Exploring the cycloheptathiophene-3-carboxamide scaffold to disrupt the interactions of the influenza polymerase subunits and obtain potent anti-influenza activity

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TITLE RUNNING HEAD: Cycloheptathiophene derivatives with anti-influenza activity.

ABSTRACT

We have recently identified various small molecules endowed with good anti-influenza activity thanks to their ability to disrupt the polymerase PA-PB1 subunits heterodimerization.

In this paper, the optimization of the cycloheptathiophene-3-carboxamide scaffold was attempted, leading to the identification of new and more potent PA-PB1 inhibitors. The presence of 2-hydroxybenzamido moiety at the C-2 position of the central core was particularly effective, giving compounds that inhibited viral growth in the low micromolar range coupled with SI values up to >1388. Computational studies suggested that the hydroxyl group induces a significant change of the binding mode of the compounds with respect to previously reported cycloheptathiophene-3-carboxamide analogues, justifying their higher potency as PA/PB1 interaction inhibitors.

Keywords: PA-PB1 interaction, influenza virus polymerase, protein-protein interaction inhibitors, influenza virus inhibitors.

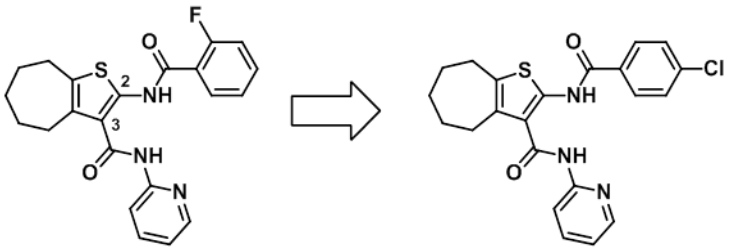
1. INTRODUCTION

Influenza virus (flu) still causes high morbidity and mortality in humans because of seasonal epidemics and global pandemics. The high mutation rate together with the genomic reassortment among different viral strains lead flu to elude vaccine-induced immunity and/or to develop resistance to the current antiviral drugs, which are limited to neuraminidase and M2 ion channel inhibitors. Therefore, the development of anti-flu therapeutics exploiting new targets and/or innovative strategies is highly desirable. To this end, the inhibition of one or more components of the viral RNA-dependent RNA polymerase (RdRp) could have a bright future [1,2]. Indeed, RdRp is essential for virus transcription, replication, and evolution, its structure is highly conserved among the different flu A, B, and C strains, and it does not possess homologues in mammalian cells [3-6]. RdRp is a heterotrimeric complex composed of polymerase acidic protein (PA), polymerase

basic protein 1 (PB1), and polymerase basic protein 2 (PB2) subunits that work together in a tightly associated and coupled fashion [7]. Targeting the RdRp complex has been already proven to be a successful approach with the PB1 inhibitor favipiravir (T-705, Avigan®) [8,9], which has been approved in Japan to treat pandemic flu, and the PB2 inhibitor VX-787, which is currently in phase II clinical development [10].

An alternative and appealing strategy to inhibit the RdRp functions is to interfere with its correct assembly through protein-protein interaction (PPI) inhibitors that may have the advantage to be less prone to drug resistance emergence [11-14].

During the last four years, several remarkable efforts were focused on the identification of small molecules as PA-PB1 subunits interaction inhibitors [14], with our groups that gave the major contribution [15-21]. Indeed, starting from a structure-based virtual screening on three million compounds using the crystallographic structure of the C-terminal domain of PA bound to a PB1-derived peptide reported by He *et al.* (PDB ID 3CM8) [22], a first series of hit compounds was identified [15]. Structural optimization studies were successively conducted leading to the identification of very promising compounds that, acting through low micromolar PA-PB1 complex inhibition, displayed good anti-flu activity against several clinical isolates of fluA, including an oseltamivir-resistant strain, and of fluB [18-20]. In particular, the structural evolution of derivative **1** (Fig. 1) allowed to improve both the antiviral and the PPI inhibitory activity, as in the *p*-chloro derivative **2** [18] (Fig. 1), together with the delineation of useful structure-activity relationship (SAR) insights. From these studies, it clearly emerged how minor structural modifications around the cycloheptathiophene-3-carboxamide (cHTC) scaffold strongly influence both the ability to interfere with the PA-PB1 subunits interaction and the anti-flu activity. Thus, a further optimization of this scaffold has been attempted in the present study, by synthesizing a new series of derivatives that were tested for their PPI inhibitory activity and anti-flu properties. Moreover, computational studies were conducted to rationalize their binding mode within the PA cavity.



1
IC_{50} = 90 µM (PA-PB1)
EC_{50} = 145 µM (Flu A replication)
CC_{50} > 250 µM (cytotoxicity)

2
IC_{50} = 32 µM (PA-PB1)
EC_{50} = 18 µM (Flu A replication)
CC_{50} > 250 µM (cytotoxicity)

Fig. 1. Structures of cHTC derivatives **1** and **2**.

2. DESIGN OF cHTC ANALOGUES

In the first optimization study of hit compound **1**, the attention was mainly focused on the C-2 position of the cHTC scaffold: different substituents replaced the *o*-fluorine atom, one or two methylene units were inserted between the C-2 phenyl ring and the core, and the phenyl ring was replaced by bulky aromatic rings or a cyclohexyl moiety. Fewer modifications involved the cycloheptane ring, whose size was reduced to cyclohexane or cyclopentane, and the C-3 pyridinyl carboxamide moiety, which was replaced by smaller groups such as primary amide, ethyl carboxylate, and carboxylic acid [18]. From this first set of compounds, clear SAR information emerged: the cycloheptane cannot be replaced by smaller rings and aromatic moieties both at C-2 and at C-3 position are required to impart potent activity.

By analyzing the predicted binding modes of the best PA-PB1 inhibitors reported so far in the PA cavity from X-ray crystal structure PDB ID 3CM8 [22], it emerged that two hydrophobic interactions, one of which essential for the interaction with W706, seem sufficient to impart inhibitory activity against PA-PB1 interaction [14]. When additional hydrophobic interactions do not occur, favorable stabilization of the PA-small molecule interaction seems to take place with the formation of one (with Q408) or more favorable H-bonds.

By aligning some PA-PB1 inhibitors, we proposed a first pharmacophore model entailing two hydrophobic moieties separated by a "polar belt" with two H-bond acceptor points and two H-bond donor points [19]. More recently, we generated a new pharmacophore model by using all the best PA-PB1 inhibitors reported to date [14]. It preserves the two hydrophobic moieties, of which one involved in the interaction with W706. On the contrary, the two H-bond donor points do not seem critical for intermolecular interactions and only a H-bond acceptor point is preserved. Thus, two hydrophobic moieties and a central H-bond acceptor point appear to be the minimum structural requirement for PA-PB1 inhibition. However, since eight out of the eleven best PA-PB1 inhibitors studied matched two H-bond interaction points, additional H-bond interactions should be exploited to achieve a more efficient PA-PB1 inhibition.

Taking into account the indications coming from the previous SAR coupled with the recent computational suggestions, a new set of derivatives has been designed by inserting a hydroxyl group to the different positions of the C-2 phenyl ring (compounds **3-5**, Table 1).

Computational studies based on the Fingerprints for Ligands and Proteins (FLAP) algorithm [23] were performed to analyze their binding poses in the PA cavity from the structure PDB ID 3CM8 [22]. To be consistent with our previous results [14-16,18-20], the FLAP software was used in the "Structure-based" mode: this approach allows to generate binding poses of a ligand in a protein cavity based on the similarity between their GRID fields [24]. Thus, although this approach is not a standard energy-based docking method, it proved to be successful in identifying or optimizing PA-PB1 inhibitors [15,18-20]. Similarity scores were used to rank the most reliable poses for compounds 3-5 (Fig. 2B-D); the FLAP binding pose previously obtained for hit compound **1** [18] was also reported as a reference in Fig. 2A. Compound **1** interacted with the first (defined by W706 and F411) and the second (defined by P710 and L666) hydrophobic pockets and with the additional central hydrophobic region (mainly generated by the aliphatic chain of E623 and the protein backbone). The similarity score for the proposed binding pose of 1 was 0.53. According to our model, the insertion of the hydroxyl group induced a significant change of the binding mode.

Indeed, the hydrophobic interaction with W706, already reported as a key residue for PA-PB1 inhibition [14], still occurred for all compounds **1** and **3-5**, even though the orientation of the cycloheptathiophene core in compounds **3-5** resulted to be inverted with respect to **1**. As a consequence, the hydroxyl-substituted phenyl ring pointed towards a region of the PA cavity defined by F411, C415, and I621. Hydrophobic and H-bond interactions contributed to obtain a more favorable binding mode for compounds **3-5**. Based on the FLAP similarity scoring, which takes into account a global estimation of hydrophobic, hydrophilic, H-bond donor and H-bond acceptor interactions, compounds 3-5 are almost equivalent (0.65, 0.63, and 065, respectively), with higher values with respect to compound **1** (0.53). Thus, the similarity scores are in agreement with the ELISA PA-PB1 interaction assay data, shown in Table 1. A deeper visual inspection of the binding poses in Figure 2 was performed. The *o*-hydroxyl derivative **3** displayed the most efficient hydrophobic interactions between the cycloheptathiophene moiety with W706 and between the hydroxyphenyl group with the hydrophobic region defined by F411, C415, and I621 (Fig. 2B). The latter hydrophobic region was recently described as another key area of PA for the interaction with potent inhibitors [25]. In addition, compound **3** resulted able to form a H-bond between I621 and its hydroxyl group, and a second one between the C-3 carbonyl group and Q408. On the contrary, the FLAP binding poses for the *meta* and *para* isomers **4** and **5** resulted shifted towards C415, weakening the hydrophobic interaction with W706 (Fig. 2C-D). This shift could allow **5** to form a new H-bond interaction between the *p*-hydroxyl group and the carbonyl group of C415, while the *m*-hydroxyl group was not involved in any specific interaction. Additional H-bonds with I621 and Q408 were still possible for both compounds **4** and **5**.

