Efficient and regioselective one-step synthesis of 7-aryl-5-methyl- and 5-aryl-7-methyl-2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidine derivatives.

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Abstract

Two facile and efficient one-step procedures for the regioselective synthesis of 7-aryl-5-methyl- and 5-aryl-7-methyl-2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidines have been developed, *via* reactions of 3,5-diamino-1,2,4-triazole with variously substituted 1-aryl-1,3-butanediones and 1-aryl-2-buten-1-ones, respectively. The excellent yield and/or regioselectivity shown by the reactions decreased when ethyl 5-amino-1,2,4-triazole-3-carboxylate was used. Being the [1,2,4]triazolo[1,5-*a*]pyrimidine a privileged scaffold, the procedure herein reported may be useful for the preparation of biologically active compounds. In this study, the preparation of a set of compounds based on the [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold let to the identification of compound **20** endowed with a very promising ability to inhibit influenza virus RNA polymerase PA-PB1 subunits heterodimerization.

Introduction

[1,2,4]triazolo[1,5-*a*]pyrimidine is a privileged structure with numerous chemical and biological applications. Beside to their great versatility in the interactions with metal ions, [1,2,4]triazolo[1,5-*a*]pyrimidines showed a wide range of biological activities both in agriculture and in medicine.[1a,b] Examples of biologically active compounds include trapidil (Rocornal®, Fig. 1), a platelet-derived growth factor antagonist that has been used to treat patients with ischemic coronary heart, liver, and kidney disease,[1c] and filibuvir (Fig. 1), a non-nucleoside inhibitor of HCV NS5B polymerase that passed stage II clinical trials, although its clinical development program was then suspended.[1d] Focusing on the most recent literature, compounds based on the [1,2,4]triazolo[1,5-*a*]pyrimidine core have been reported as phosphodiesterase 2 (PDE2a) inhibitors for the treatment of memory disorders,[2a] anti-Alzheimer's disease,[2b-d] anticancer,[2e,f] antimalarial,[2g,h] antitubercular,[2i] antileishmanial,[2j] antibacterial,[2k] antiviral,[2l] hypnotic,[2m] and CB2 cannabinoid receptor inverse agonists.[2n]

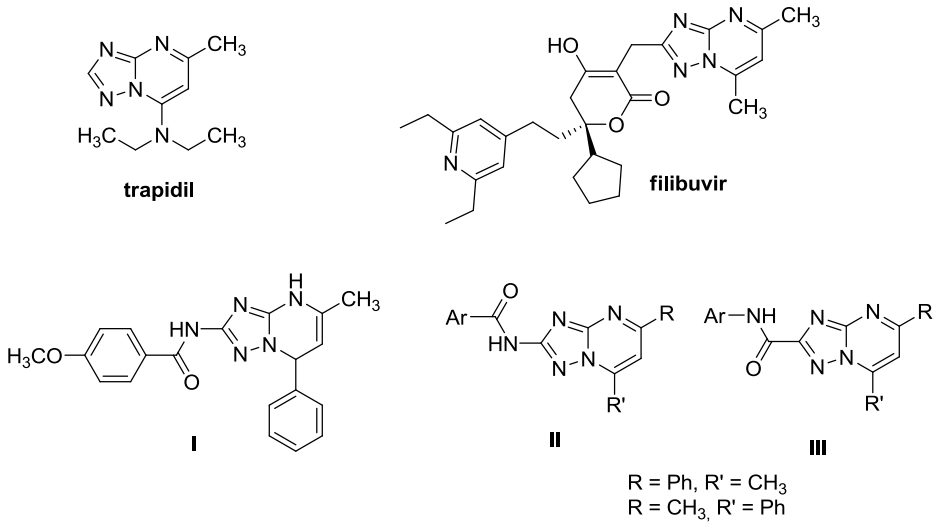


Figure 1. Examples of biologically active compounds with a [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold.

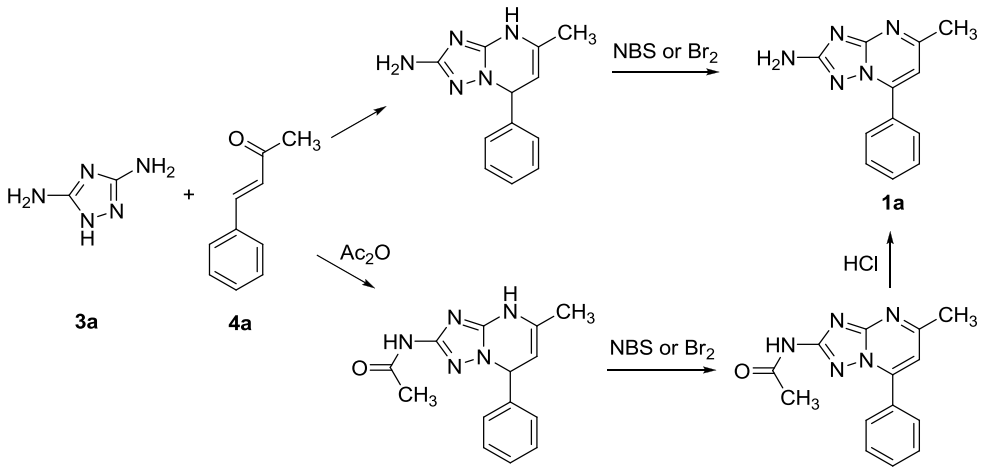
We have also been involved in the synthesis of a series of [1,2,4]triazolo[1,5-*a*]pyrimidines (compound I and structures II and III, Fig. 1)[3a,b] within our research program on the development of influenza virus (flu) RNA-dependent RNA polymerase (RdRP) PA-PB1 subunits interaction inhibitors.[3] In particular, the synthesis of the

anti-flu compounds entailed the preparation of the key intermediate 2-amino-5-methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine (**1a**) and its isomer 2-amino-7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine (**2a**) (Table 1).

A major contribution to the chemistry of 2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidines has been provided by Desenko and co-workers, who were the first to report on their synthesis,[4] and Chernyshev's research group, who investigated their synthesis further,[5a-c] their reactivity,[5d] and their use for the preparation of polycondensed heterocycles.[5e-h] Nevertheless, the synthesis of 7-aryl-5-methyl- and 5-aryl-7-methyl-2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidine derivatives has been scarcely explored. In this work, we reported two approaches for their preparation *via* reaction between 3,5-diamino-1,2,4-triazole and variously functionalized 1-aryl-1,3-butanediones and 1-aryl-2-buten-1-ones, respectively. Both strategies allowed the synthesis of the desired isomer under mild conditions, with high yields, and regioselectively.

Results and discussion

The synthetic method known for the regioselective preparation of compound **1a**, as well as of some 5,7-diaryl-2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidines, involves a two-step procedure (Scheme 1) entailing: i) cyclocondensation of 3,5-diamino-1,2,4-triazole (**3a**) with chalcone 4-phenylbut-3-en-2-one (**4a**) giving2-amino-5-methyl-7-phenyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine,[4a-c] and ii) heteroaromatization using either *N*-bromosuccinimide (NBS) or Br_2.[4a] However, the overall yields do not exceed 25-30%. Moreover, NBS and Br_2 are highly reactive and, thus, this method can only be used to prepare [1,2,4]triazolo[1,5-*a*]pyrimidines with limited substitutions at the C-5 and C-7 positions. Increased overall yields up to 40-77% were achieved by acetyl protection of the C-2 amino group, by adding Ac_2O to the reaction mixture after the completion of cyclocondensation, preventing oxidation of the amino group in the successive step.[5a] Through this three-step procedure, compound **1a** was regioselectively obtained in 77% yield (Scheme 1).



Scheme 1. Known procedures for the synthesis of **1a**.

