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Structural Investigation of Cycloheptathiophene-3-carboxamide Derivatives Targeting Influenza Virus Polymerase Assembly

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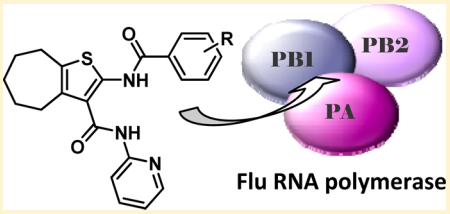
Supporting Information

ABSTRACT: The limited number of drug classes licensed for treatment of influenza virus (Flu), together with the continuous emergence of viral variants and drug resistant mutants, highlights the urgent need to find antivirals with novel mechanisms of action. In this context, the viral RNA-dependent RNA polymerase (RdRP) subunits assembly has emerged as an attractive target. Starting from a cycloheptathiophene-3-carboxamide derivative recently identified by us for its ability to disrupt the interaction between the PA and PB1 subunits of RdRP, we have designed and synthesized a series of analogues. Their biological evaluation led to the identification of more potent protein−protein interaction inhibitors, endowed with antiviral activity that also encompassed a number of clinical isolates of FluA, including an oseltamivir-resistant strain, and FluB, without showing appreciable toxicity. From this study, the cycloheptathiophene-3-carboxamide scaffold emerged as being particularly suitable to impart anti-Flu activity.



INTRODUCTION

The discovery of novel antiviral therapies[1] and the continuing development of annual vaccines[2] have not yet led to an adequate treatment for influenza virus (Flu), an important pathogen responsible for both yearly seasonal epidemics and more extensive global pandemics. This issue was clearly evident during the unexpected emergence of the 2009 H1N1 pandemic strain by the reassortment of genes from human, pig, and bird's H1N1 viruses.[3] This past pandemic, together with the ongoing circulation of highly pathogenic avian H5N1 strains and the recent emergence of the H7N9 virus, a new reassortant of avian origin isolated in China and associated with severe respiratory disease with 40% of mortality,[4] which could potentially adapt for human-to-human transmission,[5] highlighted the vulnerability of the world population to novel Flu strains. Although vaccination remains the main prophylactic strategy for controlling Flu infection, during the lagging time needed to produce a new vaccine and even during typical epidemic years, since vaccination does not prevent completely Flu infection, our only weapon against pandemic Flu are antivirals. Currently, only two classes of drugs have been approved by FDA for the treatment of Flu: the neuraminidase inhibitors oseltamivir and zanamivir, which are active on both influenza A (FluA) and influenza B (FluB) viruses, and the M2 channel blockers amantadine and rimantadine, whose spectrum of action is limited to FluA. Since Flu viruses have a high mutation rate, a major problem with both classes of drugs is the emergence of drug-resistant strains. Clearly, next-generation antivirals are needed to efficiently combat Flu, preferably with an innovative mechanism of action. The viral RNA-dependent RNA polymerase (RdRP) provides an attractive target[6−9] given its functional essentiality for viral replication and involvement in virus pathogenicity.[10−12] RdRP could be ideal for the development of new antivirals, since it is highly conserved among FluA, FluB, and FluC[13] while no homologue has been found in mammalian cells.[6,8,13] RdRP is a complex of three subunits, polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2), which are responsible for both transcription and replication.[10,14−16] The assembly of the three subunits into functional viral RdRP is an essential step for Flu RNA synthesis and virus replication.[17−19] Thus, the interference with its correct assembly through protein−protein interaction (PPI) inhibitors represents an attractive strategy to inhibit this enzyme. Although very challenging,[20−23] the feasibility of such an approach has been demonstrated by the identification of antiviral peptides able to inhibit the PA−PB1 interaction.[24−27] The recent publication of two crystallographic structures of a truncated form of PA bound to a PB1-derived peptide has

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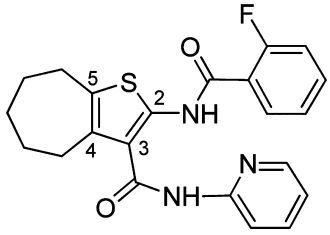
shown that relatively few residues drive the binding of PB1 to PA,[28,29] suggesting the potential for small molecule-mediated inhibition. The proof-of-principle that the PA−PB1 interaction can indeed be disrupted also by small molecular-weight compounds was recently provided by us[30−32] and a few other authors.[33,34] In our SBDD approach, an in silico screening of 3 million small molecules from the ZINC database, performed using one of the available crystal structures,[28] led to the identification of 32 virtual hits, which were then evaluated in vitro for their ability to disrupt the PA−PB1 interaction. Five of them inhibited this interaction specifically and in a dose-dependent manner with IC_{50} values in the micromolar range,[31] making all of them worthy of further investigation.

In the present study, we attempted the structural optimization of thiophene-3-carboxamide derivative **1** (Figure 1, compound **10** in ref 31). This compound was able to disrupt



compound **1**

Figure 1. Hit compound previously identified by SBDD.

the PA−PB1 interaction in vitro with an IC_{50} of 90.7 μM, and even though it exhibited antiviral activity against FluA in infected cells at EC_{50} values slightly higher than 100 μM, its peculiar mechanism of action, coupled with the lack of cytotoxicity (CC_{50} > 250 μM) evaluated in two cell lines (Mardin−Darby canine kidney (MDCK) and HEK 293T), made this compound a valid starting point. In an attempt to improve the ability to disrupt the PA−PB1 interaction and achieve a better anti-Flu activity while maintaining the lack of toxicity, a number of structural modifications were undertaken synthesizing a series of analogues (compounds **2**−**36** in Table 1). Herein we report on their design, synthesis, and biological evaluation.

DESIGN OF THIOPHENE-3-CARBOXAMIDE DERIVATIVES

Compound **1** is made up of a tetrahydrocycloheptathiophene ring bearing at both the C-2 and C-3 positions an amide moiety functionalized with an aromatic ring (Figure 1). The attention was mainly focused on modifying the *o*-fluorophenyl ring placed at the C-2 position, because of the higher synthetic accessibility. In particular, following the classical medicinal chemistry strategy, the *o*-fluoro atom was eliminated (compound **2**), shifted as in its positional isomers (compounds **3** and **4**) as well as replaced by a chlorine atom (compounds **5** and **6**). Dihalogen derivatives (compounds **7** and **8**) were also prepared. A cyclohexyl ring replaced the phenyl group in derivative **9**. Then the phenyl/cyclohexyl ring was spaced by inserting one or two methylenic units (compounds **10**−**16**).

Additional derivatives were synthesized following the suggestions of a computational study performed using the fingerprints for ligands and proteins (FLAP) algorithm. This method recently led us to identify a number of inhibitors of the PA/PB1 binding,[31] using the X-ray crystallographic structure of the PA/PB1 complex[28] as a template. Although FLAP is not a classical docking procedure based on energy minimization, it allows for evaluation of hypotheses on the binding mode of the screened compounds, looking for the best overlap of the GRID molecular interaction fields (MIFs)[35] calculated for the protein cavity and for the ligand.[36−38] Shape, hydrophobic, hydrophilic, and hydrogen-bond donor and acceptor interactions are considered. Since GRID MIFs are energy-related, a high similarity between the MIFs of the protein and of the ligands indicates an energetically favored binding mode.

Our previous study revealed the importance of hydrophobic interactions in the ligand−PA recognition process.[31] The green spots in Figure 2A represent the hydrophobic regions in the PA cavity generated by GRID force field.[35] The presence of those regions in the PA cavity is in agreement with the findings of Liu et al.,[39] who described the existence of three hydrophobic pockets. Indeed, one hydrophobic pocket is generated by W706 and P411 and is responsible for the binding of P5 from PB1. The second hydrophobic pocket is due to P710 and L666 interactions, and it is responsible for the binding of F9 from PB1. Finally, L640, V636, M595, and W619 form the third hydrophobic pocket, wherein L8 of PB1 is located. An additional hydrophobic spot is located in the middle of the cavity and mainly generated by the aliphatic chain of E623 and the protein backbone. The most favorable FLAP docking pose in the PA cavity for compound **1** is shown in Figure 2B. On the basis of our model, compound **1** interacts with the first and the second pockets and with the additional central hydrophobic region. From Figure 2B one can reason that in order to optimize the hydrophobic interactions to better fit the second pocket defined by Liu et al., the *o*-fluorophenyl ring at the C-2 position of compound **1** could be replaced with a bulkier aromatic substituent. In agreement with this hypothesis, a series of bicyclic heteroaromatic derivatives were designed looking for the best compromise between solubility and lipophilicity, according to the predicted values calculated using VolSurf+[40,41] and the synthetic accessibility. Among the selected compounds, we synthesized **17** and **18** as the first examples of such derivatives; their best poses are reported in Figure 2C and Figure 2D. Compounds **1**, **17**, and **18** display a similar orientation, with the tetrahydrocycloheptathiophene moiety being oriented toward the W706 PA residue in the first pocket likely involving $\pi-\pi$ interactions. The substituent in C-2 position is located in the second pocket, while the substituent in C-3 position fits the central hydrophobic spot. In addition, it must be noted that according to our modeling studies, all three compounds might be stabilized by a hydrogen bond between the oxygen of the C-2 amide group and the PA residue K643.

To conclusively determine the role that the simultaneous presence of both the aromatic rings at C-2 and C-3 positions plays on the biological activity, we synthesized the 2-amino derivative **19**, which lacks the *o*-fluorobenzoyl moiety, and the primary amide **20**, which lacks the C-3 pyridine ring. The ester and acid derivatives **21** and **22**, analogues of **20**, were also synthesized together with additional ethyl esters **23**−**26** and the corresponding acids **27**−**30**, characterized by various substituents at the C-2 position.

