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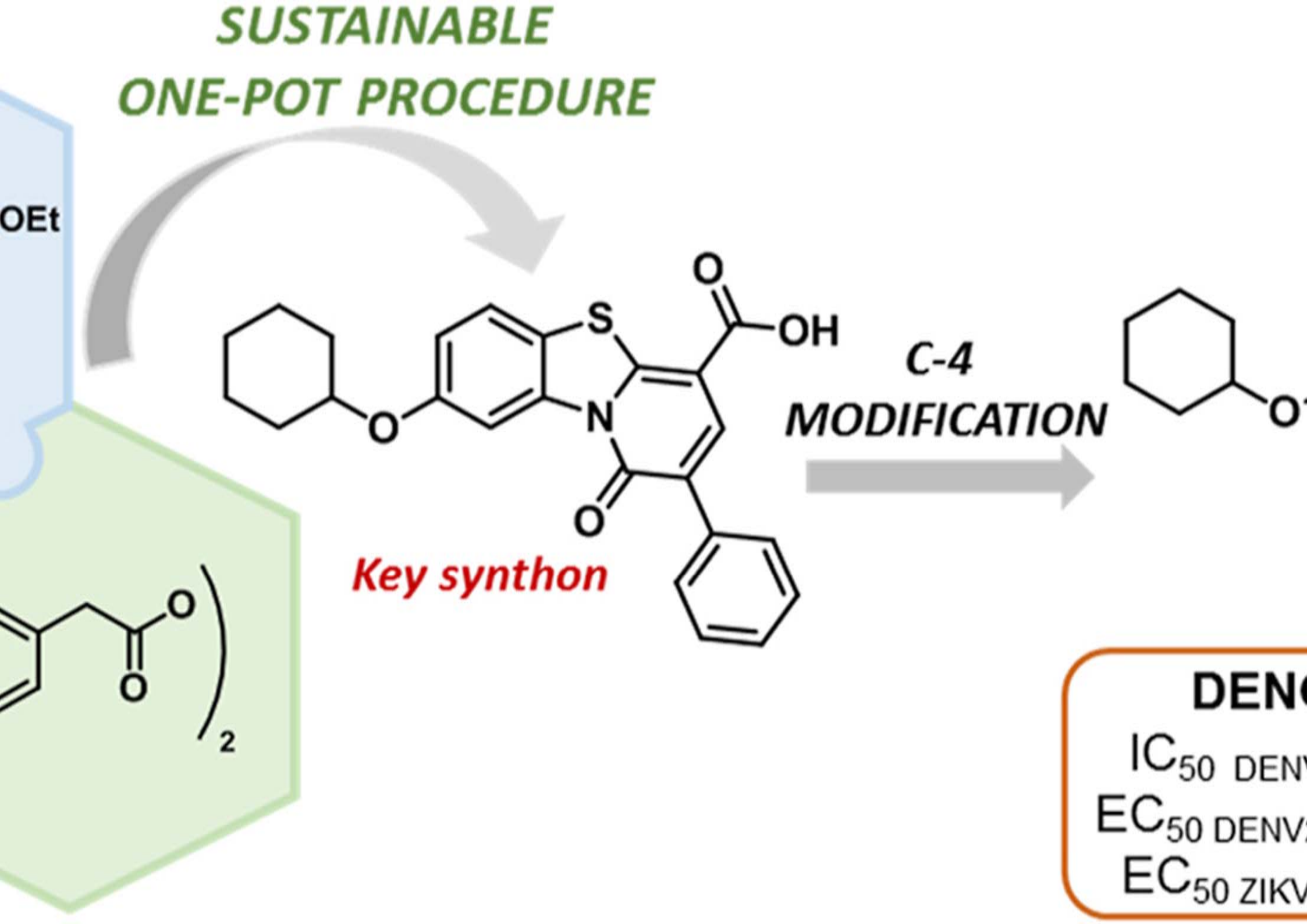
SUSTAINABLE
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MODIFICATION
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IC50 DENV
EC50 DENV
EC50 ZIKV

Sustainable, three-component, one-pot procedure to obtain active anti-flavivirus agents

Tommaso Felicetti,[1] Maria Sole Burali,[1] Chin Piaw Gwee,[2,3] Kitti Wing Ki Chan,[2] Sylvie Alonso,[3,4] Serena Massari,[1] Stefano Sabatini,[1] Oriana Tabarrini,[1] Maria Letizia Barreca,[1] Violetta Cecchetti,[1] Subhash G. Vasudevan,[2,3,5]* and Giuseppe Manfroni[1,]*

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ABSTRACT

The mosquito-borne viruses belonging to the genus Flavivirus such as Dengue virus (DENV) and Zika virus (ZIKV) cause human infections ranging from mild flu-like symptoms to hemorrhagic fevers, hepatitis, and neuropathies. To date, there are vaccines only for few flaviviruses while no effective treatments are available.

Pyridobenzothiazole (PBTZ) derivatives are a class of compounds endowed with a promising broad-spectrum anti-flavivirus activity and most of them have been reported as potent inhibitors of the flaviviral NS5 polymerase. However, synthesis of PBTZ analogues entails a high number of purification steps, the use of hazardous reagents and environmentally unsustainable generation of waste.

Considering the promising antiviral activity of PBTZ analogues which require further exploration, in this work, we report the development of a new and sustainable three-component reaction (3CR) that can be combined with a basic hydrolysis in a one-pot procedure to obtain the PBTZ scaffold, thus reducing the number of synthetic steps, improving yields and saving time. 3CR was significantly explored in order to demonstrate its wide scope by using different starting materials. In addition, taking advantage of these procedures, we next designed and synthesized a new set of PBTZ analogues that were tested as anti-DENV-2 and anti-ZIKV agents. Compound **22** inhibited DENV-2 NS5 polymerase with an IC_{50} of 10.4 μM and represented the best anti-flavivirus compound of the new series by inhibiting DENV-2- and ZIKV-infected cells with EC_{50} values of 1.2 and 5.0 μM, respectively, that translates into attractive selectivity indexes (SI - 83 and 20, respectively). These results strongly reaffirm PBTZ derivatives as promising anti-flavivirus agents that now can be synthesized through a convenient and sustainable 3CR in order to obtain more potent compounds for further pre-clinical development studies.

Keywords: three-component reaction (3CR), antiviral agents, NS5 polymerase, one-pot procedure, Dengue inhibitors, Zika inhibitors.

1. Introduction

The genus Flavivirus, family *Flaviviridae* consists of over 70 positive-sense single-stranded RNA viruses that are transmitted by insect (arthropod) vectors, mosquitos (of the genera *Aedes* and *Culex*) and ticks, and these viruses are therefore also classified as arboviruses (arthropod-borne) [1,2]. The most studied flaviviruses are Zika virus (ZIKV), Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV) [2]. About 400 million cases of DENV infections occur annually, 100 million of which develop symptoms ranging from mild flu-like illness to severe hemorrhagic fever and shock syndrome [3]. Moreover, ZIKV caused a widespread epidemic leading to congenital Zika syndrome (CZS) in the Americas that raised alarm bells as it was occurring at the same time as Brazil was preparing to host the 2016 Olympic games [4]. With about 500,000 suspected cases only between 2015 and 2016, ZIKV represents a serious threat for the public health, especially due to its association with microcephaly in newborns [4]. For these reasons, in 2019 WHO included DENV and ZIKV in the list of "10 Threats to Global Health" [5]. There are no antiviral drugs available for these infections and the live-attenuated tetravalent vaccine Dengvaxia (CYD-TDV, Sanofi-Pasteur), approved in 20 endemic countries, shows limited efficacy with some safety concerns [1]. Therefore, new anti-flavivirus drugs are required and compounds with broad-spectrum anti-flavivirus activity would be particularly desirable in the perspective to treat cases of flavivirus coinfections that can impact close to two-thirds of the world's population.

The flavivirus RNA genome encodes for structural and non-structural proteins having different roles during the viral life cycle and among them, the RNA-dependent RNA polymerase (RdRp) is particularly appealing for the development of anti-flavivirus compounds [1,6–9]. The flaviviral RdRp is located at the C-terminal portion of the NS5 protein and shows a typical right-hand orientation with three subdomains: fingers, palm and thumb [10,11]. The flaviviral RdRp is essential for the viral replication cycle, is highly conserved and lacks a eukaryotic homologue, therefore it represents an attractive target for the development of broad-spectrum anti-flavivirus agents [1,12].

We previously identified and optimized a class of 1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-ones (PBTZs) which displayed a promising broad-spectrum anti-flavivirus activity by acting within the framework of the

replication complex [13–16]. This peculiar mode of action (MoA) is characterized by the dual inhibition of NS5 RdRp and NS3-NS5 protein-protein interaction together with the production of multiple variants in the 3'-untranslated region (UTR) of DENV genome. Indeed, similarly to NS3-NS5 interaction-defective mutants that showed an impairment in the infectious virus production [17], PBTZ derivatives were also able to significantly reduce the infectivity of released virions in the second round of infection [14]. Interestingly, by RNA-Electrophoretic Mobility Shift Assay (REMSA), we also observed the appearance of unbound RNA following the addition of a PBTZ analogue to NS5/3′ UTR RNA mixture, thus suggesting that PBTZs may interfere with the NS5-RNA interaction [15]. Our studies demonstrated that the PBTZ class is very promising and further optimization is desirable to obtain molecules suitable for pre-clinical evaluation. However, the development of new PBTZ analogues faces several challenging synthetic drawbacks related to time-consuming procedures, use of hazardous reagents and room temperature instability of some intermediates.

The synthesis of the PBTZ nucleus was reported for the first time by Kato and colleagues in 1979 and its preparation entailed a two-step procedure [18]: *i*) synthesis of intermediate benzothiazole acrylate (**2**) via condensation of the in-situ prepared Vilsmeier reagent and benzothiazole acetate (**1**) in 78% yield and *ii*) cyclization via reaction of acrylate (**2**) with an anhydride, such as acetic anhydride, to give PTBZ derivative **3** in 62% yield (Scheme 1a). Criticisms related to this procedure entail waste production and hazardous reagent use such as the in-situ preparation of the Vilsmeier reagent from $POCl_3$ and DMF in an excess ratio (2:1) with respect to the benzothiazole acetate (**1**), in the first step, and a large excess of the anhydride (20:1) with respect to the benzothiazole acrylate (**2**), in the second step.

In order to synthesize anti-HCV PTBZs [19], we applied the synthetic route reported by Kato and colleagues, and, starting from cyclohexyloxy benzothiazole acetate (**4**), ethyl 1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazole ester analogue (**6**) was obtained in 68% overall yield (Scheme 1b). By applying this procedure, we sought to overcome the above-mentioned issues, by reducing, in the second step, the amount of phenylacetic anhydride from 20 to 2 equiv., obtaining compound **6** in good yield (83% yield).