The reaction of 3-amino-1,2,4-triazole bearing different substituents at the C-5 position with unsymmetrically 1,3-diketones where one of the substituents is a methyl group and the other is a group different from methyl, was known from literature to form a mixture of 5-methyl and 7-methyl isomers. The two isomers can be distinguished by NMR on the basis of the chemical shifts of the pyrimidine methyl carbon appearing at 24-25 ppm and 16-17 ppm for the 5-methyl and 7-methyl isomers, respectively.[6] Although 5-methyl isomer is always the main product of the reaction, the ratio of isomers is influenced: i) by the steric hindrance of the substituent on the 1,3-diketone, with a more bulky substituent that gives a higher ratio of 5-methyl isomer, and ii) by the inductive effect of the substituent at the C-5 position of the 3-amino-1,2,4-triazole, which influences the ratio of isomers much more than the inductive effect of the substituent on the 1,3-diketone different from the methyl.[6b]

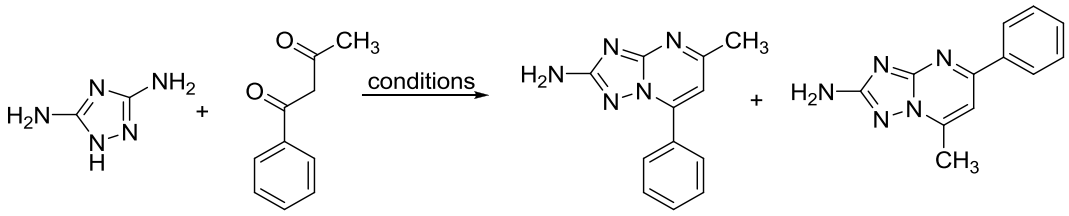
Based on these facts, we hypothesized that, the presumable high nucleophilicity of **3a** owing to the presence of a second electron-donating amino groups in the molecule,[7] might led to an efficient synthesis of 2-amino-7-aryl-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines by cyclocondensation with 1-aryl-1,3-butanediones. To test this idea, we first tried the reaction of **3a** with 1-phenyl-1,3-butanedione (**5a**) in glacial acetic acid at reflux (Table 1, entry 1). Actually, Kreutzberger and Risse reported in 1979 that this reaction condition provided a mixture of isomers **1a** and **2a** in 2% and 26% yield, respectively.[8] In contrast to what reported by Kreutzberger and Risse who mistakenly inverted the assignment of the structures, we were pleased to find that the

reaction took place rapidly (4 h), highly efficiently, and, more interestingly, highly regioselectively. Indeed, 2-amino-5-methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine (**1a**) was obtained in 88% yield while its regioisomer 2-amino-7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine (**2a**) was formed only in traces.

To further explore the reaction conditions, the reaction was then carried out in different solvents. In particular, the reaction was repeated in protic and aprotic solvents, i.e. EtOH, DMF, $CHCl_3$, THF, and acetone (Table 1), in order to understand whether the acid environment of glacial acetic acid could be essential or not for regioselectivity. We also aimed at evaluating the possible influence on tautomerism of **5a**, that is well known to be extremely sensitive to solvent effect.[9] In particular, the enolic form of **5a** is much greater in nonpolar solvents than in polar or hydrogen-bond donor solvents, since the first ones do not compete with hydrogen-bond formation.[9a] For example, in water compound **5a** is extimated to be a mixture of both tautomers, with the keto-form being more favoured than the enol one (about 60% and 40%, respectively).[9b] Although data on tautomerism of **5a** in glacial acetic acid are not available, it is reasonable to hypothesize that in this solvent the keto-form is predominant over the enol one, while, for example, in $CHCl_3$ it is known that this compound exists as two kinds of *cis*-enol forms.[9c] No significant reaction was observed in $CHCl_3$ (entry 2), acetone (entry 3), and THF (entry 4) at reflux after 24 h, because of the insolubility of **3a** in these solvents at the used concentrations. On the other hand, the reaction in EtOH (entry 5) and DMF (entry 6) led to the formation of isomer **1a** as the main product but in lower yield (57% and 66%, respectively) and much more slowly than in acetic acid. Since the reactions did not go to completion under both conditions after 24 h, we studied the effect of the equiv of **3a** (entry 7) as well as the influence of the addition of a base such as triethylamine (entries 8) in DMF. Analogously, the reactions were much more slow and less efficient (69% and 61%, respectively), but, most importantly, they showed a dramatically decreased regioselectivity. Finally, the reaction was carried out in EtOH with the addition of a catalytic amount of acetic acid (entry 9), to verify whether a protic solvent in acid conditions could lead to a similar regioselectivity compared to that obtained with glacial acetic acid. The presence of acetic acid did not influence the outcome of the reaction, meaning that the simple catalytic effect by acid conditions in the condensation reaction is not sufficient to obtain a high regioselectivity.

Thus, the best reaction conditions are treating **3a** (1 equiv) and **5a** (1 equiv) in acetic acid at reflux for 4 h. Through this one-step procedure, compound **1a** was regioselectively obtained in 88% yield.

Table 1. Optimization of reaction conditions for **1a**[a]



3a **5a** **1a** **2a**

Entry	3a (equiv)	Solvent	Time (h)	Yield (%)[b]	
				1a	2a
1	1	Acetic Acid	4	88	traces
2	1	$CHCl_3$	24	-	-
3	1	Acetone	24	-	-
4	1	THF	24	-	-
5	1	EtOH	24	57	4
6	1	DMF	24	66	3
7	2	DMF	24	39	30
8	1	DMF (1 equiv of Et_3N)	24	48	13
9	1	EtOH (0.5 mL of Acetic Acid)	24	62	5

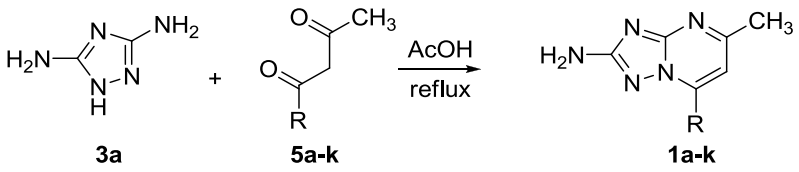
[a] The reaction was performed on 1.0 mmol scale of **5a** in 2.5 mL of solvent at reflux.
[b] Determined by ^1H NMR.

Utilizing the optimized conditions, we then studied the scope of the reaction (Table 2). Thus, 1-aryl-1,3-butanediones (**5b-k**) bearing different electron-donating and electron-withdrawing substituents on the phenyl ring were prepared and reacted with **3a**. 1-Aryl-1,3-butanediones were in turn synthesized through a

Claisen condensation by reacting aryl-methyl ketones with ethyl acetate in the presence of sodium[10] (for the synthesis and characterization of compounds **5b-k**, see SI).

The results listed in Table 2 show that 1-aryl-1,3-butanediones bearing both electron-donating (entries 2 and 3) and electron-withdrawing (entry 4) substituents on the phenyl ring are suitable substrates for this reaction, reacting smoothly with **3a** to give products **1b-d**. The effect of the position of electron-withdrawing substituents on the phenyl ring was also studied (entries 5 and 6), and the reaction gave consistently good yields for compounds **1e** and **1f**. Multiple electron-donating (entry 7) and electron-withdrawing (entry 8) substituents on the 1-aryl-1,3-butanedione phenyl ring gave goods yields. Finally, using 1-(naphthalen-1-yl)butane-1,3-dione (entry 9) and 1-(pyridin-4-yl)butane-1,3-dione (entry 10), the reaction gave compounds **1i** and **1k** in modest yields.

Table 2. Preparation of 2-amino-7-aryl-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines[a]



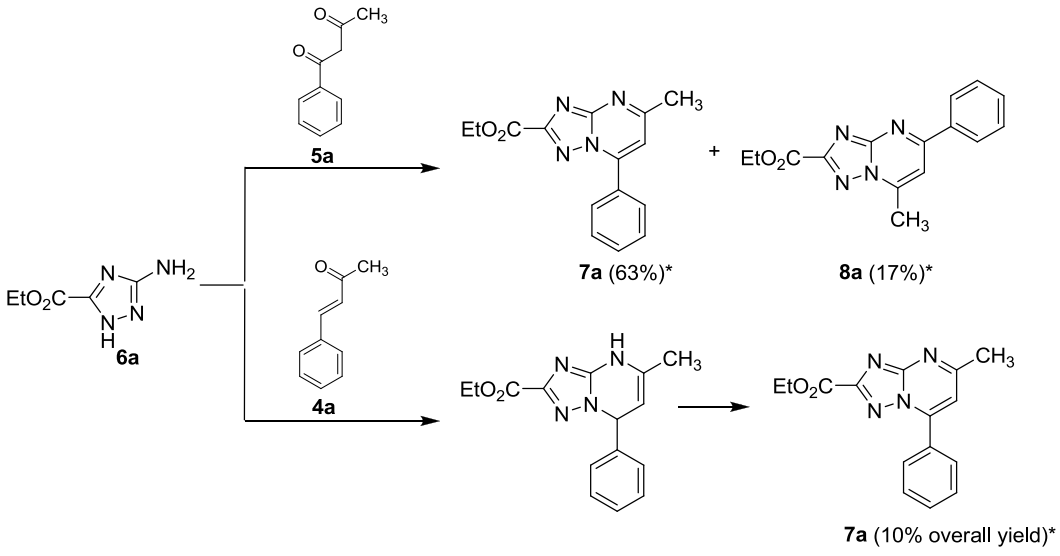
3a **5a-k** **1a-k**

Entry	R	Time (h)	Product	Yield (%)[b]
1	C_6H_5	4	**1a**	88
2	*p*-$CH_3C_6H_5$	3	**1b**	93
3	*p*-$CH_3SC_6H_5$	4	**1c**	98
4	*p*-BrC_6H_5	3	**1d**	98
5	*o*-ClC_6H_5	2	**1e**	90
6	*m*-$CF_3C_6H_5$	3	**1f**	86
7	*m,p-Di*-$CH_3OC_6H_5$	3	**1g**	80
8	*m,p-Di*-ClC_6H_5	2	**1h**	83
9	1-Naphthyl	2	**1i**	62

10	4-Pyridinyl	5	**1k**	65

[a] The reaction was performed on 1.0 mmol scale of **3a** and 1 equiv of **5** in 2.5 mL of glacial acetic acid at reflux.
[b] Isolated yields.