Few modifications involved the cycloheptane ring, whose size was reduced to cyclohexane and cyclopentane as in compounds **31** and **32**, the direct analogues of **1**, as well as in the C-3 variously functionalized derivatives **33**−**36**. However, the

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Table 1. Structure and Biological Activity of the Compounds Synthesized in This Study

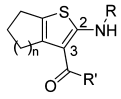
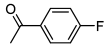
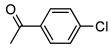
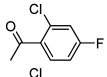
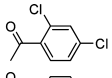
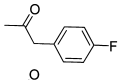
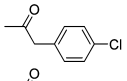
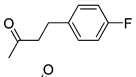
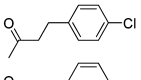
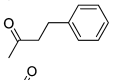
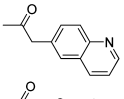
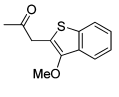
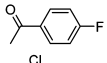
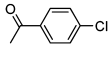
Compd				ELISA PA-PB1 Interaction Assay IC_{50}, μM[a,d]	PRA in MDCK cells EC_{50}, μM[b,d]	Cytotoxicity (MTT Assay) HEK 293T cells CC_{50}, μM[c,d]	Cytotoxicity (MTT Assay) MDCK cells CC_{50}, μM[c,d]
	n	R'	R				
1	3			145 ± 12	90 ± 13	>250	>250
2	3	"		87 ± 11	>100	>250	>250
3	3	"		>200	>100	>250	150
4	3	"		99 ± 20	58 ± 7	>250	>250
5	3	"		134 ± 21	39 ± 3	>250	>250
6	3	"		32 ± 9	18 ± 2	>250	>250
7	3	"		>200	23 ± 3	>250	>250
8	3	"		>200	31 ± 5	231 ± 7	>250
9	3	"		>200	20 ± 5	>250	180 ± 27
10	3	"		>200	16 ± 1	250 ± 16	>250
11	3	"		>200	19 ± 3	>250	>250
12	3	"		>200	19 ± 1	220 ± 16	>250
13	3	"		>200	17 ± 2	>250	>250
14	3	"		>200	>100	>250	>250
15	3	"		>200	18 ± 1	>250	246 ± 5
16	3	"		>200	21 ± 2	>250	>250
17	3	"		48 ± 12	58 ± 11	>250	>250
18	3	"		90 ± 22	61 ± 9	>250	>250
19	3	"	H	35 ± 10	26 ± 4	>250	>250
20	3	NH_2		>200	>100	>250	>250
21	3	OEt	"	200	78 ± 3	>250	>250
22	3	OH	"	200	76 ± 3	>250	>250
23	3	OEt		>200	91 ± 10	>250	>250
24	3	OEt		>200	94 ± 6	>250	>250
25	3	OEt		>200	89 ± 10	>250	>250

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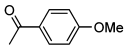
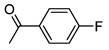
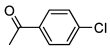
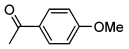
Table 1. continued

Compd	n	R	R'	ELISA PA-PB1 Interaction Assay IC_{50}, μM[a,d]	PRA in MDCK cells EC_{50}, μM[b,d]	Cytotoxicity (MTT Assay) HEK 293T cells CC_{50}, μM[c,d]	Cytotoxicity (MTT Assay) MDCK cells CC_{50}, μM[c,d]
26	3	OEt		>200	95 ± 5	>250	>250
27	3	OH		>200	81 ± 16	>250	>250
28	3	OH		>200	79 ± 16	>250	>250
29	3	OH		>200	43 ± 6	>250	>250
30	3	OH		>200	52 ± 10	>250	>250
31	2			>200	>100	60 ± 8	>250
32	1	"	"	>200	>100	150 ± 8	>250
33	2	NH_2	"	>200	>100	31 ± 5	118 ± 12
34	1	NH_2	"	>200	>100	>250	>250
35	1	OEt	"	>200	>100	>250	>250
36	1	OH	"	159 ± 15	77 ± 15	>250	>250
RBV					8 ± 2	>250	>250
Tat-$PB1_{1-15}$ peptide				35 ± 3	40 ± 5	>100	>100

[a]Activity of the compounds in ELISA PA−PB1 interaction assays. The IC_{50} value represents the compound concentration that reduces by 50% the interaction between PA and PB1. [b]Activity of the compounds in plaque reduction assays with the FluA PR8 strain. The EC_{50} value represents the compound concentration that inhibits 50% of plaque formation. [c]Activity of the compounds in MTT assays. The CC_{50} value represents the compound concentration that causes a decrease of cell viability of 50%. [d]All the reported values represent the mean ± SD of data derived from at least three independent experiments in duplicate.

increased toxicity, especially of the cyclohexane derivatives, has discouraged the synthesis of further analogues.

CHEMISTRY

The synthesis of 2-[(substituted)amino]-*N*-(2-pyridinyl)-thiophene-3-carboxamide derivatives **1**−**18**, **31**, and **32** was accomplished, as outlined in Scheme 1, by applying the two-step Gewald synthesis.[42,43] Thus, a first Knoevenagel condensation of various cycloalkyl ketones with 2-cyano-*N*-pyridin-2-ylacetamide[44] gave *α*,*β*-unsaturated nitriles. These intermediates were used without isolation in the successive cyclization step, performed in the presence of sulfur and *N*,*N*-diethylamine in EtOH to give derivatives **19**, **37**, and **38**. The target compounds **1**−**16**, **31**, and **32** were then obtained by a coupling reaction of these intermediates with the appropriate acyl chlorides in pyridine. Amide bond formation in derivatives **17** and **18** was carried out through an alternative procedure by reacting intermediate **19** with quinolin-6-ylacetic acid and (3-methoxy-1-benzothien-2-yl)acetic acid, respectively, in THF in the presence of 4-(4,6-dimethoxy-(1,3,5)-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)[45] as activating agent.

The starting materials for the preparation of the remaining derivatives were compounds **39**−**41**,[46] **42**,[47] and **43**,[42] which reacting with selected benzoyl chlorides gave thiophene-3-carboxamides **20**, **33**,[48] and **34**[48] and ethyl thiophene-3-carboxylates **21**, **23**, **24**, **25**,[47] **26**, and **35**,[49] as shown in Scheme 2. The thiophene-3-carboxylic acids **22**, **27**−**30**, and **36** were then prepared by basic hydrolysis, starting from the corresponding ethyl esters.

RESULTS AND DISCUSSION

We started the study synthesizing and testing compound **1**. The in-house synthesized **1** displayed biological activity comparable to that reported for the commercial derivative (compound **10** in ref 31). Indeed, it was endowed with a slightly lower PA−PB1 interaction inhibitory activity (IC_{50} = 145 μM) and a slightly higher anti-FluA activity in MDCK cells (EC_{50} = 90 μM). The in-house synthesized **1** was used as comparative compound in all successive studies.

The whole set of derivatives was evaluated for their ability to inhibit the physical interaction between PA and PB1 subunits by ELISA assays including the $PB1_{(1-15)}$−Tat peptide[26] as a positive control. This peptide, which contains a synthetic PB1-derived peptide (amino acids 1−15) fused to the translocating domain of HIV Tat protein, inhibited the PA−PB1 interaction with an apparent IC_{50} of 35 μM (Table 1).

In parallel, for all the synthesized compounds the antiviral activity was tested in FluA virus-infected MDCK cells by plaque reduction assays (PRA) with the A/PR/8/34 (PR8) strain. Ribavirin (RBV), a known inhibitor of RNA viruses polymerase,[50] was included as a positive control, exhibiting an EC_{50} of 8 μM (Table 1).

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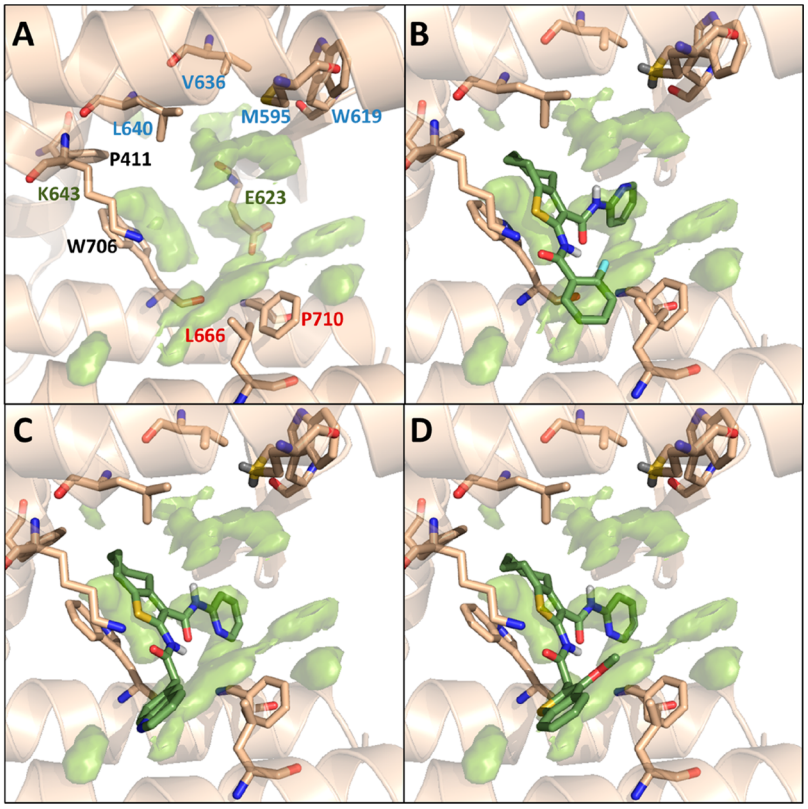
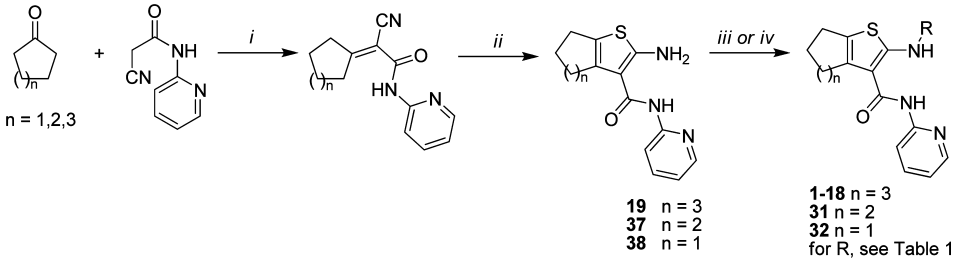


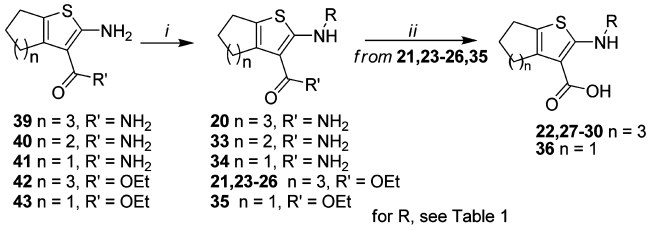
Figure 2. (A) Hydrophobic regions generated by GRID[35] in the PA cavity from the X-ray structure reported by He and co-workers.[28] The best docked poses generated by FLAP for compound **1** (B), compound **17** (C), and compound **18** (D) are also reported, after short minimization. Residues that define the first (black), the second (red), and the third (blue) cavity as defined by Liu et al. are reported. Other residues discussed in the text are reported in green.

