Bioorganic & Medicinal Chemistry xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

3-Carboxamido-5-aryl-isoxazoles as new CB_2 agonists for the treatment of colitis

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ARTICLE INFO

Article history:
Received 26 March 2013
Revised 28 May 2013
Accepted 6 June 2013
Available online xxxx

Keywords:
CB_2
Cannabinoid
Isoxazoles
IBD

ABSTRACT

Recent investigations showed that anandamide, the main endogenous ligand of CB_1 and CB_2 cannabinoid receptors, possesses analgesic, antidepressant and anti-inflammatory effects. In the perspective to treat inflammatory bowel disease (IBD), our approach was to develop new selective CB_2 receptor agonists without psychotropic side effects associated to CB_1 receptors. In this purpose, a new series of 3-carboxamido-5-aryl-isoxazoles, never described previously as CB_2 receptor agonists, was designed, synthesized and evaluated for their biological activity. The pharmacological results have identified great selective CB_2 agonists with in vivo anti-inflammatory activity in a DSS-induced acute colitis mouse model.

1. Introduction

The endocannabinoid system is an ubiquitous lipid signaling system with important regulatory functions. This system includes endogenous ligands, named endocannabinoids (ECs), two G-protein-coupled receptors (CB_1 and CB_2) that bind these ECs and several enzymes responsible for the synthesis and the degradation of these ECs.[1–3]

ECs, whose main representatives are anandamide (AEA) and 2-arichidonoylglycerol (2-AG), are polyunsaturated fatty acid ester and amide derivatives. AEA and 2-AG, synthesized by *N*-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively, are known to modulate several physiological responses, including depression, pain sensation and inflammation.[4] Immediately released from cells after their biosynthesis, ECs act on their receptors only locally.[4] However, AEA and 2-AG are rapidly eliminated by cellular uptake through a facilitated transport mechanism and principally hydrolyzed by the fatty acid amid hydrolase (FAAH) and the monoacylglycerol lipase (MAGL), respectively.[5–8]

ECs bind to both cannabinoid receptors CB_1 and CB_2. Cloning and localization of these G-protein-coupled receptors were major steps in the understanding of their physiological properties. The CB_1 receptor is mostly located in the brain and vessels.[9] On the contrary, the CB_2 receptor is mainly expressed in immune cells,[10] and has demonstrated immunomodulatory activities.[11]

It has been established that the endocannabinoid system plays an autoprotecting role in numerous diseases like pain,[12] chronic inflammation disorders[13] or cancer.[14] Thereby, modulation of this system through the activation of cannabinoid receptors consists in a promising therapeutic strategy.

Following this interesting approach, we decided to design molecules which target this endocannabinoid system and especially cannabinoid receptors. Because some CB_1 ligands cause psychotropic side effects due to the main localization of CB_1 receptors in the brain,[15] there is a fundamental need to develop selective CB_2 agonists without psychoactivity due to CB_1.

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http://dx.doi.org/10.1016/j.bmc.2013.06.010

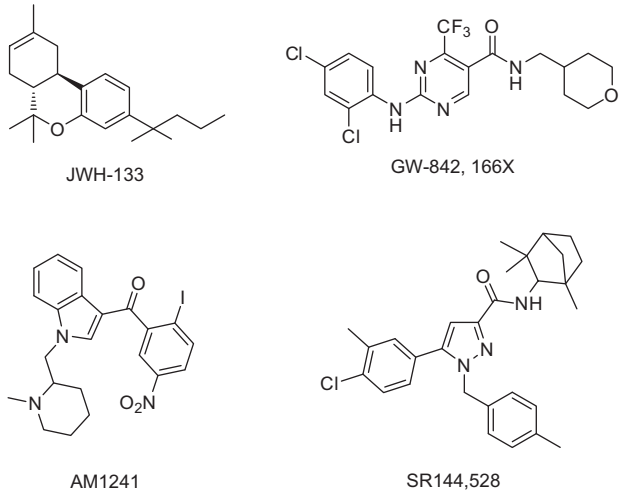


Figure 1. Structures of representatives CB_2 selective ligands.

Since the last decades, several selective CB_2 agonists have been described: aminoalkylindoles,[16] quinolones,[17] oxadiazoles[18] or diazepanes,[19] for example. Some of these selective CB_2 agonists have shown very interesting pharmacological properties like anti-inflammatory (JWH-133, GW-842,166X),[20] analgesic (AM1241)[21] or anticancer (JWH-133)[22] activities (Fig. 1). In our laboratory, we focused our research work on the therapeutic niche of inflammatory bowel disease (IBD).

Crohn's disease and ulcerative colitis are the main forms of IBD and cause, respectively, partial or complete inflammation of the gastrointestinal tract induced by an excessive immune response. Indeed, neutrophiles infiltration in the intestinal mucosal lesions with cell necrosis and ulceration of the epithelium were observed in patients suffering from IBD.[23]

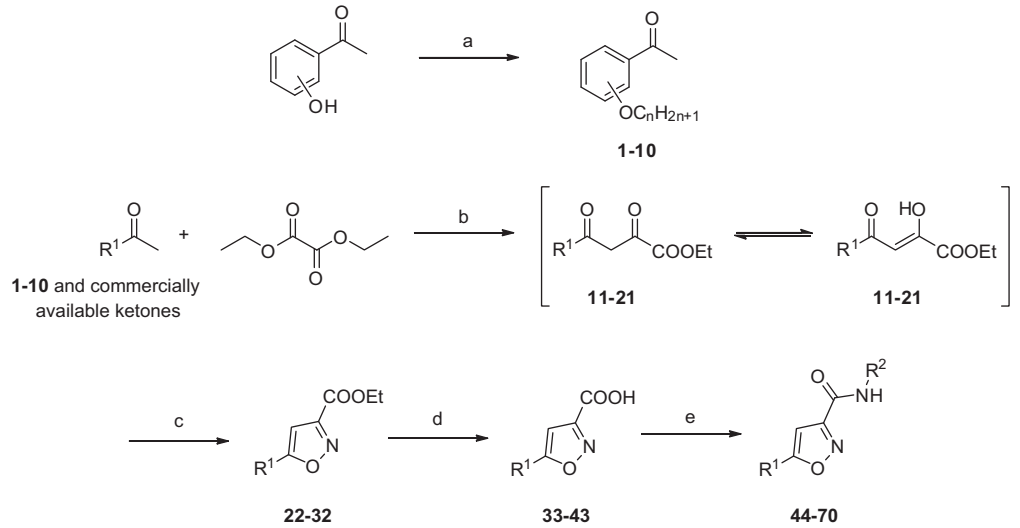
The endocannabinoid system has been identified in the gastrointestinal tract and plays an important role in intestinal motility and inflammation.[24,25] More specifically, an overexpression of cannabinoid receptors has been observed in patients suffering from IBD and in animal models of these diseases.[24,26] Several in vivo and in vitro studies have highlighted the beneficial role of CB_2 in intestinal inflammation.[20,27–29] Furthermore, Di Sabatino et al. showed that the AEA level was reduced in IBD inflamed mucosa of patients, as a consequence of both, its defective synthesis and its increased degradation.[30]

Based on the structure of SR144,528,[31] a specific CB_2 ligand (Fig. 1), we decided to design and synthesize new selective CB_2 agonists around the 3-carboxamido-5-aryl-isoxazole scaffold for the treatment of IBD. The aim of this work was to develop bioisosters of SR144,528 targeting the CB_2 receptor. For this purpose, we introduced pharmacophoric features, which have been previously identified in our group as crucial for CB_2 affinity and selectivity, that is, an alkyl chain and a bulky aliphatic group.[17,32] Following this strategy, a series of 27 new molecules was synthesized and tested for their biological activity.