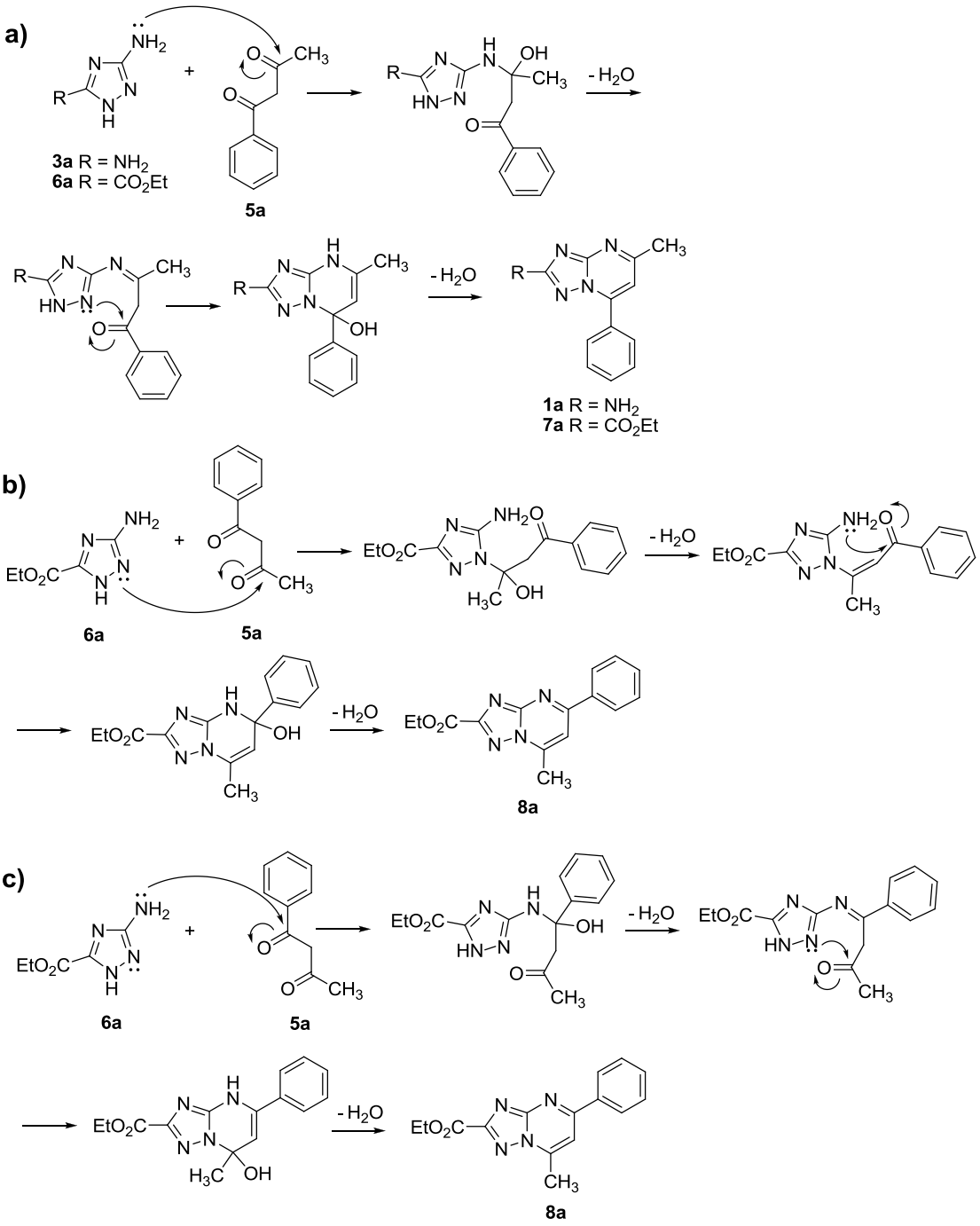
To further explore the scope of the reaction, **5a** was reacted with 5-amino-1,2,4-triazole-3-carboxylic acid, characterized by the presence of an electron-withdrawing group at the C-2 position, under the same reaction conditions. Unfortunately, the reaction was accompanied by decarboxylation, thus, it was repeated starting from ethyl 5-amino-1,2,4-triazole-3-carboxylate (**6a**). The reaction was equally rapid (4 h) and efficient (80% yield) but showed a dramatically decreased regioselectivity, in that ethyl 5-methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylate (**7a**) and ethyl 7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylate (**8a**) were obtained in the ratio of 3.7:1 (Scheme 2). Of note, although not regioselective, this reaction permitted to obtain derivatives **7a** in acceptable yield (63%) with respect to the procedure entailing the reaction of **6a** with 4-phenylbut-3-en-2-one (**4a**) followed by heteroaromatization (Scheme 2), which provided compound **7a** in a very lower yield (10%).[3a]



Scheme 2. Synthesis of **7a** *via* reaction of **6a** with **5a** and **4a**. *Isolated yields.

Plausible pathways accounting for the formation of compounds **7a** and **8a** through the reaction of **6a** with **5a** are speculatively reported in Scheme 3. In particular, an initial direct addition of the amino group at the C(5) position of **6a** on the carbonyl carbon C(3) of **5a** to give a β-aminovinyl ketone, followed by intramolecular

cyclization of the latter at the nucleophilic N(1) center of **6a** on the carbonyl C(1) of **5a** would give **7a** (Scheme 3a). The same pathway may be responsible for the formation of **1a** starting from **3a**. On the other hand, compound **8a** could be obtained by: i) an initial direct addition of N(1) of **6a** on the carbonyl carbon C(3) of **5a** followed by intramolecular cyclization of the latter by direct addition of the C(5) amino group of **6a** on the carbonyl C(1) of **5a** (Scheme 3b); or ii) an initial direct addition of the C(5) amino group of **6a** on the carbonyl carbon C(1) of **5a** followed by intramolecular cyclization of the latter by direct addition of N(1) of **6a** on the carbonyl C(3) of **5a** (Scheme 3c).



Scheme 3. Plausible reaction mechanisms toward **1a**, **7a**, and **8a**.

Although a deep investigation of the mechanisms involved in this reaction is beyond the scope of this study, the mechanism reported in Scheme 3b was hypothesized as more likely to occur compared to that in Scheme 3c. Indeed, if the steric hindrance of the phenyl group in **5a** was negligible in driving the nucleophilic attack of the amino group, the high regioselectivity observed in the reaction of **3a** could not be explained. Thus, the difference in the regioselectivity shown by the reactions of **3a** and **6a** with **5a** might depend on the different nucleophilicity of the two aminotriazoles due to the effect of the substituent. In particular, while the presence of a second electron-donating amino group in **3a** might be responsible for a higher nucleophilicity of the C(3) amino group than N(2), the electron-withdrawing ethyl carboxylate moiety in **6a** might led to a smaller difference of nucleophilicity between the C(5) amino group and N(1), resulting in a decreased selectivity in forming **7a** and **8a**. Moreover, in the acidic conditions used (pH in glacial acetic acid is reported to be 2.4) compound **3a** should be almost fully protonated, while about 34% of **6a** is in its neutral form according to MoKa predictions,[11] and this might explain why the nucleophilic attack of N(1) can occur for compound **6a** (although this remains the minor pathway). Finally, the presence of two amino groups in **3a** with a presumable comparable nucleophilicity would make their initial attack more probable in **3a** than in **6a**, as also confirmed by lost of regioselectivity observed when one of the two amino groups in **3a** was dimethylated.[6b]

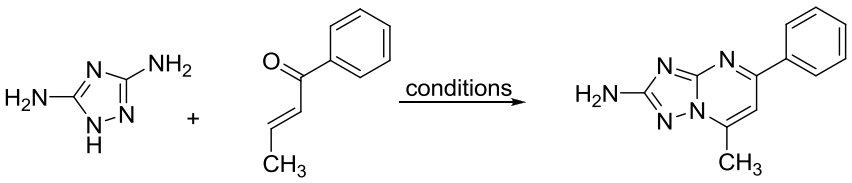
As shown above, the reaction of **3a** and **5a** under a few different conditions (entries 7 and 8 Table 1) gave a mixture of **1a** and **2a**, of which, however, the latter is always obtained in very low yield (no more than 30% yield). Thus, we searched for an alternative synthetic procedure to prepare 2-amino-5-aryl-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine derivatives, taking into account the regioselective cyclocondensation of **3a** with chalcones.