Scheme 1[a]



[a]Reagents and conditions: (i) ammonium acetate, glacial acetic acid, benzene, reflux; (ii) sulfur, *N*,*N*-diethylamine, EtOH, 40−50 °C; (iii) acyl chlorides, pyridine; (iv) $ArCH_2CO_2H$, DMTMM, THF, pyridine.

Scheme 2[a]



[a]Reagents and conditions: (i) acyl chlorides, pyridine; (ii) LiOH, H_2O/THF, 50 °C.

To exclude that the observed antiviral activities could be due to toxic effects in the target cells, all the synthesized compounds were tested by MTT assays in two cell lines, i.e., MDCK and HEK 293T (Table 1). RBV, included again as a reference compound, showed a CC_{50} value of >250 μM in both cell lines, as previously reported.[51,52]

From the activities shown in Table 1, it clearly appears that in some cases, such as in compounds **4**−**6** and **17**−**19**, the structural modifications improved the ability of the analogues to inhibit the PA−PB1 interaction. In particular, compounds **6** and **19** were the most potent with IC_{50} values of 32 and 35 μM, superimposable to that of the reference peptide. In addition, the 2-bycyclic derivatives **17** and **18**, designed to improve the inhibitory efficiency of the hit **1** by increasing the hydrophobic interactions defined by GRID MIFs, indeed proved to be better inhibitors, especially **17**, which exhibited an inhibitory activity 3-fold higher than that of compound **1**. This finding can also be considered a validation of the suggested binding pose for compound **1**. According to the FLAP docking pose reported in

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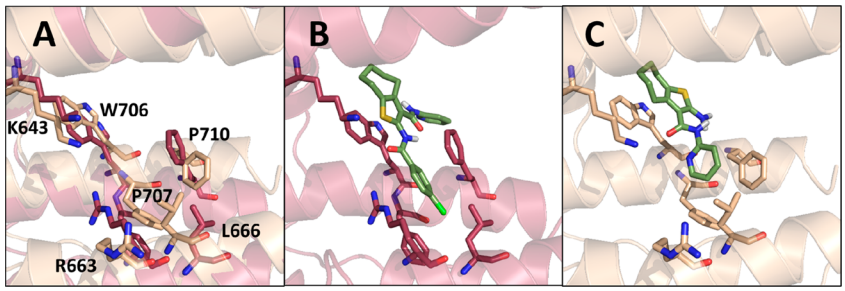

Figure 3. (A) Comparison between the crystallographic structure 3CM8 (light pink) and the dynamic snapshot (dark red) of PA.[31] Hypothesis on the binding modes for compound **6** (B) and compound **19** (C) in the PA cavity as generated by FLAP, after short minimization.

Figure 2, the different inhibitory activity among **1**, **17**, and **18** does not seem to correspond to a different binding mode of the compounds. However, considering that the three structures differ in the substituent at the C-2 position only and that **17** possesses the most hydrophobic substituent in that position, this suggests that a stronger hydrophobic interaction could be the reason for a more difficult displacement of the compound, resulting in an increased activity.

Since the FLAP-structure-based approach proved to be successful to retrieve and optimize ligand−protein complexes[31,37] and in order to make a comparison with the binding modes hypothesized for the other PA/PB1 inhibitors, we inspected the possible FLAP docking poses for the most active compounds, **6** and **19**, in the PA cavity, and results are shown in Figure 3. While for compounds **1**, **17**, and **18** reported in Figure 2 the most favorable poses were obtained upon interaction with the PA X-ray conformation (i.e., structure 3CM8 after removal of PB1, thus in the most favorable pose for PB1 binding), the docking of **6** in the same cavity was unfavorable because of a steric hindrance between the *p*-chloro and the P710 residue defining the second hydrophobic pocket. However, the analysis of the flexibility of P710 based on the dynamic studies previously reported[31] showed that this residue can rotate when the PA−PB1 complex is not formed. At the same time, W706, which is not directly involved in PB1 binding but represents the bottom of the second hydrophobic cavity, can freely rotate to further enlarge this subpocket. By use of a more relaxed snapshot of the PA structure, having the P710 and of W706 rotated to enlarge the second pocket (see Figure 3A), **6** retains a strong $\pi-\pi$ interaction with W706 and the *p*-chlorophenyl group of derivative **6** can fit the enlarged second pocket (Figure 3B). Compound **6** can be further stabilized by R663 or K643 through H-bonds. A possible explanation of the high activity of **6** can be due to the presence of the *p*-chloro substituent that may induce a change in the position of residue P710. In this new position, P710 may interfere with the PA−PB1 interaction. Although an in-depth molecular dynamics investigation was not performed, this study led to the hypothesis that conformational changes in the second hydrophobic pocket might play a role in the stabilization of the PA−ligand complex.

The high PA−PB1 inhibitory activity found for the 2-amino derivative **19** represents an intriguing result, showing that the presence of a hydrophobic moiety in the C-2 position is even not essential for activity. Concerning the possible PA−**19** interaction, the structural features of **19** do not allow the simultaneous binding to the three hydrophobic regions observed for compounds **1**, **17**, and **18**. However, despite the analogy with the other docked compounds that would suggest that **19** could occupy the first and the central hydrophobic pocket, the FLAP docking pose for this compound actually is flipped, with the pyridine group being located in the second pocket (Figure 3C). According to this hypothesis, binding energies for compounds **6** and **19**, which display similar activity despite structural differences, should be comparable. Indeed, the binding energies for the FLAP docked poses, evaluated using the GRID package, differ for 5 kcal/mol. This behavior seems to suggest that the interaction with the second pocket rather than with the central pocket should be investigated to improve activity. PA residues that are involved in the binding of **19** are W706, I666, and P710. A H-bond with K643 could also occur. For the weak PA−PB1 inhibitors **2**, **4**, and **5**, the FLAP binding poses have also been inspected (Figure S1 in Supporting Information). Compounds **2** and **4** are similarly oriented, while for compound **5** the chlorine atom in ortho position of the phenyl group seems to induce a slightly different binding mode with weaker hydrophobic interaction, in agreement with the ELISA data.

All the compounds able to inhibit PA−PB1 interaction were also active in inhibiting virus growth with EC_{50} values ranging from 18 μM for compound **6** to 61 μM for compound **18**. In addition, many of the compounds synthesized in this study were surprisingly endowed with antiviral activity in the micromolar range and at nontoxic concentrations, although they were inactive in vitro as PA−PB1 inhibitors. By examination of all the anti-influenza data reported in Table 1, some clues emerged. The cycloheptane ring of compound **1** cannot be replaced by smaller rings such as cyclohexane and cyclopentane; indeed, both derivatives **31** and **32** were inactive while displaying some cytotoxicity in 293T cells, more evident for the cyclohexane derivative **31** (CC_{50} = 60 μM). The unsuitability of smaller rings was also confirmed by the inactivity of the C-3 primary amides **33** and **34** and ester **35**. Once again, the cyclohexane derivative **33** showed a certain toxicity on both cell lines, with CC_{50} = 31 μM in 293T cells.

Maintaining the *N*-pyridinylcycloheptathiophene-3-carboxamide, the *o*-fluorophenyl ring of compound **1** has been widely modified. The fluorine atom deletion (compound **2**) and its shifting to the meta position (compound **3**) were detrimental, while the *p*-fluoro derivative **4** was characterized by an increase in the inhibition of Flu replication (EC_{50} = 58 μM). The replacement of the fluorine atom with a chlorine was productive at the ortho position as in compound **5** (EC_{50} = 39 μM) but above all at the para position; indeed, compound **6** emerged as one of the most potent (EC_{50} = 18 μM), in agreement with the PA−PB1 inhibitory activity. The addition of another halogen atom permitted the activity to be maintained as in derivatives **7** and **8**. The presence of the

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cyclohexane ring at the C-2 position improved the activity, giving compound **9** with EC_{50} = 20 μM, which was, however, endowed with a slight cytotoxicity (CC_{50} = 180 μM). Excellent antiviral activity was obtained by inserting a monomethylenic or dimethylenic chain between the C-2 amide moiety and the phenyl ring. Indeed, methylphenyl derivatives **10**, **11**, and **12**, as well as the ethylphenyl analogues **13**, **15**, and **16**, showed EC_{50} values ranging from 16 to 21 μM. On the contrary, spacing the cyclohexyl moiety from the C-2 position was deleterious, as **14** was inactive. The addition of a further aromatic ring at the C-2 position as in compounds **17** and **18** imparted a medium activity against virus replication, as expected by their anti-PA−PB1 activity. Notably, the deletion of the aromatic substituent from the C-2 position, as in the amino derivative **19**, permitted maintainance of very good anti-Flu activity with an EC_{50} value of 26 μM, similar to that observed in the ELISA assay.

When the 2-pyridine ring was deleted by placing at the C-3 position a primary amide function, the antiviral activity disappeared (compound **20**) and the same behavior was shown by cyclopentane and cyclohexane analogues **33** and **34**. On the other hand, all the ethyl cycloheptathiophene-3-carboxylates **21** and **23**−**26** as well as the corresponding acids **22** and **27**−**30** possessed antiviral activity with EC_{50} values ranging from 43 to 95 μM. In general, the acid derivatives displayed a better activity than the ester analogues, with the *p*-chlorophenyl derivative **29** emerging once again as the best compound, with an EC_{50} value of 43 μM.

Among all the synthesized compounds, *p*-chloro (compound **6**), 2-amino (compound **19**), and bicyclic derivatives (**17** and **18**), which exhibited the best inhibitory activity against both the PA−PB1 interaction and viral growth, were selected for further investigations including RBV as a control.

