Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supplementary Material

Optimization of triazole-based TGR5 agonists towards orally available agents

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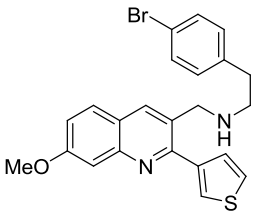
Section 1: A data set of compounds 2-9 used for a plot in Figure 2

Reported TGR5 agonists **2-9** were synthesized according to the literature (references are attached in the Table S1). Human uninduced EC_{50} as well as intrinsic clearance in human liver microsomes (HLM) were shown in Table S1.



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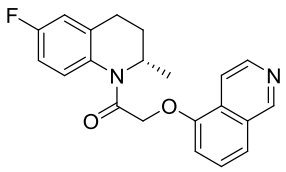
Kalypsys



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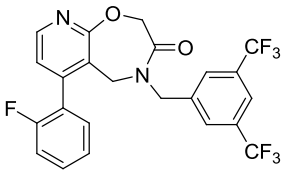

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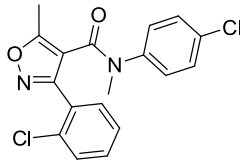

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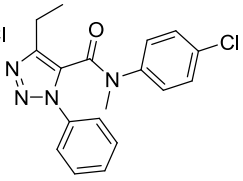
GSK



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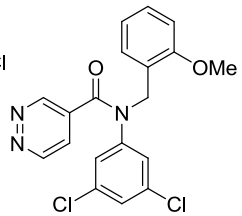

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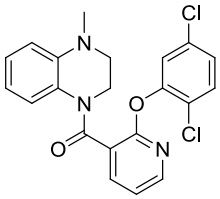

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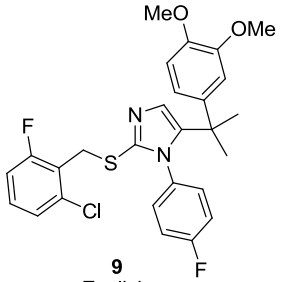
Roche



9
Exelixis

Table S1. Human uninduced EC_{50} and intrinsic clearance in HLM for compounds **2-9.**

Cpd	Human uninduced EC_{50}±SD (nM)[a]	HLM CL_{int} (μL/min/mg)[b]	Compound purity (%)	Reference for the synthesis
2[c]	3010±1170	249	98 (by HPLC)	*Bioorg. Med. Chem. Lett.*, 2010, **20**, 5718-5721
3	166±103	>320	>99 (by HPLC)	*J. Med. Chem.*, 2009, **52**, 7962-7965
4	1423±810	>320	95 (by HPLC)	See section 2 for this ESI
5[c]	94.9±84.8	280	>99 (by HPLC)	JP 2006063064
6	51.8±51	>320	>99 (by LCMS)	JP 2006056881
7	75.1±18.6	>320	97 (by HPLC)	WO08125627
8	29.0±17.4	171	>99 (by HPLC)	US 20100105906
9	45.8±11.4	186	>99 (by LCMS)	WO 2010093845

[a] Values are arithmetic means of at least three experiments unless otherwise noted.

[b] Intrinsic clearance determined in human liver microsomes

[c] Tested as HCl salts

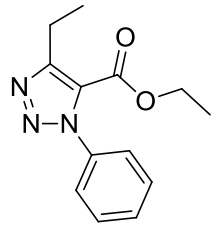
Section 2: Synthesis and characterizations of compounds 4, 10-18, and intermediates 19-21.

General Methods:

All chemicals, reagents and solvents were purchased from commercial sources and used without further purification. All reactions were performed under an atmosphere of nitrogen unless otherwise noted. Proton (^{1}H NMR) nuclear magnetic spectroscopy were recorded with 400 MHz and 500 MHz Varian spectrometers. Chemical shifts are expressed in parts per million downfield from tetramethylsilane. The peak shapes are denoted as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet. Mass spectrometry (MS) was performed via atmospheric pressure chemical ionization (APCI) or electron scatter (ESI) ionization sources. Liquid chromatography mass spectrometry (LCMS) was performed on an Agilent 1100 Series (Waters Atlantis C18 column, 4.6 x 50 mm, 5 μm; 95% water/acetonitrile linear gradient to 5% water/acetonitrile over 4 min, hold at 5% water / 95% acetonitrile to 5.0 min, trifluoroacetic acid modifier (0.05%); flow rate of 2.0 mL/ min). Silica gel chromatography was performed using a medium pressure Biotage or ISCO system using columns pre-packaged by various commercial vendors including Biotage and ISCO.

Experimental Procedures and Spectral Data:

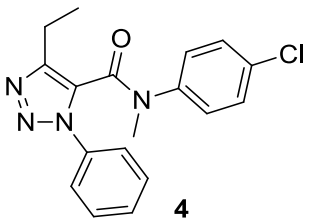
Ethyl 4-ethyl-1-phenyl-1*H*-1,2,3-triazole-5-carboxylate



Phenyl hydrazine (97%, 1.91 ml, 18.8 mmol) was dissolved in water (19 ml) and conc. HCl (3.44 ml). Ether (24 ml) was added and the mixture was cooled with an ice bath. A solution of $NaNO_2$ (1.56 g, 22.6 mmol) in water (2 ml) was added via syringe over 30 min with vigorous stirring. The solution was stirred for 1 h, after which it was filtered. The phases were separated and the aqueous layer washed with ether (3 x 80 ml). The combined ether layers were washed with brine (80 ml), dried over $MgSO_4$, filtered and concentrated to yield crude phenyl azide as 3.6 g orange oil. The crude azide was dissolved in toluene (17 ml). Ethyl 2-pentynoate (5.18 mL, 45.1 mmol) was added. The mixture was stirred at 120 °C overnight. The mixture was concentrated and the crude material purified by silica gel chromatography, eluting with a 0-40% ethyl acetate in heptane gradient, to yield ethyl 4-ethyl-1-phenyl-1*H*-1,2,3-triazole-5-carboxylate (439 mg, 9.5 %) as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54-7.50 (m, 3 H) 7.46-7.42 (m, 2 H), 4.26 (q, *J* = 7.23 Hz, 2 H), 3.05 (q, *J* = 7.62 Hz, 2 H), 1.44 (t, *J* = 7.50 Hz, 3 H), 1.22 (t, *J* = 7.08 Hz, 3 H); MS: ESI *m*/*z* 246.1 $[M+1]^+$.

The regioisomeric triazole, ethyl 5-ethyl-1-phenyl-1*H*-1,2,3-triazole-4-carboxylate (588 mg, 12.8 %) was also isolated as a light yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61-7.57 (m, 3 H), 7.46-7.42 (m, 2 H), 4.48 (q, *J* = 7.10 Hz, 2 H), 3.00 (q, *J* = 7.43 Hz, 2 H), 1.46 (t, *J* = 7.12 Hz, 3 H), 1.17 (t, *J* = 7.52 Hz, 3 H); MS: ESI *m*/*z* 246.1 $[M+1]^+$.