The study started by reacting **3a** with phenyl-1-propenyl-ketone (**9a**) in DMF at reflux and by adding Ac_2O, in order to obtain *N*-(7-methyl-5-phenyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)acetamide to be then heteroaromatized and hydrolysed, to give **2a**. After 30 min, the reaction gave a mixture compounds, of which one was the already oxidized *N*-(7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)acetamide derivative. Thus, the reaction was repeated without adding Ac_2O, in order to directly obtain **2a** (Table 3).

After 30 min, compound **2a** was obtained in 44% yield (entry 1),[3a] which increased up to 50% yield after 4 h (entry 2).

With these encouraging results, we further explored the reaction conditions. Based on the fact that atmospheric O_2 can enhance oxidation of 4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidines,[13] we hypothesized that an higher yield might be achieved by carrying out the reaction in an open flask. Thus, the reaction was initially carried out in different solvents (Table 3), i.e. toluene, dioxane, *N*-methylpyrrolidone (NMP), and DMF ($CHCl_3$, acetone, and THF have not been used because of the insolubility of **3a**), at 110 °C in an open flask. No significant reaction was observed in toluene (entry 3) and dioxane (entry 4) after 24 h, while the reaction in NMP (entry 5) provided compound **2a** in 25% yield after 4 h. On the other hand, the reaction in DMF (entry 6) led to the formation of **2a** in 57% yield after 2 h. Then, we studied the effect of the equiv of **9a** (entry 7), the presence of a base such as Et_3N (entry 8), and both of them (entry 9) in DMF, and found that the optimum reaction conditions are treating **3a** (2 equiv) and **9a** (1 equiv) in the presence of Et_3N (1 equiv) in DMF at 110 °C for 2 h (entry 9) in an open flask. Through this one-step procedure, compound **2a** was regioselectively obtained in 80% yield.

Table 3. Optimization of reaction conditions for **2a**.[a]



3a **9a** **2a**

Entry	**3a** (equiv)	**Et_3N** (equiv)	Solvent	Time (h)	Yield (%)[b]
1	1	no base	DM[c]	0.5	44
2	1	no base	DMF[c]	4	50
3	1	no base	Toluene	24	-

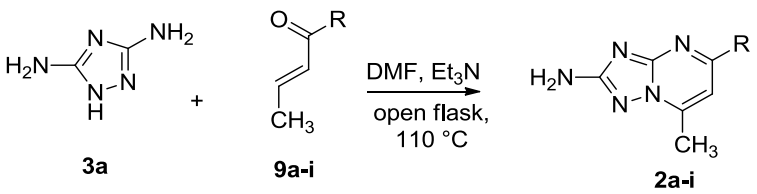
4	1	no base	Dioxane	24	-
5	1	no base	NMP	4	25
6	1	no base	DMF	2	57
7	2	no base	DMF	2	67
8	1	1	DMF	2	71
9	2	1	DMF	2	80

[a] Unless otherwise indicated, the reaction was performed on 1.0 mmol scale of **9a** in 2.5 mL of solvent in an open flask at 110 °C.
[b] Isolated yields.
[c] Using the condenser.

With these optimized reaction conditions, we next studied the scope of the reaction (Table 4). Thus, 1-aryl-2-buten-1-ones (**9b-i**) containing different electron withdrawing as well as electron-donating groups on the phenyl ring were synthesized and reacted with **3a**. 1-Aryl-2-buten-1-ones were synthesized through a Witting reaction. In particular, 2-bromoacetophenones were treated with triphenylphosphine in toluene giving triphenylphosphonium bromides,[13] which were then reacted with aqueous NaOH in dichloromethane to afford 1-aryl-2-(triphenylphosphoranylidene) ethanones, and then with acetaldehyde in a Witting reaction to give the corresponding 1-aryl-2-buten-1-ones[14] (for the synthesis and characterization of compounds **9b-i**, see SI).

The results shown in Table 4 highlight that 1-aryl-2-buten-1-ones bearing both electron-donating and electron-withdrawing substituents on the phenyl ring are suitable substrates for this reaction. Indeed, with the exception of 4-nitrophenyl- (entry 4) and 4-pyridinyl- (entry 9) 2-buten-1-ones, which gave derivatives **2d** and **2i** in modest yields, all the other studied 1-aryl-2-buten-1-ones (entries 2, 3, and 5-7) reacted efficiently and regioselectively with **3a** to give 2-amino-5-aryl-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines in consistently good yields.

Table 4. Preparation of 2-amino-5-aryl-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines.[a]

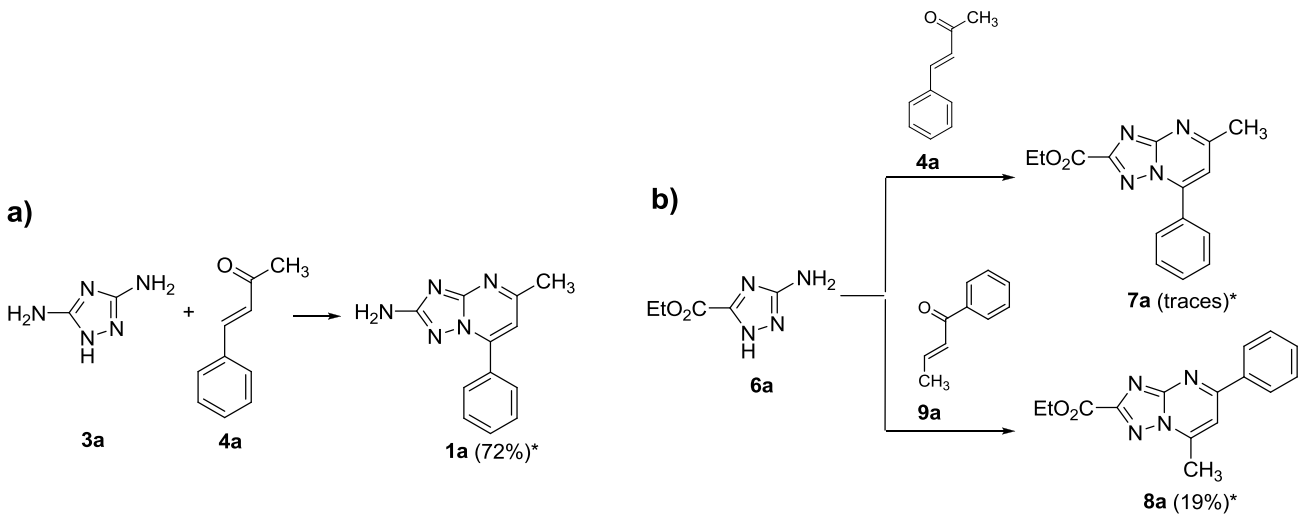


3a + **9a-i** → **2a-i**

Entry	R	Time (h)	Product	Yield (%)[b]
1	C_6H_5	2	**2a**	80
2	*p*-$CH_3C_6H_5$	3	**2b**	83
3	*p*-$CH_3OC_6H_5$	2	**2c**	92
4	*p*-$NO_2C_6H_5$	2	**2d**	73
5	*p*-ClC_6H_5	2	**2e**	80
6	*m*-BrC_6H_5	2	**2f**	87
7	*o*-FC_6H_5	3	**2g**	80
8	*o,p-Di*-FC_6H_5	2	**2h**	88
9	4-Pyridinyl	2	**2i**	60

[a] The reaction was carried out on 1.0 mmol scale of **3a**, 0.5 mmol of **9**, and 0.5 mmol of Et_3N in 2.5 mL of DMF in an open flask at 110 °C.
[b] Isolated yields.