First we evaluated the effects of the compounds in virus yield assays, where all of them showed good antiviral activity (Table 2). Then we investigated whether their ability to disrupt the PA−PB1 interaction in vitro correlated with the ability to interfere with the catalytic activity of FluA RdRP in a cellular context. To this end, a minireplicon assay was performed. 293T cells were co-transfected in the presence of the test compounds or DMSO with plasmids for the expression of influenza nucleoprotein (NP), PA, PB1, and PB2 proteins and the firefly luciferase RNA in negative-sense orientation, flanked by the noncoding regions of Flu A/WSN/33 segment 8. The expression of the firefly reporter gene indicates that a negative-sense RNA is synthesized and is reconstituted intracellularly into functional ribonucleoprotein (RNP) in which all four NP, PA, PB1, and PB2 proteins are coexpressed and interact with each other. Table 2 shows that, with the exception of compound **18**, all the thiophene-3-carboxamide derivatives had an effect on FluA polymerase activity, with compound **6** showing an EC_{50} value of 10 μM, thus being slightly more effective than RBV.

Table 2. Activity of Selected Compounds on PR8 Virus Yield and against Viral RNA Polymerase

compd	virus yield reduction assay, EC_{50}, $\mu M^{a,c}$	minireplicon assay, EC_{50}, $\mu M^{b,c}$
6	12 ± 3	10 ± 2
17	20 ± 6	67 ± 10
18	6 ± 1	>100
19	6 ± 1	14 ± 4
RBV	2 ± 1	15 ± 7

[a]This EC_{50} value represents the compound concentration that inhibits 50% of plaque formation. [b]This EC_{50} value represents the compound concentration that reduces by 50% the activity of FluA virus RNA polymerase in 293T cells. [c]All data shown represent the mean ± SD of data derived from at least two independent experiments in duplicate.

Finally, for the best derivatives, **6** and **19**, we investigated the antiviral effects against a number of clinical isolates of FluA other than PR8. PRAs were performed using influenza strains A/Parma/24/09 (H1N1), A/Wisconsin/67/05 (H3N2), and several pandemic swine-originated influenza virus (S-OIV) clinical isolates (H1N1). As shown in Table 3, the new derivatives effectively inhibited all FluA strains tested, of both H1N1 and H3N2 subtypes, including the oseltamivir-resistant clinical isolate (A/Parma/24/09). Compound **6** was slightly more potent than **19**, with EC_{50} values ranging from 15 to 23 μM.

Table 3. Activity of Selected Compounds against a Panel of Influenza A Virus Strains and against Influenza B Virus

virus strain	compound PRA, EC_{50}, μM^{a}		
	6	**19**	RBV
A/PR/8/34 (H1N1)	18 ± 2	26 ± 4	8 ± 2
A/Padova/30/2011 (H1N1)	20 ± 4	28 ± 5	7 ± 2
A/Padova/72/2011 (H1N1)	19 ± 2	22 ± 3	6 ± 1
A/Padova/253/2011 (H1N1)	21 ± 3	27 ± 2	11 ± 4
A/Parma/24/09 (H1N1) (oseltamivir-resistant)	23 ± 3	25 ± 1	17 ± 2
A/Wisconsin/67/05 (H3N2)	15 ± 1	19 ± 2	12 ± 7
B/Lee/40	21 ± 1	19 ± 1	6 ± 3

[a]The EC_{50} value represents the compound concentration that inhibits 50% of plaque formation. All data shown represent the mean ± SD of data derived from at least two independent experiments.

The same compounds were also assayed for their ability to inhibit the replication of FluB. When tested against Flu B/Lee/40 strain by PRA, compounds **6** and **19** were found to be effective against FluB, with EC_{50} values similar to those obtained with the FluA viruses (Table 3), thus displaying broad-spectrum anti-influenza activity.

CONCLUSIONS

The viral RdRP is an attractive target to design new antivirals, since it is highly conserved among Flu strains and no homologous has been found in mammalian cells. In particular, the disruption of its correct assembly through PPI inhibitors could be a promising strategy, although very few studies have been reported so far. Because of a SBDD approach, we recently identified a set of different small molecules able to disrupt PA−PB1 interaction and inhibit viral growth. In this study, some efforts have been pursued to evolve one of these molecules. Starting from thiophene-3-carboxamide derivative **1**, more potent PA−PB1 inhibitors and anti-Flu derivatives have been achieved by mainly modifying the substituent placed at the C-2 position. Among these small molecules, compounds **6** and **19** emerged as the most potent in inhibiting the physical interaction between the two viral subunits with IC_{50} values of 32 and 35 μM, better than the hit compound and comparable to that of the reference peptide. With the purpose of rationalizing the inhibition data for the most potent PA−PB1 inhibitors, their FLAP-binding modes have been compared. On the basis of this analysis, the presence of the bulky halogen

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atom in para position of compound **6** might induce a different orientation of several residues in the PA cavity, making the competition with PB1 more efficient. The good activity of **19**, the only active compound that lacks the aromatic ring at the C-2 position, is more intriguing and deserves further exploration. However, modeling studies suggest that its FLAP docking pose is flipped with respect to that of the other compounds, with the pyridine group being located in the second hydrophobic pocket. This behavior seems to suggest that the interaction with the second pocket rather than with the central pocket should be investigated to improve activity. The last finding is supported by the promising activity displayed by compounds **17** and **18**, appositely designed to increase the interaction with the second pocket, according to the optimization of the hydrophobic GRID MIFs. The PPI inhibitory activity of **6** and **19** is preserved in the cellular context, where they showed a good inhibitory effect on FluA polymerase activity and viral replication also encompassing a panel of clinically isolated strains, including a FluA oseltamivir-resistant, as well as FluB.

An unexpected result coming from this study is the identification of many derivatives able to inhibit the viral growth without affecting PA−PB1 interaction in vitro. For these compounds, an alternative mechanism of action should exist and will be the object of future investigations.

In conclusion, the cycloheptathiophene-3-carboxamide scaffold, when properly functionalized, emerged as particularly suitable to impart anti-Flu activity.

EXPERIMENTAL SECTION

Computational Methods. The design of larger compounds to optimize the hydrophobic interaction was performed using the FLAP approach[35] previously used to identify inhibitors of the PA−PB1 complex by virtual screening.[31] The FLAP software is developed and licensed by Molecular Discovery Ltd. (www.moldiscovery.com). The docking procedure used by the FLAP algorithm generates the GRID MIFs[35] for the cavity and allows visualization and quantification of the hydrophobic and hydrophilic regions as well as the regions where H-bond donors and acceptors might occur. Then MIFs are also calculated for each ligand, and FLAP docking poses are generated looking for the best overlap of the GRID MIFs of protein and those of the ligand.[36]

In this study, the main cavity of the crystallographic structure of a large C-terminal fragment of PA (aa 257−716) derived from the X-ray structure named 3CM8[28] was explored using the GRID force field.[35] By use of the PA cavity as a template, the design of new possible inhibitors was performed using FLAP in the structure-based mode,[37,38] evaluating the best pose among 50 conformers for each candidate, as previously reported.[31] The probes used to generate the molecular interaction fields were H (shape), DRY (hydrophobic interactions), N1 (H-bond donor), and O (H-bond acceptor) interactions. A second snapshot of the PA in a more relaxed form was used to dock compound **6**. The method used to generate the new conformation of PA was previously reported.[31]

FLAP binding poses reported in this study underwent a short optimization using Sybyl 2.0. Both ligand and surrounded PA residues were energy minimized using the Powell algorithm, with a convergence gradient of ≤0.1 kcal/mol and a maximum of 5000 cycles.

Chemistry. All reactions were routinely checked by TLC on silica gel $60F_{254}$ (Merck) and visualized by using UV or iodine. Flash column chromatography separations were carried out on Merck silica gel 60 (mesh 230−400). Melting points were determined in capillary tubes (Büchi Electrothermal model 9100) and are uncorrected. The purity of the compounds was determined by combustion analysis employing a Fisons elemental analyzer, model EA1108CHN, and data for C, H, and N are within 0.4% of the theoretical values (purity of ≥95%). ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 MHz (Bruker Avance DPX-200) and 400 MHz (Bruker Avance DRX-400) using residual solvents such as chloroform (δ = 7.26) or dimethylsulfoxide (δ = 2.48) as an internal standard. Chemical shifts are given in ppm (δ), and the spectral data are consistent with the assigned structures. Reagents and solvents were purchased from common commercial suppliers and were used as such. After extraction, organic solutions were dried over anhydrous Na_2SO_4, filtered, and concentrated with a Büchi rotary evaporator at reduced pressure. Yields are of purified product and were not optimized. All starting materials were commercially available unless otherwise indicated. Some of the compounds reported in this study, i.e., **1**, **2**, **20**−**24**, **26**−**30**, **32**, and **36**, are also commercially available, but being prepared by us and not reported in any previous reference, their synthesis is described below together with the description of the new derivatives.

General Procedure for the Preparation of *N*-(2-Pyridinyl)-2-aminothiophene-3-carboxamide Derivatives (Method A). A mixture of 2-cyano-*N*-pyridin-2-ylacetamide[44] (1.0 equiv), cyclopentanone, cyclohexanone, or cycloheptanone (4.0 equiv), ammonium acetate (1.3 equiv), and glacial acetic acid (3.5 equiv) in benzene was refluxed for 16 h in a Dean−Stark apparatus. After cooling, the reaction mixture was diluted with $CHCl_3$ and washed with H_2O, 10% Na_2CO_3 solution, and H_2O. The organic phase was evaporated to dryness, affording to the crude Knoevenagel product, which was used in the next step without further purification. This material was dissolved in EtOH, and to it were added sulfur (4.0 equiv) and *N*,*N*-diethylamine (4.0 equiv). The mixture was maintained at 40−50 °C for 1.5 h. After cooling, the reaction mixture was evaporated to dryness and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and crystallized by EtOH.

2-Amino-*N*-(2-pyridinyl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (19). The title compound was prepared starting from cycloheptanone by method A, in 59% yield: mp 176−178 °C (dec); ^{1}H NMR ($CDCl_3$) δ 1.70−1.90 (m, 6H, cycloheptane CH_2), 2.60−2.70 and 2.80−2.90 (m, each 2H, cycloheptane CH_2), 4.80 (bs, 2H, NH_2), 7.00 (dd, J = 1.1 and 7.0 Hz, 1H, pyridine CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.10 (bs, 1H, NH), 8.25−8.30 (m, 2H, pyridine CH); ^{13}C NMR ($CDCl_3$) δ 27.2, 27.5, 28.6, 29.2, 31.5, 113.6, 114.3, 119.2, 124.0, 135.0, 138.8, 146.9, 151.5, 155.8, 164.4. Anal. ($C_{15}H_{17}N_3OS$) C, H, N.

