Journal of
Medicinal Chemistry

Article

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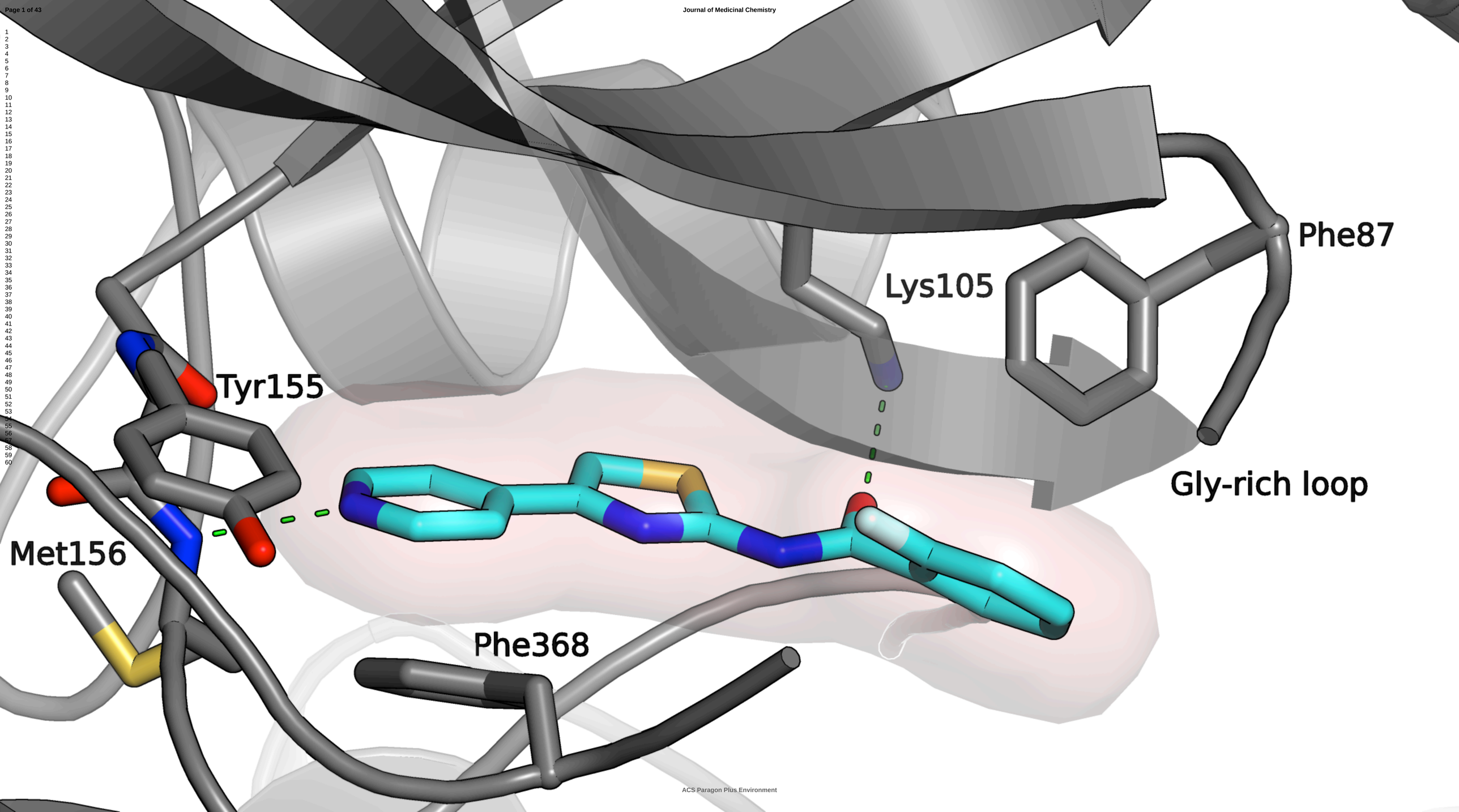
J. Med. Chem., **Just Accepted Manuscript** • Publication Date (Web): 03 Jun 2015

Just Accepted

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036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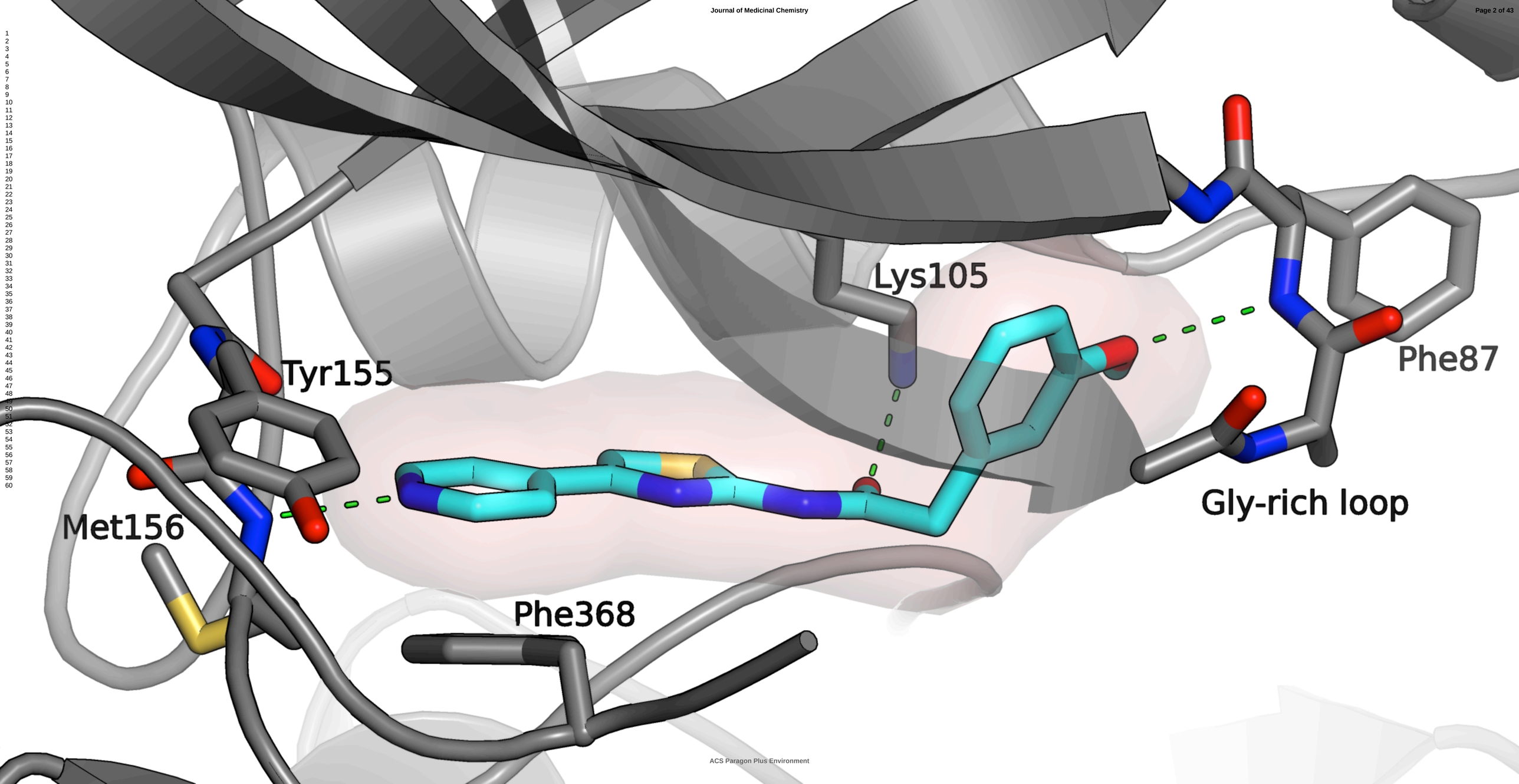
Tyr155
Gly-rich loop
Met156
Phe368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5/22/15

Design, Synthesis and Structure-Activity Relationships of Pyridine-Based Rho Kinase (ROCK) Inhibitors.

Jeremy Green[a]*, Jingrong Cao[a], Upul K. Bandarage[a], Huai Gao[a], John Court[a], Craig Marhefka[a], Marc Jacobs[a], Paul Taslimi[a,√], David Newsome[a], Tomoko Nakayama[a,†], Sundeep Shah[b], Steve Rodems[b,∫].

[a]Vertex Pharmaceuticals, Inc., 50 Northern Avenue, Boston, MA 02210, USA

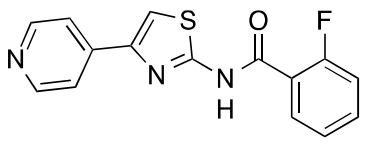
[b]Vertex Pharmaceuticals, Inc., 11010 Torreyana Road, San Diego, CA 92121, USA

* Corresponding author. Telephone: 617-341-6315; email: jeremy_green@vrtx.com

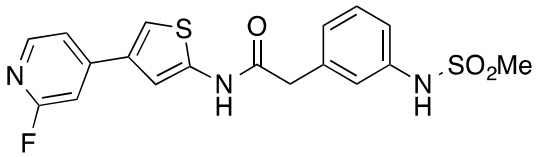
√ Present address: Ariad Pharmaceuticals, 26 Landsdowne Street, Cambridge, MA 02139
† Present address: Digilab, Inc., 100 Locke Drive, Marlborough, MA 01752
∫ Present address: Retrophin, Inc., 12255 El Camino Real, Suite 250, San Diego, CA 92130

Abstract

The Rho kinases (ROCK1 and ROCK2) are highly homologous serine/threonine kinases that act on substrates associated with cellular motility, morphology and contraction, and are of therapeutic interest in diseases associated with cellular migration and contraction, such as hypertension, glaucoma and erectile dysfunction. Beginning with compound **4**, an inhibitor of ROCK1 identified through high-throughput screening, systematic exploration of SAR and application of structure-based design, led to potent and selective ROCK inhibitors. Compound **37** represents significant improvements in inhibition potency, kinase selectivity and CYP inhibition and possesses pharmacokinetics suitable for *in vivo* experimentation.