***N*-(4-Chlorophenyl)-4-ethyl-*N*-methyl-1-phenyl-1*H*-1,2,3-triazole-5-carboxamide** (**4**)

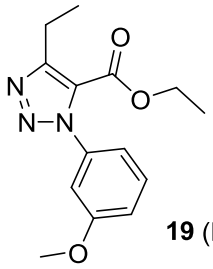


4

A solution of BuLi (2.1 M in heptane, 1.17 mL, 2.46 mmol) was added to a solution of 4-chloro-*N*-methylaniline (300 mg, 2.1 mmol) in tetrahydrofuran (16 mL) at 0 °C. The solution was then kept at -78

°C for 30 min. A suspension of ethyl 4-ethyl-1-phenyl-1*H*-1,2,3-triazole-5-carboxylate (430 mg, 1.75 mmol) in tetrahydrofuran (10 mL) was added at -78 °C. The reaction mixture was stirred for 1 h. The solution was poured into 1 N aq. HCl and diluted with EtOAc. The organic layer was washed with 1 N HCl, 1 N NaOH, brine, dried over Na_2SO_4, and concentrated to dryness. The resulting oil was purified by silica gel chromatography, eluting with a 0-60% ethyl acetate in heptanes gradient, to yield *N*-(4-chlorophenyl)-4-ethyl-*N*-methyl-1-phenyl-1*H*-1,2,3-triazole-5-carboxamide (339 mg, 57%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49-7.41 (br m, 3 H), 7.27-7.21 (br m, 2 H), 7.00 (d, *J* = 7.76 Hz, 2 H), 6.32 (d, *J* = 7.82 Hz, 2 H), 3.35 (br s, 3 H), 2.81 (q, *J* = 7.62 Hz, 2 H), 1.39 (t, *J* = 7.63 Hz, 3 H); MS: ESI *m*/*z* 341.1 $[M+1]^+$; HPLC purity (wave length: 254 nm) = 97%.

Ethyl 4-ethyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (19, R^1= Et)

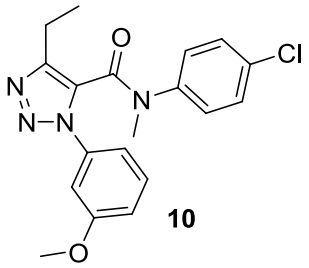


19 (R^1= Et)

3-Methoxyphenylhydrazine hydrochloride (821 mg, 4.70 mmol) was dissolved in 5 mL water and 0.86 mL conc. HCl was added. Ether (6 mL) was added and the mixture was cooled in an ice bath. A solution of sodium nitrite (389 mg, 5.64 mmol) in water (0.5 mL) was added dropwise. The mixture was stirred for 1 h. The phases were separated. The aqueous phase was extracted with ether. The combined organic phases were dried over sodium sulfate and concentrated to yield 700 mg of crude 3-methoxyphenylazide. The crude azide was dissolved in 5 mL toluene. Ethyl 2-pentynoate (1.55 mL, 11.7 mmol) was added and the mixture was stirred at 120 °C overnight. The reaction mixture was concentrated and the crude residue purified by silica gel chromatography, eluting with a 0-60% EtOAc in heptanes gradient, to give ethyl 4-ethyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (287 mg, 22.2%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43-7.38 (m, 1 H), 7.06 (ddd, *J* = 8.35, 2.40, 0.97 Hz, 1 H), 7.02-6.98 (m, 2 H), 4.27 (q, *J* = 7.14 Hz, 2 H), 3.85 (s, 3 H), 3.04 (q, *J* = 7.53 Hz, 2 H), 1.39 (t, *J* = 7.58 Hz, 3 H), 1.23 (t, *J* = 7.13 Hz, 3 H); MS: ESI *m*/*z* 276.2 $[M+1]^+$.

The regioisomeric triazole, ethyl 5-ethyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-4-carboxylate (436 mg, 33.7 %) was also isolated as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50-7.44 (m, 1H), 7.12-7.09 (m, 1 H), 7.03-6.99 (m, 1 H), 6.98-6.96 (m, 1 H), 4.48 (q, *J* = 7.15 Hz, 2 H), 3.87 (s, 3 H), 3.01 (q, *J* = 7.50 Hz, 2 H), 1.46 (t, *J* = 7.15 Hz, 3 H), 1.19 (t, *J* = 7.50 Hz, 3 H); MS: ESI *m*/*z* 276.2 $[M+1]^+$.

_N_-(4-Chlorophenyl)-4-ethyl-1-(3-methoxyphenyl)-_N_-methyl-1_H_-1,2,3-triazole-5-carboxamide (10)

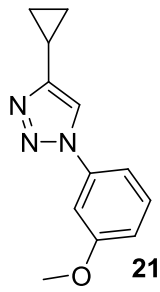


10

4-Chloro-*N*-methylaniline (26 mg, 0.18 mmol) was dissolved in 0.5 mL THF and cooled to 0 °C under N_2 atmosphere. Butyllithium (2.1 M in hexanes, 107 μL, 0.225 mmol) was added. The mixture was cooled to -78 °C and stirred for 30 min. A solution of ethyl 4-ethyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (42 mg, 0.15 mmol) in THF (1 mL) was added. The mixture was stirred at -78 °C for 1 h. Saturated aq. NH_4CO_3 (1 mL) was added, and the mixture stirred at room temperature overnight. The mixture was partitioned between with CH_2Cl_2 and 1N aq. HCl. The phases were separated. The organic phase was concentrated to yield 45 mg of crude oil. The residue was dissolved in dimethyl sulfoxide (1 mL) and purified by reversed-phase HPLC (Column: Waters Sunfire C18 19x100, 5u; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in acetonitrile (v/v); Gradient: 80.0% H_2O/20.0% Acetonitrile linear to 40% H_2O/60% Acetonitrile in 10.5min to 0% H_2O/100% MeCN to 11.0min, HOLD at 0% H_2O / 100% Acetonitrile from 11.0 to 12.0min. Flow: 25mL/min.) to provide *N*-(4-chlorophenyl)-4-ethyl-1-(3-methoxyphenyl)-*N*-methyl-1*H*-1,2,3-triazole-5-carboxamide (11 mg, 19.4%).

Analytical HPLC: Column: Waters Atlantis dC18 4.6x50mm,5um; Modifier: TFA 0.05%; Gradient: 95% H_2O/5% acetonitrile linear to 5% H_2O/95% acetonitrile over 4.0 min, HOLD at 5% H_2O/95% acetonitrile to 5.0 min; Flow: 2.0 mL/min; retention time: 3.21 min. MS: ESI *m*/*z* 371.2 $[M+1]^+$; LCMS purity >99%.

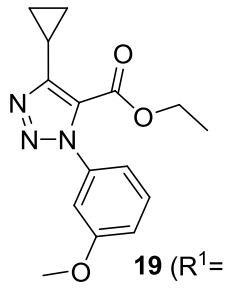
4-Cyclopropyl-1-(3-methoxyphenyl)-1_H_-1,2,3-triazole (21, R^1= cyclopropyl)



21 (R^1= cyclopropyl)

An auto cleave vessel was sequentially charged with 3-iodoanisole (60 g, 256.36 mmol, 1.0 eq.), alkyne (16.94g, 256.36, 1.0 eq,.), L-proline (5.9, 51.26 mmol, 0.2 eq), Na_2CO_3 (5.4 g, 51.26 mmol, 0.2 eq.), sodium ascorbate (10.14 g, 51.26 mmol, 0.2 eq), and 432 mL of DMSO:H2O (9:1). Then NaN_3 (20 g 307.64 mmol, 1.2 eq.,) and $CuSO_4$-$5H_2O$ (6.4 g, 25.6 mmol, 0.1 eq) were added, and the vessel was sealed. The greenish-brown suspension was stirred at 65ºC for 22 h. TLC (30% EA/Hex) showed complete consumption of starting material. Upon reaching ambient temperature, the brown suspension was poured into a stirring mixture of dilute aq.NH_4OH (500 mL) and EtOAc (700 mL). The reaction vessel was rinsed with EtOAc and water. The organic layer was separated, washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The crude was purified by silica column using 10-20% EA/Hex to afford 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole (42g, 76%) as a brownish oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (s, 1 H), 7.41-7.36 (m, 1 H), 7.33-7.30 (m, 1 H), 7.23 (dd, *J* = 7.93, 2.07 Hz, 1 H), 6.95 (dd, *J* = 8.29, 2.44 Hz, 1 H), 3.88 (s, 3 H), 2.06-1.99 (m, 1 H), 1.04-0.99 (m, 2 H), 0.96-0.91 (m, 2 H); MS: ESI *m*/*z* 216.0 $[M+1]^+$.