Nevertheless, the synthetic procedure still suffered from other issues, in particular during the first reaction (indicated in Scheme 1b as "rate-limiting step"): i) the aqueous work-up required the use of a large amount

of halogenated solvent ($CHCl_3$) to extract the benzothiazole acrylate analogue **5** from water; ii) the reaction always yielded a side product, never characterized before and resulting in a fluorescent spot under UV light on thin layer chromatography (TLC), which needed a chromatography purification to isolate the pure intermediate (**5**); and iii) compound **5** was poorly stable at room temperature and difficult to be stored over time.

Considering the great societal expectation that more environmentally sustainable chemical processes should be developed, green chemistry has become a mainstream research topic and its 12 principles pave the way towards a more sustainable chemistry [20]. In this regard, multicomponent reactions (MCRs) are reactions in which three or more starting materials react to form the desired product, thus being characterized by atom economy, limited numbers of synthetic steps, simplified-product isolation procedures, time saving and sustainability [21]. Therefore, the identification of new MCRs, to synthesize more complex scaffolds and extend the chemical space of the modern organic chemistry in respect of the environment, represents a relevant and challenging topic.

In this study, we describe a more convenient procedure for the synthesis of PBTZ core. In particular, we merged more synthetic steps into one, performing a multicomponent reaction (MCR) (Scheme 1d) aimed at reducing the reaction time, limiting the use of reagents and matching the principles of the green chemistry [20,22]. In addition, we performed an optimized synthetic procedure in which the MCR could be coupled with a basic hydrolysis. Through this one-step procedure, acid PBTZ scaffold was easily obtained and functionalized with different amino acids affording derivatives able to inhibit the DENV-2 NS5 RdRp and endowed with a potent anti-flavivirus activity.

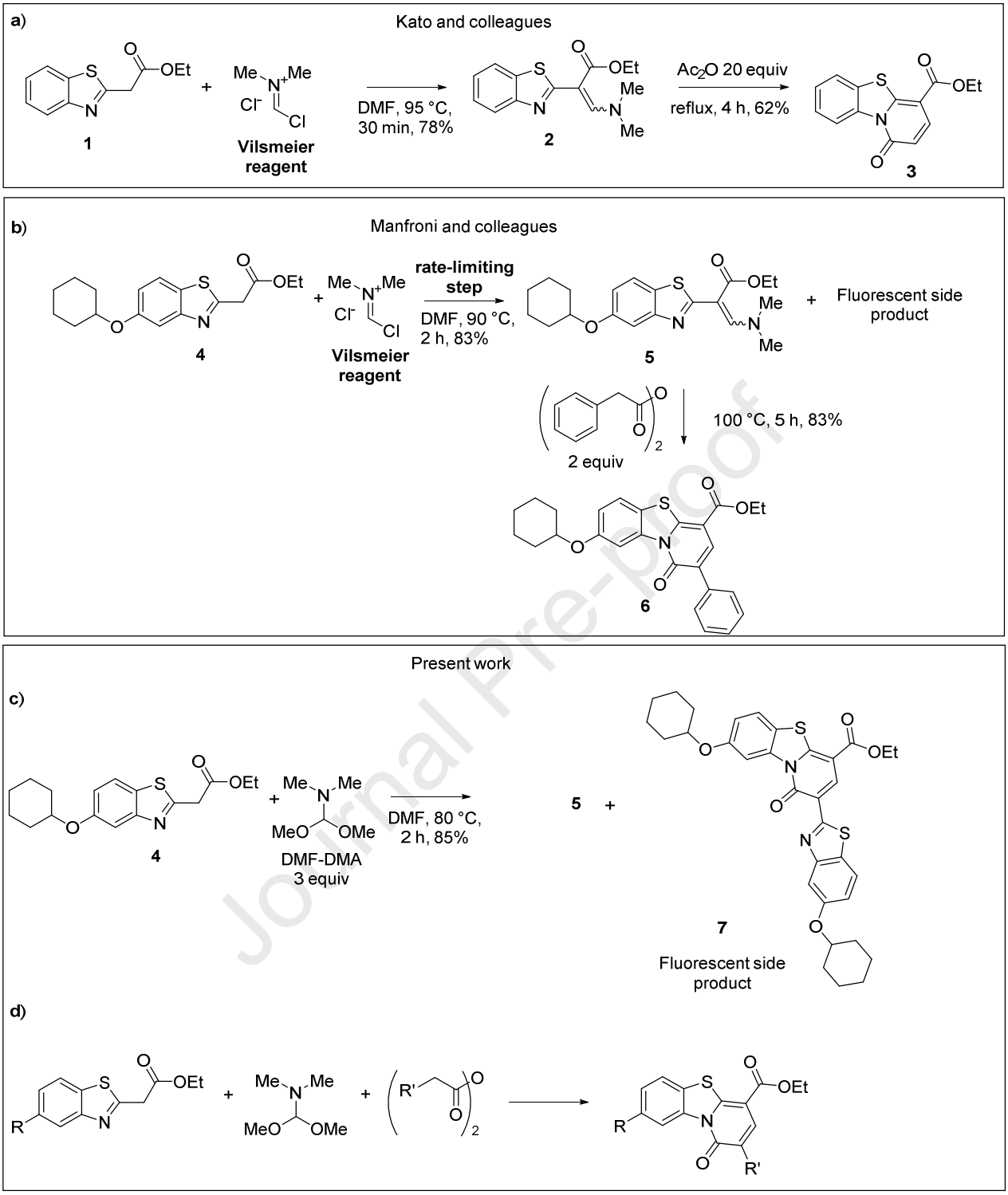
2. Results and discussion

2.1. Planning and optimization of the MCR to achieve the PBTZ key synthon

The replacement of the Vilsmeier reagent with the *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA) in the reaction with benzothiazole acetate **4** afforded acrylate intermediate (**5**) (Scheme 1c) preserving good yields (85%) and reducing the formation of the fluorescent by-product that was easily removed by treating the reaction crude with EtOAc. The characterization of the fluorescent product, never characterized until

now, ethyl 1-oxo-1*H*-pyrido[2,1-*b*][1,3]benzothiazole-4-carboxylate (**7** - Scheme 1c), suggested us that PBTZ core could be formed in the same reaction conditions as acrylate formation. Thus, we attempted to optimize the procedure to obtain PTBZ analogues via a three-component reaction (3CR) between a benzothiazole acetate, the DMF-DMA and an anhydride (Scheme 1d).

Scheme 1.



As initial survey of the 3CR, we used as a template the unsubstituted benzothiazole acetate (**1**). Thus, ethyl 2-(benzo[*d*]thiazol-2-yl)acetate (**1**), DMF-DMA and phenylacetic anhydride, prepared as previously reported [14], were mixed in a flask and dipped immediately into a pre-heated oil bath at 110 °C (Table 1, entry 1). After 6h, although in low yield (8%), the reaction furnished compound **8**. No traces of any

fluorescent spot attributable to a potential by-product derived from the reaction of benzothiazole acetate (**1**) and the acrylate intermediate were observed. In addition, since only EtOH was used to crystallize compound **8**, the use of solvents such as EtOAc and Et_2O and the need of a chromatography purification were avoided in line with a green approach.

Subsequently, in order to improve the reaction yield, we performed the 3CR by using different reaction conditions (Table 1, entries 2-12).

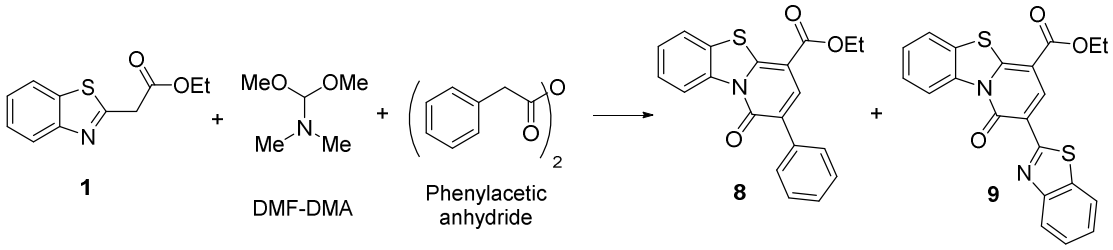
We initially evaluated the effect of different ratio between the reactants (entries 2 and 3). The reaction performed by using 2 equiv. of both DMF-DMA and phenylacetic anhydride (entry 2) was more advantageous (35% yield). On the other hand, the reaction performed with 3 equiv. of DMF-DMA (entry 3) led to the formation of **8** less efficiently (12% yield) and of traces of the side fluorescent product (compound **9**), close analogue of **7** observed in the previous reactions.

Then, we evaluated the effect of the addition of common catalysts used in organic chemistry such as AcOH, $CuBr_2$ and CuI (entries 4-6). AcOH was used because it is widely employed as acid catalyst in cyclization reactions, while $CuBr_2$ and CuI were selected due to the ability of copper to catalyze carbon–carbon and carbon–heteroatom bond formation. No significant reaction was observed in presence of the AcOH (entry 4) after 12h. Differently, the presence of $CuBr_2$ or CuI (entries 5 and 6) led to the formation of the compound **8** after 3h, as detected by TLC. We were unable to remove $CuBr_2$ during the reaction work-up by using EtOH, while the removal of CuI (entry 6) by filtering the boiling EtOH allowed to recover compound **8** in 37% yield. Although the presence of CuI as catalyst showed a slight benefit, we focused on the optimization of the reaction conditions of entry 2 with the aim to respect the atom economy.

Based on the observation that the increase of the equiv. of DMF-DMA was detrimental (entry 3) while a more efficient reaction was obtained by enhancing the equiv. of phenylacetic anhydride (entry 2), we came back to study the effect of different ratio between the reactants (entries 7-12). In particular, we investigated the effect of 1.1 equiv. of DMF-DMA and rising equiv. of anhydride (from 2 to 5 equiv., entries 7-10) and 2 equiv. of DMF-DMA with 4 or 5 equiv. of anhydride (entries 11 and 12). We were pleased to find that, with the exception of the reaction performed by using 2 equiv. of anhydride, all the reactions furnished compound **8** rapidly (2h), efficiently (from 56 to 71% yield) and without the formation of side product **9**. The best

156 results were obtained by reacting 1 equiv. of the benzothiazole acetate (**1**), 1.1 equiv. of DMF-DMA and 3
157 equiv. of phenylacetic anhydride at 110 °C (entry 8). This 3CR furnished compound **8** after 2h in 71% yield.

