<td>2d</td>
<td>Me</td>
</tr>
<tr>
<td>2e</td>
<td>Me</td>
</tr>
<tr>
<td>2f</td>
<td>Me</td>
</tr>
<tr>
<td>2g</td>
<td>Me</td>
</tr>
<tr>
<td>2h</td>
<td>Me</td>
</tr>
</tbody>
</table>

**Notes:**
- **Comp.** Compounds.
- **Ar**: Substituent at aromatic ring.
- **EC₅₀** and **CC₅₀**: Concentration at 50% effect and 50% cell viability, respectively, in micromolar (µM) and nM (n.d. = not determined).
- **SF**: Selectivity factor.

**SPECS**
- Synthesis method.
The presence and position of the two hydroxyl substituents in the structure of 1a seem to play an important role for antiviral activity, since their removal is associated with loss of activity (10a). Of particular importance is the hydroxyl substitution at position 2' of the original scaffold: while 11a shows retained activity in comparison with 1a, 12a, with the sole 3'-hydroxy group, shows complete loss of antiviral potential, thus suggesting a key role for the 2'-OH. The same negative effect is observed for 13a, where the hydroxyl function is moved to the 4'-para position of the phenyl ring. Unsubstituted hydrazine 8a does not show a significant antiviral effect, while removal of the phenylethylidene methyl group (9a) is associated with an increased cytotoxicity in comparison with 1a, with lower values of CC<sub>50</sub> and SI. The role of the hydroxyl substituent in the original scaffold was further explored by its replacement with methoxy (14a) and chloro groups (15a): these modifications are associated with activity reduction, thus confirming the importance of the 2'-OH group for the antiviral properties of 1a. The next step was the exploration of different heteroaromatic rings in the ethylidene portion of the molecule (16-24a): a successful replacement of the original hydroxyl functions is achieved with the introduction of a pyrazine (16a) and 2-benzimidazole (20a), for which the modification seems to be tolerated in terms of EC<sub>50</sub>. 16a shows the best activity profile found so far in this series, while 17a and 21a, even if showing retained EC<sub>50</sub> values, seem to be mainly cytotoxic. Comparison of the biological data found for 16a and 17a with 18a, in which the 3-pyridine substituent is correlated with a dramatic
loss of antiviral potential, suggests the same trend previously found for the aromatic hydroxyl groups also in the case of the heteroaromatic nitrogen in the phenylethylidene substituent: the loss of activity found for 18a indicates the same role for a nitrogen in position 2 and a 2'-hydroxyphenyl group. Furthermore, replacement of 6-membered heteroaromatic rings with 5-membered rings (22a, 23a, 24a), is associated with loss of activity (23a, 24a) or increased cytotoxicity (22a).

A successful replacement of the original hydrazone group is achieved with the insertion of a hydrazide, as suggested by the biological data found for 25a, 26a, 28a, 29a and 32a, which all show EC\textsubscript{50} values equal or below 1 µM. 25a and 26a are also associated with good SI values and represent promising candidates for further development, while for 28a, 29a and 32a an increased cytotoxic effect can be observed. Results obtained for 27a, 30a, 31a, 34a, 35a and 36a, which are associated with a dramatic loss of activity, reveal the same trend found in the hydrazone series of compounds for the importance of the hydroxyl group at position 2 (25a and 26a), along with its potential replacement with heteroaromatic moieties with a nitrogen atom at position 2, such as pyrazine (28a) and 2-pyridine (29a), even if these modifications are associated with an increased cytotoxicity in this series of compounds. A second attempt to replace the hydrazone bond with a sulfonylhydrazide (37) led to a dramatic loss of activity. The same consideration can be extended to the replacement of the hydrazone linker with an amine function (38-40), which had been rationally designed as a means to lock the internal hydrogen bond found for 11a. For all three compounds in this series a significant loss of activity is observed, possibly suggesting that some residual flexibility is important for the biological activity associated with this scaffold.

After having confirmed the antiviral potential associated with the structure originally found and established a preliminary SAR for the phenylethylidene and hydrazone portions, the role of the tetrahydrobenzenic substituent on the thiophene ring was also explored. Six modifications were envisaged for this part of the molecule: an unsubstituted thieno-pyrimidine system, a cyclopentane ring condensed to the thiophene, an oxidised benzene substituent, two methyl groups, and finally two unsymmetrical substitutions, methyl-ethyl and methyl-chloro. Removal of the tetrahydrobenzene substituent seems to be associated with a slight decrease of activity in the hydrazone series (1f, 11f and 18f), along with an increased cytotoxicity found for 11f and 16f. In the hydrazide series this modification is mainly associated with retained activity (28f, 29f) and significantly improved SI values in comparison with the tetrahydrobenzenic counterparts (28a, 29a), even if 26f represents an exception to this trend, with a forty-fold increase in EC\textsubscript{50} in comparison with 26a. The replacement of the tetrahydrobenzene substituent with a cyclopentyl ring is mainly associated with retained activity, both in the hydrazone and hydrazide series of analogues. With the exception of 16b, which is mainly associated with a cytotoxic effect, and 1b, which shows a higher EC\textsubscript{50} value than 1a and 1c, analogues 11b, 17b, 20b, 25b, 26b, 28b and 29b are mainly associated with a retained antiviral effect, with 26b showing the most promising profile in this series in terms of both EC\textsubscript{50} and SI values. Aromatisation of the cyclohexyl ring is associated with a retained or improved antiviral activity in comparison with the original substituent, for both hydrazone and hydrazide series. With the exception of 16g and 29g, for which an increased cytotoxic effect is observed, retained or improved activity profiles are found for 1g, 11g, 20g, 25g, 26g and 28g, with EC\textsubscript{50} values in the nanomolar range for 11g and 28g, and with a particularly interesting profile found for 11g and 26g, which are associated with good SI values. These data suggest that the
rigidity of a tricyclic aromatic system in this part of the molecule is tolerated. Biological data found for 1c, 11c, 28c, 1d, 1i, 16i, 26i and 29i, in which the cyclic substituent is replaced with smaller aliphatic or halogen groups, suggest activity retention for these three modifications: both for hydrazones (1c, 11c, 1d, 11i and 16i) and hydrazides (28c, 26i, 29i) retained or improved EC$_{50}$ and SI values are found, with 1c, 11c, 1d showing the most promising profiles and three analogues (28c, 16i and 29i) reaching nanomolar EC$_{50}$ values. A final modification on the thienopyrimidine system was planned to explore the role of the aromatic proton in the pyrimidine ring of 11a, through its replacement with a methyl group. Methylation of the pyrimidine ring is associated with an inconsistent effect among the four compounds tested: while this modification seems to induce activity retention for 16h, 26h and 29h, with EC$_{50}$ values in the nanomolar range for 16h and 29h, suggesting potential for structural expansion in this part of the molecular scaffold, antiviral activity is completely lost in 11h, which bears a 2'-hydroxyphenyl group in the original hydrazone structure.

### 2.4.2 HCV NS3 helicase enzymatic assay

A selection among the newly synthesised thieno-pyrimidines was tested for any potential interference with the HCV NS3 helicase activity. In particular, interference with the enzyme unwinding activity was analysed for 41 new analogues (Table S2), while for 11a, 37, 26b, 11g and 28g additional studies were also performed, and their potential effect of the enzyme ATPase activity and binding to DNA was also assessed. Data for those compounds for which an IC$_{50}$ can be estimated are summarised in **Table 2**. Primuline [43] and aurintricarboxylic acid [44] were included as positive controls.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$(μM)</th>
<th>Unwinding</th>
<th>ATPase (w RNA)$^a$</th>
<th>ATPase (no RNA)$^a$</th>
<th>DNA binding$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>11a</td>
<td>&gt;1000</td>
<td>780 ± 50</td>
<td>760 ±40</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>31a</td>
<td>224</td>
<td>n.d</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>n.d.</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>26b</td>
<td>250</td>
<td>333</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>1f</td>
<td>229</td>
<td>n.d</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>11g</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>n.d.</td>
<td>827 ± 240</td>
<td></td>
</tr>
<tr>
<td>28g</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>n.d.</td>
<td>949 ± 130</td>
<td></td>
</tr>
<tr>
<td>26i</td>
<td>306</td>
<td>n.d</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Primuline</td>
<td>10±2</td>
<td>40±12</td>
<td>&gt;200</td>
<td>46±10</td>
<td></td>
</tr>
<tr>
<td>Aurintricarboxylic Acid</td>
<td>0.3±0.1</td>
<td>5.1±2.7</td>
<td>3.3±1.7</td>
<td>2.0±0.9</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The IC$_{50}$ values are the mean ± standard deviations of at least 2 independent experiments.

Despite the fact that 31a, 26b, 1f and 26i show some inhibition of the HCV NS3 helicase unwinding activity at high concentrations, a trend for this effect cannot be identified as it does not seem to be correlated with any specific structural feature. 26b was also found to show inhibition of the enzyme ATPase activity at high concentrations, and this effect can be observed also for 11a, while 37, 11g and 28g were identified as weak inhibitors of DNA binding. Considering all three effect examined, the observed IC$_{50}$ values are dramatically higher than the range of activities found in the HCV replicon assay.
for most of the compounds tested. The lack of correlation between the two sets of data suggests that the antiviral effect of the thieno-pyrimidine structures presented in this study is probably due to a different target, viral or cellular, other than the HCV NS3 helicase. It should also be noted that evaluation of QU663 in similar enzymatic assays did not reveal any significant interference with the helicase ATPase or unwinding activity also for this reference compound [43].

3 Conclusions
Starting from a computer-based approach, a substituted thieno-pyrimidine scaffold was identified as promising inhibitor of the HCV replication in the subgenomic replicon assay. Its structure was the starting point for the design and synthesis of different series of new analogues, with which the antiviral potential originally found was confirmed and improved. Several new derivatives were found to be associated with EC$_{50}$ values in the sub-micromolar or nanomolar range, and different among them are also associated with good SI values. Structure-activity relationships were identified for the biological effect of the scaffold originally found: the presence of a hydrazone linker is essential for antiviral activity, but this group can be successfully replaced with a more stable hydrazide. In order to retain activity, both hydrazone and hydrazide linkers need functionalisation with a six-membered aromatic ring carrying a hydroxyl group at position 2, or a heteroaromatic ring with a nitrogen atom at position 2. The presence of an aliphatic or aromatic substituent in the thienopyrimidine nucleus appears to be beneficial for activity, and suggests expansion potential in this part of the structure. The role of the pyrimidine proton has also been explored: its replacement with a methyl group is mainly associated with activity retention, suggesting also in this case the potential for structural expansion. Preliminary enzymatic evaluations suggest that the antiviral effect associated with the newly prepared compounds is mainly unrelated to the interference with the NS3 helicase activity, and other viral or cellular targets are likely involved. Understanding the biological target and the mechanism of action of these structures, along with in vitro pre-clinical studies on the most promising new analogues found (11a, 16a, 25a, 26b, 1c, 11c, 1d, 28f, 29f, 11g, 26g), will be the main focus of future studies.

4 Experimental

4.1 Synthetic chemistry methods
All solvents used for chromatography were HPLC grade from Fisher Scientific (UK). $^1$H and $^{13}$C NMR spectra were recorded with a Bruker Avance DPX500 spectrometer operating at 500 and 125 MHz, with Me$_4$Si as internal standard. Mass spectra were determined with a Bruker microTOF spectrometer using electrospray ionization (ESI source). For mass spectra, solutions were made in HPLC grade methanol. Flash column chromatography was performed with silica gel 60 (230–400mesh) (Merck) and TLC was carried out on precoated silica plates (kiesel gel 60 F$_{254}$, BDH). Compounds were visualised by illumination under UV light (254 nm). Melting points were determined on an electrothermal instrument and are uncorrected. All solvents were dried prior to use and stored over 4 Å molecular sieves, under nitrogen. All final compounds were more than 95% pure.
Intermediates 5-8 were generally prepared according to literature procedures, which are described in detail along with compound characterisation in the Supporting Information. Details for the preparation and full characterisation of the new target compounds 9-40 are given below.

4.1.1 General method for the preparation of (thieno[2,3-d]pyrimidin-4-yl)-hydrazones 1, 9-26
The different (thieno[2,3-d]pyrimidin-4-yl)-hydrazine (8a-d or f-i) (1 eq.) and arylketone or aldehyde (1.2) were dissolved in EtOH (12 mL/mmol eq.), and the mixture was refluxed for 24 h. The reaction mixture was then cooled to r.t and placed in a fridge o.n. The resulting precipitate was filtered, washed with a cold solution of 80% EtOH in water and crystallised from EtOH unless otherwise stated. Most hydrazone products show two sets of signals in NMR experiments. All 1H-NMR spectra of the new hydrazone compounds are reported for clarity in the Supporting Information.

4.1.1.1 2-[(1-[(5,6,7,8-Tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazono]-ethyl]-benzene-1,4-diol (1a)
Obtained in 66% yield as a yellow solid. M.p. >300 °C. Two sets of signals observed in NMR experiments. 1H-NMR (DMSO-d6), δ: (first set) 1.83 (broad s, 4H), 2.45 (s, 3H), 2.76 (broad s, 2H), 3.01 (broad s, 2H), 6.75 (broad s, 2H), 6.99 (broad s, 1H), 7.72 (broad s, 1H), 8.86 (broad s, 1H), 11.39 (broad s, 1H), 11.73 (broad s, 1H). 13C-NMR (DMSO-d6), δ: (first and second set) 12.9, 13.3, 22.0, 22.2, 24.7, 26.4, 113.4, 114.1, 116.9, 117.6, 118.1, 119.4, 121.0, 126.2, 130.7, 132.5, 133.8, 143.96, 145.2, 149.2, 150.9, 152.5, 153.82, 154.4, 155.6, 162.9, 165.7. MS [ESI, m/z]: 377 [M+Na]. Anal. Calcd for C18H18N4O2S: C, 61.00; H, 5.12; N, 15.81. Found: C, 61.15; H, 5.38; N, 15.91.