2-Amino-*N*-(2-pyridinyl)-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide (37). The title compound was prepared starting from cyclohexanone by method A, in 41% yield: mp 158−159 °C; ^{1}H NMR ($CDCl_3$) δ 1.80−1.90 (m, 4H, cyclohexane CH_2), 2.50−2.60 and 2.80−2.90 (m, each 2 H, cyclohexane CH_2), 6.20 (bs, 2H, NH_2), 7.00 (dd, J = 5.0 and 7.0 Hz, 1H, pyridine CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.10 (bs, 1H, NH), 8.25−8.30 (m, 2H, pyridine CH); ^{13}C NMR ($CDCl_3$) δ 22.8, 22.9, 24.5, 27.2, 108.5, 114.0, 119.1, 119.2, 128.5, 138.1, 147.8, 151.9, 160.8, 164.4.

2-Amino-*N*-(2-pyridinyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (38). The title compound was prepared starting from cyclopentanone by method A, in 46% yield: mp 159−160 °C; ^{1}H NMR ($CDCl_3$) δ 2.45 (q, J = 7.1 Hz, 2H, cyclopentane CH_2), 2.80 and 3.10 (t, J = 7.1 Hz, each 2H, cyclopentane CH_2), 6.25 (bs, 2H, NH_2), 7.00 (dd, J = 5.0 and 7.0 Hz, 1H, pyridine CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.10 (bs, 1H, NH), 8.25−8.30 (m, 2H, pyridine CH); ^{13}C NMR ($CDCl_3$) δ 27.4, 28.6, 30.7, 104.6, 113.7, 119.1, 122.5, 138.1, 138.2, 147.9, 151.8, 164.0, 166.5.

General Procedure for C-2 Amidation (Method B). To a solution of the appropriate 2-aminothiophene derivative (1.0 equiv) in pyridine was added a suitable acyl chloride (2.0 equiv). The reaction mixture was maintained at room temperature for 1 h and worked up and purified as defined in the description of the compounds.

2-[(2-Fluorobenzoyl)amino]-*N*-(2-pyridinyl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (1). The title compound was prepared starting from **19** by method B, using 2-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **1** in 42% yield: mp 206−209 °C; ^{1}H NMR δ 1.70−1.90 (m, 6H, cycloheptane CH_2), 2.70−2.75 and 2.95−

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300 (m, each 2H, cycloheptane CH_2), 7.05 (ddd, J = 0.9, 5.0, and 7.0 Hz, 1H, pyridine CH), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.30 (dt, J = 0.9 and 7.6 Hz, 1H, aromatic CH), 7.50 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.10−8.20 (m, 2H, aromatic CH and NH), 8.20 (d, J = 4.4 Hz, 1H, pyridine CH), 8.30 (d, 1H, J = 8.4 Hz, pyridine CH), 13.00 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.3, 27.6, 28.6, 29.0, 31.7, 114.3, 116.3 (d, J_{C-F} = 23 Hz), 118.8, 119.8, 119.9, 124.9, 132.1, 133.0, 133.2, 134.1 (d, J_{C-F} = 9 Hz), 138.4, 141.4, 147.9, 151.2, 159.8, 160.6 (d, J_{C-F} = 248 Hz), 164.6. Anal. ($C_{22}H_{20}FN_3O_2S$) C, H, N.

The physical−chemical properties are comparable to those of the commercial compound (STK063428) purchased from Vitas-M (Moscow, Russia) including the purity that is 100% for both the compounds, as assessed by UV chromatogram at 280 nm.

2-(Benzoylamino)-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (2). The title compound was prepared starting from **19** by method B, using benzoyl chloride. The reaction mixture was poured into ice/water. A precipitate was obtained, which was filtered and purified by flash chromatography, eluting with EtOAc/petroleum ether (15%), and then washed with cyclohexane to give **2** in 34% yield: mp 208−210 °C; ^{1}H NMR (DMSO d_6) δ 1.45−1.70 (m, 6H, cycloheptane CH_2), 1.70−1.85 and 2.65−2.80 (m, each 2H, cycloheptane CH_2), 7.05−7.10 (m, 1H, pyridine CH), 7.40−7.60 (m, 3H, aromatic CH), 7.65−7.85 (m, 3H, aromatic CH and pyridine CH), 8.15 (d, J = 8.2 Hz, 1H, pyridine CH), 8.30 (d, J = 4.0 Hz, 1H, pyridine CH), 10.40 and 11.00 (s, each 1H, NH). Anal. ($C_{22}H_{21}N_3O_2S$) C, H, N.

2-[(3-Fluorobenzoyl)amino]-*N*-(2-pyridinyl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (3). The title compound was prepared starting from **19** by method B, using 3-fluorobenzoyl chloride. The reaction mixture was poured into ice/water and extracted with EtOAc. The organic layer was evaporated to dryness, obtaining a solid which was purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **3** in 39% yield: mp 215−216 °C; ^{1}H NMR ($CDCl_3$) δ 1.75−1.80 (m, 6H, cycloheptane CH_2), 1.85−1.90, 2.80−2.85 and 3.00−3.05 (m, each 2H, cycloheptane CH_2), 7.10 (ddd, J = 0.9, 5.0, and 7.0 Hz, 1H, pyridine CH), 7.30 (dt, J = 2.5 and 8.9 Hz, 1H, aromatic CH), 7.50 (dq, J = 2.5 and 5.5 Hz, 1H, aromatic CH), 7.70−7.80 (m, 3H, pyridine CH and aromatic CH), 8.25 (d, J = 4.4 Hz, 1H, pyridine CH), 8.30 (bs, 1H, NH), 8.35 (d, J = 8.3 Hz, 1H, pyridine CH), 12.00 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.2, 27.5, 28.6, 29.1, 31.5, 114.3, 114.8 (d, J_{C-F} = 28 Hz), 117.9, 119.3 (d, J_{C-F} = 21 Hz), 120.0, 122.7 (d, J_{C-F} = 3 Hz), 130.5 (d, J_{C-F} = 8 Hz), 132.8, 132.9, 134.9 (d, J_{C-F} = 7 Hz), 138.3, 142.8, 148.1, 151.0, 162.1, 162.9 (d, J_{C-F} = 248 Hz), 164.9. Anal. ($C_{22}H_{20}FN_3O_2S$) C, H, N.

2-[(4-Fluorobenzoyl)amino]-*N*-(2-pyridinyl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (4). The title compound was prepared starting from **19** by method B, using 4-fluorobenzoyl chloride. The reaction mixture was poured into ice/water and extracted with EtOAc. The organic layer was evaporated to dryness, obtaining a solid which was purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) to give **4** in 44% yield: mp 192−193 °C; ^{1}H NMR ($CDCl_3$) δ 1.70−1.80 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80 and 3.00−3.05 (m, each 2H, cycloheptane CH_2), 7.10 (ddd, J = 0.9 and 5.0 and 7.0 Hz, 1H, pyridine CH), 7.15−7.20 (m, 2H, aromatic CH), 7.75 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.95−8.05 (m, 2H, aromatic CH), 8.15−8.20 (m, 2H, pyridine CH and NH), 8.30 (d, J = 8.3 Hz, 1H, pyridine CH), 12.00 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.2, 27.5, 28.5, 29.1, 31.5, 114.3, 115.9 (d, J_{C-F} = 22 Hz), 117.6, 120.0, 128.8, 129.9 (d, J_{C-F} = 9 Hz), 132.6, 132.8, 138.3, 143.2, 148.1, 151.0, 162.3, 163.9, 165.2 (d, J_{C-F} = 252 Hz), 166.5. Anal. ($C_{22}H_{20}FN_3O_2S$) C, H, N.

2-[(2-Chlorobenzoyl)amino]-*N*-(2-pyridinyl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (5). The title compound was prepared starting from **19** by method B, using 2-chlorobenzoyl chloride. The reaction mixture was poured into ice/water and extracted with EtOAc. The organic layer was evaporated to dryness, obtaining a solid which was purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **5** in 31% yield: mp 160−161 °C; ^{1}H NMR ($CDCl_3$) δ 1.65−1.75 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 2.90−2.95 (m, each 2H, cycloheptane CH_2), 7.05 (ddd, J = 0.9 and 5.0 and 7.0 Hz, 1H, pyridine CH), 7.30−7.45 (m, 3H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.75 (dd, J = 1.6 and 7.5 Hz, 1H, aromatic CH), 8.15 (d, J = 4.4 Hz, 1H, pyridine CH), 8.25 (d, J = 8.3 Hz, 1H, pyridine CH), 8.40 (bs, 1H, NH), 11.40 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.3, 27.5, 28.6, 29.0, 31.7, 114.2, 118.8, 120.0, 127.1, 130.6, 130.7, 131.4, 132.0, 133.0, 133.1, 133.3, 138.3, 141.4, 148.0, 151.1, 162.8, 164.6. Anal. ($C_{22}H_{20}ClN_3O_2S$) C, H, N.

2-(4-Chlorobenzamido)-*N*-(pyridin-2-yl)-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (6). The title compound was prepared starting from **19** by method B, using 4-chlorobenzoyl chloride. The reaction mixture was poured into ice/water obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (20%) to give **6** in 29% yield: mp 222−223 °C; ^{1}H NMR ($CDCl_3$) δ 1.70−1.80 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.00−3.05 (m, each 2H, cycloheptane CH_2), 7.10 (ddd, J = 0.9 and 5.0 and 7.0 Hz, 1H, pyridine CH), 7.45 (d, J = 8.4 Hz, 2H, aromatic CH), 7.75 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.90 (d, J = 8.4 Hz, 2H, aromatic CH), 8.10 (bs, 1H, NH), 8.25−8.30 (m, 2H, pyridine CH), 12.00 (s, 1H, NH). Anal. ($C_{22}H_{20}ClN_3O_2S$) C, H, N.

2-[(2-Chloro-4-fluorobenzoyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (7). The title compound was prepared starting from **19** by method B, using 2-chloro-4-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) to give **7** in 25% yield: mp 162−163 °C; ^{1}H NMR ($CDCl_3$) δ 1.70−1.80 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.00−3.05 (m, each 2H, cycloheptane CH_2), 7.05−7.15 (m, 2H, aromatic CH and pyridine CH), 7.20 (dd, J = 2.4 and 8.4 Hz, 1H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.80 (dd, J = 6.0 and 8.4 Hz, 1H, aromatic CH), 8.00 (bs, 1H, NH), 8.20 (d, J = 8.4 Hz, 1H, pyridine CH), 8.30 (d, J = 4.6 Hz, 1H, pyridine CH), 11.50 (s, 1H, NH). Anal. ($C_{22}H_{19}ClFN_3O_2S$) C, H, N.