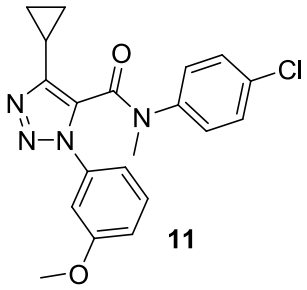
Ethyl 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (19, R^1=cyclopropyl)



19 (R^1= cyclopropyl)

A solution of 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole (15 g, 70 mmol) in THF (500 ml) was cooled to -78 °C. n-BuLi (1.7 M in hexane, 49.2 ml, 83.6 mmol) was added dropwise, and the mixture was allowed to stir at -78 °C for 30 min and then at -60 °C for 15 min. The reaction mixture was cooled to -78 °C and a solution of ethyl chloroformate (6.94 ml, 73.2 mmol) in THF (160 mL) was added dropwise. The mixture was stirred for at -78 °C for 30 min. The solution was allowed to warm to -60 °C and stirred for 15 min. The reaction was quenched by addition of saturated aq. NH_4Cl solution (250 ml). The phases were separated. The organic phase was concentrated under vacuum. The crude material was purified by silica gel chromatography, eluting with a 10-20% EtOAc in hexanes gradient, to afford ethyl 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (9 g, 45%) as a solid. ^{1}H NMR (500 MHz, CD_3OD) δ 7.49-7.44 (m, 1 H), 7.16 (dd, *J* = 8.56, 2.30 Hz, 1 H), 7.07-7.05 (m, 1 H), 7.05-7.02 (m, 1 H), 4.27 (q, *J* = 7.07 Hz, 2 H), 3.87 (s, 3 H), 2.59-2.51 (m, 1 H), 1.19 (t, *J* = 7.04 Hz, 3 H), 1.14-1.10 (m, 4 H); MS: ESI *m*/*z* 288.2 $[M+1]^+$.

_N_-(4-Chlorophenyl)-4-ethyl-1-(3-methoxyphenyl)-_N_-methyl-1_H_-1,2,3-triazole-5-carboxamide (11)

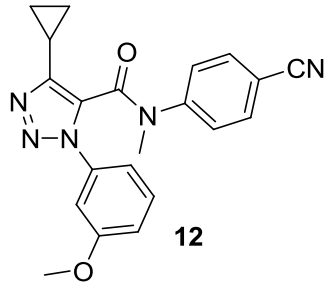


11

A solution of n-BuLi (2.0 M in heptane, 0.1 mL, 0.20 mmol) was added to a solution of 4-chloro-*N*-methylaniline (24 mg, 0.17 mmol) in THF (0.5 mL) at 0 °C. The solution was then cooled and kept at -78 °C for 30 min. A suspension of ethyl 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (40 mg, 0.14 mmol) in THF (1 mL) was then added. The reaction mixture was stirred for 1 h. Saturated aq. NH_4Cl and EtOAc were added. The phases were separated and the organic phase concentrated to dryness. The residue was dissolved in dimethyl sulfoxide (1 mL) and purified by reversed-phase HPLC (Column: Waters Sunfire C18 19x100, 5u; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in acetonitrile (v/v); Gradient: 80.0% H20/20.0% acetonitrile linear to 30% H_2O/70% acetonitrile in 10.5 min to 0% H_2O/100% MeCN to 11.0 min, HOLD at 0% H_2O / 100% acetonitrile from 11.0 to 12.0 min. Flow: 25mL/min.) to give *N*-(4-chlorophenyl)-4-ethyl-1-(3-methoxyphenyl)-*N*-methyl-1*H*-1,2,3-triazole-5-carboxamide (26.1 mg, 49%).

Analytical HPLC: Column: Waters Atlantis dC18 4.6x50mm,5um; Modifier: TFA 0.05%; Gradient: 95% H_2O /5% acetonitrile linear to 5% H_2O/95% acetonitrile over 4.0 min, HOLD at 5% H_2O/95% acetonitrile to 5.0 min; Flow: 2.0mL/min; retention time: 3.31 min. MS: ESI *m*/*z* 383.2 $[M+1]^+$; LCMS purity >99%.

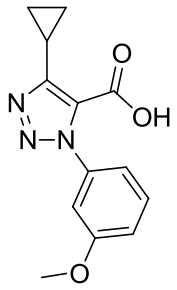
_N_-(4-Cyanophenyl)-4-cyclopropyl-1-(3-methoxyphenyl)-_N_-methyl-1_H_-1,2,3-triazole-5-carboxamide (12)



12

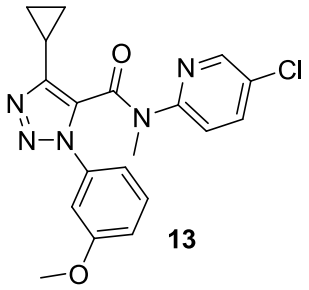
A solution of 4-cyano-*N*-methylaniline (2.75 g, 20.9 mmol) in THF (42 ml) was cooled to -78 °C. n-BuLi (1.7M in hexane, 15.4 ml, 26.1 mmol) was added dropwise. The mixture was stirred at -78 °C for 1 h and allowed to warm to -60 °C stirring for additional 30 min. The reaction mixture was cooled to -78 °C and a solution of ethyl 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (5 g, 17.4 mmol) in THF (84 mL) was added dropwise. The mixture was stirred at -78 °C for 30 min. The solution was allowed to warm to -10 °C stirring for additional 1 h. The reaction was quenched by addition of saturated aq. NH_4Cl solution (250 ml). The phases were separated. The organic phase was concentrated under vacuum. The crude material was purified by silica gel chromatography eluting with 10-20% EtOAc in hexanes gradient to afford *N*-(4-cyanophenyl)-4-cyclopropyl-1-(3-methoxyphenyl)-*N*-methyl-1*H*-1,2,3-triazole-5-carboxamide (3.5 g, 53%) as a solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.51 (d, *J* = 8.4 Hz, 2 H), 7.44-7.40 (m, 1 H), 7.01 (dd, *J* = 8.2, 2.3 Hz, 1 H), 6.87-6.78 (m, 3 H), 6.68 (br s, 1 H), 3.82 (s, 3 H), 3.39 (s, 3 H), 2.03-2.00 (m, 1 H), 1.04-0.80 (m, 4 H); MS: ESI *m*/*z* 374.2 $[M+1]^+$; HPLC purity 98 - >99 % (tested for multiple lots).