Table 1. Optimization of 3CR conditions for **8**.[a]



Entry	Ratio[b]	Temperature	Catalyst	Time (h)	Yield (8)/(9)[c]
1	1:1:1	110 °C	-	6	8%/ND[d]
2	1:2:2	110 °C	-	6	35%/ND
3	1:3:1.2	110 °C	-	4	12%/trace
4	1:2:2	110 °C	AcOH (1 eq)	12	ND/ND
5	1:2:2	110 °C	$CuBr_2$ (1 eq)	3	ND/ND
6	1:2:2	110 °C	CuI (1 eq)	3	37%/ND
7	1:1.1:2	110 °C	-	2	45%/trace
8	**1:1.1:3**	**110 °C**	**-**	**2**	**71%**/ND
9	1:1.1:4	110 °C	-	2	56%/ND
10	1:1.1:5	110 °C	-	2	62%/ND
11	1:2:4	110 °C	-	2	62%/ND
12	1:2:5	110 °C	-	2	56%/ND
13	1:1.1:3	90 °C	-	2	59%/trace
14	1:1.1:3	rt	-	2	46%/ND

[a]The reaction was performed on 0.9 mmol scale of **1**. [b]Referred to the ratio between **1**:DMF-DMA:phenylacetic anhydride. [c]Isolated yields. [d]ND: not detected by TLC.

158 In order to undertake a greener approach, we also tried to reduce the temperature of the reaction (entries
159 13 and 14). Interestingly, both the reactions performed at 90 °C and rt were equally rapid (2h) although less
160 efficient (59% and 46% yield, respectively) and traces of the side product **9** were observed in the reaction
161 conducted at 90 °C.

Of note, the reaction crudes were only treated with EtOH (25 mL) and refluxed, and the target compounds were then collected as pure solids after precipitations. When traces of the insoluble **9** were detected, filtration of the boiling EtOH enabled us to collect pure target compounds. To compare the yield of the 3CR using our optimized conditions (Table 1, entry 8) with the classic two-step procedure, we synthesized **8** starting from benzothiazole acetate **1**, by using the classic method. Initially, benzothiazole acetate **1** (1 equiv.) was reacted with 3 equiv. of DMF-DMA in dry DMF at 90 °C. After 2h, intermediate benzothiazole acrylate (**2**) was isolated in 76% yield by treating the crude with EtOAc and filtering. Then, acrylate **2** was reacted with 2 equiv. of phenylacetic anhydride for 2h at 110 °C and, after treatment with Et_2O and following filtration, target compound **8** was obtained in 53% yield.

These data highlighted significant advantages of the 3CR over the multi-step procedure (MSP): i) it was more efficient showing a 71% yield with respect to the MSP showing an overall yield of 40%; ii) it was more rapid (2h of the 3CR *vs* 4h of the MSP); iii) it required a lower use of solvents. In particular, no solvent was used in the 3CR and only 25 mL of EtOH were needed for the work-up to obtain pure compound **8**. On the contrary, MSP necessitated the use of solvents such as DMF during the reaction and EtOAc and Et_2O during the work-up to remove the side product **9** and recover compound **8**, respectively.

In order to compare the optimized 3CR and MSP by green parameters, indicators commonly applied in sustainable green chemistry such as atom economy (AE), reaction mass efficiency (RME), process mass intensity (PMI), simple E-factor (sEF) and complete E-factor (cEF) were evaluated.

The first parameter (AE), regarding the ratio between the molecular weight (MW) of the target compound and the MW of the starting materials for the used equivalents, shows a similar behavior between MSP (32%) and 3CR (31%). Conversely, when masses of reactants and products are considered in the RME, differences between the two procedures starts getting significant (MSP = 13%; 3CR = 22%). To be noted, when solvents used for the reaction and work-up are taken into account, green parameters (PMI, sEF and cEF) widely support the use of the 3CR (92.96, 3.50 and 91.96, respectively) with respect to the MSP (292.44, 14.29, and 291.44, respectively). Therefore, 3CR seems more favored than MSP even considering approved green parameters.

In a successive step of this study, we decided to investigate the role of both anhydride and DMF-DMA in the 3CR. Thus, we repeated the reaction by using the optimized conditions and replacing phenylacetic anhydride with the corresponding ethyl 2-phenylacetate or replacing DMF-DMA with trimethyl orthoformate. Both reactions did not furnish compound **8** after 2h, while led to the formation of the side product **9** in traces and in 58% yield, respectively. These data clearly suggested that both the anhydride and DMF-DMA are required for the positive outcome of the reaction and, in particular, to allow cyclization. Moreover, the replacement of DMF-DMA with trimethyl orthoformate strongly promoted the formation of the side product **9**.

2.2. Scope evolution of the 3CR and one-pot procedure

2.2.1. Variation of the anhydride

Utilizing the optimized conditions, we then studied the scope of the reaction (Table 2). The first step was to replace the phenylacetic anhydride with the acetic anhydride (**10a**), firstly used by Kato and colleagues for the PBTZ formation [18], with the aim to further compare 3CR with MSP. Interestingly, acetic anhydride **10a** (entry 1) was a suitable substrate for this reaction, reacting smoothly with **1** and DMF-DMA to give product **3** in 55% yield after 2h. The same reaction performed through the MSP (entry 2) was both less rapid (2 + 8h) and efficient (43% yield). In addition, the evaluation of the three key green parameters PMI, sEF and cEF (Table 2) highlighted the greater sustainability of the 3CR than the MSP.

Subsequently, succinic anhydride (**10b**) and 3-phenylpropanoic anhydride (**10c**) were chosen to extend the scope of the reaction and a set of phenylacetic anhydrides (**10d-i**) was selected in view of their use for medicinal chemistry purpose such as functionalization of the phenyl at PBTZ C-2 position.

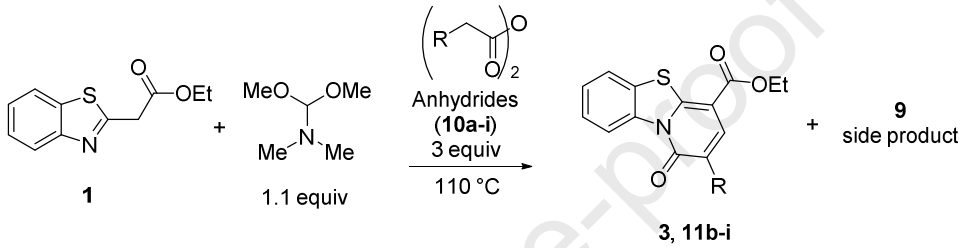
Anhydrides **10c-i** were prepared starting from the corresponding acids according to the literature protocols [14,23–25], while succinic anhydride (**10b**) was purchased.

When succinic anhydride **10b** was used (entry 3), no significant reaction was observed after 12h. However, the reaction did not even occur by MSP, showing that compound **11b** could not be obtained regardless of the conditions used. On the contrary, 3-phenylpropanoic anhydride **10c** and differently substituted phenylacetic anhydrides (**10d-i**) (entries 4-10) afforded target compounds **11c-i** in moderate to

214 good yields (from 19 to 58%). In particular, 3-phenylpropanoic anhydride **10c** furnished the worst results
215 (entry 4), suggesting that a greater reactivity of the α carbon of the anhydride is required during the reaction.

216 The substituted phenylacetic anhydrides (**10d**, **10e**, **10g** and **10i**) afforded target compounds without
217 traces of by-product **9** (entries 5, 6, 8 and 10), differently from anhydrides **10f** and **10h** (entries 7 and 9), both
218 having an ortho substituent that likely impaired the reaction owing to the steric hindrance. However, with the
219 exception of compound **11c**, all the target compounds **11d-i** were obtained with acceptable green parameters
220 (Table 2) and collected as pure solids after crystallization of the reaction crude by EtOH.

Table 2. Scope of 3CR using different anhydrides **10a-i**.[a]



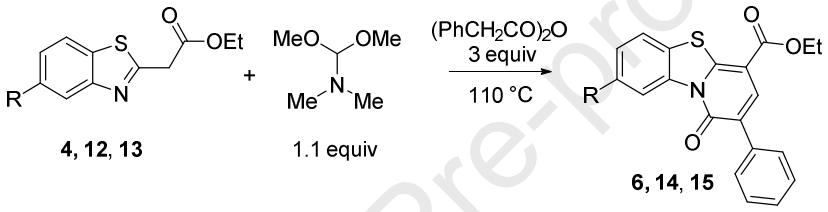
Entry		Anhydride	Compd. (R)	Time (h)	Yield (3, 11b-i)/(9)[b]	PMI	sEF/cEF
1	3CR	**10a** (acetic)	**3** R = H	2	55%/ND[c]	150.18	3.38/149.18
2	MSP			2+8	43%//ND	345.79	14.65/344.79
3	3CR	**10b** (succinic)	**11b** R = (-CH_2CO_2H)	12	ND/ND	-	-/-
4	3CR	**10c**, R = -CH_2-C_6H_5	**11c**	3	19%[d]/ND	334.76	16.36/333.76
5	3CR	**10d**, R = 4Cl-C_6H_4	**11d**	2	53%/ND	114.23	5.50/113.23
6	3CR	**10e**, R = 4OMe-C_6H_4	**11e**	2	46%/ND	132.99	6.42/131.99
7	3CR	**10f**, R = 2F-C_6H_4	**11f**	2	45%/trace	139.76	6.39/138.76
8	3CR	**10g**, R = 3F-C_6H_4	**11g**	2	58%/ND	108.43	4.73/107.43
9	3CR	**10h**, R = 2Me-C_6H_4	**11h**	2	36%/trace	176.68	8.16/175.68
10	3CR	**10i**, R = 3Me-C_6H_4	**11i**	3	44%/ND	144.55	6.50/143.55

[a]The reaction was performed on 0.9 mmol scale of **1**. [b]All yields related to 3CRs have been calculated after crystallization by EtOH. [c]ND: not detected by TLC. [d]The yield of the reaction has been calculated after purification by chromatography.

221 2.2.2. Variation of the benzothiazole

Subsequently, we investigated the role of some substituents on the benzothiazole core (Table 3). Thus, using our optimized conditions, we performed the 3CR starting from C-5 substituted benzothiazole acetate derivatives **4**, **12** and **13** (entries 1-3), prepared according to the literature protocols [19], to obtain the PBTZ derivatives **6**, **14** and **15**. The use of cyclohexyloxy benzothiazole acetate **4** was prompted by the need to develop a suitable procedure for the synthesis of compound **6**, a key intermediate useful for the synthesis of anti-flavivirus PBTZs. Interestingly, the 3CRs showed good yields (ranging from 63% to 80%) and ideal green parameters, bringing out that also substituted benzothiazole acetate derivatives are suitable substrates for the 3CR aimed at building substituted PBTZ core.