4
ROCK K_i = 1300 nM



37
ROCK K_i = 10 nM

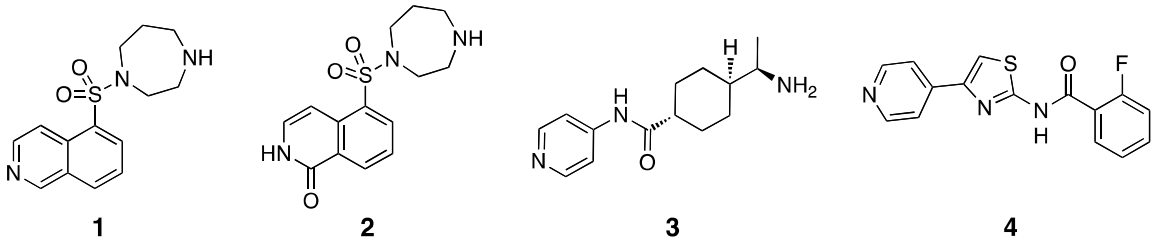
Introduction

The Rho kinases (ROCK1 and ROCK2) are 160 kDa serine/threonine kinases consisting of an N-terminal kinase domain, a central coiled-coil region containing the Rho binding domain and a C-terminal pleckstrin homology (PH) domain, containing an internal cysteine-rich region.[1] The C-terminal region negatively regulates ROCK activity,[2] however upon binding of activated RhoA-GTP, the interaction between the C-terminal inhibitory region and the kinase domain is disrupted and kinase function is activated. ROCK1 and ROCK2 are highly homologous in their kinase domains (92% sequence identity) with 65% overall sequence identity[3] and both are ubiquitously expressed in rat and mouse tissues. ROCK phosphorylates a variety of cellular substrates, mainly associated with actin-cytoskeletal reorganization affecting cell adhesion, cell morphology, cell motility and smooth muscle contraction.[4] Notable substrates include myosin light chain (MLC) and the myosin binding subunit of myosin phosphatase (MYPT). Other substrates include proteins acting on actin, such as the LIM kinases, adducin and the ERM proteins.[5] The action of ROCK on these substrates implicates ROCK as a potential therapeutic target for indications wherein intervention in smooth muscle contraction is desired, such as hypertension and cardiovascular disease,[6] glaucoma,[7] Raynaud's disease[8] or erectile dysfunction.[9]

A number of molecules have been reported that inhibit ROCK, the most widely studied of which are compound **1** (Fasudil)[10] and compound **3** (Y-27632).[11] Fasudil, is approved for clinical use in Japan for the treatment of sub-arachnoid hemorrhage.[12] Interestingly, compound **1** is metabolized *in vivo* to the quinolone, **2** (widely referred to as hydroxyfasudil),[13] which is also a ROCK inhibitor, even though the mode of binding

to ROCK is changed.[14] Compound **3**[11] has also been widely utilized as a ROCK inhibitor standard in a large number of studies.[15] Compounds **1**-**3** are modest inhibitors of ROCK, with reported comparable K_i values of 530, 150 and 150 nM respectively.[14] More recently, numerous publications have reported potent ROCK inhibitors based on derivatives of indazoles, isoquinolines, phthalimides, aminofurazans, pyrazoles, aminopyrimidines, 7-azaindoles and pyridines. Much of this work has been reviewed.[16] In particular, a number of pyridine-containing inhibitors of ROCK have been published,[17] prompting us to report our findings in this area.

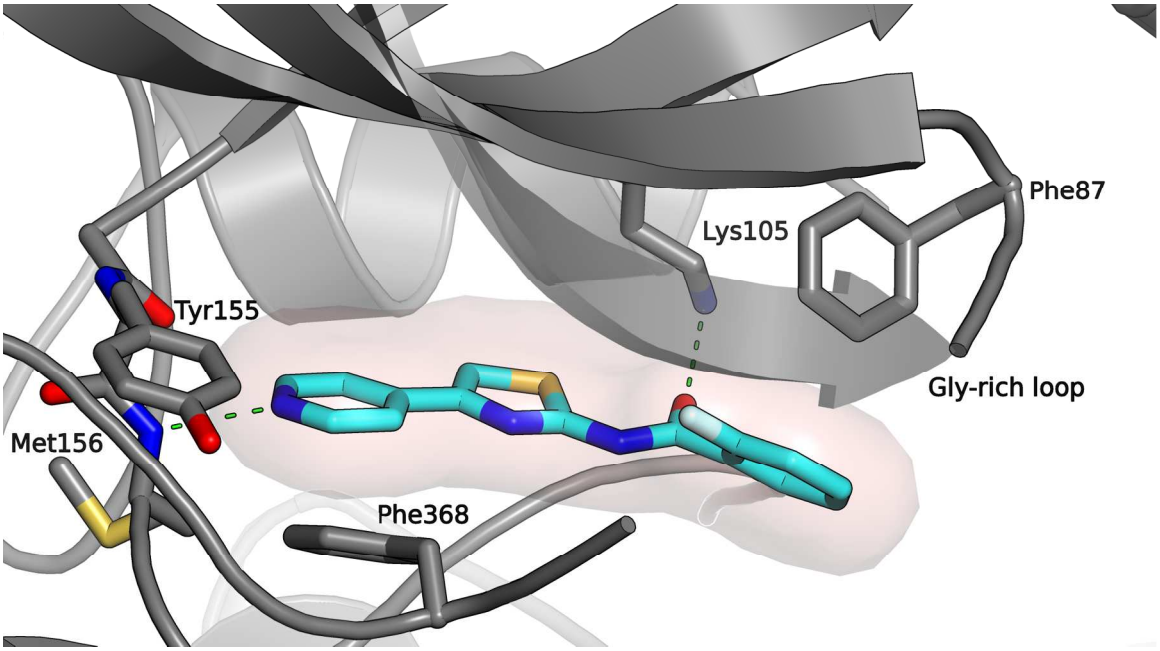
Figure 1. Structures of ROCK inhibitors



High throughput screening of our compound collection identified compound **4** as an interesting inhibitor of ROCK1, with K_i = 1.1 μM. This compound had not been previously identified in our other kinase screens and thus we felt that it may offer opportunities to develop both potent and selective inhibitors. It was also less active against PKAα (K_i > 5 μM), a closely related member of the AGC kinase subfamily,[18] suggesting that selectivity within this subfamily might also be achieved. An X-ray crystallographic structure of **4** bound to ROCK-1 (PDB ID: 4YVC) showed several notable features, illustrated in Figure 2. The pyridine group is hydrogen bonded to the backbone NH of hinge residue Met156. In contrast to most other kinases, the hinge

region constitutes a deep, distinct binding pocket as the lower region is blocked by the sidechains of residues Tyr155 and Phe368, blocking access to solvent from the pyridine 2-position. The amide carbonyl of **4** is within H-bonding distance of catalytic Lys105. The 2-fluorophenyl ring lies unproductively beneath the glycine-rich loop and suggests an obvious area for the design of improved inhibitors. We reasoned that insertion of one or more* *sp3*-hybridized atoms next to the carbonyl (for example, an N-phenylacetyl group) would allow this ring (or any other group) to occupy space beneath the glycine-rich loop and for substituents to consummate further enzyme-inhibitor interactions with loop residues.

Figure 2. X-ray crystallographic structure of **4** bound to ROCK1 (PDB ID: 4YVC)



* Extending the chain length between aryl group and carbonyl was also investigated and results are summarized in Supporting Material Table S2. Although the parent phenylpropionyl compound offered potency and selectivity, the crystal structure of **10** suggested that less space is available for substituents to make productive interactions.

Results and Discussion

Exploration of acyl substituents showed that extending the substituent into the pocket defined by the glycine-rich loop enhanced ROCK inhibition potency relative to **4**, and established a baseline for selectivity against PKA (Table 1). The X-ray crystal structure of compound **10** (K_i = 100 nM) (Figure 3, PDB ID: 4YVE) indicated that aryl substituents, such as the 3-methoxy of **10**, are capable of additional, productive interactions with the glycine-rich loop. Using this structure as a guide, substitutions capable of forming hydrogen-bonds with the main chain amide of Phe87 were explored. The 3-hydroxy- and 3,4-methylenedioxy-compounds **13** and **15** were similarly potent at 28 nM and 100 nM respectively, while combination of a 2-chloro substituent, with the 4,5-methylenedioxy in compound **16** further enhances potency into the single digit nanomolar range, presumably as a result of conformational constraint and reduced entropic contribution. Other 3-substituents capable of H-bonding interaction with the Phe87 NH were also investigated. Among these, compound **18** bearing a 3-methanesulfonylamino group, provided the best overall combination of potency (K_i = 26 nM) and selectivity (127-fold *vs* PKAα).