4.1.1.2 2-[(5,6,7,8-Tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazonomethyl]-benzene-1,4-diol (9a)
Obtained in 61% yield as a yellow solid. M.p. 235-237 °C. Two sets of signals observed in NMR experiments. 1H-NMR (CDCl3), δ: (first set) 1.75-1.88 (m, 4H), 2.71-2.86 (broad m, 2H), 2.97-3.14 (broad m, 2H), 6.71-6.76 (m, 2H), 6.92 (s, 1H), 7.19 (s, 1H), 7.76 (s, 1H), 8.48 (s, 1H), 8.91 (broad s, 1H), 9.60 (broad s, 1H), 11.48 (broad s, 1H). 13C-NMR (CDCl3), δ: (second set) 1.75-1.88 (broad m, 4H), 2.71-2.86 (broad m, 2H), 2.97-3.14 (broad m, 2H), 6.71-6.76 (m, 2H), 6.92 (s, 1H), 8.48 (s, 1H), 8.91 (broad s, 1H), 10.09 (broad s, 1H), 10.75 (broad s, 1H). 13C-NMR (CDCl3), δ: (first and second set) 21.9, 22.1, 22.3, 24.6, 25.0, 25.5, 26.5, 114.1, 114.4, 116.6, 116.9, 118.3, 118.8, 119.4, 120.4, 126.4, 130.8, 132.2, 133.1, 143.9, 146.4, 147.5, 149.8, 149.9, 152.5, 153.3, 156.9. MS [ESI, m/z]: 341 [M+H]. Anal. Calcd for C17H16N4O2S: C, 59.98; H, 5.74; N, 16.46. Found: C, 59.64; H, 5.89; N, 16.43.

4.1.1.3 N-(1-Phenyl-ethylidene)-N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (10a) [38]
Obtained in 62% yield as yellow crystals. M.p. 190-192 °C (lit. 150-152 °C [33]). Two sets of signals observed in NMR experiments. 1H-NMR (CDCl3), δ: (first set) 1.85-1.91 (m, 4H), 2.55 (s, 3H), 2.79-2.82
(m, 2H), 3.10-3.14 (m, 2H), 7.38-7.43 (m, 5H), 7.66 (s, 1), 10.36 (broad s, 1H). $^1$H-NMR (CDCl$_3$), $\delta$: (second set) 1.94-2.06 (m, 4H), 2.38 (s, 3H), 2.86-2.90 (m, 2H), 3.04-3.07 (m, 2H), 7.83-7.87 (m, 5H), 8.50 (broad s, 1H), 8.64 (s, 1H). $^1$C-NMR (CDCl$_3$), $\delta$: (first and second set) 13.5, 15.1, 22.2, 22.3, 22.6, 22.7, 25.3, 25.4, 26.6, 26.7, 120.5, 124.5, 126.5, 126.7, 128.4, 129.1, 129.2, 129.5, 129.8, 131.4, 133.7, 134.4, 135.8, 135.3, 137.9, 139.3, 141.3, 148.0, 149.2, 153.4, 156.7, 159.5. MS [ESI, m/z]: 345. [M+Na]. Anal. Calcd for C$_{18}$H$_{18}$N$_4$S: C, 67.05; H, 5.63; N, 17.38. Found: C, 67.02; H, 5.95; N, 17.37.

4.1.1.4 2-{1-[(5,6,7,8-Tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazono]-ethyl}-phenol (11a)

Obtained in 98% yield as a pale yellow solid. M.p. 250-252°C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: (first set) 1.76-1.91 (broad m, 4H), 2.49 (s, 3H), 2.73-2.85 (broad m, 2H), 2.97-3.01 (m, 2H), 6.89-6.93 (m, 2H), 7.27-7.31 (m, 1H), 7.60-7.63 (m, 1H), 7.77 (s, 1H), 11.43 (broad s, 1H), 12.50 (broad s, 1H). $^1$H-NMR (DMSO-d$_6$), $\delta$: (second set) 1.76-1.91 (broad m, 4H), 2.49 (s, 3H), 2.73-2.85 (broad m, 2H), 3.16-3.19 (broad m, 2H), 6.89-6.93 (m, 2H), 7.27-7.31 (m, 1H), 7.64-7.67 (m, 1H), 8.53 (s, 1H), 9.38 (broad s, 1H), 13.33 (broad s, 1H). $^1$C-NMR (DMSO-d$_6$), $\delta$: (first and second set) 12.9, 14.8, 22.0, 22.3, 24.6, 25.0, 26.5, 116.5, 117.2, 118.6, 119.4, 120.9, 126.3, 128.0, 128.7, 130.6, 132.6, 133.9, 144.0, 145.3, 152.6, 153.9, 156.6, 158.2, 163.2. MS [ESI, m/z]: 339 [M+H]. Anal. Calcd for C$_{18}$H$_{18}$N$_4$OS: C, 63.88; H, 5.36; N, 16.56. Found: C, 63.75; H, 5.64; N, 16.51.

4.1.1.5 3-{1-[(5,6,7,8-Tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazono]-ethyl}-phenol (12a)

Obtained in 99% yield as a light orange solid. M.p. 307-309°C. Single set of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: 1.76-1.84 (m, 4H), 2.39 (s, 3H), 2.71-2.75 (m, 2H), 2.99-3.03 (m, 2H), 6.79-6.83 (m, 1H), 7.19-7.22 (m, 1H), 7.37-7.39 (m, 1H), 7.49 (d, J= 7.9 Hz, 1H), 7.76 (s, 1H), 9.39 (broad s, 1H). $^1$C-NMR (DMSO-d$_6$), $\delta$: 14.4, 22.1, 22.3, 24.6, 26.4, 113.5, 115.9, 117.5, 119.6, 128.8, 130.8, 132.2, 140.1, 144.0, 147.2, 156.7, 157.1, 157.7. MS [ESI, m/z]: 339 [M+H]. Anal. Calcd for C$_{18}$H$_{18}$N$_4$OS: C, 63.88; H, 5.36; N, 16.56. Found: C, 63.04; H, 5.31; N, 16.66.

4.1.1.6 4-{1-[(5,6,7,8-Tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazono]-ethyl}-phenol (13a)

Obtained in 66% yield as an orange solid. M.p. 263-265°C. Single set of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: 1.74-1.83 (m, 4H), 2.39 (s, 3H), 2.71-2.75 (m, 2H), 2.99-3.03 (m, 2H), 6.80 (d, J= 8.7 Hz, 2H), 7.74 (s, 1H), 7.90 (d, J= 8.7 Hz, 2H), 9.68 (broad s, 1H), 11.48 (broad s, 1H). $^1$C-NMR (DMSO-d$_6$), $\delta$: 14.0, 22.1, 22.4, 24.6, 26.3, 114.8, 119.6, 128.1, 129.7, 130.8, 131.9, 144.0, 146.7, 156.3, 157.4, 158.5. MS [ESI, m/z]: 339 [M+H]. Anal. Calcd for C$_{18}$H$_{18}$N$_4$OS: C, 63.88; H, 5.36; N, 16.56. Found: C, 64.08; H, 5.83; N, 16.67.

4.1.1.7 4-(2-(1-(2,5-Dimethoxyphenyl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo [4,5]thieno[2,3-d]pyrimidine (14a)

Purified by crystallisation from DCM/n-hexane. Obtained in 61% yield as a grey solid. M.p. 143-145 °C.
Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): (first set) 1.57-1.63 (m, 2H), 1.68-1.77 (m, 2H), 2.29 (s, 3H), 2.69-2.75 (m, 2H), 3.75 (s, 3H), 6.91-6.95 (m, 1H), 7.06-7.12 (m, 1H), 7.18 (d, \(J= 9.0\) Hz, 1H), 8.38 (s, 1H), 8.40 (bs). \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): (second set) 1.45-1.51 (m, 2H), 1.68-1.77 (m, 2H), 2.29 (s, 3H), 2.32-2.39 (m, 2H), 2.61-2.65 (m, 2H), 3.65 (s, 3H), 6.70-6.73 (m, 1H), 6.83-6.87 (m, 1H), 6.91-6.95 (m, 1H), 7.63 (s, 1H), 11.43 (broad s, 1H). 

\(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): (first and second set) 21.7, 21.8, 22.3, 23.8, 23.9, 24.4, 24.7, 24.8, 25.8, 55.4, 55.6, 55.8, 56.2, 112.1, 113.4, 113.5, 114.3, 114.6, 115.9, 119.2, 123.4, 125.0, 129.3, 130.8, 131.3, 133.3, 143.8, 145.6, 148.8, 149.4, 150.4, 152.5, 152.7, 153.2, 153.8, 155.8, 157.8, 165.2. MS [ESI, m/z]: 383 [M+H]. Anal. Calcd for C\(_{20}\)H\(_{22}\)N\(_4\)O\(_2\)S: C, 62.80; H, 5.80; N, 14.65. Found: C, 62.48; H, 6.12; N, 14.58.

4.1.1.8 \(N\)-(1-(2,5-Dichloro-phenyl)-ethylidene)-\(N'\)-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (15a)

Purified by flash column chromatography eluting with \(n\)-hexane-EtOAc 100:0 v/v increasing to 70:30 v/v. Obtained in 46\% yield as a yellow solid. TLC (1:1 \(n\)-hexane –EtOAc, Rf: 0.57). M.p. 82-84 °C.

Single set of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): 1.76-1.84 (m, 4H), 2.38 (s, 3H), 2.74-2.77 (m, 2H), 3.01-3.04 (m, 2H), 7.49 (dd, \(J_1= 8.6\) Hz, \(J_2= 2.5\) Hz, 1H), 7.55 (d, \(J= 8.6\) Hz, 1H), 7.61 (d, \(J= 2.5\) Hz, 1H), 7.71 (s, 1H), 11.58 (broad s, 1H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): 18.4, 22.1, 22.3, 24.6, 26.3, 119.2, 129.4, 130.0, 130.3, 130.8, 131.3, 131.6, 132.4, 141.3, 143.7, 148.2, 157.2, 157.6. MS [ESI, m/z]: 391, 393 [M+H]. Anal. Calcd for C\(_{18}\)H\(_{16}\)Cl\(_2\)N\(_4\)S: C, 55.25; H, 4.12; N, 14.32. Found: C, 55.21; H, 4.23; N, 14.27.

4.1.1.9 \(N\)-(1-Pyrazin-2-yl-ethylidene)-\(N'\)-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (16a)

Obtained in 55\% yield as a yellow solid. M.p. 201-203 °C. Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): (first set) 1.86-191 (m, 4H), 2.59 (s, 3H), 2.74-2.77 (m, 2H), 3.01-3.04 (m, 2H), 7.49 (dd, \(J_1= 8.6\) Hz, \(J_2= 2.5\) Hz, 1H), 7.55 (d, \(J= 8.6\) Hz, 1H), 7.61 (d, \(J= 2.5\) Hz, 1H), 7.71 (s, 1H), 11.58 (broad s, 1H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): (second set) 18.4, 22.1, 22.3, 24.6, 26.3, 119.2, 129.4, 130.0, 130.3, 130.8, 131.3, 131.6, 132.4, 141.3, 143.7, 148.2, 157.2, 157.6. MS [ESI, m/z]: 325 [M+H]. Anal. Calcd for C\(_{16}\)H\(_{16}\)N\(_6\)S: C, 55.25; H, 4.12; N, 14.32. Found: C, 55.21; H, 4.23; N, 14.27.

4.1.1.10 \(N\)-(1-Pyridin-2-yl-ethylidene)-\(N'\)-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (17a)

Purified by crystallisation from EtOH/H\(_2\)O. Obtained in 81\% yield as an orange solid. M.p. 195-197 °C. Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\(_6\)), \(\delta\): (first set) 1.86-191 (m, 4H), 2.59 (s, 3H), 2.74-2.77 (m, 2H), 3.01-3.04 (m, 2H), 7.49 (dd, \(J_1= 1.6\) Hz, \(J_2= 5.1\) Hz, 1H), 8.46 (d, \(J= 2.7\) Hz, 1H), 8.53 (dd, \(J_1= 2.7\) Hz, \(J_2= 1.6\) Hz, 1H), 9.32 (s, 1H). \(^{13}\)C-NMR (DMSO-d\(_6\)), \(\delta\): (second set) 10.8, 13.4, 22.6, 22.7, 22.8, 25.3, 25.6, 26.6, 26.6, 116.2, 120.5, 124.4, 131.4, 134.3, 136.0, 141.1, 142.9, 143.2, 143.3, 143.4, 143.8, 147.6, 149.0, 150.4, 152.0, 153.7, 157.9, 158.3, 167.3. MS [ESI, m/z]: 325 [M+H]. Anal. Calcd for C\(_{16}\)H\(_{16}\)N\(_6\)S: C, 59.14; H, 4.79; N, 25.89. Found: C, 59.24; H, 4.97; N, 25.89. Found: C, 59.14; H, 4.79; N, 25.95.
(second set) 1.82-1.86 (m, 2H), 1.91-1.97 (m, 2H), 2.42 (s, 3H), 2.75-2.78 (m, 2H), 3.06-3.09 (m, 2H), 7.52 (dd, J = 7.8 Hz, J = 5.1 Hz, 1H), 7.74 (d, J = 8.0 Hz, 1H), 8.01-8.03 (m, 1H), 8.41 (s, 1H), 8.57-8.61 (m, 1H), 9.73 (broad s, 1H).\textsuperscript{13}C-NMR (DMSO-d\textsubscript{6}), \(\delta\): (first and second set) 13.1, 21.8, 22.1, 22.2, 22.3, 24.6, 24.9, 26.1, 26.3, 119.6, 120.8, 123.5, 124.0, 124.3, 130.8, 132.4, 132.7, 135.8, 143.8, 147.1, 148.0, 148.4, 156.0, 157.3, 158.6. MS [ESI, m/z]: 324 [M+H]. Anal. Calcd for C\textsubscript{17}H\textsubscript{17}N\textsubscript{5}S: C, 63.13; H, 5.30; N, 21.65. Found: C, 63.20; H, 5.55; N, 21.60.