Table 3. Scope of 3CR using three different benzothiazole acetates (**4**, **12** and **13**).[a]



Entry		R	Benzothiazole	Compd.	Time (h)	Yield[b]	PMI	sEF/cEF
1	3CR	-OCy	**4**	**6**	6	71%	72.79	2.82/71.79
2	3CR	-OMe	**12**	**14**	2	63%	96.48	3.79/95.48
3	3CR	-Cl	**13**	**15**	2	80%	75.12	2.74/74.12

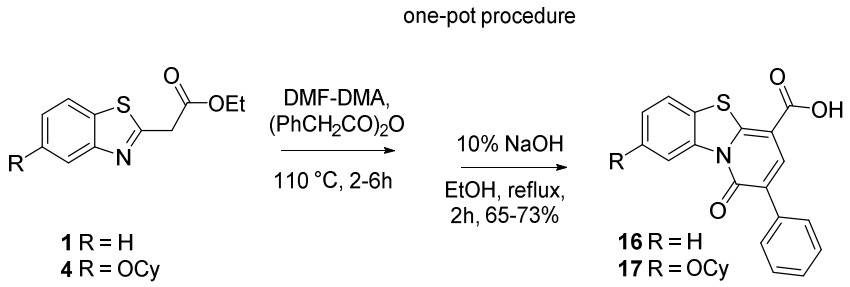
[a]The reaction was performed on 0.9 mmol scale of **4**, **12** or **13**. [b]All yields related to 3CRs have been calculated after crystallization by EtOH.

2.3. One-pot procedure: 3CR and in-situ basic hydrolysis

Strongly motivated from these good results, we also attempted a one-pot procedure to directly obtain the corresponding PBTZ acid derivatives **16** and **17** (Scheme 2), key intermediates useful to perform different kinds of reaction couplings. Indeed, compound **17** represents a key synthon to afford potent anti-flavivirus PBTZ analogues by amide functionalization at C-4 position. The achievement of the key synthon **17** in a one-pot procedure would be time saving and may strongly reduce the amount of solvents used for multi-step reactions and purifications of chemical intermediates. Thus, once performed the 3CR to obtain **6** and **8**, we directly added EtOH and 10% aqueous NaOH in the reaction vessel and after 2h, we observed complete

238 conversion. Compound **16** and **17** were smoothly recovered following acidification and filtration as a pure
239 solid in good 65 and 73% overall yields, respectively.

240 **Scheme 2.**



241

242 **3. Design, synthesis and anti-flavivirus evaluation of new PBTZ derivatives**

243 Based on the promising results of the recently published PBTZ analogue **18** having a *β*-alanine moiety at
244 C-4 amide position [15], we decided to investigate the introduction of some chemical modifications on the *β*-
245 alanine chain and how those could impact on the antiviral activity of the new derivatives (**19-26** – Table 4).
246 Intriguingly, since the removal of the carboxylic function from the PBTZ C-4 portion led to the loss of RdRp
247 inhibition while retaining good cell-based antiviral activity, as for derivative **27** [15], we also designed three
248 different alcohol PBTZ derivatives (**28-30**).

249 Synthetic procedures to afford the target compounds **19-26** and **28-30** starting from synthons **6** or **17**,
250 obtained by 3CR and one-pot procedures, have been reported in the Supplementary Material (Schemes S1
251 and S2). For a first survey of biological evaluation, compounds **19**, **21**, **22**, **24**, **25** and **28** having a chiral
252 centre, were synthesized using racemic amino acids.

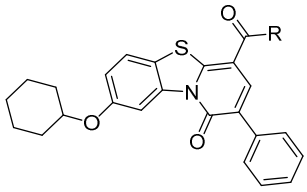
253 All compounds were initially subjected to *in vitro* DENV-2 *de novo* initiation and elongation polymerase
254 assays as previously described [26] employing purified recombinant NS5 full-length protein (Table 4). After
255 evaluation of IC_{50} values of RdRp elongation inhibition for those compounds exhibiting an inhibition ≥ 70%
256 at 30 µM, single point inhibition experiments at 10 µM against both DENV-2 and ZIKV infected Huh7 cells
257 were performed for all PBTZ analogues. For compounds that were unable to reduce the viral titer of at least
258 70%, the percentage of inhibition at 10 µM is reported in Table 4, while the active compounds (viral titer
259 reduction > 70%) were subjected to a dose−response testing from 0.01 to 100 µM in order to calculate the

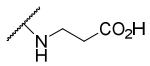
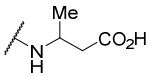
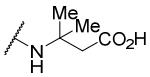
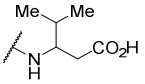
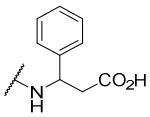
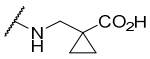
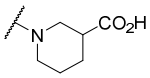
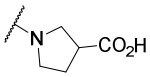
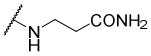
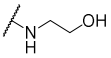
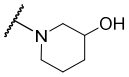
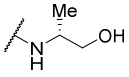
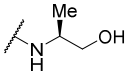
effective concentration that results in 50% reduction in infective virus particles (EC_{50}) for both DENV-2 and ZIKV. In parallel, toxicity was determined on Huh7 cells to obtain CC_{50} values, and the selectivity index (SI; CC_{50}/EC_{50}) was calculated (Table 4).

The introduction of a methyl group on the *β* carbon of the *β*-alanine portion afforded derivative **19** with a comparable RdRp inhibition (just below the 70% threshold in elongation inhibition and no *de novo* initiation inhibition) as the hit **18** (77.6%). On the other hand, a reduction of the anti-DENV-2 and -ZIKV activity in cell-based assays was observed, with compound **19** that did not reach in both cases the 70% of viral titer reduction at 10 μM, differently from **18** showing EC_{50} values of 3.5 and 1.0 μM, respectively. When the size of the group on the *β* carbon was increased (derivatives **20**-**22**), a significant improvement in terms of RdRp inhibition was obtained. All three derivatives reached almost the 100% inhibition at 30 μM in elongation activity and gained RdRp inhibition also in *de novo* phase (≥70% at 30 μM) with respect to **18** (no significant inhibition observed). Corresponding IC_{50} values of the elongation polymerase activity resulted from 1.6 to 3.6-fold better than **18**. However, improvements with respect to **18** in the cell-based anti-DENV-2 activity were observed only for the phenyl derivative **22** (EC_{50} = 1.2 μM, CC_{50} = 100 μM, SI = 83).

When shifting the introduction of an alkyl group to the *α* carbon of the *β*-alanine portion, compound **23** having a cyclopropyl exhibited a comparable activity to **21** (isopropyl on the *β* carbon) both in RdRp inhibition and cell-based anti-DENV-2 activity. Therefore, there are evidences that the introduction of lipophilic moieties on the *β*-alanine chain led to an improvement in RdRp inhibition, which was not translated in an increase in anti-DENV-2 activity with regard to **18**, except for compound **22**. However, the introduction of these lipophilic portions significantly impaired anti-ZIKV activity, with only phenyl derivative **22** reaching the 70% viral titer reduction and showing an EC_{50} of 5.0 μM (SI = 20), 5-fold higher than that of **18** (EC_{50} = 1.0 μM, SI > 100).

Table 4. DENV-2 RdRp biochemical assay and antiviral (DENV-2 and ZIKV) evaluation in HuH7 cells of compounds **19**-**26**, **28**-**30** and reference compounds **18**, **27** and NITD008.



Compd	R	Polymerase assay against DENV-2 full-length NS5 Inhibition %[a] De novo	Inhibition %[a] Elong.	Elong. IC_{50} (μM)[b]	CC_{50} (μM)[c] HuH7 cells	Infection cell-based assay EC_{50} (μM),[d](SI)[e] DENV-2	ZIKV
18[15]		ND[f]	77.6	37.7 ± 1.75	>100	3.5 ± 0.45 (>30)	1.0 ± 0.44 (>100)
19		22.7	69.3	ND[f]	ND[f]	68.1%[g]	29.0%[g]
20		81.5	95.9	23.2 ± 2.60	~100	3.1 ±0.38 (32)	10.6%[g]
21		75.9	92.8	22.1 ± 0.09	~100	8.4 ± 0.48 (12)	47.4%[g]
22		79.3	96.2	10.4 ± 0.09	~100	1.2 ± 0.36 (83)	5.0 ±4.2 (20)
23		68.1	96.7	17.8 ± 1.14	~100	7.5 ± 3.48 (13.3)	15.8%[g]
24		ND[f]	64.9	ND[f]	ND[f]	65.3%[g]	26.3%[g]
25		11.0	64.6	ND[f]	>100	6.4 ± 2.8 (15.6)	8.2 ± 0.08(12.2)
26		ND[f]	37.2	ND[f]	52.3	0.9 ± 0.02 (58)	7.3 ± 4.20 (7.1)
27[15]		10	63.3	ND[f]	86.3	1.9 ± 0.03 (43)	1.8 ± 0.68 (43)
28		ND[f]	36.9	ND[f]	22.2	1.3 ± 0.13 (17)	11.1 ± 1.13 (2)
29		ND[f]	42.7	ND[f]	17.9	5.8 ± 0.81 (3)	7.3 ± 0.49 (2.4)
30		ND[f]	42.5	ND[f]	21.7	1.1 ± 0.62 (20)	8.1 ± 3.13 (2.6)
NITD-008	-	-	-	-	100	1.0 (100)	0.2 (>100)

[a] % inhibitory activity against RdRp in *de novo* (primer independent) and elongation (primer dependent) polymerase assays at single 30 μM concentration. [b]Elong. IC_{50} is the concentration that inhibits 50% of RdRp activity in elongation assay; values represent average ± SE from a single experiment carried out with duplicates. [c] CC_{50} is the cytotoxic concentration that affects 50% of cell viability as determined by CellTiter-Glo Luminescent Assay (Promega). [d] EC_{50} is the effective concentration that inhibits 50% virus infection as determined by plaque assay against DENV-2 EDEN 3295 (GenBank accession: EU081177.1) after 48h treatment and ZIKV H/PF/2013 (GenBank accession: KJ776791.2) after 24h treatment; values represent average ± SE from a single experiment carried out with duplicates. [e] SI is selectivity index calculated as ratio CC_{50}/EC_{50}. [f] ND: not determined due to poor or no activity in the corresponding assay. [g] Percentage of viral inhibition at 10μM.

