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Identification of thieno[3,2-*b*]pyrroles as allosteric inhibitors of hepatitis C virus NS5B polymerase

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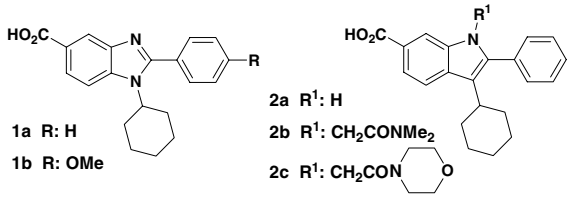
Abstract—Thieno[3,2-*b*]pyrroles are a novel class of allosteric inhibitors of HCV NS5B RNA-dependent RNA polymerase which show potent affinity for the NS5B enzyme. Introduction of a polar substituent in the position N1 led to a compound that efficiently blocks subgenomic HCV RNA replication in HUH-7 cells with an EC_{50} of 2.9 μM.

Hepatitis C virus (HCV) is a major world health problem that affects an estimated 170 million individuals worldwide.[1] Despite considerable reduction of the incidence of new infections due to the improvements in blood screening in many countries,[2] HCV has become the leading cause of liver transplantation worldwide and results in up to 10,000 deaths annually in the U.S. alone.[3] Current treatments based on interferon-α are effective in only a fraction of the patient population who are infected with HCV genotype 1 that predominates in industrialized nations.[4] These treatments are also poorly tolerated and expensive, adding urgency to the need for novel therapeutic agents to combat HCV.

The virally encoded HCV non-structural proteins are attractive targets for antiviral therapy as they have been shown to play an essential role in the replication of the virus.[5] In particular, NS5B RNA-dependent RNA polymerase has emerged as an especially attractive target for drug discovery efforts.[6] The absence of a functional counterpart to NS5B in mammalian cells, where DNA is usually the template during DNA transcription, may favor the development of selective and nontoxic inhibitors. Toward this goal several series of NS5B inhibitors that show activity in the replicon assay have been reported.[7,8] Chain terminating nucleoside analogues that bind at the active site of NS5B have been found to show potency in the submicromolar range.[7] Conversely, allosteric inhibition by small-molecule inhibitors of NS5B has emerged as a bona fide route toward inhibition of subgenomic HCV RNA replication, and a number of structurally diverse inhibitor classes have now been identified.[8]

In initial SAR studies on small-molecule inhibitors with a benzimidazole core we and others identified benzimidazoles such as **1a** as inhibitors of the NS5B polymerase.[9] This molecule possesses a benzimidazole core with pendant aryl functionality at C2, cycloalkyl functionality at N1, and a carboxylic acid group at C5. All three groups were identified as essential for the activity of this series of inhibitors. Replacement of the central core by the more lipophilic indole ring led to **2a** which displays nanomolar potency against NS5B polymerase and shows efficacy in the replicon assay.[9a]



1a IC_{50} 6.5 μM EC_{50} >50 μM
1b IC_{50} 11.2 μM EC_{50} >50 μM
2a IC_{50} 0.048 μM EC_{50} 6.7 μM
2b IC_{50} 0.059 μM EC_{50} 1.5 μM
2c IC_{50} 0.026 μM EC_{50} 0.8 μM