2-[(2,4-Dichlorobenzoyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (8). The title compound was prepared starting from **19** by method B, using 2,4-dichlorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **8** in 30% yield: mp 190−191 °C; ^{1}H NMR ($CDCl_3$) δ 1.70−1.80 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.00−3.05 (m, each 2H, cycloheptane CH_2), 7.05 (ddd, J = 0.9 and 5.0 and 7.0 Hz, 1H, pyridine CH), 7.35 (dd, J = 1.8 and 8.4 Hz, 1H, aromatic CH), 7.45 (d, J = 1.8 Hz, 1H, aromatic CH), 7.65−7.75 (m, 2H, aromatic CH and pyridine CH), 8.10 (bs, 1H, NH), 8.20−8.25 (m, 2H, pyridine CH), 11.50 (s, 1H, NH). Anal. ($C_{22}H_{19}Cl_2N_3O_2S$) C, H, N.

2-[(Cyclohexylcarbonyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (9). The title compound was prepared starting from **19** by method B, using cyclohexanecarbonyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/petroleum ether (15%) to give **9** in 20% yield: mp 201−204 °C; ^{1}H NMR (DMSO d_6) δ 1.00−1.80 (m, 17H, cyclohexane CH_2, cyclohexane CH, and cycloheptane CH_2), 2.50−2.65 (m, 4H, cycloheptane CH_2), 7.05 (dd, J = 5.0 and 7.3 Hz, 1H, pyridine CH), 7.75 (dt, J = 2.0 and 9.2 Hz, 1H, pyridine CH), 8.10 (d, J = 8.1 Hz, 1H, pyridine CH), 8.25 (d, J = 3.6 Hz, 1H, pyridine CH), 10.20 and 10.30 (s, each 1H, NH). Anal. ($C_{22}H_{27}N_3O_2S$) C, H, N.

2-{[(4-Fluorophenyl)acetyl]amino}-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (10). The title compound was prepared starting from **19** by method B, using (4-fluorophenyl)acetyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) to give

10 in 25% yield: mp 159−160 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 2.90−2.95 (m, each 2H, cycloheptane CH_2), 3.75 (s, 2H, CH_2), 7.00−7.10 (m, 3H, aromatic CH and pyridine CH), 7.30−7.35 (m, 2H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.90 (bs, 1H, NH), 8.15 (d, J = 8.3 Hz, 1H, pyridine CH), 8.25 (d, J = 4.6 Hz, 1H, pyridine CH), 10.75 (s, 1H, NH). Anal. ($C_{23}H_{22}FN_3O_2S$) C, H, N.

2-{[(4-Chlorophenyl)acetyl]amino}-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (11). The title compound was prepared starting from **19** by method B, using (4-chlorophenyl)acetyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) to give **11** in 27% yield: mp 169−170 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 2.90−2.95 (m, each 2H, cycloheptane CH_2), 3.75 (s, 2H, CH_2), 7.05 (ddd, J = 0.9, 5.0, and 7.0 Hz, 1H, pyridine CH), 7.25 and 7.30 (d, J = 8.3 Hz, each 2H, aromatic CH), 7.75 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 7.85 (bs, 1H, NH), 8.15 (d, J = 8.3 Hz, 1H, pyridine CH), 8.25 (d, J = 4.6 Hz, 1H, pyridine CH), 10.75 (s, 1H, NH). Anal. ($C_{23}H_{22}ClN_3O_2S$) C, H, N.

2-[(Phenylacetyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (12). The title compound was prepared starting from **19** by method B, using phenylacetyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **12** in 20% yield: mp 165−167 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 2.90−2.95 (m, each 2H, cycloheptane CH_2), 3.75 (s, 2H, CH_2), 7.10 (ddd, J = 0.9, 5.0, and 7.0 Hz, 1H, pyridine CH), 7.25−7.40 (m, 5H, aromatic CH), 7.75 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.15−8.20 (m, 2H, pyridine CH), 8.40 (bs, 1H, NH), 10.75 (s, 1H, NH). Anal. ($C_{23}H_{23}N_3O_2S$) C, H, N.

2-{[3-(4-Fluorophenyl)propanoyl]amino}-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (13). The title compound was prepared starting from **19** by method B, using 3-(4-fluorophenyl)propanoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized twice by EtOH to give **13** in 24% yield: mp 132−133 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85 (m, 2H, cycloheptane CH_2), 2.70−2.75 and 2.90−3.00 (m, each 4H, cycloheptane CH_2 and CH_2CH_2), 6.85−6.95 (m, 2H, aromatic CH and pyridine CH), 7.10−7.20 (m, 3H, aromatic CH), 7.80 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.20 (d, J = 8.3 Hz, 1H, pyridine CH), 8.30 (d, J = 4.6 Hz, 1H, pyridine CH), 8.75 (bs, 1H, NH), 10.70 (s, 1H, NH). Anal. ($C_{24}H_{24}FN_3O_2S$) C, H, N.

2-[(Cyclohexylacetyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (14). The title compound was prepared starting from **19** by method B, using cyclohexylacetyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized twice by EtOH to give **14** in 38% yield: mp 181−182 °C (dec); ^{1}H NMR ($CDCl_3$) δ 0.95−1.30 (m, 5H, cyclohexane CH_2), 1.60−1.75 (m, 10H, cycloheptane CH_2 and cyclohexane CH_2), 1.80−1.85 (m, 2H, cycloheptane CH_2), 2.25 (d, J = 7.0 Hz, 2H, CH_2), 2.70−2.75 and 2.95−3.00 (m, each 2H, cycloheptane CH_2), 7.10−7.20, 7.75−7.80, and 8.20−8.25 (m, each 1H, pyridine CH), 8.30 (d, J = 4.6 Hz, 1H, pyridine CH), 8.45 (bs, 1H, NH), 10.75 (s, 1H, NH). Anal. ($C_{23}H_{29}N_3O_2S$) C, H, N.

2-{[3-(4-Chlorophenyl)propanoyl]amino}-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (15). The title compound was prepared starting from **19** by method B, using 3-(4-chlorophenyl)propanoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **15** in 42% yield: mp 125−126 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85 (m, 2H, cycloheptane CH_2), 2.70−2.75 (m, 4H, cycloheptane CH_2 and CH_2CH_2), 2.80−2.85 (m, 2H, cycloheptane CH_2), 3.00 (t, J = 7.5 Hz, 2H, CH_2CH_2), 7.10 (ddd, J = 0.9, 5.0, and 7.0 Hz, 1H, pyridine CH), 7.05−7.10 and 7.15−7.20 (m, each 2H, aromatic CH), 7.75 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.20 (d, J = 8.3 Hz, 1H, pyridine CH), 8.25 (d, J = 4.6 Hz, 1H, pyridine CH), 8.40 (bs, 1H, NH), 10.75 (s, 1H, NH). Anal. ($C_{24}H_{24}ClN_3O_2S$) C, H, N.

2-[(3-Phenylpropanoyl)amino]-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (16). The title compound was prepared starting from **19** by method B, using 3-phenylpropanoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **16** in 28% yield: mp 135−136 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85 (m, 2H, cycloheptane CH_2), 2.70−2.75 (m, 4H, cycloheptane CH_2 and CH_2CH_2), 2.80−2.85 (m, 2H, cycloheptane CH_2), 3.00 (t, J = 7.5 Hz, 2H, CH_2CH_2), 7.10−7.25 (m, 6H, aromatic CH and pyridine CH), 7.85 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.20 (d, J = 8.3 Hz, 1H, pyridine CH), 8.30 (d, J = 4.6 Hz, 1H, pyridine CH), 8.75 (bs, 1H, NH), 10.70 (s, 1H, NH). Anal. ($C_{24}H_{25}N_3O_2S$) C, H, N.

***N*-Pyridin-2-yl-2-[(quinolin-6-ylacetyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (17).** To a solution of **19** (0.5 g, 1.75 mmol) in THF (40 mL) was added quinolin-6-ylacetic acid (0.3 g, 1.59 mmol) and DMTMM.[45] After 24 h, the reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by cyclohexane/EtOAc to give **17** (0.4 g, 55%): mp 122−123 °C; ^{1}H NMR ($CDCl_3$) δ 1.50−1.55 (m, 4H, cycloheptane CH_2), 1.55−2.00, 2.40−2.60, and 2.80−3.00 (m, each 2H, cycloheptane CH_2), 3.90 (s, 2H, CH_2), 7.00−7.10 (m, 1H, pyridine CH), 7.25 (s, 1H, quinoline CH), 7.40−7.50 (m, 1H, quinoline CH), 7.60−7.75 (m, 2H, quinoline CH and pyridine CH), 7.85 (s, 1H, quinoline CH), 7.95 (d, J = 8.0 Hz, 1H, quinoline CH), 8.10−8.30 (m, 3H, quinoline CH and pyridine CH), 9.00 and 11.00 (s, each 1H, NH). Anal. ($C_{26}H_{24}N_4O_2S$) C, H, N.

2-{[(3-Methoxy-1-benzothien-2-yl)acetyl]amino}-*N*-pyridin-2-yl-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (18). The title compound was prepared by using the same procedure used for the synthesis of **17**, replacing quinolin-6-ylacetic acid with (3-methoxy-1-benzothien-2-yl)acetic acid, giving 16% yield after purification by flash chromatography, eluting with EtOAc/cyclohexane (20%): mp 153−155 °C; ^{1}H NMR (DMSO-d_6) δ 1.50−1.70 (m, 4H, cycloheptane CH_2), 1.75−1.80 (m, 2H, cycloheptane CH_2), 2.60−2.70 (m, 4H, cycloheptane CH_2), 3.85 (s, 3H, OCH_3), 4.00 (s, 2H, CH_2), 7.05−7.15 (m, 1H, pyridine CH), 7.30−7.40 (m, 2H, benzothiene CH), 7.65−7.70 (m, 1H, pyridine CH), 7.75−7.90 (m, 2H, benzothiene CH), 1.15 (d, J = 8.36 Hz, 1H, pyridine CH), 8.25−8.30 (m, 1H, pyridine CH), 10.45 and 10.70 (s, each 1H, NH). Anal. ($C_{26}H_{25}N_3O_3S$) C, H, N.