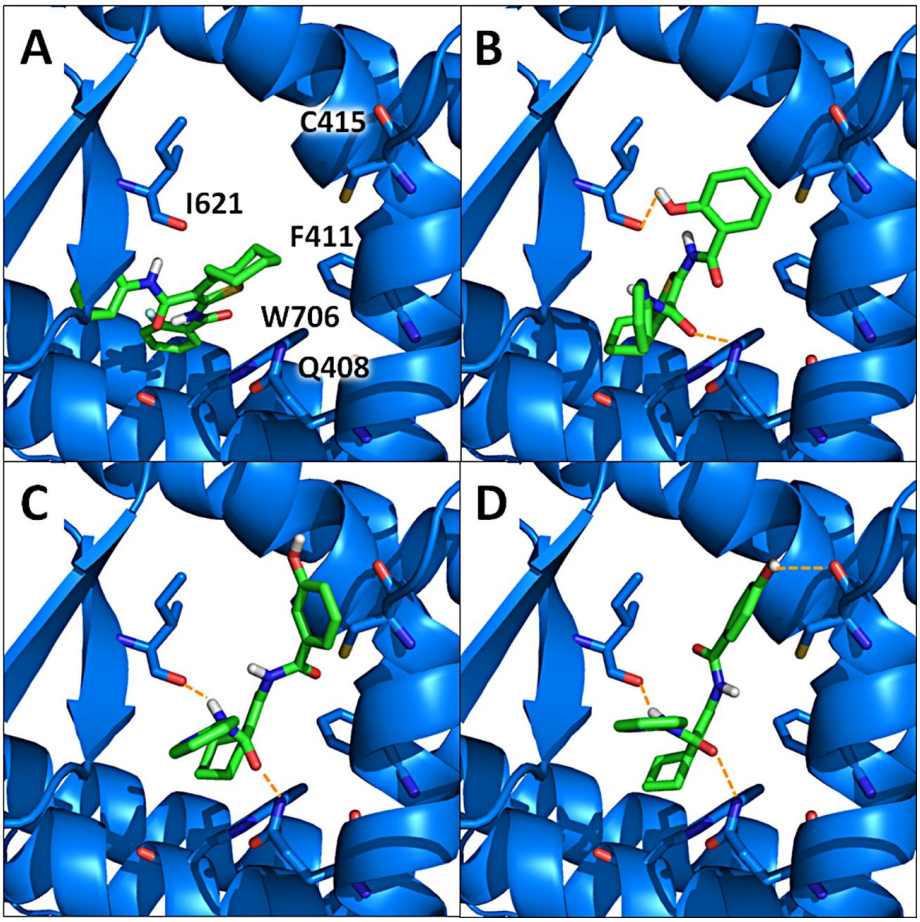


Fig. 2. FLAP binding poses for compounds **1 (A)**, **3 (B)**, **4 (C)**, and **5 (D)**. Some of the PA key residues located in the cavity are highlighted in stick-mode.

In agreement with the computational study, the biological evaluation of the three isomers (**3**, **4**, and **5**) showed that the hydroxyl group resulted useful to impart PA-PB1 interaction inhibitory activity especially when placed at *ortho* position (IC_{50} = 15 μM), and also allowed to achieve a really potent anti-flu activity (EC_{50} = 2.6 μM).

Given the potent antiviral profile of compound **3**, the *o*-hydroxyphenyl moiety was maintained at the C-2 position while the C-3 position was thoroughly explored in a successive set of derivatives (compounds **6-16**, Table 1). In particular, the 2-pyridinyl ring was replaced by a phenyl (**6**), *p*-chlorophenyl (**7**), *p*-fluorophenyl (**8**), and also by a thiazole (**9**) ring. A methylene unit was inserted between the aromatic ring and amide moiety at the C-3 position, as in compounds **10** and **11**. Aliphatic alkyl moieties, i.e. isopropyl, *tert*-butyl, cyclohexyl, piperazine, and 2-pyridilpiperazine

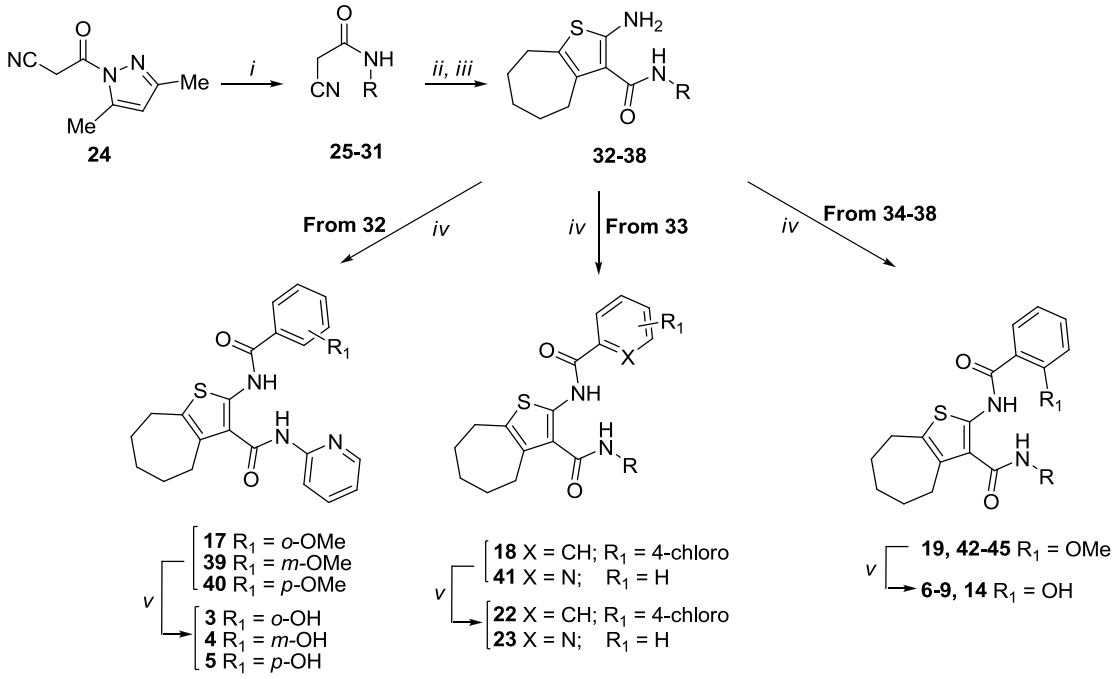
(**12-16**), were also used as C-3 substituents. Methoxy intermediates **17** [26], **18**, and **19** (Table 1) were also assayed to enrich the SAR. The replacement of the hydroxyl group at the C-2 phenyl ring was also investigated with the *o*-amino and *p*-amino derivatives **20** [26] and **21** [26] (Table 1). Finally, the C-2 and C-3 aromatic groups were interchanged synthesizing compounds **22** and **23** (Table 1).

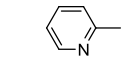
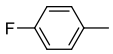
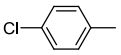
3. CHEMISTRY

The synthesis of the target compounds **3-9**, **14**, **18**, **19**, **22**, and **23** was accomplished by applying the two-steps Gewald synthesis, as outlined in Scheme 1. Thus, the 2-cyano-*N*-(substituted)-acetamides **25** [27], **26** [28], **27**, **28** [27], and **29-31**, prepared by reacting 3-(3,5-dimethyl-1*H*-pyrazol-1-yl)-3-oxopropanenitrile **24** [27] with the appropriate amines in toluene, were used in a first Knoevenagel condensation with cycloheptanone. The successive cyclization step performed in the presence of sulfur and *N*,*N*-diethylamine in EtOH gave synthones **32** [18], **33** [29], and **34-38**. The reaction of synthones **32-38** with the appropriate acyl chlorides in dry pyridine led to the methoxyl derivatives **17** [26], **18**, **19**, and **39-45**. The successive *O*-demethylation performed with BBr_3 in dry CH_2Cl_2 provided the hydroxyl target compounds **3-9**, **14**, **22**, and **23**.

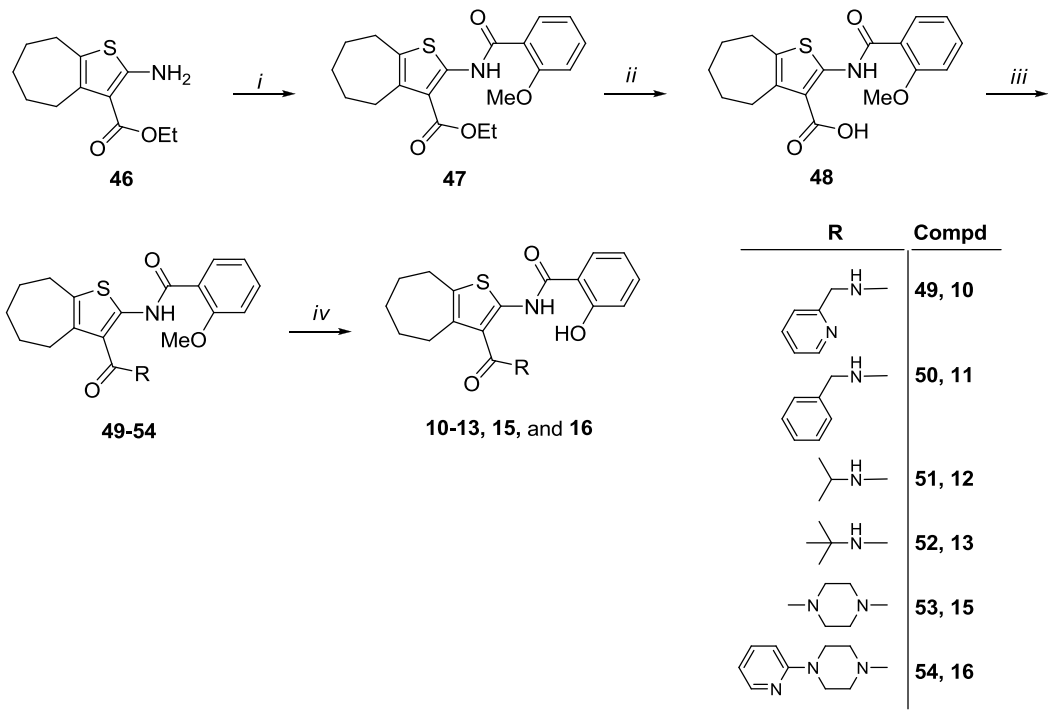
An alternative strategy was used for the synthesis of the target compounds **10-13**, **15** and **16** (Scheme 2), all characterized by the *o*-hydroxyl phenyl ring at C-2 position, entailing the preparation of the key intermediate **48** suitable to be widely functionalized at the C-3 position. Thus, synthone **48** was prepared by reacting ethyl 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxylate **46** [30] with the 2-methoxybenzoyl chloride in dry pyridine, to obtain derivative **47** that was then hydrolyzed under basic conditions. Coupling reaction of 3-carboxylic acid **48** with the appropriate alkyl amines in presence of benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and collidine in dry CH_2Cl_2 gave the desired 2-methoxybenzamido derivatives **49-54**. Of note, the coupling reaction carried out in the same conditions but using anilines for the synthesis of the target compounds shown in

Scheme 1, did not furnish the desired compounds but gave only the cyclized cyclohepta[4,5]thieno[2,3-*d*][1,3]oxazin-4-one derivatives. The *o*-hydroxy target derivatives **10-13**, **15**, and **16** were then obtained by *O*-demethylation of methoxyl intermediates **49-54** in the presence of BBr_3 in dry CH_2Cl_2.