To further explore the scope of the reaction, by using the same reaction conditions, **3a** was reacted with 4-phenylbut-3-en-2-one (**4a**) giving isomer **1a** regioselectively in 72% yield after 6 h (Scheme 4a). Of note, although in lower yield (72%) than the reaction of **3a** with **5a** (88% yield), the reaction of **3a** with **4a** under these conditions is an alternative one-step procedure for the regioselective synthesis of **1a**, which was obtained with comparable yield to that of the known three-step procedure (77% yield).[5a] Finally, by using the same reaction conditions, ethyl 5-amino-1,2,4-triazole-3-carboxylate (**6a**) was also reacted with both **4a** and **9a**. Surprisingly, isomers **7a** and **8a** were obtained in traces and 19% yield, respectively, after 24 h (Scheme 4b).



Scheme 4 a) Synthesis of **1a** *via* reaction of **3a** with **4a**; b) Synthesis of **7a** and **8a** *via* reaction of **3a** with **4a** and **8a** respectively. *Isolated yields.

All the compounds herein reported, with the exception of **1a**, were not described previously and their structures were fully characterized by spectra data of ^{1}H NMR, ^{13}C NMR, and HRMS. It is worthwhile to underline that all the products obtained from both the procedures were purified by simple crystallization without the involvement of chromatography.

[1,2,4]triazolo[1,5-*a*]pyrimidines as anti-flu compounds

As mentioned above, we have recently identified a series of potent anti-flu compounds based on the [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold that act by inhibiting flu RdRP PA-PB1 subunits interaction.[3a] In particular, within the optimization of compound **I** (Fig. 1) we prepared a large series of analogues, along with a few derivatives characterized by the oxidized [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold (structure II and III, Fig. 1).[3a] Among them, derivatives **10** and **11** (Table 5) showed a good ability to inhibit flu replication (EC_{50} = 42 and 25 μM, respectively) at non toxic concentrations (CC_{50} > 250 μM). Compound **11** also inhibited PA-PB1 interaction with a comparable IC_{50} (26 μM).

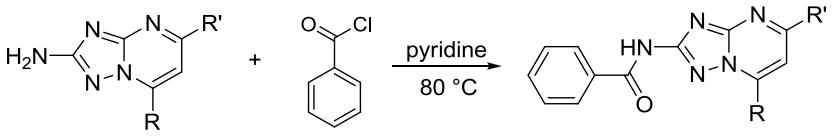
In order to add structure-activity relationship insights, we decided to exploit the scaffolds herein synthesized by preparing an additional set of compounds to study the effect of modifications on the C-5, C-7, and C-2

positions of the [1,2,4]triazolo[1,5-*a*]pyrimidine core. Thus, 2-amino derivatives **1e**, **1f**, **2c**, **2d**, and **2e** variously functionalized at the C-5 or C-7 position were reacted with benzoyl chloride in pyridine at 80 °C providing target derivatives **12-16** (Table 5). To study the C-2 position, target compounds **19** and **20** (Table 5) were prepared starting from 2-carboxylate scaffolds **7a** and **8a**, which were hydrolyzed to give intermediates **17** and **18**, chlorinated, and then reacted with 2-aminobenzamide in CH_2Cl_2 in the presence of DIPEA.

The synthesized compounds were first evaluated for the ability to inhibit the physical interaction between fluA PA and PB1 subunits by ELISA including the Tat-$PB1_{1-15}$ peptide[15] 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,[16] was also included. To exclude that the observed antiviral activities could be due to toxic effects on the target cells, the compounds were also tested by MTT assays in MDCK cells.

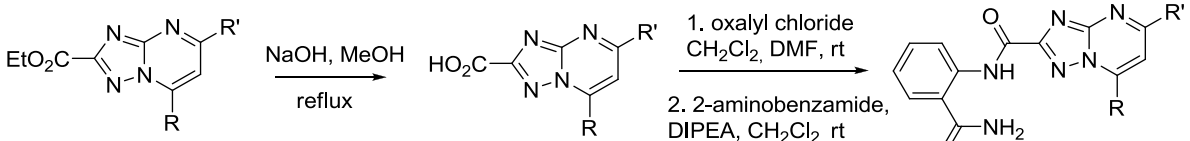
As shown in Table 5, derivatives **12-16**, which were functionalized on the phenyl ring at the C-5 or C-7 position, although nontoxic, resulted unable to inhibit the viral growth at low micromolar concentrations. Nevertheless, *p*-nitrophenyl and *p*-chlorophenyl derivatives **15** and **16** showed a good ability to interfere with PA-PB1 heterodimerization (IC_{50} = 25 and 40 μM, respectively). The lack of antiviral activity was shown also by compound **19**, in which however the presence of the benzamide moiety at the C-2 position led to increase of about 15 folds the anti-PA-PB1 activity (IC_{50} = 11 μM) with respect to the strict analogue compound **10** (IC_{50} = 160 μM). The best and most balanced results was achieved with compound **20**, which showed both the ability to inhibit viral replication and PA-PB1 heterodimerization at non toxic concentrations. In particular, derivative **20** showed a slightly decreased anti-flu activity (EC_{50} = 31 μM) but an enhanced ability to inhibit PA-PB1 complex formation (IC_{50} = 11 μM) with respect to its analogue **11** (IC_{50} = 25 μM), and even better than the reference $PB1_{1–15}$–Tat peptide (IC_{50} = 41 μM).

Table 5. Synthesis and biological activity of [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives.



1e,f and **2c,d,h** **12-16 (10,11)**

Compd	R	R'	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]
10	C_6H_5	CH_3	160 ± 11	42 ± 5	>250
11	CH_3	C_6H_5	26 ± 5	25 ± 1	>250
12	*o*-ClC_6H_5	CH_3	>200	>100	>250
13	*m*-$CF_3C_6H_5$	CH_3	163 ± 20	>100	>250
14	CH_3	*p*-$CH_3OC_6H_5$	160 ± 1	92 ± 5	>250
15	CH_3	*p*-$NO_2C_6H_5$	25 ± 2	92 ± 5	>250
16	CH_3	*p*-ClC_6H_5	40 ± 4	99 ± 2	>250



7a and **8a** **17** and **18** **19** and **20**

Compd	R	R'	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]
19	C_6H_5	CH_3	11 ± 3	>100	>250
20	CH_3	C_6H_5	7 ± 1	31 ± 10	>250
Tat-PB1$_{1-15}$ peptide			35 ± 4	41 ± 5	>100
RBV				10 ± 2	>250

[a] Compounds activity in ELISA PA–PB1 interaction assays. The IC_{50} value is defined as the compound concentration that reduces the PA-PB1 interaction by 50%. [b] Antiviral activity of the compounds against the fluA A/PR/8/34 strain in plaque reduction assays. The EC_{50} value represents the effective compound concentration required to reduce virus plaque formation by 50%. [c] Citotoxicity of the compounds in MTT assays. The CC_{50} value represents the compound concentration resulting in 50% inhibition of MDCK cell viability. All the reported values represent the means ± SD of data obtained from at least three independent experiments in duplicate.

Conclusions

In summary, two facile and efficient one-step procedures for the regioselective synthesis of 7-aryl-5-methyl- and 5-aryl-7-methyl-2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidines have been developed. These procedures have proven to be suitable for 1-aryl-1,3-butanediones and 1-aryl-2-buten-1-ones with different substitution patterns on the phenyl ring, permitting to obtain 2-amino-[1,2,4]triazolo[1,5-*a*]pyrimidines variously functionalized at the C-5 and C-7 positions, respectively. The synthesized derivatives may be useful for the preparation of biologically active compounds. In this study, they have been used for the synthesis of a set of [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives as anti-flu compounds. From this study, derivative **20** emerged as a new potential antiviral compound endowed with a very good ability to inhibit flu RNA polymerase complex formation.