4.1.1.11 4-(2-(1-(Pyridin-3-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno [2,3-d]pyrimidine (18a)
Obtained in 61% yield as yellow crystals. M.p. 188-191 °C. Single set of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\textsubscript{6}), \(\delta\): 1.76-1.83 (m, 4H), 2.46 (s, 3H), 2.73-2.76 (m, 2H), 3.01-3.05 (m, 2H), 7.42-7.45 (m, 1H), 7.80 (d, J = 3.6 Hz, 1H), 8.33-8.36 (m, 1H), 8.57 (dd, J\(_1\) = 4.7 Hz, J\(_2\) = 1.5 Hz, 1H), 9.28 (s, 1H), 11.74 (broad s, 1H). \textsuperscript{13}C-NMR (DMSO-d\textsubscript{6}), \(\delta\): 13.8, 22.1, 22.3, 24.6, 26.3, 119.5, 123.1, 130.8, 132.4, 133.6, 133.9, 143.9, 147.8, 148.0, 149.4, 155.4, 157.2. MS [ESI, m/z]: 324 [M+H]. Anal. Calcd for C\textsubscript{17}H\textsubscript{17}N\textsubscript{5}S: C, 63.13; H, 5.30; N, 21.65. Found: C, 63.32; H, 5.51; N, 21.73.

4.1.1.12 N-[1-(1H-Indol-2-yl)-ethylidene]-N'-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (19a)
Obtained in 53% yield as a yellow solid. M.p. 213-215°C. Single set of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\textsubscript{6}), \(\delta\): 1.77-1.85 (m, 4H), 2.41 (s, 3H), 2.74-2.78 (m, 2H), 3.00-3.06 (m, 2H), 6.87 (s, 1H), 7.01-7.03 (m, 1H), 7.18-7.20 (m, 1H), 7.44 (d, J = 7.9 Hz, 1H), 7.96 (s, 1H), 11.35 (broad s, 1H), 11.98 (broad s, 1H). \textsuperscript{13}C-NMR (DMSO-d\textsubscript{6}), \(\delta\): 13.5, 22.1, 22.3, 24.6, 26.3, 103.3, 110.9, 119.1, 119.6, 120.7, 123.0, 128.1, 130.8, 132.4, 136.8, 137.7, 143.5, 147.9, 150.3, 156.9. MS [ESI, m/z]: 362 [M+H]. Anal. Calcd for C\textsubscript{20}H\textsubscript{19}N\textsubscript{5}S: C, 66.46; H, 5.30; N, 19.37. Found: C, 66.74; H, 5.51; N, 19.27.

4.1.1.13 N-[1-(1H-Benzimidazol-2-yl)-ethylidene]-N'-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazine (20a)
Obtained in 42% yield as a light brown solid. M.p. 287-289 °C. Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-d\textsubscript{6}), \(\delta\): (first set) 1.74-1.84 (m, 4H), 7.17-7.23 (m, 1H), 7.25-7.31 (m, 1H), 7.54-7.61 (m, 1H), 7.63-7.73 (m, 1H), 8.04 (s, 1H), 12.10 (broad s, 1H), 12.57 (broad s, 1H). \(^1\)H-NMR (DMSO-d\textsubscript{6}), \(\delta\): (second set) 1.91-1.96 (m, 2H), 2.02-2.10 (m, 2H), 2.83-2.87 (m, 2H), 3.42-3.46 (m, 2H), 4.08 (s, 3H), 7.36-7.43 (m, 2H), 7.63-7.73 (m, 2H), 8.50 (s, 1H), 13.03 (broad s, 1H), 14.31 (broad s, 1H). \textsuperscript{13}C-NMR (DMSO-d\textsubscript{6}), \(\delta\): (first and second set) 13.1, 22.0, 22.3, 24.6, 26.2, 48.5, 111.0, 119.3, 119.5, 121.4, 123.5, 130.8, 132.9, 133.5, 143.4, 143.7, 149.2, 149.7, 151.7, 157.9. MS [ESI, m/z]: 363 [M+H]. Anal. Calcd for C\textsubscript{20}H\textsubscript{19}N\textsubscript{6}S: C, 62.96; H, 5.01; N, 23.19. Found: C, 62.85; H, 4.85; N, 23.37.

4.1.1.14 4-(2-(1-(Quinolin-2-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno [2,3-d]pyrimidine (21a)
Purified by crystallisation from EtOH/H₂O. Obtained in 71% yield as an orange solid. M.p. 21-123 °C. Single set of signals observed in NMR experiments. ¹H-NMR (DMSO-d₆), δ: 1.79-1.83 (m, 4H), 2.62 (s, 3H), 2.74-2.78 (m, 2H), 3.05-3.09 (m, 2H), 7.58-7.61 (m, 1H), 7.74-7.78 (m, 1H), 7.89 (s, 1H), 7.94 (d, J= 8.3 Hz, 1H), 8.04 (d, J= 8.3 Hz, 1H), 8.34 (d, J= 8.7 Hz, 1H), 8.77 (d, J= 8.7 Hz, 1H), 11.90 (broad s, 1H). ¹³C-NMR (DMSO-d₆), δ: 12.9, 22.1, 22.3, 24.7, 26.3, 119.2, 121.9, 126.6, 127.6, 127.7, 129.0, 129.4, 130.9, 132.7, 135.2, 143.8, 146.9, 148.4, 156.2, 158.0, 159.1. MS [ESI, m/z]: 374 [M+H]. Anal. Calcd for C₂₁H₁₉N₅S: C, 67.53; H, 5.13; N, 18.75. Found: C, 67.31; H, 5.36; N, 18.62.

4.1.1.15 4-(2-(1H-Pyrrol-2-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (22a)
Obtained in 62% yield as a yellow solid. M.p. 84-89 °C. Two sets of signals observed in NMR experiments. ¹H-NMR (DMSO-d₆), δ: (first set) 1.76-1.86 (broad m, 4H), 2.27 (s, 3H), 2.71-2.75 (broad m, 2H), 2.97-3.01 (broad m, 2H), 6.11-6.14 (m, 1H), 6.47-6.49 (m, 1H), 7.00-7.02 (m, 1H), 7.82 (s, 1H), 11.31 (broad s, 1H), 11.80 (broad s, 1H). ¹³C-NMR (DMSO-d₆), δ: (first and second set) 13.4, 22.1, 22.4, 24.6, 26.2, 108.7, 110.2, 119.7, 120.7, 130.8, 131.8, 131.9, 143.6, 146.8, 150.4, 156.1. MS [ESI, m/z]: 312 [M+H]. Anal. Calcd for C₁₆H₁₇N₅S: C, 61.71; H, 5.50; N, 22.49. Found: C, 61.57; H, 5.38; N, 22.59.

4.1.1.16 4-(2-(1-(Furan-2-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (23a)
Obtained in 36% yield as a yellow solid. M.p. 134-139 °C. Single set of signals observed in NMR experiments. ¹H-NMR (DMSO-d₆), δ: 1.75-1.82 (m, 4H), 2.33 (s, 3H), 2.72-2.75 (m, 2H), 2.98-3.01 (m, 2H), 6.63 (dd, J₁= 3.4 Hz, J₂= 1.7 Hz, 1H), 7.08 (d, J= 3.4 Hz, 1H), 7.66-7.79 (m, 2H), 11.47 (broad s, 1H). ¹³C-NMR (DMSO-d₆), δ: 13.7, 22.1, 22.3, 24.6, 26.3, 108.7, 110.2, 119.7, 120.7, 130.8, 131.8, 131.9, 143.6, 146.8, 150.4, 156.1. MS [ESI, m/z]: 313 [M+H]. Anal. Calcd for C₁₆H₁₆N₄OS: C, 61.52; H, 4.97; N, 17.52.

4.1.1.17 4-(2-(1-(Thiophen-2-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (24a)
Obtained in 44% yield as yellow crystals. M.p. 129-132 °C. Single set of signals observed in NMR experiments. ¹H-NMR (DMSO-d₆), δ: 1.76-1.83 (m, 4H), 2.42 (s, 3H), 2.72-2.75 (m, 2H), 2.98-3.01 (m, 2H), 7.11 (dd, J₁= 5.2 Hz, J₂= 3.7 Hz, 1H), 7.51 (dd, J₁= 3.7 Hz, J₂= 0.9 Hz, 1H), 7.58 (dd, J₁= 5.2 Hz, J₂= 0.9 Hz, 1H), 7.80 (s, 1H), 11.24 (broad s, 1H). ¹³C-NMR (DMSO-d₆), δ: 14.9, 22.1, 22.3, 24.6, 26.3, 119.5, 127.0, 127.5, 127.9, 130.8, 132.3, 144.0, 144.4, 146.8, 153.7, 156.7. MS [ESI, m/z]: 329 [M+H]. Anal. Calcd for C₁₆H₁₆N₅S₂ (328.4): C, 58.51; H, 4.91; N, 17.06. Found: C, 58.70; H, 5.17; N, 17.15.

4.1.1.18 2-[1-[(2,3-Dihydro-1H-8-thia-5,7-diaza-cyclopenta[a]inden-4-yl)-hydrazono]-ethyl]-benzene-1,4-diol (1b)
Obtained in 74% yield as a yellow solid. M.p. 308-310 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-$d_6$), $\delta$: (first set) 2.41 (s, 3H), 2.44-2.49 (m, 2H), 6.74-6.76 (m, 2H), 6.98-7.00 (m, 1H), 8.52 (s, 1H), 8.88 (broad s, 1H), 9.51 (broad s, 1H), 12.38 (broad s, 1H). $^1$H-NMR (DMSO-$d_6$), $\delta$: (second set) 2.41 (s, 3H), 2.44-2.49 (m, 2H), 6.74-6.76 (m, 2H), 6.98-7.00 (m, 1H), 7.73 (s, 1H), 8.88 (broad s, 1H), 11.54 (broad s, 1H), 11.79 (broad s, 1H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$: (first and second set) 13.0, 14.4, 27.3, 28.6, 29.0, 29.3, 29.5, 112.9, 113.5, 114.1, 116.9, 117.6, 118.0, 118.2, 119.7, 135.4, 139.2, 143.8, 144.7, 149.1, 151.0, 152.5, 153.5, 154.9, 171.3. MS [ESI, m/z]: 341 [M+H]. Anal. Calcd for C$_{17}$H$_{16}$N$_4$O$_2$S: C, 59.98; H, 4.74; N, 16.46. Found: C, 59.71; H, 5.01; N, 16.39.

4.1.1.19 2-{1 -[(2,3-Dihydro-1$H$-8-thia-5,7-diaza-cyclopenta[a]inden-4-yl)-hydrazono]-ethyl}-phenol (11b)

Obtained in 71% yield as a pale yellow solid. M.p. 267-269 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-$d_6$), $\delta$: (first set) 2.37-2.48 (broad m, 5H), 2.98-3.07 (broad m, 2H), 3.18-3.25 (broad m, 2H), 6.88-6.92 (m, 2H), 7.27-7.32 (m, 1H), 7.59-7.67 (m, 1H), 8.54 (s, 1H), 9.59 (broad s, 1H), 13.16 (broad s, 1H). $^1$H-NMR (DMSO-$d_6$), $\delta$: (second set) 2.37-2.48 (broad m, 5H), 2.90-2.94 (broad m, 2H), 2.98-3.07 (broad m, 2H), 6.88-6.92 (m, 2H), 7.27-7.32 (m, 1H), 7.59-7.67 (m, 1H), 7.75 (s, 1H), 11.49 (broad s, 1H), 12.55 (broad s, 1H). $^{13}$C-NMR (DMSO-$d_6$), $\delta$: (first and second set) 13.0, 14.4, 27.3, 28.6, 29.1, 29.3, 29.5, 112.9, 113.5, 114.1, 116.9, 117.6, 118.0, 118.2, 119.7, 135.4, 139.2, 143.8, 144.7, 149.1, 151.0, 152.5, 153.5, 154.9, 171.3. MS [ESI, m/z]: 325 [M+H]. Anal. Calcd for C$_{17}$H$_{16}$N$_4$OS: C, 62.94; H, 4.97; N, 17.26. Found: C, 62.92; H, 4.83; N, 17.36.

4.1.1.20 4-(2 - (1 -(Pyrazin-2-yl)ethylidene)hydrazinyl)-6,7-dihydro-5$H$-cyclopenta[4,5] thieno[2,3-d]pyrimidine (16b)

Obtained in 42% yield as a yellow solid. M.p. 182-185 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (CDCl$_3$), $\delta$: (first set) 2.48-2.56 (broad m, 5H), 3.05-3.09 (m, 2H), 3.11-3.17 (m, 2H), 3.31-3.35 (m, 2H), 7.40-7.43 (m, 1H), 7.62-7.64 (m, 1H), 7.91-7.95 (m, 1H), 8.65 (s, 1H), 8.68-8.70 (m, 1H), 8.53-8.56 (m, 1H), 9.34 (d, J= 1.4 Hz, 1H), 10.39 (broad s, 1H). $^1$H-NMR (CDCl$_3$), $\delta$: (second set) 2.48-2.56 (m, 2H), 2.61 (s, 3H), 2.97-3.01 (m, 2H), 3.11-3.17 (m, 2H), 3.12-3.16 (m, 2H), 7.75 (s, 1H), 8.49 (d, J= 2.5 Hz, 1H), 8.53-8.56 (m, 1H), 8.53-8.56 (m, 1H), 9.34 (d, J= 1.4 Hz, 1H), 10.39 (broad s, 1H). $^{13}$C-NMR (CDCl$_3$), $\delta$: (first and second set) 10.6, 13.0, 27.9, 28.1, 29.7, 29.8, 29.9, 30.9, 113.5, 134.8, 140.8, 141.2, 142.9, 143.0, 143.2, 143.3, 143.4, 143.7, 147.3, 150.5, 152.3, 153.8, 173.6. MS [ESI, m/z]: 311 [M+H]. Anal. Calcd for C$_{15}$H$_{14}$N$_6$S: C, 58.05; H, 4.55; N, 27.08. Found: C, 57.91; H, 4.89; N, 27.05.