4-Cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylic acid



Ethyl 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylate (246mg, 0.856 mmol) was dissolved in MeOH (6 mL) and water (1 mL). Lithium hydroxide (47 mg, 1.9 mmol) was added. The reaction mixture was heated at 70 °C for 2 h. The mixture was concentrated to remove methanol. 1N HCl was added to adjust the pH to ~1. The mixture was extracted multiple times with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The residue was dried under high vacuum to give 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylic acid (215 mg, 97%) as a solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43-7.38 (m, 1 H), 7.07 (ddd, *J* = 8.43, 2.51, 0.99 Hz, 1 H), 7.00 (ddd, *J* = 7.82, 2.00, 0.99 Hz, 1 H), 6.99-6.96 (m, 1 H), 3.85 (s, 3 H), 2.58-2.50 (m, 1 H), 1.30-1.25 (m, 2 H), 1.15-1.09 (m, 2 H); MS: ESI *m*/*z* 260.2 $[M+1]^+$.

_N_-(5-Chloropyridin-2-yl)-4-cyclopropyl-1-(3-methoxyphenyl)-_N_-methyl-1_H_-1,2,3-triazole-5-carboxamide (13)



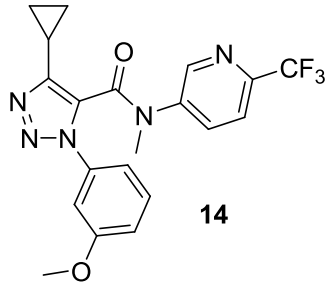
13

To 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylic acid (64.3 mg, 0.248 mmol) was added dichloromethane (1 mL), DMF (70 µL) and thionyl chloride (50 µL, 0.7 mmol). The yellow mixture was stirred at room temperature overnight. To the reaction mixture was added a solution of 5-chloro-*N*-methylpyridin-2-amine (37 mg, 0.23 mmol) and triethylamine (140 µL, 1 mmol) in dichloromethane (1 mL), and the resulting mixture was stirred at room temperature for 2 h. Water was added to the reaction mixture. The organic layer was separated, dried over $MgSO_4$, filtered, concentrated and purified by silica gel chromatography, followed by reversed-phase HPLC to afford the title compound (10.4 mg, 12% yield).

Analytical HPLC: Column: Waters Atlantis dC18 4.6x50mm,5um; Modifier: TFA 0.05%; Gradient: 95% H_2O /5% acetonitrile linear to 5% H_2O/95% acetonitrile over 4.0 min, HOLD at 5% H_2O/95% acetonitrile to 5.0 min; Flow: 2.0mL/min; retention time: 3.15 min. MS: ESI *m*/*z* 384.0 $[M+1]^+$; LCMS purity >99%.

Preparative HPLC: Column: Waters XBridge C18 19x100, 5u; Mobile phase A: 0.03% NH_4OH in water (v/v); Mobile phase B: 0.03% NH_4OH in acetonitrile (v/v); Gradient: 85.0% H_20/15.0% Acetonitrile linear to 0% H_20/100% Acetonitrile in 8.5min, HOLD at 0% H_20/100% Acetonitrile to 10.0min. Flow: 25mL/min.) .

4-Cyclopropyl-1-(3-methoxyphenyl)-*N*-methyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)-1*H*-1,2,3-triazole-5-carboxamide (14)

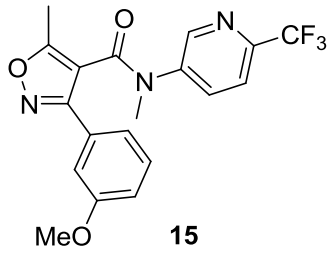


14

To 4-cyclopropyl-1-(3-methoxyphenyl)-1*H*-1,2,3-triazole-5-carboxylic acid (47.9 mg, 0.185 mmol) was added dichloromethane (1 mL), DMF (50 µL) and thionyl chloride (26 µL, 0.36 mmol). The yellow mixture was stirred at room temperature for 1 h. 6-(Trifluoromethyl)pyridin-3-amine (37 mg, 0.23 mmol) and triethylamine (80uL,0.6 mmol) were added as a solution in 0.5 mL DMF. The mixture was stirred at room temperature overnight. Water and saturated aq. $NaHCO_3$ were added. The mixture was extracted with EtOAc. The organic phases were combined, dried over $MgSO_4$, filtered, concentrated and purified by silica gel chromatography to give 15 mg of 4-cyclopropyl-1-(3-methoxyphenyl)-*N*-(6-(trifluoromethyl)pyridin-3-yl)-1*H*-1,2,3-triazole-5-carboxamide that was dissolved in DMSO (0.5 mL). Powdered KOH (7.2 mg, 0.111 mmol) was added, followed by iodomethane (5 µL, 0.074 mmol). The mixture was stirred at 50 °C for 3 h. The mixture was filtered and purified by reversed-phase HPLC (Column: Waters Sunfire C18 19x100, 5u; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in acetonitrile (v/v); Gradient: 60.0% H_2O/40.0% acetonitrile linear to 40% H_2O/60% acetonitrile in 10.5min to 0% H_2O/100% MeCN to 11.0min, HOLD at 0% H_2O / 100% acetonitrile from 11.0 to 12.0min. Flow: 25mL/min.) to provide 4-cyclopropyl-1-(3-methoxyphenyl)-*N*-methyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)-1*H*-1,2,3-triazole-5-carboxamide (3.3 mg, 4.3%) as a solid.

Analytical HPLC: Column: Waters Atlantis dC18 4.6x50mm,5um; Modifier: TFA 0.05%; Gradient: 95% H_2O /5% acetonitrile linear to 5% H_2O/95% acetonitrile over 4.0 min, HOLD at 5% H_2O/95% acetonitrile to 5.0 min; Flow: 2.0mL/min; retention time: 3.12 min. MS: ESI *m/z* 418.0 $[M+1]^+$; LCMS purity >99%.

3-(3-Methoxyphenyl)-*N*,5-dimethyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (15)

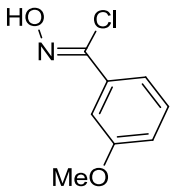


15

To a mixture of 3-(3-methoxyphenyl)-5-methylisoxazole-4-carboxylic acid (92 mg, 0.40 mmol) in DCM (5 mL) was added DMF (1 drop) at room temperature. Oxalyl chloride (102 mg, 0.80 mmol) was added to the reaction mixture at 0 ℃. The mixture was allowed to warm to room temperature and stirred for 1 h. The mixture was concentrated in vacuo to give crude acid chloride (100 mg) as yellow solid. To a mixture of 6-(trifluoromethyl)pyridin-3-amine (71 mg, 0.40 mmol) and triethylamine (81 mg, 0.8 mmol) in DME (2 mL) was added a solution of the above acid chloride (100 mg, 0.40 mmol) in DME (1 mL) at room temperature. The resulting mixture was irradiated in the microwave at 190 ℃ for 4 min. The mixture was concentrated in vacuo and the residue was purified by preparative HPLC to give 3-(3-methoxyphenyl)-N,5-dimethyl-N-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (52.4 mg, 33%) as a white solid.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (s, 1 H), 7.30-7.25 (m, 2 H), 6.99-6.97 (m, 2 H), 6.77-6.76 (m, 1 H), 6.65 (s, 1H), 3.76 (s, 3H), 3.39 (s, 3H), 2.63 (s, 3H); MS: ESI *m*/*z* 392.0 $[M+1]^+$; LCMS purity >99%.