Keywords: HCV NS5B polymerase; Allosteric inhibitors; Thieno[3,2-*b*]-pyrrole.
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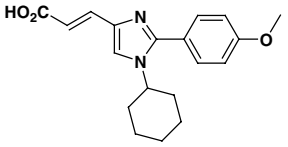
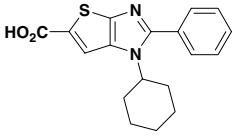
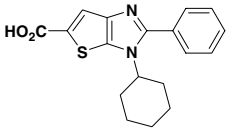
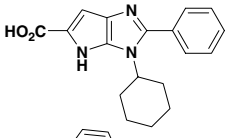
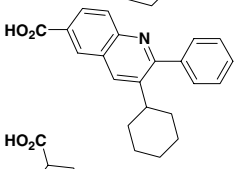
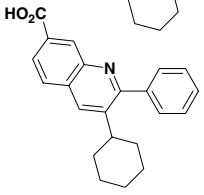
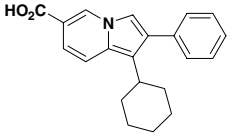
Introduction of an acetamide side chain at the position N1 of the indole ring, as in **2b**, led to a 5-fold improvement in cell-based activity over the N-unsubstituted indole **2a**.[9a] Benzimidazole inhibitors were shown by us[10,11] and others[6b] to bind to the thumb domain of NS5B and replicons resistant to this class of inhibitors harbor a single escape mutation of Pro495, located in the upper thumb region, to either Leu or Ala.[11] This was confirmed by the crystal structure of NS5B complexed with indole **2c**, where it occupies a well-defined lipophilic site with the indole ring stacked against the side chain of Pro495, the cyclohexyl and phenyl ring substituents filling two closely spaced pockets, and the carboxylate substituent forms a salt bridge with the side chain of Arg503.[10] Binding of the inhibitors to the site in the thumb domain prevents interactions with loops extending from the finger domain. It is hypothesized that this locks the polymerase in an unproductive conformation.[10]

In parallel to the work done in the indole series, we also explored the replacement of the 6,5-bicyclic core of benzimidazole **1a** with other scaffolds, and present here the initial results of this work. Trisubstituted imidazole **3** (Table 1), where the 6-membered ring is replaced by a double bond, shows a 7-fold decrease in activity with respect to benzimidazole **1b**.[12] This loss in potency can be explained by two factors: the higher flexibility of the acid moiety with respect to **1b**, that would weaken a critical hydrogen bond interaction with the protein, and the absence of the carbons of the benzo moiety, which engage in a lipophilic interaction in the binding pocket.[10] Next, we focused our work on the study of other bicyclic systems exploring the effect of the ring size and position of the heteroatoms. Introduction of a 5-membered ring, as in thieno[2,3-*d*]imidazole **4**, led to 2-fold loss in enzymatic activity which may be due to a slightly different orientation of the carboxylic acid. Similar potency was observed in the regioisomeric thieno[2,3-*d*]imidazole **5**. Interestingly, introduction of a pyrrole ring as in **6** led to a complete loss of activity probably due to the basic character (calcd pK_a 9.6) of the 3,4-dihydropyrrolo[2,3-*d*]imidazole **6** compared with benzimidazole **1a** (calcd pK_a 5.4). The replacement of the benzimidazole core by fused 6,6-bicyclic scaffolds gave quinoline **7** which showed similar potency to our lead compound **1a**. By contrast, the isomeric quinoline **8**, with the carboxylic acid differently positioned in space, was not active. An overlay of quinoline **7** with benzimidazole **1a** showed a good overlap between the three key functional groups which we believe to be important for activity (see Fig. 1). Quinoline **8** presented the same good overlap of the cyclohexyl and phenyl groups, but the acid is in a different position, possibly explaining the difference in potency. Finally, indolizine **9**, with a 6,5-bicyclic scaffold, displayed a 70-fold increase in potency with respect to the benzimidazole **1a**. This is a result of a less polar core favoring interaction at a lipophilic site of the enzyme as was observed previously for indole **2a**.[9a]

Unfortunately, all the compounds from Table 1 were not active up to 100 μM concentration in a HCV RNA replication assay. This can be explained by insufficient potency in the case of compounds **3**–**8**, whereas indolizine **9**, which is only 2-fold less active than **2a**, was found to be unstable in the medium used for the cell-based assay.

Table 1. SAR in the central core: NS5B enzyme inhibition (IC_{50})[12]

Compound		IC_{50} (μM)[a]
3		77.3
4		13.8
5		17.2
6		na
7		4.8
8		na
9		0.094

[a] na means IC_{50} > 50 μM.

A first hint of cell-based activity was observed when we made and tested the 5-azaindole **10a** (Table 2). This compound showed activity in the low micromolar range on the enzyme, but measurable inhibition of the replicon (EC_{50} = 48.2 μM). The loss of potency with respect to **2a** was thought to be due to the position and the basicity of the newly introduced nitrogen (calcd pK_a 8.6) in a lipophilic environment. Conversely, the less basic 7-azaindole **11a** (calcd pK_a 5.4) is 12-fold more active on the enzyme, confirming the hypothesis, but only 3-fold more active in the replicon assay with respect to **10a**.

We have found that the introduction of a *N*,*N*-dimethylacetamide chain in indole **2b** improved cell-based potency, probably by modulating the physicochemical properties of the molecule. For example, it was noted

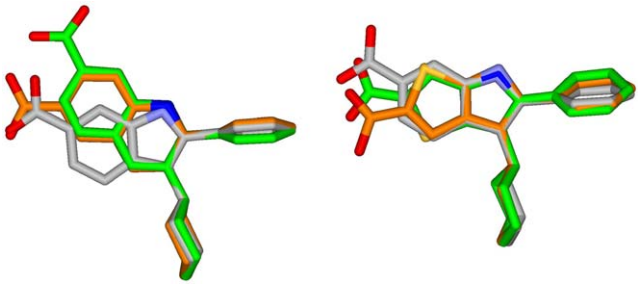
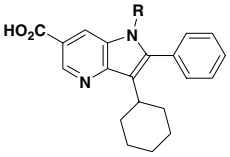
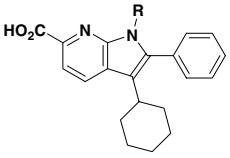
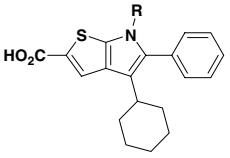
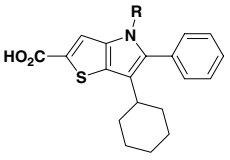


Figure 1. Overlapping of quinolines **7** (orange) and **8** (green) with indole **2a** (gray) (left) and of thienopyrroles **12a** (orange) and **13a** (green) with indole **2a** (gray) (right).

Table 2. Trisubstituted azaindoles and thienopyrroles: NS5B enzyme inhibition (IC_{50}) and cell-based efficacy (EC_{50})[12]

Compound[a]		IC_{50} (μM)	EC_{50}[b] (μM)	log D[c,13]
10		a 1.9 b 2.2	48.2 na	2.5 0.8
11		a 0.15 b 0.32	16.8 9.8	3.3 1.1
12		a 0.12 b 0.23	19.5 9.5	nd nd
13		a 0.066 b 0.058	11.2 2.9	3.4 1.6

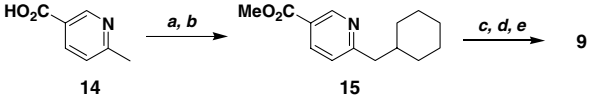
[a] a R: H, b R: CH_2CONMe_2.
[b] na means $EC_{50} > 50$ μM.
[c] nd means not determined.

that the log D decreased by two units (compare **2b** log D 2.1 with **2a** calcd log D 4.2).[13] This same behavior was observed for the 7-azaindole series, where the acetamide **11b** (log D 1.1), despite the 2-fold loss in intrinsic potency, is nearly 2-fold more active in the cell-based assay with respect to **11a** (log D 3.3). However, this was not the case for the 5-azaindole scaffold, where the introduction of the acetamide lowered the log D from 2.5 for **10a** to 0.8 for **10b**, but not the basicity of the azaindole scaffold (calcd pK_a 7.6), with the consequence that no improvement in replicon activity was noted for **10b** with respect to **10a**.

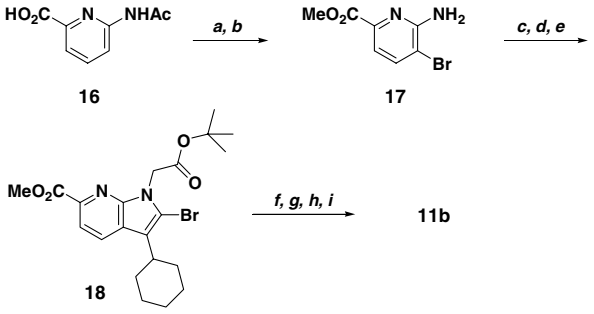
As shown in thieno[2,3-*d*]imidazole **4**, replacement of the phenyl ring by a thiophene in the bicyclic scaffold is tolerated, and consequently this was also applied to the indole scaffold. Thieno[2,3-*b*]pyrrole **12a** is a potent inhibitor of NS5B polymerase, being only 3-fold less active than indole **2a**. Interestingly, the regioisomeric thieno[3,2-*b*]pyrrole **13a** is nearly as potent as indole **2a**. Due to the bigger length of the C-S versus the C–C bond, the spatial orientation of the carboxylic acid is different between the two regioisomers and also in comparison to **2a**. The overlap is better for the more active thieno[3,2-*b*]pyrrole **13a** (Fig. 1). Compound **13a** showed a 170-fold loss in potency in the cell-based assay (EC_{50} = 11.2 μM) with respect to the enzyme assay. As shown previously, introduction of the *N*,*N*-dimethylacetamide group resulted in a less lipophilic compound **13b** (log D 1.6 vs 3.4 for **13a**) and a 4-fold improvement in replicon activity (EC_{50} = 2.9 μM). For the less active thieno[2,3-*b*]pyrrole **12a** a 2-fold gain in the replicon assay was seen with the acetamide group. In order to determine the mechanism of inhibition of this series of inhibitors, **13a** was tested against NS5B bearing the P495L mutation. Compound **13a** lost around two orders of magnitude in potency (IC_{50} = 4 μM), showing to bind at the same binding site as indole **2c**.[9a,10]

Some compounds described in Table 1 were accessed by synthetic procedures described in the literature.[14] Indolizine **9** was prepared as outlined in Scheme 1. Alkylation of 2-methylpyridine-5-carboxylic acid **14** in the substituent in position 2 was followed by hydrogenation of the cyclohexene ring and in situ esterification to give the intermediate **15**. Reaction of compound **15** with 2-bromoacetophenone followed by treatment with $NaHCO_3$ led to the formation of the indolizine system.[15] Final hydrolysis of the ester with KOH led to compound **9**.

Compound **11b** was synthesized starting from commercially available pyridine **16**. Esterification of the carboxylic group with AcCl/MeOH led to concomitant deprotection of the amide in position 2. Subsequent regioselective bromination afforded the intermediate **17**. Construction of the azaindole system was done by Sonogashira reaction with 1-phenyl-2-(trimethylsilyl)acetylene and subsequent ring closure in the presence of Pd catalyst.[16] Subsequent bromination with NBS in the position 2 followed by N-alkylation with *tert*-butyl bromoacetate gave the intermediate **18**. Reaction of **18** with phenylboronic acid under Suzuki-type cross-coupling conditions led to the introduction of the substituent at the position C2 as described in Scheme 2. Elaboration of the N1 chain by selective ester deprotection and amide coupling, followed by



Scheme 1. Reagents and conditions: (a) *n*-BuLi, 3-bromocyclohexene, THF, −78 °C; (b) H_2, Pd/C; $TMSCHN_2$ (30% over two steps); (c) 2-bromoacetophenone, acetone, reflux; (d) $NaHCO_3$; (e) KOH (50% over three steps).

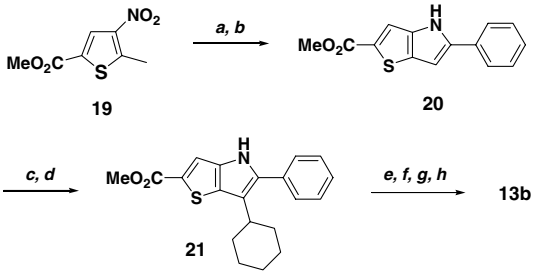


Scheme 2. Reagents and conditions: (a) AcCl, MeOH, reflux (64%); (b) Br_2, $CHCl_3$, rt (28%); (c) $C_6H_{11}C{\equiv}CTMS$, $Pd(dppf)Cl_2$, LiCl, Na_2CO_3, DMF, 110 °C (63%); (d) $BrCH_2CO_2{}^tBu$, NaH, DMF, 60 °C; (e) NBS, DCM, rt (48% over two steps); (f) $C_6H_5B(OH)_2$, $Pd(PPh_3)_4$, K_3PO_4, toluene, 110 °C (61%); (g) TFA, DCM (100%); (h) Me_2N-H·HCl, HATU, DIPEA, DMF, RT (100%); (i) BBr_3, DCM (42%).

hydrolysis of the methyl ester in the presence of BBr_3, gave the trisubstituted azaindole **11b**.

Compound **13b** was prepared as described in Scheme 3. The thieno[3,2-*b*]pyrrole core was synthesized by condensation of methyl 5-methyl-4-nitrothiophene-2-carboxylate **19** with benzaldehyde followed by ring-closure with triethyl phosphite as described in the literature.[17] Introduction of the cyclohexyl ring was performed by condensation of intermediate **20** with cyclohexanone and reduction with triethylsilane giving precursor **21**. Introduction of the *N*,*N*-dimethylacetamide chain at the position N1 was done as described in the literature.[9a] Subsequent hydrolysis of the ester group afforded compound **13b**.

In summary, we have described here the SAR performed in the central core of our initial NS5B polymerase inhibitor lead **1a**. This work led to the discovery of a novel series of thieno[3,2-*b*]pyrroles that are potent allosteric inhibitors of the HCV NS5B polymerase. Introduction of a polar substituent in the position N1 led to compound **13b** which efficiently blocks subgenomic HCV RNA replication in HUH-7 cells at low micromolar concentration. Further optimization of this new series of inhibitors will be described in the near future.



Scheme 3. Reagents and conditions: (a) PhCHO, pyrrolidine, MeOH, reflux; (b) $(EtO)_3P$, MW (55% over two steps); (c) cyclohexanone, H_3PO_4, Ac_2O, AcOH, 80 °C (70%); (d) Et_3SiH, TFA, rt (100%); (e) $BrCH_2CO_2{}^tBu$, NaH, DMF, rt; (f) TFA, DCM (95% over two steps); (g) Me_2NH·HCl, HATU, DIPEA, DMF, rt; (h) NaOH 2 N, MeOH/THF (40% over two steps).

Acknowledgments

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12. The compounds described herein were assessed for activity (IC_{50}) against the purified BK strain of NS5BΔC55 enzyme in the presence of heterogenic template RNA cells using a modification of the procedure described in Ref. 11. Inhibition of the replication of subgenomic HCV RNA was measured in HUH-7 cells using a modification of the procedure described in Lohmann, V.; Korner, F.; Koch, J.; Henan, U.; Theilmann, L.; Bartenschlager, R. *Science* **1999**, *285*, 110. IC_{50} and EC_{50} values are the average of at least two independent determinations. Cell-based data (EC_{50}) were measured in the presence of 10% fetal calf serum.
13. The partition coefficient of the compounds between 1-octanol and buffer, log *D*, was determined at pH 7.4 using a modification of the procedure described in Hitzel, L.; Watt, A. P.; Locker, K. L. *Pharm. Res.* **2000**, *17*, 1389. log *D* values are the average of at least two independent determinations.
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