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Discovery and Optimization of an Azetidine Chemical Series As a Free Fatty Acid Receptor 2 (FFA2) Antagonist: From Hit to Clinic

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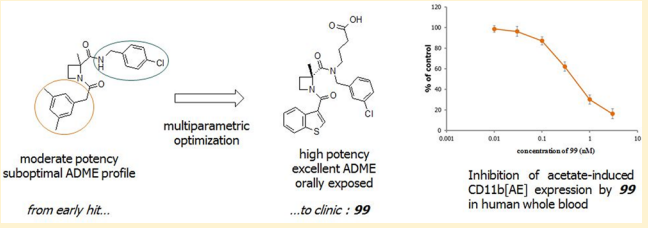
Supporting Information

ABSTRACT: FFA2, also called GPR43, is a G-protein coupled receptor for short chain fatty acids which is involved in the mediation of inflammatory responses. A class of azetidines was developed as potent FFA2 antagonists. Multiparametric optimization of early hits with moderate potency and suboptimal ADME properties led to the identification of several compounds with nanomolar potency on the receptor combined with excellent pharmacokinetic (PK) parameters. The most advanced compound, 4-[[(*R*)-1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid **99** (GLPG0974), is able to inhibit acetate-induced neutrophil migration strongly in vitro and demonstrated ability to inhibit a neutrophil-based pharmacodynamic (PD) marker, CD11b activation-specific epitope [AE], in a human whole blood assay. All together, these data supported the progression of **99** toward next phases, becoming the first FFA2 antagonist to reach the clinic.



INTRODUCTION

Lipids play an important role in many biological processes and are crucial in the pathogenesis of numerous common diseases. Especially, fatty acids (FAs), which are derived from triglycerides and phospholipids, are essential sources of energy in complex organisms and animals, yielding large quantities of ATP when metabolized. FAs are also key signals for metabolic regulation, acting through enzymatic and transcriptional networks to modulate gene expression, growth, and survival pathways, as well as inflammatory and metabolic responses. There is now a substantial body of evidence that disturbances in FAs are strongly involved in several chronic pathologies including metabolic syndrome, inflammatory diseases, and some cancers.[1] Fatty acid chains differ by length, often categorized as short to very long. Short-chain fatty acids (SCFA, carbon length C2−C6: acetate, propionate, and butyrate) are byproducts of anaerobic bacterial fermentation of fibers in the gut.[2] It was discovered that SCFAs bind to FFA2 receptor, also known as GPR43.[3] FFA2 is highly expressed in immune cells (e.g., neutrophils, peripheral blood mononuclear cells (PBMC), purified monocytes, eosinophils, and B lymphocytes)[3,4] and also in other tissues that may play a role in the propagation of the inflammatory response (e.g., intestinal cells or endothelial cells).[5]

It was demonstrated that neutrophilic granulocytes isolated from FFA2-deficient mice lost the ability to migrate in response to SCFAs, indicating that FFA2 has a major role in neutrophilic granulocyte migration.[6] As neutrophil migration into the gut mucosal tissue is considered a driver of tissue damage in IBD and hence neutrophilic markers (myeloperoxidase (MPO), calprotectin) are also used as markers for disease progression in IBD, reducing neutrophil migration by FFA2 inhibition could be considered as a therapeutic option in IBD. Reports on the outcome of models for IBD in GPR43-deficient mice, however, describe conflicting results, ranging from reduced disease severity, associated with reduced polymorphonuclear leukocytes (PMN) migration in the inflamed gut of mice subjected to the chronic dextran sodium sulfate (DSS)-induced colitis model, to exacerbated or unresolving inflammation in the DSS and trinitrobenzoic sulfonic acid (TNBS)-induced model for IBD.[6,7] These different outcomes can be explained by different parameters (genetic background of the mice used, differences in model setup, food).[8] The potential of FFA2

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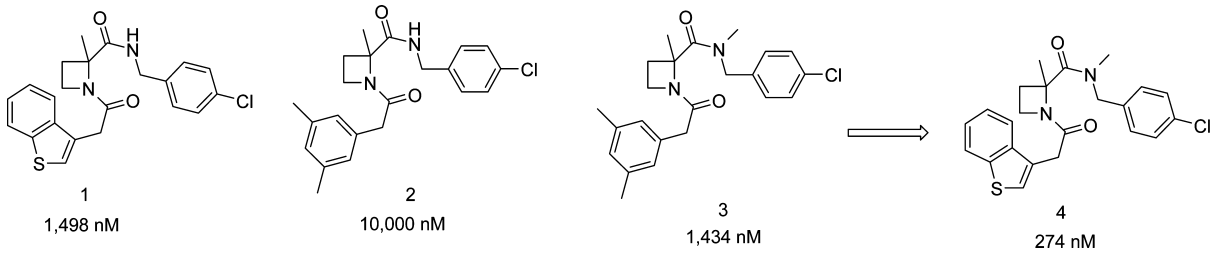
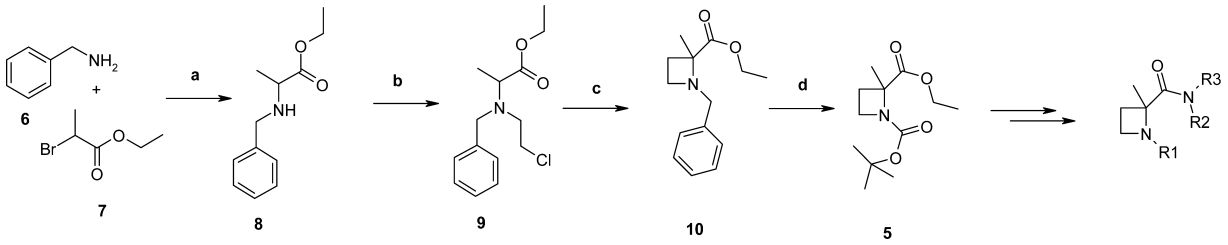


Figure 1. IC_{50} (CaFlux assay) of three representative compounds from HTS (**1**, **2**, **3**) and the *N*-methylated analogue **4**.

Scheme 1. Synthesis of the Common Azetidine Core[a]



[a]Reagents and conditions: (a) K_2CO_3, MeCN, reflux, 4 h, 97%; (b) chloroacetaldehyde, $MgSO_4$, sodium triacetoxyborohydride, AcOH, DCM, 0 °C, 1 h, 85%; (c) KHMDS −78°C/−70°C, THF, 1 h, 79%; (d) H_2, Boc_2O, Pd/C 10%, EtOH, 20 °C, 18 h, 79%.

inhibition for the treatment of IBD, is currently explored in pharmacological testing.

Considering the key function of FFA2 in inflammatory processes, blocking the downstream signaling of this receptor by a small molecule could be useful in the treatment of inflammatory conditions, infectious diseases, autoimmune diseases, and impairments of immune cell functions.[9] At the moment we started our investigations, only FFA2 agonists were reported in the literature.[10] Since that time, several agonist compound series have been published as well as one series of FFA2 antagonists.[11,12]

The strategy developed to discover a new FFA2 antagonist was based on a high throughput screening of a diverse compound collection. Compounds were evaluated in a calcium flux assay performed in antagonist mode, measuring the change, induced by sodium acetate (FFA2 agonist), in free intracellular calcium concentration in a HEK293 cell line stably overexpressing the human FFA2 receptor. This calcium flux assay was successful in identifying different compounds bearing an azetidine scaffold, and displaying moderate potencies in the micromolar range (compounds **1**, **2** and **3**, Figure 1). Interestingly, it was observed that simple methylation of the secondary carboxamide remarkably increased potency (**3** vs **2**). The same modification was also considered for compound **1**. Compound **4** was synthesized for that purpose and evaluated, leading to a first breakthrough with a potency of 274 nM. With a submicromolar starting point, the azetidine scaffold was therefore considered as a tractable series for SAR exploration, enabling possible access to three substitutions of the core to increase the chemical space.

CHEMISTRY

Different synthetic approaches were developed to facilitate the SAR exploration of the three main structural subunits.

Synthesis of the Boc-protected central core **5** was performed in four steps following a straightforward sequence depicted in Scheme 1. Benzylamine **6** was condensed with 2-bromo-propionic acid ethyl ester **7** to yield secondary amine **8**. Reductive amination with chloroacetaldehyde gave tertiary amine **9**, which was then cyclized under basic condition to give the racemic 1-benzyl-2-methyl-azetidine-2-carboxylic acid ethyl ester **10**. Benzyl group was removed by hydrogenolysis and directly replaced by a Boc-protecting group to give **5**.

Boc-protected central core **5** was a pivotal intermediate in our different synthetic strategies that allowed us to explore indifferently R1, R2, and R3.[13]

Different substitutions at R1 were introduced by amide coupling of the corresponding carboxylic acids and the HCl salt of azetidine **11** obtained by the removal of the Boc-protecting group under acidic conditions. R2 and R3 functionalities were installed by amide coupling of secondary amines having corresponding moieties or a direct precursor already in place with carboxylic acid **11** obtained by saponification of azetidine ethyl ester **5** (Scheme 2).

With two orthogonal protecting groups installed on the central core, the order of the two amide couplings provided a flexible access to a wide range of compounds for a broad range of SAR modulations. When necessary, final modifications on the R3 side chains were performed to set the appropriate functionality.

IDENTIFICATION OF LEAD COMPOUNDS

Early in the discovery process, the hits were tested against mouse and rat FFA2 receptors. Unexpectedly, consistently across our series, the compounds were found inactive on the rodent orthologues in different cellular assays. A recent publication reports differences in potencies for SCFA between species orthologues, pointing to an "ionic lock" between extracellular loop 2 and the ligand binding pocket regulating constitutive activity.[14] This could rationalize the absence of activity of our series on rodent FFA2 receptors. As a consequence of the lack of orthology, the efficacy of the FFA2 inhibitors generated could not be assessed in rodent disease models. The drug discovery program was run by in vitro/ex vivo optimization of the compound potencies, in addition to in vivo pharmacokinetics parameters with the purpose of achieving a very long predicted target coverage in human. To this end, during the lead optimization process, the in vitro liver microsomal stability was first evaluated in rat and further checked in human microsomes.