Experimental

Material and methods

Commercially available starting materials, reagents, and solvents were used as supplied. Compounds 4-phenylbut-3-en-2-one (**4a**), 1-phenyl-1,3-butanedione (**5a**), and phenyl-1-propenyl-ketone (**9a**) were purchased from Alfa Aesar and Apollo Scientific. Synthesis of 1-aryl-1,3-butanediones (**5b-k**) and 1-aryl-2-buten-1-ones (**9b-i**) was reported in the SI. Compound 5-amino-1,2,4-triazole-3-carboxylate (**6a**) was synthesized as reported in literature.[17] Hydrolysis of compounds **8a** and **9a** to [1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylic acid **16** and **17**, respectively, was carried out as previously reported by us.[3a]

All reactions were routinely monitored 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. Purities of compounds **12-16**, **19**, and **20** were determined by UHPLC on Agilent Technologies 6540 UHD Accurate Mass Q-TOF LC/MS, HPLC 1290 Infinity with DAD detector and evaluated to be 100% pure. 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 254 nm; oven temperature, 30 °C; gradient of acetonitrile in water containing 0.1% of formic acid (0−100% in 10 min). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance DRX-400MHz using residual solvents such as dimethylsulfoxide (δ = 2.48) or chloroform (δ = 7.26) 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).

General procedure for the synthesis of 2-amino-7-aryl-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines (1a-k). A mixture of the appropriate 1-aryl-1,3-butanedione (**5a-k**) (1 mmol) and **3a** (1 mmol) in glacial acetic acid (2.5 mL) was refluxed until no starting material was detected by TLC (2-5h). After cooling, the reaction mixture was poured into ice/water and neutralized with 10% NaOH, obtaining a precipitate that was filtered and crystallized by EtOH/DMF.

5-Methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1a). White crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.52 (s, 3H, CH_3), 6.32 (s, 2H, NH_2), 7.14 (s, 1H, H-6), 7.56-7.57 (m, 3H, aromatic CH), 8.10-8.12 (m, 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 107.3, 128.4, 129.1, 130.3, 131.0, 143.8, 155.7, 161.6, 167.1; HRMS: *m/z* calcd for $C_{12}H_{11}N_5$ 226.1093 (M + H^+), found 226.1021 (M + H^+).

5-Methyl-7-*p*-tolyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1b). White crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.38 and 2.51 (s, each 3H, CH_3), 6.32 (s, 2H, NH_2), 7.15 (s, 1H, H-6), 7.37 and 8.06 (d, *J* = 8.0 Hz, each 2H,

aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 21.1, 24.1, 106.9, 127.4, 129.0, 129.1, 141.2, 143.8, 155.8, 161.5, 167.1; HRMS: *m/z* calcd for $C_{13}H_{13}N_5$ 240.1250 (M + H^+), found 240.1249 (M + H^+).

5-Methyl-7-(4-(methylthio)phenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1c). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.51 and 2.54 (s, each 3H, CH_3), 6.32 (s, 2H, NH_2), 7.17 (s, 1H, H-6), 7.41 and 8.13 (d, *J* = 8.5 Hz, each 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 14.1, 24.1, 106.7, 124.9, 126.1, 129.5, 142.7, 143.3, 155.8, 161.5, 167.0; HRMS: *m/z* calcd for $C_{13}H_{13}N_5S$ 272.0971 (M + H^+), found 272.0968 (M + H^+).

7-(4-Bromophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1d). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.52 (s, 3H, CH_3), 6.35 (s, 2H, NH_2), 7.19 (s, 1H, H-6), 7.78 and 8.09 (d, *J* = 8.5 Hz, each 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 107.3, 124.7, 129.5, 131.2, 131.5, 142.6, 155.7, 161.6, 167.1; HRMS: *m/z* calcd for $C_{12}H_{10}BrN_5$ 304.0199 (M + H^+), found 304.0199 (M + H^+).

7-(2-Chlorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1e). Light yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.53 (s, 3H, CH_3), 6.31 (s, 2H, NH_2), 6.99 (s, 1H, H-6), 7.51-7.65 (m, 4H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 109.0, 127.4, 129.5, 130.3, 131.4, 131.9, 132.1, 142.2, 154.9, 161.5, 167.2; HRMS: *m/z* calcd for $C_{12}H_{10}ClN_5$ 260.0704 (M + H^+), found 260.0702 (M + H^+).

5-Methyl-7-(3-(trifluoromethyl)phenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1f). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.54 (s, 3H, CH_3), 6.38 (bs, 2H, NH_2), 7.29 (s, 1H, H-6), 7.82 (t, *J* = 7.8 Hz, 1H, aromatic CH), 7.95 and 8.40 (d, *J* = 7.8 Hz, each 1H, aromatic CH), 8.48 (s, 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 107.9, 125.8 (q, $J_{C\text{-}F}$ = 3.03 Hz), 126.6 (q, $J_{C\text{-}F}$ = 2.72.7 Hz), 127.5 (q, $J_{C\text{-}F}$ = 3.03 Hz), 129.2 (d, $J_{C\text{-}F}$ = 32.3 Hz), 129.7, 131.4, 133.2, 142.2, 155.6, 161.8, 167.0; HRMS: *m/z* calcd for $C_{13}H_{10}F_3N_5$ 294.0961 (M + H^+), found 294.0967 (M + H^+).

7-(3,4-Dimethoxyphenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1g). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.50 (s, 3H, CH_3), 3.84 (s, 6H, OCH_3), 6.28 (s, 2H, NH_2), 7.11 (d, *J* = 8.5 Hz, 1H, aromatic CH), 7.20 (s, 1H, H-6), 7.77 (d, *J* = 1.8 Hz, 1H, aromatic CH), 7.90 (dd, *J* = 1.8 and 8.5 Hz, 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 55.7, 55.7, 106.5, 111.2, 112.6, 122.3, 122.9, 143.6, 148.3, 151.1, 155.9, 161.3, 166.9; HRMS: *m/z* calcd for $C_{14}H_{15}N_5O_2$ 286.1305 (M + H^+), found 286.1303 (M + H^+).

7-(3,4-Dichlorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1h). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.52 (s, 3H, CH_3), 6.41 (s, 2H, NH_2), 7.27 (s, 1H, H-6), 7.85 (d, J = 8.5 Hz, 1H, aromatic CH), 8.12 (dd, J = 2.0 and 8.5 Hz, 1H, aromatic CH) 8.47 (d, J = 2.0 Hz, 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.1, 107.6, 129.3, 130.7, 131.7, 131.0, 131.3, 133.7, 141.1, 155.7, 161.7, 167.1; HRMS: *m/z* calcd for $C_{12}H_9Cl_2N_5$ 294.0314 (M + H^+), found 294.0315 (M + H^+).

5-Methyl-7-(naphthalen-1-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1i). White powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.56 (s, 3H, CH_3), 6.17 (s, 2H, NH_2), 7.03 (s, 1H, H-6), 7.36 (d, J = 8.3 Hz, 1H, naphthalene CH), 7.45-7.49 and 7.54-7.58 (m, each 1H, naphthalene CH), 7.62-7.69 (m, 2H, naphthalene CH), 8.03 and 8.12 (d, J = 8.0 Hz, each 1H, naphthalene CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.3, 110.0, 125.2, 125.5, 126.6, 127.3, 127.9, 128.5, 129.0, 129.8, 130.6, 133.0, 144.2, 155.1, 161.9, 167.1; HRMS: *m/z* calcd for $C_{16}H_{13}N_5$ 276.125 (M + H^+), found 276.1247 (M + H^+).

5-Methyl-7-(pyridin-4-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (1k). White crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.54 (s, 3H, CH_3), 6.42 (s, 2H, NH_2), 7.30 (s, 1H, H-6), 8.10 and 8.80 (d, J = 4.6 Hz, each 2H, pyridine CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.2, 107.7, 123.0, 137.7, 141.1, 150.1, 155.6, 161.8, 167.2; HRMS: *m/z* calcd for $C_{11}H_{10}N_6$ 227.1046 (M + H^+), found 227.1044 (M + H^+).