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Figure 3. X-ray crystallographic structure of **10** bound to ROCK1 (PDB ID: 4YVE)

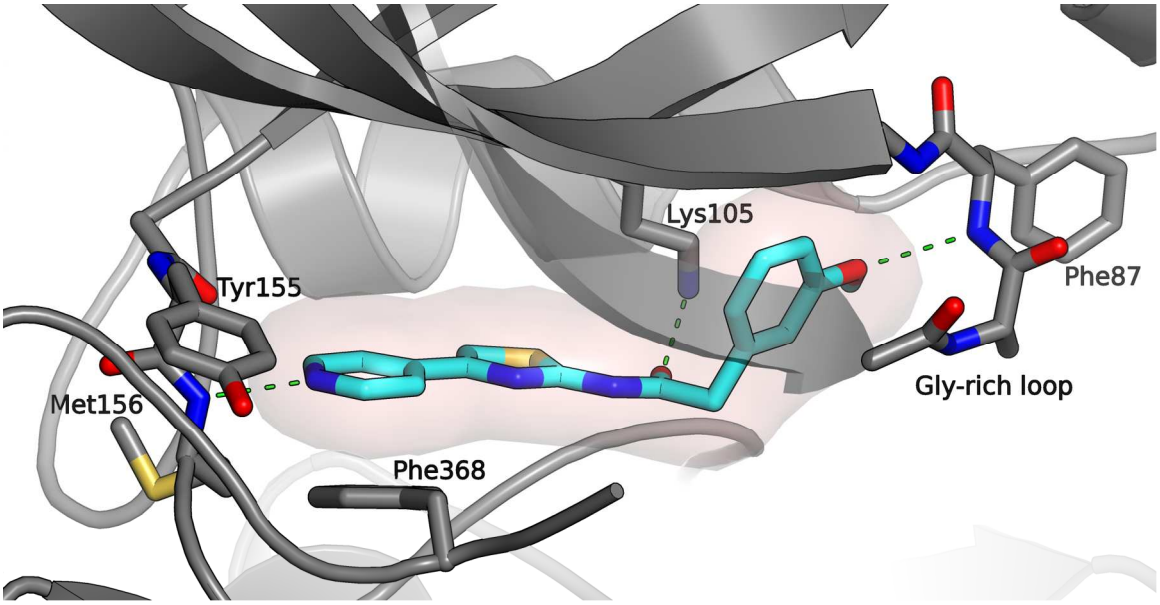
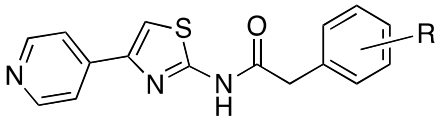


Table 1. SAR of substituted phenylacetyl series

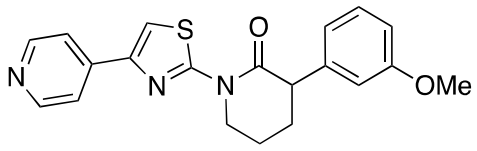


Compound	R	ROCK K_i, nM[a]	PKA K_i, nM	Selectivity
5	H	170	3500	21
6	2-F	130	2090	16
7	3-F	120	2000	17
8	4-F	81	1580	20
9	2-OMe	>2500	>5000	NC
10	3-OMe	100	2500	25
11	4-OMe	540	>5000	9
12	2-OH	900	>5000	>6
13	3-OH	28	1700	61
14	4-OH	70	>5000	>71
15	3,4-OCH_2O-	100	>5000	>50
16	2-Cl-3,4-OCH_2O-	7	310	23
17	3-NH_2	350	>5000	>14
18	3-$NHSO_2CH_3$	26	>3300	127

[a] Minimum Significant Ratio (MSR)[19] = 3.6: i.e. compounds that have a difference in K_i of at least a factor of 3.6 are considered significantly different.

Substitution at the methylene of the phenylacetyl series was next investigated to improve PKA selectivity over ROCK. In most cases, neither ROCK potency nor PKA selectivity was improved significantly, though lactam **19**, showed improved potency for ROCK and 135-fold selectivity against PKA (Figure 4). Other attempts to capitalize on N- or C-substitution did not lead to significant improvements in potency or selectivity (see Supporting Information Table S3).

Figure 4.



19

ROCK K_i = 17 nM
PKA K_i = 2300 nM

Replacement of the thiazole ring with other 5-membered rings was investigated and the results are summarized in Table 2. Non-sulfur-containing heterocycles such as pyrazole, triazole and oxadiazole derivatives showed relatively weak ROCK inhibition (data not shown), while thiophenes, regioisomeric thiazoles and thiadiazoles showed good ROCK potency. For these sulfur-containing heterocycles, those with NH adjacent to S atom (**10**, **22**, **25**) showed better activity than the corresponding isomers in which the 4-pyridyl system was adjacent to sulfur (**20**, **23**), while systems in which sulfur bridged the two substituted carbons was least effective (**21**, **24**, **26**). Thus, thiazole **10** and thiophene **22** were identified as the best leads in this series, with the latter showing some improvements in potency and selectivity.

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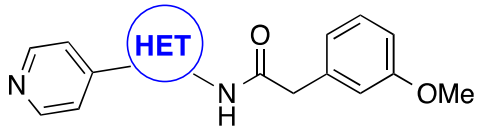
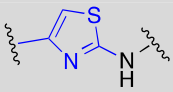
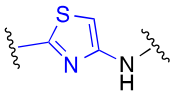
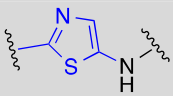
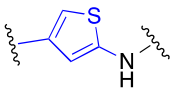
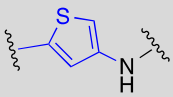
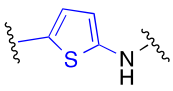
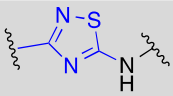
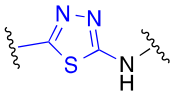
Table 2. SAR of central heterocycle replacements



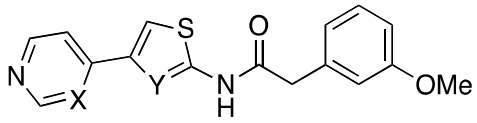
Cpd	HET	ROCK Ki, nM	PKA Ki, nM	Selectivity
10		100	>2500	>25
20		530	>5000	>10
21		490	520	1
22		10	600	60
23		61	2400	39
24		140	1070	7
25*		89	500	6
26		>2500	1700	<1

* 4-fluorophenylacetyl derivative

Despite the pyridine ring providing a simple template for binding to the hinge pocket of ROCK, compounds such as **10** and **22** also possessed less desirable qualities, notably pan-cytochrome P450 inhibition. These compounds inhibited CYP 3A4 with IC_{50} = 1.9 and 0.80 μM respectively. Applying lessons from p38 inhibitors, in which a pyridine

hinge binding ring was successfully replaced with an unsubstituted pyrimidine to maintain target affinity and reduce CYP inhibition,[20] we investigated the analogous modification (Table 3). However this tactic did not succeed. Compound **27** containing the pyrimidinyl-thiazole system, lessened CYP 3A4 inhibition (CYP 3A4 IC_{50} = 10 μM), but ROCK potency was lost (Ki > 2.5 μM).

Table 3. SAR and CYP 3A4 inhibition of the 4-Pyridyl and 4-Pyrimidinyl Substituted Thiazole and Thiophenes



Cpd	X	Y	ROCK K_i, nM	PKA K_i, nM	Selectivity	CYP 3A4 IC_{50}, μM
10	CH	N	100	>2500	>25	0.75
22	CH	CH	10	600	60	0.48
27	N	N	>2500	>5000	-	10
28	N	CH	120	2900	24	2.0

Our interpretation for the loss of ROCK inhibition is that the low energy conformation orients the two aromatic rings such that the nitrogen atoms are separated by 180° as shown in Figure 5A. In the case of **27** this places the pyrimidine nitrogen in an unfavourable hydrophobic location in the binding site. This energy barrier is remedied by replacing thiazole with thiophene, since the rotational difference is minimal, and ROCK inhibition is restored. Unfortunately the thiophene compound **28** retained significant CYP inhibitory activity (CYP 3A4 IC_{50} = 3.7 μM).

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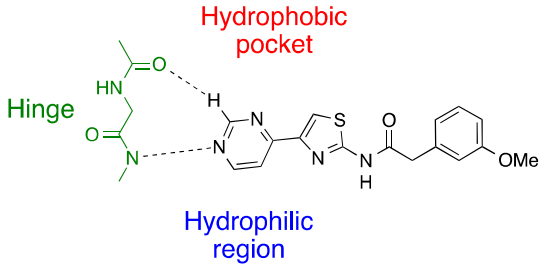
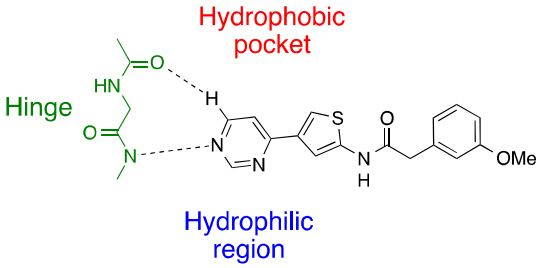
Figure 5. Rationale for observed pyrimidine activities.

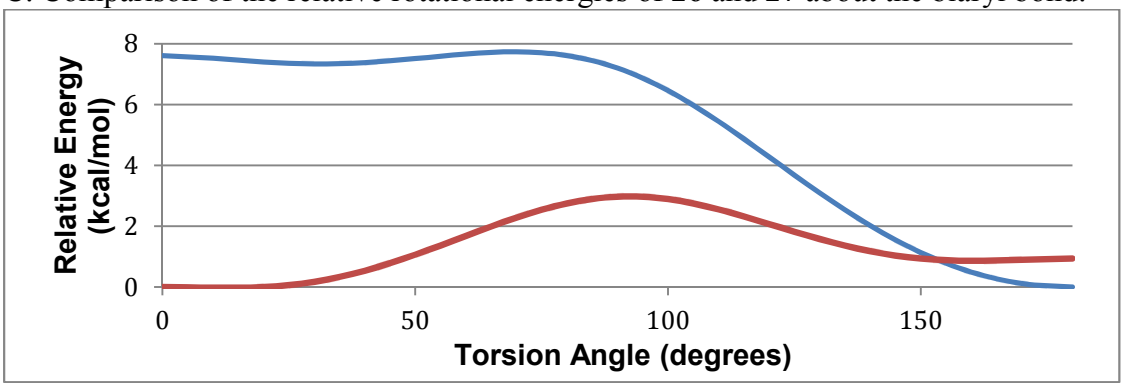
A. Unfavourable orientation of low energy conformation of **27**



B. Favourable orientation of thiophene **28**



C. Comparison of the relative rotational energies of **26** and **27** about the biaryl bond.

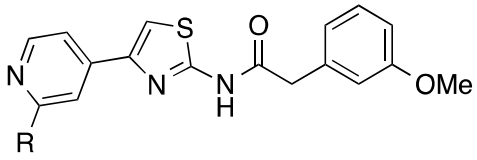


blue = **27** red = **28**

One strategy to circumvent CYP inhibition is to interfere with the interaction of the heme group of the CYP enzyme with the pyridine.[21] Therefore, we next investigated substitution of the pyridine ring, focusing on position 2. The results are summarized in Table 4. In general, pyridine 2-substitution had a desirable effect on reducing CYP inhibition to acceptable levels. The 2-aminopyridine compound **30** demonstrated 35-fold less CYP 3A4 inhibition liability compared with unsubstituted compound **10** (IC_{50} = 26 μM and 0.75 μM respectively) while maintaining the ROCK potency (K_i = 170 nM). However, most of the pyridine 2-substitutions had little significant effect on ROCK inhibitory activity, with 2-methyl- (**29**), 2-fluoro- (**34**) and 2-chloro-pyridines (**35**) within 2-fold of the unsubstituted system, **10**. Larger substituents such as 2-methylamino- (**31**),

2-dimethylamino- (**32**) and 2-methoxy-pyridine (**33**) significantly decreased ROCK potency. In the case of compound **31**, modeling suggested that even the relatively small methylamino group requires sufficient space to result in steric overlap with residues Tyr155 and Phe368 at the base of the pyridine-binding pocket. In compound **33,** potential unfavourable interactions between the methoxy oxygen and carbonyls of hinge residues Gln 154 and Met156 explains the poor ROCK potency.