R	Compd	R	Compd
	25, 32, 3-5		29, 36, 43, 8
	26, 33, 18, 41		30, 37, 44, 9
	27, 34, 19, 7		31, 38, 45, 14
	28, 35, 42, 6		22, 23

Scheme 1. Synthesis of compounds **3-9**, **14**, **18**, **19**, **22**, and **23**. *Reagents and conditions:* (i) amines, toluene, reflux; (ii) cycloheptanone, ammonium acetate, glacial acetic acid, benzene, reflux; (iii) sulfur, *N*,*N*-diethylamine, EtOH, 40−50 °C; (iv) acyl chlorides, pyridine, rt; (v) BBr_3, dry CH_2Cl_2, rt.

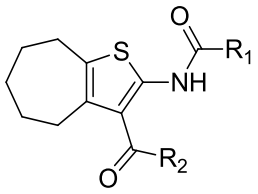


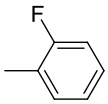
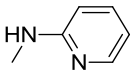
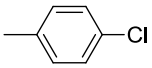
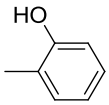
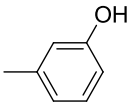
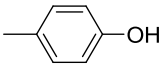
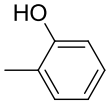
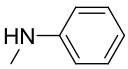
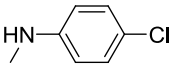
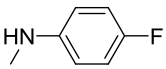
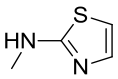
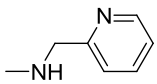
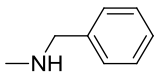
Scheme 2. Synthesis of compounds **10-13**, **15**, and **16**. *Reagents and conditions:* (i) 2-methoxybenzoyl chloride, pyridine, rt; (ii) LiOH, H_2O/THF, 50 °C; (iii) amines, BOP, collidine, dry CH_2Cl_2, rt; (iv) BBr_3, dry CH_2Cl_2, rt.

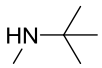
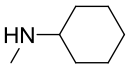
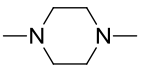
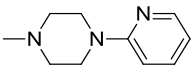
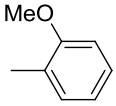
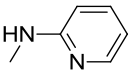
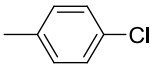
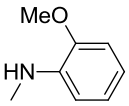
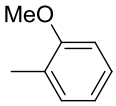
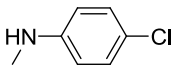
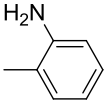
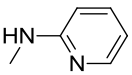
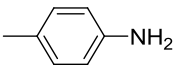
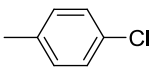
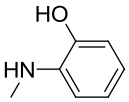
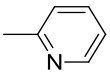
4. RESULTS AND DISCUSSION

The whole set of derivatives were first evaluated for the ability to inhibit the physical interaction between fluA PA and PB1 subunits by ELISA including the $PB1_{(1–15)}$–Tat peptide [31] as a positive control of inhibition. In parallel, for all the synthesized compounds the antiviral activity was tested by plaque reduction assays (PRA) in Mardin-Darby canine kidney (MDCK) cells infected with a reference fluA virus, the A/PR/8/34 strain. Ribavirin (RBV), a known broad-spectrum inhibitor of RNA viruses polymerase [32], was included as a positive control of inhibition. To exclude that the observed antiviral activities could be due to toxic effects on the target cells, all the synthesized compounds were also tested by MTT assays in MDCK cells. The antiviral activity and toxicity data for the tested compounds, as well as their ability to disrupt the PA-PB1 interaction in ELISA, are reported in Table 1.

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



Compd	R_1	R_2	ELISA PA-PB1 Interaction Assay IC_{50}, μM[a]	PRA in MDCK cells EC_{50}, μM[b]	Cytotoxicity (MTT Assay) in MDCK cells CC_{50}, μM[c]
1			145 ± 12	90 ± 13	>250
2		"	32 ± 9	18 ± 2	>250
3		"	15 ± 2	2.6 ± 0.5	>250
4		"	23 ± 6	22 ± 9	248 ± 1
5		"	26 ± 4	19 ± 2	>250
6			58 ± 16	30 ± 1	>250
7	"		18 ± 1	1.2 ± 0.3	>250
8	"		20 ± 5	1.2 ± 0.4	>250
9	"		69 ± 15	0.18 ± 0.07	>250
10	"		33 ± 6	>100	>250
11	"		191 ± 21	>100	172 ± 18
12	"		>200	>100	>250

13	"		156 ± 8	>100	>250
14	"		114 ± 21	>100	172 ± 39
15	"		>200	94 ± 6	>250
16	"		197 ± 15	>100	145 ± 7
17[d]			22 ± 2	86 ± 8	147 ± 39
18			45 ± 14	24 ± 6	>250
19			78 ± 11	82 ± 3	>250
20[d]			>200	101 ± 6	>250
21[d]		"	65 ± 20	0.26 ± 0.13	>250
22			26 ± 6	1.8 ± 1.0	28 ± 8
23		"	118 ± 21	8.2 ± 1.1	>250
RBV				10 ± 2	>250
Tat-PB1$_{1\text{-}15}$ peptide			35 ± 4	41 ± 5	>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 flu A A/PR/8/34 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%. All the reported values represent the means ± SD of data derived from at least three independent experiments in duplicate. [d] Reported in reference [26].

With the aim to exploit polar groups able to establish important H-bonds, the hydroxyl derivatives **3-5** were initially prepared. The *ortho* hydroxyl derivative **3** was the most active, being able to efficiently interfere with PA-PB1 interaction (IC_{50} = 15 μM) and exhibiting potent antiviral activity (EC_{50} = 2.6 μM) without showing cytotoxicity (CC_{50} > 250 μM), resulting in a selectivity index (SI) > 96. The shifting of the hydroxyl group to *meta* and *para* positions (compounds **4** and **5**, respectively) decreased both the PA-PB1 interaction inhibitory activity and mainly the antiviral effect. Within the C-2 *o*-hydroxyl phenyl derivatives variously functionalized at C-3 position, *para* halogenated derivatives **7** and **8** showed a good anti-flu activity in the cellular context, even better than compound **3**, with EC_{50} values of 1.2 μM; in addition, the absence of cytotoxicity up to 250 μM led to SIs > 200. Also the C-3 thiazole ring improved the anti-flu activity; indeed, compound **9** emerged as the most potent of the series with an EC_{50} of 0.18 μM, which coupled with the lack of cytotoxicity up to 250 μM led to the impressive SI > 1388; however, compound **9** showed a decreased ability to inhibit the PPI. Conversely, compound **6**, having a nude phenyl ring as C-3 substituent, was found to be a weak inhibitor of both PA-PB1 interaction and flu growth. The introduction of a methylene unit as a spacer between the aromatic ring and the scaffold (compounds **10** and **11**) as well as the replacement of the aromatic ring with aliphatic substituents (compounds **12-16**) at the C-3 position were in all cases detrimental.

The *o*-hydroxyl phenyl moiety still conferred anti-PA-PB1 and above all anti-flu activity when moved to the C-3 position and coupled with the C-2 *p*-chlorophenyl ring, as in compound **22**, even though it caused cytotoxicity. The similar anti-PA-PB1 effect displayed by compound **22** and its analogue **2** was also confirmed by FLAP-computational studies, suggesting that the two compounds exhibit a similar binding pose in the PA cavity, with the *p*-chloro substituent likely oriented into an enlarged hydrophobic pocket generated by L666 and a rotated P710 (not shown). For what concerns the other C-3 hydroxyphenyl compound **23**, although showing good ability to inhibit viral growth at nontoxic concentrations, only a weak anti-PA-PB1 activity was observed.

The ability to interfere with PA-PB1 binding and mainly the antiviral activity dramatically decreased when the hydroxyl group was replaced with a methoxyl group, such as in compounds **17-19**, as compared with their counterparts **3**, **22**, and **7**, respectively. Also the replacement of the hydroxyl with an amino group both in *ortho* (compound **20**) and *para* (compound **21**) position was detrimental for what concerns the PA-PB1 inhibition. However, the *p*-amino derivative **21** exhibited a very potent anti-flu activity with EC_{50} of 0.26 μM and it was found to be not cytotoxic in MDCK cells (CC_{50} > 250), reaching an SI > 961.

5. CONCLUSIONS

The continuous need to identify innovative anti-flu compounds led us to explore the subunits assembly of RdRp as a possible alternative antiviral target. This approach has already proven to be successful with the identification of effective PA-PB1 interaction inhibitors endowed with the ability to inhibit flu growth.

In this study we came back to the cHTC scaffold, which was functionalized with a hydroxyl moiety with the aim of exploiting additional H-bond interactions with key residues of the PA cavity, as suggested by recent computational studies [14]. As a result, new very interesting compounds were identified. In particular, C-2 *o*-hydroxyphenyl derivatives **3**, **7**, and **8** emerged as the best in inhibiting PA-PB1 interaction that well translated into a potent anti-flu activity. Two additional compounds, C-3 thiazole derivative **9** and C-2 *p*-aminophenyl compound **21**, although showing a weaker ability to inhibit PA-PB1 interaction, were endowed with a very potent anti-flu activity (IC_{50} = 0.18 and 0.26 μM), reaching SI values >1388 and >961, respectively. For these compounds, further studies are in progress to uncover whether an additional mechanism of action might contribute to their potent anti-influenza activity.