The decrease in the degree of flexibility of the aliphatic side chain, obtained by two different constrains, furnished the 3-carboxy piperidine derivative **24** and the *β*-proline derivative **25**, both showing a moderate RdRp elongation inhibition (≈ 65%) while not exhibiting any inhibition of the *de novo* polymerase activity, similarly to **18**. An opposite trend was instead observed for the two analogues when considering the cell-based anti-flavivirus activity. Piperidine derivative **24** at 10uM showed a 65.3% of DENV-2 viral titer reduction but only a 26.3% of reduction for ZIKV. On the other hand, pyrrolidine analogue **25** at 10 μM reached the 70% of viral titer reduction against both viruses and exhibited EC_{50} values of 6.4 and 8.2 μM against DENV-2 and ZIKV, respectively, and a tolerable SI (15.6 and 12.2, respectively).

Interestingly, when carboxylic function of **18** was replaced with an amide portion resulting in the close analogue **26**, we observed the loss of RdRp inhibition but an increase in anti-DENV-2 activity (EC_{50} = 0.9 μM). However, anti-ZIKV activity (EC_{50} = 7.3 μM) was about 7-fold less than that of **18**. Despite cytotoxicity evaluation of **26** on Huh7 cells displayed a lower CC_{50} (52.3 μM) than **18** (>100 μM), the resulting SI is suitable for anti-DENV-2 activity (58) and slightly under the accepted threshold for anti-ZIKV activity (7.1).

As expected, derivatives having the alcoholic functionality (**28-30**) did not show any significant RdRp inhibition at 30 μM, as their analogue **27** [15], thus confirming the key role of the carboxylic group in the PBTZ derivatives to inhibit flavivirus RdRp. Regarding the cell-based anti-flavivirus activity of **28-30**, low EC_{50} values were obtained for all three compounds but the evaluation of CC_{50} values often yielded unsuitable SIs (ranging from 2 to 20). Best results were obtained against DENV-2, with compounds **28** and **30** displaying EC_{50} values of 1.3 (SI = 17) and 1.1 μM (SI = 20), respectively. However, all three derivatives showed an anti-flavivirus profile worse than **27** ($EC_{50\ DENV\text{-}2}$ = 1.9 μM, SI = 43; $EC_{50\ ZIKV}$ = 1.8 μM, SI = 43). Taken together, these results highlighted that the introduction of a methyl group or an increase of the rigidity on the ethanolic chain of **27** reduced the antiviral activity.

5. Conclusions

In conclusion, we report on the exploration and optimization of the synthesis of the ethyl 1-oxo-1*H*-pyrido[2,1-*b*][1,3]benzothiazole-4-carboxylate and its corresponding acid, a scaffold found on several broad-

spectrum anti-flavivirus agents. Through a 3CR and a one-pot procedure, both environment-friendly approaches, we quickly and easily obtained the PBTZ scaffold that was further functionalized to synthesize a set of eleven PBTZ analogues. Through the evaluation of the NS5 RdRp inhibition and antiviral activity against DENV-2- and ZIKV-infected cells of the PBTZ derivatives, we identified compound **22**, having a phenyl moiety on the β carbon of the β-alanine chain, as the best compound of the series since endowed with a potent antiviral activity against both DENV-2 and ZIKV (EC_{50} = 1.2 and 5.0 μM, respectively) and a suitable SI (83 and 20, respectively). Worthy mentioning, the amidic derivative **26** showed the best anti-DENV-2 activity (EC_{50} = 0.9 μM, SI = 58) among the series of the PBTZ analogues. Lacking the free carboxylic function, compound **26** did not inhibit the NS5 RdRp, but its very promising activity on DENV-2-infected cells deserves further investigation to understand its mechanism of action.

4. Experimental section

4.1. Chemistry

Unless otherwise indicated, all starting materials were commercially available. Reagents and solvents were purchased from common commercially suppliers and were used as received. Organic solutions were dried over anhydrous Na_2SO_4 and concentrated with a Büchi rotary evaporator under reduced pressure. All reactions were routinely checked by TLC on silica gel 60F254 (Merck) and visualized by using UV and iodine. Flash column chromatography separations were carried out on Merck silica gel 60 (mesh 230-400). Yields are referred to purified products and are not optimized. Melting points were determined in capillary tubes using a Stuart SMP3. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 101 MHz, respectively, with a Bruker Advance DRX- 400 instrument. Chemical shifts (δ) are reported in ppm relative to TMS and calibrated using residual undeuterated solvent as internal reference. Coupling constants (*J*) are reported in Hz. Spectra were acquired at 298 K. Data processing were performed with MestReNova software, and the spectral data are consistent with the assigned structures. Compounds **19-26** and **28-30** were ≥95% pure as determined by LC/MS using an Agilent 1290 Infinity System machine equipped with DAD detector from 190 to 640 nm. The purity was revealed at 254 nm using a Phenomenex AERIS Widepore C4, 4.6 mm, 100 mm (6.6 lm) with flow rate: 0.85 ml/min; acquisition time: 10 min; gradient: acetonitrile in water containing

0.1% of formic acid (0 100% in 10 min); oven temperature, 30 C. Peak retention time is given in minutes. Detection was based on electrospray ionization (ESI) in negative polarity using Agilent 1290 Infinity System equipped with a MS detector Agilent 6540UHD Accurate Mass Q-TOF. Optical rotatory power was determined using DIP-360 JASCO digital polarimeter.

4.1.1. General procedure for the 3CR of compounds 3, 6, 11c-i, 14 and 15 (Method A). In a round-bottom flask, benzothiazole acetate derivatives (**1**, **4**, **12** and **13**) (0.90 mmol), DMF-DMA (0.99 mmol) and phenyl acetic anhydride or anhydrides **10a-i** (2.70 mmol) were added and the mixture was placed in a 110 °C pre-heated oil bath under stirring. After the disappearance of benzothiazole acetate derivatives by monitoring TLC, EtOH (25 mL) was added. When traces of **9** were detected by TLC, the boiling EtOH was immediately filtered and the desired compound was collected after evaporation of the filtrate as pure yellow solids. When traces of **9** were absent by TLC, after cooling of the reaction mixture, crystallization was allowed, and pure yellow compounds were collected following filtration.

4.1.2. General Procedure for the amidation reaction for compounds 25, **26 and 40-44 (Method B).** Under N_2 atmosphere, to a mixture of the appropriate acid (1.0 mmol), and the appropriate amino acid methyl ester hydrochloride or the desired amine (1.3 mmol) in dry DMSO (15 mL), TBTU coupling reagent (1.3 mmol), and DIPEA (4.6 mmol) were added. After stirring at room temperature, the reaction mixture was poured into ice/water and acidified with 2N HCl (pH = 4-5) to give a precipitate which was filtered under vacuum to give the relative solid which was purified, as described below for each compound, in order to obtain the desired PBTZ derivative.

4.1.3. 1-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}pyrrolidine-3-carboxylic acid (25). Following the general procedure (Method B), starting from acid (**17**) and using methyl pyrrolidine-3-carboxylate hydrochloride (**37**), compound **25** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (100 to 97:3), as a yellow solid in 27% yield. Reaction time: 4h; m.p. 118-120 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.71 (brs, 1H, COOH), 8.85 (d, *J* = 2.3 Hz, 1H, H-9), 8.03 (s, 1H, H-3), 7.88 (d, *J* = 8.7 Hz, 1H, H-6), 7.72 (d, *J* = 7.5 Hz, 2H, H-2' and H-6'), 7.41 (t, *J* = 7.8 Hz, 2H, H-3' and H-5'), 7.32 (t, *J* = 7.3 Hz, 1H, H-4') 7.19 (dd, *J* =

2.4 and 8.8 Hz, 1H, H-7), 4.41-4.36 (m, 1H, OC*H*), 3.77-3.66 (m, 4H, pyrrolidine CH_2 x 2), 3.10-3.03 (m, 1H, pyrrolidine C*H*), 2.15-1.92 (m, 2H, pyrrolidine CH_2), 1.90 (m, 2H, cyclohexyloxy CH_2), 1.70-1.67 (m, 2H, cyclohexyloxy CH_2), 1.50-1.23 (m, 6H, cyclohexyloxy CH_2 x 3). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 174.41, 165.51, 160.97, 156.55, 151.77, 139.34, 136.86, 135.04, 129.18, 128.48, 127.70, 123.01, 121.74, 120.35, 116.81, 108.18, 107.41, 76.13, 50.45, 47.82, 42.73, 31.66, 28.80, 25.55, 23.42. HPLC: r_t 6.344, HRMS (ESI) calculated for $C_{29}H_{29}N_2O_5S$ $[M+H]^+$ 517.1796 found 517.1802.

4.1.4. *N*-(3-amino-3-oxopropyl)-8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-benzo[4,5]thiazolo[3,2-*a*]pyridine-4-carboxamide (26). Following the general procedure (Method B), starting from acid **17** and using *β*-alaninamide hydrochloride (**38**), compound **26** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 25% yield. Reaction time: 4h; m.p. 204-205 °C (d). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.84 (d, *J* = 2.0 Hz, 1H, H-9), 8.72 (t, *J* = 5.4 Hz, 1H, CON*H*), 8.38 (s, 1H, H-3), 7.87 (d, *J* = 8.7 Hz, 1H, H-6), 7.76 (d, *J* = 7.6 Hz, 2H, H-2' and H-6'), 7.43 (t, *J* = 7.4 Hz, 2H, H-3' and H-5'), 7.35-7.31 (m, 2H, H-4' and $CONH_2$ x $^1/_2$), 7.16 (dd, *J* = 2.1 and 8.7 Hz , 1H, H-7), 6.84 (brs, 1H, $CONH_2$ x $^1/_2$), 4.37-4.33 (m, 1H, OC*H*), 3.44 (q, *J* = 6.65 Hz, 2H, NH$CH_2$$CH_2CONH_2$), 2.34 (t, *J* = 7.1 Hz, 2H, $NHCH_2CH_2CONH_2$), 1.89 (m, 2H, cyclohexyloxy CH_2), 1.68-1.66 (m, 2H, cyclohexyloxy CH_2) 1.46-1.23 (m, 6H, cyclohexyloxy CH_2 x 3). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 172.82, 164.31, 161.25, 156.18, 151.34, 138.88, 136.74, 133.86, 129.27, 128.33, 127.69, 123.02, 122.22, 121.02, 116.40, 107.46, 105.84, 75.58, 36.44, 35.44, 31.50, 25.46, 23.41. HPLC: r_t 5.976, HRMS (ESI) calculated for $C_{27}H_{28}N_3O_4S$ $[M+H]^+$ 490.1800 found 490.1796.