Table 4. SAR of 2-Substituted pyridines

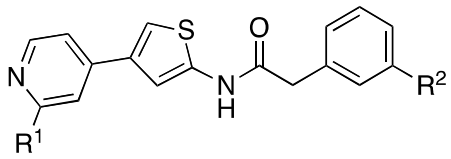


Cpd	R	ROCK Ki, nM	PKA Ki, nM	Selectivity	CYP 3A4, IC_{50}, μM
10	2-H	100	>2500	>25	0.75
29	2-CH_3	210	>3300	>15	7.4
30	2-NH_2	170	1850	11	26
31	2-NHMe	770	>3300	>4	11.4
32	2-NMe_2	>2500	>3300	-	3.5
33	2-OMe	>2500	>3300	-	8.3
34	2-F	110	>3300	>30	1.5
35	2-Cl	120	>3300	>27	3.6

The above described scaffold modifications, taken *in toto*, led to a set of molecules that encapsulate the key findings, and that offered the best opportunities to secure ROCK potency and PKA selectivity while minimizing CYP inhibition. Compounds containing the optimal phenylacetyl, thiophene and 2-pyridine substitutions are summarized in Table 5. In addition Table 5 includes cellular activity of the top compounds, representing the

effects of ROCK inhibitors on cellular migration of THP-1 cells towards the chemokine attractant MCP-1.

Table 5. Activity profiles of combinations of preferred substituents



Cpd	R^1	R^2	ROCK K_i, nM	PKA K_i, nM	Selectivity, fold	THP-1 migration, IC_{50}	CYP 3A4, IC_{50}, μM
36	F	3-OMe	18	780	43	840	3.8
37	F	3-$NHSO_2Me$	10	3000	300	240	>30
38	Cl	3-OMe	44	2200	50	1600	5.0
39	Cl	3-$NHSO_2Me$	45	>3300	>73	650	7.5
40	NH_2	3-OMe	22	140	6	590	5.6
41	NH_2	3-$NHSO_2Me$	30	850	28	450	>15

The 2-fluoro- and 2-amino-substituted pyridines **37** and **41** showed reduced CYP inhibition while maintaining good ROCK potency and PKA selectivity. The 2-chloropyridines **38** and **39** were less potent than the analogous 2-fluoropyridines **36** and **37**. Compound **37** represented the best overall combination of ROCK potency, PKA selectivity, cellular efficacy and CYP inhibition for further investigation. The crystal structure of **37** bound to the active ste of ROCK is illustrated in Figure 6 and shows additional contact between the methylsulfonamide and the glycine-rich loop, contacting both Phe87, as previously seen with compound **10**, as well as a new interaction with the NH of Ala86. Kinase selectivity screening of **37** at 2 μM against a panel of 46 kinases, revealed that only three kinases, all from the AGC family kinases, were inhibited at

greater than 25%, namely MSK1 (54%), PRK2 (90%) and ROCK2 (99%). Further CYP profiling showed that potent inhibition had been abrogated by the combination of the thiophene and 2-fluoropyridine (Table 5). *In vivo* rat pharmacokinetics of compound **37** showed excellent properties and is suitable for oral administration (Table 6).

Figure 6. X-ray crystallographic structure of **37** bound to ROCK1 (PDB ID: 5BML)

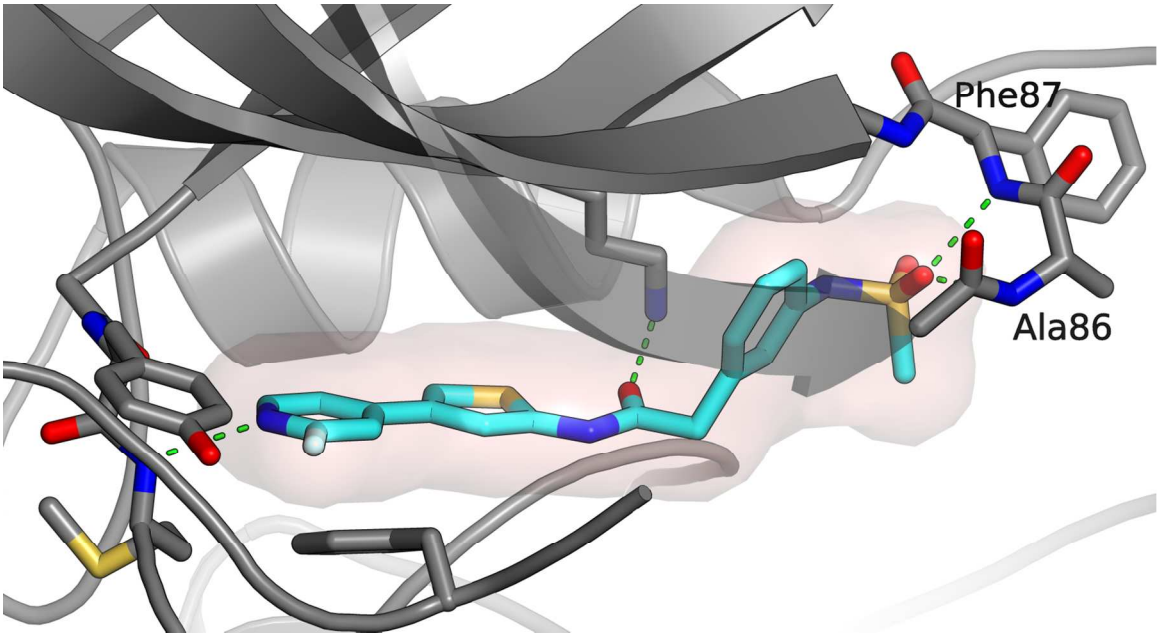


Table 5. CYP inhibition profile of **37**

CYP isozyme	IC_{50}, μM
1A2	>100
2C9	12
2C19	7.3
2D6	8.7
3A4	>30

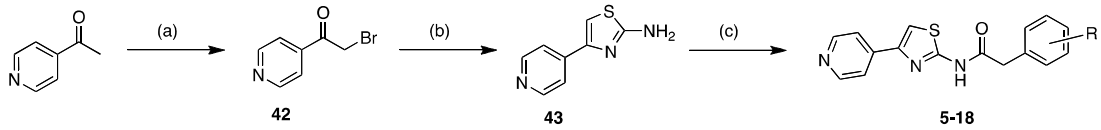
Table 6. Rat pharmacokinetic profile of compound **37**

Pharmacokinetics (i.v.)	
CL (L/min/kg)	3.45
$T_{1/2}$ (h)	2.6
V_{dss} (L/kg)	0.675
C_{max} (μg/mL)	2.92
AUC (μg h/mL)	4.31
Pharmacokinetics (p.o.)	
Formulation	0.5% aqueous methyl cellulose
C_{max} (μg/mL)	3.2
AUC (μg h/mL)	32.6
T_{max} (h)	3
$T_{1/2}$ (h)	3.4
%F	69

Chemistry

Acylated aminothiazoles were synthesized as shown in Scheme 1. The synthesis began with α-bromination of 4-acetylpyridine with bromine, followed by reaction of α-bromoketone **42** with thiourea to obtain 4-(pyridin-4-yl)thiazol-2-amine **43**. The weak nucleophilicity of **43** required a convenient and consistent method for acylation for which we identified the coupling agent *N*-(1-methanesulfonyl)benzotriazole ($MeSO_2Bt$) reported by Katritzky *et al.*[22] Microwave heating of a mixture of **43** with carboxylic acids in the presence of $MeSO_2Bt$ and Et_3N in THF at 160°C reliably afforded compounds **5-18** in good yields.

Scheme 1. Preparation of N-Acyl-2-Aminothiazole Derivatives

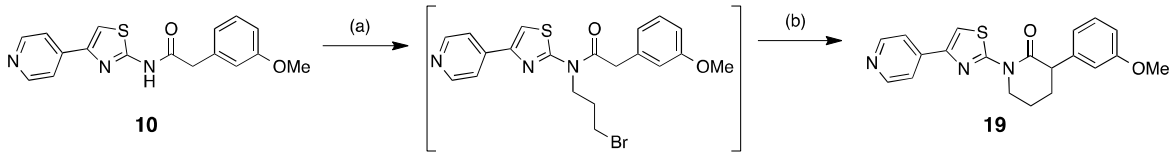


(a) Br_2, 48% HBr, 70°C; (b) thiourea, EtOH, reflux; (c) carboxylic acid, $MeSO_2Bt$, Et_3N, THF, μW, 160°C, 10 min

Compound **19** was synthesized by Mitsunobu reaction with 3-hydroxybromopropane and compound **10** and followed by sodium hydride-mediated cyclization as shown in Scheme 2.