2. Results and discussion

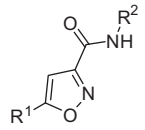
2.1. Chemistry

The targeted 3-carboxamido-5-aryl-isoxazoles **44–70** were obtained in four steps from diverse ketones, as described in Scheme 1. The non-commercial *O*-alkyl aromatic ketones were synthesized by an alkylation of the phenol function of the corresponding hydroxyacetophenone (compounds **1–10**, 59–95%). The ethyl 2,4-dioxobutanoates **11–21** were obtained by a Claisen condensation of the corresponding ketones with diethyl oxalate in presence of sodium ethoxide, as described by Marvel and Dreger,[33] in variable yields (23–93%). It was demonstrated that these ethyl 2,4-dioxobutanoates **11–21** are in their enol form due to the formation of an internal hydrogen bond.[34] The obtained intermediates **11–21** were cyclized into ethyl isoxazole-3-carboxylates (compounds **22–32**) by addition of hydroxylamine hydrochloride in ethanol at reflux (27–82%).[35] The desired compounds **44–70** were finally obtained in moderate to good yields (13–77%), by saponification of the ethyl ester function of compounds **22–32** with sodium hydroxide in ethanol at room temperature followed by amidation under peptide coupling conditions (HOBt/HBTU/DIEA in chloroform at room temperature). Structures of original compounds **44–70** are summarized in Table 1.



Scheme 1. Synthesis of 3-carboxamido-5-aryl-isoxazoles **44–70**. Reagents and conditions: (a) alkyl bromide, K_2CO_3, DMF, reflux, 16 h; (b) sodium ethoxide, EtOH, reflux, 2 h; (c) hydroxylamine hydrochloride, EtOH, reflux, 2 h; (d) NaOH, EtOH, rt, 24 h; (e) R-NH_2, HOBt, HBTU, DIEA, $CHCl_3$, rt, 24 h.

Table 1
Structures of the new synthesized compounds



Compounds	R^1	R^2
44	Phenyl	1-Adamantyl
45	Phenyl	2-Adamantyl
46	Phenyl	1-(Adamantyl)methyl
47	2-Methoxyphenyl	1-Adamantyl
48	2-Ethoxyphenyl	1-Adamantyl
49	2-Propoxyphenyl	1-Adamantyl
50	2-Butoxyphenyl	1-Adamantyl
51	2-Pentoxyphenyl	1-Adamantyl
52	2-Hexoxyphenyl	1-Adamantyl
53	2-Heptoxyphenyl	1-Adamantyl
54	2-Octoxyphenyl	1-Adamantyl
55	3-Pentoxyphenyl	1-Adamantyl
56	4-Pentoxyphenyl	1-Adamantyl
57	2-Pentoxyphenyl	2-Adamantyl
58	2-Pentoxyphenyl	3-Noradamantyl
59	2-Pentoxyphenyl	Cyclopropyl
60	2-Pentoxyphenyl	Cyclobutyl
61	2-Pentoxyphenyl	Cyclopentyl
62	2-Pentoxyphenyl	Cyclohexyl
63	2-Pentoxyphenyl	1-Piperidinyl
64	2-Pentoxyphenyl	Cycloheptyl
65	2-Pentoxyphenyl	Cyclooctyl
66	2-Pentoxyphenyl	Phenyl
67	2-Pentoxyphenyl	Benzyl
68	2-Pentoxyphenyl	2-(5-Methyl)-1,3,4-thiadiazolyl
69	2-Pentoxyphenyl	1-Naphtyl
70	2-Pentoxyphenyl	Diisobutyl

2.2. In vitro CB_1 and CB_2 receptor affinity and structure–activity relationships

The affinities for the human CB_1 and CB_2 receptors of the new synthesized compounds **44**–**70** were determined by a competitive radioligand displacement assay using [^{3}H]-SR141716A and [^{3}H]-CP55,940 as radioligands, respectively.[36] Membrane from Chinese Hamster Ovary (CHO) cells expressing respectively hCB$_1$ or hCB$_2$ were used in these experiments. All compounds were first screened at a concentration of 10 μM for their affinity toward the cannabinoid receptors. Inhibition constant (K_i) values were determined for compounds exhibiting a specific displacement superior to 60% for hCB$_2$ and hCB$_1$. Selectivity indices (hCB$_2$ vs hCB$_1$) were calculated whenever possible.

As shown in Table 2, 15 compounds possess a nanomolar affinity for the hCB$_2$ receptor. Among these 15 CB$_2$ ligands, compound **58** showed the best affinity for the hCB$_2$ receptor with a K_i value of 9.0 nM.

First, we noted that compounds **44**–**46** with a non-substituted phenyl group at the C-5 position (R^1) showed no affinity for hCB$_2$. On the other hand, when previously described CB$_2$ affinity and selectivity crucial pharmacophoric features, that is, an *n*-pentyl chain on the phenyl group and a 1-adamantyl group on the carboxamide function[17,32,37] were introduced, compound **51** showed a good affinity for hCB$_2$ with a K_i value of 36.0 nM.

Thus, we decided to study the best position of the *O*-pentyl chain on the phenyl group by synthesizing position's isomers of compound **51**. Interestingly, neither compound **55** with an *O*-pentyl at the meta position nor compound **56** with an *O*-pentyl at the para position revealed any affinity for the hCB$_2$ receptor, contrary to compound **51** with an *O*-pentyl at the ortho position (K_i = 36.0 - nM). These results indicate that the best position for the *O*-alkyl chain on the phenyl group is the ortho position.