6. Experimental section

6.1. Computational methods

The binding poses in the PA cavity were generated using the FLAP software in the structure-based mode (Molecular Discovery Ltd., UK; www.moldiscovery.com). The procedure has been extensively described elsewhere [16]. As in the previous studies, the main cavity of the crystallographic structure of a large C-terminal fragment of PA (aa 257-716) (pdb code: 3CM8) [22] was used as a template. A total of 50 conformers for each ligand were generated to mimic the compound flexibility, and the most abundant protonation state of each molecule was used, as predicted by MoKa [33]. The probes used to generate the GRID Molecular Interaction Fields were H (shape), DRY (hydrophobic interactions), N1 (H-bond donor) and O (H-bond acceptor) interactions. The global similarity score (Glob-Pro) was used for ranking.

6.2. Chemistry

6.2.1. General experimental procedures

Commercially available starting materials, reagents, and solvents were used as supplied. All reactions were routinely checked by TLC on silica gel 60F254 (Merck) and visualized by using UV or iodine. Flash column chromatography was performed on Merck silica gel 60 (mesh 230-400). 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. HRMS spectra were registered on Agilent Technologies 6540 UHD Accurate Mass Q-TOF LC/MS, HPLC 1290 Infinity. Purity of the target compounds was determined by LC/MS on Agilent Technologies 6550 iFUNNEL Q-TOF equipped with HPLC 1290 Infinity with DAD detector. HPLC conditions to assess the purity of final compounds were as follows: column, Phenomenex AERIS Widepore C4, 4.6mm × 100 mm (6.6 μm); flow rate, 0.85 mL/min; acquisition time, 10 min; DAD 190−650 nm; oven temperature, 30 °C; gradient of acetonitrile in water containing 0.1% of formic acid (0−100% in 10 min). Analyses indicated by the symbols of

the elements or functions were within ± 0.4 % of the theoretical values. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance DPX-200 and Bruker Avance DRX-400MHz using residual solvents such as dimethylsulfoxide (δ = 2.48) as an internal standard. Chemical shifts were recorded in ppm (δ) and the spectral data are consistent with the assigned structures. The spin multiplicities are indicated by the symbols s (singolet), d (doublet), t (triplet), q (quartet), m (multiplet), and bs (broad singolet).

The synthesis of some compounds reported in this study, i.e., **17** [26], **20** [26], **21** [26], **24** [27], **28** [27], **32** [18], **33** [29], and **46** [30], has been previously described. Derivatives **34**-**36**, **38**, and **48** are commercially available but their synthesis has been not published before and is herein reported. The synthesis of compounds **27** [28], **29** [34], **30** [35], and **31** [36] has been previously described but their preparation is herein reported through a different synthetic strategy.

*6.2.2. General procedure for the preparation of 2-cyano-N-(substituted)acetamides **27** and **29-31** (**Method A**).* To a solution of the appropriate amine (1.0 equiv) in toluene, **24** [27] (1.0 equiv) was added. The mixture was refluxed for 5 min, and then stirred at room temperature to obtain a precipitate, which was filtered, washed with Et_2O, and dried.

*6.2.3. N-(4-Chlorophenyl)-2-cyanoacetamide (**27**).* The tile compound was prepared starting from *p*-chloroaniline through Method A, in 84% yield as white solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.80 (s, 2H, CH_2), 7.35 (d, J = 8.8 Hz, 2H, aromatic CH), 7.50 (d, J = 8.8 Hz, 2H, aromatic CH), 10.25 (bs, 1H, NH).

*6.2.4. 2-Cyano-N-(4-fluorophenyl)acetamide (**29**).* The tile compound was prepared starting from *p*-fluoroaniline through Method A, in 87% yield as white solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.75 (s, 2H, CH_2), 7.00-7.20 (m, 2H, aromatic CH), 7.40-7.50 (m, 2H, aromatic CH), 10.50 (bs, 1H, NH).

*6.2.5. 2-Cyano-N-(thiazol-2-yl)acetamide (**30**).* The tile compound was prepared starting from 2-aminothiazole through Method A, in 87% yield as white solid. White solid. ^{1}H NMR (200 MHz,

DMSO-d_6,): δ 4.00 (s, 2H, CH_2), 7.20 and 7.45 (d, *J* = 3.5 Hz, each 1H, aromatic CH) 12.50 (bs, 1H, NH).

*6.2.6. 2-Cyano-N-cyclohexylacetamide (**31**)* The tile compound was prepared starting from cyclohexylamine through Method A, in 72% yield as white solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.00-1.45 and 1.50-1.80 (m, each 5H, cyclohexyl CH_2), 3.45-3.50 (m, 1H, cyclohexyl CH), 3.55 (s, 1H, CH_2), 8.05 (d, *J* = 7.6 Hz, 1H, NH).

*6.2.7. General procedure for the preparation of 2-amino-N-(substituted)-cycloheptathiophene-3-carboxamide synthones **34-38** (**Method B**).* By applying a two-steps Gewald synthesis, a mixture of the appropriate 2-cyano-*N*-(substituted)acetamide (1.0 equiv), cycloheptanone (4.0 equiv), ammonium acetate (1.3 equiv), and glacial acetic acid (3.5 equiv) in benzene (10 ml/mmol) was heated at reflux for 16 h in a Dean-Stark apparatus. After cooling, the mixture was diluted with $CHCl_3$ and then washed with H_2O, 10% $NaCO_3$ solution, and finally H_2O. The organic layer was concentrated to dryness to afford the crude Knoevenagel product, which was used directly in the successive step without further purification. Thus, to the crude Knoevenagel product (1.0 equiv) dissolved in EtOH, sulfur (4.0 equiv) and *N,N*-diethylamine (4.0 equiv) were added. The mixture was maintained at 40-50 °C for 2 h and then concentrated to dryness to yield a residue, which was treated with a mixture of cyclohexane/Et_2O and filtered.

*6.2.8. 2-Amino-N-(4-chlorophenyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**34**).*The title compound was prepared starting from **27** through Method B, in 61% yield as red-brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.40-1.60 (m, 4H, cycloheptane CH_2), 1.65-1.80 (m, 2H, cycloheptane CH_2), 2.40-2.55 and 2.60-2.70 (m, each 2H, cycloheptane CH_2), 5.80 (bs, 2H, NH_2), 7.25 (d, *J* = 8.8 Hz, 2H, aromatic CH), 7.60 (d, *J* = 8.8 Hz, 2H, aromatic CH), 9.55 (bs, 1H, NH).

*6.2.9. 2-Amino-N-phenyl-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**35**).* The title compound was prepared starting from **28** [27] through Method B, in 64% yield as red-brown

solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.40-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.50-2.60 and 2.75-2.85 (m, each 2H, cycloheptane CH_2), 5.05 (s, 2H, NH_2), 7.05 (t, *J* = 7.2 Hz, 1H, aromatic CH), 7.20-7.35 (m, 2H, aromatic CH), 7.45 (d, *J* = 7.9 Hz, 2H, aromatic CH), 9.50 (s, 1H, NH).

*6.2.10. 2-Amino-N-(4-fluorophenyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**36**).* The title compound was prepared starting from **29** through Method B, in 41% yield as red-brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.40-1.60 (m, 4H, cycloheptane CH_2), 1.60-1.90 (m, 2H, cycloheptane CH_2), 2.50-2.70 (m, 4H, cycloheptane CH_2), 5.75 (bs, 2H, NH_2), 6.90-7.10 (m, 2H, aromatic CH), 7.70-7.85 (m, 2H, aromatic CH), 9.50 (s, 1H, NH).

*6.2.11. 2-Amino-N-(thiazol-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**37**).* The title compound was prepared starting from **30** through Method B, in 40% yield as yellow solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.50-2.60 and 2.75-2.85 (m, each 2H, cycloheptane CH_2), 5.15 (s, 2H, NH_2), 6.90 and 7.30 (d, *J* = 3.6 Hz, each 1H, aromatic CH), 10.00 (s, 1H, NH).

*6.2.12. 2-Amino-N-cyclohexyl-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**38**).* The title compound was prepared starting from **31** through Method B, in 55% yield as light-brown solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.00-1.25 and 1.40-1.55 (m, each 5H, cyclohexyl CH_2), 1.60-1.75 (m, 8H, cycloheptane CH_2), 2.50-2.60 (m, 2H, cycloheptane CH_2), 3.50-3.60 (m, 1H, cyclohexyl CH), 5.50 (bs, 2H, NH_2), 7.20 (d, *J* = 7.6 Hz, 1H, NH).

*6.2.13. General procedure for C-2 amidation for derivatives **18**, **19**, and **39-45** (**Method C**).* A solution of the appropriate synthones (1.0 equiv) in dry pyridine was added of the suitable benzoyl chloride (2.0 equiv). The reaction mixture was maintained at room temperature until no starting material was detected by TLC. After cooling, the reaction mixture was poured into ice/water, obtaining a precipitate which was filtered and purified as described below.

*6.2.14. 2-(4-Chlorobenzamido)-N-(2-methoxyphenyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**18**).* The title compound was prepared starting from **33** [29] through Method C and purified by crystallization by EtOH, in 29% yield as light-yellow solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50-1.80 (m, 4H, cycloheptane CH_2), 1.80-2.00 (m, 2H, cycloheptane CH_2), 2.70-2.80 and 2.90-3.00 (m, each 2H, cycloheptane CH_2), 3.90 (s, 3H, OCH_3), 6.80 (dd, *J* = 1.8 and 7.3 Hz, 1H, aromatic CH), 7.05 (dt, *J* = 2.0 and 7.5 Hz, 2H, aromatic CH), 7.45 (d, *J* = 8.7 Hz, 2H, aromatic CH), 7.90 (d, *J* = 8.7 Hz, 2H, aromatic CH), 8.10 (s, 1H, NH), 8.45 (dd, *J* = 1.8 and 7.4 Hz, 1H, aromatic CH), 12.00 (s, 1H, NH). HRMS: *m/z* calcd for $C_{24}H_{23}ClN_2O_3S$ 455.1197 $(M+H)^+$, found 455.1198.