4.1.1.21 4-(2 - (1 -(Pyridin-2-yl)ethylidene)hydrazinyl)-6,7-dihydro-5$H$-cyclopenta[4,5] thieno[2,3-d]pyrimidine (17b)

Obtained in 36% yield as an orange solid. M.p. 155-157 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (CDCl$_3$), $\delta$: (first set) 2.59 (s, 3H), 2.60-2.65 (m, 2H), 3.04-3.08 (m, 2H), 3.31-3.35 (m, 2H), 4.70-4.73 (m, 1H), 7.40-7.44 (m, 1H), 7.62-7.64 (m, 1H), 7.91-7.95 (m, 1H), 8.65 (s, 1H), 8.68-8.70 (m, 1H), 15.07 (broad s, 1H). $^1$H-NMR (CDCl$_3$), $\delta$: (second set) 2.54 (s, 3H), 2.60-2.65 (m, 2H), 3.04-3.08 (m, 2H), 3.12-3.16 (m, 2H), 7.27-7.29 (m, 1H), 7.71-7.75 (m, 1H), 8.20-8.22 (m, 1H), 8.48 (broad s, 1H),
8.61 (s, 1H), 8.62-8.63 (m, 1H). $^{13}$C-NMR (CDCl$_3$), $\delta$: (first and second set) 10.9, 22.6, 27.9, 29.8, 29.8, 30.5, 113.5, 121.2, 123.6, 123.7, 134.9, 136.2, 137.7, 139.4, 139.8, 140.6, 146.9, 148.5, 149.7, 152.6, 153.4, 153.6, 153.9, 155.0, 172.4. MS [ESI, m/z]: 310 [M+H]. Anal. Calcd for C$_{16}$H$_{15}$N$_5$S: C, 62.11; H, 4.89; N, 22.64. Found: C, 61.97; H, 5.02; N, 22.59.

4.1.1.22 4-(2-((1H-Benzol[d]imidazol-2-yl)ethyliedene)hydrazinyl)-6,7-dihydro-5$^H$-cyclopenta[4,5]thieno[2,3-d]pyrimidine (20b)
Obtained in 58% yield as a light brown solid. M.p. charring > 230 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: (first set) 2.36-2.42 (m, 2H), 2.52 (s, 3H), 2.89-2.94 (m, 2H), 2.99-3.07 (m, 2H), 7.16-7.24 (m, 2H), 7.52-7.60 (m, 1H), 7.62-7.71 (m, 1H), 8.04 (s, 1H), 12.09 (broad s, 1H), 12.54 (broad s, 1H). $^1$H-NMR (DMSO-d$_6$), $\delta$: (second set) 2.29-2.33 (m, 2H), 2.52 (s, 3H), 2.89-2.94 (m, 2H), 2.99-3.07 (m, 2H), 7.35-7.41 (m, 2H), 7.62-7.71 (m, 1H), 7.73-7.76 (m, 1H), 8.47 (s, 1H), 13.10 (broad s, 1H), 14.40 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: (first and second set) 12.6, 18.5, 27.1, 29.3, 29.4, 29.7, 111.1, 111.9, 113.7, 117.2, 118.7, 119.2, 119.3, 121.4, 123.4, 124.5, 132.3, 137.5, 139.7, 141.6, 143.3, 143.4, 143.5, 148.6, 151.8, 155.2, 172.7. MS [ESI, m/z]: 349 [M+H]. Anal. Calcd for C$_{18}$H$_{16}$N$_6$S: C, 62.05; H, 4.63; N, 24.12. Found: C, 61.89; H, 4.51; N, 24.33.

4.1.1.23 2-(1-((2-(5,6-Dimethylthieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)benzene-1,4-diol (1c)
Obtained in 71% yield as a yellow solid. M.p. > 300 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: (first set) 2.36 (s, 3H), 2.47 (s, 3H), 2.49 (s, 3H), 6.73-6.77 (m, 2H), 6.97-7.02 (m, 1H), 7.74 (s, 1H, H-2), 8.87 (broad s, 1H), 11.36 (broad s, 1H), 11.71 (broad s, 1H). $^1$H-NMR (DMSO-d$_6$), $\delta$: (second set) 2.47 (s, 3H), 2.49 (s, 3H), 2.61 (s, 3H), 6.73-6.77 (m, 2H), 6.97-7.02 (m, 1H), 8.52 (s, 1H), 8.87 (broad s, 1H), 9.51 (broad s, 1H), 12.60 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: (first and second set) 12.8, 14.1, 15.0, 112.5, 114.2, 116.9, 118.1, 121.1, 128.8, 129.5, 143.8, 144.6, 149.2, 150.8, 155.1, 163.0. MS [ESI, m/z]: 329 [M+H]. Anal. Calcd for C$_{16}$H$_{16}$N$_4$O$_2$S: C, 58.52; H, 4.63; N, 24.12. Found: C, 61.89; H, 4.51; N, 24.33.

4.1.1.24 2-(1-((2-(5,6-Dimethylthieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)phenol (11c)
Obtained in 54% yield as a pale yellow solid. M.p. 222-226 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), $\delta$: (first set) 2.36 (s, 3H), 2.47 (s, 3H), 2.53 (s, 3H), 6.89-6.93 (m, 2H), 7.27-7.30 (m, 1H), 7.61 (d, J = 7.5 Hz, 1H), 7.75 (s, 1H), 11.38 (broad s, 1H), 12.49 (broad s, 1H). $^1$H-NMR (DMSO-d$_6$), $\delta$: (second set) 2.47 (s, 3H), 2.53 (s, 3H), 2.61 (s, 3H), 6.89-6.93 (m, 2H), 7.27-7.30 (m, 1H), 7.64-7.67 (m, 1H), 8.54 (s, 1H), 9.54 (broad s, 1H), 13.37 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), $\delta$: (first and second set) 12.8, 13.0, 13.2, 13.5, 14.1, 14.9, 116.5, 117.2, 118.5, 118.6, 119.4, 120.1, 121.0, 124.2, 128.0, 128.7, 128.8, 129.5, 130.6, 143.8, 145.4, 152.5, 153.9, 155.3, 155.8, 158.1, 158.4, 163.3, 165.1. MS [ESI, m/z]: 313 [M+H]. Anal. Calcd for C$_{16}$H$_{16}$N$_4$O$_2$: C, 58.52; H, 4.91; N, 17.06. Found: C, 58.46; H, 4.89; N, 17.25.

4.1.1.25 2-(1-(2-(6-Ethyl-5-methylthieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)benzene-1,4-diol (1d)
Obtained in 53% yield as a yellow solid. M.p. 235-237 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), δ: (first set) 1.22 (t, J = 7.4 Hz, 3H), 2.46 (s, 3H), 2.52 (s, 3H), 2.78 (q, J = 7.4 Hz, 2H), 6.73-6.76 (m, 2H), 6.97-6.99 (m, 1H), 7.74 (s, 1H), 8.87 (broad s, 1H), 11.36 (broad s, 1H), 11.70 (broad s, 1H). $^1$H-NMR (DMSO-d$_6$), δ: (second set) 1.26 (t, J = 7.4 Hz, 3H), 2.45 (s, 3H), 2.63 (s, 3H), 2.89 (q, J = 7.4 Hz, 2H), 6.73-6.76 (m, 2H), 7.00-7.02 (m, 1H), 8.53 (s, 1H), 8.87 (broad s, 1H), 9.53 (broad s, 1H), 12.60 (broad s, 1H).

$^{13}$C-NMR (DMSO-d$_6$), δ: (first and second set) 14.0, 15.0, 15.3, 20.6, 114.2, 116.9, 118.0, 120.7, 121.1, 127.9, 137.0, 143.8, 144.7, 149.2, 150.8, 155.2, 163.0. MS [ESI, m/z]: 343 [M+H]. Anal. Calcd for C$_{17}$H$_{18}$N$_4$O$_2$S: C, 59.63; H, 5.30; N, 16.35. Found: C, 59.69; H, 5.31; N, 16.51.

4.1.1.26 2-(1-([Thieno[2,3-d]pyrimidin-4-yl]-hydrazono)-ethyl)-benzene-1,4-diol (1f)
Obtained in 81% yield as a yellow solid. M.p. 267-269 °C. Single set of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), δ: 2.47 (s, 3H), 6.75-6.76 (m, 2H), 6.98-7.00 (m, 1H), 7.73 (d, J = 5.8 Hz, 1H), 7.93 (d, J = 5.8 Hz, 1H), 7.93 (broad s, 1H), 8.90 (s, 1H), 10.74 (broad s, 1H), 12.10 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), δ: 14.3, 113.4, 115.3, 117.5, 118.3, 120.8, 121.2, 123.9, 149.2, 150.6, 152.9, 153.3, 154.4, 167.4. MS [ESI, m/z]: 301 [M+H]. Anal. Calcd for C$_{14}$H$_{12}$N$_4$O$_2$: C, 55.99; H, 4.03; N, 18.65. Found: C, 56.13; H, 4.33; N, 18.71.

4.1.1.27 2-(1-([Thieno[2,3-d]pyrimidin-4-yl]-hydrazono)-ethyl)-phenol (11f)
Obtained in 61% yield as pale yellow crystals. M.p. 178-180 °C. Single set of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), δ: 2.54 (s, 3H), 6.89-6.94 (m, 2H), 7.27-7.31 (m, 1H), 7.61 (dd, J$_1$ = 7.9 Hz, J$_2$ = 1.3 Hz, 1H), 7.72-7.76 (m, 1H), 7.91-7.95 (m, 1H), 8.60 (s, 1H), 10.79 (broad s, 1H), 12.93 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), δ: 14.2, 115.4, 117.0, 118.5, 120.1, 120.5, 124.0, 128.2, 130.7, 153.3, 154.2, 154.7, 158.0, 167.3. MS [ESI, m/z]: 285 [M+H]. Anal. Calcd for C$_{14}$H$_{12}$N$_2$O$_2$: C, 59.14; H, 4.25; N, 19.70. Found: C, 58.91; H, 5.51; N, 19.64.

4.1.1.28 4-(2-([Pyrazin-2-yl]ethylidene)hydrazinyl)thieno[2,3-d]pyrimidine (16f)
Obtained in 96% yield as a white solid. M.p. 239-243 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), δ: (first set) 2.52 (s, 3H), 7.79 (d, J = 5.9 Hz, 1H), 8.08 (d, J = 5.9 Hz, 1H), 8.60 (s, 1H), 8.64-8.67 (m, 1H), 8.69-8.70 (m, 1H), 9.26 (s, 1H). $^1$H-NMR (DMSO-d$_6$), δ: (second set) 2.52 (s, 3H), 7.47 (d, J = 5.3 Hz, 1H), 7.62 (d, J = 5.3 Hz, 1H), 7.98-8.00 (m, 1H), 8.60 (s, 1H), 8.64-8.67 (m, 1H), 9.81 (s, 1H), 12.06 (broad s, 1H). $^{13}$C-NMR (DMSO-d$_6$), δ: (first and second set) 11.9, 12.3, 115.0, 121.1, 122.3, 124.2, 141.8, 142.1, 143.3, 143.5, 143.6, 143.8, 144.6, 144.7, 148.3, 150.7, 152.6, 156.0. MS [ESI, m/z]: 271 [M+H]. Anal. Calcd for C$_{12}$H$_{10}$N$_6$S: C, 53.32; H, 3.73; N, 31.09. Found: C, 53.27; H, 3.91; N, 31.05.

4.1.1.29 2-(1-([Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl]hydrazono)ethyl)benzene-1,4-diol (1g)
Obtained as a yellow solid in 73% yield. M.p. 246-249 °C. Two sets of signals observed in NMR experiments. $^1$H-NMR (DMSO-d$_6$), δ: (first set) 2.62 (s, 3H), 6.77-6.79 (m, 2H), 7.04-7.07 (m, 1H), 7.50-7.52 (m, 1H), 7.57-7.63 (m, 1H), 8.03-8.08 (m, 2H), 8.74 (d, J = 7.9 Hz, 1H), 8.91 (broad s, 1H), 11.66
(broad s, 1H), 12.06 (broad s, 1H). ^1^H-NMR (DMSO-d$_6$), δ: (second set) 2.62 (s, 3H), 6.77-6.79 (m, 2H), 7.04-7.07 (m, 1H), 7.57-7.63 (m, 1H), 7.66-7.71 (m, 1H), 8.15-8.19 (m, 1H), 8.33-8.37 (m, 1H), 8.74 (d, J= 7.9 Hz, 1H), 8.91 (broad s, 1H), 10.42 (broad s, 1H), 12.70 (broad s, 1H). ^1^C-NMR (DMSO-d$_6$), δ: (first and second set) 15.2, 114.1, 114.3, 117.0, 118.3, 121.1, 122.7, 125.2, 125.6, 125.7, 133.2, 135.1, 145.0, 147.0, 149.3, 150.8, 160.8, 163.3. MS [ESI, m/z]: 351 [M+H]. Anal. Calcd for C$_{16}$H$_{14}$N$_4$O$_2$S: C, 61.70; H, 4.03; N, 15.99. Found: C, 61.58; H, 4.29; N, 15.87.

4.1.1.30 2-(1 - (2 -(Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)phenol (11g)
Obtained in 81% yield as a yellow solid. M.p. 197-199 °C. Two sets of signals observed. ^1^H-NMR (DMSO-d$_6$), δ: (first set) 2.66 (s, 3H), 6.92-6.97 (m, 2H), 7.30-7.34 (m, 1H), 7.50-7.52 (m, 1H), 7.58-7.60 (m, 1H), 7.65-7.70 (m, 1H), 8.04-8.09 (m, 2H), 8.73-8.79 (m, 1H), 12.04 (broad s, 1H), 12.39 (broad s, 1H).

^1^H-NMR (DMSO-d$_6$), δ: (second set) 2.66 (s, 3H), 6.92-6.97 (m, 2H), 7.30-7.34 (m, 1H), 7.58-7.60 (m, 1H), 7.65-7.70 (m, 1H), 8.15-8.19 (m, 1H), 8.34-8.38 (m, 1H), 8.73-8.79 (m, 1H), 10.46 (broad s, 1H), 13.46 (broad s, 1H).

^1^C-NMR (DMSO-d$_6$), δ: (first and second set) 15.1, 114.1, 116.6, 118.7, 121.1, 122.7, 125.2, 125.6, 125.7, 128.9, 130.8, 133.2, 135.2, 145.1, 146.9, 158.1, 160.9, 163.4. MS [ESI, m/z]: 335 [M+H]. Anal. Calcd for C$_{18}$H$_{14}$N$_4$OS: C, 64.65; H, 4.22; N, 16.75. Found: C, 64.74; H, 4.10; N, 16.89.