Table 2
Affinities (K_i values) of compounds **47**–**70** and reference compounds (WIN-55,212-2, CP55,940) towards hCB$_2$ and hCB$_1$ cannabinoid receptors,[a] selectivity ratios hCB$_2$ versus hCB$_1$, and cytotoxicity on HT29 cells[b]

Compounds	Binding affinity K_i (nM)		Selectivity ratio hCB$_2$ versus hCB$_1$	Cytotoxicity (HT29) at 10 μM
	hCB$_2$	hCB$_1$		
44	>1000			
45	>1000			
46	>1000			
47	70.1 ± 5.4	>3000	>42	ND
48	>1000			
49	79.6 ± 5.8	>1000	>12	8%
50	369 ± 62	>3000	>8	2%
51	36.0 ± 3.4	>3000	>83	0%
52	30.5 ± 6.4	>3000	>98	17%
53	>1000			
54	>1000			
55	>1000			
56	>1000			
57	60.1 ± 6.2	>3000	>49	0%
58	9.0 ± 0.6	>1000	>111	20%
59	>1000			
60	412 ± 71	>1000	>2	46%
61	60.9 ± 0.9	>1000	>16	13%
62	22.8 ± 4.6	>3000	>131	37%
63	600 ± 76			
64	60.8 ± 6.9	>3000	>49	34%
65	73.5 ± 6.8	>3000	>40	17%
66	>1000			
67	>3000			
68	>3000			
69	>1000			
70	22.1 ± 3.9	33.0 ± 5.3		
WIN-55,212-2	9.1 ± 0.8	16.1 ± 6.0		
CP-55,940	15.4 ± 1.4	1.3 ± 0.4[38]		

[a] The K_i values were obtained from nonlinear analysis of competition curves using [^{3}H]-SR141716A and [^{3}H]-CP-55,940 as radioligands for hCB$_1$ and hCB$_2$ cannabinoid receptors, respectively, and are expressed as mean ± SEM of at least four experiments performed in duplicate.

[b] The cytotoxicity values are expressed as the percentage of cellular proliferation inhibition of at least four experiments performed in duplicate.

Then, we decided to modulate the *O*-alkyl chain length on the phenyl group. Compounds **49**–**52** with a medium *O*-alkyl chain (between 3 to 6 carbons) at the ortho position showed good to moderate affinity for hCB$_2$ (K_i = 30.5 to 369 nM). However, no hCB$_2$ affinity was observed for compounds with a shorter (less than 3 carbons, compound **48**) or longer (more than 6 carbons, compounds **53**–**54**) *O*-alkyl chain, except for compound **47** with an *O*-methyl chain (K_i = 70.1 nM). Thus among the compounds bearing an ortho-substituted phenyl group, compounds **51** and **52** showed the best affinity for hCB$_2$ (**51**: K_i = 36.0 ± 3.4 nM; **52**: K_i = 30.5 ± 6.4 nM).

Pharmacomodulations of the substituent on the carboxamide function (R^2) was also carried out. Compounds substituted by an aromatic moiety (**66**–**69**) demonstrated no affinity for hCB$_2$, contrary to compounds **57**–**65** with an aliphatic substituent (except for the cyclopropyl group, compound **59**) on the carboxamide function which showed affinities ranging from 9.0 to 600 nM. To maintain the hCB$_2$ affinity, a bulky aliphatic substituent on the carboxamide function (R^2) seems to be essential. Indeed, neither compounds bearing an aromatic, nor small aliphatic group (cyclopropyl) showed any hCB$_2$ affinity. The best substituents are cyclohexyl (**62**), *N*-*N*-diisobutyl (**70**) and adamantyl (**51**, **57**–**58**) groups.

In order to determine the selectivity ratio of our CB$_2$ ligands, 13 of these molecules (K_i < 500 nM) were tested for their CB1 affinity. Amongst these CB2 ligands, only compound **70** presented a CB1 affinity with a 90% displacement at 10 μM.

2.3. CB_2 receptor functional activity

The functional activity of our best compounds (**49**–**52**, **57**–**58**, **60**–**62**, **64** and **65**) was determined using a guanosine-5′-*O*-(3-[^{35}S]-GTPγS) binding assay and *h*CB_2-CHO cells membranes, as previously described.[32] This assay consists in a functional measurement of the interaction between the receptor and the G-protein, which constitutes the first step of the G-protein coupled receptor activation. In this assay, antagonists do not affect [^{35}S]-GTPγS interaction whereas agonists and inverse agonists increase or decrease the binding, respectively. The functional activity of the reference cannabinoid agonist, WIN-55,212-2 (CB_1 and CB_2 agonist), was also determined. Maximum efficacy (E_{max}) and half-maximal effective concentration (EC_{50}) values of the new synthesized compounds and references are gathered in Table 3. This assay showed that our 12 selective CB_2 ligands are all agonists of the CB_2 receptor.

Starting from the SR144,528 structure, the replacement of the pyrazole by an isoxazole, inducing the suppression of the benzyl group resulted in the switch of the functional activity from inverse agonist to agonist. Moreover, we demonstrated once more that the introduction of an alkyl chain and a bulky aliphatic group on the central heterocycle are crucial pharmacophoric features for the design of selective CB_2 agonists.[17,32]

Table 3
Half-maximal effective (EC_{50}) and maximum efficacy (E_{max}) of selected compounds and reference *h*CB_2 ligands[a]

Compounds	[^{35}S]-GTPγS(*h*CB_2)	
	EC_{50} (nM)	E_{max}[b] (%)
47	2.56 ± 0.3	155 ± 11
49	2.21 ± 0.3	144 ± 8
50	2.88 ± 0.3	147 ± 10
51	2.9 ± 0.3	151 ± 12
52	3.4 ± 0.3	157 ± 10
57	14.8 ± 3.8	270 ± 16
58	4.8 ± 0.3	234 ± 13
60	4.4 ± 0.4	184 ± 15
61	2.1 ± 0.3	160 ± 11
62	1.9 ± 0.3	161 ± 12
64	11.1 ± 3.4	233 ± 14
65	1.8 ± 0.3	167 ± 10
WIN-55,212-2	2.04 ± 0.3	243 ± 14

[a] The results are expressed as mean ± SEM of at least four experiments performed in duplicate.
[b] Basal constitutive activity of the receptor has been set at a value of 100%, E_{max} values above 100% indicated that the compound behaves as an agonist, E_{max} values under 100% indicated that the compound behaves as an inverse agonist, and E_{max} values equal to 0% indicated that the compound behaves as an antagonist.

2.4. Cell proliferation assay

Cytotoxicity of our 12 selective CB_2 agonists was determined (at 10 μM) using a cell proliferation assay on human colorectal adenocarcinoma cells HT29. This test is based on a colorimetric method, which measures the activity of cellular enzymes that reduce the tetrazolium dye (MTS, uncolored) to its insoluble formazan giving a purple color. This assay measures cellular metabolic activity via NADPH-dependent cellular oxidoreductase enzymes and reflects, under defined conditions, the number of viable cells. No cytotoxicity was observed for our new selective CB_2 agonists (Table 2).