Analytical HPLC method: Column: Xtimate C18,2.1*30mm.3um
Retention Time: 1.050 min;
Mobile phase: from 10% MeCN (0.06% TFA) in water (0.06% TFA) to 80% MeCN (0.06% TFA) in water (0.06% TFA);
Wavelength: 220 nm

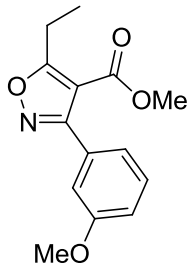
(Z)-*N*-Hydroxy-3-methoxybenzimidoyl chloride



To a solution of (*E*)-3-methoxybenzaldehyde oxime (14 g, 92.6 mmol) in dry chloroform (120 mL) and THF (60 mL) was added *N*-chlorosuccinimide (13.4 g, 101.8 mmol) and pyridine (0.6 mL) at 0 °C. The resulting mixture was stirred at 30 °C for 2 h. The solvent was removed under reduced pressure. The resulting mass was poured into ice water and extracted with MTBE (2 x 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated to give (Z)-*N*-hydroxy-3-methoxybenzimidoyl chloride (15.5 g, 90%) as an oil.

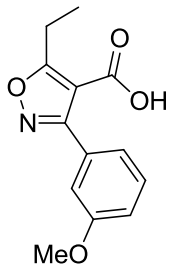
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (br s, 1 H), 7.37-7.23 (m, 3 H), 6.94-6.93 (m, 1 H), 3.77 (s, 3H).

Methyl 3-(3-methoxyphenyl)-5-ethylisoxazole-4-carboxylate



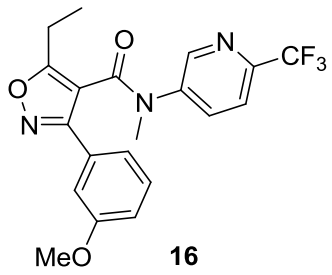
A mixture of methyl 3-oxopentanoate (13 g, 100 mmol) and pyrrolidine (10.7 g, 150 mmol) was stirred at 25 °C overnight. The solvent was removed under reduced pressure to give a crude enamine (18.3 g) as an oil, which was used in the next step directly. To a solution of (Z)-*N*-hydroxy-3-methoxybenzimidoyl chloride (9.25 g, 50 mmol) in EtOH (120 mL) was added the crude enamine (9.15 g, 50 mmol) and Et_3N (7.5 g, 75 mmol) at 0 °C. The resulting mixture was stirred at 30 °C overnight. The solvent was removed under reduced pressure and the residue was extracted with EtOAc (2 x 100 mL). The organic layer was washed with aqueous 1 N HCl solution (100 mL), dried over anhydrous Na_2SO_4 and concentrated to give a crude product, which was purified by silica gel chromatography to give methyl 3-(3-methoxyphenyl)-5-ethylisoxazole-4-carboxylate (10.5 g, 80%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.28 (t, *J* = 8.0 Hz, 1 H), 7.13-7.10 (m, 2 H), 6.94 (dd, *J* = 1.6, 8.0 Hz, 1 H), 3.77 (s, 3 H), 3.70 (s, 3 H), 3.08 (q, *J* = 7.6 Hz, 2 H), 1.31 (t, *J* = 7.6 Hz, 3 H).

5-Ethyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid



To a solution of methyl 3-(3-methoxyphenyl)-5-ethylisoxazole-4-carboxylate (10.5 g, 40 mmol) in THF (100 mL) was added 2N aqueous LiOH (100mL, 200 mmol) at 0 °C. The resulting mixture was stirred under reflux overnight. The solvent was removed under reduced pressure. The mixture was acidified with 1 N aqueous HCl to pH ~3-4 and extracted with DCM (2 x 100 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated to give crude material, which was purified by silica gel chromatography to give 5-ethyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid (7.8 g, 78%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.35 (t, *J* = 6.8 Hz, 1 H), 7.19-7.18 (m, 2 H), 7.06-7.03 (m, 1 H), 3.83 (s, 3 H), 3.18 (q, *J* = 7.6 Hz, 2 H), 1.37 (t, *J* = 7.6 Hz, 3 H).

5-Ethyl-3-(3-methoxyphenyl)-*N*-methyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (**16**)



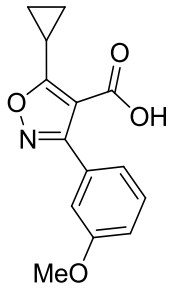
16

To a solution of 5-ethyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid (137.1 mg, 0.5 mmol) in DCM (5 mL) was added oxalyl chloride (190.5 mg, 1.5 mmol) at 0 °C. DMF (1 drop) was added to the reaction at the same temperature. After stirring for 1 h, TLC (petroleum ether: EtOAc = 5: 1) showed the reaction was complete. The reaction solution was concentrated under reduced pressure to give the crude acid chloride (140 mg) as an oil, which was used in the next step directly. To a solution of crude acid chloride (133 mg, 0.5 mmol) in DCM (5 mL) was added 6-(trifluoromethyl)pyridin-3-amine (97 mg, 0.55 mmol) followed by addition of Et_3N (156.5 mg, 1.5 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 12 h. The reaction mixture was washed with NH_4Cl and extracted with EtOAc (3 x 10

mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuum to give crude, which was purified by preparative HPLC to give 5-ethyl-3-(3-methoxyphenyl)-*N*-methyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (20.5 mg, 18%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 8.01 (s, 1 H), 7.50-7.48 (m, 1 H), 7.38-7.31 (m, 2 H), 7.10-7.07 (m, 1 H), 6.79-6.64 (m, 2 H), 3.85 (s, 3 H). 3.44 (s, 3 H), 3.07-3.03 (q, *J* = 8.0 Hz, 2 H), 1.40 (t, *J* = 8.0 Hz, 3 H); ESI *m*/*z* 406.1 $[M+1]^+$; HPLC purity 97- >99% (tested for two lots).

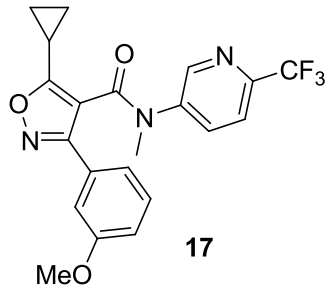
Prep HPLC Method: Column: DIKMA Diamonsil(2) C18 200*20mm*5um
Mobile phase: from 40 % MeCN in water (0.225% FA) to 60 % MeCN in water (0.225% FA), 35 ml/min
Wavelength: 220 nm

5-Cyclopropyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid



To a solution of (Z)-*N*-hydroxy-3-methoxybenzimidoyl chloride (162.9 mg, 1.25 mmol) in THF (20 mL) was added KHMDS (274.7 mg, 1.37 mmol) at 0 °C. After 10 minutes, methyl 3-cyclopropyl-3-oxopropanoate (270 mg, 1.25 mmol) was added, and the reaction was stirred at room temperature for 12 h. The reaction was quenched with aqueous NH_4Cl and extracted with EtOAc (2 x 20 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give ethyl 5-cyclopropyl-3-(3-methoxyphenyl)isoxazole-4-carboxylate (170 mg) as a yellow oil which was used in the next step directly. This ester (170 mg, 0.42 mmol) was dissolved in water (5 mL) and THF (5 mL) and LiOH (176.2 mg, 2N) was added at 0 °C.The mixture was stirred at room temperature for 2 h. The mixture was neutralized with 1N aqueous HCl and extracted with EtOAc (3 x 5 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to give the crude product, which was purified by combi-flash to give 5-cyclopropyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid (160 mg, 98 %) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 7.44 (t, *J* = 8.0 Hz, 1 H), 7.21 (s, 2 H), 7.14 (t, *J* = 8.0 Hz, 1 H), 3.93 (s, 3 H), 3.07-3.04 (m, 1 H), 1.32-1.26 (m, 4 H); MS: ESI *m*/*z* 259.9 $[M]^+$.

5-Cyclopropyl-3-(3-methoxyphenyl)-N-methyl-N-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (17)

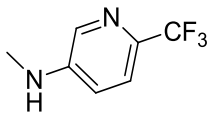


17

To a solution of 5-cyclopropyl-3-(3-methoxyphenyl)isoxazole-4-carboxylic acid (98.3 mg, 0.379 mmol) in DCM (5 mL) was added oxalyl chloride (144.4 mg, 1.137 mmol) at 0 °C. DMF (1 drop) was added to the reaction at the same temperature. After stirring for 1 h, the reaction solution was concentrated under reduced pressure to give the crude acid chloride (105 mg) as an oil, which was used in the next step directly. To a solution of the acid chloride (105 mg, 0.379 mmol) in DCM (5 mL) was added 6-(trifluoromethyl)pyridin-3-amine (73.4 mg, 0.417 mmol) followed by addition of Et_3N (1115 mg, 1.37 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 12 h. The reaction was washed with NH_4Cl and extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give crude material, which was purified by preparative HPLC to give 5-cyclopropyl-3-(3-methoxyphenyl)-*N*-methyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)isoxazole-4-carboxamide (33.5 mg, 20%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 8.05 (s, 1 H), 7.51 (d, *J* = 8.0 Hz, 1 H), 7.34 (t, *J* = 8.0 Hz, 2 H), 7.05 (d, *J* = 8.0 Hz ,1 H), 6.81 (d, *J* = 8.0 Hz, 1 H), 6.69 (s, 1 H), 3.79 (s, 3 H), 3.44 (s, 3 H), 2.38 (t, *J* = 8.0 Hz, 1 H), 1.22-1.20 (m , 4 H); MS: ESI *m/z* 418.2 $[M+1]^+$; HPLC purity = 97%.

Prep HPLC method: Column: Phenomenex Synergi C18 150*30mm*4um
Mobile phase: from 50% MeCN in water (0.225% FA) to 70% MeCN in water (0.225% FA)
Wavelength: 220 nm

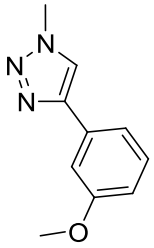
***N*-Methyl-6-(trifluoromethyl)pyridin-3-amine**



Acetic anhydride (2.1 mL, 22 mmol) was added to formic acid (1.05g, 22.8 mmol) at 0 °C. The mixture was warmed to room temperature then heated at 50 °C for 2 h. The mixture was cooled to room

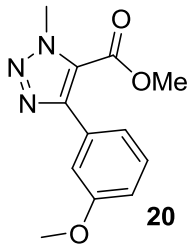
temperature and 5-amino-2-(trifluoromethyl)pyridine (1.2g, 7.4 mmol) was added. The resulting solution was stirred for 1 h. LCMS shows conversion to product after 30 min. The mixture was concentrated under reduced pressure. The residue was purified by chromatography, eluting with 0-100% EtOAc in heptane to afford an *N*-formyl intermediate as a colorless solid (1.37 g, 98 %). This intermediate was dissolved in tetrahydrofuran (34 mL) and cooled to 0 °C. A 1 M solution of lithium aluminum hydride in tetrahydrofuran (14 mL, 14 mmol) was added dropwise via syringe. Vigorous gas evolution was observed during the addition, and the mixture changes from yellow to a dark red. The solution was then warmed to reflux and stirred for 4 h, at which time TLC (1:1 heptane:EtOAc) showed complete consumption of starting material. The solution was cooled to 0 °C. Water (500 uL) was added dropwise, followed by the addition of 3 N potassium hydroxide (500 µL). The resulting thick yellow slurry was stirred at room temperature for 1 h. The mixture was filtered through celite and concentrated to give 754 mg of an orange oil. Purification by silica gel chromatography (0-50% EtOAc in heptane) afforded the title amine as a yellow oil (492 mg, 41%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.05 (d, *J* = 2.9 Hz, 1 H), 7.47 (d, *J* = 8.6, 1 H), 6.87 (dd, *J* = 8.6, 2.7, 1 H), 4.18 (br.s., 1 H), 2.90 (d, *J* = 5.3, 3H).

4-(3-Methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole



Copper(II) acetate (18 mg, 0.095 mmol) was added to a mixture of 3-ethylnylanisole (480 uL, 3.78 mmol), sodium azide (246 mg, 3.78 mmol), and iodomethane (240 uL, 3.85 mmol) in 4 mL of water, and the mixture was heated at 55 °C for 24 h. The reaction mixture was diluted with ethyl acetate (20 mL) and washed with water (10 mL) and saturated aqueous ammonium chloride. The organic layer was dried over magnesium sulfate and concentrated to afford a brown oil. Purification by silica gel chromatography (0-30% EtOAc in heptane) affordred the title compound as a colorless oil (374 mg, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 1 H), 7.45 – 7.42 (m, 1 H), 7.34 – 7.29 (m, 1 H), 6.89 – 6.85 (m, 1 H), 4.14 (s, 3 H), 3.86 (s, 3 H).

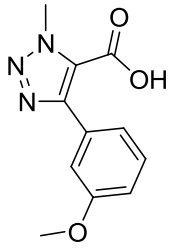
Methyl 4-(3-methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole-5-carboxylate (20)



20

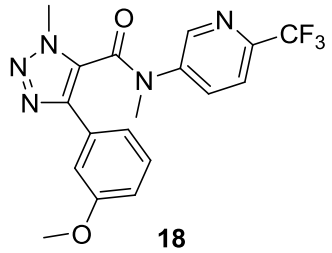
To a solution of 4-(3-methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole (370 mg, 1.96 mmol) in tetrahydrofuran (20 mL) at –78 °C was added a solution of *n*-butyllithium (850 uL of 2.3 M solution in hexanes, 2.0 mmol). The mixture was stirred at –78 °C for 30 min. Methyl chloroformate (200 uL, 2.6 mmol) was added neat in one portion, and the mixture was allowed to warm to room temperature. Saturated aqueous ammonium chloride (5 mL) was then added, followed by additional water to dissolve the salts. The mixture was extracted with ethyl acetate (3 x 20 mL). The organic layer was dried over magnesium sulfate and concentrated to a yellow oil. Purification by silica gel chromatography (0-50% EtOAc in heptane) afforded the title compound as a colorless oil (347 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36 – 7.32 (m, 1 H), 7.31 – 7.27 (m, 2 H), 6.96 (ddd, *J* = 8.0, 2.5, 1.4 Hz, 1 H), 4.33 (s, 3 H), 3.87 (s, 3 H), 3.84 (s, 3 H).

4-(3-Methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole-5-carboxylic acid



To a solution of methyl 4-(3-methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole-5-carboxylate (347 mg, 1.40 mmol) in methanol (5 mL) at room temperature was added aqueous sodium hydroxide (2.8 mL, 1 N). The colorless solution was stirred for 16 h. The mixture was then concentrated, and the residue was dissolved in water and acidified to ~ pH 3 by the addition of aqueous hydrochloric acid (1 N), which caused a colorless precipitate to form. The mixture was extracted with ethyl acetate (3 x 25 mL). The organic layers were dried over magnesium sulfate and concentrated to afford the title acid as a colorless solid (111 mg, 34%), which was used without further purifications. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.39 – 7.30 (m, 3 H), 7.01 – 6.96 (m, 1 H), 4.36 (s, 3 H), 3.83 (s, 3 H).

4-(3-Methoxyphenyl)-*N*,1-dimethyl-*N*-(6-(trifluoromethyl)pyridin-3-yl)-1*H*-1,2,3-triazole-5-carboxamide (18)



18

To a solution of 4-(3-methoxyphenyl)-1-methyl-1*H*-1,2,3-triazole-5-carboxylic acid (47 mg, 0.20 mmol) in dicholoromethane (2 mL) was added oxalyl chloride (20 uL, 0.23 mmol) at 0 °C. A drop of *N*,*N*-dimethylformamide was then added. The mixture was allowed to reach room temperature over 4 h, at which time the mixture was concentrated to a viscous residue (90 mg, assumed 100%), containing the acid chloride intermediate. This crude acid chloride was combined in dichloromethane (1 mL) with *N*-methyl-6-(trifluoromethyl)pyridin-3-amine (50 mg, 0.28 mmol) at 0 °C, followed by the addition of triethylamine (85 µL, 0.60 mmol). The resulting mixture was stirred at room temperature for 16 h. The mixture was diluted with dichloromethane and washed with aqueous sodium bicarbonate. The organic layer was dried over magnesium sulfate and concentrated to a colorless oil, containing crude title compound. This residue was purified by Prep-HPLC to give the title compound (6.5 mg, 8%) as a white solid.

Prep HPLC method: Column: DIKMA Diamonsil(2) C18 200*20mm*5µm; Gradient: 40- 70% acetonitrile in water (0.225% formic acid) over 8 min followed by 1.5 min hold at 100% acetonitrile. 35 mL/min flow rate.

Analytical HPLC method: Column: Xbridge c18 2.1*50mm*5 µm; Mobile Phase A 0.0375% TFA in water; Mobile Phase B 0.01875% TFA in acetonitrile. Gradient 1% B to 5% B over 0.60 min, 5% B to 100 % over 3.40 min, 100% B to 1% B over 0.30 min, hold 1% B for 0.40 min. Flow rate 0.8 ml/min. Retention time 2.85 min. MS: ESI *m*/*z* 392.1 $[M+1]^+$; LCMS purity >99%.

Section 3: *in vitro* cAMP assay protocols

TGR5 is a Gαs-protein coupled that, when stimulated, induces the activation of Adenylate Cyclase (AC), thereby resulting in increases of intracellular cAMP. Agonists of TGR5 were identified using an *in-vitro* HTRF® (Homogeneous Time-Resolved Fluorescence) competitive immunoassay (HTRF® cAMP dynamic 2 Assay Kit; Cis Bio cat # 62AM4PEC) which compares basal cAMP level in whole cells with cAMP levels reached after stimulation with compound. A tracer molecule, d2-labeled cAMP, acts as an acceptor for a Europium (Eu^{3+}) cryptate donor. A monoclonal anti-cAMP antibody has been labeled with Eu-cryptate so that when d2-cAMP binds to the Mab, energy is transferred from the donor to the acceptor. The complex is excited by light at a wavelength of 340 nm and d2-cAMP binding is detected by emission at wavelength 665 nm. In this assay, intracellular cAMP generated by TGR5 activation competes with the d2-cAMP tracer molecule for binding to the labeled antibody, thus resulting in a change in fluorescence due to the prevention of energy transfer from the donor to the tracer. The fluorescent signal is therefore inversely proportional to the concentration of cellular cAMP resulting from TGR5 activation. Detecting and calculating the ratio of 665 nm/620 nm emissions allowed sources of interference to be minimized (e.g. medium, colored compounds).

The two cell lines used in this assay, Flp-In™-CHO-TO-humanTGR5 and Flp-In™-CHO-TO-canineTGR5, were both constructed using the Flp-In™ T-REx™ System (pcDNA™5/FRT/TO Vector Kit Invitrogen cat# V6520-20). This expression system utilized the tetracycline repressor gene to tightly regulate transcription of the gene of interest (GOI) which, in our case, was human and canine TGR5. In the absence of tetracycline, transcription is blocked and therefore little or no TGR5 is expressed. However, when cells are induced with doxycycline (an analog of tetracycline), transcription of our GOI occurs and very high levels of TGR5 are expressed. The screening cascade we developed utilized both the induced and uninduced cell lines. The induced cell line, with a high level of receptor expression, provided high sensitivity and a large dynamic range which allowed us to screen compounds with widely varying potencies. The lower-expressing, uninduced cell line provided a more conservative estimate of agonist potency and intrinsic activity.

The growth and assay conditions for both human and canine TGR5 cell lines were identical (induced canine TGR5 cells were not used in this publication). To prepare the cells for screening, one vial of cryo-preserved Flp-In™-CHO-TO-humanTGR5 (or Flp-In™-CHO-TO-canineTGR5,

respectively) was rapidly thawed in a 37°C water bath, transferred drop-wise into 10mL of Growth Media (F-12 Nutrient Mixture Ham's (Invitrogen, cat# 11765-054), 10% Tet System Approved Fetal Bovine Serum, US Sourced (Gamma Irradiated) (Clonetech, cat# 631101), 1% Penicillin/Streptomycin (Gibco, cat# 15140), 2mM L-Glutamine (Gibco cat# 25030), 550µg/mL Hygromycin B (Invitrogen cat#10687-010), 15µg/mL Blasticidin S HCl (Invitrogen cat# R210-01) in a 50mL conical tube, centrifuged at 1000rpm for 5min then gently resuspended in 15mL of fresh Growth Media before added to a T75 flask to incubate at 37°C, in a 5% CO^2 incubator. When cells reached ~80% confluence, they were expanded into two new flasks, one for induced cells and one for uninduced cells. The media was removed from the T75 flask and cells were washed with room temperature Dulbecco's Phosphate Buffered Saline (PBS) (Sigma cat# D8537). The PBS was aspirated and 5ml 0.05% Trypsin-EDTA solution (Gibco cat# 25300) was added to the flask and incubated for 2-3 minutes at 37°C/5% CO_2. The cells were detached by gently tapping the flask, then adding back 10mL pre-warmed Growth Media to deactivate Trypsin-EDTA. The cell density was adjusted to $1.5x10^7$ cells/ T175 flask so that 80% confluence could be reached 48 hours after seeding each flask. The cells were allowed to attach for 24 hours, after which one flask was induced with 4µg/mL doxycycline (prepared in 100% ethanol at 10mg/mL) before returning the flasks for overnight incubation. On the day of the assay, the cells were harvested and resuspended in Assay Media containing F-12 Nutrient Mixture Ham's (Invitrogen, cat# 11765-054), 0.1% Bovine Serum Albumin Fraction V (heat shock) (Roche Applied Bioscience, cat# 03116999001), Penicillin/Streptomycin (Gibco, cat# 15140), 2mM L-Glutamine (Gibco cat# 25030), 400µM isobutylmethylxanthine (IBMX) (Tocris Bioscience, cat#2845). Cells were re-suspended at a density of 2-4 x 10^5 cells/ml. A total of 5µl/well of cell suspension was dispensed to all wells of white Greiner 384-well, low-volume assay plate (VWR cat # 82051-458) using a Thermo Multidrop Combi.

Test compounds were serially diluted in 100% dimethysulfoxide (DMSO) and spotted 0.5µL/well to an empty, 384-well, polypropylene plate (Costar #3654). A reported TGR5 agonist (*S*)-1-(6-fluoro-2-methyl-3,4-dihydroquinolin-1(2H)-yl)-2-(isoquinolin-5-yloxy)ethanone (JP 2006063064) was used as a high control while DMSO was used as the low control. Reference compounds were also used in the assay. All wells of the spotted compound plate were diluted 1:120 with 60µL/well of assay media. 5µL was transferred from the compound plate to the assay plate containing 5µL/well of cells (final compound dilution = 1:240). After 30 minutes incubation at 22ºC, 5 µL of d2-labeled cAMP and 5 µL of anti-cAMP antibody (both diluted 1:20 in cell lysis buffer as described in the manufacturers assay protocol) were dispensed to each well of the assay plate using a Thermo Multidrop Combi. The plates were incubated at

room temperature for 60 minutes and then read with a Perkin-Elmer Envision™ 2104 multilabel plate reader using excitation wavelength of 330 nm and emission wavelengths of 615 nm and 665 nm to detect changes in the HTRF® signal. EC_{50} determinations were made from an agonist response curves analyzed with a curve fitting program using a 4-paramter logistic dose response equation. The intrinsic activity is calculated as the percent of maximal activity of the test compound, relative to the activity of a reported TGR5 agonist (*S*)-1-(6-fluoro-2-methyl-3,4-dihydroquinolin-1(2H)-yl)-2-(isoquinolin-5-yloxy)ethanone (JP 2006063064).

Section 4: Human *ex vivo* whole blood assay protocol

Whole blood from human donors was treated with heparin (1ml heparin added to 50 ml blood). After indomethacin to a final concentration of 1μM and compound **12** (final DMSO concentration of 0.1%) were added to 96 well plates, 500 μl of blood per well were aliquotted into 96 well plates and incubated at 37 °C for 30 minutes. LPS to a final concentration of 100 ng/ml was added and then incubated at 37 °C for 4 to 5 hours, mixing every 1 hour to prevent settling of blood. The plates were centrifuged at 2500 rpm for 10 minutes and 100 μL from each well assayed for TNFα following the manufacturer's instructions for the Millipore Milliplex MAP Human Cytokine/Chemokine Panel kit (catalog number MPXHCYTO-60K). Results were analyzed using GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. For human blood donations, documented informed consent of the donor was obtained in accordance with recognized international standards for the protection of human research subject(s).

Section 5: Procedures for in vitro clearance/plasma protein binding/ in vivo dog PK

Microsomal Incubations

Pooled human liver microsomes and pooled male dog liver microsomes were purchased from BD Biosciences (Woburn, MA). Reaction was conducted in a high throughput screen. Briefly, stock solutions of compound **12** (1 mM in dimethylsulfoxide) were diluted in 50:50 methanol:water (100 μM) and finally to 10 μM working stock in 0.1 M potassium phosphate buffer. The final concentration of dimethylsulfoxide in the incubation media was less than 0.1% (v/v). Microsomal stability assessments

were determined after incubation of compound **12** (1 μM) with human or dog liver microsomes (P450 concentration, 0.25 μM) in 0.1 M potassium phosphate buffer (pH 7.4), containing 1 mM magnesium chloride, at 37 °C. Incubations were conducted in the presence of NADPH (1 mM). Reaction mixture was quenched with acetonitrile at 0, 5, 10, 20, 30 and 60 minutes post initialization of the reaction. Samples were analyzed via LC-MS/MS for the disappearance of compound **12**. Incubation half-lives and corresponding incubation intrinsic clearance values were determined from analysis of the substrate depletion (Obach et al 1997).

In Vivo Pharmacokinetics

All animal care and in vivo procedures conducted were in accordance with guidelines of the Pfizer Animal Care and Use Committee. Male beagle dogs (~9-12 kg) were used as animal models for pharmacokinetic studies. Dogs were housed one per cage in an American Animal Association Laboratory Animal Care accredited facility with a 12 h light/dark cycle (7:00 AM - 7:00 PM). This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were allowed ad libitum access to water and were fasted overnight before dosing and food was returned following the 4 hr blood collection timepoint. Compound **12** was administered intravenously via the cephalic vein (n = 3). For oral studies, **12** was administered by oral gavage to dogs (n = 2). Compound **12** was administered at 1.0 mg/kg i.v. and 3.0 mg/kg p.o.. Compound **12** was formulated as a solution in polyethylene glycol-400 and a 30% sulfobutylether-β-cyclodextrin (40:60, v/v) and filtered through a 0.22 μm sterile filter for intravenous administration and as a suspension in 0.5% (w/v) methylcellulose for oral studies. After dosing, serial plasma samples were collected at appropriate times and kept frozen at −20°C until LC-MS/MS analysis. Urine samples (0–7.0 and 7.0–24 h) were also collected after intravenous administration.

Protein binding

Frozen plasma in K3EDTA from male beagle dog was purchased from Bioreclamation, Inc. (Westbury, NY). Dulbecco's phosphate buffered saline was purchased from Sigma (Saint Louis, MO). A 96-well equilibrium dialysis apparatus from HTDialysis (Gales Ferry, CT) was used to determine the fu,plasma (Pacifini and Viani 1992; Banker et al. 2003). Spectra-Por 2 membranes with molecular cutoff of 12 to 14 kDa, obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA), were used for the dialysis. The Spectra-Por 2 membranes were conditioned in HPLC grade water for 15 min followed by 30% ethanol for 15 min and 0.10 M sodium phosphate pH 7.4 buffer for 15 min. Frozen plasmas were thawed on the day of the experiment. Untreated plasma and plasma treated with compound **12** (1 μM) and aliquots (150 μl) were loaded into the 96-well equilibrium dialysis plate and dialyzed versus 150 μl of Dulbecco's phosphate-buffered saline buffer. Equilibrium was achieved by incubating the 96-well

equilibrium dialysis apparatus on a plate shaker (set at 155 rpm) for 4 hr in a 37 °C CO2 incubator. After reaching equilibrium, 20 µl of plasma sample and 100 µl of buffer sample aliquots were taken from the 96-well equilibrium dialysis apparatus and added to HPLC vials containing 200 µl of acetonitrile and Terfenadine as an internal standard. The appropriate amount of control buffer was added to the plasma and the appropriate amount of control plasma was added to the buffer samples to yield identical matrix between buffer and non-buffer samples. The samples were centrifuged and the supernatant was assayed by LC-MS/MS. The percentage of **12** bound to plasma proteins was calculated as: 100 – [(concentration of **12** in buffer/concentration of **12** in plasma) x 100%] (Maurer et al 2005; Kalvass and Maurer 2002).

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