4.1.5. Methyl 3-({[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)butanoate (40). Following the general procedure (Method B), starting from acid **17** and using methyl 3-aminobutanoate hydrochloride (**31**), compound **40** was obtained, after purification by flash column chromatography eluting with Cy/EtOAc (8:2), as a yellow solid in 59% yield. Reaction time: 4h; m.p. 178-180 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.85 (d, *J* = 1.9 Hz, 1H, H-9), 8.76 (t, *J* = 5.5 Hz, 1H, CON*H*), 8.38 (s, 1H, H-3), 7.89 (d, *J* = 8.7 Hz, 1H, H-6), 7.76 (d, *J* = 7.9 Hz, 2H, H-2' and H-6'), 7.46-7.42 (m, 2H, H-3' and H-5'), 7.36-7.32 (m, 1H, H-4'), 7.18 (dd, *J* = 1.7 and 8.8 Hz, 1H, H-7), 4.39-4.35 (m,

1H, OC*H*), 3.57 (s, 3H, CO_2CH_3), 3.51-3.44 (m, 1H, $CH_3CHCH_aH_b$), 3.36-3.29 (m, 1H, $CH_3CHCH_aH_b$), 2.79-2.70 (m, 1H, NH*CH*CH_2), 1.91 (m, 2H, cyclohexyloxy CH_2), 1.78-1.67 (m, 2H, cyclohexyloxy CH_2), 1.56-1.18 (m, 6H, cyclohexyloxy CH_2 x 3), 1.08 (d, J = 7.0 Hz, 3H, CH_3CHCH_2).

4.1.6. Methyl 3-({[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-3-methylbutanoate (41). Following the general procedure (Method B), starting from acid **17** and using methyl 3-amino-3-methylbutanoate hydrochloride (**32**), compound **41** was obtained, after purification by flash column chromatography eluting with Cy/EtOAc (8:2), as a yellow solid in 39% yield. Reaction time: 3h; m.p. 176-178 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.56 (brs, 1H, H-9), 8.40 (s, 1H, H-3), 7.99 (s, 1H, CON*H*), 7.86 (d, J = 8.6 Hz, 1H, H-6), 7.73 (d, J = 7.5 Hz, 2H, H-2' and H-6'), 7.45-7.41 (m, 2H, H-3' and H-5'), 7.35-7.32 (m, 1H, H-4'), 7.17 (d, J = 8.2 Hz, 1H, H-7), 4.36-4.28 (m, 1H, OC*H*), 3.50 (s, 3H, CO_2CH_3), 2.89 (s, 2H, CH_2), 1.94 (m, 2H, cyclohexyloxy CH_2), 1.68-1.58 (m, 2H, cyclohexyloxy CH_2), 1.46-1.17 (m, 12H, cyclohexyloxy CH_2 x 3 and CH_3 x 2).

4.1.7. Methyl 3-({[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-4-methylpentanoate (42). Following the general procedure (Method B), starting from acid **17** and using methyl 3-amino-4-methylpentanoate hydrochloride (**33**), compound **42** was obtained, after purification by flash column chromatography eluting Cy/EtOAc (8:2), as a yellow solid in 77% yield. Reaction time: 3h; m.p. 162-164 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.89 (brs, 1H, H-9), 8.39 (s, 1H, H-3), 8.29 (d, J = 8.5 Hz, 1H, CONH), 7.87 (d, J = 8.7 Hz, 1H, H-6), 7.29 (d, J = 7.5 Hz, 2H, H-2' and H-6'), 7.46-7.42 (m, 2H, H-3' and H-5'), 7.36-7.32 (m, 1H, H-4'), 7.18-7.16 (m, 1H, H-7), 4.36 (m, 1H, OC*H*), 4.23-4.22 (m, 1H, NH*CH*CH_2), 3.51 (s, 3H, CO_2CH_3), 2.68-2.59 (m, 2H, NHCHCH_2), 1.88 (m, 2H, cyclohexyloxy CH_2), 1.81 (m, 1H, *i*Pr CH), 1.77-1.68 (m, 2H, cyclohexyloxy CH_2), 1.47-1.18 (m, 6H, cyclohexyloxy CH_2 x 3), 0.86 (d, J = 6.7 Hz, 6H, *i*Pr CH_3 x 2).

4.1.8. Methyl 3-({[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-3-phenylpropanoate (43). Following the general procedure (Method B), starting from acid **17** and using methyl 3-amino-3-phenylpropanoate hydrochloride (**34**), compound **43** was obtained, after purification by column chromatography eluting with Cy/EtOAc (8:2), as a yellow solid in 60% yield.

Reaction time: 4h; m.p. 143-145 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.96 (d, *J* = 8.0 Hz, 1H, CON*H*), 8.84 (d, *J* = 2.4 Hz, 1H, H-9), 8.44 (s, 1H, H-3), 7.87 (d, *J* = 8.8 Hz, 1H, H-6), 7.75-7.72 (m, 2H, Ar-H), 7.48-7.46 (m, 2H, Ar-H), 7.44-7.36 (m, 3H, Ar-H), 7.34-7.29 (m, 2H, Ar-H), 7.24-7.22 (m, 1H, Ar-H), 7.14 (dd, *J* = 2.4 and 8.7 Hz, 1H, H-7), 5.52-5.46 (m, 1H, NH*CH*CH_2), 4.39-4.35 (m, 1H, OC*H*), 3.54 (s, 3H, CO_2CH_3) 3.01-2.95 (m, 1H, NHCHC$H_a$$H_b$), 2.91-2.86 (m, 1H, NHCHC$H_a$$H_b$), 1.95-1.90 (m, 2H, cyclohexyloxy CH_2), 1.70-1.67 (m, 2H, cyclohexyloxy CH_2), 1.51-1.21 (m, 6H, cyclohexyloxy CH_2 x 3).

4.1.9. Methyl 1-({[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)cyclopropanecarboxylate (44). Following the general procedure (Method B), starting from acid **17** and using methyl 1-(aminomethyl)cyclopropanecarboxylate hydrochloride (**35**), compound **44** was obtained, after purification by flash column chromatography eluting with Cy/EtOAc (5:5), as a yellow solid in 16% yield. Reaction time: 3h; m.p. 235-237 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.85 (d, *J* = 2.3 Hz, 1H, H-9), 8.57 (t, *J* = 5.2 Hz, 1H, CON*H*), 8.42 (s, 1H, H-3), 7.87 (d, *J*= 8.7 Hz, 1H, H-6), 7.74 (d, *J* = 7.3 Hz, 2H, H-2' and H-6') 7.43 (t, *J* =7.5 Hz, 2H, H-3' and H-5'), 7.34 (m, 1H, H-4'), 7.17 (dd, *J* = 2.3 and 7.2 Hz, 1H, H-7), 4.39-4.35 (m, 1H, OC*H*), 3.57-3.54 (m, 5H, CO_2CH_3 and NHCH_2), 1.90 (m, 2H, cyclohexyloxy CH_2), 1.70-1.67(m, 2H, cyclohexyloxy CH_2) 1.50-1.25 (m, 6H, cyclohexyloxy CH_2 x 3), 1.09-1.06 (m, 2H, cyclopropyl CH_2), 1.00-0.97 (m, 2H, cyclopropyl CH_2).

4.1.10. Ethyl 1-{[8-(cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}piperidine-3-carboxylate (45). Under N_2 atmosphere, to a solution of acid **17** (0.3 g 1.4 mmol) in dry CH_2Cl_2, EDCI*HCl (0.32 g, 2.1 mmol), HOBt (0.19 g, 1.4 mmol), DIPEA (0.7 mL, 4.2 mmol) and ethyl nipecotate **36** (0.2 mL, 1.4 mmol) were subsequently added and the reaction was stirred at room temperature for 4h. After evaporation of the solvent under vacuum, the reaction mixture was poured into ice/water and the resulting solid was filtered under vacuum to give a yellow solid in 53% yield. Reaction time: 4h; m.p. 133-135 °C (d). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.83 (d, *J* = 2.2 Hz, 1H, H-9), 7.87 (d, *J* = 8.7 Hz, 1H, H-6), 7.83 (s, 1H, H-3), 7.71 (d, *J* = 7.4 Hz, 2H, H-2' and H-6'), 7.42-7.38 (m, 2H, H-3' and H-5'), 7.33-7.30 (m, 1H, H-4'), 7.18 (dd, *J* = 2.3 and 8.8 Hz, 1H, H-7), 4.39-4.35 (m, 1H, OC*H*), 4.09-4.03 (m, 1H, piperidine C*H*), 3.98-3.91 (m, 2H, $CO_2CH_2CH_3$), 3.81-3.78 (m, 1H, piperidine C*H*), 2.65-2.62 (m,

1H, piperidine C*H*), 1.95-1.93 (m, 3H, cyclohexyloxy CH_2 and piperidine C*H*), 1.70-1.66 (m, 3H, cyclohexyloxy CH_2 and piperidine C*H*), 1.49-1.20 (m, 10H, cyclohexyloxy CH_2 x 3 and piperidine CH_2 x 2), 1.07 (t, *J* = 7.1 Hz, 3H, $CO_2CH_2CH_3$).

4.1.11. 8-(Cyclohexyloxy)-4-[(3-hydroxypiperidin-1-yl)carbonyl]-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-one (28). Following the procedure used for **45** and using 3-hydrossipiperidine hydrochloride **39** (0.03 g, 0.24 mmol), compound **28** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (99:1), as a yellow solid in 32% yield. Reaction time: 8h; m.p. 167-169 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.84 (d, *J* = 1.8 Hz, 1H, H-9), 7.88-7.86 (m, 2H, H-3 and H-6), 7.73 (d, *J* = 7.8 Hz, 2H, h-2' and H-6'), 7.41 (t, *J* = 7.6 Hz, 2H, H-3' and H-5'), 7.31 (t, *J* = 7.3 Hz, 1H, H-4'), 7.17 (dd, *J* = 1.9 and 8.7 Hz, 1H, H-7), 4.89 (brs, 1H, OH), 4.40-4.36 (m, 1H, OC*H*), 3.74 (m, 1H, piperidine C*H*), 3.73-3.56 (m, 2H, piperidine CH_2), 3.49-3.43 (m, 1H, piperidine C*H*), 3.15 (m, 1H, piperidine C*H*), 1.95-1.90 (m, 2H, cyclohexyloxy CH_2), 1.77-1.68 (m, 4H, piperidine CH_2 and cyclohexyloxy CH_2), 1.55-1.24 (m, 8H, cyclohexyloxy CH_2 x 3 and piperidine CH_2). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 167.26, 160.98, 156.31, 150.77, 139.48, 136.68, 135.82, 129.16, 128.52, 127.75, 123.26, 122.2, 119.31, 116.45, 107.78, 107.27, 75.66, 65.23, 53.02, 45.35, 33.79, 32.77, 25.52, 23.47, 22.50. HPLC: r_t 6.377, HRMS (ESI) calculated for $C_{29}H_{31}N_2O_4S$ $[M+H]^+$ 503.2004 found 503.2004.