2.5. In vivo study

Considering their good affinity for *h*CB_2, selectivity versus *h*CB_1 and agonist property, compounds **58** and **64** have been selected for the in vivo study. Specific Pathogen Free male 7 weeks old C57/Bl6 mice received 2.5% dextran sodium sulfate (DSS) in drinking water during 7 days. Concomitantly, they were dosed intraperitoneally with compounds **58** or **64** in hydroxypropyl β cyclodextrine (150 mM) at the dosage of 10 mg/kg body weight. Control mice were injected with vehicle only. Mice receiving vehicle developed progressive weight loss (Fig. 2A) and increased Disease Activity Index based on weight loss, stool consistency and rectal bleeding (Fig. 2B). There were no overt reactions following 7 days of daily IP treatment with the compounds **58** and **64**. At day 7, mice body weights relative to their day 0 weights were significantly higher in mice treated with **58** and **64** as compared to vehicle-treated mice (**58**: 92.8 ± 1.2% and **64**: 91.8 ± 2.2% vs vehicle: 83.1 ± 2.2%, $p \leqslant 0.001$ for both). Accordingly, **58** and **64** reduced significantly the Disease Activity Index, from 15.6 ± 0.5 for vehicle controls to 8.3 ± 0.9 for **58** ($p = 0.0004$) and 10.6 ± 1.0 for **64** ($p = 0.001$). Another disease indicator measured was colon length because DSS typically results in shortening of the colon. Mice treated with **58** and **64** presented a colon length significantly higher than vehicle mice (4.6 ± 0.2 cm for vehicle, 5.6 ± 0.2 cm for **58** ($p = 0.003$), 5.4 ± 0.1 cm for **64** ($p = 0.002$), Fig. 2C). Furthermore, histology sections of colon from mice treated with vehicle, **58** and **64** were scored for histological damage (Fig. 3). The extent of colon inflammation was reduced in mice treated with **58** (2.3 ± 0.7, p = 0.01, Fig. 3A and D) and **64** (4.0 ± 0.5, ns, Fig. 3A and C) relative to mice treated with vehicle (5.1 ± 0.4, Fig. 3A and B). Finally, colon levels of major inflammatory cytokines were quantified in mice by real-time PCR. Compounds **58** and **64** did not show any significant effect on TNF-α mRNA levels (Fig. 4A). Colon levels of IL-1β were significantly reduced in **58** and **64** treated mice (vehicle: 105.1 ± 17.0, **58**: 19.9 ± 8.7, $p = 0.0003$, **64**: 13.2 ± 4.2, $p < 0.0001$, Fig. 4B). Similarly, **58** and **64** treated mice have colon Kc mRNA levels significantly lower as compared with control mice (vehicle: 68.9 ± 9.8, **58**: 11.5 ± 2.2, $p < 0.0001$, **64**: 12.3 ± 2.8, $p < 0.0001$, Fig. 4C). Taken together, these different parameters demonstrate that both **58** and **64** treatments inhibit the development of DSS-induced acute colitis in mice.

3. Conclusion

We have synthesized the first series of selective CB_2 receptor ligands based on a 3-carboxamido-5-aryl-isoxazoles scaffold. 12 selective CB_2 receptor agonists without any cytotoxicity were identified. Amongst these 12 compounds, compounds **58** and **64** have shown in vivo anti-inflammatory activities in a colitis mouse model, with a significantly reduction of the Disease Activity Index, and the IL-1β and Kc mRNA colon levels. In conclusion, the 3-carboxamido-5-aryl-isoxazole scaffold was highlighted as a highly effective scaffold for the design of new CB_2 receptor agonists.

4. Experimental section

4.1. Chemistry

All commercial reagents and solvents were used without further purification. Analytical thin-layer chromatography was performed on pre-coated Polygram Sil G/UV254 plates (Macherey-Nagel®); the spots were located by UV (254 and 366 nm) and the compounds were extracted from the silica using cyclohexane/AcOEt (7:3, v/v). Silica gel 60 230–400 mesh purchased from Merck® was used for column chromatography. All melting points were determined with a Büchi 535® capillary apparatus and remain uncorrected. ^{1}H NMR spectra were obtained using a Brüker® 300 MHz spectrometer, chemical shifts (δ) are expressed in ppm relative to tetramethylsilane used as an internal standard, *J* values are in hertz, and the splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. All compounds were

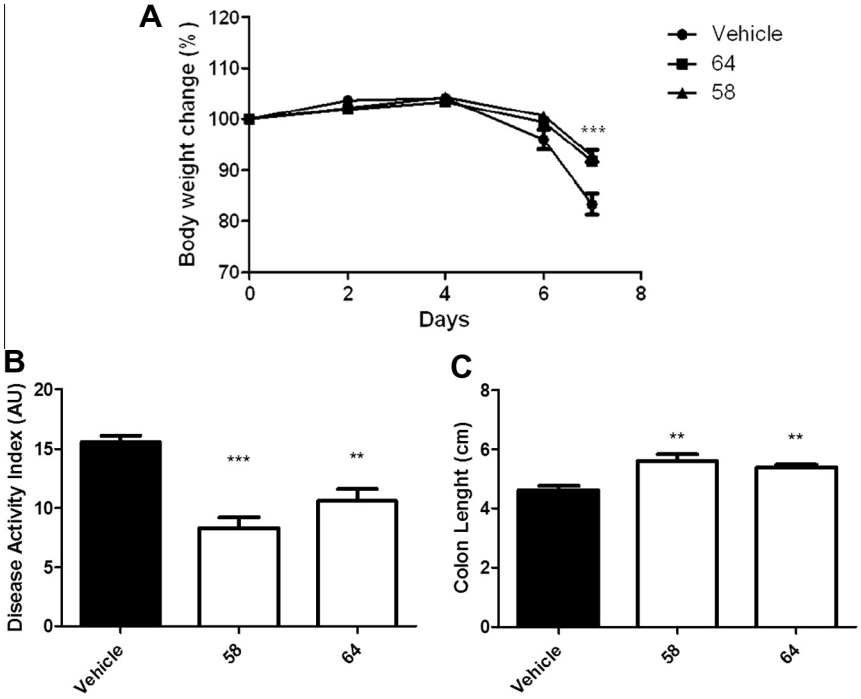


Figure 2. Effects of **58** and **64** daily treatment (10 mg/kg, IP) on body weight (A), Disease Activity Index on day 7 (B) and colon length (C) during DSS-induced acute colitis. Values are expressed as a mean ± SEM, $n = 10$. $^{**}p < 0.01$, $^{***}p < 0.001$.

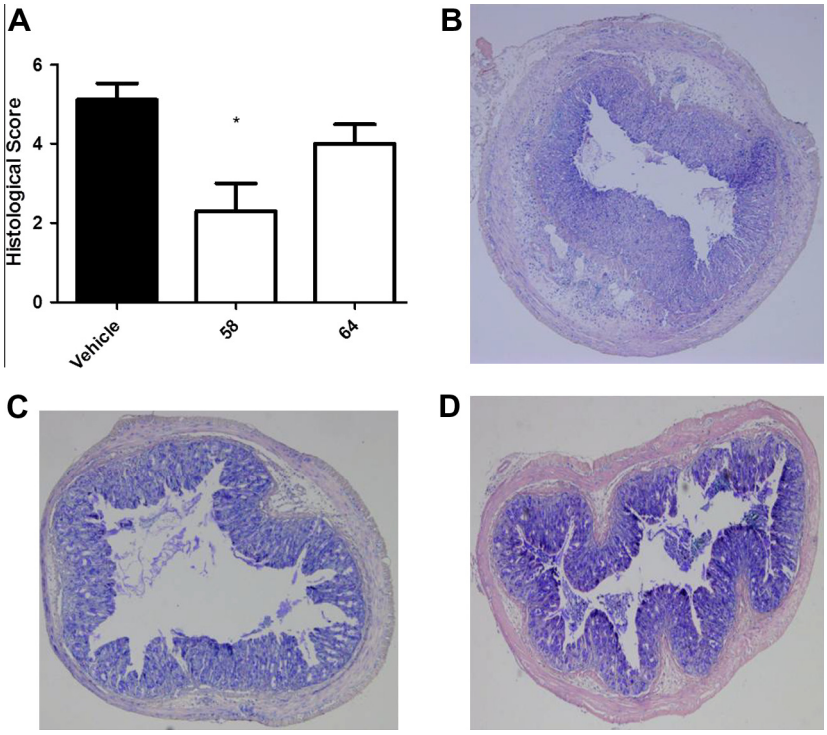


Figure 3. Histological scoring of colons from DSS-treated mice administrated with vehicle, **58** and **64** (A). Values are expressed as a mean ± SEM, $n = 10$. $^{*}p < 0.05$. Representative MGG stained sections of distal colon of vehicle (B), **64** (C) and **58** (D) treated mice (original magnification ×5).