4.1.1.31 4-(2 - (1 -(Pyrazin-2-yl)ethylidene)hydrazinyl)benzo[4,5]thieno[2,3-d]pyrimidine (16g)
Obtained as a yellow solid in 73% yield. M.p. 218-221 °C. Single set of signals observed in NMR experiments. ^1^H-NMR (DMSO-d$_6$), δ: 2.62 (s, 3H), 7.50-7.54 (m, 1H), 7.58-7.62 (m, 1H), 8.08 (d, J= 7.9 Hz, 1H), 8.15 (d, J= 3.7 Hz), 8.61 (s, 1H), 8.65-8.66 (m, 1H), 8.78 (d, J= 7.9 Hz), 9.87 (d, J= 1.4 Hz, 1H), 12.44 (broad s, 1H).

^1^C-NMR (DMSO-d$_6$), δ: 12.8, 114.4, 122.7, 125.5, 125.6, 125.7, 128.9, 130.8, 133.2, 135.2, 145.1, 146.9, 151.1, 157.5, 161.8. MS [ESI, m/z]: 321 [M+H]. Anal. Calcd for C$_{16}$H$_{12}$N$_6$O: C, 59.98; H, 3.78; N, 26.23. Found: C, 59.85; H, 3.66; N, 26.31.

4.1.1.32 4-(2 - (1 - (1H-Benzol[d]imidazol-2-yl)ethylidene)hydrazinyl)benzo[4,5]thieno[2,3-d]pyrimidine (20g)
Obtained in 62% yield as a yellow solid. M.p. charring > 250 °C. Two sets of signals observed in NMR experiments. ^1^H-NMR (DMSO-d$_6$), δ: (first set) 2.67 (s, 3H), 7.22-7.32 (m, 2H), 7.52-7.55 (m, 1H), 7.61-7.72 (m, 3H), 8.10 (d, J= 7.9 Hz, 1H), 8.36 (s, 1H), 8.80 (d, J= 7.9 Hz, 1H), 12.60 (broad s, 1H), 12.68 (broad s, 1H). ^1^H-NMR (DMSO-d$_6$), δ: (second set) 2.63 (s, 3H), 7.45-7.48 (m, 2H), 7.61-7.72 (m, 2H), 7.91-8.00 (m, 2H), 8.22 (d, J= 7.9 Hz, 1H), 8.75 (s, 1H), 9.31 (d, J= 7.9 Hz, 1H), 13.21 (broad s, 1H), 13.49 (broad s, 1H).

^1^C-NMR (DMSO-d$_6$), δ: (first and second set) 13.3, 114.3, 121.5, 123.2, 124.8, 125.7, 125.8, 126.9, 130.4, 133.1, 134.8, 135.2, 146.3, 148.6, 150.4, 151.6, 155.2, 161.8. MS [ESI, m/z]: 359 [M+H]. Anal. Calcd for C$_{19}$H$_{16}$N$_6$S: C, 63.67; H, 3.94; N, 23.45. Found: C, 63.61; H, 3.79; N, 23.56.

4.1.1.33 2-(1-(2-(Methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)phenol (11h)
Obtained in 86% yield as a pale yellow solid. M.p. 257-260 °C. Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): (first set) 1.77-1.82 (m, 4H), 2.33 (s, 3H), 2.49 (s, 3H), 2.72-2.75 (m, 2H), 2.97-3.01 (m, 2H), 6.88-6.96 (m, 2H), 7.26-7.31 (m, 1H), 7.58-7.65 (m, 1H), 10.96 (broad s, 1H), 12.73 (broad s, 1H). \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): (second set) 1.85-1.89 (m, 4H), 2.49 (s, 3H), 2.56 (s, 3H), 2.79-2.84 (m, 2H), 3.12-3.16 (m, 2H), 6.88-6.96 (m, 2H), 7.26-7.31 (m, 1H), 7.58-7.65 (m, 1H), 9.32 (broad s, 1H), 13.35 (broad s, 1H). 

\(^{13}\)C-NMR (DMSO-\(d_6\)), \(\delta\): (first and second set) 12.8, 14.6, 21.2, 22.0, 22.1, 22.2, 22.4, 24.6, 24.9, 25.5, 26.4, 113.6, 116.5, 117.3, 117.4, 118.4, 118.5, 119.6, 121.0, 126.1, 127.9, 128.5, 130.5, 131.3, 132.4, 145.9, 152.8, 153.7, 154.5, 157.0, 158.4, 161.4, 162.6, 166.6. MS [ESI, m/z]: 353 [M+H]. Anal. Calcd for C\(_{19}\)H\(_{20}\)N\(_4\)OS: C, 64.75; H, 5.72; N, 15.90. Found: C, 64.83; H, 5.76, N, 16.03.

4.1.1.34 2-Methyl-4-(2-(1-(pyrazin-2-yl)ethylidene)hydrazinyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine (16h)

Obtained in 59% yield as a yellow solid. M.p. 189-191 °C. Single set of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): 1.76-1.82 (m, 4H), 2.44 (s, 3H), 2.46 (s, 3H), 2.72-2.74 (m, 2H), 3.00-3.03 (m, 2H), 8.58 (d, \(J= 2.6\) Hz, 1H), 8.61 (dd, \(J_1= 2.6\) Hz, \(J_2= 1.4\) Hz, 1H), 9.82 (d, \(J= 1.4\) Hz, 1H), 11.18 (broad s, 1H). 

\(^{13}\)C-NMR (DMSO-\(d_6\)), \(\delta\): 12.8, 21.5, 22.1, 22.3, 24.6, 26.4, 117.7, 130.6, 131.5, 143.1, 143.4, 143.6, 148.9, 151.2, 152.7, 157.0, 158.2. MS [ESI, m/z]: 339 [M+H]. Anal. Calcd for C\(_{17}\)H\(_{18}\)N\(_6\)S: C, 60.33; H, 5.36; N, 24.83. Found: C, 60.42; H, 5.28; N, 24.87.

4.1.1.35 2-(1-(2-(6-Chloro-5-methylthieno[2,3-d]pyrimidin-4-yl)hydrazono)ethyl)phenol (11i)

Obtained in 77% yield as a light grey solid. M.p. 220-223 °C. Two sets of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): (first set) 2.51 (s, 3H), 2.54 (s, 3H), 6.89-6.94 (m, 2H), 7.29-7.31 (m, 1H), 7.60-7.67 (m, 1H), 7.82 (s, 1H), 11.61 (broad s, 1H), 12.29 (broad s, 1H). \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): (second set) 2.54 (s, 3H), 2.69 (s, 3H), 6.89-6.94 (m, 2H), 7.29-7.31 (m, 1H), 7.60-7.67 (m, 1H), 8.63 (s, 1H), 9.68 (broad s, 1H), 13.26 (broad s, 1H). 

\(^{13}\)C-NMR (DMSO-\(d_6\)), \(\delta\): 14.3, 15.1, 116.5, 118.7, 119.2, 120.7, 121.0, 128.8, 130.9, 144.5, 145.5, 155.9, 158.1, 164.0. MS [ESI, m/z]: 332.9, 334.9 [M+H]. Anal. Calcd for C\(_{15}\)H\(_{13}\)ClN\(_4\)OS: C, 54.13; H, 3.94; N, 16.83. Found: C, 53.96; H, 4.17; N, 16.77.

4.1.1.36 6-Chloro-5-methyl-4-(2-(1-(pyrazin-2-yl)ethylidene)hydrazinyl)thieno[2,3-d]pyrimidine (16i)

Obtained in 64% yield as a yellow solid. M.p. 235-237 °C. Single set of signals observed in NMR experiments. \(^1\)H-NMR (DMSO-\(d_6\)), \(\delta\): 2.48 (s, 3H), 2.58 (s, 3H), 7.95 (s, 1H), 8.61 (d, \(J= 2.4\) Hz, 1H), 8.64-8.65 (m, 1H), 9.79 (s, 1H), 12.09 (broad s, 1H). \(^{13}\)C-NMR (DMSO-\(d_6\)), \(\delta\): 12.7, 14.2, 115.7, 120.0, 143.2, 143.5, 143.7, 144.9, 147.4, 150.8, 155.0, 164.9. MS [ESI, m/z]: 318.9, 320.9 [M+H]. Anal. Calcd for C\(_{13}\)H\(_{11}\)ClN\(_4\)S: C, 48.98; H, 3.48; N, 26.36. Found: C, 48.94; H, 3.76, N, 26.32.

4.1.2 General method for the preparation of (thieno[2,3-d]pyrimidin-4-yl)hydrazides 25-36

The aryl carboxylic acid (1.1 eq.) and TBTU (1.2) were suspended in anhydrous THF (11 mL/mmol eq.) at r.t. under \(N_2\) atmosphere. DiPEA (2.4 eq.) was then added dropwise to the reaction mixture, followed
by the different (thieno[2,3-d]pyrimidin-4-yl)-hydrazine (8a-d or f-i) (1 eq.) dissolved in anhydrous THF (11 mL/mmol eq.). The mixture was stirred at r.t. for 4 h, then concentrated under vacuum. The residue was dissolved in EtOAc (30 mL/mmol eq.), washed with water (30 mL/mmol eq.), saturated NaHCO₃ solution (30 mL/mmol eq.), and finally with brine (30 mL/mmol eq.). The organic phase was concentrated under vacuum after drying over MgSO₄. The crude residue was purified by re-crystallisation or flash column chromatography.

4.1.2.1 2,5-Dihydroxy-benzoic acid N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazide (25a)

Purified by crystallisation from EtOH. Obtained in 28% yield as a light grey solid. M.p. 261-263 °C.

1H-NMR (DMSO-d₆), δ: 1.81-1.91 (m, 4H), 2.80-2.86 (m, 2H), 2.99-3.08 (m, 2H), 6.82 (d, J= 8.7 Hz, 1H), 6.91 (dd, J₁= 8.7 Hz, J₂= 1.6 Hz, 1H), 7.37 (d, J= 1.6 Hz, 1H), 8.36 (s, 1H), 8.77 (broad s, 1H), 9.09 (broad s, 1H), 10.77 (bs 1H), 11.17 (broad s, 1H). 13C-NMR (DMSO-d₆), δ: 21.9, 22.1, 24.9, 25.6, 114.0, 115.1, 115.4, 117.8, 121.4, 126.4, 132.9, 149.6, 151.1, 152.3, 156.4, 165.2, 166.4. MS [ESI, m/z]: 357 [M+H].

Anal. Calcd for C₁₇H₁₆N₄O₃S: C, 57.29; H, 4.52; N, 15.72. Found: C, 57.12; H, 4.73; N, 15.66.

4.1.2.2 2-Hydroxy-benzoic acid N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazide (26a) [45]

Purified by crystallisation from EtOH/H₂O. Obtained in 35% yield as a light orange solid. M.p. 327-329 °C.

1H-NMR (DMSO-d₆), δ: 1.84-1.88 (m, 4H), 2.79-2.88 (m, 2H), 2.98-3.08 (m, 2H), 6.94-7.01 (m, 2H), 7.74 (t, J= 7.7 Hz, 1H), 8.00 (d, J= 7.7 Hz, 1H), 8.37 (s , 1H), 8.79 (broad s, 1H), 10.85 (broad s, 1H), 10.77 (bs 1H), 11.99 (broad s, 1H). 13C-NMR (DMSO-d₆), δ: 21.9, 22.1, 24.9, 25.6, 114.7, 117.3, 119.0, 126.4, 128.3, 133.0, 134.0, 149.4, 151.2, 152.3, 156.6, 159.2. MS [ESI, m/z]: 341 [M+H].


4.1.2.3 3-Hydroxy-N’-(5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)benzo hydrazide (27a)

Purified by crystallisation from EtOH. Obtained in 29% yield as a white solid. M.p. 241-243 °C.

1H-NMR (CDCl₃), δ: 1.90-2.00 (m, 4H), 2.84-2.87 (m, 2H), 3.06-3.10 (m, 2H), 8.19 (broad s, 1H), 8.51 (s, 1H), 8.61-8.62 (m, 1H), 8.81 (d, J= 2.4 Hz, 1H), 9.39 (d, J= 1.3 Hz, 1H), 10.68 (broad s, 1H). 13C-NMR (CDCl₃), δ: 21.9, 22.1, 24.9, 25.7, 114.5, 115.1, 117.9, 118.6, 126.4, 129.4, 132.7, 134.1, 152.4, 157.3, 157.5, 165.3, 165.9. MS [ESI, m/z]: 341 [M+H].


4.1.2.4 Pyrazine-2-carboxylic acid N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazide (28a)

Purified by crystallisation from DCM/n-hexane. Obtained in 29% yield as a pale yellow solid. M.p. 200-202 °C.

1H-NMR (CDCl₃), δ: 1.83-1.87 (m, 4H), 2.81-2.84 (m, 2H), 3.01-3.04 (m, 2H), 6.96-6.99 (m, 1H), 7.29-7.33 (m, 1H), 7.34-7.35 (m, 1H), 7.40 (dt, J₁= 7.7 Hz, J₂= 1.2 Hz, 1H), 8.34 (s, 1H), 8.58 (broad s, 1H), 9.70 (broad s, 1H), 10.48 (broad s, 1H). 13C-NMR (CDCl₃), δ: 21.9, 22.1, 24.9, 25.7, 114.5, 115.1, 117.9, 118.6, 126.4, 129.4, 132.7, 134.1, 152.4, 157.3, 157.5, 165.3, 165.9. MS [ESI, m/z]: 341 [M+H].

25.75. Found: C, 55.13; H, 4.22; N, 25.83.

4.1.2.5 Pyridine-2-carboxylic acid N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazide (29a)

Purified by crystallisation from EtOH. Obtained in 67% yield as a light grey solid. M.p. 232-234 °C. 
\[ ^1H-NMR \ (CDCl_3), \delta: 1.89-2.00 \ (m, 4H), 2.83-2.87 \ (m, 2H), 3.07-3.11 \ (m, 2H), 7.47-7.51 \ (m, 1H), 7.89 \ (td, J_1 = 7.7 \ Hz, J_2 = 1.6 \ Hz, 1H), 8.15-8.20 \ (m, 2H), 8.52 \ (s, 1H), 8.65 \ (d, J = 4.7 \ Hz, 1H), 10.77 \ (broad \ s, 1H). \]
\[ ^13C-NMR \ (CDCl_3), \delta: 22.4, 22.5, 25.4, 26.1, 115.7, 122.3, 125.3, 126.7, 135.1, 137.4, 148.3, 148.7, 152.4, 154.5, 160.5, 166.2. MS \ [ESI, m/z]: 326 \ (M+H). \]

Anal. Calcd for C_{16}H_{15}N_{5}OS: C, 59.06; H, 4.65; N, 21.51. Found: C, 58.99; H, 4.61; N, 21.51.

4.1.2.6 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)nicotinohydrazide (30a)

Purified by crystallisation from EtOH/H_2O. Obtained in 23% yield as a pale yellow solid. M.p. 137-140 °C. 
\[ ^1H-NMR \ (DMSO-d_6), \delta: 1.84-1.89 \ (m, 4H), 2.81-2.85 \ (m, 2H), 3.02-3.06 \ (m, 2H), 7.58 \ (dd, J_1 = 8.4 \ Hz, J_2 = 4.8 \ Hz), 8.28-8.31 \ (m, 1H), 8.36 \ (s, 1H), 8.69-8.71 \ (m, 1H), 8.78-8.80 \ (m, 1H), 9.12 \ (broad \ s, 1H), 10.80 \ (broad \ s, 1H). \]
\[ ^13C-NMR \ (DMSO-d_6), \delta: 21.9, 22.1, 24.9, 25.7, 115.2, 123.6, 126.4, 128.3, 132.9, 135.2, 148.4, 152.4, 157.2, 164.5, 165.4. MS \ [ESI, m/z]: 326 \ [M+H]. \]

Anal. Calcd for C_{16}H_{15}N_{5}OS: C, 59.02; H, 4.79; N, 21.60.

4.1.2.7 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-1H-indole-2-carbohydrazide (31a)

Purified by crystallisation from EtOH. Obtained in 36% yield as a pale yellow solid. M.p. 232-235 °C. 
\[ ^1H-NMR \ (DMSO-d_6), \delta: 1.83-1.89 \ (m, 4H), 2.80-2.84 \ (m, 2H), 3.03-3.06 \ (m, 2H), 7.06-7.09 \ (m, 1H), 7.20-7.24 \ (m, 1H), 7.34 \ (s, 1H), 7.47 \ (d, J = 8.2 \ Hz, 1H), 7.68 \ (d, J = 8.0 \ Hz, 1H), 8.35 \ (s, 1H), 8.70 \ (broad \ s, 1H), 10.58 \ (broad \ s, 1H), 11.69 \ (broad \ s, 1H). \]
\[ ^13C-NMR \ (DMSO-d_6), \delta: 22.0, 22.1, 24.9, 25.8, 103.4, 112.3, 115.1, 119.8, 121.6, 123.6, 126.4, 127.0, 129.7, 132.7, 136.6, 152.4, 157.5, 160.9, 165.3. MS \ [ESI, m/z]: 364 \ [M+H]. \]

Anal. Calcd for C_{19}H_{17}N_{5}OS: C, 62.79; H, 4.71; N, 19.27. Found: C, 62.87; H, 4.56; N, 19.36.

4.1.2.8 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-1H-benzo[d]imidazole-2-carbohydrazide (32a)

Purified by crystallisation from EtOH. Obtained in 26% yield as a yellow solid. M.p. >300 °C. 
\[ ^1H-NMR \ (DMSO-d_6), \delta: 1.83-1.86 \ (m, 4H), 2.80-2.84 \ (m, 2H), 3.00-3.04 \ (m, 2H), 7.31-7.37 \ (m, 2H), 7.55-7.56 \ (m, 1H), 7.76-7.82 \ (m, 1H), 8.34 \ (s, 1H), 8.79 \ (broad \ s, 1H), 10.89 \ (broad \ s, 1H), 13.40 \ (broad \ s, 1H). \]
\[ ^13C-NMR \ (DMSO-d_6), \delta: 21.9, 22.1, 24.9, 25.7, 112.5, 115.2, 120.0, 122.7, 124.3, 126.4, 132.8, 134.3, 142.5, 144.3, 152.3, 156.9, 158.4, 165.3. MS \ [ESI, m/z]: 365 \ [M+H]. \]

Anal. Calcd for C_{18}H_{16}N_{6}OS: C, 59.32; H, 4.43; N, 23.06. Found: C, 59.51; H, 4.27; N, 23.15.

4.1.2.9 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)quinoline-2-carbohydrazide (33a)

Purified by crystallisation from EtOH. Obtained in 68% yield as a white solid. M.p. 261-263 °C.
1H-NMR (DMSO-d₆), δ: 1.85-1.89 (m, 4H), 2.82-2.86 (m, 2H), 3.04-3.08 (m, 2H), 7.75-7.78 (m, 1H), 7.90-7.94 (m, 1H), 8.12 (d, J = 8.1 Hz, 1H), 8.17 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 8.34 (s, 1H), 8.61 (d, J = 8.4 Hz, 1H), 8.76 (broad s, 1H), 10.84 (broad s, 1H).

13C-NMR (DMSO-d₆), δ: 21.9, 22.1, 24.9, 25.7, 115.2, 118.8, 122.3, 126.5, 128.1, 128.3, 128.9, 129.3, 130.6, 132.8, 137.9, 146.0, 149.7, 152.4, 158.6, 164.2. MS [ESI, m/z]: 376 [M+H]. Anal. Calcd for C₂₀H₁₇N₅OS: C, 63.98; H, 4.56; N, 18.65. Found: C, 64.11; H, 4.77; N, 18.82.

4.1.2.10 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-1H-pyrrole-2-carbohydrazide (34a)

Purified by crystallisation from MeOH. Obtained in 65% yield as a pale yellow solid. M.p. charring > 300 °C. 1H-NMR (DMSO-d₆), δ: 1.83-1.87 (m, 4H), 2.80-2.84 (m, 2H), 3.00-3.04 (m, 2H), 6.14-6.17 (m, 1H), 6.93-6.95 (m, 1H), 6.98-7.00 (m, 1H), 8.32 (s, 1H), 8.52 (broad s, 1H), 10.08 (broad s, 1H), 11.56 (broad s, 1H).

13C-NMR (DMSO-d₆), δ: 22.0, 22.1, 24.9, 25.8, 108.7, 110.9, 115.0, 122.0, 124.3, 126.4, 132.6, 152.4, 157.4, 160.4, 165.2. MS [ESI, m/z]: 314 [M+H]. Anal. Calcd for C₁₅H₁₅N₅O₂S: C, 57.49; H, 4.82; N, 22.35. Found: C, 57.62; H, 5.01; N, 22.46.

4.1.2.11 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)furan-2-carbohydrazide (35a)

Purified by crystallisation from MeOH. Obtained in 35% yield as white crystals. M.p. 216-219 °C.

1H-NMR (DMSO-d₆), δ: 1.83-1.86 (m, 4H), 2.80-2.84 (m, 2H), 2.99-3.03 (m, 2H), 6.69 (dd, J₁ = 3.5 Hz, J₂ = 1.7 Hz, 1H), 7.29 (dd, J₁ = 3.5 Hz, J₂ = 0.4 Hz, 1H), 7.92-7.93 (m, 1H), 8.34 (s, 1H), 8.59 (broad s, 1H), 10.45 (broad s, 1H).

13C-NMR (DMSO-d₆), δ: 21.9, 22.1, 24.9, 25.7, 111.8, 114.4, 115.1, 126.4, 132.6, 145.6, 146.4, 152.3, 157.3, 157.5, 165.3. MS [ESI, m/z]: 315 [M+H]. Anal. Calcd for C₁₅H₁₄N₄O₂S: C, 57.31; H, 4.49; N, 4.41.

4.1.2.12 N’-(5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)thiophene-2-carbohydrazide (36a)

Purified by crystallisation from MeOH. Obtained in 15% yield as a white solid. M.p. charring > 248 °C. 1H-NMR (DMSO-d₆), δ: 1.82-1.89 (m, 4H), 2.42-2.48 (m, 2H), 2.99-3.05 (m, 2H), 2.91-2.95 (m, 1H), 7.21-7.25 (m, 1H), 7.84-7.85 (m, 1H), 7.94-7.98 (m, 1H), 8.35 (s, 1H), 8.64 (broad s, 1H), 10.60 (broad s, 1H).

13C-NMR (DMSO-d₆), δ: 21.9, 22.1, 24.9, 25.7, 115.1, 126.4, 128.1, 128.9, 131.4, 132.8, 137.6, 152.4, 157.3, 160.9, 165.4. MS [ESI, m/z]: 331 [M+H]. Anal. Calcd for C₁₅H₁₄N₄O₂S: C, 54.52; H, 4.27; N, 19.96. Found: C, 54.48; H, 4.49; N, 19.93.

4.1.2.13 N’-(6,7-dihydro-5H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-yl)-2,5-dihydroxy benzoxyhydrazide (25b)

Purified by crystallisation from EtOH. Obtained in 26% yield as a light grey solid. M.p. charring > 248 °C. 1H-NMR (DMSO-d₆), δ: 2.42-2.48 (m, 2H), 2.99 (t, J = 7.1 Hz, 2H), 3.12 (t, J = 6.9 Hz, 2H), 6.82 (d, J = 8.8 Hz, 1H), 6.92 (dd, J₁ = 8.8 Hz, J₂ = 2.8 Hz, 1H), 7.37 (d, J = 2.8 Hz, 1H), 8.35 (s, 1H), 8.98 (broad s, 1H), 9.09 (broad s, 1H), 10.66 (broad s, 1H), 11.18 (broad s, 1H).

13C-NMR (DMSO-d₆), δ: 27.4, 29.2, 111.9, 113.8, 115.2, 117.8, 121.6, 135.3, 138.5, 149.5, 151.4, 152.2, 156.4, 167.3, 170.9. MS [ESI, m/z]:
4.1.2.14 \( N^\prime -(6,7\text{-Dihydro-5\text{-H-cyclopenta}[4,5]thieno[2,3-d]pyrimidin-4-yl])-2\text{-hydroxybenzo hydrazide (26b)} \)

Purified by flash column chromatography eluting with \( n\text{-hexane-EtOAc 100:0 v/v increasing to n-hexane-EtOAc 0:100 v/v. Obtained in 30\% yield as a white solid. TLC (EtOAc, Rf: 0.70). M.p charring > 230 °C. \)

\( ^1\text{H-NMR (DMSO-d}_6, \delta: 2.43-2.49 (m, 2H), 3.00 (t, J=7.1 Hz, 2H), 3.13 (J=7.1 Hz, 2H), 6.96-7.00 (m, 2H), 7.46-7.50 (m, 1H), 7.99 (dd, J_1= 7.9 Hz, J_2= 1.2 Hz, 1H), 8.36 (s, 1H), 9.01 (broad s, 1H), 10.79 (bs 1H), 11.98 (broad s, 1H). \)

\( ^{13}\text{C-NMR (DMSO-d}_6, \delta: 27.4, 29.2, 29.2, 111.9, 114.5, 117.4, 119.0, 128.2, 134.1, 135.3, 138.6, 152.3, 156.5, 159.4, 168.1, 171.0. MS [ESI, m/z]: 327 \text{[M+H]. Anal. Calcd for C}_{16}\text{H}_{14}\text{N}_4\text{O}_3\text{S: C, 56.13; H, 4.12; N, 16.36. Found: C, 56.07; H, 3.99; N, 16.45.} \)

4.1.2.15 \( N^\prime -(6,7\text{-Dihydro-5\text{-H-cyclopenta}[4,5]thieno[2,3-d]pyrimidin-4-yl])pyrazine-2-carbohydrazide (28b) \)

Purified by crystallisation from EtOH. Obtained in 15\% yield as a light brown solid. M.p 174-178 °C.

\( ^1\text{H-NMR (DMSO-d}_6, \delta: 2.41-2.47 (m, 2H), 2.98 (t, J= 7.1 Hz, 2H), 3.12 (, J= 6.7 Hz, 2H), 8.33 (s, 1H), 8.82-8.83 (m, 1H), 8.95 (d, J= 2.4 Hz, 1H), 9.00 (broad s, 1H), 9.22 (d, J= 1.2 Hz, 1H), 10.93 (bs 1H). \)

\( ^{13}\text{C-NMR (DMSO-d}_6, \delta: 27.4, 29.2, 29.3, 111.9, 135.3, 138.4, 143.6, 144.3, 148.0, 152.2, 156.6, 162.5, 169.9. MS [ESI, m/z]: 313 \text{[M+H]. Anal. Calcd for C}_{14}\text{H}_{12}\text{N}_6\text{O}_2\text{S: C, 53.83; H, 3.87; N, 26.91. Found: C, 53.76; H, 3.71; N, 27.13.} \)

4.1.2.16 \( N^\prime -(6,7\text{-Dihydro-5\text{-H-cyclopenta}[4,5]thieno[2,3-d]pyrimidin-4-yl])picolinohydrazide (29b) \)

Purified by crystallisation from EtOH. Obtained as a grey solid in 30\% yield. M. p. 219-222 °C.