2-[(2-Fluorobenzoyl)amino]-*N*-(2-pyridinyl)-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide (31). The title compound was prepared starting from **37** by method B, using 2-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) to give **31** in 35% yield: 204−205 °C; ^{1}H NMR ($CDCl_3$) δ 1.80−1.90 (m, 4H, cyclohexane CH_2), 2.70−2.75 and 2.85−2.90 (m, each 2H, cyclohexane CH_2), 7.05 (dd, J = 5.0 and 7.0 Hz, 1H, pyridine CH), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.30 (t, J = 7.6 Hz, 1H, aromatic CH), 7.50 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.20 (dt, J = 1.4 and 7.7 Hz, 1H, aromatic CH), 8.25 (d, J = 4.4 Hz, 1H, pyridine CH), 8.30−8.35 (m, 2H, pyridine CH and NH), 13.00 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 22.5, 22.9, 24.3, 26.7, 114.5, 115.1, 116.4 (d, J_{C-F} = 23 Hz), 119.8, 120.1 (d, J_{C-F} = 11 Hz), 124.8 (d, J_{C-F} = 3 Hz), 127.0, 128.8, 132.1, 134.1 (d, J_{C-F} = 9 Hz), 138.3, 146.3, 147.9, 151.2, 160.0, 160.7 (d, J_{C-F} = 249 Hz) 164.2. Anal. ($C_{21}H_{18}FN_3O_2S$) C, H, N.

2-[(2-Fluorobenzoyl)amino]-*N*-(2-pyridinyl)-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxamide (32). The title compound was prepared starting from **38** by method B, using 2-fluorobenzoyl chloride. The reaction mixture was poured into ice/

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water and extracted with EtOAc. The organic layer was evaporated to dryness, obtaining a solid which was purified by flash chromatography, eluting with EtOAc/cyclohexane (15%) and then crystallized by EtOH to give **32** in 37% yield: mp 184−185 °C; ^{1}H NMR ($CDCl_3$) δ 2.55 (q, J = 7.1 Hz, 2H, cyclopentane CH_2), 2.80 and 3.20 (t, J = 7.1 Hz, each 2H, cyclopentane CH_2), 7.00 (dd, J = 5.0 and 7.0 Hz, 1H, pyridine CH), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.30 (t, J = 7.6 Hz, 1H, aromatic CH), 7.55 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 7.70 (dt, J = 1.4 and 7.0 Hz, 1H, pyridine CH), 8.20 (dt, J = 1.4 and 7.7 Hz, 1H, aromatic CH), 8.25 (d, J = 4.4 Hz, 1H, pyridine CH), 8.30−8.35 (m, 2H, pyridine CH and NH), 13.00 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 28.2, 28.6, 30.3, 111.2, 114.2, 116.4 (d, J_{C-F} = 23 Hz), 119.8, 120.0, 124.8 (d, J_{C-F} = 3 Hz), 132.1, 134.2 (d, J_{C-F} = 9 Hz), 134.4, 137.1, 138.2, 148.0, 150.9, 151.2, 159.9, 160.7 (d, J_{C-F} = 249 Hz), 163.6. Anal. ($C_{20}H_{16}FN_3O_2S$) C, H, N.

2-[(2-Fluorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (20). The title compound was prepared starting from 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide **39**[46] by method B, using 2-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **20** in 40% yield: mp 233−234 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 2.85−2.90 (m, each 2H, cycloheptane CH_2), 5.75 (bs, 2H, NH_2), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.30 (dt, J = 0.9 and 7.6 Hz, 1H, aromatic CH), 7.50 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 8.25 (dt, J = 1.7 and 7.8 Hz, 1H, aromatic CH), 12.25 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.1, 27.6, 28.5, 28.8, 31.7, 116.3 (d, J_{C-F} = 23 Hz), 117.5, 121.8, 124.8 (d, J_{C-F} = 3 Hz), 132.1, 132.6, 133.4, 134.0 (d, J_{C-F} = 9 Hz), 141.8, 159.8, 160.7 (d, J_{C-F} = 249 Hz), 168.2. Anal. ($C_{17}H_{17}FN_2O_2S$) C, H, N.

Ethyl 2-[(2-Fluorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate (21). The title compound was prepared starting from ethyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate **42**[47] by method B, using 2-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **21** in 100% yield: mp 117−118 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (t, J = 7.1 Hz, 3H, CH_2CH_3), 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 3.05−3.10 (m, each 2H, cycloheptane CH_2), 4.35 (q, J = 7.1 Hz, 2H, CH_2CH_3), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.35 (dt, J = 0.9 and 7.6 Hz, 1H, aromatic CH), 7.55 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 8.20 (dt, J = 1.7 and 7.8 Hz, 1H, aromatic CH), 12.25 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 14.3, 26.9, 27.8, 28.2, 28.6, 32.2, 60.7, 114.0, 116.3 (d, J_{C-F} = 24 Hz), 119.9 (d, J_{C-F} = 11 Hz), 124.9 (d, J_{C-F} = 3 Hz), 131.7, 132.2 (d, J_{C-F} = 1 Hz), 134.1 (d, J_{C-F} = 9 Hz), 137.0, 144.5, 159.7 (d, J_{C-F} = 3 Hz), 160.6 (d, J_{C-F} = 248 Hz), 166.1. Anal. ($C_{19}H_{20}FNO_3S$) C, H, N.

Ethyl 2-[(4-Fluorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate (23). The title compound was prepared starting from **42**[47] by method B, using 4-fluorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **23** in 64% yield: mp 117−118 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, J = 7.1 Hz, 2H, CH_2CH_3), 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 3.05−3.10 (m, each 2H, cycloheptane CH_2), 4.35 (q, J = 7.1 Hz, 3H, CH_2CH_3), 7.10−7.20 and 7.95−8.05 (m, each 2H, aromatic CH), 12.25 (s, 1H, NH). Anal. ($C_{19}H_{20}FNO_3S$) C, H, N.

Ethyl 2-[(2-Chlorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate (24). The title compound was prepared starting from **42**[47] by method B, using 2-chlorobenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **24** in 46% yield: mp 91−92 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (t, J = 7.1 Hz, 2H, CH_2CH_3), 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 3.05−3.10 (m, each 2H, cycloheptane CH_2), 4.30 (q, J = 7.1 Hz, 3H, CH_2CH_3), 7.30−7.45 (m, 3H, aromatic CH), 7.75 (dd, J = 1.5 and 7.5 Hz, 1H, aromatic CH), 11.80 (s, 1H, NH). Anal. ($C_{19}H_{20}ClNO_3S$) C, H, N.

Ethyl 2-[(4-Methoxybenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate (26). The title compound was prepared starting from **42**[47] by method B, using 4-methoxybenzoyl chloride. The reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and crystallized by EtOH to give **26** in 45% yield: mp 127−128 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (t, J = 7.1 Hz, 3H, CH_2CH_3), 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.80−1.85, 2.70−2.75, and 3.05−3.10 (m, each 2H, cycloheptane CH_2), 3.85 (s, 3H, CH_3), 4.35 (q, J = 7.1 Hz, 2H, CH_2CH_3), 6.95−7.00 and 7.90−7.95 (m, each 2H, aromatic CH), 12.10 (s, 1H, NH). Anal. ($C_{20}H_{23}NO_4S$) C, H, N.

General Procedure for Hydrolysis (Method C). A suspension of the appropriate ethyl thiophene-3-carboxylate (1.0 equiv) and LiOH (4.0 equiv) in a mixture H_2O/THF (1:1) was maintained at 50 °C for 48 h. After cooling, the reaction mixture was acidified (pH 6) with 2 N HCl and the precipitate was filtered, washed with water, and crystallized by EtOH.

2-[(2-Fluorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic Acid (22). The title compound was prepared starting from **21** by method C in 65% yield: mp 186−187 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.15−3.20 (m, each 2H, cycloheptane CH_2), 7.20 (dd, J = 8.3 and 12.0 Hz, 1H, aromatic CH), 7.35 (dt, J = 0.9 and 7.6 Hz, 1H, aromatic CH), 7.55 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 8.20 (dt, J = 1.7 and 7.8 Hz, 1H, aromatic CH), 12.25 (d, J = 11.0 Hz, 1H, NH); ^{13}C NMR ($CDCl_3$) δ 27.0, 27.7, 27.9, 28.6, 32.3, 112.8, 116.3 (d, J_{C-F} = 24 Hz), 119.7 (d, J_{C-F} = 10 Hz), 125.0 (d, J_{C-F} = 3 Hz), 132.0, 132.4, 134.3 (d, J_{C-F} = 9 Hz), 137.5, 146.7, 159.7, 160.6 (d, J_{C-F} = 248 Hz), 170.4. Anal. ($C_{17}H_{16}FNO_3S$) C, H, N.

2-[(4-Fluorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic Acid (27). The title compound was prepared starting from **23** by method C in 33% yield: mp 205−206 °C (dec); ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.15−3.20 (m, each 2H, cycloheptane CH_2), 7.20−7.30 and 7.95−8.05 (m, each 2H, aromatic CH), 12.00 (s, 1H, NH). Anal. ($C_{17}H_{16}FNO_3S$) C, H, N.

2-[(2-Chlorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic Acid (28). The title compound was prepared starting from **24** by method C in 39% yield: mp 226−227 °C (dec); ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.15−3.20 (m, each 2H, cycloheptane CH_2), 7.35−7.45 (m, 3H, aromatic CH), 7.80 (d, J = 7.5 Hz, 1H, aromatic CH), 11.75 (s, 1H, NH). Anal. ($C_{17}H_{16}ClNO_3S$) C, H, N.

2-[(4-Chlorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic Acid (29). The title compound was prepared starting from ethyl 2-[(4-chlorobenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate **25**[47] by method C in 29% yield: mp 208−209 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.15−3.20 (m, each 2H, cycloheptane CH_2), 7.45−7.50 and 7.85−7.90 (d, J = 8.3 Hz, each 2H, aromatic CH), 12.00 (s, 1H, NH). Anal. ($C_{17}H_{16}ClNO_3S$) C, H, N.