*6.2.15. N-(4-Chlorophenyl)-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**19**).* The title compound was prepared starting from **34** through Method C and purified by flash chromatography eluting with cyclohexane:EtOAc 6:4, in 21% yield as red-brown solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.55-1.65 (m, 4H, cycloheptane CH_2), 1.65-1.75 (m, 2H, cycloheptane CH_2), 2.55-2.65 and 2.65-2.80 (m, each 2H, cycloheptane CH_2), 4.00 (s, 3H, OCH_3), 6.95 (d, *J* = 8.3 Hz, 1H, aromatic CH), 7.10 (t, *J* = 7.2 Hz, 1H, aromatic CH), 7.35 (d, *J* = 8.8 Hz, 2H, aromatic CH), 7.40-7.50 (m, 2H, aromatic CH and NH), 7.60 (d, *J* = 8.7 Hz, 2H, aromatic CH), 8.25 (dd, *J* = 8.07 and 1.9 Hz, 1H, aromatic CH), 12.10 (s, 1H, NH). HRMS: *m/z* calcd for $C_{24}H_{23}ClN_2O_3S$ 455.1197 $(M+H)^+$, found 455.1196.

*6.2.16. 2-(3-Methoxybenzamido)-N-(pyridin-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**39**).* The title compound was prepared starting from **32** [18] through Method C and purified by flash chromatography eluting with cyclohexane:EtOAc 7:3, in 41% yield as red-brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.60 (m, 4H, cycloheptane CH_2), 1.80-1.90 (m, 2H, cycloheptane CH_2), 2.70-2.85 (m, 4H, cycloheptane CH_2), 3.75 (s, 3H, OCH_3), 7.05-7.15 (m, 2H, aromatic CH and pyridine CH), 7.35-7.45 (m, 3H, aromatic CH), 7.70-7.80 (m, 1H, pyridine CH),

8.10 (d, *J* = 8.2 Hz, 1H, pyridine CH), 8.25-8.30 (d, 1H, pyridine CH), 10.30 and 10.90 (s, each 1H, NH).

*6.2.17. 2-(4-Methoxybenzamido)-N-(pyridin-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**40**).* The title compound was prepared starting from **32** [18] through Method C and purified by treatment with Et_2O, in 34% yield, as red-brown solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.40-1.60 (m, 4H, cycloheptane CH_2), 1.65-1.80 (m, 2H, cycloheptane CH_2), 2.60-2.80 (m, 4H, cycloheptane CH_2), 3.80 (s, 3H, OCH_3), 6.95 (d, *J* = 8.7 Hz, 2H, aromatic CH), 7.05-7.15 (m, 1H, pyridine CH), 7.70-7.85 (m, 3H, aromatic CH and pyridine CH), 8.15 (d, *J* = 8.3 Hz, 1H, pyridine CH), 8.25-8.35 (m, 1H, pyridine CH), 10.30 and 10.90 (s, each 1H, NH).

*6.2.18. N-(3-((2-Methoxyphenyl)carbamoyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)picolinamide (**41**).* The title compound was prepared starting from **33** [29] through Method C and purified by flash chromatography eluting with $CHCl_3$:MeOH 98:2, in 34% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.55-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.70-2.80 and 2.80-2.90 (m, each 2H, cycloheptane CH_2), 3.80 (s, 3H, OCH_3), 6.95 (t, *J* = 7.3 Hz, 1H, aromatic CH), 7.05 (d, *J* = 7.4 Hz, 1H, aromatic CH), 7.10 (t, *J* = 7.1 Hz, 1H, aromatic CH), 7.55-7.65 (m, 1H, pyridine CH), 7.90 (d, *J* = 7.7 Hz, 1H, pyridine CH), 8.00-8.20 (m, 2H, pyridine and aromatic CH), 8.70 (d, *J* = 4.7 Hz, 1H, pyridine CH), 8.80 (s, 1H, NH), 12.20 (s, 1H, NH).

*6.2.19. 2-(2-Methoxybenzamido)-N-phenyl-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**42**).* The title compound was prepared starting from **35** through Method C and purified by flash chromatography eluting with cyclohexane:EtOAc 7:3, in 32% yield as white solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.45-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.80 (m, 4H, cycloheptane CH_2), 3.80 (s, 3H, OCH_3), 7.00-7.20 (m, 3H, aromatic CH), 7.30 (t, *J* = 7.6 Hz, 2H, aromatic CH), 7.50 (t, *J* = 7.1 Hz, 1H, aromatic CH), 7.70 (d,

J = 7.5 Hz, 2H, aromatic CH), 8.00 (d, *J* = 6.7 Hz, 1H, aromatic CH), 10.10 and 11.60 (s, each 1H, NH).

*6.2.20. N-(4-Fluorophenyl)-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**43**).* The title compound was prepared starting from **36** through Method C and purified by treatment with Et_2O, in 74% yield as light-brown solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.60-2.00 (m, 6H, cycloheptane CH_2), 2.60-2.75 (m, 4H, cycloheptane CH_2), 3.75 (s, 3H, OCH_3), 7.00-7.25 (m, 4H, aromatic CH), 7.50-7.60 (m, 1H, aromatic CH), 7.70-7.80 (m, 2H, aromatic CH), 8.00 (d, *J* = 7.5 Hz, 1H, aromatic CH), 10.20 and 11.75 (s, each 1H, NH).

*6.2.21. 2-(2-Methoxybenzamido)-N-(thiazol-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**44**).* The title compound was prepared starting from **37** through Method C and purified by flash chromatography eluting with cyclohexane:EtOAc 6:4, in 32% yield as yellow-orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55-1.65 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.70-2.80 and 2.85-2.90 (m, each 2H, cycloheptane CH_2), 4.15 (s, 3H, OCH_3), 6.90 (d, *J* = 3.5 Hz, 1H, aromatic CH), 6.95-7.00 (m, 2H, aromatic CH), 7.15 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.45 (dt, *J* = 1.7 and 8.6 Hz, 1H, aromatic CH), 8.25 (dd, *J* = 1.7 and 7.8 Hz, 1H, aromatic CH), 11.40 and 12.40 (s, each 1H, NH).

*6.2.22. N-Cyclohexyl-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**45**).* The title compound was prepared starting from **38** through Method C and purified by treatment with Et_2O, in 89% yield as light-brown solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.00-1.30 (m, 5H, cyclohexyl CH_2), 1.50-1.90 (m, 13H, cyclohexyl CH_2 and cycloheptane CH_2), 2.50-2.60 (m, 2H, cycloheptane CH_2), 3.50-3.60 (m, 1H, cyclohexyl CH), 4.00 (s, 3H, OCH_3), 7.10 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.20 (d, *J* = 8.5 Hz, 1H, NH), 7.55 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.75 (d, *J* = 8.1 Hz, 1H, aromatic CH), 8.00 (d, *J* = 7.7 Hz, 1H, aromatic CH), 11.75 (s, 1H, NH).

*6.2.23. General procedure for O-demethylathion reaction for target derivatives **3-16**, **22**, and **23**. **(Method D)*** To a solution of the appropriate methoxy derivative (1.0 equiv) in dry CH_2Cl_2, 1M solution of BBr_3 in CH_2Cl_2 (6.0 equiv) was added dropwise maintaining the temperature at 0 °C. The reaction mixture was stirred at room temperature for 3-6 h and then quenched with MeOH and water. The organic solvent was removed under vacuum affording a residue, which was filtered and purified as described below.

*6.2.24. 2-(2-Hydroxybenzamido)-N-(pyridin-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**3**).* The title compound was prepared starting from **17** [26] through Method D and purified by crystallization by EtOH/H_2O, in 48% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.65 (m, 4H, cycloheptane CH_2), 1.70-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.70 and 2.70-2.80 (m, each 2H, cycloheptane CH_2), 6.85-6.95 (m, 2H, aromatic CH), 7.05-7.15 (m, 1H, pyridine CH), 7.35 (t, *J* = 7.7 Hz, 1H, aromatic CH), 7.80 (t, *J* = 7.1 Hz, 1H, pyridine CH), 7.95 (d, *J* = 7.7 Hz, 1H, aromatic CH), 8.05 (d, *J* = 8.1 Hz, 1H, pyridine CH), 8.30 (d, *J* = 4.6 Hz, 1H, pyridine CH), 10.40 (s, 1H, NH), 11.65 (bs, 1H, OH), 11.95 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.5, 28.1, 28.7, 28.8, 32.1, 115.1, 117.1, 117.5, 120.1, 120.2, 121.8, 130.9, 131.4, 134.2, 135.3, 137.5, 138.4, 148.5, 152.1, 156.7, 162.0, 164.9. HRMS: *m/z* calcd for $C_{22}H_{21}N_3O_3S$ 408.1383 $(M+H)^+$, found 408.1380.

*6.2.25. 2-(3-Hydroxybenzamido)-N-(pyridin-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**4**).* The title compound was prepared starting from **39** through Method D and purified by flash chromatography eluting with $CHCl_3$:MeOH 98:2, in 13% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 1.50-1.60 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.80 (m, 4H, cycloheptane CH_2), 6.90-7.00 (m, 1H, aromatic CH), 7.00-7.10 (m, 1H, aromatic CH), 7.20-7.30 (m, 3H, aromatic CH and pyridine CH), 7.80 (t, *J* = 7.0 Hz, 1H, pyridine CH), 8.10 (d, *J* = 8.3 Hz, 1H, pyridine CH), 8.3 (d, *J* = 4.1 Hz, 1H, pyridine CH), 9.80 (s, 1H, OH), 10.25 and 10.90 (s, each 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.5, 28.1, 28.5,

28.8, 32.1, 114.7, 115.0, 118.4, 119.5, 120.1, 124.1, 130.1, 132.0, 134.7, 136.2, 136.9, 138.4, 148.4, 152.3, 157.9, 164.3, 164.4. HRMS: *m/z* calcd for $C_{22}H_{21}N_3O_3S$ 408.1383 $(M+H)^+$, found 408.1377.