4.1.12. General procedure for hydrolysis of methyl esters for compounds 19-24. (Method C). A solution of the appropriate methyl ester compound (**40-45**) (1 mmol) in 1,4 dioxane and 1N LiOH (5 mmol) was stirred at room temperature for 3/4h. Then, the reaction mixture was poured into ice/water, acidified with 2N HCl (pH = 3-4) to give a precipitate that was filtered and purified as described below for each product.

4.1.13. 3-({[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)butanoic acid (19). Following the general procedure (Method C), starting from derivative **40**, compound **19** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 50% yield. Reaction time: 3h; m.p.: 198 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.30 (brs, 1H, CO_2H), 8.85 (d, *J* = 2.3 Hz, 1H, H-9), 8.75 (t, *J* = 5.8 Hz, 1H, CON*H*), 8.40

(s, 1H, H-3), 7.88 (d, *J* = 8.7 Hz, 1H, H-6), 7.75 (d, *J* = 7.3 Hz, 2H, H-2' and H-6'), 7.44 (t, *J* = 7.8 Hz, 2H, H-3' and H-5'), 7.33 (m, 1H, H-4'), 7.10 (dd, *J* = 2.4 and 8.7 Hz, 1H, H-7), 4.39-4-35 (m, 1H, OC*H*), 3.50-3.23 (m, 1H, NHCHC$H_a$$H_b$), 3.42-3.23 (m, 1H, NHCHC$H_a$$H_b$), 2.68-2.63 (m, 1H, NH*CH*CH_2), 1.96-1.92 (m, 2H, cyclohexyloxy CH_2), 1.70-1.66 (m, 2H, cyclohexyloxy CH_2), 1.49-1.23 (m, 6H, cyclohexyloxy CH_2 x 3), 1.06 (d, *J* = 7.1 Hz, CH_3). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 176.43, 164.50, 161.27, 156.22, 151.50, 138.91, 136.75, 133.85, 129.30, 128.35, 127.72, 123.06, 122.28, 120.99, 116.43, 107.48, 105.73, 75.60, 52.58, 42.81, 31.52, 25.47, 23.43, 15.42. HPLC: r_t 6.574, HRMS (ESI) calculated for $C_{28}H_{29}N_2O_5S$ $[M+H]^+$ 505.1796 found 505.1797.

4.1.14. 3-({[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-3-methylbutanoic acid (20). Following the general procedure (Method C), starting from derivative **41**, compound **20** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 46% yield. Reaction time: 3h; m.p. 168-170 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.02 (brs, 1H, CO_2*H*), 8.85 (d, *J* = 2.2 Hz, 1H, H-9), 8.40 (s, 1H, H-3), 8.04 (brs, 1H, CON*H*), 7.85 (d, *J* = 8.6 Hz, 1H, H-6), 7.73 (d, *J* = 7.3 Hz, 2H, H-2' and H-6'), 7.43 (t, *J* = 7.3 Hz, 2H, H-3' and H-5'), 7.33 (t, *J* = 6.7 Hz, 1H, H-4'), 7.17 (dd, *J* = 1.7 and 8.8 Hz, 1H, H-7), 4.39-4.30 (m, 1H, OC*H*), 2.79 (s, 2H, CH_2), 1.93-1.90 (m, 2H, cyclohexyloxy CH_2), 1.70-1.67 (m, 2H, cyclohexyloxy CH_2), 1.49-1.17 (m, 12H, cyclohexyloxy CH_2 x 3 and CH_3 x 2). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 172.70, 164.35, 161.28, 156.22, 151.25, 138.87, 136.85, 134.46, 129.43, 128.32, 127.63, 122.98, 122.15, 121.09, 116.46, 107.49, 106.67, 75.64, 52.66, 43.67, 31.53, 27.47, 25.46, 23.41. HPLC: r_t 6.893, HRMS (ESI) calculated for $C_{29}H_{31}N_2O_5S$ $[M+H]^+$ 519.1953 found 519.1958.

4.1.15. 3-({[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-4-methylpentanoic acid (21). Following the general procedure (Method C), starting from derivative **42**, compound **21** was obtained, after purification flash by column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 41% yield. Reaction time: 3h; m.p. 146-148 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.18 (brs, 1H, CO_2*H*), 8.86 (d, *J* = 2.4 Hz, 1H, H-9), 8.43 (s, 1H, H-3), 8.33 (d, *J* = 8.4 Hz, 1H, CON*H*), 7.88 (d, *J* = 8.7 Hz, 1H, H-6), 7.73 (d, *J* = 7.4 Hz, 2H, H-2' and H-6'), 7.46-7.42

(m, 2H, H-3' and H-5'), 7.36-7.32 (m, 1H, H-4'), 7.18 (dd, *J* = 2.4 and 8.7 Hz, 1H, H-7), 4.40-4.35 (m, 1H, OC*H*), 4.27-4.20 (m, 1H, NHC*H*CH_2), 2.57-2.42 (m, 2H, NHCH*CH_2*), 1.92-1.91 (m, 2H, cyclohexyloxy CH_2), 1.85-1.80 (m, 1H, *i*Pr C*H*), 1.71-1.56 (m, 2H, cyclohexyloxy CH_2), 1.50-1.24 (m, 6H, cyclohexyloxy CH_2 x 3), 0.86 (d, *J* = 6.8 Hz, 6H, *i*Pr CH_3 x 2). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 173.34, 136.88, 161.28, 156.23, 151.57, 138.94, 136.84, 133.83, 129.39, 128.36, 127.69, 122.99, 122.39, 121.12, 116.44, 107.51, 105.93, 75.66, 52.24, 36.62, 32.24, 31.53, 25.46, 23.42, 19.22, 19.07. HPLC: r_t 6.951, HRMS (ESI) calculated for $C_{30}H_{33}N_2O_5S$ $[M+H]^+$ 533.2109 found 533,2111.

4.1.16. 3-({[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)-3-phenylpropanoic acid (22). Following the general procedure (Method C), starting from derivative **43**, compound **22** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow in 58% yield. Reaction time: 3h; m.p. 155-158 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.19 (brs, 1H, CO_2*H*), 9.07 (d, *J* = 7.7 Hz 1H, CON*H*), 8.81 (d, *J* = 2.4 Hz, 1H, H-9), 8.47 (s, 1H, H-3), 7.87 (d, *J* = 8.8 Hz, 1H, H-6), 7.76-7.71 (m, 2H, H-2' and H-6'), 7.46 (t, *J* = 7.4 Hz, 2H, H-3' and H-5'), 7.41-7.36 (m, 3H, H-4', H-2'' and H-6''), 7.34-7.29 (m, 2H, H-3'' and H-5''), 7.24-7.20 (m, 1H, H-4''), 7.17 (dd, *J* = 2.4 and 8.8 Hz, 1H, H-7), 5.48-5.39 (m, 1H, NH*C*HCH_2), 4.39-4.34 (m, 1H, OC*H*), 2.91-2.79 (m, 1H, NHCH*CH_a*H_b), 2.76-2.75 (m, 1H, NHCHCH_a*H_b*), 1.91-1.90 (m, 2H, cyclohexyloxy CH_2), 1.74-1.68 (m, 2H, cyclohexyloxy CH_2), 1.49-1.21 (m, 6H, cyclohexyloxy CH_2 x 3). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 172.20, 163.59, 161.28, 156.24, 151.83, 143.04, 138.91, 136.82, 133.78, 129.42, 128.77, 128.42, 127.77, 127.47, 126.99, 123.06, 122.54, 120.96, 116.46, 107.42, 105.52, 75.61, 50.60, 40.99, 31.51, 25.46, 23.43. HPLC: r_t 6.964, HRMS (ESI) calculated for $C_{33}H_{31}N_2O_5S$ $[M+H]^+$ 567.1953 found 567.1960.

4.1.17. 1-[({[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}amino)methyl]cyclopropanecarboxylic acid (23). Following the general procedure (Method C), starting from derivative **44**, compound **23** was obtained, after purification by column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 19% yield. Reaction time: 3h; m.p. 250-253 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.07 (brs, 1H, CO_2*H*), 8.83 (d, *J* = 2.4 Hz, 1H, H-9), 8.58 (t, *J* = 5.3 Hz,

1H, CON*H*), 8.43 (s, 1H, H-3), 7.87 (d, *J* = 8.7 Hz, 1H, H-6), 7.75-7.73 (m, 2H, H-2' and H-6'), 7.43 (t, *J* = 7.8 Hz, 2H, H-3' and H-5'), 7.35-7.31 (m, 1H, H-4'), 7.15 (dd, *J* = 2.3 and 8.8 Hz, 1H, H-7), 4.38-4.32 (m, 1H, OC*H*), 3.53 (d, *J* = 5.2 Hz, 2H, CH_2), 1.92-1.89 (m, 2H, cyclohexyloxy CH_2), 1.69-1.66 (m, 2H, cyclohexyloxy CH_2), 1.42-1.16 (m, 6H, cyclohexyloxy CH_2 x 3), 1.04-1.01 (m, 2H, cyclopropryl CH_2), 0.94-0.91 (m, 2H, cyclopropyl CH_2). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 175.83, 164.68, 161.28, 156.19, 151.60, 138.92, 136.81, 134.05, 129.38, 128.33, 127.67, 123.01, 122.27, 121.03, 116.42, 107.43, 105.76, 75.59, 42.14, 31.50, 25.46, 23.42, 23.29, 13.77. HPLC: r_t 6.697, HRMS (ESI) calculated for $C_{29}H_{29}N_2O_5S$ $[M+H]^+$ 517.1796 found 517.1800.