analyzed by HPLC-MS on a HPLC combined with a Surveyor MSQ (Thermo Electron®) equipped with an APCI-source. The mass of **58** and **64** were obtained by HRMS with Q Exactive Benchtop LC–MS/MS (Thermo Scientific®). All tested compounds showed a purity superior at 96% in $APCI^+$ mode. Elemental analyses for target compounds were performed by the 'Service Central d'Analyses' at the CNRS, Vernaison (France) and the data were within ±0.4% of the theoretical values.

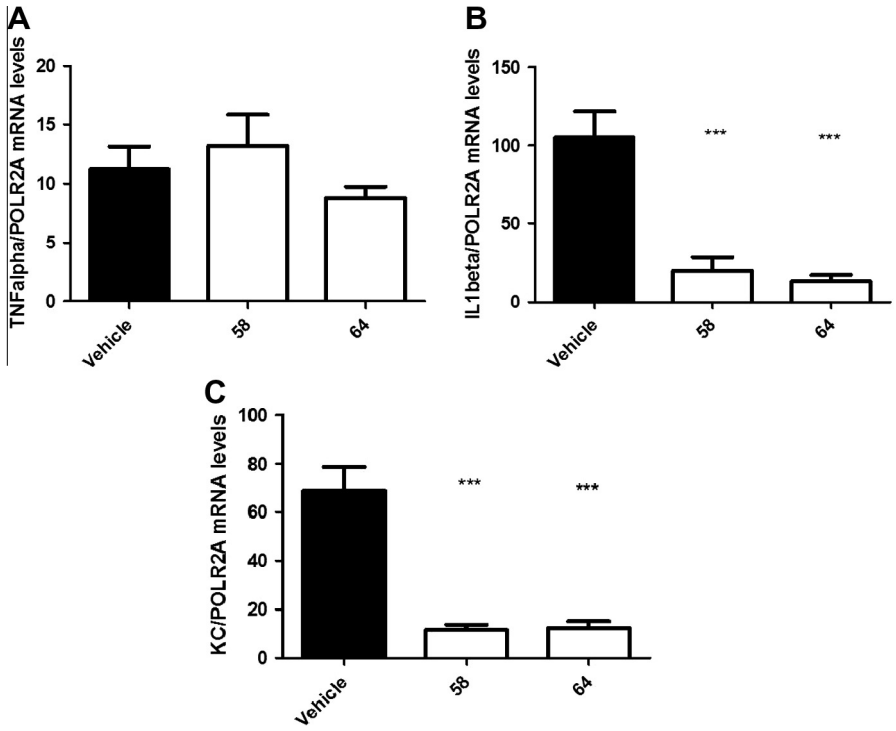


Figure 4. Quantification by real-time PCR of colon TNF-α (A), IL1-β (B) and Kc (C) mRNA levels in mice with DDS-induced colitis treated with vehicle, 803 and 809. Values are expressed as a mean ± SEM, $n = 10$. $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$.

4.1.1. General procedure for the preparation of 1-(2-alkyloxyphenyl)ethanones (1–10)

A solution of 2-hydroxyacetophenone, 3-hydroxyacetophenone or 4-hydroxyacetophenone (3.00 g, 22 mmol, 1 equiv), potassium carbonate (4.57 g, 33.1 mmol, 1.5 equiv) and the corresponding alkylbromide (24 mmol, 1.1 equiv) in 20 mL of DMF was stirred and refluxed for 16 h. After reaction, the DMF was evaporated and 30 mL of 2 N aqueous NaOH were added to the crude. Then, the solution was extracted with EtOAc (2 × 20 mL) and washed with water. The organic layers was dried over $MgSO_4$ and evaporated under reduce pressure. Crystallization in absolute EtOH gave the pure desired compounds, except for compounds **1**, **4** and **6**–**9** which were purified by TLC using the appropriate eluent (cyclohexane/EtOAc 7:3, v/v).

4.1.1.1. 1-(2-Methyloxyphenyl)ethanone (1). Yellow oil (93%). ^{1}H NMR ($CDCl_3$) δ 7.72 (d, J = 7.6 Hz, 1H), 7.45 (t, J = 7.8 Hz, 1H), 7.01–6.95 (m, 2H), 3.90 (s, 3H), 2.60 (s, 3H). LC–MS (APCI$^+$) m/z 151.2 (MH$^+$).

4.1.1.2. 1-(2-Ethyloxyphenyl)ethanone (2). Yellow solid (82%); mp 28 °C. ^{1}H NMR (DMSO) δ 7.57–7.47 (m, 2H), 7.15 (d, J = 8.2 Hz, 1H), 6.99 (t, J = 7.6 Hz, 1H), 4.14 (q, J = 7.0 Hz, 2H), 2.58 (s, 3H), 1.39 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 165.1 (MH$^+$).

4.1.1.3. 1-(2-Propyloxyphenyl)ethanone (3). White solid (84%); mp 28 °C. ^{1}H NMR (DMSO) δ 7.58–7.47 (m, 2H), 7.13 (d, J = 8.4 Hz, 1H), 6.99 (t, J = 7.6 Hz, 1H), 4.04 (t, J = 6.3 Hz, 2H), 2.57 (s, 3H), 1.78 (m, 2H), 1.01 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 179.2 (MH$^+$).

4.1.1.4. 1-(2-Butyloxyphenyl)ethanone (4). Brown oil (79%). ^{1}H NMR (DMSO) δ 7.57 (d, J = 6.4 Hz, 1H), 7.49 (t, J = 7.3 Hz, 1H), 7.11 (d, J = 8.5 Hz, 1H), 6.97 (t, J = 7.6 Hz, 1H), 4.06 (t, J = 6.4 Hz, 2H), 2.82 (s, 3H), 1.74 (m, 2H), 1.45 (m, 2H), 0.92 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 193.1 (MH$^+$).

4.1.1.5. 1-(2-Pentyloxyphenyl)ethanone (5). White solid (95%); mp 30 °C. ^{1}H NMR (DMSO) δ 7.57 (d, J = 7.3 Hz, 1H), 7.48 (t, J = 7.9 Hz, 1H), 7.10 (d, J = 8.2 Hz, 1H), 6.97 (t, J = 7.3 Hz, 1H), 4.04 (t, J = 6.1 Hz, 2H), 2.55 (s, 3H), 1.75 (m, 2H), 1.44–1.23 (m, 4H), 0.87 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 207.1 (MH$^+$).