\( ^1\text{H-NMR (DMSO-d}_6, \delta: 2.40-2.46 (m, 2H), 2.98 (t, J= 7.1 Hz, 2H), 3.12 (, J= 6.9 Hz, 2H), 7.66-7.69 (m, 1H), 8.02-8.05 (m, 1H), 8.31 (s, 1H), 8.72-8.74 (m, 1H), 8.96 (broad s, 1H), 10.72 (bs 1H). \)

\( ^{13}\text{C-NMR (DMSO-d}_6, \delta: 27.4, 29.2, 29.3, 111.9, 114.8, 122.7, 126.9, 135.4, 137.8, 138.2, 148.6, 149.3, 152.2, 156.2, 169.7. MS [ESI, m/z]: 312 \text{[M+H]. Anal. Calcd for C}_{15}\text{H}_{13}\text{N}_5\text{O}_2\text{S: C, 57.86; H, 4.21; N, 22.49. Found: C, 57.99; H, 4.30; N, 22.66.} \)

4.1.2.17 \( N^\prime -(6,7\text{-Dihydro-5\text{-H-cyclopenta}[4,5]thieno[2,3-d]pyrimidin-4-yl])-1\text{-H-benzo[d] imidazole-2-carbohydrazide (32b)} \)

Purified by crystallisation from EtOH. Obtained in 47\% yield as a white solid. M.p. 220-224°C. \( ^1\text{H-NMR (DMSO-d}_6, \delta: 2.40-2.47 (m, 2H), 2.96-3.00 (m, 2H), 3.11-3.14 (, m, 2H), 7.30-7.38 (m, 2H), 7.56-7.60 (m, 1H), 7.78-7.82 (m, 1H), 8.33 (s, 1H), 9.04 (broad s, 1H), 10.96 (bs 1H), 13.40 (broad s, 1H). \)

\( ^{13}\text{C-NMR (DMSO-d}_6, \delta: 27.4, 29.2, 29.3, 111.9, 112.5, 120.1, 122.7, 124.4, 135.2, 138.4, 142.3, 144.3, 152.2, 156.6, 158.7, 171.0. MS [ESI, m/z]: 373 \text{[M+Na]. Anal. Calcd for C}_{17}\text{H}_{14}\text{N}_6\text{OS: C, 58.27; H, 4.03; N, 23.98. Found: C, 58.41; H, 3.86; N, 24.11.} \)

4.1.2.18 \( \text{N}^\prime -(5,6\text{-Dimethylthieno[2,3-d]pyrimidin-4-yl])pyrazine-2-carbohydrazide (28c) } \)
Obtained in 35% yield as a white solid. M.p. 194-197 °C. 1H-NMR (DMSO-d$_6$), δ: 2.44 (s, 3H), 2.53 (s, 3H), 8.31 (s, 1H), 8.81-8.82 (m, 1H), 8.94 (d, J = 2.3 Hz, 1H), 8.96 (broad s, 1H), 9.22 (d, J = 1.2 Hz, 1H), 10.90 (bs 1H). 13C-NMR (DMSO-d$_6$), δ: 13.1, 14.0, 116.5, 124.6, 127.1, 129.7, 143.6, 144.4, 146.7, 147.6, 147.9, 151.8, 161.9. MS [ESI, m/z]: 301 [M+H]. Anal. Calcd for C$_{13}$H$_{12}$N$_6$O$_4$: C, 51.99; H, 4.03; N, 27.98. Found: C, 51.96; H, 4.11; N, 27.95.

4.1.2.19 2,5-Dihydroxy-N'-[(thieno[2,3-d]pyrimidin-4-yl)benzohydrazide (25f)

Purified by crystallisation from EtOH. Obtained in 15% yield as a light brown solid. M.p. >300 °C. 1H-NMR (DMSO-d$_6$), δ: 6.83 (d, J = 8.8 Hz, 1H), 6.93 (dd, J$_1$ = 8.8 Hz, J$_2$ = 2.8 Hz, 1H), 7.35 (d, J = 2.8 Hz, 1H), 7.69-7.72 (m, 2H), 8.43 (s, 1H), 8.44 (s, 1H), 10.18 (broad s, 1H), 10.73 (bs 1H), 11.07 (broad s, 1H). 13C-NMR (DMSO-d$_6$), δ: 113.9, 114.7, 115.3, 117.9, 119.0, 121.6, 124.1, 128.5, 149.4, 149.6, 151.2, 153.4, 167.2. MS [ESI, m/z]: 303 [M+H]. Anal. Calcd for C$_{13}$H$_{10}$N$_4$O$_3$S: C, 51.65; H, 3.33; N, 18.53. Found: C, 51.57; H, 3.27; N, 18.57.

4.1.2.20 2-Hydroxy-N'-[(thieno[2,3-d]pyrimidin-4-yl)benzohydrazide (26f)

Purified by crystallisation from EtOH. Obtained in 19% yield as a white solid. M.p. 180-183 °C. 1H-NMR (DMSO-d$_6$), δ: 6.96-7.01 (m, 2H), 7.46-7.50 (m, 1H), 7.68-7.74 (m, 2H), 7.97 (dd, J$_1$ = 7.9 Hz, J$_2$ = 1.0 Hz, 1H), 8.44 (s, 1H), 10.18 (broad s, 1H), 10.85 (broad s, 1H), 11.85 (broad s, 1H). 13C-NMR (DMSO-d$_6$), δ: 114.7, 117.3, 118.9, 119.0, 120.6, 128.3, 134.1, 134.4, 153.4, 159.1, 167.9. MS [ESI, m/z]: 287 [M+H]. Anal. Calcd for C$_{13}$H$_{10}$N$_4$O$_3$S: C, 54.54; H, 3.52; N, 19.57. Found: C, 54.49; H, 3.33; N, 19.61.

4.1.2.21 N'-[(Thieno[2,3-d]pyrimidin-4-yl)pyrazine-2-carbohydrazide (28f)

Purified by crystallisation from EtOH. Obtained in 34% yield as a white solid. M.p. 193-197 °C. 1H-NMR (DMSO-d$_6$), δ: 7.68-7.72 (m, 2H), 8.41 (s, 1H), 8.83 (dd, J$_1$ = 2.5 Hz, J$_2$ = 1.5 Hz, 1H), 8.96 (d, J = 2.5 Hz, 1H), 9.23 (d, J = 1.5 Hz, 1H), 10.14 (broad s, 1H), 11.06 (bs 1H). 13C-NMR (DMSO-d$_6$), δ: 114.8, 119.0, 124.0, 123.1, 138.4, 143.6, 143.7, 144.1, 148.1, 153.4, 162.6. MS [ESI, m/z]: 273 [M+H]. Anal. Calcd for C$_{11}$H$_8$N$_6$OS: C, 48.52; H, 2.96; N, 30.86. Found: C, 48.46; H, 2.99; N, 30.95.

4.1.2.22 N'-[(Thieno[2,3-d]pyrimidin-4-yl)picolinohydrazide (29f)

Purified by crystallisation from EtOH. Obtained in 30% yield as a white solid. M.p. 191-194 °C. 1H-NMR (DMSO-d$_6$), δ: 7.67-7.71 (m, 2H), 8.03-8.09 (m, 2H), 8.40 (s, 1H), 8.74 (d, J = 4.6 Hz, 1H), 10.09 (broad s, 1H), 10.87 (bs 1H). 13C-NMR (DMSO-d$_6$), δ: 114.7, 119.1, 121.1, 122.4, 123.9, 127.0, 137.8, 138.0, 148.7, 149.2, 153.4, 163.5. MS [ESI, m/z]: 272 [M+H]. Anal. Calcd for C$_{12}$H$_9$N$_5$OS: C, 53.13; H, 3.34; N, 25.81. Found: C, 53.22; H, 3.31; N, 25.89.

4.1.2.23 N'-[(Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-2,5-dihydroxybenzohydrazide (25g)

Purified by flash column chromatography eluting with n-hexane-EtOAc 100:0 v/v increasing to n-hexane-EtOAc 30:70 v/v. Obtained in 17% yield as a light grey solid. TLC (EtOAc, Rf: 0.65). M.p. 241-244 °C. 1H-NMR (DMSO-d$_6$), δ: 6.84 (d, J = 8.8 Hz, 1H), 6.93 (dd, J$_1$ = 8.8 Hz, J$_2$ = 2.8 Hz, 1H), 7.41 (d, J = 2.8 Hz, 1H).
J= 2.8 Hz, 1H), 7.58-7.66 (m, 2H), 8.16 (d, J= 7.6 Hz, 1H), 8.56-8.61 (m, 2H), 9.11 (broad s, 1H), 9.64 (broad s, 1H), 10.92 (broad s, 2H). 13C-NMR (DMSO-d$_6$), δ: 110.0, 113.9, 115.4, 117.9, 121.6, 123.3, 124.5, 125.6, 126.7, 130.7, 134.5, 149.6, 151.5, 154.8, 167.1. MS [ESI, m/z]: 353 [M+H]. Anal. Calcd for C$_{17}$H$_{12}$N$_4$O$_3$S: C, 57.95; H, 3.43; N, 15.90. Found: C, 57.80; H, 3.31; N, 15.92.

4.1.2.24 N’-(Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-2-hydroxybenzohydrazide (26g)

Purified by crystallization from EtOH. Obtained in 14% yield as a pale yellow solid. M.p. >300 °C.

1H-NMR (DMSO-d$_6$), δ: 6.98-7.03 (m, 2H), 7.48-7.52 (m, 1H), 7.58-7.67 (m, 2H), 8.07 (d, J= 7.5 Hz, 1H), 8.18 (d, J= 7.3 Hz, 1H), 8.59-8.63 (m, 2H), 9.65 (broad s, 1H), 11.00 (broad s, 1H), 12.00 (broad s, 1H).

13C-NMR (DMSO-d$_6$), δ: 110.0, 114.7, 117.4, 119.0, 123.3, 124.5, 125.6, 126.7, 128.3, 130.5, 134.1, 134.5, 154.9, 157.4, 159.3, 167.9. MS [ESI, m/z]: 337 [M+H]. Anal. Calcd for C$_{17}$H$_{12}$N$_4$O$_2$S: C, 60.70; H, 3.60; N, 16.66. Found: C, 60.63; H, 3.51; N, 16.78.

4.1.2.25 N’-(Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)pyrazine-2-carbohydrazide (28g)

Purified by crystallization from EtOH/H$_2$O. Obtained in 21% yield as a pale yellow solid. M.p. 115-118 °C.

1H-NMR (DMSO-d$_6$), δ: 7.57-7.65 (m, 2H), 8.15 (d, J= 7.6 Hz, 1H), 8.56 (s, 1H), 8.63 (d, J= 7.3 Hz, 1H), 8.84-8.85 (m, 1H), 8.96 (d, J= 2.3 Hz, 1H), 9.26 (d, J= 1.3 Hz, 1H), 9.64 (broad s, 1H), 11.14 (broad s, 1H).

13C-NMR (DMSO-d$_6$), δ: 110.1, 123.2, 124.5, 125.6, 125.9, 126.6, 130.7, 134.4, 143.6, 143.7, 144.0, 144.3, 148.0, 167.8. MS [ESI, m/z]: 323 [M+H]. Anal. Calcd for C$_{15}$H$_{10}$N$_6$OS: C, 55.89; H, 3.13; N, 26.07. Found: C, 55.80; H, 2.97; N, 26.20.

4.1.2.26 N’-(Benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)picolinohydrazide (29g)

Purified by crystallization from EtOH. Obtained in 28% yield as a white solid. M.p. 220-223 °C.

1H-NMR (DMSO-d$_6$), δ: 1.82-1.87 (m, 4H), 2.42 (s, 3H), 2.77-2.81 (m, 2H), 2.99-3.03 (m, 2H), 6.95-7.02 (m, 2H), 7.44-7.48 (m, 1H), 7.98 (d, J= 7.0 Hz, 1H), 8.69 (broad s, 1H), 10.92 (broad s, 1H).

13C-NMR (DMSO-d$_6$), δ: 21.9, 22.1, 24.8, 25.4, 25.6, 112.8, 115.2, 117.2, 119.0, 126.1, 128.4, 131.6, 133.8, 156.3, 158.8, 161.0, 166.1, 166.8. MS [ESI, m/z]: 322 [M+H]. Anal. Calcd for C$_{16}$H$_{11}$N$_2$OS: C, 59.80; H, 3.45; N, 21.79. Found: C, 59.83; H, 3.49; N, 21.92.

4.1.2.27 2-Hydroxy-N’-(2-methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl) benzohydrazide (26h)

Purified by crystallization from MeOH/H$_2$O. Obtained in 36% yield as a light orange solid. M.p. 209-212 °C.

1H-NMR (DMSO-d$_6$), δ: 1.82-1.87 (m, 4H), 2.42 (s, 3H), 2.77-2.81 (m, 2H), 2.99-3.03 (m, 2H), 6.95-7.02 (m, 2H), 7.44-7.48 (m, 1H), 7.98 (d, J= 7.0 Hz, 1H), 8.69 (broad s, 1H), 10.92 (broad s, 1H), 11.89 (bs 1H).

13C-NMR (DMSO-d$_6$), δ: 21.9, 22.1, 24.8, 25.4, 25.6, 112.8, 115.2, 117.2, 119.0, 126.1, 128.4, 131.6, 133.8, 156.3, 158.8, 161.0, 166.1, 166.8. MS [ESI, m/z]: 355 [M+H]. Anal. Calcd for C$_{16}$H$_{10}$N$_2$OS: C, 61.00; H, 5.12; N, 15.81. Found: C, 61.11; H, 5.06; N, 15.90.

4.1.2.28 N’-(2-Methyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-yl)picolino hydrazide (29h)
Purified by crystallisation from MeOH/H₂O. Obtained in 38% yield as a pale yellow solid. M.p. 99-101 °C. ¹H-NMR (DMSO-ᵈ, _, δ): 1.83-1.86 (m, 4H), 2.38 (s, 3H), 2.78-2.81 (m, 2H), 2.98-3.02 (m, 2H), 7.65-7.69 (m, 1H), 8.02-8.08 (m, 2H), 8.58 (broad s, 1H), 8.72-8.74 (m, 1H), 10.63 (broad s, 1H). ¹³C-NMR (DMSO-ᵈ, δ): 22.0, 22.2, 24.8, 25.4, 25.6, 112.9, 122.2, 126.2, 126.9, 131.3, 137.8, 148.7, 149.3, 156.6, 161.0, 162.7, 166.0. MS [ESI, m/z]: 340 [M+H]. Anal. Calcd for C₁₇H₁₇N₅O: C, 60.16; H, 5.05; N, 20.63. Found: C, 60.11; H, 20.61; N, 5.12.