2-[(4-Methoxybenzoyl)amino]-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylic Acid (30). The title compound was prepared starting from **26** by method C in 32% yield: mp 187−188 °C; ^{1}H NMR ($CDCl_3$) δ 1.60−1.70 (m, 4H, cycloheptane CH_2), 1.85−1.90, 2.75−2.80, and 3.15−3.20 (m, each 2H, cycloheptane CH_2), 3.90 (s, 3H, CH_3), 6.95−7.00 and 7.90−7.95 (d, J = 8.5 Hz, each 2H, aromatic CH), 12.00 (s, 1H, NH). Anal. ($C_{18}H_{19}NO_4S$) C, H, N.

2-[(2-Fluorobenzoyl)amino]-5,6-dihydro-4*H*-cyclopenta[*b*]-thiophene-3-carboxylic Acid (36). The title compound was prepared starting from ethyl 2-[(2-fluorobenzoyl)amino]-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carboxylate **35**[48] by method C in 63% yield: mp 219−220 °C; ^{1}H NMR (DMSO-d_6) δ 2.25 (quintet, J = 7.5 Hz, 2H, cyclopentane CH_2), 2.80 (q, J = 7.5 Hz, 4H, cyclopentane CH_2), 7.35−7.45 (m, 2H, aromatic CH), 7.70 (dq, J = 1.4 and 7.3 Hz, 1H, aromatic CH), 8.00 (dt, J = 1.7 and 7.8 Hz, 1H, aromatic CH),

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12.10 (d, J = 10.5 Hz, 1H, NH), 13.10 (bs, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 27.8, 28.8, 30.2, 110.1, 117.2 (d, J_{C-F} = 23 Hz), 119.5 (d, J_{C-F} = 11 Hz), 125.9, 132.0, 132.6, 135.5 (d, J_{C-F} = 9 Hz), 142.2, 150.0, 159.3, 160.3 (d, J_{C-F} = 247 Hz), 166.9. Anal. ($C_{15}H_{12}FNO_3S$) C, H, N.

Biology. Compounds and Peptide. RBV (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was obtained from Roche. Each test compound was dissolved in DMSO 100%. The PB1$_{(1-15)}$−Tat peptide was synthesized and purified by the Peptide Facility of CRIBI Biotechnology Center (University of Padua, Padua, Italy). This peptide possesses a C-terminal sequence from the HIV Tat protein (amino acids 47−59), which has been shown to mediate cell entry.[53]

Plasmids. Plasmids pcDNA-PB1, pcDNA-PB2, pcDNA-PA, and pcDNA-NP, containing cDNA copies of the influenza A/PR/8/34 virus *PB1*, *PB2*, *PA*, and *NP* genes, respectively, were created as described elsewhere[54] and kindly provided by P. Digard (Roslin Institute, University of Edinburgh, U.K.). Plasmid pPolI-Flu-ffLuc, which contains an influenza-virus-based luciferase minireplicon vRNA under the control of the human RNA polymerase I promoter, was provided by L. Tiley (University of Cambridge, U.K.). Plasmid pRL-SV40 expressing the *Renilla* luciferase was purchased from Promega.

Cells and Virus. Human embryonic kidney (HEK) 293T and Mardin−Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle medium (DMEM, Life Biotechnologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Life Technologies). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Influenza A/PR/8/34 virus (H1N1, Cambridge lineage) was obtained from P. Digard (Roslin Institute, University of Edinburgh, U.K.). Influenza A/Wisconsin/67/05 was provided by R. Cusinato (Clinical Microbiology and Virology Unit, Padua University Hospital, Padua, Italy). The clinical isolate A/Parma/24/09 was kindly provided by I. Donatelli (Istituto Superiore di Sanità, Rome, Italy). Local strains of the new pandemic variant H1N1 FluA virus (A/Padova/30/2011, A/Padova/72/2011, and A/Padova/253/2011) were provided by C. Salata and A. Calistri (University of Padua, Padua, Italy). Influenza B/Lee/40 virus was obtained from W. S. Barclay (Imperial College, London, U.K.).

Protein Expression and Purification. To obtain the 6His-PA$_{(239-716)}$ protein, the pET28a-PA239-716 plasmid was transformed into *E. coli* strain BL21(DE3)pLysS (Stratagene). Typically, cells were grown in Luria−Bertani (LB) medium containing 50 μg/mL kanamycin until the OD$_{600}$ was 0.8 and then induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, ICN) overnight (O/N) at 16 °C. Cells were pelleted, resuspended in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 500 mM urea, 10 mM β-mercaptoethanol, 25 mM imidazole, 1 mg/mL lysozyme, and Complete protease inhibitors (Roche Molecular Biochemicals), and then lysed by two freeze/thaw cycles and by sonication. The lysate was centrifuged at 13 000 rpm for 30 min, applied to a 0.5 mL Ni-NTA agarose resin column (Qiagen) that had been equilibrated in resuspension buffer. Protein was eluted with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 500 mM urea, 10 mM β-mercaptoethanol, 250 mM imidazole. The GST-PB1$_{(1-25)}$ fusion protein and GST alone were purified from *E. coli* BL21(DE3)/pLysS harboring the pD15-PB1$_{1-25}$ or pD15-GST plasmid, respectively. Cells were grown in LB medium containing 100 μg/mL ampicillin until the OD$_{600}$ was 0.8 and then induced by the addition of 0.5 mM IPTG O/N at 16 °C. Cells were pelleted, resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20% glycerol, 5 mM DTT, 1 mg/mL lysozyme, and Complete protease inhibitors, and then lysed by two freeze/thaw cycles followed by sonication. The lysate was centrifuged at 13 000 rpm for 30 min, applied to a 0.5 mL glutathione-Sepharose 4 FastFlow column (Amersham Pharmacia Biotech) that had been equilibrated in lysis buffer. Finally, protein was eluted with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20% glycerol, 5 mM DTT, and glutathione 40 mM. Both 6His-PA$_{(239-716)}$ and GST-PB1$_{(1-25)}$ purified proteins were dialyzed against 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 30% glycerol, 5 mM DTT and stored at −80 °C.

PA−PB1 Interaction Enzyme-Linked Immunosorbent Assay (ELISA). Microtiter plates (Nuova Aptca) were coated with 400 ng of purified 6His-PA$_{(239-716)}$ for 3 h at 37 °C and then blocked with 2% BSA (Sigma) in PBS for 1 h. After washes with PBS containing 0.3% Tween 20, an amount of 200 ng of GST-PB1$_{(1-25)}$, or GST alone as a control, in the absence or the presence of test compounds at various concentrations (10, 50, 100, 200 μM) was added and incubated O/N at room temperature. After being washed, samples were incubated with horseradish peroxidase (HRP) conjugated anti-GST monoclonal antibody (GenScript; diluted 1:3000 in PBS containing 2% FBS). Following washes with PBS plus 0.3% Tween 20, the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB, KPL) was added and absorbance was read at 450 nm on an ELISA plate reader (Tecan Sunrise).

Cytotoxicity Assays. Cytotoxicity of compounds was tested in MDCK and HEK 293T cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, cells were seeded at a density of 2 × 10^4 per well into 96-well plates. The next day, cell medium was removed, and fresh medium containing DMEM plus test compounds at various concentrations (from 250 to 1.9 μM) was added to each well. After 24 and 48 h for HEK 293T and MDCK cells, respectively, 5 mg/mL of MTT solution was added to each well and plates were incubated for 4 h at 37 °C in a CO_2 incubator. Successively, a solubilization solution was added to lyse cells. After 3 h of incubation at 37 °C, absorbance was measured at 620 nm using an ELISA plate reader (Tecan Sunrise).

Plaque Reduction Assays (PRA). A confluent monolayer of MDCK cells was prepared in 12-well plates. Cells were infected with the FluA or FluB virus at 40 PFU/well in DMEM supplemented with 1 μg/mL of TPCK-treated trypsin (Worthington Biochemical Corporation) and 0.14% BSA in the presence of various concentrations (0, 25, 50, 100 μM) of test compounds for 1 h at 37 °C. Medium containing 1 μg/mL of TPCK-treated trypsin, 0.14% BSA, 1.2% Avicel, and test compounds at the same concentrations was then added. After 2 days of incubation, cell monolayers were fixed with 4% formaldehyde and stained with 0.1% toluidine blue, and viral plaques were counted.

Virus Yield Reduction Assays. MDCK cells were seeded at a density of 2 × 10^5 cells per well in 24-well plates. The next day, cells were infected with influenza A/PR/8/34 virus at an MOI of 0.01 in DMEM plus 0.14% BSA and 1 μg/mL TPCK-treated trypsin, in the presence of various concentrations (0, 3, 10, 30, 100 μM) of test compounds for 1 h at 37 °C. Cells were then incubated with DMEM containing 1 μg/mL of TPCK-treated trypsin, 0.14% BSA, and test compounds at the same concentrations. At 12 h postinfection (pi), cell culture supernatants were collected and viral progeny was titrated by plaque assays on MDCK monolayers.

Minireplicon Assays. HEK 293T cells were seeded at a density of 10^5 per well into 24-well plates. After 24 h, cells were transfected with pcDNA-PB1, pcDNA-PB2, pcDNA-PA, pcDNA-NP plasmids (100 ng/well of each) along with pPolI-Flu-ffLuc plasmid (50 ng/well). The transfection mixture also contained pRL-SV40 plasmid (50 ng/well) to normalize variations in transfection efficiency. Transfections were performed using calcium phosphate protocol in the presence of the test compounds or DMSO. Cell medium was replaced 4 h post-transfection with DMEM containing compounds or DMSO. At 24 h post-transfection, cells were harvested and both firefly luciferase and *Renilla* luciferase expression were determined using the dual luciferase assay kit from Promega.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table containing elemental analysis data for target compounds **1**−**24**, **26**−**32**, and **36**; FLAP binding poses for compounds **2**, **4**, and **5** (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥There are two sets of equal contributions: (1) S.M. and G.N. contributed equally to this work; (2) A.L. and O.T. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CC_{50}, concentration that causes a decrease of cell viability by 50%; Flu, influenza virus; FluA, influenza A virus; FluB, influenza B virus; MDCK, Mardin–Darby canine kidney; NP, nucleoprotein; O/N, overnight; PA, polymerase acidic protein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2; PPI, protein–protein interaction; PRA, plaque reduction assay; RBV, ribavirin; RdRP, RNA-dependent RNA polymerase; RNP, ribonucleoprotein

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