*6.2.26. 2-(4-Hydroxybenzamido)-N-(pyridin-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**5**).* The title compound was prepared starting from **40** through Method D and purified by crystallization by EtOH/DMF followed by flash chromatography eluting with $CHCl_3$:MeOH 98:2, in 32% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.45-1.65 (m, 4H, cycloheptane CH_2), 1.70-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.70 and 2.70-2.80 (m, each 2H, cycloheptane CH_2), 6.75 (d, *J* = 8.0 Hz, 2H, aromatic CH), 7.10 (t, *J* = 7.0 Hz, 1H, pyridine CH), 7.70 (d, *J* = 8.0 Hz, 2H, aromatic CH), 7.80 (t, *J* = 7.2 Hz, 1H, pyridine CH), 8.15 (d, *J* = 7.7 Hz, 1H, pyridine CH), 8.25-8.35 (m, 1H, pyridine CH), 10.20 (s, 1H, OH), 10.30 and 10.90 (s, each 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.5, 28.1, 28.5, 28.8, 32.1, 115.0, 115.6, 120.1, 123.5, 123.8, 130.1, 131.7, 136.1, 137.5, 138.4, 148.3, 152.3, 161.5, 163.9, 164.5. HRMS: *m/z* calcd for $C_{22}H_{21}N_3O_3S$ 408.1383 $(M+H)^+$, found 408.1380.

*6.2.27. 2-(2-Hydroxybenzamido)-N-phenyl-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**6**).* The title compound was prepared starting from **42** through Method D and purified by crystallization by EtOH, in 30% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.80 (m, 4H, cycloheptane CH_2), 6.80-6.90 (m, 2H, aromatic CH), 7.05 (t, *J* = 7.3 Hz, 1H, aromatic CH), 7.20-7.40 (m, 3H, aromatic CH), 7.60 (d, *J* = 7.7 Hz, 2H, aromatic CH), 7.95 (d, *J* = 7.1 Hz, 1H, aromatic CH), 10.00 (s, 1H, NH), 11.60 (s, 1H, OH), 11.80 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.6, 28.1, 28.8, 28.9, 32.2, 117.1, 117.5, 120.1, 120.5, 122.6, 124.1, 129.1, 131.1, 131.4, 134.2, 135.2, 136.4, 139.2, 156.7, 161.9, 164.2. HRMS: *m/z* calcd for $C_{23}H_{22}N_2O_3S$ 407.1430 $(M+H)^+$, found 407.1427.

*6.2.28. N-(4-Chlorophenyl)-2-(2-hydroxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**7**).* The title compound was prepared starting from **19** through Method D and purified by crystallization by EtOH, in 55% yield as light-yellow solid. ^{1}H

NMR (200 MHz, DMSO-d_6): δ 1.40-1.60 (m, 4H, cycloheptane CH_2), 1.60-1.80 (m, 2H, cycloheptane CH_2), 2.60-2.70 (m, 4H, cycloheptane CH_2), 6.80-6.90 (m, 2H, aromatic CH), 7.30-7.40 (m, 3H, aromatic CH), 7.65 (d, *J* = 8.8 Hz, 2H, aromatic CH), 7.90 (d, *J* = 8.0 Hz, 1H, aromatic CH), 10.20 (s, 1H, NH), 11.80 and 11.90 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ27.6, 28.1, 28.8, 32.1, 117.2, 117.5, 120.1, 121.9, 127.7, 129.0, 131.2, 131.3, 134.2, 135.2, 136.5, 138.2, 156.6, 162.0, 164.2. HRMS: *m/z* calcd for $C_{23}H_{21}ClN_2O_3S$ 441.1040 $(M+H)^+$, found 441.1034.

*6.2.29. N-(4-Fluorophenyl)-2-(2-hydroxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**8**).* The title compound was prepared starting from **43** through Method D and purified by flash chromatography eluting with cyclohexane:EtOAc 8:2, in 16% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.65 (m, 4H, cycloheptane CH_2), 1.70-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.80 (m, 4H, cycloheptane CH_2), 6.85-6.95 (m, 2H, aromatic CH), 7.15 (t, *J* = 8.8 Hz, 2H, aromatic CH), 7.30 (t, *J* = 7.7 Hz, 1H, aromatic CH), 7.60-7.70 (m, 2H, aromatic CH), 7.90 (d, *J* = 8.0 Hz, 1H, aromatic CH), 10.20 (s, 1H, NH), 11.70 and 11.90 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.6, 28.1, 28.8, 32.2, 115.7 (d, J_{C-F} = 22 Hz), 117.1, 117.5, 120.1, 122.2 (d, J_{C-F} = 8 Hz), 122.4, 131.1, 131.4, 134.2, 135.2, 135.6, 136.4, 157.1(d, J_{C-F} = 87 Hz), 159.9, 161.9, 164.1. HRMS: *m/z* calcd for $C_{23}H_{21}FN_2O_3S$ 425.1336 $(M+H)^+$, found 425.1335.

*6.2.30. 2-(2-Hydroxybenzamido)-N-(thiazol-2-yl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**9**).* The title compound was prepared starting from **44** through Method D and purified by flash chromatography eluting with CH_2Cl_2:Acetone 98:2, in 35% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.85 (m, 4H, cycloheptane CH_2), 6.80-7.05 (m, 2H, aromatic CH), 7.20-7.30 (m, 1H, aromatic CH), 7.35 (dt, *J* = 1.3 and 8.0 Hz, 1H, aromatic CH), 7.50 (d, *J* = 2.9 Hz, 1H, aromatic CH), 7.90 (d, *J* = 7.0 Hz, 1H, aromatic CH), 11.50 (bs, 1H, NH), 11.75 (bs, 1H, OH),

12.25 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 26.7, 27.6, 28.1, 28.6, 28.7, 32.2, 117.2, 117.5, 120.1, 120.4, 131.2, 131.3, 134.3, 135.6, 137.9, 156.8, 162.2. HRMS: *m/z* calcd for $C_{20}H_{19}N_3O_3S_2$ 414.0868 $(M+H)^+$, found 414.0941.

*6.2.31. N-Cyclohexyl-2-(2-hydroxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**14**).* The title compound was prepared starting from **45** through Method D and purified by flash chromatography eluting with cyclohexane:EtOAc 7:3, in 16% yield as light-brown solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.20-1.30 and 1.45-1.60 (m, each 5H, cyclohexyl CH_2), 1.60-1.85 (m, 6H, cycloheptane CH_2), 2.60-2.70 (m, 4H, cycloheptane CH_2), 3.50-3.60 (m, 1H, cyclohexyl CH), 6.80-6.90 (m, 2H, aromatic CH and NH), 7.35 (t, *J* = 7.6 Hz, 1H, aromatic CH), 7.80 (d, *J* = 7.6 Hz, 1H, aromatic CH), 7.90 (d, *J* = 7.6 Hz, 1H, aromatic CH), 11.75 and 11.90 (s, 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 25.1, 25.6, 26.7, 17.6, 28.2, 28.7, 32.2, 32.6, 48.6, 117.1, 117.7, 120.0, 122.9, 130.7, 131.3, 134.1, 135.1, 135.5, 156.7, 161.7, 164.5. HRMS: *m/z* calcd for $C_{23}H_{28}N_2O_3S$ 413.1900 $(M+H)^+$, found 413.1899.

*6.2.32. 2-(4-Chlorobenzamido)-N-(2-hydroxyphenyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**22**).* The title compound was prepared starting from **18** through Method D and purified by crystallization by EtOH, in 64% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.45-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.70 and 2.70-2.80 (m, each 2H, cycloheptane CH_2), 6.70-7.00 (m, 3H, aromatic CH), 7.60 (d, *J* = 7.6 Hz, 2H, aromatic CH), 7.80-7.90 (m, 3H, aromatic CH), 9.00 (s, 1H, NH), 9.95 and 11.30 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.4, 28.1, 28.4, 28.7, 32.1, 115.8, 119.4, 123.1, 124.0, 125.6, 126.3, 129.3, 129.8, 131.9, 132.8, 135.9, 136.5, 137.5, 148.6, 163.1, 163.7. HRMS: *m/z* calcd for $C_{23}H_{21}ClN_2O_3S$ 441.1040 $(M+H)^+$, found 441.1037.

*6.2.33. N-(3-((2-Hydroxyphenyl)carbamoyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)picolinamide (**23**).* The title compound was prepared starting from **41** through Method D and purified by flash chromatography eluting with $CHCl_3$, in 10% yield as light-yellow solid. ^{1}H NMR

(400 MHz, DMSO-d_6): δ 1.55-1.65 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.70-2.80 and 2.85-3.95 (m, each 2H, cycloheptane CH_2), 6.80 (t, *J* = 7.6 Hz, 1H, aromatic CH), 6.90 (d, *J* = 7.8 Hz, 1H, aromatic CH), 7.00 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.60 (m, 1H, pyridine CH), 7.90 (d, *J* = 7.2Hz, 1H, pyridine CH), 8.05 (t, *J* = 7.5 Hz, 1H, aromatic CH), 8.15 (d, *J* = 7.7 Hz, 1H, pyridine CH), 8.75 (d, *J* = 4.6 Hz, 1H, pyridine CH), 8.80 (s, 1H, NH), 9.95 (s, 1H, OH), 12.15 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.3, 27.9, 28.6, 28.7, 31.9, 115.8, 119.5, 121.4, 122.8, 122.9, 125.6, 126.2, 127.9, 132.0, 134.8, 138.3, 138.8, 148.3, 148.5, 149.5, 161.1, 163.9. HRMS: *m/z* calcd for $C_{22}H_{21}N_3O_3S$ 408.1383 $(M+H)^+$, found 408.1381.

*6.2.34. Ethyl 2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxylate (**47**).* The title compound was prepared starting from ethyl 2-amino-5,6,7,8-tetrahydro-4H-cyclohepta[*b*]thiophene-3-carboxylate **46** [30] by Method C (2h) and purified by treatment with cyclohexane, in 100% yield as light-yellow solid. ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.35 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 1.50-1.65 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.65-2.75 and 2.95-3.05 (m, each 2H, cycloheptane CH_2), 4.10 (s, 3H, OCH_3), 4.35 (q, *J* = 7.1 Hz, 2H, CH_2CH_3), 6.90-7.20 (m, 2H, aromatic CH), 7.40-7.50 (m, 1H, aromatic CH), 8.25 (dd, *J* = 1.8 and 7.8 Hz, 1H, aromatic CH), 13.00 (s, 1H, NH).