4.1.18. 1-{[8-(Cyclohexyloxy)-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazol-4-yl]carbonyl}piperidine-3-carboxylic acid (24). Following the general procedure (Method C), starting from derivative **45**, compound **24** was obtained, after purification by flash column chromatography eluting with $CHCl_3$/MeOH (97:3), as a yellow solid in 44% yield. Reaction time: 3h; m.p. 119-120 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 12.21 (brs, 1H, CO_2H), 8.83 (d, *J* = 1.8 Hz, 1H, H-9), 7.87 (d, *J* = 8.7 Hz, 1H, H-6), 7.79 (s, 1H, H-3), 7.71 (d, *J* = 7.4 Hz, 2H, H-2' and H-6'), 7.40 (t, *J* = 7.5 Hz, 2H, H-3' and H-5'), 7.31 (t, *J* = 7.2 Hz, 1H, H-4'), 7.18 (dd, *J* = 1.9 and 8.6 Hz, 1H, H-7), 4.37-4.29 (m, 1H, OC*H*), 4.08-4.05 (m, 1H, piperidine C*H*), 3.81-3.72 (m, 1H, piperidine C*H*), 3.23-3.16 (m, 2H, piperidine CH_2), 2.51-2.46 (m, 1H, piperidine C*H*), 1.93 (m, 3H, cyclohexyloxy CH_2 and piperidine C*H*), 1.66 (m, 4H, cyclohexyloxy CH_2 and piperidine CH_2), 1.47-1.23 (m, 7H, cyclohexyloxy CH_2 x 3 and piperidine C*H*). ^{13}C NMR (101 MHz, [D_6]DMSO): δ = 174.73, 167.15, 160.98, 156.33, 150.91, 139.50, 136.67, 135.61, 129.20, 128.53, 127.80, 123.26, 121.90, 119.29, 116.46, 107.79, 107.06, 75.67, 47.59, 45.85, 41.14, 31.55, 27.23, 25.53, 24.52, 23.48. HPLC: r_t 6.485, HRMS (ESI) calculated for $C_{30}H_{31}N_2O_5S$ $[M+H]^+$ 531.1953 found 531.1959.

4.1.19. 8-(Cyclohexyloxy)-*N*-[(1*R*)-2-hydroxy-1-methylethyl]-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazole-4-carboxamide (29). A mixture of starting derivative **6** (0.3 g, 0.67 mmol) and (*R*)-2-aminopropan-1-ol **46** (2 mL) was heated at reflux in neat conditions for 5h. Then, the reaction mixture was poured into ice/water and acidified with 2N HCl (pH = 5) to give a precipitate which was filtered under vacuum. After purification by flash column chromatography eluting with $CHCl_3$/MeOH (98:2), compound

29 was obtained as a yellow solid in 39% yield. Reaction time: 4h; m.p. 139-140 °C. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.86 (d, *J* = 2.4 Hz, 1H, H-9), 8.44 (s, 1H, H-3), 7.91 (d, *J* = 8.7 Hz, 1H, H-6), 7.76-7.74 (m, 2H, H-2' and H-6'), 7.44 (t, *J* = 7.7 Hz, 2H, H-3' and H-5'), 7.34-7.32 (m, 1H, H-4'), 7.17 (dd, *J* = 2.4 and 8.8 Hz, 1H, H-7), 4.76 (t, *J* = 5.8 Hz, 1H, CHCH$_2$*OH*), 4.39-4.35 (m, 1H, OC*H*), 4.07-3.99 (m, 1H, *CH*CH$_2$OH), 3.48-3.40 (m, 2H, CH*CH$_2$*OH), 1.92-1.90 (m, 2H, cyclohexyloxy CH$_2$), 1.69-1.67 (m, 2H, cyclohexyloxy CH$_2$), 1.50-1.26 (m, 6H, cyclohexyloxy CH$_2$ x 3), 1.11 (d, *J* = 6.7 Hz, 3H, CH$_3$).^{13}C NMR (101 MHz, [D_6]DMSO): δ = 163.83, 161.30, 156.17, 151.42, 138.89, 136.81, 134.05, 129.38, 128.33, 127.67, 122.99, 122.30, 121.11, 166.42, 107.45, 105.99, 75.59, 64.71, 47.76, 31.50, 25.45, 23.41, 17.51. HPLC: r_t 6.430, HRMS (ESI) calculated for $C_{27}H_{29}N_2O_4S$ $[M+H]^+$ 477.1847 found 477.1847. $[\alpha]_D$ = - 0.036 (0.5 % p/v, DMSO).

4.1.20. 8-(cyclohexyloxy)-*N*-[(1*S*)-2-hydroxy-1-methylethyl]-1-oxo-2-phenyl-1*H*-pyrido[2,1-*b*][1,3]benzothiazole-4-carboxamide (30). Following procedure reported above for compound **29** and using (*S*)-2-aminopropan-1-ol **47** (2 mL), compound **30** was obtained, after purification by flash column chromatography $CHCl_3$/ MeOH (98:2), as a yellow solid in 11% yield. Reaction time: 4h; m.p. 139-140 °C. ^{1}H NMR (400MHz, MeOD): δ = 8.83 (d, *J* = 2.4 Hz, 1H, H-9), 8.28 (s, 1H, H-3), 7.76-7.74 (m, 2H, H-2' and H-5'), 7.62 (d, *J* = 8.7 Hz, 1H, H-6), 7.42-7.36 (m, 2H, H-3' and H-5'), 7.33-7.30 (m, 1H, H-4'), 7.05 (dd, *J* = 2.4 and 8.7 Hz, 1H, H-7), 4.35-4.29 (m, 1H, OC*H*), 4.19-4.13 (m, 1H, *CH*CH$_2$OH), 3.62-3.53 (m, 2H, CH*CH$_2$*OH), 2.00-1.97 (m, 2H, cyclohexyloxy CH$_2$), 1.78-1.75 (m, 2H, cyclohexyloxy CH$_2$), 1.57-1.32 (m, 6H, cyclohexyloxy CH$_2$ x 3), 1.23 (d, *J* = 6.8 Hz, 3H, CH$_3$). ^{13}C NMR (101 MHz, DMSO-*d_6*): δ 164.71, 161.84, 156.43, 151.52, 138.50, 136.38, 133.52, 128.76, 127.67, 127.11, 122.94, 121.53, 120.47, 116.35, 106.79, 105.96, 75.53, 64.64, 47.62, 31.26, 25.23, 23.15, 15.74. HPLC: r_t 6.425, HRMS (ESI) calculated for $C_{27}H_{29}N_2O_4S$ $[M+H]^+$ 477.1848 found 477.1848. $[\alpha]_D$ = + 0.036 (0.5 % p/v, DMSO).

4.2. Biological part

4.2.1 Cell lines and viruses

BHK-21 cells (baby hamster kidney fibroblast; ATCC) were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (P/S) at 37 ^{o}C in 5% CO_2. HuH-7 cells (human hepatocarcinoma; ATCC) were cultured in DMEM medium (Gibco) with 4.5g/L glucose, 10% FBS and 1% P/S at 37^{o}C in 5% CO_2.

DENV-2 EDEN3295 (GenBank accession: EU081177) was obtained from the Early Dengue infection and outcome (EDEN) study [27]. The ZIKV strain H/PF/2013 (GenBank accession: KJ776791.2) was a gift from Cécile Baronti at Aix Marseille Université. These viruses used in this study were grown in C6/36 cells, titered in BHK-21 cells and stored at -80^{o}C. Institutional approval has been granted by Duke-NUS Medical School to perform experiments with ZIKV.

4.2.2 *in vitro* NS5 polymerase assays

The *de novo* initiation/elongation assay was performed as previously described [15,17]. Briefly, the initiation assay reaction comprised 100 nM DENV-2 NS5, 100 nM in vitro transcribed DENV-2 5'UTR-3'UTR RNA, 20 μM ATP, 20 μM GTP, 20 μM UTP, 5 μM Atto-CTP (Trilink Biotechnologies), in a volume of 15 μL of reaction buffer comprising 50 Mm Tris-HCL, Ph7.5, 10 Mm KCl, 1mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.001% Triton X-100 and 10 μM cysteine. The elongation assay reaction comprised of 100 nM DENV-2 NS5, 100 nM U30-3' UTR (Integrated DNA Technologies), 3 μM Atto-ATP, in a volume of 15 μL of reaction comprised of 50 mM Tris-HCL, pH 7.5, 10 mM KCl, 0.5 mM $MnCl_2$, 0.001% Triton and 10 μM cysteine. All reactions were incubated at 37°C for 1 h and 10 μL of STOP buffer (200 mM NaCl, 25 mM $MgCl_2$, 1.5 M DEA, pH10; Promega) with 25 nM calf intestine alkaline phosphatase was added to the wells to stop the reactions. The plate was centrifuged briefly at 1200 rpm, followed by incubation at RT for 60 mins and the release AttoPhos was monitored by reading on a Tecan machine at excitation$_{max}$ and emission$_{max}$ wavelengths 422 nm and 566 nm respectively.

4.2.3 Cell cytotoxicity test

HuH-7 cells were seeded in a 96-well white opaque plate (Grenier) at a density of 2×10^4. Compounds were diluted to the indicated concentrations in the medium and incubated with cells for 48h at 37 □.

Cytotoxicity was determined by CellTiter Glo® Luminescent Assay (Promega) kit according to manufacturer's instructions. Cell viability curve presented as percentage of luminescence derived from treated samples to that of the untreated control. The 50% cytotoxic concentration (CC_{50}; concentration at which 50% of the cells are dead) was determined using GraphPad Prism.

4.2.4 Dose-dependent inhibition assay

Huh-7 cells was seeded at a density of 1×10^5 in a 24-well plate. Cells were infected with DENV-2 EDEN 3295 at MOI 0.3 and ZIKV H/PF/2013 at MOI 0.5 for 1 h at 37 □. Following infection, the cells were then treated with the compounds at indicated concentrations ranging from 100 µM to 0.01 µM for 24h (ZIKV infection) and 48h (DENV infection). Supernatants were collected, clarified and subjected to plaque quantification by BHK-21 plaque assay. The efficacy of the compounds (EC_{50}: concentration at which the virus infection is reduced by 50%) was determined by the sigmoidal dose response curve of virus titer against concentration in GraphPad Prism.

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Abbreviations

3CR	three-component reaction
AE	atom economy
cEF	complete E-factor
DENV	Dengue virus
DMF-DMA	*N*,*N*-dimethylformamide dimethyl acetal
JEV	Japanese encephalitis virus
MCR	multicomponent reaction
MoA	mode of action

MSP	multi-step procedure
MW	molecular weight
PBTZ	1*H*-pyrido[2,1-*b*][1,3]benzothiazol-1-one
PMI	process mass intensity
RdRp	RNA-dependent RNA polymerase
REMSA	RNA-Electrophoretic Mobility Shift Assay
RME	reaction mass efficiency
sEF	simple E-factor
TBEV	tick-borne encephalitis virus
TLC	thin layer chromatography
UTR	untranslated region
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

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HIGHLIGHTS

- Three-component reaction for preparing biologically active pyridobenzothiazolones
- Three-component reaction with wide scope for fast functionalization of the scaffold
- Pyridobenzotiazolones were quickly obtained by an optimized one-pot procedure
- New pyridobenzothiazolones displayed a potent anti-flavivirus activity
- New insights on the anti-flavivirus activity of pyridobenzothiazolone series

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships, which may be considered as potential competing interests: