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Novel selective inhibitor of *Leishmania (Leishmania) amazonensis* arginase

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Abstract

Arginase is a glycosomal enzyme in *Leishmania* that is involved in polyamine and trypanothione biosynthesis. The central role of arginase in *L. (L.) amazonensis* was demonstrated by the generation of two mutants: one with an arginase lacking the

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glycosomal addressing signal and one in which the arginase coding gene was knocked out. Both of these mutants exhibited decreased infectivity. Thus, arginase seems to be a potential drug target for *Leishmania* treatment. In an attempt to search for arginase inhibitors, twenty-nine derivatives of the [1,2,4]triazolo[1,5-*a*]pyrimidine system were tested against *L. (L.) amazonensis* arginase *in vitro*. The [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold containing R_1 = CF_3 exhibited greater activity against the arginase rather than when the substituent R_1 = CH_3 in the 2-position. The novel compound 2-(5-methyl-2-(trifluoromethyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)hydrazinecarbothioamide **(30)** was the most potent, inhibiting arginase by a non-competitive mechanism, with the Ki and IC_{50} values for arginase inhibition estimated to be 17 ± 1 μM and 16.5 ± 0.5 μM, respectively. These results can guide the development of new drugs against leishmaniasis based on [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives targeting the arginase enzyme.

Keywords: triazolopyrimidine; trifluoromethyl; arginase; polyamines; trypanothione; *Leishmania*

1. Introduction

Leishmaniasis is considered by the World Health Organization (WHO) as one of the world's most neglected diseases (WHO 2010). Because there are different causative species (1, 2) and various clinical manifestations of the disease, the treatment of leishmaniasis is complicated. Pentavalent antimonial compounds (3), amphotericin B (1, 2), pentamidine (4), miltefosine (5) have been used in the treatment of leishmaniasis. The misuse of those drugs, incomplete treatment, inappropriate prescription guidelines and the occurrence of serious adverse effects have led to the emergence of drug-resistant *Leishmania* parasites (6-8).

An important approach to the rational development of new drug candidates is the selection of a specific target with which the candidate compounds can interact. Knowledge of parasite-host relationships is essential to the development of new drugs against parasites. The arginase enzyme of *Leishmania* has been shown to be a promising target, and simple molecules have achieved a good inhibition of arginase (9-12).

The metabolism of the amino acid L-arginine during *Leishmania* infection is crucial because it is required for both the parasite and host (10, 13). Parasites sense the internal/external pool of L-arginine and regulate the expression of two genes coding for transporters in response (14). The amino acid L-arginine is hydrolyzed by arginase (E.C. 3.5.3.1), yielding L-ornithine and urea in the first step of polyamine biosynthesis in *Leishmania*. The L-ornithine used in the synthesis of polyamines plays an important role in cellular processes such as growth and the antioxidant activity mediated by trypanothione (13, 15, 16). The *Leishmania* arginase is a glycosomal enzyme (17, 18), and this compartmentalization is important for L-ornithine production and optimal infectivity (19).

The deletion of the arginase gene demonstrated the importance of this enzyme for *Leishmania major*, which becomes auxotrophic for polyamines (20). The *Leishmania* arginase exhibits significant differences from human arginase that can be exploited in the design of specific inhibitors (11, 12, 21). Thus, arginase seems to be a pharmacological target of interest for the development of new anti-leishmaniasis drugs.

Many heterocyclic systems such as quinolines (22), isoquinoline (23), indoles (24), purines (25), pyrazolopyrimidines (26), pyrazolopyridines (27) and thienopyridine (28, 29) have been developed in an attempt to find new drugs to treat leishmaniasis. Iyamu and co-workers reported chloroquine as the first example of an anti-malarial agent displaying competitive inhibition of arginase (28, 29).

Recently, we described a series of twenty-six [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives (**4–7, 9–16, 18–29, 31-32**), which were planned as bioisosteres of chloroquine. This series was evaluated *in vitro* against *Plasmodium falciparum* and showed good to excellent activities (28). As triazolopyrimidine is bioisostere of quinoline, chloroquine is an arginase inhibitor, and arginase is a possible target for leishmaniasis, we decided to test twenty nine [1,2,4]triazolo[1,5-*a*]pyrimidines against the arginase from *L. amazonensis*.

Different arylamines and aliphatic amines were incorporated into the [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold to investigate the importance of the substituent at the 7-position. The trifluoromethyl moiety, one of the most widespread fluorine-containing functional groups in bioactive molecules, is a highly electronegative substituent that can exert a significant electronic influence on neighboring groups. It is also one of the most lipophilic groups known, making it useful for improving the targeting of molecules to the active sites of enzymes (28). Therefore, we tested a [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold with 2-CF_3 (**4**-**11**, **13**-**15**, **17**, **19**-**30** and **32**), 2-CH_3 (**12**, **16** and **18**) and 2-H (**31**) to compare the importance of these substituents to the enzymatic activity. Compounds **8** and **17** were not synthesized in our previous work and now were obtained. The literature has described structural units such as N(C=N)N, N(C=S)N or C(C=N)N, as required for leishmanicidal activity (Figure 1) (28). Additionally, expecting that the introduction of a thiosemicarbazide structural unit could enhance the leishmanicidal activity, we synthesized the new compound **30** (Figure 2).

2. Materials and Methods

2.1 Chemistry

The synthetic route used to produce [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives **4–7**, **9–16**, **18–29**, **31**, **32** was previously described (28) and was reproduced for the synthesis of the compounds **8**, **17** and **30** (Figure 3).

2.2 Experimental

^{1}H-, ^{13}C- and ^{19}F-Nuclear Magnetic Resonance (NMR) spectra were obtained at 400.00 MHz, 100.00 MHz and 376.00 MHz, respectively, on a Bruker Avance instrument equipped with a 5 mm probe, using tetramethylsilane as the internal standard. Chemical shifts (δ) are reported in ppm, and coupling constants (*J*) are reported in Hertz. Fourier transform infrared (FT-IR) absorption spectra were recorded on a Shimadzu mode IR Prestige-21 spectrophotometer by reflectance in KBr. GC/MS experiments were conducted using a model 6,890 N gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a 7,683 B autosampler coupled with a model MS 5,973 N single quadrupole mass spectrometer (Agilent). Electron-ionization mass spectra (EI-MS, scan ES+ Capillary (3.0 kV) / cone (30 V) / extractor (1 V) / RF lens (1.0 V) / source temperature (150 °C) / desolvation temperature (300 °C)) were recorded using a Micromass/Waters Spectrometer (model: ZQ-4000). The GC was equipped with a HP-5MS capillary column 30 m in length and 0.25 mm in diameter, with a 0.25 μm film thickness. The initial temperature was 50 °C, and was then increased at 10 °C/min to 300 °C, where it was held for 10 minutes. The helium flow rate was 0.5 mL/min. Melting points (m.p.) were determined with a Büchi model B-545 apparatus. TLC was performed using silica gel F-254 glass plates (20 × 20 cm). All other reagents and solvents were of analytical grade.

2.3 General procedure for preparing 5-Methyl-7-arylamine/hydrazinecarbothioamide[1,2,4]triazolo[1,5-*a*]pyrimidines 8, 17 and 30.

A mixture of a 7-chloro[1,2,4]triazolo[1,5-*a*]pyrimidine derivative **3c** and the appropriate amine or thiosemicarbazide (1 equivalent) in ethanol (10 mL) was stirred at room temperature for 6–18 h. The reaction mixture was concentrated and poured into 50 mL of ice-cold water. The precipitate was collected by filtration and washed with water.

7-(4-Chlorophenylamine)-5-methyl-2-(trifluoromethyl)[1,2,4]triazolo[1,5-*a*]pyrimidine **(8)**. Yield: 80%; m.p. 104-106 °C. IR (KBr, cm^{-1}): 3468; 3047; 2887; 2306; 1664; 1612, 1589; 1487; 1365; 1338; 1325; 1200, 1190, 1170; 1157; 1089; 893; 819; 763. ^{1}H-NMR (400 MHz, DMSO-d6, δ in ppm): 2.64 (s; 3H; CH_3); 6.66 (s, 1H, H-6); 7.52 (d, *J* = 8.5 Hz, 2H, H-2'; H-6'); 7.62 (d; *J* = 8.5 Hz; 2H; H-3'; H-5'). ^{13}C-NMR (100 MHz, DMSO-d_6, δ in ppm): 21,11; 93,60; 120,35 (q; *J* = 269 Hz; C-9); 128,56; 131,63; 134,56; 135,89; 151,42; 152,18; 157,37 (q; *J* = 40 Hz; C-2); 162,07. ^{19}F-NMR (376 MHz, DMSO-d_6, δ in ppm): - 67.45 (s; 3F). GC/ME (70 *eV*) *m/z* (%): 327 (100); 328 (M+1); 329; 312; 258; 190; 177; 111.

5-methyl-7-(*4-trifluorophenylamine*)*-2-*(*trifluoromethyl*)[*1,2,4*]*triazolo*[*1,5-a*]*pyrimidine* **(17)**. Yield: 80%; m.p. 100-103 °C. IR (KBr, cm^{-1}): 3633; 3005; 2357; 1631; 1608; 1570; 1516; 1431; 1373; 1323; 1296; 1195; 1149; 848; 806. ^{1}H-NMR (400 MHz, DMSO-d6, δ in ppm): 2.54 (s; 3H; CH_3); 6.71 (s; 1H; H-6); 7,67 (d; *J* = 8.5 Hz; 2H; H-2'; H-6'); 7,81 (d; *J* = 8.5 Hz; 2H; H-3'; H-5'). ^{13}C-NMR (100 MHz, DMSO-d_6, δ in ppm): 25.25; 92.82; 121.01 (q, *J* = 268 Hz, C-9); 125.59 (q, *J* = 269 Hz, C-18); 125.75; 128.14; 129.61 (q, *J* = 33 Hz, C-15); 141.53; 147.97; 156.85 (q, *J* = 39 Hz, C-2);157.40; 169.10. ^{19}F-NMR (376 MHz, DMSO-d_6, δ in ppm):- 67.22 (s; 3F); - 63.90 (s; 3F). GC/ME (70 *eV*) *m/z* (%): 361 (100); 362 (M+1); 346; 292; 190; 177; 145.

2-(5-methyl-2-(trifluoromethyl)-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)hydrazinecarbothioamide **(30)**. Yield: 98%; m.p. 195-197 °C. IR (KBr, cm^{-1}): 1620; 1585; 1512; 1188; 1157; 1000; 756. ^{1}H-NMR (400 MHz, DMSO-d_6, δ in ppm): 2.50 (s; 3H; CH_3); 6.30 (s; 1H; H-6); 7.88 (s; 1H; NH_2); 8.17 (s; 1H; NH_2); 9.87 (s; 1H; NH); 10.81 (s; 1H; NH). ^{13}C-NMR (100 MHz, DMSO-d_6, δ in ppm): 24.87; 90.11; 119.45 (q; J = 268 Hz; C-9); 148.58; 154.40 (q, J= 40 Hz, C-2); 155.60; 166.45; 182.10. ^{19}F-NMR (376 MHz, DMSO-d_6, δ in ppm): - 64,47 (s; 3F). EI/MS *m/z* (%):290 (100).

2.4 Expression and purification of arginase

Recombinant ARG-L was expressed and purified as previously describe (30).

2.5 Inhibitor screening and determination of IC_{50}

Arginase inhibition, IC_{50} and the mode of inhibition were determined as previously described (31, 32). Compounds were screened for inhibition using 1 mM of each prepared in MeOH at 10 mM. The inhibition experiments were performed at pH 9.5 in 50 mM CHES buffer, 50 mM L-arginine (pH 9.5) and enzyme at 0.006 µM. The samples were incubated in a water bath at 37 °C for 15 minutes. Urea was quantified as described by Berthelot (33). Briefly, the catalytic activity of the arginase reactions was stopped by transferring 10 µL of reaction mixture into 750 µL of reagent A (20 mM phosphate buffer, pH 7, containing 60 mM salicylate, 1 mM sodium nitroprusside and >500 IU of urease). This mixture was incubated at 37°C for 5 minutes. Next, 750 µL of reagent B (sodium hypochlorite 10 mM and NaOH 150 mM) were added, and then the samples were incubated at 37°C for 10 min. The positive and negative controls were performed under the same conditions in the absence of inhibitor. At least three independent experiments were performed with duplicate samples.

The IC_{50} (the concentration that inhibits 50% of the catalytic activity of the enzyme) was determined by varying the inhibitor concentration with a 1:10 dilution factor. The experiments were performed in duplicate in at least three independent experiments until a non-linear regression coefficient $R^2 \geq 0.85$ was obtained. The reactions were performed under the same conditions described above. A sigmoidal model (log IC_{50}) was used to determine the IC_{50} in Origin 8.0 software.

2.6 Determination of the constants Ki, Ki' and the mechanism of inhibition

All reactions were performed in 50 mM CHES buffer, pH 9.5, containing variable concentrations of the substrate L-arginine (12.5, 25, 50 and 100 mM) at pH 9.5. Inhibitors were tested at three different concentrations close to the IC_{50}. All reactions were performed in duplicate in at least three independent experiments.

The inhibitor association constant (Ki) was determined by plotting the reciprocal initial velocity of catalysis (1/Vo) as a function of inhibitor concentration (34). For non-competitive inhibition, y = 0 was used to determine the values of the constants Ki and Ki'.

2.7 Cell Cytotoxicity

The cytotoxicity of new compounds was determined as previous described (28).

3. Results and Discussion

3.1 Arginase inhibition by trifluoromethyltriazolopyrimidine

The compounds synthesized with different substituents in the 2-, 5-, and 7-positions of the [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold were previously tested with HepG2 cells and were shown to induce no toxicity (28), and in this study they were tested at 1 mM against the arginase enzyme from *Leishmania (Leishmania) amazonensis* (Table 1).

Twelve compounds (**5**, **7-8**, **11**-**13**, **15**, **17**, **21**, **26**, **29** and **30**) inhibited arginase in the range of 23-79% at 1 mM. The [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold containing R_1 = CF_3 at the 2-position exhibited greater activity against the arginase enzyme than when the substituent R_1 = CH_3.. This finding can be confirmed by comparing the derivatives **11** and **12** (3',5'-diCl); **15** and **16** (3',5'-diOCH_3); **17** and **18** (4'-CF_3). The trifluoromethylated derivative **11** inhibited 71% of arginase activity and was approximately 3 times more active than analog **12**. Compounds **15** (39%) and **17** (52%) became inactive when the CF_3 groups were replaced by CH_3 in derivatives **16** and **18**.

Derivatives **22**-**25**, **31**, and **32** without aromatic amines at the 7-position were inactive with the exception of the pyrrolidinyl derivative **25**, which exhibited a low level of inhibition (10%).

Compound **30** (Figure 4) with the structural units thiosemicarbazide inhibited the arginase activity by 79% corresponding our expectations. However, compounds **5**, **7**-**8**, **11**-**13**, **15**, **17**, **21**, **26** and **29** also showed activity despite lacking these structural units. Compound **30** showed selectivity for parasite enzyme because it did not inhibit rat liver arginase, used as model of mammalian arginase. The compound **11** did not inhibit arginase at 100 μM and we did not consider it in kinetics analysis.

3.2 Kinetic analysis of arginase inhibition by compound 30

Based on the results of the experiments with trifluoromethyl[1,2,4]triazolo[1,5-*a*]pyrimidine derivatives at 1 mM described above, we designed, synthesized and tested the novel compound **30** against the arginase enzyme from *L. (L.) amazonensis*. Compound **30** has a CF_3 group in the 2-position and a hydrazinecarbothioamide in the 7-position, and is the most active compound of the series tested here. Compound **30** showed an IC_{50} of 16.5 ± 0.5 μM calculated by a nonlinear regression of the dose response curve (Figure 5). The mechanism of inhibition was determined by varying the concentration of inhibitor and substrate in the reaction. The Dixon and Cornish-Bowden plots (28) reveal that compound **30** inhibits the arginase of *L. (L.) amazonensis* by a non-competitive mechanism (Ki = Ki') (Figure 6). Taking 1/V or S/V (y-axis) as zero in the equations obtained by linear regression for the Dixon and Cornish-Bowden plots, the values of the constants Ki were estimated as 17 ± 1 μM. This result shows that compound **30** binds with equal affinity to the free enzyme or the enzyme-substrate complex. The compound **30** showed an IC_{50} closed related with a natural arginase inhibitor orientin (IC_{50} = 16 ± 2 μM) (32) and quercetin (IC_{50} = 4.30 ± 0.03 μM) (30). Quercetin was used as a control drug and showed an IC_{50} of the 4.0 ± 0.5 μM.

3.3 Toxicity

The toxicity of the active compound **30** was evaluated and showed MDL_{50} >1000 µg/mL (> 3.4 mM) against BGM cell line. The compounds 8 and 17 showed a MDL_{50} of the 0.6 and 0.4 mM, respectively, against a human hepatocyte cell line (HepG2). The toxicity of others compounds was determined previous (28).

Conclusions

These results show that the [1,2,4]triazolo[1,5-*a*]pyrimidine scaffold is an effective starting point for the inhibition of the arginase enzyme and that the CF_3 group increases the activities of these derivatives. The compound **30** with thiosemicarbazide structural units were crucial to enhance the inhibition of activity. However, compounds **5**, **7-8**, **11-13**, **15**, **17**, **21**, **26** and **29** have also exhibited activity but lack these structural units. The novel compound **30** is a selective and non-competitive inhibitor of the arginase from *L. (L.) amazonensis*. These data suggest that these inhibitors of the enzyme arginase from *L. amazonensis* can be used as lead compounds for the design of new agents against *Leishmania*.

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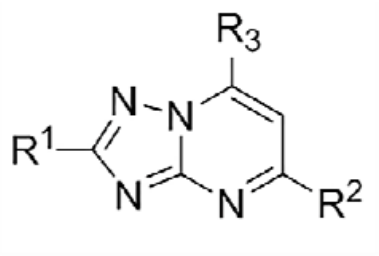
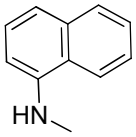
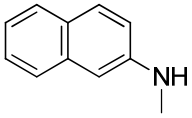
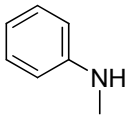
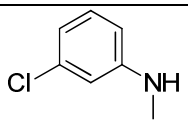
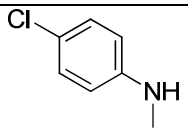
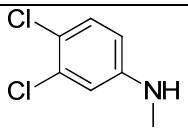
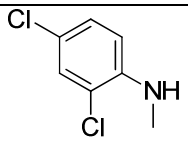
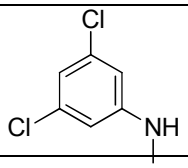
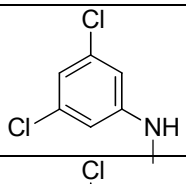
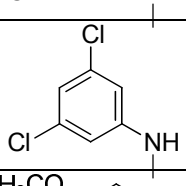
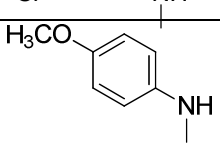
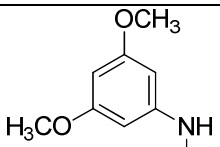
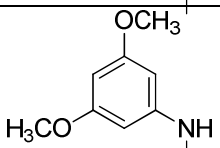
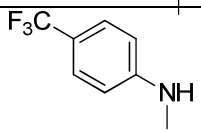
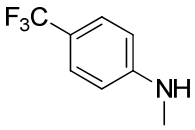
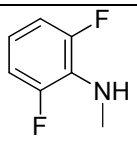
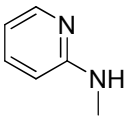
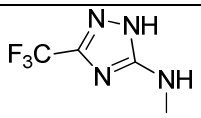
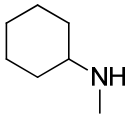
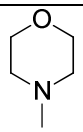
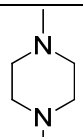
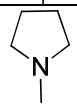
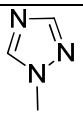
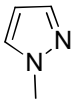
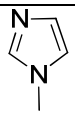
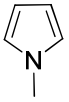
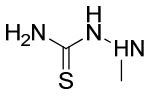
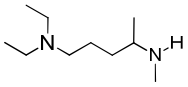
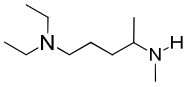
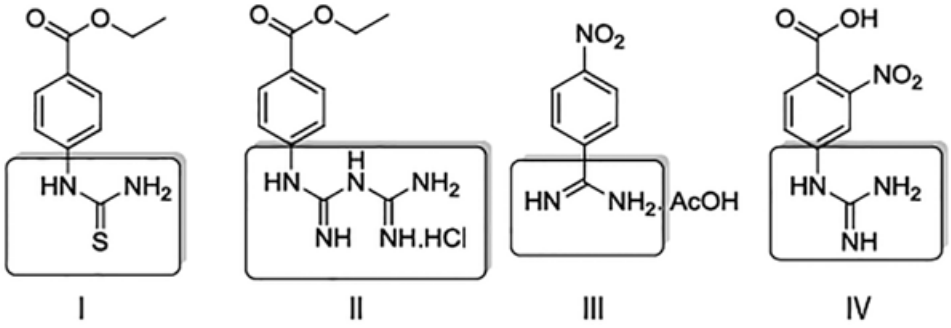
Table 1. Preliminary screening of arginase inhibition by [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives **4**–**32**.

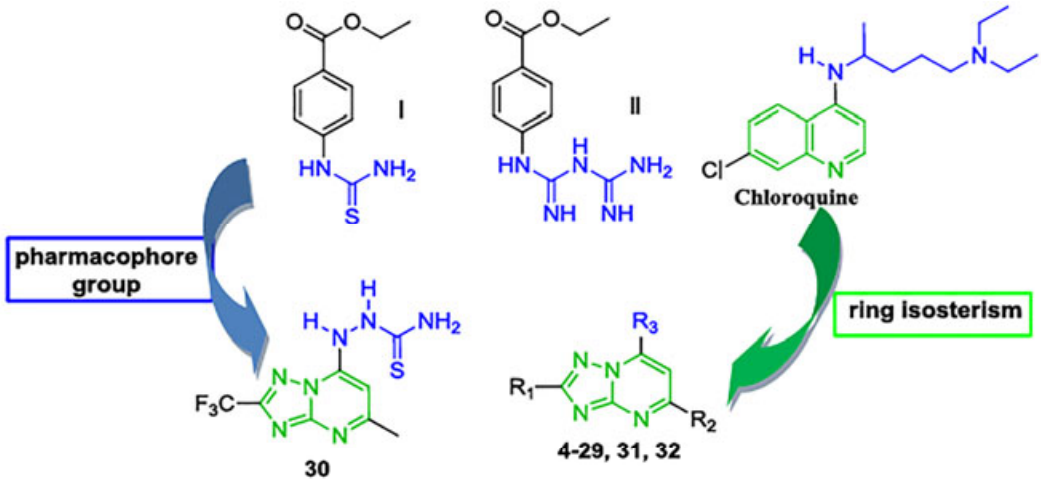


Compounds	R_1	R_2	R_3	Arginase Inhibition (1 mM)
4	CF_3	CH_3		0%
5	CF_3	CH_3		29%
6	CF_3	CH_3		1%
7	CF_3	CH_3		51%
8	CF_3	CH_3		42%
9	CF_3	CH_3		3%
10	CF_3	CH_3		0%
11	CF_3	CH_3		71%

12	CH_3	CH_3		23%
13	CF_3	CF_3		47%
14	CF_3	CH_3		5%
15	CF_3	CH_3		39%
16	CH_3	CH_3		0%
17	CF_3	CH_3		52%
18	CH_3	CH_3		0%
19	CF_3	CH_3		8%
20	CF_3	CH_3		0%
21	CF_3	CH_3		57%
22	CF_3	CH_3		0%

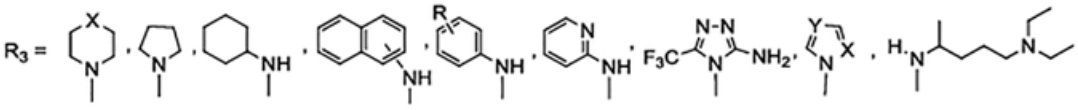
23	CF_3	CH_3		0%
24	CF_3	CH_3		0%
25	CF_3	CH_3		10%
26	CF_3	CH_3		47%
27	CF_3	CH_3		0%
28	CF_3	CH_3		0%
29	CF_3	CH_3		38%
30	CF_3	CH_3		79%
31	H	CH_3		0%
32	CF_3	CH_3		0%

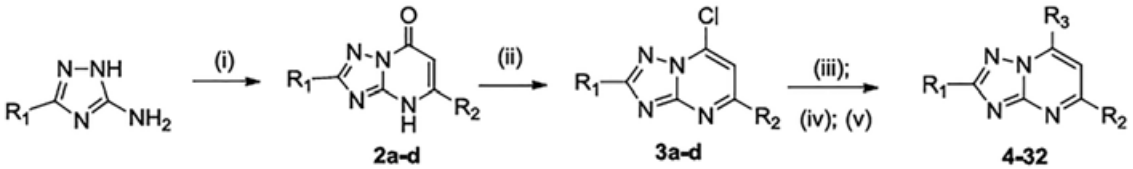




R_1 = H, CH_3, CF_3 R_2 = CH_3, CF_3

R_3 =





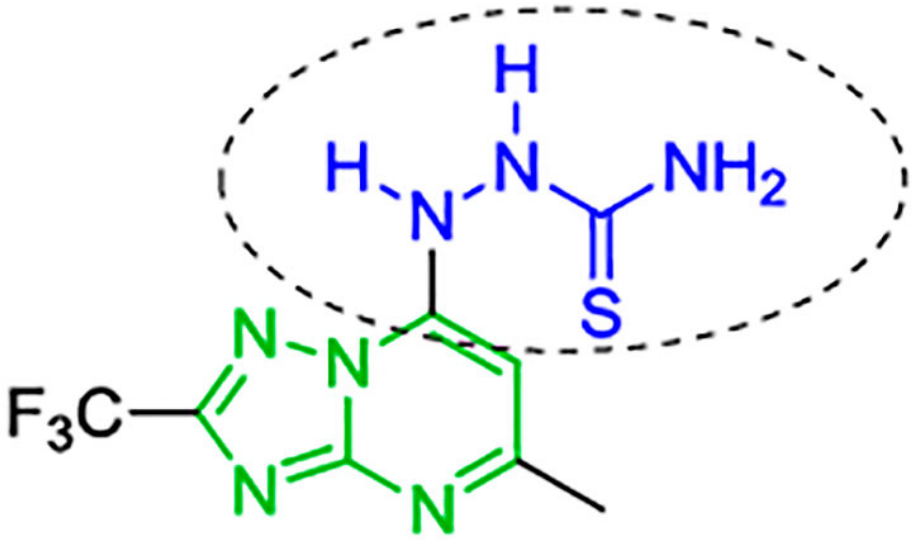
1a: R_1= H;
1b: R_1= CH_3;
1c: R_1= CF_3

2a; **3a**: R_1= H, R_2= CH_3;
2b; **3b**: R_1= CH_3, R_2= CH_3;

2c; **3c**: R_1= CF_3, R_2= CH_3;
2d; **3d**: R_1= CF_3, R_2= CF_3

R_1, R_2, R_3 shown in Table 1

Reagents and conditions: (i) ethyl acetoacetate or ethyl 4,4,4-trifluoroacetoacetate, TsOH (cat.), toluene, reflux, 20 h; (ii) $POCl_3$, reflux, 4 h; (iii) appropriate amine, EtOH, RT, 16-18 h; (iv) appropriate amine/azol, DMF, 120 °C, 12 h; (v) appropriate amine, EtOH, RT, 43 h.



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