*6.2.35. 2-(2-Methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxylic acid (**48**).* A suspension of **47** (3.75 mmol, 1.40 g) and LiOH monohydrate (14.99 mmol, 0.63 g) in a mixture H_2O:THF 1:1 (60 ml) was maintained at 50 °C overnight. After cooling, the reaction mixture was acidified (pH = 6) with 2N HCl yielding a precipitate, which was filtered, washed with water, and purified by treatment with Et_2O to give **47** as light-yellow solid (0.6 g, 46%). ^{1}H-NMR (200 MHz, DMSO-d_6): δ 1.45-1.60 (m, 4H, cycloheptane CH_2), 1.70-1.80 (m, 2H, cycloheptane CH_2), 2.60-2.70 and 2.95-3.05 (m, each 2H, cycloheptane CH_2), 4.00 (s, 3H, OCH_3), 7.10 (t, *J* = 7.7 Hz, 1H, aromatic CH), 7.20 (d, *J* = 8.3 Hz, 1H, aromatic CH), 7.55 (dt, *J* = 1.7 and 8.5 Hz, 1H, aromatic CH), 8.00 (dd, *J* = 1.7 and 7.7 Hz, 1H, aromatic CH), 13.00 (s, 1H, NH).

*6.2.36. General procedure for C-3 amidation for derivatives **49-54** (**Method E**).* To a solution of **48** (1.0 equiv) in dry CH_2Cl_2 was added the appropriate alkyl amine (3 equiv) followed by BOP (1.5 equiv) and finally collidine (4.0 equiv). The mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc and successively washed with: water (twice), 0.5 M HCl, saturated $NaHCO_3$ solution, water, and finally brine. The organic layer was dried over Na_2SO_4, filtered and then evaporated to dryness providing a residue that was purified as described below.

*6.2.37. 2-(2-Methoxybenzamido)-N-(pyridin-2-ylmethyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**49**).* The title compound was prepared through Method E and purified by flash chromatography eluting with cyclohexane:EtOAc 5:5, in 15% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.70 and 2.75-2.90 (m, each 2H, cycloheptane CH_2), 3.75 (s, 3H, CH_3), 4.60 (d, *J* = 5.7 Hz, 2H, CH_2), 7.00-7.25 (m, 3H, aromatic CH), 7.35 (d, *J* = 7.8 Hz, 1H, aromatic CH), 7.55 (t, *J* = 7.1 Hz, 1H, aromatic CH), 7.75 (t, *J* = 7.6 Hz, 1H, aromatic CH), 8.05 (dd, *J* = 1.5 and 7.7 Hz, 1H, aromatic CH), 8.35 (d, *J* = 3.9 Hz, 1H, NH), 8.45-8.55 (m, 1H, aromatic CH), 12.00 (s, 1H, NH).

*6.2.38. N-Benzyl-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**50**).* The title compound was prepared through Method E and purified by treatment with Et_2O, in 93% yield as white solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.45-1.65 (m, 4H, cycloheptane CH_2), 1.70-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.75 (m, 4H, cycloheptane CH_2), 3.80 (s, 3H, CH_3), 4.45 (d, *J* = 5.7 Hz, 2H, CH_2), 7.00-7.30 (m, 7H, aromatic CH and NH), 7.45-7.60 (m, 1H, aromatic CH), 8.00 (dd, *J* = 1.7 and 7.8 Hz, 1H, aromatic CH), 8.40 (t, *J* = 5.7 Hz, 1H, aromatic CH), 12.00 (s, 1H, NH).

*6.2.39. N-Isopropyl-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**51**).* The title compound was prepared through Method E and purified by treatment with Et_2O, in 54% yield as light-yellow solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.10 and 1.15 (s, each 3H, CH_3), 1.45-1.65 (m, 4H, cycloheptane CH_2), 1.70-1.80 (m, 2H, cycloheptane CH_2), 2.60-

2.75 (m, 4H, cycloheptane CH_2), 4.00 (s, 3H, OCH_3), 4.00-4.10 (m, 1H, CH), 7.10 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.20 (d, *J* = 8.3 Hz, 1H, aromatic CH), 7.45-7.60 (m, 1H, aromatic CH), 7.75 (d, *J* = 7.9 Hz, 1H, NH), 8.00 (dd, *J* = 1.7 and 7.8 Hz, 1H, aromatic CH), 11.90 (s, 1H, NH).

*6.2.40. N-(tert-Butyl)-2-(2-methoxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**52**).* Compound **52** was prepared by Method E and purified by treatment with Et_2O, in 40% yield as light-yellow solid. ^{1}H NMR (200 MHz, DMSO-d_6): δ 1.30 (s, 9H, CH_3), 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.70-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.75 (m, 4H, cycloheptane CH_2), 4.05 (s, 3H, OCH_3), 7.10 (t, *J* = 7.5 Hz, 1H, aromatic CH), 7.20 (d, *J* = 8.3 Hz, 1H, aromatic CH), 7.35 (s, 1H, NH), 7.50-7.60 (m, 1H, aromatic CH), 8.00 (dd, *J* = 1.6 and 7.7 Hz, 1H, aromatic CH), 11.90 (s, 1H, NH).

*6.2.41. 2-Methoxy-N-(3-(4-methylpiperazine-1-carbonyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)benzamide (**53**).* The title compound was prepared through Method E purified by flash chromatography eluting with $CHCl_3$:MeOH 97:3, in 26% yield as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.70-1.90 (m, 2H, cycloheptane CH_2), 2.25 (s, 3H, CH_3), 2.30-2.40 (m, 2H, cycloheptane CH_2), 2.45-2.65 (m, 4H, piperazine and cycloheptane CH_2), 2.75-2.85 and 3.25-3.35 (m, each 2H, piperazine CH_2), 3.55-3.75 (m, 2H, piperazine CH_2), 4.00 (s, 3H, OCH_3), 6.90 (d, J = 8.3 Hz, 1H, aromatic CH), 7.10 (t, J = 7.4 Hz, 1H, aromatic CH), 7.45 (t, J = 7.3 Hz, 1H, aromatic CH), 8.25 (d, J = 7.4 Hz, 1H, aromatic CH).

*6.2.42. 2-Methoxy-N-(3-(4-(pyridin-2-yl)piperazine-1-carbonyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)benzamide (**54**).* The title compound was prepared through Method E and purified by flash chromatography eluting with cyclohexane:EtOAc 4:6, in 67% yield as brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.50-2.70 and 2.80-2.90 (m, each 2H, cycloheptane CH_2), 3.25-3.60 (m, 2H, piperazine CH_2), 3.65-3.80 (m, 2H, piperazine CH_2), 3.90-4.05 (m, 4H, piperazine CH_2), 4.10 (s, 3H, OCH_3), 6.60-6.70 (m, 2H, pyridine CH), 6.90 (d, *J* = 8.1 Hz, 1H, aromatic CH), 7.10 (t, *J* = 8.0

Hz, 1H, aromatic CH), 7.40-7.50 (m, 2H, aromatic and pyridine CH), 8.10-8.20 (m, 1H, pyridine CH), 8.25 (dd, *J* = 1.8 and 7.8 Hz, 1H, aromatic CH), 11.40 (s, 1H, NH).

*6.2.43. 2-(2-Hydroxybenzamido)-N-(pyridin-2-ylmethyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**10**).* The title compound was prepared starting from **49** through Method D and purified by crystallization by EtOH, in 53% yield as light-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6,): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.60-2.75 and 2.75-2.90 (m, each 2H, cycloheptane CH_2), 4.65 (d, *J* = 5.3 Hz, 2H, CH_2), 6.85-7.00 (m, 2H, aromatic CH), 7.35 (t, *J* = 7.4 Hz, 1H, aromatic CH), 7.45-7.55 (m, 2H, pyridine CH), 7.90 (d, *J* = 7.7 Hz, 1H, aromatic CH), 8.05 (t, *J* = 7.0 Hz, 1H, pyridine CH), 8.55-8.65 (m, 2H, pyridine CH and NH), 11.75 (s, 1H, OH), 12.10 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.6, 28.1, 28.5, 28.6, 32.0, 43.4, 117.0, 117.5, 120.2, 121.3, 122.9, 123.9, 131.3, 131.4, 134.3, 135.1, 136.6, 141.0, 146.3, 156.4, 156.8, 162.0, 165.8. HRMS: *m/z* calcd for $C_{23}H_{23}N_3O_3S$ 422.1539 $(M+H)^+$, found 422.1539.

*6.2.44. N-Benzyl-2-(2-hydroxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**11**).* The title compound was prepared starting from **50** through Method D and purified by flash chromatography eluting with cyclohexane:EtOAc 8:2, in 9% yield as white solid. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 1.45-1.70 (m, 4H, cycloheptane CH_2), 1.75-2.00 (m, 2H, cycloheptane CH_2), 2.60-2.90 (m, 4H, cycloheptane CH_2), 4.50 (d, *J* = 5.8 Hz, 2H, CH_2), 6.80-7.10 (m, 2H, aromatic CH), 7.20-7.50 (m, 6H, aromatic CH), 8.00 (d, *J* = 7.8 Hz, 1H, aromatic CH), 8.50-8.60 (m, 1H, NH), 11.75 and 12.00 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.6, 28.1, 28.6, 28.7, 32.1, 42.8, 117.0, 117.5, 120.2, 122.2, 127.2, 127.6, 128.7, 131.2, 131.3, 134.2, 135.1, 135.7, 139.6, 156.4, 161.8, 165.4. HRMS: *m/z* calcd for $C_{24}H_{24}N_2O_3S$ 421.1587 $(M+H)^+$, found 421.1586.

*6.2.45. 2-(2-Hydroxybenzamido)-N-isopropyl-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**12**).* The title compound was prepared starting from **51** through Method D and purified by flash chromatography eluting with $CHCl_3$, in 18% yield as white solid. ^{1}H NMR (400

MHz, DMSO-d_6,): δ 1.10 (d, *J* = 6.5 Hz, 6H, CH_3), 1.50-1.60 (m, 4H, cycloheptane CH_2), 1.70-1.80 (m, 2H, cycloheptane CH_2), 2.60-2.75 (m, 4H, cycloheptane CH_2), 3.95-4.05 (m, 1H, CH), 6.90-7.00 (m, 2H, aromatic CH), 7.30 (dt, *J* = 1.6 and 7.2 Hz, 1H, aromatic CH), 7.80 (d, *J* = 7.7 Hz, 1H, NH), 7.90 (dd, *J* = 1.7 and 7.8 Hz, 1H, aromatic CH), 11.75 and 11.0 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 22.6, 27.6, 28.2, 28.6, 28.7, 32.1, 41.4, 117.1, 117.7, 120.0, 123.0, 130.7, 131.3, 134.0, 135.0, 135.2, 156.7, 161.8, 164.6. HRMS: *m/z* calcd for $C_{20}H_{24}N_2O_3S$ 373.1587 $(M+H)^+$, found 373.1587.