Ethyl 5-methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylate (7a) and ethyl 7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylate (8a). The title compounds were prepared through the general procedure for the synthesis of 2-amino-5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidines by replacing **3a** with ethyl 5-amino-1,2,4-triazole-3-carboxylate (**6a**),[15] and were separated by flash chromatography eluting with $CHCl_3$/acetone (9:1). **7a**: white solid (63% yield); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.31 (t, J = 7.0 Hz, CH_2CH_3), 2.69 (s, 3H, CH_3), 4.38 (q, J = 7.0 Hz, CH_2CH_3), 7.63-7.65 (m, 4H, H-6 and aromatic CH), 8.07-8.09 (m, 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 14.1, 24.8, 61.7, 112.2, 128.7, 129.3, 129.5, 131.7, 146.7, 155.6, 155.9, 160.0, 166.9; HRMS: *m/z* calcd for $C_{15}H_{14}N_4O_2$ 283.1196 (M + H^+), found 283.1196 (M + H^+); **8a**: white solid (17% yield); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.35 (t, J = 7.0 Hz, CH_2CH_3), 2.84 (s, 3H, CH_3), 4.41 (q, J = 7.0 Hz, CH_2CH_3), 7.59-7.60 (m, 3H, aromatic CH), 8.10 (s, 1H, H-6), 8.25-8.27 (m, 2H, aromatic CH); ^{13}C NMR

(101 MHz, DMSO-d_6) δ: 14.1, 17.0, 61.7, 108.9, 127.7, 129.2, 131.7, 135.7, 149.1, 155.0, 156.3, 159.9, 161.3; HRMS: *m/z* calcd for $C_{15}H_{14}N_4O_2$ 283.1196 (M + H^+), found 283.1194 (M + H^+).

General procedure for the synthesis of 2-amino-5-aryl-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidines (2a-i). To a mixture of the appropriate 1-aryl-2-buten-1-ones (**9a-i**) (0.5 mmol) and **3a** (1 mmol) in dry DMF (2.5 mL), dry Et_3N (0.5 mmol) was added and the reaction mixture was heated at 110 °C until no starting material was detected by TLC (2-3h). After cooling, the reaction mixture was poured into ice/water, obtaining a precipitate that was filtered and crystallized by EtOH/DMF.

7-Methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2a). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.64 (s, 3H, CH_3), 6.41 (s, 2H, NH_2), 7.49-7.54 (m, 3H, aromatic CH), 7.59 (s, 1H, H-6), 8.13-8.15 (m, 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.1, 104.7, 127.0, 128.9, 130.4, 136.7, 145.4, 155.1, 156.8, 167.5; HRMS: *m/z* calcd for $C_{12}H_{11}N_5$ 226.1093 (M + H^+), found 266.1088 (M + H^+).

7-Methyl-5-*p*-tolyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2b). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.36 and 2.63 (s, each 3H, CH_3), 6.38 (s, 2H, NH_2), 7.32 (d, *J* = 6.0 Hz, each 1H, aromatic CH), 7.55 (s, 1H, H-6), 8.05 (d, *J* = 6.0 Hz, each 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.4, 21.3, 104.7, 127.2, 129.8, 134.2, 140.6, 145.7, 155.4, 157.2, 167.7; HRMS: *m/z* calcd for $C_{13}H_{13}N_5$ 240.1250 (M + H^+), found 240.1251 (M + H^+).

5-(4-Methoxyphenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2c). Yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.62 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 6.34 (s, 2H, NH_2), 7.06 (d, *J* = 8.8 Hz, 2H, aromatic CH), 7.51 (s, 1H, H-6), 8.11 (d, *J* = 8.8 Hz, 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.0, 55.4, 104.0, 114.0, 128.6, 129.0, 145.2, 155.1, 156.7, 161.2, 167.3; HRMS: *m/z* calcd for $C_{13}H_{13}N_5O$ 256.1199 (M + H^+), found 256.1193 (M + H^+).

7-Methyl-5-(4-nitrophenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2d). Yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.66 (s, 3H, CH_3), 6.54 (s, 2H, NH_2), 7.71 (s, 1H, H-6), 8.33 and 8.38 (d, *J* = 8.9 Hz, each 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.3, 105.7, 124.2, 128.3, 142.7, 146.0, 148.4, 154.2, 155.1, 167.9; *m/z* calcd for $C_{12}H_{10}N_6O_2$ 271.0944 (M + H^+), found 271.0944 (M + H^+).

5-(4-Chlorophenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2e). Yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.64 (s, 3H, CH_3), 6.45 (s, 2H, NH_2), 7.57-7.61 (m, 3H, aromatic CH and H-6), 8.17 (d, J = 8.4 Hz, 2H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.1, 104.6, 128.8, 129.0, 135.2, 135.5, 145.6, 155.0, 155.4, 167.6; HRMS: *m/z* calcd for $C_{12}H_{10}ClN_5$ 260.0704 (M + H^+), found 260.0681 (M + H^+).

5-(3-Bromophenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2f). Light brown crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.63 (s, 3H, CH_3), 6.42 (s, 2H, NH_2), 7.47 (t, J = 7.8 Hz, 1H, aromatic CH), 7.61 (s, 1H, H-6), 7.67 and 8.12 (d, J = 7.8 Hz, each 1H, aromatic CH), 8.29 (s, 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.3, 105.2, 122.6, 126.2, 129.7, 131.4, 133.3, 139.1, 146.1, 155.2, 155.4, 167.7; HRMS: *m/z* calcd for $C_{12}H_{10}BrN_5$ 304.0199 (M + H^+), found 304.0194 (M + H^+).

5-(2-Fluorophenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2g). Yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.66 (s, 3H, CH_3), 6.49 (s, 2H, NH_2), 7.33-7.51 (m, 3H, aromatic CH and H-6), 7.52-7.57 (m, 1H, aromatic CH), 7.97 (dt, J = 1.5 and 7.8 Hz, 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.1, 108.2 (d, J = 10.1 Hz), 116.5 (d, J = 22.2 Hz), 125.0, 125.1 (d, J = 22.2 Hz), 130.9 (d, J = 3.0 Hz), 132.1 (d, J = 8.0 Hz), 145.2, 153.4, 154.9, 160.0 (d, J = 251.4 Hz), 167.6; HRMS: *m/z* calcd for $C_{12}H_{10}FN_5$ 244.0999 (M + H^+), found 244.0997 (M + H^+).

5-(2,4-Difluorophenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2h). Light yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.65 (s, 3H, CH_3), 6.49 (s, 2H, NH_2), 7.24-7.29 (m, 2H, aromatic CH), 7.31 (s, 1H, H-6), 7.41-7.47 and 8.02-8.08 (m, each 1H, aromatic CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.1, 104.9 (t, $J_{C\text{-}F}$ = 26.7 Hz), 107.9 (d, $J_{C\text{-}F}$ = 10.1 Hz), 112.4 (dd, $J_{C\text{-}F}$ = 3.0 and 21.2 Hz), 122.0 (dd, $J_{C\text{-}F}$ = 4.0 and 13.1 Hz), 132.4 (dd, $J_{C\text{-}F}$ = 4.0 and 11.1 Hz), 145.3, 152.5, 154.9, 160.3 (dd, $J_{C\text{-}F}$ = 13.1 and 251.4 Hz), 163.2 (dd, $J_{C\text{-}F}$ = 13.1 and 252.5 Hz), 167.3; HRMS: *m/z* calcd for $C_{12}H_9F_2N_5$ 262.0905 (M + H^+), found 262.0899 (M + H^+).

7-Methyl-5-(pyridin-4-yl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-amine (2i). Yellow crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.68 (s, 3H, CH_3), 6.56 (s, 2H, NH_2), 7.73 (s, 1H, H-6), 8.07 and 8.73 (dd, J = 1.6 and 4.6 Hz, each 2H, pyridine CH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.2, 105.2, 120.9, 143.7, 145.9, 150.5, 154.0, 155.0, 167.8; HRMS: *m/z* calcd for $C_{11}H_{10}N_6$ 227.1046 (M + H^+), found 277.1041 (M + H^+).

General procedure for the synthesis of compounds 12-16 by amidation. A solution of benzoyl chloride (2.0 mmol) in dry pyridine (5 mL) was added dropwise to a solution of the appropriate [1,2,4]triazolo[1,5-*a*]pyrimidine-2-amine (**1e**, **1f**, **2c**, **2d**, or **2e**) (1.0 mmol) in dry pyridine (15 mL), and then the reaction mixture was maintained at 80 °C overnight. After cooling, it was poured into ice/water, obtaining a precipitate that was filtered and purified as described below.

***N*-(7-(2-chlorophenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)benzamide (12).** The title compound was prepared starting from **1e** and purified by flash chromatography eluting with CH_2Cl_2/MeOH (98:2) in 71% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.67 (s, 3H, CH_3), 7.34 (s, 1H, H-6), 7.46-7.50 and 7.57-7.58 (m, each 2H, aromatic CH), 7.64 (t, *J* = 7.2 Hz, 1H, aromatic CH), 7.70-7.72 (m, 2H, aromatic CH), 7.96 (d, *J* = 7.5 Hz, 2H, aromatic CH), 11.30 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.6, 112.0, 127.5, 128.0, 128.3, 129.6, 129.7, 131.5, 132.1, 132.3, 132.3, 133.4, 143.9, 154.0, 160.1, 164.6, 164.8.; HRMS: *m/z* calcd for $C_{19}H_{14}ClN_5O$ 364.0966 (M + H^+), found 364.0965 (M + H^+).