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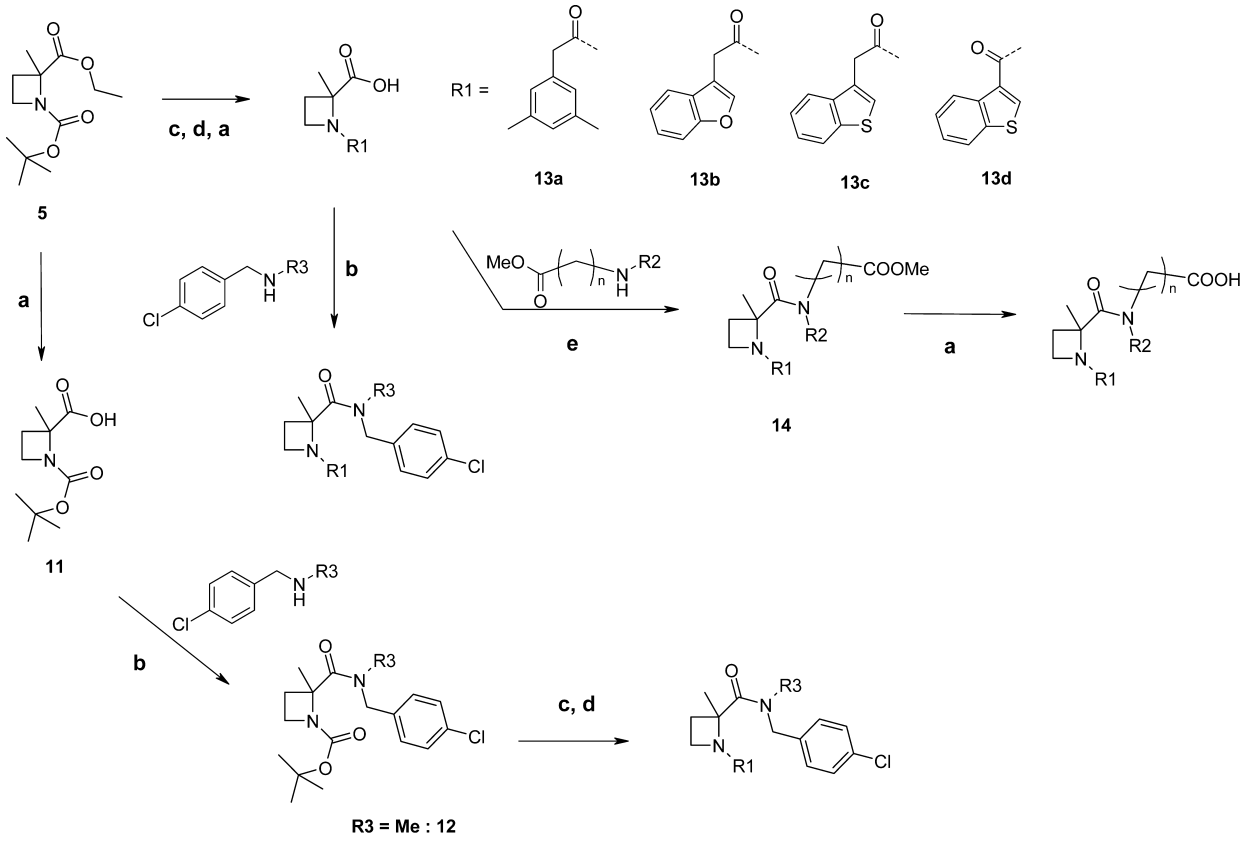
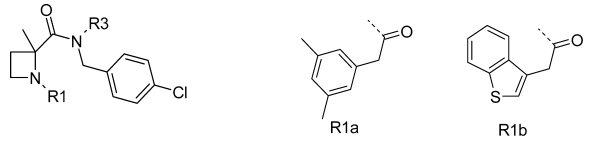
Scheme 2. General Synthetic Access to Azetidine FFA2 Antagonists[a]



[a]Reagents and conditions: (a) NaOH, MeOH; (b) EDC/HOBt, chlorobenzylamine; (c) HCl, dioxane; (d) R_1CO_2H, EDC/HOBt, NEt_3; (e) $(COCl)_2$, NEt_3, DCM, DMF.

Table 1. Optimization of R3



compd	R1	R3	IC_{50} (nM)	RLM (% remaining)	compd	R1	R3	IC_{50} (nM)	RLM (% remaining)
2	R1a	H	10000	0[b]	**24**	R1a	$CH_2CONHMe$	442	5[b]
1	R1b	H	1498	11[b]	**25**	R1a	CH_2CONMe_2	1584	0[b]
3	R1a	Me	1434	0[b]	**26**	R1a	$(CH_2)_3CONH_2$	158	22[a]
4	R1b	Me	274	0[b]	**27**	R1a	$(CH_2)_2CONH_2$	510	
15	R1a	Et	473	7[b]	**28**	R1a	CH_2CO(morpholine)	4673	38[a]
16	R1b	$(CH_2)_2OH$	3350	1[b]	**29**	R1a	$(CH_2)_3CO$(morpholine)	388	31[a]
17	R1a	$(CH_2)_2NH_2$	10000		**30**	R1b	$(CH_2)_3CO$(morpholine)	76	2[a]
18	R1a	$(CH_2)_2NHCONHMe$	10000	0[a]	**31**	R1a	$(CH_2)_2NHCOMe$	337	12[a]
19	R1a	$(CH_2)_2OMe$	1208		**32**	R1a	$(CH_2)_2NHSO_2Me$	429	
20	R1b	CH_2CN	247	2[b]	**33**	R1a	$(CH_2)_3SO_2NH_2$	245	11[a]
21	R1b	$(CH_2)_3CN$	143	0[a]	**34**	R1b	$(CH_2)_3SO_2NH_2$	76	0[a]
22	R1a	$(CH_2)_3CO_2Me$	361	0[b]	**35**	R1b	$(CH_2)_3SO_2NHMe$	44	0[a]
23	R1a	CH_2CONH_2	123	0[b]	**36**	R1a	$(CH_2)_3SO_2NHMe$	263	16[a]

[a]Rat liver microsomal (RLM) stability % remaining after 30 min. [b]Rat liver microsomal (RLM) stability % remaining after 60 min.

As a first step in our hit series optimization, a wide range of side chains in the R3 position was explored (Table 1). On the basis of HTS results, dimethylphenylacetamido and benzothiophene 3-acetamido substitutions at R1 were chosen. Short alkyl chains substitutions in R3 seemed to improve significantly potency (compound **15**). Adding polar groups like ethylhydroxy (**16**), ethylamino (**17**), or urea (**18**) led to a drop in potency, whereas the ethylmethoxy derivative was moderately active (**19**).

Introduction of a nitrile side chain resulted in a potency improvement comparable to simple methylation (**20** and **21**). A large variety of esters, amides, and sulfonamides was explored, all showing moderate to high potency (IC_{50} below 100 nM for

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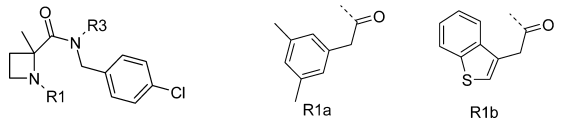
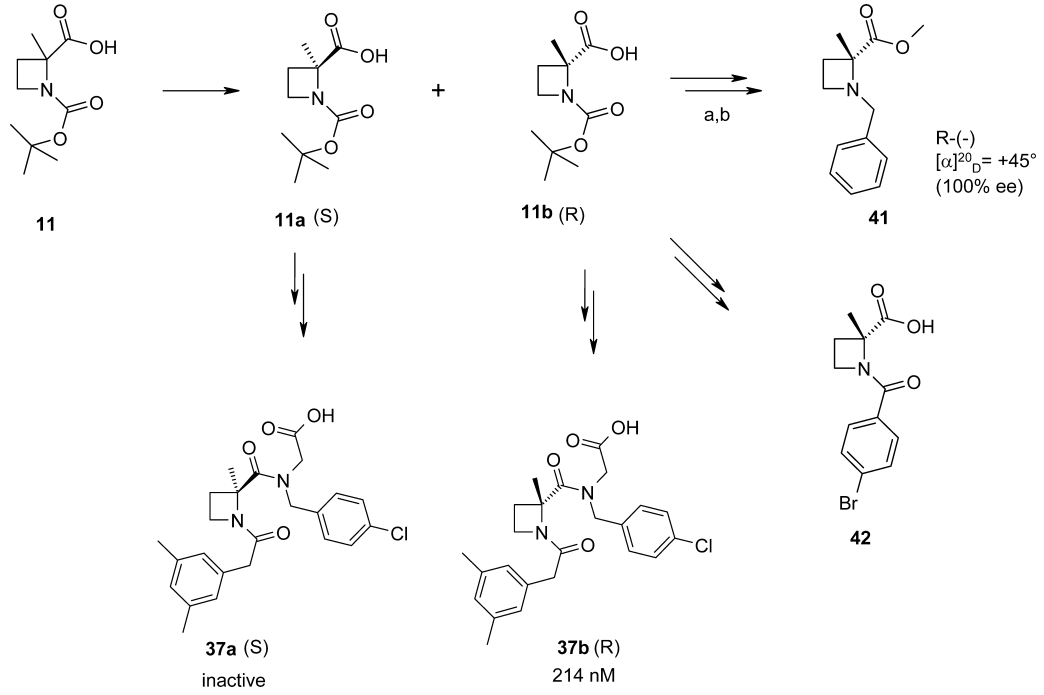
Table 2. Introduction of a Carboxylic Acid Side Chain at R3



compd	R1	R3	IC_{50} (nM)	RLM (% remaining)	AUC (0–24 h) (ng·h/mL)[c]
37	R1a	CH_2COOH	278	100[b]	1160
38	R1b	CH_2COOH	36	56[b]	362
39	R1b	$(CH_2)_2COOH$	317	100[a]	
40	R1b	$(CH_2)_3COOH$	42	68[a]	542

[a]Rat microsomal stability % remaining after 30 min. [b]Rat microsomal stability % remaining after 60 min. [c]Oral plasma exposure, 5 mg/kg, Sprague–Dawley rat.

Scheme 3. Synthesis of Both Enantiomers of 37 and Determination of Their Absolute Configuration



several compounds like **30**, **34**). The optimal length of this spacer emerging from this SAR exploration was one or three carbon atoms, depending on the functionality and also on R1. Overall, compounds showed a low stability when incubated (30 or 60 min) in rat liver microsomes. Carboxamides and sulfonamides were also found generally unstable with few noticeable exceptions (**28**, **29**).

To tackle this issue, introduction of carboxylic acid side chains was considered. The carboxylic acid moiety is known to be poorly accommodated by the CYP450 active site and therefore often leads to compounds with improved metabolic stability.[15] In addition, as a short-chain fatty acid receptor is targeted, an additional interaction could be obtained by introduction of a carboxylic acid functionality on the molecule.

Our hypothesis was validated by a series of compounds depicted in Table 2. Good potencies as well as good microsomal stabilities (more than 50% remaining after 30 or 60 min incubation) were obtained (**37**, **38**, **39**, and **40**). This key finding turned out to be a general feature throughout the series for compounds bearing a carboxylic acid moiety. Three compounds were evaluated in pharmacokinetic studies (PO administration in Sprague–Dawley rats at 5 mg/kg). Oral plasma exposure was acceptable for **37**, **38** and **40**, demonstrating that an increase in microsomal stability translated well to improved in vivo oral exposure.

Among the initial compounds evaluated, compounds **38** and **40** displayed attractive profiles, both in terms of potency and oral exposure. In addition, no undesired inhibition of CYP450 isoforms or of the hERG channel was reported.

Data generated with this subset of compounds were strong enough to further explore the series by keeping the carboxylic acid as a key residue in the R3 position.

For synthetic ease, all the SAR studies previously described were performed on racemic mixtures. To assess the influence of the absolute configuration of the quaternary center on the activity of our FFA2 antagonists, we prepared and evaluated potency of both enantiomers of compound **37**.

For that purpose, separation of racemic intermediate **11** was performed by preparative chiral chromatography, thus affording **11a** and **11b** in their enantiopure forms (Scheme 3); synthesis of both enantiomers of **37** was then carried on following the previously described pathway. They were both evaluated in the CaFlux assay. *R* enantiomer **37a** was found active (214 nM), whereas *S* enantiomer **37b** was found totally inactive.

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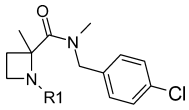
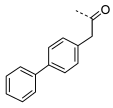
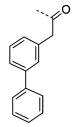
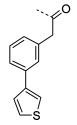
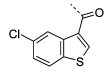
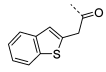
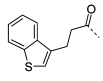
Table 3. Optimization of R1



Compd	R1	IC50 (nM)	Compd	R1	IC50 (nM)
3		274	**51**		>10,000
43		>10,000	**52**		>10,000
44		7,820	**53**		5,045
45		>10,000	**54**		>10,000
46		7,614	**55**		>10,000
47		5,800	**56**		142
48		3,350	**57**		3,350
49		3,350	**58**		5,002
50		4,469	**59**		>20,000

Absolute configuration of active enantiomer was determined by converting its precursor **11b** in methyl ester **41** whose $[\alpha]_D$ and absolute configuration are described in the literature.[16]

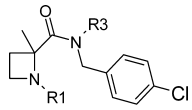
Absolute configuration was finally unambiguously confirmed by an X-ray structure of a single crystal of compound **42** derived from compound **11b** (see Supporting Information)

LEAD OPTIMIZATION

Considering the profile of lead compounds **38** and **40**, we focused our efforts on both potency and oral exposure improvements. Lead optimization was directed by modifications of the two other subunits R1 and R2.

A wide variety of changes to the R1 part of the molecule was explored, and a selection of the most informative is included in Table 3. For synthetic reasons, we decided first to set R3 as a methyl group for exploration of the SAR. Replacing R1 by benzyl residues or biphenyl moieties did not lead to any affinity improvement (compounds **43**, **48**). Heterocyclic replacements of R1 were investigated, substituted heterocycles like thiophene **49** were moderately potent, whereas 5−6 fused heterocycles **50** and **51** were inactive. An interesting improvement in potency could be achieved with benzothiophene analogue **56**. The position of the heteroatom as well as substitutions on the benzothiophene moiety proved crucial for potency and could lead to reduction in activity as demonstrated by compounds **54**, **55**, and **57**. Adding a carbon between the amide linkage and the benzothiophene (compound **59**) was detrimental to activity.

Table 4. Optimization of R1



Compd	R1	R3	IC_{50} (nM)	RLM (% compd)	AUC (0-24h) (ng.h/mL)
37		CH_2COOH	278	100[b]	1,160
38		CH_2COOH	36	56[b]	362
60		$(CH_2)_3COOH$	118	82[a]	1,012
40		$(CH_2)_3COOH$	42	68[a]	1,022
61		$(CH_2)_3COOH$	166	90[a]	1,982
62		CH_2COOH	227	81[b]	575
63		CH_2COOH	5,005	100[b]	-
64		CH_2COOH	>10,000	-	-

[a]Rat liver microsomal (RLM) stability % remaining after 30 min. [b]Rat liver microsomal (RLM) stability % remaining after 60 min. [c]Plasma exposure in rat after oral administration at 5 mg/kg.

Because these SAR studies allowed us to identify some benzothiophene patterns as key R1 subunits to improve potency, we decided to further explore fused heterocycles bearing an acid moiety on the R3 side chain. Results are depicted in Table 4. As previously observed, this substitution had a positive impact on potency and microsomal stability (compounds **37**, **60**). Interestingly, benzofuran derivatives (compounds **61**, **62**) were found to be active, whereas other 5−6 fused heterocycles like benzimidazole **64** and oxindole **63** were much less active.

Different potent and stable compounds bearing this key carboxylic side chain were evaluated in pharmacokinetic studies

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Table 5. Optimization of R1 and R2

R2 \ R1												
	Cpd	IC_{50} (nM)	AUC (0-24h) (ng.h/mL)	Cpd	IC_{50} (nM)	AUC (0-24h) (ng.h/mL)	Cpd	IC_{50} (nM)	AUC (0-24h) (ng.h/mL)	Cpd	IC_{50} (nM)	AUC (0-24h) (ng.h/mL)
Me	**65**	>10,000	-									
Bu	**66**	>10,000	-									
Cy	**67**	>10,000	-									
	**60**	118	1,012	**81**	179	-	**42**	42	363	**64**	166	1,460
	**68**	1,144	-									
	**69**	63	-									
	**70**	347	-									
	**71**	256		**82**	551	-	**88**	57	378	**94**	261	-
	**72**	25	1,020	**83**	3,350	-	**89**	500	-	**95**	>10,000	-
	**73**	63	-	**84**	85	-	**90**	48	-	**96**	90	-
	**74**	115	-	**85**	781	-	**91**	36	35	**97**	116	-
	**75**	130	-	**86**	156	-	**92**	40	26	**98**	138	-
	**76**	321	-	**87**	711	-	**93**	95	-			
	**77**	>10,000	-									
	**78**	579	-									
	**79**	663	-									
	**80**	2,853										

(oral administration in Sprague−Dawley rats at 5 mg/kg). Good profiles were generally observed, and interestingly the longest acid side chain in the R3 position (3 carbons vs 1 carbon) seemed to have a positive impact on oral exposure (**40** vs **38** and **61** vs **62**).

However, the differences in both activity and oral exposure found for the different potent moieties identified at R1 ((3,5-dimethyl-phenyl)-acetamide, benzothiophene-3-carboxamide, benzothiophene-3-acetamide, and benzofuran-3-acetamide) were not significant enough to restrict our lead optimization strategy to one of these patterns. Therefore, for the optimization of the R2 subunit, we decided to keep these four interesting groups in R1 in combination with the 3C-long acid side chain in R3 identified from the previous SAR.

A large exploration of the SAR in R2 was undertaken and summarized in Table 5.

First, several R2 substitutions were explored having R1 set as benzothiophene-3-carboxamide (first column). Replacement of the 4-Cl benzylamine moiety by alkyl groups led to complete loss of potency (compounds **65**−**67**). Compared to **60**, extending the linker (**68**) reduced significantly activity whereas removing the benzylic CH_2 (**69**) slightly improved potency. However, this was not observed for 3-Cl phenyls (**70** being 10-times less potent than **72**). Different substitutions patterns on the benzylamine and introduction of heterocycles were also evaluated. We observed that indole substitution led to a potent analogue (**73**), as well as benzofuran and indazole moieties (**74**, **75**). Neither benzothiazole (**76**) nor 4-trifluoromethylbenzyl derivative (**71**) showed any increase on potency.

The most striking aspect of this study was the unexpected results observed for the 3-chloro benzyl substitution at R2 (compound **72**). It appeared to be, by far, the most potent pattern when combined to benzothiophene-3-acetamide, whereas it proved to be detrimental when combined to other R1 substitutions. However, other combinations of the three other R1 groups with the best R2 moiety identified confirmed that 6-indole, indazole, and benzofuran could replace 4-chlorobenzyl R2 moiety and retain potency.

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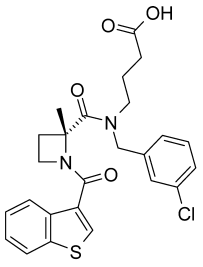
A selection of the best molecules was evaluated in PK in Sprague−Dawley rats at 5 mg/kg. Oral plasma exposure of these compounds can be found in Table 5. Heterocyclic substitutions at R2 had a detrimental impact on oral exposure as demonstrated with compounds **91** and **92**, whereas the position of the chlorine atom had no impact on PK parameters (**72** vs **60**).

It appears that both in terms of oral exposure and potency, a single compound clearly emerged from this table. Compound **72** was the most potent compound and one of the best orally exposed in rat.

The corresponding *R*-enantiomer of **72** was prepared. Enantiopure compound **99** was synthesized following the usual route starting from chiral Boc-protected azetidine carboxylic acid **11b**. As expected, an improvement in potency for **99** was observed, with an IC_{50} of 9 nM. A thorough investigation of the selectivity profile of **99** was performed. The close receptor homologue FFA3 (also called GPR41) is not inhibited by **99** up to 30 μM. Less than 50% inhibition at 10 μM was observed against all the 55 receptors, ion channels, and transporters from the Cerep ExpresSProfile. A full evaluation of its ADMET parameters was performed (Figure 2). Solubility, microsomal



99 (GLPG0974)

Thermodynamic solubility (pH 3) : 15 µg/mL
Thermodynamic solubility (pH 7.4) : 837 µg/mL
Rat liver microsom. stability (30 min incubation) : 69.5 % remaining
Human liver microsom. stability (30 min incubation) : 76.3 % remaining
Rat liver hepatocyte stability (90 min incubation) : 72.2 % remaining
Human liver hepatocyte stability (90 min incubation) : 85.6 % remaining
human plasma protein binding: 98.6 %
hERG (% inhibition at 10 µM) : 0%

Figure 2. ADME parameters of **99**.

stability, and plasma protein binding were all in the acceptable range. No inhibition of CYP450 isoforms was observed, and no time-dependent inhibition of CYP3A4 was noted. Compound **99** did not activate pregnane X receptor (PXR), suggesting a low induction potential for CYP3A4.[17] Compound **99** was found negative in the AMES-II test and exhibited no inhibition of the hERG channel.

Moreover, **99** showed excellent pharmacokinetic properties in rat with a bioavailability of 47% and a linear increase of the plasma exposure after oral dosing at 5 and 30 mg/kg (Table 6). The extended half-life observed following the increase of oral dose was consistent with the project objective to obtain long target coverage in human. Moreover, this assumption was further strengthened by allometric human prediction using additional pharmacokinetics studies in nonrodent species (data not shown).

NEUTROPHIL BIOLOGY

The potential of our FFA2 antagonists for inhibiting inflammatory processes has been further assessed. FFA2 was described as a potential essential player in the process of neutrophil

Table 6. Plasma PK Parameter of 99 in Sprague−Dawley Rat[a]

rat PK parameters	IV (1 mg/kg)	PO (5 mg/kg)	PO (30 mg/kg)
C0 (ng/mL)	968		
half-life (h)	1.7	1.7	2.8
clearance (L/h/kg)	2.1		
V_{ss} (L/kg)	3.2		
F (%)		47	40
AUC (0−24 h) (ng·h/mL)	517	1213	6170
C_{max} (ng/mL)		705	2230
T_{max} (h)		0.4	0.25

[a]PEG400/MC 0.5% (10/90; v/v) for oral route (PO), PEG200/NaCl 0.9% (60/40; v/v) for intravenous route (IV).

recruitment during intestinal inflammation. It has been shown that sodium acetate and sodium propionate, two natural ligands of FFA2, act as chemoattractants and induce neutrophil migration.[6] Consequently, several FFA2 antagonists were evaluated for their ability to inhibit human neutrophil migration induced by acetate (Table 7). Most of the compounds displayed

Table 7. Potency of Representative Compounds for Inhibition of Acetate-Induced Human Neutrophil Migration

compd	**61**	**60**	**73**	**99**	**75**	**38**	**40**
IC_{50} (nM)	561	75	58	27	24	23	5

a good ability to block neutrophil migration in this phenotypic assay, thus demonstrating their potential toward inhibition of inflammatory processes.

Moreover the specificity of this mechanism has been assessed by determining the impact of FFA2 antagonists on both fMLP- and IL8-mediated chemotaxis. None of the compounds inhibited the neutrophil migration, induced by these two chemotactic agents (data not shown).

A general trend throughout our chemical series was to exhibit a high protein binding, typically higher than 95% for most of our compounds bearing a carboxylic acid side chain at R3. As mentioned earlier, the lack of an animal model to assess efficacy did drive the decision to aim for a very long target coverage in human. To figure out whether plasma protein binding might limit the efficacy at the target level in vivo, the human neutrophil migration assay previously set up was performed in plasma instead of chemotaxis buffer (compound preincubation in whole blood and then incubation with neutrophils in 90% plasma). Similar potencies were obtained in both assays, in buffer or in plasma (compound **99** IC_{50} being 27 and 43 nM, respectively).