*6.2.46. N-(tert-Butyl)-2-(2-hydroxybenzamido)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophene-3-carboxamide (**13**).* The title compound was prepared starting from **52** through Method D and purified by flash chromatography eluting with benzene:$CHCl_3$ 5:5, in 21% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35 (s, 9H, CH_3), 1.50-1.60 (m, 4H, cycloheptane CH_2), 1.70-1.80 (m, 2H, cycloheptane CH_2), 2.60-2.70 (m, 4H, cycloheptane CH_2), 6.90 (t, *J* = 7.4 Hz, 1H, aromatic CH), 7.00 (d, *J* = 8.0 Hz, 1H, aromatic CH), 7.35 (t, *J* = 7.1 Hz, 1H, aromatic CH), 7.55 (s, 1H, NH), 7.90 (d, *J* = 7.1 Hz, 1H, aromatic CH), 11.50 and 11.60 (s, each 1H, OH and NH); ^{13}C NMR (101 MHz, DMSO-d_6): δ 27.5, 28.2, 28.7, 28.8, 28.9, 32.2, 51.2, 117.2, 117.8, 120.1, 124.4, 130.7, 131.4, 134.0, 134.4, 135.2, 156.6, 161.7, 165.1. HRMS: *m/z* calcd for $C_{21}H_{26}N_2O_3S$ 387.1743 $(M+H)^+$, found 387.1743.

*6.2.47. 2-Hydroxy-N-(3-(4-methylpiperazine-1-carbonyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)benzamide (**15**).* The title compound was prepared starting from **53** through Method D and purified by flash chromatography eluting with $CHCl_3$:MeOH 98:2, in 47% yield as light-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50-1.70 (m, 4H, cycloheptane CH_2), 1.75-1.85 (m, 2H, cycloheptane CH_2), 2.25 (s, 3H, CH_3), 2.30-2.60 (m, 6H, cycloheptane CH_2 and piperazine CH_2), 2.60-2.70 (m, 2H, piperazine CH_2), 3.40-3.80 (m, 4H, cycloheptane CH_2 and piperazine CH_2), 6.85-6.95 (m, 2H, aromatic CH), 7.40 (t, *J* = 7.6 Hz, 1H, aromatic CH), 7.60 (d, *J* = 7.8 Hz, 1H, aromatic CH), 10.75 (bs, 1H, NH); ^{13}C NMR (101 MHz, $CDCl_3$): δ 27.6, 27.9, 28.8,

29.3, 32.5, 45.8, 113.9, 118.4, 119.2, 120.7, 126.5, 133.1, 134.2, 134.5, 135.5, 161.1, 166.0, 167.2. HRMS: *m/z* calcd for $C_{22}H_{27}N_3O_3S$ 414.1852 $(M+H)^+$, found 414.1849.

*6.2.48. 2-Hydroxy-N-(3-(4-(pyridin-2-yl)piperazine-1-carbonyl)-5,6,7,8-tetrahydro-4H-cyclohepta[b]thiophen-2-yl)benzamide (**16**).* The title compound was prepared starting **54** through Method D and purified by crystallization by EtOH, in 39% yield as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50-1.70 (m, 6H, cycloheptane CH_2), 1.80-1.90 (m, 2H, piperazine CH_2), 2.60-2.70 and 2.75-2.85 (m, each 2H, cycloheptane CH_2), 3.30-3.55 (m, 2H, piperazine CH), 3.55-3.75 (m, 4H, piperazine CH_2), 6.60-6.70 (m, 2H, aromatic CH), 6.85 (t, *J* = 7.7 Hz, 1H, pyridine CH), 6.95 (d, *J* = 7.9 Hz, 1H, pyridine CH), 7.40 (dt, *J* = 1.1 and 8.3 Hz, 1H, aromatic CH), 7.45-7.55 (m, 2H, pyridine CH), 8.20 (dd, *J* = 1.8 and 4.9 Hz, 1H, aromatic CH), 10.25 and 11.75 (s, each 1H, NH and OH); ^{13}C NMR (101 MHz, $CDCl_3$,): δ 27.7, 28.0, 29.0, 29.3, 32.6, 107.3, 113.5, 114.1, 118.6, 119.2, 120.6, 126.0, 133.3, 134.1, 134.8, 136.3, 137.7148.0, 158.9, 161.6, 166.1, 167.5. HRMS: *m/z* calcd for $C_{26}H_{28}N_4O_3S$ 477.1961 $(M+H)^+$, found 477.1957.

6.3. Biology

6.3.1. Compounds and peptide. RBV (1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was purchased from Roche. Each test compound was dissolved in 100% DMSO. 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 corresponds to the first 15 amino acids of PB1 protein fused to a short sequence of HIV Tat protein (amino acids 47−59), which allows the delivery into the cell [37].

6.3.2. Cells and virus. Mardin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's 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 virus strain A/PR/8/34 (H1N1, Cambridge lineage) was kindly provided by P. Digard (Roslin Institute, University of Edinburgh, United Kingdom).

6.3.3. PA-PB1 interaction enzyme-linked immunosorbent assay (ELISA). The PA−PB1 interaction was detected by a procedure previously described [15]. Briefly, 96-well microtiter plates (Nuova Aptca) were coated with 400 ng of 6His-$PA_{(239–716)}$ for 3 h at 37 °C and then blocked with 2% BSA (Sigma) in PBS for 1 h at 37 °C. The 6His-$PA_{(239–716)}$ protein was expressed in *E. coli* strain BL21(DE3)pLysS and purified as already described [15]. After washing, 200 ng of GST-$PB1_{(1–25)}$, or of GST alone as a control, in the absence or the presence of test compounds at various concentrations, were added and incubated O/N at room temperature. *Escherichia coli*-expressed, purified GST and GST-$PB1_{(1–25)}$ proteins were obtained as previously described [15, 38]. After washing, the interaction between 6His-$PA_{(239–716)}$ and GST-$PB1_{(1–25)}$ was detected with a horseradish peroxidase-coupled anti-GST monoclonal antibody (GenScript) diluted 1:4,000 in PBS supplemented with 2% FBS. Following washes, the substrate 3,3′,5,5′tetramethylbenzidine (TMB, KPL) was added and absorbance was measured at 450 nm by an ELISA plate reader (Tecan Sunrise™). Values obtained from the samples treated with only DMSO were used to set as 100% of PA−PB1 interaction.

6.3.4. Cytotoxicity assay. Cytotoxicity of compounds was tested in MDCK cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, as previously reported [15, 39]. Briefly, MDCK cells (seeded at density of 2 x 10^4 per well) were grown in 96-well plates for 24 h and then treated with serial dilutions of test compounds, or DMSO as a control, in DMEM supplemented with 10% FBS. After incubation at 37 °C for 48 h, 5 mg/mL of MTT (Sigma) in PBS was added into each well and incubated at 37 °C for further 4 h. Successively, a solubilization solution was added to lyse the cells and incubated O/N at 37 °C. Finally, optical density was read at the wavelength of 620 nm on a microtiter plate reader.

6.3.5. Plaque reduction assay (PRA). The antiviral activity of test compounds against influenza A virus was tested by PRA as previously described [15]. MDCK cells were seeded at 5 x 10^5 cells/well into 12-well plates, and incubated at 37°C for 24 h. The following day, the culture medium was removed and the monolayers were first washed with serum-free DMEM and then

infected with the flu A/PR/8/34 strain at 40 PFU/well in DMEM supplemented with 1 μg/mL of TPCK-treated trypsin (Worthington Biochemical Corporation) and 0.14% BSA and incubated for 1 h at 37 °C. The influenza virus infection was performed in the presence of different concentrations of test compounds or solvent (DMSO) as a control. After virus adsorption, DMEM containing 1 μg/mL of TPCK-treated trypsin, 0.14% BSA, 1.2% Avicel, and DMSO or test compounds was added to the cells. At 48 h post-infection, cells were fixed with 4% formaldehyde and stained with 0.1% toluidine blue. Viral plaques were counted, and the mean plaque number in the DMSO-treated control was set at 100%.

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Appendix A. Supplementary data

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Figure legends

Fig. 1. Structures of cHTC derivatives **1** and **2**.

Fig. 2. FLAP binding poses for compounds **1 (A)**, **3 (B)**, **4 (C)**, and **5 (D)**. Some of the PA key residues located in the cavity are highlighted in stick-mode.

Scheme Footnotes

Scheme 1. Synthesis of compounds **3-9**, **14**, **18**, **19**, **22**, and **23**. *Reagents and conditions:* (i) amines, toluene, reflux; (ii) cycloheptanone, ammonium acetate, glacial acetic acid, benzene, reflux; (iii) sulfur, *N*,*N*-diethylamine, EtOH, 40−50 °C; (iv) acyl chlorides, pyridine, rt; (v) BBr_3, dry CH_2Cl_2, rt.

Scheme 2. Synthesis of compounds **10-13**, **15**, and **16**. *Reagents and conditions:* (i) 2-methoxybenzoyl chloride, pyridine, rt; (ii) LiOH, H_2O/THF, 50 °C; (iii) amines, BOP, collidine, dry CH_2Cl_2, rt; (iv) BBr_3, dry CH_2Cl_2, rt.

Abbreviations:

Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate, (BOP); cycloheptathiophene-3-carboxamide, (cHTC); fingerprints for ligands and proteins, (FLAP); Influenza virus (flu), mardin-darby canine kidney, (MDCK); plaque reduction assays, (PRA); polymerase acidic protein, (PA); polymerase basic protein 1, (PB1); polymerase basic protein 2, (PB2); protein-protein interaction, (PPI); ribavirin, (RBV); RNA-dependent RNA polymerase, (RdRp); structure-activity relationship (SAR).

Highlights

- Cycloheptathiophene-3-carboxamide scaffold is a suitable to achieve PA-PB1 interaction inhibition;
- Compounds **9** and **21** showed potent anti-influenza activity;
- The *o*-hydroxyphenyl moiety induces a significant change of the binding mode of the compounds

Graphical abstract