4.1.2.29 N’-(6-Chloro-5-methylthieno[2,3-d]pyrimidin-4-yl)-2-hydroxybenzohydrazide (26i)

Purified by crystallisation from MeOH. Obtained in 19% yield as a light grey solid. M.p. 322-324 °C. ¹H-NMR (DMSO-ᵈ, δ): 2.62 (s, 3H), 6.96-7.00 (m, 2H), 7.45-7.50 (m, 1H), 8.00 (d, J= 7.7 Hz, 1H), 8.45 (s, 1H), 9.17 (broad s, 1H), 10.87 (broad s, 1H), 11.93 (bs 1H). ¹³C-NMR (DMSO-ᵈ, δ): 14.1, 114.7, 114.8, 117.3, 119.0, 123.7, 126.8, 128.4, 134.0, 153.4, 159.0, 168.1. MS [ESI, m/z]: 334.9, 336.9 [M+H]. Anal. Calcd for C₁₄H₁₁ClN₄O₂S: C, 50.23; H, 3.31; N, 16.73. Found: C, 50.12; H, 3.57; N, 16.36.

4.1.2.30 N’-(6-Chloro-5-methylthieno[2,3-d]pyrimidin-4-yl)picolinohydrazide (29i)
Purified by crystallisation from MeOH. Obtained in 61% yield as a pale yellow solid. M.p. 210-212 °C. ¹H-NMR (DMSO-ᵈ, δ): 2.60 (s, 3H), 7.66-7.69 (m, 1H), 8.03-8.09 (m, 2H), 8.37 (s, 1H), 8.72-8.74 (m, 1H), 9.26 (broad s, 1H), 10.77 (broad s, 1H). ¹³C-NMR (DMSO-ᵈ, δ): 14.2, 115.0, 121.4, 122.3, 126.9, 131.9, 137.8, 144.2, 148.6, 149.3, 162.7. MS [ESI, m/z]: 319.9, 321.9 [M+H]. Anal. Calcd for C₁₃H₁₀ClN₅O₂S: C, 48.83; H, 3.15; N, 21.90. Found: C, 48.79; H, 3.12; N, 21.97.

4.1.3 Synthesis of 2-Pyridine sulfonyl acid N’-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d]pyrimidin-4-yl)-hydrazide (37)

Hydrazine 8a (0.20 g, 0.9 mmol) was suspended in pyridine (7 mL) under N₂ atmosphere. 2-Pyridinesulfonyl chloride [46] (0.7 mmol) dissolved in pyridine (3 mL) was then added dropwise to the reaction mixture under ice-cooling. The reaction was stirred at 0 °C for 1 h and then at r.t. for 24 h. The reaction mixture was then diluted with EtOAc (40 mL) and washed with 0.5 M HCl solution (2 x 50 mL). The water phase was then extracted with EtOAc (2 x 50 mL) and the organic layers were collected, dried over MgSO₄ and concentrated under vacuum. The crude residue was purified by crystallisation from EtOH giving a white solid in 34% yield. M.p. 189-191 °C. ¹H-NMR (DMSO-ᵈ, δ): 1.78-1.84 (m, 4H), 2.75-2.79 (m, 2H), 2.90-2.94 (m, 2H), 7.59-7.62 (m, 1H), 7.90 (d, J= 7.7 Hz, 1H), 7.94-8.00 (m, 1H), 8.02 (s, 1H), 8.63-8.67 (m, 1H), 8.83 (broad s, 1H), 10.10 (broad s, 1H). ¹³C-NMR (DMSO-ᵈ, δ): 21.8, 22.0, 24.9, 25.2, 115.4, 122.7, 126.3, 127.2, 133.3, 138.2, 149.6, 151.4, 156.3, 156.8, 165.2. MS [ESI, m/z]: 362 [M+H]. Anal. Calcd for C₁₅H₁₁N₂O₂S₂: C, 49.84; H, 4.18; N, 19.38. Found: C, 49.81; H, 4.11; N, 19.45.

4.1.4 General method for the preparation of N-aryl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amines 38-40
Hydrazine 8a (0.20 g, 0.9 mmol), the different arylamine (1.8 mmol) and NaHCO$_3$ (0.15 g, 1.8 mmol) were heated under reflux in iPrOH (8 mL) for 96 h. The reaction mixture was then cooled to r.t. and concentrated under vacuum. The crude residue was purified by flash column chromatography.

4.1.4.1  N-(1H-Benzo[d]imidazol-2-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (38)
Purified by flash column chromatography eluting with n-hexane-EtOAc-MeOH 50:50:0 v/v increasing to n-hexane-EtOAc:MeOH 0:95:5 v/v. Obtained in 12% yield as a pale yellow solid. TLC (9:1 EtOAc-MeOH, Rf: 0.65). M.p. 122-125 °C. $^1$H-NMR (CDCl$_3$), $\delta$: 1.52-1.60 (m, 1H), 1.61-1.68 (m, 1H), 1.76-1.84 (m, 1H), 1.86-1.93 (m, 1H), 2.01-2.09 (m, 1H), 2.28-2.34 (m, 1H), 2.89-2.93 (m, 2H), 5.54 (broad s, 2H), 6.79 (d, J= 7.9 Hz, 1H), 6.99-7.02 (m, 1H), 7.16-7.19 (m, 1H), 7.46 (d, J= 7.9 Hz, 1H), 9.01 (s, 1H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 21.9, 22.4, 24.3, 26.1, 108.8, 116.8, 120.6, 122.8, 126.7, 127.1, 134.9, 141.0, 142.0, 148.0, 152.4, 171.7. MS [ESI, m/z]: 322 [M+H]. Anal. Calcd for C$_{17}$H$_{15}$N$_5$S: C, 63.53; H, 4.70; N, 21.79. Found: C, 63.48; H, 4.50; N, 21.81.

4.1.4.2  N-(Quinolin-8-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (39)
Purified by flash column chromatography eluting with n-hexane-EtOAc 100:0 v/v increasing to n-hexane-EtOAc 85:15 v/v. Obtained in 14% yield as a pale yellow solid. TLC (7:3 n-hexane-EtOAc, Rf: 0.59). M.p. 193-196 °C. $^1$H-NMR (CDCl$_3$), $\delta$: 1.95-1.99 (m, 2H), 2.03-2.08 (m, 2H), 2.86-2.90 (m, 2H), 3.32-3.37 (m, 2H), 7.40-7.44 (m, 2H), 7.55-7.59 (m, 1H), 8.14 (d, J= 7.8 Hz, 1H), 8.61 (s, 1H), 8.78-8.81 (m, 1H), 9.16 (d, J= 7.5 Hz, 1H), 10.24 (broad s, 1H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 22.5, 22.8, 25.6, 26.5, 116.2, 117.9, 119.9, 121.3, 125.8, 127.4, 128.0, 134.1, 135.7, 136.2, 138.9, 147.8, 152.4, 154.4, 166.0. MS [ESI, m/z]: 333 [M+H]. Anal. Calcd for C$_{19}$H$_{16}$N$_4$S: C, 68.65; H, 4.85; N, 16.85. Found: C, 68.47; H, 4.95; N, 16.91.

4.1.2.3  N-(Naphthalen-1-yl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4-amine (40)
Purified by flash column chromatography eluting with n-hexane-EtOAc 100:0 v/v increasing to n-hexane-EtOAc 70:30 v/v. Obtained in 21% yield as a pale yellow solid. M.p. 169-172 °C. TLC (7:3 n-hexane-EtOAc, Rf: 0.46). $^1$H-NMR (CDCl$_3$), $\delta$: 1.97-2.02 (m, 2H), 2.03-2.08 (m, 2H), 2.91-2.94 (m, 2H), 3.20-3.23 (m, 2H), 7.38 (broad s, 1H), 7.52-7.56 (m, 2H), 7.57-7.59 (m, 1H), 8.14 (d, J= 7.8 Hz, 1H), 8.61 (s, 1H), 8.78-8.81 (m, 1H), 9.16 (s, 1H), 10.24 (broad s, 1H). $^{13}$C-NMR (CDCl$_3$), $\delta$: 22.5, 22.8, 25.6, 26.8, 116.7, 121.3, 122.2, 125.0, 125.8, 126.1, 126.1, 126.4, 128.7, 128.8, 133.4, 134.4, 134.6, 152.9, 156.3, 166.5. MS [ESI, m/z]: 332 [M+H]. Anal. Calcd for C$_{20}$H$_{17}$N$_3$S: C, 72.48; H, 5.17; N, 12.68. Found: C, 72.40; H, 5.16; N, 12.73.

4.2 Molecular Modelling
All molecular modelling studies were performed on a MAC pro 2.66 GHz Quad-Core Intel Xeon, running Ubuntu. Molecular Operating Environment (MOE) 2014.10 [28] and Maestro (Schrodinger version 9.0) [29] were used as molecular modelling softwares. All minimisations were performed with MOE until RMSD gradient of 0.001 Kcal mol$^{-1}$ Å$^{-1}$ with the AMBER99 force field. Partial charges were
automatically calculated. Conformational analyses were performed with MOE 2014.10; conformers with a strain energy >4 kcal/mol were discarded, and the maximum number of conformations per ligand was set to 500. In every step MOE default settings were applied. Pharmacophoric filters were created within MOE 2010.10 choosing the PCH (polar-charged-hydrophobic) scheme. Docking experiments were carried out using GlideSP module in Maestro [29] with the default options. A 12 Å docking grid was generated using as centroid defined by Arg393, Glu493 and Trp501 in the 3KQH crystal structure. Docking results were rescored using Plants ChemPLP [31], FlexX [32] and Glide XP [30] scoring functions.

4.3 HCV replicon assay

The compounds were dissolved in dimethyl sulfoxide, stored at -20 °C protected from light and further diluted in culture medium prior to use. The Huh 5-2 HCV subgenomic replicon-containing cells were provided by Prof. R. Bartenschlager (University of Heidelberg, Heidelberg, Germany). Huh 5.2 cells, containing the hepatitis C virus genotype 1b I389Iuc-ubi-neo/NS3-3'/5.1 replicon were sub-cultured in DMEM supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin and 2% Geneticin at a ratio of 1:3 to 1:4, and grown for 3-4 days in 75 cm2 tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in assay medium (DMEM, 10% FCS, 1% non-essential amino acids, 1% penicillin/streptomycin) at a density of 6 500 cells/well (100 μL/well) in 96-well tissue culture microtiter plates for evaluation of anti-metabolic effect and CulturPlate (Perkin Elmer) for evaluation of the antiviral effect. The microtiter plates were incubated overnight (37 °C, 5% CO2, 95-99% relative humidity), yielding a nonconfluent cell monolayer. The evaluation of the anti-metabolic as well as antiviral effect of each compound was performed in parallel.

Four-step, 1-to-5 compound dilution series were prepared for the first screen, to collect data for a more detailed dose-response curve, an eight-step, 1-to-2 dilution series was used. Following assay setup, the microtiter plates were incubated for 72 hours (37 °C, 5% CO2, 95-99% relative humidity). For the evaluation of anti-metabolic effects, the assay medium was aspirated, replaced with 75 μL of a 5% MTS solution in phenol red-free medium and incubated for 1.5 hours (37 °C, 5% CO2, 95-99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan), and optical densities (OD values) were converted to percentage of untreated controls. For the evaluation of antiviral effects, assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 μL of Glo Lysis Buffer (Promega) was added allowing for cell lysis to proceed for 5 min at room temperature. Subsequently, 50 μL of Luciferase Assay System (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire, Tecan). Relative luminescence units were converted into percentage of untreated controls.

The EC50 and EC90 (values calculated from the dose-response curve) represent the concentrations at which 50% and 90% inhibition, respectively, of viral replication is achieved. The CC50 (value calculated from the dose-response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50% as compared to untreated cells.

4.4 HCV NS3 helicase enzymatic assays
Molecular beacon-based NS3 helicase assays were performed as described by Hanson et al. [47]. Reactions contained 25mM MOPS pH 6.5, 1.25mM MgCl₂, 5% DMSO, 5 µg/ml BSA, 0.01% (v/v) Tween20, 0.05 mM DTT, 5 nM fluorescent DNA substrate, 12.5 nM NS3h, and 1 mM ATP. The ability of each compound to displace NS3 from a DNA oligonucleotide was monitored as described by Mukherjee et al. [48]. Each assay contained 15 nM NS3h, 25 mM MOPS, pH 7.5, 1.25 mM MgCl₂, 0.0025 mg/ml BSA, 0.005% (v/v) Tween20, 0.025 mM DTT and 12.5nM NS3h_(1b). The ability of NS3 to hydrolyze ATP was monitored as described by Sweeney et al. [49]. Reactions performed in the presence of RNA contained 25mM MOPS pH 6.5, 1.25mM MgCl₂, 15 µM Poly U RNA (Sigma), 6 nM NS3h_1b(con1) in 5 µg/mL BSA, 0.001% Tween 20. Reactions performed in the absence of RNA contained all the same reagents except that 300 nM NS3h _1b(con1) was included. To determine the compound concentration needed to reduce helicase catalyzed ATP hydrolysis by 50 % (IC₅₀), reactions were performed in duplicate two-fold dilution series such that final compound concentrations ranged from 0.5 mM to 0.78 µM. Data obtained from all reactions within the linear range of the assays were normalized to controls lacking inhibitor (100%) and controls lacking enzyme (0%), and fit to a normalized dose response equation with a variable Hill slope using GraphPad Prism (v. 6). Reactions were performed in duplicate and each titration was fit to the above concentration response equation. Average IC₅₀ values ± standard deviations are reported.

Acknowledgements
The authors would like to acknowledge Biovitas Capital Inc (Rome) for funding (M.B.).

References

8 FDA approves Sovaldi for chronic hepatitis C in FDA New Release, U.S. Food and Drug Administration, 2013-12-06.

Anna Suk-Fong Lok. HCV NS5A Inhibitors in Development. Clinics in Liver Disease, 2013, 17 (1), 111-121.


www.gilead.com/~/media/Files/pdfs/medicines/liver-disease/harvoni/harvoni_pi


26 Gu, M.; Rice, C.M. Three conformational snapshots of the hepatitis C virus NS3 helicase reveal a ratchet translocation mechanism. PNAS 2010, 107, 521-528;


44 Shadrick, W.R.; Mukherjee, S.; Hanson, A.M.; Sweeney, N.L.; Frick, D.N. Aurintricarboxylic acid modulates the affinity of hepatitis C virus NS3 helicase for both nucleic acid and ATP. Biochemistry 2013, 52, 6151-6159.