4.1.1.6. 1-(2-Hexyloxyphenyl)ethanone (6). Yellow oil (59%). ^{1}H NMR (DMSO) δ 7.52 (d, J = 7.6 Hz, 1H), 7.48 (t, J = 8.5 Hz, 1H), 7.09 (d, J = 8.2 Hz, 1H), 6.96 (t, J = 7.6 Hz, 1H), 4.03 (t, J = 6.4 Hz, 2H), 2.55 (s, 3H), 1.74 (m, 2H), 1.43–1.22 (m, 6H), 0.84 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 221.2 (MH$^+$).

4.1.1.7. 1-(2-Heptyloxyphenyl)ethanone (7). Brown oil (94%). ^{1}H NMR ($CDCl_3$) δ 7.73 (d, J = 7.6 Hz, 1H), 7.43 (t, J = 7.8 Hz, 1H), 6.99–6.92 (m, 2H), 4.05 (t, J = 6.4 Hz, 2H), 2.63 (s, 3H), 1.85 (m, 2H), 1.53–1.42 (m, 2H), 1.41–1.25 (m, 6H), 0.89 (m, 3H). LC–MS (APCI$^+$) m/z 235.3 (MH$^+$).

4.1.1.8. 1-(2-Octyloxyphenyl)ethanone (8). Brown oil (69%). ^{1}H NMR (DMSO) δ 7.57 (d, J = 7.8 Hz, 1H), 7.49 (t, J = 7.8 Hz, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.97 (t, J = 7.4 Hz, 1H), 4.05 (t, J = 6.3 Hz, 2H), 2.52 (s, 3H), 1.75 (m, 2H), 1.47–1.37 (m, 2H), 1.33–1.21 (m, 8H), 0.84 (m, 3H). LC–MS (APCI$^+$) m/z 249.2 (MH$^+$).

4.1.1.9. 1-(3-Pentyloxyphenyl)ethanone (9). Brown oil (90%). ^{1}H NMR (DMSO) δ 7.57 (d, J = 7.6 Hz, 2H), 7.43–7.40 (m, 2H), 7.17 (d, J = 8.1 Hz, 1H), 3.99 (t, J = 6.4 Hz, 2H), 3.98 (s, 3H), 1.12 (m, 2H), 1.42–1.23 (m, 4H), 0.88 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 207.2 (MH$^+$).

4.1.1.10. 1-(4-Pentyloxyphenyl)ethanone (10). White solid (92%); mp 32 °C. ^{1}H NMR (DMSO) δ 7.93 (d, J = 8.7 Hz, 2H), 6.92 (d, J = 8.7 Hz, 2H), 4.02 (t, J = 7.1 Hz, 2H), 2.52 (s, 3H), 1.81 (m, 2H), 1.50–1.38 (m, 4H), 0.94 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 207.2 (MH$^+$).

4.1.2. General procedure for the preparation of ethyl 2-hydroxy-4-oxo-4-aryl-2-butenoates (11–21)

To a stirred solution of sodium ethanolate, freshly prepared by reacting Na (66 mmol, 2 equiv) with 50 mL absolute EtOH, the corresponding aryl ketone (33 mmol, 1 equiv) and diethyl oxalate (66 mmol, 2 equiv) diluted in 30 mL of absolute EtOH were added dropwise at 50 °C. The mixture was refluxed for 2 h. The solvent was evaporated under reduce pressure and the residue was dissolved in 1 N aqueous HCl (20 mL) and stirred for additional 1 h. Then, the solution was extracted with EtOAc (2 × 20 mL) and washed with distilled water (20 mL). The organic layers were dried over $MgSO_4$ and evaporated under reduce pressure. Finally, the residue was triturated in cyclohexane to give compounds **11**–**21**.

4.1.2.1. Ethyl 2-hydroxy-4-oxo-4-phenyl-2-butenoate (11). Orange solid (75%); mp 45 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 8.02 (d, J = 7.7 Hz, 2H), 7.67 (t, J = 7.9 Hz, 1H), 7.54 (t, J = 7.8 Hz, 2H), 7.08 (s, 1H), 3.98 (q, J = 6.4 Hz, 2H), 1.12 (t, J = 6.4 Hz, 3H). LC–MS (APCI$^+$) m/z 221.1 (MH$^+$).

4.1.2.2. Ethyl 2-hydroxy-4-(2-methoxyphenyl)-4-oxo-2-butenoate (12). Orange solid (74%); mp 29 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.98 (d, J = 6.4 Hz, 1H), 7.63 (t, J = 7.3 Hz, 1H), 7.24–7.08 (m, 3H), 4.28 (q, J = 7.3 Hz, 2H), 3.92 (s, 3H), 1.29 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 251.0 (MH$^+$).

4.1.2.3. Ethyl 4-(2-ethoxyphenyl)-2-hydroxy-4-oxo-2-butenoate (13). Yellow solid (90%); mp 76 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.84 (d, J = 6.7 Hz, 1H), 7.61 (t, J = 7.3 Hz, 1H), 7.40 (s, 1H), 7.19 (d, J = 8,5 Hz, 1H), 7.09 (t, J = 7.3 Hz, 1H), 4.32–4.16 (m, 4H), 1.42 (t, J = 7.0 Hz, 3H), 1.29 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 265.0 (MH$^+$).

4.1.2.4. Ethyl 2-hydroxy-4-oxo-4-(2-propoxyphenyl)-2-butenoate (14). Orange solid (59%); mp 67 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.84 (d, J = 6.7 Hz, 1H), 7.61 (t, J = 7.0 Hz, 1H), 7.38 (s, 1H), 7.20 (d, J = 8.5 Hz, 1H), 7.09 (t, J = 7.6 Hz, 1H), 4.27 (q, J = 7.0 Hz, 2H), 4.10 (t, J = 6.1 Hz, 2H), 1.82 (m, 2H), 1.28 (t, J = 7.3 Hz, 3H), 1.05 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 279.2 (MH$^+$).

4.1.2.5. Ethyl 4-(2-butoxyphenyl)-2-hydroxy-4-oxo-2-butenoate (15). Brown solid (33%); mp 86 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.91 (d, J = 6.1 Hz, 1H), 7.52–7.46 (m, 2H), 7.03–6.96 (m, 2H), 4.36 (q, J = 7.3 Hz, 2H), 4.09 (t, J = 6.1 Hz, 2H), 1.88 (m, 2H), 1.57 (m, 2H), 1.37 (t, J = 8.2 Hz, 3H), 1.03 (t, J = 6.2 Hz, 3H). LC–MS (APCI$^+$)m/z 293.2 (MH$^+$).

4.1.2.6. Ethyl 2-hydroxy-4-oxo-4-(2-pentoxyphenyl)-2-butenoate (16). Brown solid (62%); mp 106 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.84 (d, J = 7.6 Hz, 1H), 7.81 (t, J = 7.0 Hz, 1H), 7.61 (s, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.23 (t, J = 7.6 Hz, 1H), 4.28 (q, J = 7.4 Hz, 2H), 4.13 (t, J = 6.4 Hz, 2H), 1.79 (m, 2H), 1.35 (m, 7H), 0.88 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 307.3 (MH$^+$).

4.1.2.7. Ethyl 4-(2-hexoxyphenyl)-2-hydroxy-4-oxo-2-butenoate (17). Brown solid (31%); mp 59 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.92 (d, J = 6.1 Hz, 1H), 7.52–7.47 (m, 2H), 7.06–6.96 (m, 2H), 4.37 (q, J = 7.4 Hz, 2H), 4.09 (t, J = 6.4 Hz, 2H), 1.90 (m, 2H), 1.44–1.34 (m, 9H), 0.90 (t, J = 6.7 Hz, 3H). LC–MS (APCI$^+$) m/z 321.3 (MH$^+$).

4.1.2.8. Ethyl 4-(2-heptoxyphenyl)-2-hydroxy-4-oxo-2-butenoate (18). Brown solid (90%); mp 40 °C. ^{1}H NMR (DMSO) δ 15.02 (s, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.60 (t, J = 7.7 Hz, 1H), 7.34 (s, 1H), 7.20 (d, J = 8.5 Hz, 1H), 7.08 (t, J = 7.5 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 4.12 (t, J = 6.2 Hz, 2H), 1.79 (m, 2H), 1.50–1.41 (m, 2H), 1.33–1.21 (m, 9H), 0.84 (m, 3H). LC–MS (APCI$^+$) m/z 335.2 (MH$^+$).

4.1.2.9. Ethyl 2-hydroxy-4-(2-octoxyphenyl)-4-oxo-2-butenoate (19). Brown solid (93%); mp 64 °C. ^{1}H NMR (DMSO) δ 7.82 (d, J = 7.8 Hz, 1H), 7.60 (t, J = 7.9 Hz, 1H), 7.34 (s, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.08 (t, J = 7.4 Hz, 1H), 4.28 (q, J = 7.0 Hz, 2H), 4.11 (t, J = 6.2 Hz, 2H), 1.79 (m, 2H), 1.50–1.41 (m, 2H), 1.31–1.22 (m, 11H), 0.83 (m, 3H). LC–MS (APCI$^+$) m/z 349.2 (MH$^+$).

4.1.2.10. Ethyl 2-hydroxy-4-oxo-4-(3-pentoxyphenyl)-2-butenoate (20). Yellow solid (31%); mp 46 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.63 (s, 1H), 7.46 (m, 2H), 7.25 (d, J = 7.9 Hz, 1H), 7.11 (s, 1H), 4.19 (q, J = 7.4 Hz, 2H), 4.04 (t, J = 6.1 Hz, 2H), 1.38 (m, 4H), 1.05 (m, 5H), 0.89 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 307.3 (MH$^+$).

4.1.2.11. Ethyl 2-hydroxy-4-oxo-4-(4-pentoxyphenyl)-2-butenoate (21). Brown solid (23%); mp 38 °C. ^{1}H NMR (DMSO) δ 10.68 (s, 1H), 7.84 (d, J = 7.1 Hz, 2H), 7.81 (d, J = 7.0 Hz, 2H), 7.61 (s, 1H), 4.28 (q, J = 7.3 Hz, 2H), 4.13 (t, J = 6.4 Hz, 2H), 1.45–1.35 (m, 7H), 1.12 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 307.3 (MH$^+$).

4.1.3. General procedure for the preparation of ethyl 5-arylisoxazole-3-carboxylates (22–32)

A solution of compound **11**–**21** (1 equiv) and hydroxylamine hydrochloride (1 equiv) in absolute EtOH (50 mL) was stirred and refluxed for 2 h. After reaction, the solvent was removed and the residue was purified by flash chromatography (cyclohexane/EtOAc 8:2, v/v) followed by crystallization in absolute EtOH.

4.1.3.1. Ethyl 5-phenylisoxazole-3-carboxylate (22). White solid (82%); mp 60 °C. ^{1}H NMR (DMSO) δ 7.52–7.48 (m, 5H), 7.13 (s, 1H), 4.29 (q, J = 7.3 Hz, 2H), 1.30 (t, J = 7.2 Hz, 3H). LC–MS (APCI$^+$) m/z 218.1 (MH$^+$).

4.1.3.2. Ethyl 5-(2-methoxyphenyl)isoxazole-3-carboxylate (23). White solid (74%); mp 63 °C. ^{1}H NMR (DMSO) δ 7.90 (dd, J = 7.9, 1.4 Hz, 1H), 7.55 (td, J = 8.7, 1.4 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.16–7.11 (m, 2H), 4.38 (q, J = 7.0 Hz, 2H), 3.97 (s, 3H), 1.34 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 248.0 (MH$^+$).

4.1.3.3. Ethyl 5-(2-ethoxyphenyl)isoxazole-3-carboxylate (24). White solid (29%); mp 65 °C. ^{1}H NMR (DMSO) δ 7.92 (d, J = 8.0 Hz, 1H), 7.58 (t, J = 8.5 Hz, 1H), 7.28 (d, J = 8.4 Hz, 1H), 7.16–7.11 (m, 2H), 4.43 (q, J = 7.2 Hz, 2H), 4.36 (q, J = 6.7 Hz, 2H), 1.44 (t, J = 6.4 Hz, 3H), 1.34 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/zC 262.0 (MH$^+$).

4.1.3.4. Ethyl 5-(2-propoxyphenyl)isoxazole-3-carboxylate (25). White solid (65%); mp 66 °C. ^{1}H NMR (DMSO) δ 7.92 (dd, J = 7.6, 1.2 Hz, 1H), 7.52 (td, J = 8.4, 1.4 Hz, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.14–7.09 (m, 2H), 4.38 (q, J = 7.0 Hz, 2H), 4.14 (t, J = 6.4 Hz, 2H), 1.83 (m, 2H), 1.33 (t, J = 7.3 Hz, 3H), 1.04 (t, J = 7.6 Hz, 3H). LC–MS (APCI$^+$) m/z 276.1 (MH$^+$).

4.1.3.5. Ethyl 5-(2-butoxyphenyl)isoxazole-3-carboxylate (26). White solid (54%); mp 69 °C. ^{1}H NMR (DMSO) δ 7.91 (d, J = 6.7 Hz, 1H), 7.52 (t, J = 7.3 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.13–7.11 (m, 2H), 4.38 (q, J = 7.0 Hz, 2H), 4.17 (t, J = 6.1 Hz, 2H), 1.81 (m, 2H), 1.49 (m, 2H), 1.33 (t, J = 7.3 Hz, 3H), 0.96 (t, J = 7.6 Hz, 3H). LC–MS (APCI$^+$) m/z 290.2 (MH$^+$).

4.1.3.6. Ethyl 5-(2-pentyloxyphenyl)isoxazole-3-carboxylate (27). White solid (38%); mp 59 °C. ^{1}H NMR (DMSO) δ 7.91 (d, J = 6.7 Hz, 1H), 7.52 (t, J = 7.3 Hz, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.13–7.11 (m, 2H), 4.38 (q, J = 7.3 Hz, 2H), 4.16 (t, J = 6.4 Hz, 2H),

1.83 (m, 2H), 1.49–1.31 (m, 7H), 0.92 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 304.1 (MH$^+$).