To support clinical development, a biomarker was developed enabling monitoring of target engagement in healthy volunteers and patients in future studies. Efforts were focused on CD11b activation-specific epitope (AE), considered as a hallmark of neutrophil activation during the migration process. Indeed, CD11b forms together with CD18 the membrane activated complex-1 (Mac-1), a cell surface receptor found on neutrophils. Through interaction with ICAM-1, MAC-1 mediates firm adhesion and arrest of neutrophils on endothelium. This is an essential step in neutrophil extravasation, leading to neutrophil migration into tissues at foci of inflammation and infection. For interaction with ICAM-1, a conformational change of CD11b (CD11b activation-specific epitope [AE]) is required. Different inflammatory stimuli up-regulate CD11b[AE] expression, which is considered a more relevant marker of neutrophil activation in blood than the total C11Db level.[18−20]

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Hence, the capacity of acetate to increase CD11b[AE] expression on neutrophils in human whole blood by flow cytometry was identified as a potential readout. Our FFA2 antagonists dose-dependently inhibited the acetate-induced conformational active CD11b[AE] expression on neutrophils, indicating that this event is driven by FFA2 (Figure 3). This observation represents the basis

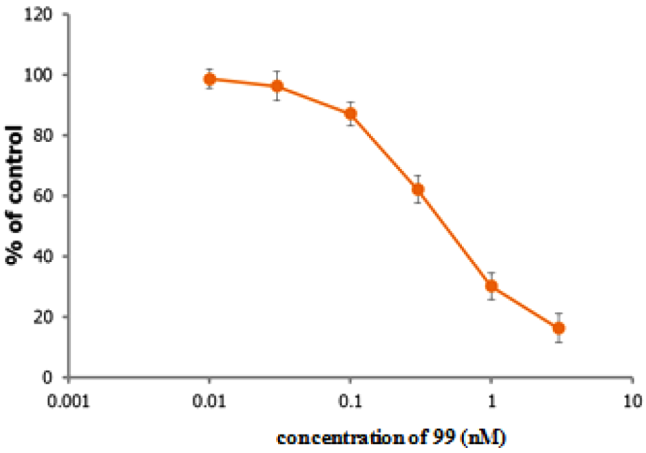

Figure 3. Dose-dependent inhibition of acetate-induced CD11b[AE] expression by compound **99**, measured by flow cytometry in human whole blood obtained from 16 different donors.

for the first biomarker assay described to date for FFA2.[21] In this assay, compound **99** is a potent inhibitor of the FFA2-induced CD11b[AE] expression (IC_{50} = 483 nM ± 72, n = 16).

As such, compound **99** showed high potency in all in vitro assays, even in whole blood context. On the basis of its overall profile, this molecule **99**, 4-[[(*R*)-1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid, was selected as preclinical candidate (GLPG0974), predicted to be able to block the acetate-induced neutrophil migration process in human for a long period of time. Additional pharmacokinetics and safety studies were performed after candidate selection to allow progression of **99** to clinical phases, becoming the first FFA2 antagonist reported to reach the clinic. From a drug design point of view, the course of the project illustrated once again that the combination of SAR improvements, although often not additive, could lead to unpredicted potent compounds. At the late stage of a lead optimization program, a systematic combination approach must not be neglected.

EXPERIMENTAL SECTION

Calcium Mobilization Assay. The following assay can be used for determination of GPR43 activation. The assay measures intracellular calcium release induced by activation of GPR43. On day 1, GPR43 stably expressing cells (Multispan) were seeded at 6000 cells/25 μL medium (DMEM supplemented with 10% FBS, 10 μg/mL puromycin, and 1% Pen/Strep) in poly-D-lysine coated 384-well plates and incubated overnight at 37 °C 10% CO_2. On day 2, 25 μL of a fluorescent Ca^{2+} indicator (Calcium 4 assay; Molecular Devices) supplemented with 2.5 mM probenecid was added on top of the cells and incubated for 2h at 37 °C. Subsequently, compounds at increasing concentration (10 μL/well) were added to the plate and incubated for 15 min at 37 °C. Finally the cells were triggered with sodium acetate at EC_{80} concentration (concentration which gives 80% of the activity of the GPR43) and the Ca^{2+}-flux was measured simultaneously to the agonist injection on a fluorescence plate reader (FlexStation3), measuring the antagonist activity of the compounds. To measure agonist activity of the compounds, compound was injected to the well and the Ca^{2+}-flux was measured simultaneously to the injection of compound.

Neutrophil Migration Assay. In the neutrophil migration assay, neutrophils, isolated from buffy coats from human volunteers, were treated with a compound for 30 min in chemotaxis buffer (RPMI 1640 medium, supplemented with 10 mM HEPES). Subsequently, the neutrophils were transferred to the upper wells of a HTS transwell 96 permeable support system (with 5.0 μm pore size polycarbonate membrane (Corning)), of which the lower wells were filled with chemotaxis buffer containing compound and sodium acetate solution. After 1 h of incubation, migration of the neutrophils toward the sodium acetate was quantified by measuring the ATP-content of the lower wells using the ATPlite luminescence ATP detection assay system (PerkinElmer, cat. no. 436110). The detected luminescent signal was linearly related to the number of migrated cells.

Whole Blood CD11b[AE] Assay. Human blood was collected by venipuncture from human volunteers, who gave informed consent, in acid-citrate-dextrose (ACD) vacutubes, mixed well, and processed as soon as possible, the latest within 90 min after blood collection. Blood was diluted 1:1 in RPMI1640. Diluted blood was treated simultaneously with a compound in DMSO solution or with DMSO (max 0.3% of DMSO) and with 20 μg/mL cytochalasin B, 2 ng/mL TNFalpha at 37 °C for 15 min without shaking. Then GPR43 agonist (sodium acetate) was added to blood. Blood was gently mixed and incubated at 37 °C for 30 min without shaking. Neutrophil surface expression of CD45, CD66b, and CD11b[AE] markers was determined by flow cytometry. Unspecific binding of antibodies was blocked by addition of 2 μg/sample of normal mouse IgG (Invitrogen) and incubation for 10 min at +4 °C in the dark. Then 2 μL of CD45 V450 antibody (BD Biosciences), 2.5 μL of CD11b APC Mouse Anti-Human CD11b[AE] 1 antibody (eBiosciences), and 10 μL of CD66b FITC antibody (BD Biosciences) were added to blood at the same time and samples incubated for 30 min at +4 °C in the dark. Red blood cells were lysed and samples fixed by addition of RBC lysing Buffer (1× solution from BD FACS Lysing Solution 10×, BD Pharmigen) and incubated for 10 min at room temperature in the dark. The cells were centrifuged at 550*g* for 10 min at +4 °C and washed once with PBS. The cells were resuspended in cold 300 μL of wash/staining buffer (PBS + 2% FBS), transferred to polystyrene 5 mL tube, and placed on ice protected from light until analyzed with FACScan/CytekDev XDP8 and FlowJo Collector's, FlowJo V7.6 software. Neutrophils were gated and selected according to morphological characteristics using FSC and SSC parameters and immunophenotype as $CD45^{high}$ $CD66b^{high}$ $CD11b[AE]^{+}$ cells. Cellular expression of CD11b[AE] was measured by determination of mean fluorescence intensity units (MFI).

Aqueous Solubility. A serial dilution of test compound was prepared in DMSO and further diluted in 0.1 M phosphate buffer, pH 7.4, or 0.1 M citrate buffer pH 3.0 to obtain final concentrations of 299, 149, 74.6, 37.3, and 18.8 μM, with all solutions having a total percentage of DMSO of 3% (v/v). The assay plates were incubated for 1 h at 37 °C while shaking at 230 rpm. The plates were scanned with a white light microscope, yielding individual pictures (50×) per concentration. The precipitate was analyzed and converted into a number by a custom-developed software tool. The lowest concentration at which the compound appears completely dissolved is reported.

Thermodynamic Solubility. Two individual solutions of about 2 mg/mL test compound were prepared in a 0.1 M phosphate buffer pH 7.4 or a 0.1 M citrate buffer pH 3.0 at room temperature in glass vials. The samples were stirred for 24 h at room temperature. The vials were centrifuged shortly, and the supernatant was filtered. Per sample, two dilutions (factor 10 and 100) were made in DMSO and analyzed on LC/MS-MS (API2000 from Applied Biosystems). A serial dilution of the compound was made in DMSO and diluted in 50−50 water/methanol to obtain final concentrations of 400, 200, 100, 50, 10, 2, 1, and 0.4 ng/mL with a total percentage of DMSO of 1.25% (v/v). These dilutions were also analyzed on LC/MS-MS. The peak areas of the serial dilutions were plotted in a graph, and a linear or polynomial of the second-order equation was used to calculate the unknown concentrations of the test compound.

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Microsomal Stability. A 10 mM stock solution of test compound in DMSO was diluted to 6 μM in a 105 mM phosphate buffer pH 7.4 and prewarmed at 37 °C. This dilution was added to microsomal/cofactormix in the same proportion (50 μL of compound diluted in buffer and 50 μL of the mix of microsome and cofactor) at 37 °C under shaking at 300 rpm. Final reaction conditions were: 3 μM of compound (n = 2), 0.5 mg/mL microsomes, 0.4 U/mL GDPDH, 3.3 mM $MgCl_2$, 3.3 mM glucose-6-phosphate, and 1.3 mM NADP+, incubation volume 100 μL, with all solutions having a total percentage of DMSO of 0.2% (v/v). After 0 and 30 min of incubation, the reaction was stopped with MeOH (1:1). The samples were mixed, centrifuged, and filtered and the supernatant analyzed by LC-MS/MS (API2000 from Applied Biosystems). The instrument responses (i.e., peak areas) were referenced to the zero time-point samples (considered as 100%) in order to determine the percentage of compound remaining.

Hepatocyte Stability. Test compound (1 μM initial concentration, n = 2) was incubated in Williams' Medium E, containing 4 mM L-glutamine and 2 mM magnesium sulfate, with pooled cryopreserved hepatocytes (Celsis International) in suspension at cell density of 0.5 million viable cells/mL. The incubations were performed at 37 °C in a shaking water bath, with samples taken from the incubation at 0, 10, 20, 45, and 90 min, and reactions terminated by addition of an equal amount of acetonitrile containing carbamazepine as analytical internal standard. Samples were centrifuged and the supernatant fractions analyzed by LC-MS/MS (Waters TQD instruments). The instrument responses (i.e., peak heights) were referenced to the zero time-point samples (considered as 100%) in order to determine the percentage of compound remaining. Ln plots of the % remaining for each compound were used to determine the half-life for the hepatocyte incubations.

Plasma Protein Binding (Equilibrium Dialysis). A 5 μM solution of test compound was made in freshly thawed human, rat, mouse, or dog plasma plasma (BioReclamation INC) with a total percentage of DMSO of 0.5% (v/v). A Pierce Red Device plate with inserts (ThermoScientific) was filled with 450 μL of PBS (buffer chamber) and 300 μL of the spiked plasma (plasma chamber) and incubated at 37 °C while shaking at 100 rpm. After 4 h incubation, 120 μL from each chamber was transferred into 480 μL of methanol. The samples were mixed and immediately centrifuged for 30 min at 1400 rcf at 4 °C. The supernatant was filtered and analyzed by LC-MS/MS (API2000 from Applied Biosystems). Addition of test compound peak area in the buffer chamber and the plasma chamber were considered to be 100% compound. The percentage bound to plasma is derived from these results and is reported as percentage bound to plasma.

hERG Patch Clamp. Whole-cell patch-clamp recordings were performed on HEK293 cells stably expressing hERG channels. Cells were cultured on 12 mm round coverslips (German glass, Bellco) anchored in the recording chamber using two platinum rods (Goodfellow). All compound concentrations were made in DMSO prior to being added to extracellular buffer with all solutions having a total percentage of DMSO of 1% (v/v). The external bathing solution contained: 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4. The internal patch pipet solution contains: 100 mM potassium gluconate, 20 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na_2ATP, 2 mM glutathione, 11 mM EGTA, 10 mM HEPES, pH 7.2. Compound was perfused using an Biologic MEV-9/EVH-9 rapid perfusion system. hERG currents were evoked using an EPC10 amplifier controlled by Pulse v8.77 software (HEKA) with an activating pulse to +40 mV for 1000 ms followed by a tail current pulse to −50 mV for 2000 ms, holding potential was −80 mV. Pulses were applied every 15 s, and all experiments were performed at room temperature.

Single Dose Pharmacokinetic Study in Rats. All animal protocols were reviewed and approved by Galapagos Animal Care and Use Committee registered at "Comité National de Réflexion Ethique sur l'Expérimentation Animale" (France). Compounds were formulated in PEG200/NaCl 0.9% (60/40; v/v) for the intravenous route and in PEG400/0.5% methylcellulose (10/90 v/v) for the oral route. Test compounds were orally dosed as a single esophageal gavage at 5−10 mg/kg and intravenously dosed as a bolus via the caudal vein at 1 mg/kg to male Sprague−Dawley rats. Each group consisted of three rats. Blood samples were collected either via the jugular vein using cannulated rats or at the retro-orbital sinus with lithium heparin as anticoagulant at 4−7 time points in the following range: 0.05−8 h (intravenous route) and 0.25−6 or 24 h (oral route). Whole blood samples were centrifuged at 5000 rpm for 10 min, and the resulting plasma samples were stored at −20 °C pending analysis. Quantification of compound levels in plasma: An API4000 mass spectrometer (ABSciex) operating in positive TurboIonSpray mode was used for the detection of the test compound in plasma, and Analyst 1.4.2 software (ABSciex) was used for samples injection, peaks integration, and plasma level quantification. Pharmacokinetic parameters were calculated using Winnonlin (Pharsight, US, version 5.2.1).

Chemistry. All reagents were of commercial grade and were used as received without further purification unless otherwise stated. Commercially available anhydrous solvents were used for reactions conducted under inert atmosphere. Reagent grade solvents were used in all other cases unless otherwise specified. Column chromatography was performed on silica standard (35−70 μm). Thin layer chromatography was carried out using precoated silica gel 60 F-254 plates (thickness 0.25 mm). ^{1}H NMR spectra were recorded on a Bruker Advance 400 NMR spectrometer (400 MHz). Chemical shifts (δ) for ^{1}H NMR spectra are reported in parts per million (ppm) relative to tetramethylsilane (δ 0.00) or the appropriate residual solvent peak as internal reference. Multiplicities are given as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). ^{13}C NMR spectrum of **99** was recorded on a Varian Unity Inova 400 NMR spectrometer at 80 °C. Electrospray MS spectra were obtained on a Waters platform LC/MS spectrometer. Unless otherwise specified the purity of all intermediates and final compounds was determined to be >95% by analytic LCMS: columns used, Waters Acquity UPLC BEH C18 1.7 μm, 2.1 mm ID × 50 mm L or Waters Acquity UPLC BEH C18 1.7 μm, 2.1 mm ID × 30 mm L. All the methods are using MeCN/H_2O gradients. MeCN and H2O contain either 0.1% formic acid or NH_3 (10 mM). Preparative LCMS: column used, Waters XBridge Prep C18 5 μm ODB 30 mm ID × 100 mm L. All the methods are using MeOH/H_2O gradients. MeOH and H_2O contain either 0.1% formic acid or 0.1% siethylamine. CHN analysis was determined using the PerkinElmer 2400 CHNS/O series II elemental analyzer, which uses a combustion method in a pure oxygen environment to convert the accurately weighed sample into the simple gases CO_2, H_2O, and N_2. After reduction through pure copper, the resulting CHN gases are then controlled to exact conditions of pressure, temperature, and volume whereupon the system uses a steady state wavefront approach to separate the controlled gases. This approach involves separating a continuous homogenized mixture of gases through a chromatographic column. The gases eluting off the column are measured as a function of their thermal conductivity. Microwave heating was performed with a Biotage Initiator.

2-Methyl-azetidine-1,2-dicarboxylic Acid 1-*tert*-Butyl Ester 2-Ethyl Ester (5). *Step 1: 2-Benzylamino-propionic Acid Ethyl Ester (**8**).* To a solution of 2-bromo-propionic acid ethyl ester **7** (17.9 mL, 1 equiv) in MeCN (250 mL) was added benzylamine **6** (13.5 mL, 0.9 equiv) and potassium carbonate (28.6 g, 1.5 equiv). The reaction was refluxed for 4 h then cooled to 20 °C and diluted with EtOAc. The crude was filtered, and the filtrate was concentrated under reduced pressure then purified by chromatography on silica gel (elution with heptane/EtOAc: 70/30) to afford 2-benzylamino-propionic acid ethyl ester **8** (24.7 g, 97% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.37−7.29 (4H, m), 7.28−7.22 (1H, m), 4.20 (2H, q), 3.82 (1H, d), 3.69 (1H, d), 3.38 (1H, q), 1.33 (3H, d), 1.29 (3H, t). MW (calcd): 207.2. MW (obsd): 208.1 (M + 1).

*Step 2: 2-[Benzyl-(2-chloro-ethyl)-amino]-propionic Acid Ethyl Ester (**9**).* To a solution of chloroacetaldehyde (56 mL, 45% w/w in water, 3 equiv) in DCM (75 mL) under nitrogen was added $MgSO_4$ (55 g, 4 equiv). The mixture was stirred for 15 min at 20 °C under nitrogen. The solid was filtered, washed with dry DCM, and the resulting filtrate (solution I) rapidly used in the following reaction. To a solution of **8** (27.7 g, 1 equiv) in dry DCM (114 mL) was added $MgSO_4$ (10.8 g, 0.75 equiv). The reaction was cooled to 0 °C then the above solution of chloroacetaldehyde in dry DCM (solution I) and acetic acid (1 equiv) was added. Sodium triacetoxyborohydride (38 g, 1.5 equiv) was added portionwise. The reaction was stirred for 1 h at 0 °C. The crude was carefully quenched with a saturated aqueous solution of $NaHCO_3$. Then

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aqueous NaOH (2N) was added. The aqueous layer was extracted with DCM. The combined organic layers were washed with an aqueous saturated solution of $NaHCO_3$, then dried over $MgSO_4$ and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (elution with heptane/EtOAc 98/2 to 95/5) to afford 2-[benzyl-(2-chloro-ethyl)-amino]-propionic acid ethyl ester **9** (27.4 g, 85% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.40−7.29 (4H, m), 7.29−7.22 (1H, m), 4.24−4.14 (2H, m), 3.90 (1H, d), 3.77 (1H, d), 3.50 (1H, q), 3.43−3.36 (2H, m), 3.13−2.94 (2H, m), 1.34 (3H, d), 1.31 (3H, t). MW (calcd): 269.8. MW (obsd): 270.1 (M + 1, ^{35}Cl).

*Step 3: 1-Benzyl-2-methyl-azetidine-2-carboxylic Acid Ethyl Ester (**10**).* A solution of **9** (27.4 g, 1 equiv) in dry THF (253 mL) was cooled to −78 °C under nitrogen. KHMDS (170.4 g, 15% w/w in toluene, 1.25 equiv) was slowly added so that temperature was kept below −65 °C. The reaction was stirred for 1 h at −70 °C, then acetic acid (1.75 mL, 0.3 equiv) was added. The reaction was warmed to 20 °C and stirred for 10 min, then quenched with a saturated aqueous solution of $NaHCO_3$ and partially concentrated under reduced pressure. The crude was extracted twice with DCM. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (elution with heptane/EtOAc 100/0 then 95/5) to afford 1-benzyl-2-methyl-azetidine-2-carboxylic acid ethyl ester **10** (18.75 g, 79% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.34−7.19 (5H, m), 4.24−4.16 (2H, m), 3.79 (1H, d), 3.58 (1H, d), 3.30−3.22 (1H, m), 3.15−3.05 (1H, m), 2.63−2.54 (1H, m), 1.97−1.88 (1H, m), 1.49 (3H, s), 1.29 (3H, t). MW (calcd): 233.3. MW (obsd): 234.1 (M + 1).

*Step 4: 2-Methyl-azetidine-1,2-dicarboxylic Acid 1-tert-butyl Ester 2-Ethyl Ester (**5**).* To a solution of **10** (18,75 g, 1 equiv) in EtOH (1,500 mL) was added Boc_2O (75g, 1.2 equiv) and Pd/C (7.3 g, 0.05 equiv). The flask was evacuated and backfilled with argon, then the reaction was evacuated and backfilled with H_2 and stirred for 15 h at 20 °C under atmospheric pressure. The crude was filtered through a pad of Celite and washed with EtOH and DCM. The filtrate was concentrated under reduced pressure to afford 2-methyl-azetidine-1,2-dicarboxylic acid 1-*tert*-butyl ester 2-ethyl ester **5** (57 g, 79% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 4.12−3.92 (2H, m), 3.86−3.71 (1H, m), 3.66−3.54 (1H, m), 2.17−2.00 (1H, m), 1.99−1.83 (1H, m), 1.33 (3H, s), 1.21 (9H, s), 1.11 (3H, t). MW (calcd): 243.3. MW (obsd): 266$^+$ (M + Na^+).

First Route: Introduction of R2 First. *General Procedure A1 for Synthesis of Compounds **3**, **4**, **12**, **43**, **44**, **48**, **49**, **51**, **52**, **53**, **54**, **55**, **56**, **57**, **58**, and **59**. Steps 1 and 2*: Synthesis of **12**.

*2-[(4-Chloro-benzyl)-methyl-carbamoyl]-2-methyl-azetidine-1-carboxylic Acid tert-Butyl Ester (**12**). Step 1*: To a solution of **5** (63 g, 1 equiv) in EtOH was added an aqueous solution of NaOH 2N (260 mL, 2 equiv). The reaction was stirred at 20 °C for 15 h. The solvent was removed under reduced pressure, and the crude was partitioned between water and EtOAc. The organic layer was discarded, and the aqueous layer was acidified by addition of a solution of citric acid 10% in water until pH = 3 and thoroughly extracted three times with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford 2-methyl-azetidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **11** (50 g, 89% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 3.93−3.81 (1H, m), 3.81−3.68 (1H, m), 2.85−2.71 (1H, m), 2.12−2.00 (1H, m), 1.72 (3H, s), 1.48 (9H, s).

Step 2: Amide coupling. To a solution **11** (250 mg, 1 equiv) in DCM (2 mL) and THF (2 mL) was added HOBt (157 mg, 1 equiv) and EDC·HCl (334 mg, 1.5 equiv), then (4-chloro-benzyl)-methyl-amine (270 mg, 1 equiv) and TEA (660 μL, 4 equiv) were added. The reaction was stirred at 20 °C for 15 h. The crude was concentrated under reduced pressure and partitioned between a saturated aqueous solution of $NaHCO_3$ and EtOAc. The aqueous layer was extracted three times with EtOAc. The organic layers were combined, washed with water then with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford 2-[(4-chloro-benzyl)-methyl-carbamoyl]-2-methyl-azetidine-1-carboxylic acid *tert*-butyl ester **12** (283 mg, 70% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.39−7.31 (2H, m), 7.26−7.15 (2H, m), 4.77−4.44 (2H, m), 3.94−3.74 (2H, m), 2.94 (3H, s), 2.55−2.39 (1H, m), 2.31−2.18 (1H, m), 1.82 (3H, s), 1.48 (9H, s).

Steps 3 and 4: Synthesis of **3**.

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-methyl-amide (**3**). Step 3*: BOC removal. To a solution of **12** (283 mg, 1 equiv) in dioxane under argon was added a solution of HCl (4N in dioxane, 10 equiv). The reaction was stirred for 16 h at 20 °C, then concentrated under reduced pressure. The crude was triturated with a mixture of DCM/iPr_2O, and the resulting solid was filtered and dried to afford the corresponding free amine as a hydrochloride salt (230 mg, quantitative yield). ^{1}H NMR δ (ppm) (MeOD-d_4): 7.46−7.40 (2H, m), 7.38−7.32 (2H, m), 4.78−4.58 (2H, m), 4.21−4.07 (1H, m), 3.87−3.77 (1H, m), 3.11−2.98 (1H, m), 2.93 (3H, s), 2.76−2.65 (1H, m), 1.97 (3H, s).

Step 4: Amide coupling. To a solution of (3,5-dimethyl-phenyl)-acetic acid (65 mg, 1.5 equiv) in DCM (1 mL) and THF (1 mL) was added HOBt (35 mg, 1.1 equiv) and EDC·HCl (77 mg, 1.5 equiv). Then the above prepared azetidine intermediate as a hydrochloride salt (75 mg, 1 equiv) and TEA (70 μL, 3 equiv) were added. The reaction was stirred at 20 °C for 15 h. The crude was concentrated under reduced pressure and partitioned between a saturated aqueous solution of $NaHCO_3$ and EtOAc. The aqueous layer was extracted three times with EtOAc. The organic layers were combined, washed with water then with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-methyl-amide **3** (77 mg, 74% yield). ^{1}H NMR δ (ppm) (MeOD-d_4): 7.49−7.25 (4H, m), 7.02−6.89 (3H, m), 4.75−4.49 (2H, m), 4.22−4.05 (1H, m), 3.95−3.81 (1H, m), 3.51−3.35 (2H, m), 3.00−2.88 (3H, m), 2.70−2.36 (2H, m), 2.35−2.30 (6H, m), 2.00−1.87 (3H, m). MW (calcd): 398.2. MW (obsd): 399.4 (M + 1).

*General Procedure A2 for the Synthesis of Compounds **45**, **46**, and **47**. Step 1*: Following general procedure A1 and using (4-iodo-phenyl)-acetic acid or (3-iodo-phenyl)-acetic acid in step 4 gave the corresponding iodo intermediate.

Step 2: Suzuki coupling. To a solution of aryl boronic acid (1.5 equiv) in dioxane/H_2O (9/1) were added Na_2CO_3 (4 equiv) and the corresponding iodo intermediate (1 equiv). The flask was evacuated and backfilled with argon. Then $Pd(PPh_3)_4$ was added and the reaction was heated at 90 °C until completion. The crude was partitioned between water and EtOAc. The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (elution with heptane/EtOAc: 100/0 to 20/80) to afford the corresponding carboxamide.

*1-(2-Biphenyl-4-yl-acetyl)-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-methyl-amide (**45**).* Following general procedure A2 and using (4-iodo-phenyl)-acetic acid (30 mg, 1 equiv) in step 1 and phenyl boronic acid (11 mg, 1.5 equiv) in step 2 gave 1-(2-biphenyl-4-yl-acetyl)-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-methyl-amide **45** (12 mg, 35% yield over two steps). ^{1}H NMR δ (ppm) (MeOD-d_4): 7.88−7.68 (4H, m), 7.68−7.36 (9H, m), 4.89−4.61 (2H, m), 4.42−4.19 (1H, m), 4.13−3.63 (3H, m), 3.06 (3H, d), 2.87−2.49 (2H, m), 2.08 (3H, d). MW (calcd): 446.2. MW (obsd): 447.4 (M + 1).

*General Procedure A3 for the Synthesis of Compounds **74**, **76**, **85**, **87**, **93**, and **97**. Step 1*: Following general procedure A1 and using the corresponding secondary amine intermediate in step 2 and the corresponding R1 carboxylic acid in step 4 gave the corresponding ester intermediate.

Step 2: Saponification. To a solution the above prepared ester (1 equiv) in EtOH was added an aqueous solution of 2N NaOH (2 equiv). The reaction was stirred at 20 °C for 15 h. The solvent was removed under reduced pressure, and the crude was partitioned between water and EtOAc. The organic layer was discarded, and the aqueous layer was acidified by addition of a solution of citric acid 10% in water until pH = 3 and thoroughly extracted by EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the corresponding carboxylic acid.

*4-{[1-(Benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-benzofuran-6-ylmethylamino}-butyric Acid (**74**).* Following

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general procedure A3 and using 4-[(benzofuran-6-ylmethyl)-amino]-butyric acid ethyl ester and benzo[*b*]thiophene-3-carboxylic acid in step 1 gave the corresponding carboxylic acid 4-{[1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-benzofuran-6-ylmethylamino}-butyric acid **74** (36 mg, 35% yield over four steps). ^{1}H NMR δ (ppm) (MeOD-d_4): 8.33−8.04 (1H, m), 8.02−7.90 (1H, m), 7.81 (1H, d), 7.72−7.62 (1H, m), 7.61−7.40 (4H, m), 7.35−7.14 (1H, m), 6.89 (1H, m), 5.12−4.69 (2H, m), 4.39−3.95 (2H, m), 3.82−3.18 (2H, m), 2.85−2.46 (2H, m), 2.45−2.28 (2H, m), 2.23−1.86 (5H, m). MW (calcd): 490.2. MW (obsd): 491.4 (M + 1).

Second Route: Introduction of R1 First. *Synthesis of **13a, 13b, 13c, and 13d**. Step 1*: BOC removal. To a solution of intermediate **7** (1 equiv) in dioxane under argon was added a solution of HCl (4N in dioxane,10 equiv). The reaction was stirred for 16 h at 20 °C, then concentrated under reduced pressure. The crude was triturated with a mixture of DCM/iPr_2O, and the resulting solid was filtered and dried to afford 2-methyl-azetidine-2-carboxylic acid ethyl ester hydrochloride. ^{1}H NMR δ (ppm) ($CDCl_3$): 9.48 (1H, br s), 4.35 (2H, q), 4.16−4.00 (2H, m), 2.82−2.70 (1H, m), 2.60−2.45 (1H, m), 1.95 (3H, s), 1.36 (3H, t).

Step 2: Amide coupling. To a solution of the corresponding carboxylic acid (1 equiv) in either DCM or THF was added HOBt (1.1 equiv), EDC·HCl (1.5 equiv). Then the above prepared azetidine intermediate as a hydrochloride salt (1 equiv) and TEA (4 equiv) were added. The reaction was stirred at 20 °C for 15 h. The crude was concentrated under reduced pressure and partitioned between a saturated aqueous solution of $NaHCO_3$ and EtOAc. The aqueous layer was extracted three times with EtOAc. The organic layers were combined, washed with water then with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the corresponding carboxamide.

Step 3: Saponification. To a solution of the corresponding above prepared carboxamide (1 equiv) in EtOH or MeOH was added an aqueous solution of 2N NaOH (2 equiv). The reaction was stirred at 20 °C for 15 h. The solvent was removed under reduced pressure, and the crude was partitioned between water and EtOAc. The organic layer was discarded, and the aqueous layer was neutralized by addition of aqueous HCl (2N) and thoroughly extracted with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the corresponding carboxylic acid intermediates **13a**, **13b**, **13c**, and **13d**.

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (**13a**).* ^{1}H NMR δ (ppm) (MeOD-d_4): 6.98−6.90 (3H, m), 4.26−4.12 (2H, m), 3.47 (2H, bs), 2.53−2.43 (1H, m), 2.34 (6H, bs), 2.29−2.18 (1H, m), 1.73 (3H, s). MW (calcd): 261.3. MW (obsd): 262.0 (M + 1).

*1-(2-Benzofuran-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic Acid (**13b**).* ^{1}H NMR δ (ppm) (DMSO): 7.86 (1H, s), 7.65−7.52 (2H, m), 7.34−7.19 (2H, m), 4.24−4.13 (2H, m), 3.49 (2H, dd), 2.39−2.26 (1H, m), 2.21−2.07 (1H, m), 1.56 (3H, s). MW (calcd): 273.3. MW (obsd): 274.1 (M + 1).

*1-(2-Benzo[b]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic Acid (**13c**).* ^{1}H NMR δ (ppm) ($CDCl_3$): 87.92 (1H, d), 7.78 (1H, d), 7.50−7.40 (2H, m), 7.35 (1H, s), 4.05−3.93 (2H, m), 3.79 (2H, s), 2.96−2.76 (1H, m), 2.19−2.08 (1H, m), 1.80 (3H, s). MW (calcd): 289.3. MW (obsd): 290.0 (M + 1).

*1-(2-Benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carboxylic Acid (**13d**).* ^{1}H NMR δ (ppm) ($CDCl_3$): δ 8.41 (1H, d), 7.92 (1H, d), 7.87 (1H, s), 7.57−7.44 (2H, m), 4.49−4.39 (1H, m), 4.31−4.21 (1H, m), 3.10−3.00 (1H, m), 2.36−2.25 (1H, m), 2.01 (3H, s).

*General Procedure B1 for the Synthesis of Compounds **1, 2, 15, 16, 19, 20, 21, 22, 23, 27, and 50**.* To a solution of the relevant carboxylic acid (**13a**, **13b**, **13c**, or **13d**) (1 equiv) in DCM under nitrogen was added DMF (0.01 equiv) then oxalyl chloride (2 equiv). The solution was stirred at 20 °C for 30 min, then cooled to 0 °C. A solution of the corresponding amine (1.5 equiv) and either TEA or DIPEA (2 to 7 equiv) in DCM was then added to the previous mixture. The solution was stirred at 0 °C for 1 h then at 20 °C until completion (1 h). The reaction was quenched with a saturated aqueous solution of $NaHCO_3$ and extracted three times with DCM. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel to afford the desired compound.

*1-(2-Benzo[b]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic Acid 4-Chloro-benzylamide (**1**).* Following general procedure B1 and using intermediate **13c** (100 mg) and 4-chloro-benzylamine (63 μL) gave 1-(2-benzo[*b*]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic acid 4-chloro-benzylamide **1** (64 mg, 45% yield). ^{1}H NMR δ ppm ($CDCl_3$): 8.74 (1H, br s), 7.91 (1H, d), 7.74 (1H, d), 7.45−7.35 (2H, m), 7.27−7.21 (3H, m), 7.15 (2H, d), 4.39 (2H, ddd), 4.10−3.94 (2H, m), 3.71 (2H, s), 2.96−2.86 (1H, m), 2.15−2.06 (1H, m), 1.81 (3H, s). MW (calcd): 412.1. MW (obsd): 413.3 (M + 1).

*General Procedure B2 and Synthesis of Compound **32**.* To a solution of 1-chloro-*N*,*N*-2-trimethylpropenylamine (2 equiv) in DCM under nitrogen was added the corresponding carboxylic acid (1 equiv). The solution was stirred at 20 °C for 1 h, then added to a solution of appropriate amine (1.2 equiv) in DCM at 0 °C. TEA (2 equiv) was then added, and the mixture was stirred at 0 °C for 3 h. The crude was diluted with DCM, washed twice with a saturated aqueous solution of $NaHCO_3$, twice with aqueous HCl (0.5N), twice with water, dried over $MgSO_4$, filtered, concentrated under reduced pressure, and purified by chromatography on silica gel to afford the corresponding carboxamide.

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-(2-methanesulfonylamino-ethyl)-amide (**32**).* Following general procedure B2 and using intermediate **13a** (120 mg) and *N*-[2-(4-chloro-benzylamino)-ethyl]-methanesulfonamide gave (130 mg) **32** (80 mg, 35% yield). ^{1}H NMR δ ppm ($CDCl_3$): 7.45−7.31 (2H, m), 7.27−7.11 (1H, m), 7.10−6.85 (4H, m), 5.25−4.70 (1H, m), 4.64−4.21 (2H, m), 4.16−3.63 (3H, m), 3.50−3.21 (4H, m), 3.03−2.89 (4H, m), 2.77−2.49 (1H, m), 2.33 (6H, s), 1.86 (3H, s). MW (calcd): 505.2. MW (obsd): 506.1 (M + 1).

*General Procedure B3 for the Synthesis of Compounds **33, 34, 35, and 36**. 1-(2-Benzo[b]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-(3-sulfamoyl-propyl)-amide (**34**). Step 1*: Following general procedure B2 using intermediate **13c** (50 mg) with 3-(4-chloro-benzylamino)-propane-1-sulfonic acid 2,4-dimethoxy-benzylamide (70 mg) gave the corresponding sulfonamide intermediate (60 mg, 52% yield).

Step 2: Sulfonamide cleavage. The above prepared protected sulfonamide (52 mg) was dissolved in TFA (10 mL) and stirred at 20 °C for 15 h. The crude was concentrated under reduced pressure then partitioned between water and EtOAc. The organic layer was washed twice with water, then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel to give 1-(2-benzo[*b*]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-(3-sulfamoyl-propyl)-amide **34** (16 mg, 39% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.90−7.71 (2H, m), 7.46−7.26 (5H, m), 7.19−6.89 (2H, m), 5.20−4.88 (2H, m), 4.70−4.34 (1H, m), 4.08−3.85 (2H, m), 3.80−3.44 (3H, m), 3.29−2.88 (3H, m), 2.59−2.37 (1H, m), 2.36−2.13 (1H, m), 2.09−1.98 (1H, m), 1.86 (3H, br s). MW (calcd): 533.1. MW (obsd): 534.4 (M + 1).

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (3-Carbamoyl-propyl)-(4-chloro-benzyl)-amide (**26**). Step 1*: Following general procedure B1 using intermediate **13a** (300 mg) and 4-(4-chloro-benzylamino)-butyric acid ethyl ester (353 mg) in the last step gave the corresponding ester intermediate.

Step 2: Amide synthesis. To a solution of the above prepared ester intermediate in MeOH (2 mL) was added an aqueous solution of NH_3 (20% in water). The reaction was stirred at 60 °C for 15 h then cooled to 20 °C. The solvents were evaporated under reduced pressure, and the crude was purified by preparative LCMS to afford 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (3-carbamoyl-propyl)-(4-chloro-benzyl)-amide **26** (100 mg, 18% yield over two steps). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.47−7.27 (2H, m), 7.24−6.98 (2H, m), 6.97−6.56 (3H, m), 5.61−5.20 (1H, m), 4.97−4.52 (1H, m), 4.49−4.06 (1H, m), 4.04−3.52 (3H, m), 3.43−2.97 (2H, m), 2.62−2.43 (1H, m), 2.40−2.10 (7H, m), 2.09−1.73 (5H, m), 1.72−1.51 (2H, m). MW (calcd): 469.2. MW (obsd): 470.1 (M + 1).

*General Procedure B4 for the Synthesis of Compounds **37, 38, 39, 40, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 75, 81, 83, 88, 89, 90, 91, 95, 96, and 98**. Step 1*: Following general procedure B1 or

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B2 and using the corresponding acids **13a−13d** and secondary amines gave the corresponding ester intermediate.

Step 2: Saponification. To a solution of the above prepared ester (1 equiv) in EtOH was added an aqueous solution of 2N NaOH (2 equiv). The reaction was stirred at 20 °C for 15h. The solvent was removed under reduced pressure and the crude was partitioned between water and EtOAc. The organic layer was discarded, and the aqueous layer was acidified by addition of a solution of citric acid 10% in water until pH = 3 and thoroughly extracted by EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the corresponding carboxylic acid.

*[[1-(2-Benzo[b]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carbonyl]-(4-chloro-benzyl)-amino]-acetic Acid (**38**).* Following general procedure B4 and using intermediate **13c** (100 mg) and (4-chloro-benzylamino)-acetic acid ethyl ester (115 mg) (B1-step1) gave [[1-(2-benzo[*b*]thiophen-3-yl-acetyl)-2-methyl-azetidine-2-carbonyl]-(4-chloro-benzyl)-amino]-acetic acid **38** (20% yield over two steps). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.91−7.69 (2H, m), 7.48−7.29 (5H, m), 7.17 (2H, d), 4.71−4.33 (3H, m), 4.22−3.92 (3H, m), 3.86−3.61 (2H, m), 2.79−2.17 (2H, m), 2.01−1.69 (3H, m). MW (calcd): 470.1. MW (obsd): 471.3 (M + 1).

*General Procedure B5 for the Synthesis of Compounds **77**, **78**, **79**, and **80**. Step 1*: Following general procedure B2 and using intermediate **13c** and the corresponding secondary amine gave the corresponding ester intermediate.

Step 2: Saponification. To a solution of the above prepared ester in THF/water (1/1) LiOH monohydrate (1 equiv) was added. The mixture was stirred at 20 °C for 16 h. The pH was adjusted to pH = 7−8 by addition of aqueous HCl 0.1N, and the mixture was concentrated. The crude was purified by preparative LCMS to the give the corresponding carboxylic acid.

*4-(1-(Benzo[b]thiophene-3-carbonyl)-N-(6-chloropyridin-3-yl)-2-methylazetidine-2-carboxamido)butanoic Acid (**80**).* Following general procedure B5 and using 4-(6-chloro-pyridin-3-ylamino)-butyric acid ethyl ester in step 1 gave **80**. ^{1}H NMR δ (ppm) ($CDCl_3$): 8.46 (1H, s), 8.29 (1H, d), 7.84 (1H, d), 7.67 (1H, dd), 7.45−7.38 (2H, m), 7.12−7.03 (HH, m), 4.03−3.92 (1H, m), 3.90−3.76 (2H, m), 3.67−3.58 (1H, m), 2.72−2.65 (1H, m), 2.48 (2H, t), 2.45−2.18 (1H, m), 2.01−1.75 (5H, m). MW (calcd): 471.1. MW (obsd): 472.0 (M + 1).

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (2-Amino-ethyl)-(4-chloro-benzyl)-amide (**17**). Step 1*: Following general procedure B1 intermediate **13a** (100 mg, 1 equiv) and [2-(4-chloro-benzylamino)-ethyl]-carbamic acid *tert*-butyl ester (163 mg, 1.5 equiv) gave the corresponding BOC protected amino intermediate (139 mg, 69% yield).

Step 2: NBOC deprotection. The above prepared protected amino compound was dissolved in DCM and TFA (30%) and stirred at 20 °C for 15 h. The crude was then partitioned between a saturated $NaHCO_3$ solution and DCM. The organic layer was washed with water, then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel to afford 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (2-amino-ethyl)-(4-chloro-benzyl)-amide **17** (72 mg, 68% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 8.53−8.41 (1H, m), 7.28−7.23 (3H, m), 6.89 (1H, bs), 6.85 (1H, bs), 4.06−3.87 (2H, m), 3.75 (2H, s), 3.46−3.27 (3H, m), 2.88−2.69 (3H, m), 2.28 (6H, s), 2.09−1.97 (1H, m), 1.92−1.78 (1H, m), 1.72 (3H, s). MW (calcd): 480.2. MW (obsd): 481.1 (M + 1).

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-[2-(3-methyl-ureido)-ethyl]-amide (**18**).* To a solution of **17** (10 mg, 1 equiv) in MeCN (1 mL) was added *N*-succinimidyl-*N*-methylcarbamate (4 mg, 1.1 equiv). The reaction was stirred overnight at 20 °C before adding 2 additional equiv of *N*-succinimidyl-*N*-methylcarbamate. Stirring was continued for 24 h until completion. The reaction mixture was then concentrated under reduced pressure and partitioned between water and EtOAc. The organic layer was washed twice with water, then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel to afford 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-[2-(3-methyl-ureido)-ethyl]-amide **18** (4 mg, 35% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 8.67−8.58 (1H, m), 7.31−7.27 (1H, m), 7.23−7.16 (2H, m), 6.91 (1H, s), 6.83 (2H, s), 4.48 (2H, dd), 4.06−3.87 (2H, m), 3.37 (2H, s), 3.23−3.06 (4H, m), 2.88 (3H, s), 2.85−2.74 (1H, m), 2.29 (6H, s), 2.10−1.99 (1H, m), 1.70 (3H, s). MW (calcd): 485.0. MW (obsd): 486.2 (M + 1).

*General Procedure B6 for the Synthesis of Compounds **29** and **30**. Step 1*: Following general procedure B4 and using the corresponding acid **13a−13d** and secondary amine gave the corresponding acid intermediate.

Step 2: Amide formation. To a solution of the above prepared carboxylic acid (1 equiv) in either DCM or THF was added HOBt (1.1 equiv) and EDC·HCl (1.5 equiv). Then the corresponding amine (1 equiv) and TEA (4 equiv) were added. The reaction was stirred at 20 °C for 15 h. The crude was concentrated under reduced pressure and partitioned between a saturated aqueous solution of $NaHCO_3$ and EtOAc. The aqueous layer was extracted three times with EtOAc. The organic layers were combined, washed with water then with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford the corresponding carboxamide.

*1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-(4-morpholin-4-yl-4-oxo-butyl)-amide (**29**).* Following general procedure B6 and using intermediate **13a**, 4-(4-chloro-benzylamino)-butyric acid methyl ester in step 1, and morpholine in step 2 gave 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-(4-morpholin-4-yl-4-oxo-butyl)-amide **29**. ^{1}H NMR δ (ppm) (MeOD-d_4): 7.52−7.20 (4H, m), 7.02−6.83 (3H), 4.86−4.49 (2H, m), 4.22−3.73 (2H, m), 3.73−3.43 (10H, m), 3.35−3.12 (2H, m), 2.69−2.27 (10H, m), 2.06−1.81 (5H, m). MW (calcd): 539.3. MW (obsd): 540.4 (M + 1).

*General Procedure B7 for the Synthesis of Compounds **24**, **25**, and **28**. 1-[2-(3,5-Dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic Acid (4-Chloro-benzyl)-methylcarbamoylmethyl-amide (**24**).* To a solution of **37** (46 mg, 1 equiv) in DMF (1 mL) were added a solution of methylamine in ethanol (33% w/w, 10 μL, 1 equiv), TEA (14 μL, 1 equiv), and TBTU (33 mg, 1 equiv). The reaction was stirred at 20 °C for 15 h. The reaction was carried on with addition of methylamine (1 equiv), TEA (1 equiv), and TBTU (1 equiv), then stirring at 20 °C for 15 h. The crude was concentrated under reduced pressure then partitioned between water and EtOAc. The organic layer was washed twice with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by preparative LCMS to afford 1-[2-(3,5-dimethyl-phenyl)-acetyl]-2-methyl-azetidine-2-carboxylic acid (4-chloro-benzyl)-methylcarbamoyl-methyl-amide **24**. ^{1}H NMR δ (ppm) (MeOD-d_4): 7.50−7.21 (4H, m), 7.01−6.85 (3H, m), 4.84−4.74 (1H, m), 4.68−4.44 (1H, m), 4.24−4.00 (2H, m), 3.95−3.71 (2H, m), 3.65−3.50 (1H, m), 3.42−3.34 (3H, m), 2.85−2.74 (3H, m), 2.32 (6H, s), 2.01−1.86 (3H, m). MW (calcd): 455.2. MW (obsd): 456.4 (M + 1).

*General Procedure B8 for the Synthesis of Compounds **84** and **86**. Step 1*: Following general procedure B1 or B2 and using corresponding carboxylic acids **13a−d** and appropriate secondary amines gave the related *N*-protected heterocycles.

Step 2: BOC removal. Following general procedure A1 (step 1) gave the corresponding esters.

Step 3: Saponification. Following general procedure B4 (step 2) gave the corresponding carboxylic acids.

*4-[[1-(2-Benzofuran-3-yl-acetyl)-2-methyl-azetidine-2-carbonyl]-(1H-indol-6-ylmethyl)-amino]-butyric Acid (**84**).* Following general procedure B8 and using intermediate **13c** and 6-[(3-ethoxycarbonyl-propylamino)-methyl]-indole-1-carboxylic acid *tert*-butyl ester in step 1 gave **82**. ^{1}H NMR δ (ppm) (MeOD-d_4): 7.64−7.55 (1H, m), 7.40−7.28 (2H, m), 7.03−6.87 (4H, m), 6.48 (1H, bs), 4.81−4.58 (2H, m), 4.12 (1H, bs), 3.91−3.85 (1H, m), 3.81−3.77 (1H, m), 3.64−3.41 (1H, m), 3.34−3.15 (2H, m), 2.78−2.50 (1H, m), 2.43−2.23 (8H, m), 2.01−1.92 (5H, m), 1.35 (1H, s). MW (calcd): 476. MW (obsd): 477 (M + 1).

*4-[[(R)-1-(Benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric Acid (**99**). Step 1*: Chiral separation of **11** was performed by preparative chiral chromatography using the following conditions: column, Chiralpak AD-H, (20 mm × 250 mm), 5 μm; mobile phase, hexane/ethanol/formic acid (95:5:0.05);

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flow rate of 9.5 mL/min, at 20 °C, thus affording **11a** (*S* isomer, rt: 6.42 mn) and **11b** (*R* isomer, rt: 9.00 mn) in their enantiopure forms (ee = 99%).

Step 2: to a solution of (*R*)-2-methyl-azetidine-1,2-dicarboxylic acid 1-*tert*-butyl ester **11b** (13 g, 1 equiv) in DCM (300 mL) under nitrogen was added dropwise a solution of 1-chloro-*N*,*N*-2-trimethylpropenylamine (16 mL, 2 equiv) in DCM (50 mL). The solution was stirred at 20 °C for 30 min, then cooled to −5 °C. A solution of 4-(3-chlorobenzylamino)-butyric acid methyl ester hydrochloride (18.5 g, 1.1 equiv) and TEA (25 mL, 3 equiv) in DCM (200 mL) was added slowly to the previous mixture. The reaction was stirred at 20 °C for 2.5 h. The mixture was added to 500 mL of a saturated aqueous solution of $NaHCO_3$. The crude was extracted three times with DCM. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered, concentrated under reduced pressure. The crude was purified by chromatography on silica gel (heptane/EtOAc: 90/10 to 50/50) to afford (*R*)-2-[(3-chloro-benzyl)-(3-methoxycarbonyl-propyl)-carbamoyl]-2-methyl-azetidine-1-carboxylic acid *tert*-butyl ester (27 g, quantitative yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.35−7.04 (4H, m), 5.13−4.29 (2H, m), 3.96−3.74 (1H, m), 3.68 (3H, s), 3.13−2.90 (4H, m), 2.65−2.40 (1H, m), 2.39−2.15 (2H, m), 2.00−1.65 (5H, m), 1.58−1.30 (9H, m). MW (calcd): 439.0. MW (obsd): 439.4 (M + l).

Step 3: To a solution of (*R*)-2-[(3-chloro-benzyl)-(3-methoxycarbonyl-propyl)-carbamoyl]-2-methyl-azetidine-1-carboxylic acid *tert*-butyl ester, (17.6 g, 1 equiv) in dioxane (200 mL) under nitrogen was added a solution of HCl (4N) in dioxane (50 mL, 5 equiv). The reaction was stirred for 16 h at 20 °C, then concentrated under reduced pressure. The crude was solubilized in DCM, poured into Et_2O, and the resulting solid was filtered, washed with Et_2O and pentane, and dried to afford 4-[(3-chloro-benzyl)-((*R*)-2-methyl-azetidine-2-carbonyl)-amino]-butyric acid methyl ester hydrochloride (12.5 g, 81% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 8.82 (1H, br s), 7.41−7.26 (2H, m), 7.25−7.11 (2H, m), 4.83−4.53 (1H, m), 4.47−4.31 (1H, m), 4.31−3.95 (2H, m), 3.72−3.64 (3H, m), 3.51−3.26 (1H, m), 3.17−3.07 (1H, m), 3.02−2.86 (1H, m), 2.78−2.52 (1H, m), 2.40−2.30 (2H, m), 2.16−2.01 (3H, m), 1.97−1.83 (2H, m). MW (calcd): 338.8. MW (obsd): 339.3 (M + 1).

Step 4: To a suspension of benzo[*b*]thiophene-3-carboxylic acid (7.12 g, 1.2 equiv) in DCM (210 mL) and THF (210 mL) were added HOBt (5.4 g, 1.2 equiv), EDC·HCl (9.6 g, 1.5 equiv), and TEA (18.5 mL, 4 equiv). The reaction was stirred at 20 °C for 15 h, then a solution of 4-[(3-chloro-benzyl)-((*R*)-2-methyl-azetidine-2-carbonyl)-amino]-butyric acid methyl ester hydrochloride (12.5 g, 1 equiv) in DCM (105 mL) and THF (105 mL) was added. The reaction was stirred at 20 °C for 4.5 h. The crude was diluted with DCM. A saturated aqueous solution of $NaHCO_3$ was added. The aqueous layer was extracted three times with DCM. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (elution with heptane/EtOAc: 90/10 to 50/50) to afford 4-[[(*R*)-1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid methyl ester (14.53 g, 87% yield). ^{1}H NMR δ(ppm) ($CDCl_3$): 8.37−8.07 (1H, m), 7.92−7.77 (1H, m), 7.51−7.36 (2H, m), 7.34−6.67 (5H, m), 5.49−4.72 (1H, m), 4.57−4.33 (1H, m), 4.05−3.76 (2H, m), 3.69 (3H, s), 3.29−2.66 (2H, m), 2.50−2.19 (3H, m), 2.15−1.83 (5H, m), 1.74−1.57 (1H, m). MW (calcd): 499.0. MW (obsd): 499.3 (M + 1).

Step 5: To a solution of 4-[[(*R*)-1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid methyl ester, (25 g, 1 equiv) in 750 mL of MeOH was added aqueous NaOH (2N) (75 mL, 3 equiv). The reaction was stirred at 20 °C for 15 h. The solvent was removed under reduced pressure, and the crude was partitioned between water and EtOAc. The organic layer was discarded. The aqueous layer was acidified by addition of HCl (2N) until pH = 2 and thoroughly extracted three times with EtOAc. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford 4-[[(*R*)-1-(benzo[*b*]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino]-butyric acid (23.7 g, 97% yield); $[\alpha]^{20}_D$ = +176 (*c* = 1.01, $CHCl_3$); ee >99.0%. ^{1}H NMR δ (ppm) (DMSO-d_6) at 80 °C: 8.14−8.08 (1H, m), 7.99−7.93 (1H, m), 7.90 (1H, s), 7.44−7.31 (3H, m), 7.30−7.25 (2H, m), 7.22−7.16 (1H, m), 4.73−4.54 (2H, m), 4.11−3.98 (2H, m), 3.44−3.34 (1H, m), 3.26−3.15 (1H, m), 2.58−2.51 (1H, m), 2.41−2.32 (1H, m), 2.18 (2H, t), 1.85−1.76 (5H, m). MW (calcd): 485.0. MW (obsd): 485.4 (M + 1). ^{13}C NMR δ (ppm) (DMSO-d_6) at 80 °C: 173.1 (C), 171.4 (C), 164.2 (C), 140,0 (C), 138.7 (C), 137.1 (C), 132.9 (C), 130.0 (C), 129.7 (CH), 128.4 (CH), 126.5 (CH), 126.5 (CH), 125.1 (CH), 124.1 (CH), 123.6 (CH), 121.9 (CH), 70.6 (C), 48.4 (CH_2), 46.5 (CH_2), 45.9 (CH_2), 30.6 (CH_2), 28.9 (CH_2), 23.3 (CH_3), 22.4 (CH_2). Anal. Calcd for $C_{25}H_{25}ClN_2O_4S$: C, 61.91; H, 5.20; N, 5.78. Found: C, 61.82; H, 5.03; N, 5.72.

*(R)-1-Benzyl-2-methyl-azetidine-2-carboxylic Acid Methyl Ester (**41**). Step 1*: To a solution of **11b** (125 mg, 0.58 mmol) in MeOH (5 mL) at 0 °C under nitrogen was added dropwise thionyl chloride (100 μL, 2.5 equiv), and the reaction was stirred at 20 °C for 15 h. The solvents were concentrated under reduced pressure to afford quantitatively (*R*)-2-methyl-azetidine-2-carboxylic acid methyl ester hydrochloride that was used as such without further purification.

Step 2: To a solution of (*R*)-2-methyl-azetidine-2-carboxylic acid methyl ester (50 mg, 1 equiv) in THF (2 mL) were added TEA (84 μL, 2 equiv) and benzyl bromide (36 μL, 1 equiv). The reaction was heated at 50 °C for 15 h. The solvent was concentrated under reduced pressure, and the crude was purified by chromatography on silica gel (heptane/EtOAc 100/0 to 50/50) to afford (*R*)-1-benzyl-2-methyl-azetidine-2-carboxylic acid methyl ester **41** (31 mg, 47% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.42−7.21 (5H, m), 3.86−3.74 (4H, m), 3.66−3.54 (1H, m), 3.34−3.07 (2H, m), 2.68−2.55 (1H, m), 2.02−1.91 (1H, m), 1.53 (3H, s). $[\alpha]^{20}_D$= +45 (*c* = 1.55, $CHCl_3$). Reported value for (*R*) enantiomer: $[\alpha]^{20}_D$ = +36° (*c* = 0.5, $CHCl_3$, 91% ee).[16]

*(R)-1-(4-Bromo-benzoyl)-2-methyl-azetidine-2-carboxylic Acid (**42**). Step 1*: To a solution of **11b** (550 mg, 1 equiv) in dioxane (30 mL) was added a solution of HCl (4N) in dioxane (5 mL), and the reaction was stirred at 20 °C for 15 h. The solvent was removed under reduced pressure, and the crude was crystallized in a mixture of DCM/iPr_2O/pentane to afford quantitatively (*R*)-2-methyl-azetidine-2-carboxylic acid hydrochloride that was used as such without further purification.

Step 2: To a solution of (*R*)-2-methyl-azetidine-2-carboxylic acid methyl ester (1 equiv) in water were added aqueous NaOH (1M) (7.5 mL, 3 equiv) then 4-bromo-benzoyl-chloride (550 μL, 1 equiv) dropwise. The reaction was stirred for 4 h at 20 °C. The reaction was neutralized with acetic acid. The solvent was partially removed under reduced pressure, and the crude was extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (elution with DCM/MeOH/AcOEt/AcOH/iPr_2O: 95/5/98/2/300). The resulting white solid was then recrystallized in isopropyl ether to afford (*R*)-1-(4-bromo-benzoyl)-2-methyl-azetidine-2-carboxylic acid **42** (355 mg, 48% yield). ^{1}H NMR δ (ppm) ($CDCl_3$): 7.65 (2H, d), 7.59 (2H, d), 4.48−4.39 (1H, m), 4.28−4.19 (1H, m), 3.07−2.97 (1H, m), 2.34−2.24 (1H, m), 1.95 (3H, s).

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, analytical data of compounds not described in the experimental part, detailed results of Cerep ExpresSprofile as well as crystallographical data of **42**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

GPR43, G-protein coupled receptor 43; CD11b, cluster of differentiation molecule 11B; AE, activation-specific epitope; FA, fatty acid; SCFA, short-chain fatty acid; PBMC, peripheral blood mononuclear cell; DSS, dextran sodium sulfate; TNBS, trinitrobenzoic sulfonic acid; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; HOBt, hydroxybenzotriazole; KHMDS, potassium bis(trimethylsilyl)amide; ICAM-1, intercellular adhesion molecule 1; MAC-1, macrophage 1 antigen; TEA, triethylamine

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