***N*-(5-methyl-7-(3-(trifluoromethyl)phenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)benzamide (13).** The title compound was prepared starting from **1f** and purified by flash chromatography eluting with CH_2Cl_2/MeOH (98:2) in 45% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.67 (s, 3H, CH_3), 7.48-7.52 (m, 2H, aromatic CH), 7.57-7.61 (m, 1H, aromatic CH), 7.63 (s, 1H, H-6), 7.87 (t, *J* = 7.8 Hz, 1H, aromatic CH), 7.99-8.01 (m, 3H, aromatic CH), 8.51 (d, *J* = 7.9 Hz, 1H, aromatic CH), 8.59 (s, 1H, aromatic CH), 11.34 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 24.5, 110.3, 123.8 (q, *J* = 273.7 Hz), 126.2 (q, *J* = 3.0 Hz), 127.9 (q, *J* = 3.0 Hz), 128.0, 128.3, 129.4 (q, *J* = 32.3 Hz), 129.8, 130.7, 132.1, 133.4, 133.5, 154.6, 159.9, 164.6, 164.8; HRMS: *m/z* calcd for $C_{20}H_{14}F_3N_5O$ 398.1229 (M + H^+), found 398.1227 (M + H^+).

***N*-(5-(4-methoxyphenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)benzamide (14).** The title compound was prepared starting from **2c** and purified by flash chromatography eluting with CH_2Cl_2/acetone (8:2) in 62% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.78 and 3.84 (s, each 3H, CH_3), 7.11 (d, *J* = 8.8 Hz, 2H), 7.50-7.54 (m, 2H, aromatic CH), 7.60 (d, *J* = 7.2 Hz, 1H, aromatic CH), 7.84 (s, 1H, H-6), 8.02 (d, *J* = 7.4 Hz, 2H, aromatic CH), 8.22 (d, *J* = 8.8 Hz, 2H, aromatic CH), 11.34 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.4,

55.8, 106.6, 114.8, 128.4, 128.8, 128.8, 129.4, 132.5, 134.0, 147.7, 154.5, 159.4, 160.5, 162.1, 165.2; HRMS: *m/z* calcd for $C_{20}H_{17}N_5O_2$ 360.1461 (M + H^+), found 360.1460 (M + H^+).

***N*-(7-methyl-5-(4-nitrophenyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)benzamide (15).** The title compound was prepared starting from **2d** and purified by flash chromatography eluting with CH_2Cl_2/acetone (8:2) in 64% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.82 (s, 3H, CH_3), 7.50-7.54 (m, 2H, aromatic CH), 7.60 (t, *J* = 7.2 Hz, 1H, aromatic CH), 8.01-8.03 (m, 3H, H-6 and aromatic CH), 8.39 and 8.46 (d, *J* = 8.8 Hz, each 2H aromatic CH), 11.47 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.2, 107.6, 124.1, 128.1, 128.4, 128.6, 132.2, 133.6, 142.0, 148.4, 148.7, 153.9, 156.7, 160.9, 164.8; HRMS: *m/z* calcd for $C_{19}H_{14}N_6O_3$ 375.1206 (M + H^+), found 375.1201 (M + H^+).

***N*-(5-(4-chlorophenyl)-7-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-2-yl)benzamide (16).** The title compound was prepared starting from **2e** and purified by flash chromatography eluting with CH_2Cl_2/acetone (8:2) in 52% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.78 (s, 3H, CH_3), 7.48-7.52 (m, 2H, aromatic CH), 7.57-7.63 (m, 3H, aromatic CH), 7.90 (s, 1H, H-6), 8.00 and 8.23 (d, *J* = 8.2 Hz, each 2H, aromatic CH), 11.38 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.1, 106.8, 128.1, 128.4, 129.1, 129.1, 132.1, 133.6, 135.0, 136.0, 147.9, 153.9, 158.0, 160.5, 164.8; HRMS: *m/z* calcd for $C_{19}H_{13}F_2N_5O$ 366.1167 (M + H^+), found $C_{19}H_{14}ClN_5O$ 364.0966 (M + H^+), found 364.0963 (M + H^+).

General procedure for the synthesis of compounds 19 and **20 by amidation.** To a solution of the appropriate [1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxylic acid (**17**[3a] or **18**[3a]) (2 mmol) in well dry CH_2Cl_2 (20 mL), oxalyl chloride (12 mmol) was added and after 30 min dry DMF (2 drops) was added. After 2 h, the reaction mixture was evaporated to dryness to give a residue that was dissolved in well dry CH_2Cl_2 and added of the appropriate aniline (2 mmol) and DIPEA (2 mmol). The reaction was maintained at rt until no starting material was detected by TLC (4h for **19** and 1h for **20**). The work up of the reaction and compound purification are reported below.

***N*-(2-carbamoylphenyl)-5-methyl-7-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxamide (19).** The reaction mixture was evaporated to dryness and treated with ice/water obtaining a precipitate that was filtered and purified by flash chromatography eluting with CH_2Cl_2/MeOH (98:2), to give **19** in 52% yield; ^{1}H-

NMR (DMSO-d_6) δ: 2.70 (s, 3H, CH_3), 7.20 and 7.57 (t, J = 7.5 Hz, each 1H, aromatic CH), 7.69-7.62 (m, 4H, H-6 and aromatic CH), 7.75 (bs, 1H, CONH_2), 7.84 (d, J = 7.7 Hz, 1H, aromatic CH), 8.25-8.19 (m, 2H, aromatic CH), 8.33 (bs, 1H, CONH_2), 8.66 (d, J = 8.2 Hz, 1H, aromatic CH), 13.14 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 25.0, 111.9, 120.5, 120.8, 123.6, 128.9, 129.0, 129.4, 129.8, 132.0, 132.6, 138.7, 146.8, 155.8, 157.4, 158.6, 167.0, 170.6; HRMS: *m/z* calcd for $C_{20}H_{16}N_6O_2$ 373.1414 (M + H^+), found 373.1411 (M + H^+).

***N*-(2-carbamoylphenyl)-7-methyl-5-phenyl-[1,2,4]triazolo[1,5-*a*]pyrimidine-2-carboxamide (20).** The reaction mixture was filtered and the precipitate was washed with Et_2O, and then purified by flash chromatography eluting with CH_2Cl_2/MeOH (98:2), to give **20** in 59% yield as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.88 (s, 3H, CH_3), 7.22 (t, J = 7.5 Hz, 1H, aromatic CH), 7.57-7.60 (m, 4H, aromatic CH), 7.78 (bs, 1H, CONH_2), 7.86 (d, J = 7.8 Hz, 1H, aromatic CH), 8.10 (s, 1H, H-6), 8.38 – 8.25 (m, 3H, aromatic CH and CONH_2), 8.73 (d, J = 8.3 Hz, 1H, aromatic CH, 13.16 (s, 1H, NH); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 17.0, 108.7, 120.4, 120.6, 123.3, 127.7, 128.7, 129.2, 131.6, 132.3, 135.8, 138.6, 149.1, 154.9, 157.2, 158.9, 161.2, 170.4; HRMS: *m/z* calcd for $C_{20}H_{16}N_6O_2$ 373.1414 (M + H^+), found 373.1413 (M + H^+).

Biological assays

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.[18]

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).

PA-PB1 interaction enzyme-linked immunosorbent assay (ELISA). The PA–PB1 interaction was detected as described,[3c] with some modifications.[19] 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.[3c]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 incubated in serum-free DMEM O/N at room temperature as described.[19] *Escherichia coli*-expressed, purified GST and GST-$PB1_{(1–25)}$ proteins were obtained as previously described.[3c,20] 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.

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.[3c,21] 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.

Plaque reduction assay (PRA). The antiviral activity of test compounds against influenza A virus was tested by PRA as previously described.[3c] 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%.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This work was supported by University of Perugia, Ricerca di base 2015 – cod. 3RICBASEOT (to OT and SM), by the University of Padova, Progetto di Ricerca di Ateneo 2014 grant n. CPDA141311 and Ex-60% (to AL), and by Associazione Italiana per la Ricerca sul Cancro (AIRC), Investigator Grant n. 18855 (to AL).

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