5/22/15

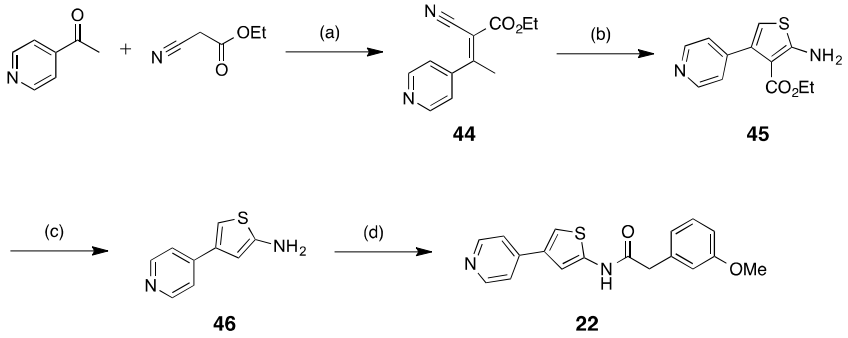
Scheme 2. Aminothiazole Alkylation via Mitsunobu Reaction and Subsequent Cyclization.



(a) $Br(CH_2)_3OH$, Ph_3P, DIAD, THF, 0°C; (b) NaH, THF, 0°C.

2-Amino-4-(pyridin-4-yl)thiophene **46** was synthesized in 3 steps as shown in Scheme 3. Knoevenagel condensation of 4-pyridylacetophenone with ethyl cyanoacetate, followed by Gewald synthesis of thiophene using elemental sulfur in morpholine afforded compound **45** in good yield.[23] Hydrolysis and decarboxylation of compound **45** afforded amino thiophene **46**.

Scheme 3. Preparation of 2-Amino-4-(pyridin-4-yl)thiophene.

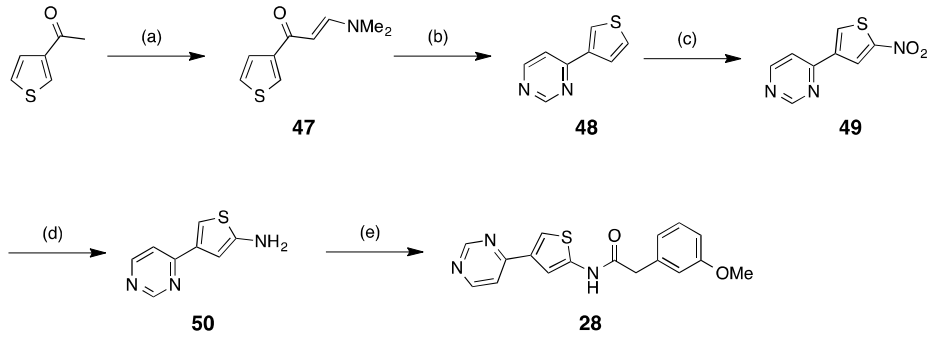


(a) NH_4OAc, AcOH, benzene, reflux; (b) S_8, morpholine, EtOH, RT; (c) KOH (20% aq), EtOH, reflux; (d) 3-methoxyphenylacetic acid, $MeSO_2Bt$, Et_3N, THF, μW, 160°C, 10 min

4-(Pyrimidin-4-yl)thiophen-2-amine **50** was synthesized in 4 steps as illustrated in Scheme 4. 3-Acetylthiophene was treated with DMF-DMA, followed by formamidine to

give the 3-(pyrimidine-4-yl)-thiophene **48**, which was subjected to nitration and followed by reduction to give the desired aminothiophene **50**.

Scheme 4. Preparation of 4-(pyrimidin-4-yl)thiophen-2-amine.

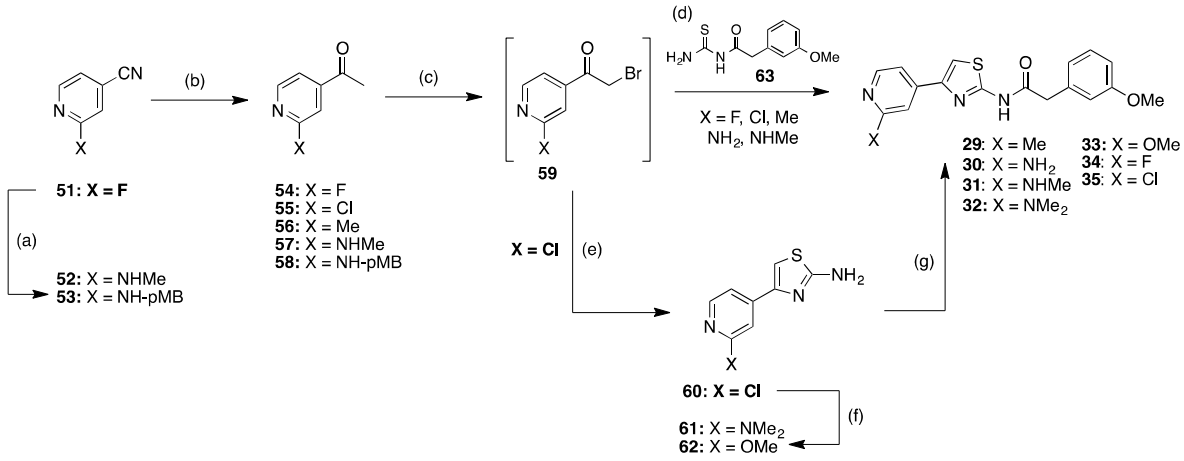


(a) DMF-DMA, reflux; (b) $CH(NH)NH_2.HCl$, K_2CO_3, DMF, 80°C; (c) KNO_3, H_2SO_4, 0°C; (d) H_2, Pd-C, RT; (e) 3-methoxyphenylacetic acid, $MeSO_2Bt$, Et_3N, THF, μW, 160°C, 10 min

2-Substituted 4-(pyridin-4-yl)-2-aminothiazoles were generally prepared by the method previously reported for 2-aminopyridines (Scheme 5).[24] Starting from either 2-substituted-4-acetylpyridines (**54-58**), or the precursor 4-nitriles (**51-53**) (converted to 4-acetyl by the action of methylmagnesium bromide), treatment with bromine in hydrobromic acid affords the 4-bromomethylketones **59**, which were converted directly to the acyl aminothiazoles **29-35** (X = F, Cl, Me, NH_2, NH_2Me) by the modified Hantzsch reaction with the phenylacetylthioureas. In the case of the 2-NH_2 system, *in situ* cleavage of the *p*-methoxybenzyl group under brominating conditions occurs, allowing direct access to the desired products on condensation of the crude product with the phenylacetylthioureas. For the 2-methoxy and 2-dimethylamino substitutions, the 2-

chloropyridin-4-yl-2-aminothiazole **60** was treated with sodium methoxide or dimethylamine respectively to afford compounds **61** and **62** respectively then by subsequent acylation gave final acylated products.

Scheme 5. Synthesis of 2-Methyl-, 2-Fluoro-, 2-Chloro-, 2-Methoxy, 2-Amino, 2-Methylamino and 2-Dimethylamino Substituted Pyridines

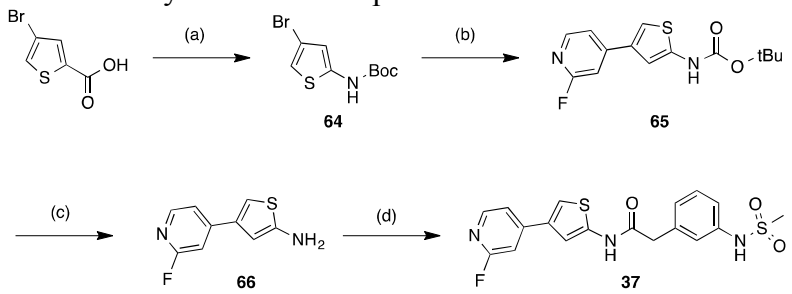


(a) X = *p*MBNH$_2$: DMA, 130°C, 1 hr. X = NHMe: 40% CH_3NH_2, DMA, 130°C, 1 hr; (b) (i) 3M MeMgBr, (ii) 6N HCl; (c) Br_2, 48% HBr, AcOH, 70°C; (d) 3-(MeO)-$PhCH_2CONHCSNH_2$, EtOH, 70°C; (e) NH_2CSNH_2, EtOH, 70°C; (f) X=NMe_2: $Me_2NH.HCl$, EtOH, Et_3N, µW, 160°C. X = OMe: 25% NaOMe, MeOH, toluene, 110°C; (g) 3-methoxyphenylacetic acid, $MeSO_2Bt$, Et_3N, THF, µW, 160°C, 10 min

Compound **37** was synthesized in four steps as outlined in Scheme 6. Curtius rearrangement of 4-bromothiophene-2-carboxylic acid afforded compound **63**, which was coupled with 2-fluoropyridine-4-boronic acid to give compound **64**. N-Boc deprotection afforded amino thiophene **66**. Coupling of **65** with 2-(3-methyl-

sulfonylaminophenyl)acetic acid by means of of $MeSO_2Bt$ afforded compound **37** in good yield.

Scheme 6. Synthesis of Compound **37**



(a) DPPA, *t*-BuOH, Et_3N, 100°C, 5 h; (b)(i) 2-fluoro-pyridine-4-boronic acid, $Pd(PPh_3)_4$, K_2CO_3, dioxane, water, 100°C, 4 h; (c) TFA, DCM, RT, 4 h; (d) 3-(methylsulfonyl-amino)-phenylacetic acid, $MeSO_2Bt$, Et_3N, THF, 85°C, 18 h.

Conclusion

We have synthesized a series of N-acylated aminothiazoles and evaluated their biological activity against the Rho kinase. These compounds possess high ROCK potency and moderate-to-excellent PKA selectivity. X-ray crystallography was used to help guide productive interaction with the target enzyme, as well as understand and forecast unproductive interactions in the closely related kinase PKA. The critical liability of the pyridine system, CYP inhibition, was addressed through pyridine replacement and substitution, leading to small 2-substituents that reduced CYP inhibition, yet did not affect ROCK inhibition. Compound **37** was identified as an optimal development of this series, with excellent potency, selectivity against a panel of kinases, good cellular activity and excellent pharmacokinetics. Thus, compound **37** is an excellent compound for further

study of the pharmacologic effects of ROCK inhibition *via* oral dosing experiments, results of which will be reported elsewhere.

Acknowledgements

The authors thank their colleagues Cameron Moody and Christine Memmott for the high throughput screen and assay development, Mike Arnost and John Cochran for synthesis support, and Barry Davis, Frank Holland and Leonard Kalkevijc for analytical chemistry support.

Experimental Section

Reagents and anhydrous solvents were obtained from commercial sources and were used without further purification. Reactions heated by microwave were performed in sealed tubes on a Biotage Initiator microwave synthesis reactor (Biotage, Uppsala, Sweden).

LCMS data was acquired by the following methods:

Method A: Samples were analyzed on a Micro Mass ZQ mass spectrometer operated in a single MS mode with electrospray ionization. Samples were introduced into the mass spectrometer by direct (flow) injection (FIA-MS) or following HPLC chromatography on an Agilent 1100 system (LC-MS). Separation was effected on a YMC ODS-Aq C18 column (3.0 × 150 mm) eluted with a linear gradient from 10% to 90% CH_3CN in H_2O (each containing v/v 0.2% formic acid) over 7 min at a flow rate of 1.5 mL/min.

Method B: Separations were performed on a tandem Waters Acquity Classic UPLC-MS system using a single quadrupole mass spectrometer (model SQ2, Waters) and a 50x 2.1 mm BEH C18, 1.7 mm column from Waters Corporation. The column was heated at

50°C and eluted with a gradient of 1% to 99% CH_3CN in H_2O (each containing 0.05% v/v TFA) over 2.9 minutes at 1.2 mL/min.

High resolution mass spectra were acquired on a Thermo QExactive Orbitrap mass spectrometer following chromatography on a Waters Acquity UPLC system. Preparative HPLC was performed on a Gilson HPLC system using a gradient of 10-90% MeCN in H_2O (0.1% TFA) at a flow rate of 15 mL/min over 10 min on either YMC ODS-AQ C18 or Kromasil C8 columns (10 micron particle size; 21 x 150 mm). ^1H NMR spectra (δ, ppm) were recorded using a Bruker DRX-500 (500 MHz), Bruker Avance 300 (300 MHz) or Bruker Avance-III (400 MHz) instrument. Exemplary methods are described for key compounds. The purity of compounds was determined by LC absorbance at two wavelengths, typically 220 nm and 254 nm, and all tested compounds were >95% pure by peak area. Additional compound characterization data is available in Table S1.

ROCK Enzyme Assay

Compounds were screened for their ability to inhibit ROCK I (AA 6-553) activity using a standard coupled enzyme system.[25] Reactions were carried out in a solution containing 100 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 25 mM NaCl, 2 mM DTT and 1.5% DMSO. Final substrate concentrations in the assay were 45 μM ATP (Sigma Chemicals, St Louis, MO) and 200 μM peptide (Lys-Lys-Arg-Asn-Arg-Thr-Leu-Ser-Val) (American Peptide, Sunnyvale, CA). Reactions were carried out at 30 °C and 45 nM ROCK1. Final concentrations of the components of the coupled enzyme system were 2.5 mM phosphoenolpyruvate, 350 μM NADH, 30 μg/ml pyruvate kinase and 10 μg/ml lactate dehydrogenase. The minimum significant ratio (MSR)[19] for this assay is 3.6, determined

using a retrospective analysis of 150 compounds whose K_i's were determined at least twice. Compounds that have a difference in K_i of at least a factor of 3.6 are considered significantly different.

PKA Enzyme Assay

The assay for PKAα was run according to the method described for ROCK, but using 10 μM ATP, 100 μM substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and 20 nM PKA (AA 1-351).

THP-1 Cellular Migration Assay

Compounds were tested for the ability to block THP-1 cell migration using a filter plate assay. Briefly, 150,000 cells in Iscoves media with 1% FBS are placed in the top filter well of a 96 well filter plate (5 micron filter, Millipore MAMIC5S10) and incubated with a bottom plate containing a 10 ng/mL MCP-1 as a chemoattractant in the same media. Compound was pre-added to both the top and bottom chambers of the plate in the same media (100 μl and 150 μl final volumes respectively). After a 1 hr incubation period at 37°C, 100 μl from the bottom chamber was transferred to a fresh 96 well plate, and cells were quantified using an ATP luciferase kit (Promega G7571). Luminescence was read using an LJL instrument with standard luminescence settings. The outer edge wells are not used for data collection due to inconsistent preliminary data. Instead the outer wells are filled with media to prevent evaporation in assay wells.

General procedure for acylation of aminothiazoles: 2-(2-Fluorophenyl)-N-(4-pyridin-4-yl-thiazol-2-yl)-acetamide (6)

4-(4-Pyridyl)-2-aminothiazole (**43**) (329 mg, 1.86 mmol), 2-fluorophenylacetic acid (377 mg, 2.25 mmol) and N-(1-methanesulfonyl)benzotriazole ($MeSO_2Bt$) (440 mg, 2.23 mmol) were placed in a microwave reaction vessel. THF (2 mL) was added followed by Et_3N (0.52 mL, 3.73 mmol) and the mixture heated in the sealed tube at 160 °C for 10 minutes. The product precipitated upon cooling to room temperature. The precipitate was filtered, washed with acetonitrile and dried to afford compound **6** (462 mg, 76%).

^1H NMR (DMSO-d6) δ 12.68 (1H, s), 8.63 (2H, d), 8.00 (1H, s), 7.84 (2H, d), 7.43-7.17 (4H, m), 3.90 (2H, s).

LC-MS (Method B) Rt = 2.09 min, $[M+H]^+ = 314$, $[M-H]^- = 312$.

HRMS: m/z calcd. for $C_{16}H_{12}FN_3OS$ $[M+H]^+$ 314.07579; found, 314.07581.

3-(3-Methoxyphenyl)-1-(4-pyridin-4-yl-thiazol-2-yl)-piperidin-2-one (19)

2-(3-Methoxy-phenyl)-N-(4-pyridin-4-yl-thiazol-2-yl)-acetamide (360 mg, 1 mmol: prepared from 4-(4-pyridyl)-2-aminothiazole and 3-methoxyphenylacetic acid as described above), triphenylphosphine (315 mg, 1.2 mmol), diisopropyl azodicarboxylate (239 mg, 1.2 mmol) and THF (10 mL) were stirred overnight at room temperature. The mixture was then cooled to 0°C and NaH (60% dispersion: 48 mg, 1.2 mmol) added and the reaction mixture was stirred at 0 °C for 30 minutes. MeOH was added to quench the reaction. The solvent was evaporated and the residue purified by chromatography on silica gel (EtOAc/hexane gradient) to afford compound **19** in 60% yield.

^{1}H NMR (DMSO-d6)□ δ 8.67 (d, 2H), 7.80 (d, 2H), 7.46 (s, 1H), 7.30 (m, 1H), 6.85 (m, 3H), 4.57 (m, 1H), 4.32 (m, 1H), 3.93 (m, 1H), 3.82 (s, 3H), 2.36 (m, 1H), 2.25 (m, 1H), 2.15 (m, 2H).

LC-MS (Method B) Rt = 2.43 min, $[M+H]^+$ = 366, $[M-H]^-$ = 364.

HRMS: m/z calcd. for $C_{20}H_{19}N_3O_2S$ $[M+H]^+$ 366.12708; found, 366.12704.

2-Amino-4-pyridin-4-yl-thiophene-3-carboxylic acid ethyl ester (45)

Ethyl cyanoacetate (6.78 g, 60 mmol) and 4-acetylpyridine (7.26 g, 60 mmol) were dissolved in dry benzene (35 mL) to which NH_4OAc (0.54 g, 7 mmol) and glacial acetic acid (1.5 mL) were added. The mixture was refluxed under a Dean-Stark trap until the formation of H_2O ceased. The mixture was cooled, diluted with benzene, and washed with water. The organic layer was dried with Na_2SO_4, filtered, and concentrated in vacuo to give compound **44** in quantitative yield. To a stirred solution of **44** in EtOH (100 mL) was added sulfur (1.9 g, 60 mmol) and morpholine (1 mL). The mixture was stirred at room temperature overnight. The precipitate was filtered, washed with cold EtOH and hexane to give compound **45** as a light yellow solid. The filtrate was concentrated and redissolved in cold ethanol, filtered and washed with hexane to give more product **45** in overall yield of 60% (2 steps).

^{1}H NMR ($CDCl_3$)□ δ 8.57 (2H, m), 7.31 (2H, m), 6.25 (2H, br), 6.17 (1H, s), 4.10 (2H, q), 0.99 (3H, t).

LC-MS (Method A) Rt = 2.26 min, $[M+H]^+$ = 248.

4-(Pyridin-4-yl)-thiophen-2-ylamine 46

A solution of compound **45 (**2.49g, 10 mmol) in 20% KOH and EtOH (10 mL) was refluxed for 18 hours. The mixture was cooled to room temperature and acidified with 2N H_2SO_4. The mixture was stirred at RT until no further evolution of gas was observed and the precipitate was filtered, washed with water, and dried to afford compound **46** as a light yellow solid (60% yield).

^{1}H NMR (400 MHz, DMSO-d6) δ 8.81 (2H, d), 8.22 (2H, d), 7.81 (1H, s), 6.71 (1H, s)

LC-MS (Method B) Rt = 0.28 min, $[M+H]^+$ = 177.

2-(3-Methoxyphenyl)-*N*-(4-(pyridin-4-yl)thiophen-2-yl)acetamide (22).

Compound **46** (1.75 g, 9.9 mmol), 3-methoxyphenylacetic acid (1.98 g, 11.9 mmol), Et_3N (25 mmol) and $MeSO_2Bt$ (2.34 g, 11.9 mmol) were placed in a microwave reaction vessel in THF (20 mL) and the mixture heated at 160 °C for 10 minutes. Upon cooling to room temperature the solvent was evaporated and the residue was taken in EtOAc and washed with 2 N NaOH. The organic layer was dried (Na_2SO_4) and evaporated to afford **22** as an oil. The product was converted to the HCl salt by redissolving in EtOAc and addition of 4N HCl in dioxane. (3.25 g, 90%)

^{1}H NMR (CD_3OD) δ 8.82 (2H, s), 8.27 (2H, d), 7.99 (1H, s), 7.32 (1H, s), 7.25 (1H, m), 6.92 (2H, m), 6.83 (1H, m), 3.80 (3H, s), 3.72 (2H, s).

LC-MS (Method B) Rt = 2.07 min, $[M+H]^+$ = 325

HRMS: m/z calcd. for $C_{18}H_{16}N_2O_2S$ $[M+H]^+$ 325.10053; found, 325.10041.

3-Dimethylamino-1-thiophen-3-yl-propenone 47

3-Acetylthiophene (42.8 g, 339 mmol) was mixed with DMF-DMA (250 mL) and heated to reflux 18 hr. The red solution was evaporated *in vacuo*. The resulting oil was dissolved in CH_2Cl_2 and washed with water. The organic solution was dried ($MgSO_4$), and evaporated in vacuo to afford compound **47** (61.04 g, 99%) as a brown oil that crystallized upon standing.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.93 (1H, dd), 7.79 (1H, d), 7.55 (1H, dd), 7.28 (1H, m), 5.60 (1H, d), 3.15 (3H, brs), 2.93 (3H, brs).

LC-MS (Method B) Rt = 0.63 min, $[M+H]^+$ = 182.

4-Thiophen-3-yl-pyrimidine (48)

A solution of compound **47** (7 g, 38.62 mmol), formamidine hydrochloride (11 g, 136.6 mmol) and DIPEA (13.5 mL, 77 mmol) in DMF (50 mL) were heated at 80°C for 4 days. The brown solution was cooled to room temperature and poured into water (200 mL). The aqueous layer was extracted with EtOAc (3x 100 mL) and the combined organic layers were dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the crude product was purified by silca gel chromatography (80g, 0-80% EtOAc in hexanes) to afford compound **48** (4.6 g, 73%) as white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.21 (1H, d), 8.74 (1H, d), 8.17 (1H, dd), 7.72 (1H, dd), 7.58 (1H, dd), 7.47 (1H, dd).

LC-MS (Method B) Rt = 0.67 min, $[M+H]^+$ = 163.

4-(5-Nitrothiophen-3-yl)-pyrimidine (49)

Potassium nitrate (686 mg, 6.78 mmol) was added to a stirred solution of compound **48** (1 g, 6.2 mmol) in conc. H_2SO_4 (15 mL) at 0°C. The pale brown solution was stirred at room temperature for 2 hr and poured into water (50 mL). The aqueous layer was extracted with EtOAc (3x 25 mL), dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by silica gel chromatography (80g, 0-80% gradient EtOAC in hexanes) to afford compound **49** (0.50 g, 40%) as off white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.27 (1H, d), 8.85 (1H, d), 8.52 (1H, d), 8.37 (1H, d), 7.60 (1H, dd).

LC-MS (Method B) Rt = 0.80 min, $[M+H]^+$ = 208.

4-Pyrimidin-4-yl-thiophen-2-ylamine hydrochloride (50)

10% Palladium on carbon (50 mg) was added to N_2 purged solution of compound **49** (0.20 g, 0.97 mmol) in EtOAc:MeOH (4 mL, 3:1). The reaction was stirred at ambient temperature under 1 atmosphere of H_2 for 4 hours. The catalyst was filtered through a celite plug and the plug was washed with EtOAc. The combined filtrate was cooled to 0°C and the filtrate volume was doubled with Et_2O, and then to the solution was added a solution of 4N HCl in dioxane (0.5 □L, 2 mmol). A light yellow solid precipitated immediately, which was stirred for 5 minutes at 0°C, and then filtered. The solids were washed with copious amounts of Et_2O with care taken not to expose the compound to air, and quickly transferred to high vacuum for drying. The solid was dried *in vacuo* to give 4-pyrimidin-4-yl-thiophen-2-ylamine hydrochloride **50** (160 mg, 77%) as a pale yellow solid.

^{1}H NMR (DMSO-d6) δ 9.17 (1H, dd), 8.82-8.79 (1H, m), 8.10 (1H, s), 7.94-7.90 (1H, m), 7.3 (2H, br).

FIA-MS (Method A) $[M+H]^+$ = 178

2-(3-Methoxyphenyl)-*N*-(4-(pyrimidin-4-yl)thiophen-2-yl)acetamide (28)

A solution of 4-(pyrimidin-4-yl)thiophen-2-amine (**50**) (240 mg, 1.35 mmol) was dissolved in a 9:1 mixture of THF-DMA (3 mL), and placed in a microwave tube. To the mixture was added 2-(3-methoxyphenyl)acetic acid (240 mg, 1.49 mmol), $MeSO_2Bt$ (294 mg, 34 mmol), and Et_3N (500 μL, 3.58 mmol). The reaction vessel was sealed and heated using microwave at 160°C for 10 minutes, and then cooled to room temperature. The mixture reduced in volume to 1 mL, and was purified by preparative HPLC to afford the title compound **86** (308 mg, 70%).

^{1}H NMR (CD_3OD) δ 9.08 (1H, s), 8.70 (1H, d), 7.87 (1H, s), 7.80 (1H, d), 7.39 (1H, s), 7.24 (1H, dd), 6.92 (2H, m), 6.83 (1H, m), 3.80 (3H, s), 3.71 (2H, s)

LC-MS (Method B) Rt = 2.65 min, $[M+H]^+$ = 326

HRMS: m/z calcd. for $C_{17}H_{15}N_3O_2S$ $[M+H]^+$ 326.09578; found, 326.09595.

2-(4-Methoxybenzylamino)pyridine-4-carbonitrile 53

2-Fluoropyridine-4-carbonitrile **(51)** (1.0 g, 8.2 mmol) and 4-methoxybenzylamine (1.1 g, 8.2 mmol) in DMA (5 mL) were combined and heated at 130°C for 1 hour. The crude product was partitioned between H_2O and EtOAc (50 mL). The aqueous layer was extracted with EtOAc and the combined organics dried over Na_2SO_4, concentrated, and recrystallized from EtOAc /hexanes to provide 2-(4-methoxybenzylamino)pyridine-4-carbonitrile **53** 1.7 g (87%) as a white solid.

^{1}H NMR ($CDCl_3$) δ 8.23 (1H, d), 7.32 - 7.22 (2H, d), 6.91 □(2H, d), 6.77 (1H, dd), 6.57 (1H, s), 5.14 (1H, br s), 4.46 (2H, d), 3.83 (3H, s).

LC-MS (Method B) Rt = 0.69 min, $[M+H]^+$ = 240

1-(2-(4-Methoxybenzylamino)pyridin-4-yl)ethanone 58

Compound **53** (1.6 g, 6.7 mmol) was dissolved in anhydrous Et_2O (50 mL) and 3M methyl magnesium bromide (9 mL) in Et_2O was added. The reaction mixture was stirred at room temperature for 16 hours and poured over ice (*ca*. 50 g), which was subsequently acidified with 6M HCl, followed by basicification with 1M NH_4OH. The resulting mixture was extracted with EtOAc (3 x 50 mL). The combined organics were concentrated *in vacuo* and purified by silica gel chromatography to provide 1-(2-(4-methoxybenzylamino)pyridin-4-yl)ethanone **58** (1.2 g, 70%) as a yellow solid.

^{1}H NMR ($CDCl_3$) δ 8.27 (1H, d), 7.31 (2H, d), 7.01 (1H, dd), 6.90 (2H, d), 6.85 (1H, s), 5.00 (1H, br s), 4.50 (2H, d), 3.82 (3H, s), 2.55 (3H, s).

FIA-MS (Method A) $[M+H]^+$ = 257.

1-(2-(3-Methoxyphenyl)acetyl)thiourea (63)

A solution of oxalyl chloride in CH_2Cl_2 (2M, 4.5 mL, 9 mmol) was added to a stirred solution of 3-methoxyphenylacetic acid (1.0 g, 6.02 mmol) in DMF (0.1 mL) and CH_2Cl_2 (25 mL) and the resulting clear solution was stirred at room temperature for 1 hr. The solvent was removed under reduced pressure and the acid chloride residue was dissolved in THF (25 mL) and thiourea (1.82 g, 24 mmol) was added. The mixture was refluxed for 6 h and then cooled to room temperature. The solution was poured into water (100 mL)

and extracted with EtOAc (3 x 50 mL). The organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexanes-EtOAc) to give 1-(2-(3-methoxyphenyl)acetyl)-thiourea, **63** (0.48 g, 35%) as a white solid.

^{1}H NMR (DMSO-d6) δ 11.23 (1H, s), 9.55 (1H, br s), 9.36 (1H, br s), 7.23 (1H, t, *J* = 7.9 Hz), 6.82-6.87 (2H, m), 3.73 (3H, s), 3.66 (2H, s).

FIA-MS (Method A) $[M+H]^+ = 225$

_N_-(4-(2-Aminopyridin-4-yl)thiazol-2-yl)-2-(3-methoxyphenyl)acetamide 30

Bromine (0.01 mL, 0.195 mmol) was added to stirred solution of **58** (50 mg, 0.195 mmol) in 48% HBr (0.2 mL) and HOAc (0.4 mL) and the solution was heated at 70°C for 2 hrs. A solution of **63** (44 mg, 0.195 mmol) in EtOH (3 mL) was then added and the heating was continued for 1 hr. The solution was poured into water (10 mL) and made basic with concentrated NH_4OH and the product was extracted with EtOAc (3x10 mL). The crude product was purified by preparative HPLC (10%-90% water/CH_3CN, 0.1% TFA, 15 min) to give compound **30** (0.37 mg, 56%).

^{1}H NMR (DMSO-d6) δ 12.6 (1H, br s), 7.93 (1H, d), 7.86 (1H, s), 7.24 (1H, t), 7.09 (2H, s), 6.83-6.93 (5H, m), 3.77 (2H, s), 3.73 (3H, s).

LC-MS (Method A) Rt = 2.20 $[M+H]^+ = 341$.

HRMS: m/z calcd. for $C_{17}H_{16}N_4O_2S$ $[M+H]^+$ 341.10668; found, 341.10632.

4-Bromo-2-(*t*-butoxycarbonylamino)-thiophene 64

A mixture of 4-bromothiophene-2-carboxylic acid (53 g, 255 mmol), diphenylphosphoryl azide (70 mL, 323 mmol), Et_3N (71 mL, 510 mmol) in *t*-BuOH (675 mL) was heated at 100°C for 5 h and then cooled to room temperature. The solvent was evaporated to give brown gum which was dissolved in EtOAc (500 mL). The organic solution was washed with saturated $NaHCO_3$ (500 mL), water (500 mL) dried (Na_2SO_4) and concentrated. The crude product was dissolved in CH_2Cl_2 (75 mL) and purified by flash column chromatography on silica gel (10%-15% EtOAc/hexanes) to afford **64** (47 g, 66%) as a white solid.

^{1}H NMR (DMSO-d6) δ 10.65 (1H, s), 6.97 (1H, d, J = 1.7 Hz), 6.43 (1H, d), 1.46 (9H, s).

LC-MS (Method B) Rt = 1.05 min, $[M+H]^+$ = 223

4-(2-Fluoropyrid-4-yl)-2-(*t*-butoxycarbonylamino)-thiophene 65

A mixture of compound **64** (42 g, 150 mmol), 2-fluoropyridinyl-4-boronic acid (27 g, 190 mmol), $Pd(PPh_3)_4$ (11 g, 9.5 mmol) and powdered K_2CO_3 (52 g, 380 mmol) in 1,4-dioxane (500 mL) and water (150 mL) was heated at 100°C for 5 h and then cooled to room temperature. The inorganic solid was filtered and the dark brown solution was evaporated under reduced pressure. The crude product was dissolved in EtOAc (1000 mL) and washed with 10% HCl (200 mL) and brine (500 mL). The organic solution was dried (Na_2SO_4) and concentrated. The crude material was purified by flash chromatography on silica gel eluting with EtOAC/ hexanes (1:1) to afford **65** (29.5 g, 67%) as a white solid.

^{1}H NMR ($CDCl_3$) δ 8.21 (1H, d), 7.32 (1H, 2 d), 7.21 (1H, d), 7.15 (1H, br s), 7.04 (1H, s), 6.85 (1H, d), 1.56 (9H, s).

LC-MS (Method B) Rt = 1.03 min, $[M+H]^+$ = 295

4-(2-Fluoropyridinyl)thiophen-2-amine 66

Trifluoroacetic acid (50 mL) was added to a stirred suspension of compound **65** (29 g, 98 mmol) in CH_2Cl_2 (50 mL). The resulting brown solution was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure to give a brown gum which was poured into ice/ water. The solution was basified with 2N NaOH (125 mL) to produce a yellow precipitate which was extracted with EtOAc (5 x 250 mL). The organic solution was dried (Na_2SO_4) and concentrated to afford **66** (15 g, 80%) as a pale brown solid.

^{1}H NMR (DMSO-d6) δ 8.15 (1H, s), 7.50 (2H, dd), 7.16 (1H, d), 6.36 (1H, d), 5.72 (2H, br s).

LCMS (Method A) Rt = 1.9 min, $[M+H]^+$ = 195.

***N*-(4-(2-Fluoropyridin-4-yl)thiophen-2-yl)-2-(3-(methylsulfonamido)phenyl)-acetamide 37**

A mixture of compound **66** (14.7 g, 76 mmol), 3-methylsulfonamidephenylacetic acid (17.4 g, 76 mmol), $MeSO_2Bt$ (17 g, 86.3 mmol), Et_3N (20 mL) in THF (250 mL) was heated at 85°C for 16 h and then cooled to room temperature. The solvent was evaporated and the crude material was dissolved in EtOAc (500 mL). The organic solution was washed with saturated $NaHCO_3$ (200 mL), brine (500 mL), dried (Na_2SO_4) and concentrated. The crude material was purified by flash chromatography on silica gel

eluting with 85% EtOAc in hexanes to afford compound **37** (22 g, 74.6%) as an off white solid.

^{1}H NMR (DMSO-d6) δ 11.55 (1H, s), 9.85 (1H, s), 8.20 (1H, s), 7.75 (1H, s), 7.65 (1H, s), 7.45 (1H, s), 7.3-7.1 (5H, m), 3.7 (2H, s), 3.3 (1H, s), 3.0 (3H, s).

LCMS (Method B) Rt = 2.94 min, $[M+H]^+$ = 406.0.

HRMS: m/z calcd. for $C_{18}H_{16}FN_3O_3S_2$ $[M+H]^+$ 406.06899; found, 406.06903.

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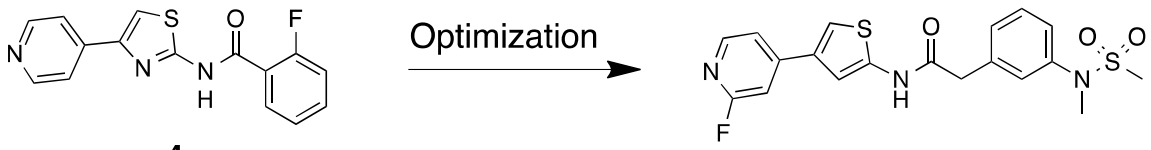

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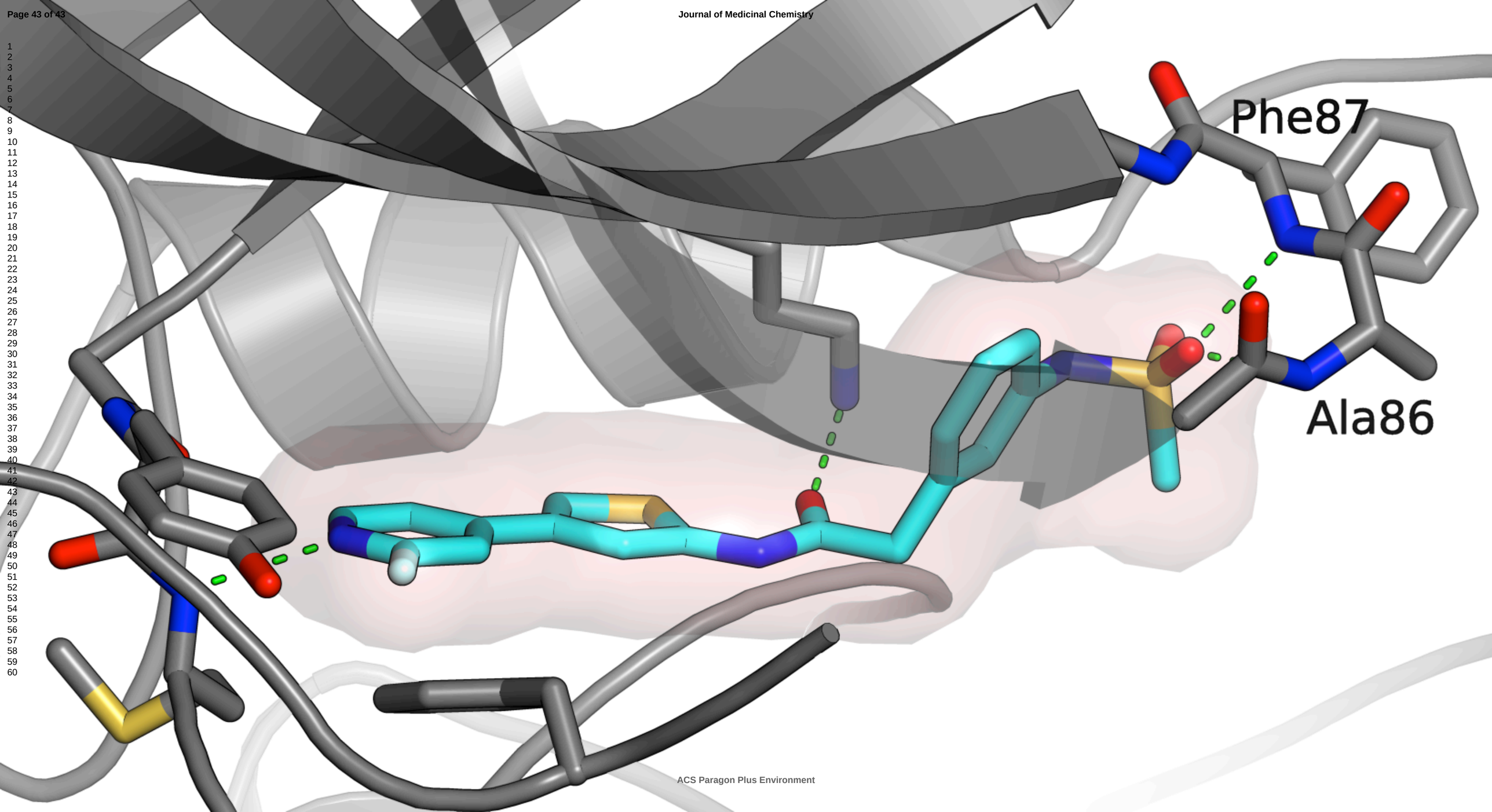
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