4.1.3.7. Ethyl 5-(2-hexyloxyphenyl)isoxazole-3-carboxylate (28). White solid (53%); mp 42 °C. ^{1}H NMR (DMSO) δ 7.91 (d, J = 6.4 Hz, 1H), 7.52 (t, J = 7.3 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.13–7.11 (m, 2H), 4.38 (q, J = 7.0 Hz, 2H), 4.17 (t, J = 6.1 Hz, 2H), 1.82 (m, 2H), 1.52–1.46 (m, 9H), 0.85 (t, J = 6.7 Hz, 3H). LC–MS (APCI$^+$) m/z 318.3 (MH$^+$).

4.1.3.8. Ethyl 5-(2-heptyloxyphenyl)isoxazole-3-carboxylate (29). White solid (34%); mp 35 °C. ^{1}H NMR (DMSO) δ 7.91 (d, J = 7.9 Hz, 1H), 7.52 (t, J = 7.7 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.14–7.09 (m, 2H), 4.39 (q, J = 7.0 Hz, 2H), 4.17 (t, J = 6.2 Hz, 2H), 1.86–1.77 (m, 2H), 1.52–1.43 (m, 2H), 1.38–1.24 (m, 9H), 0.85 (t, J = 6.6 Hz, 3H). LC–MS (APCI$^+$) m/z 332.2 (MH$^+$).

4.1.3.9. Ethyl 5-(2-octyloxyphenyl)isoxazole-3-carboxylate (30). Beige solid (66%); mp 52 °C. ^{1}H NMR (DMSO) δ 7.88 (d, J = 7.7 Hz, 1H), 7.49 (m, 1H), 7.21 (m, 1H), 7.14–7.07 (m, 2H), 4.42–4.33 (m, 2H), 4.12 (m, 2H), 1.78 (m, 2H), 1.44 (m, 2H), 1.36–1.30 (m, 5H), 1.28–1.21 (m, 6H), 0.82 (m, 3H). LC–MS (APCI$^+$) m/z 346.2 (MH$^+$).

4.1.3.10. Ethyl 5-(3-pentyloxyphenyl)isoxazole-3-carboxylate (31). White solid (27%); mp 61 °C. ^{1}H NMR (DMSO) δ 7.58 (d, J = 6.6 Hz, 1H), 7.54–7.48 (m, 3H), 7.08 (d, J = 8.5 Hz, 1H), 4.39 (q, J = 7.2 Hz, 2H), 4.05 (t, J = 6.4 Hz, 2H), 1.72 (m, 2H), 1.47–1.35 (m, 7H), 0.89 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$)m/z 304.2 (MH$^+$).

4.1.3.11. Ethyl 5-(4-pentyloxyphenyl)isoxazole-3-carboxylate (32). White solid (60%); mp 43 °C. ^{1}H NMR (DMSO) δ 7.73 (d, J = 8.9 Hz, 2H), 6.97 (d, J = 8.7 Hz, 2H), 6.79 (s, 1H), 4.47 (q, J = 7.3 Hz, 2H), 4.02 (t, J = 6.4 Hz, 2H), 1.82 (m, 2H), 1.52–1.45 (m, 7H), 0.95 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 304.2 (MH$^+$).

4.1.4. General procedure for the preparation of 5-arylisoxazole-3-carboxylic acids (33–43)

To a stirred solution of ester **22**–**32** (1 equiv) in 95% EtOH (50 mL), was added sodium hydroxide in pellets (10 equiv). The mixture was stirred at room temperature for 24 h. EtOH was removed under reduced pressure and the residue was acidified (1 N HCl, pH 2) and extracted with EtOAc (2 × 20 mL). The organic layers were washed with water (20 mL) and brine (20 mL), dried over $MgSO_4$ and evaporated under reduced pressure to afford pure carboxylic acids **33**–**43**.

4.1.4.1. 5-Phenylisoxazole-3-carboxylic acid (33). White solid (79%); 164 °C. ^{1}H NMR (DMSO) δ 12.79 (s, 1H), 7.96–7.93 (m, 2H), 7.59–7.53 (m, 3H), 7.41 (s, 1H). LC–MS (APCI$^+$) m/z 190.0 (MH$^+$).

4.1.4.2. 5-(2-Methoxyphenyl)isoxazole-3-carboxylic acid (34). White solid (68%); mp 197 °C. ^{1}H NMR (DMSO) δ 12.79 (s, 1H), 7.91 (dd, J = 7.9, 1.5 Hz, 1H), 7.55 (td, J = 8.5, 1.5 Hz, 1H), 7.23 (d, J = 8.2 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 7.06 (s, 1H), 3.96 (s, 3H). LC–MS (APCI$^+$)m/z 220.0 (MH$^+$).

4.1.4.3. 5-(2-Ethoxyphenyl)isoxazole-3-carboxylic acid (35). White solid (45%); mp 192 °C. ^{1}H NMR (DMSO) δ 12.79 (s, 1H), 7.93 (dd, J = 7.9, 1.5 Hz, 1H), 7.54 (td, J = 8.4, 1.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 1H), 7.15–7.07 (m, 2H), 4.26 (q, J = 6.7 Hz, 2H), 1.46 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 234.1 (MH$^+$).

4.1.4.4. 5-(2-Propoxyphenyl)isoxazole-3-carboxylic acid (36). White solid (69%); mp 172 °C. ^{1}H NMR (DMSO) δ 12.78 (s, 1H), 7.91 (dd, J = 7.6, 1.1 Hz, 1H), 7.51 (td, J = 8.5, 1.5 Hz, 1H), 7.22 (d, J = 8.4 Hz, 1H), 7.14–7.07 (m, 2H), 4.13 (t, J = 6.4 Hz, 2H), 1.80 (m, 2H), 1.04 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 248.2 (MH$^+$).

4.1.4.5. 5-(2-Butoxyphenyl)isoxazole-3-carboxylic acid (37). White solid (71%); mp 149 °C. ^{1}H NMR (DMSO) δ 12.79 (s, 1H), 7.91 (d, J = 7.7 Hz, 1H), 7.53 (t, J = 7.0 Hz, 1H), 7.25 (d, J = 8.1 Hz, 1H), 7.12 (t, J = 7.7 Hz, 1H), 7.08 (s, 1H), 4.17 (t, J = 6.3 Hz, 2H), 1.82 (m, 2H), 1.48 (m, 2H), 0.96 (t, J = 7.1 Hz, 3H). LC–MS (APCI$^+$)m/z 262.2 (MH$^+$).

4.1.4.6. 5-(2-Pentyloxyphenyl)isoxazole-3-carboxylic acid (38). White solid (55%); mp 129 °C. ^{1}H NMR (DMSO) δ 12.78 (s, 1H), 7.89 (d, J = 7.9 Hz, 1H), 7.48 (t, J = 7.3 Hz, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.10 (t, J = 7.3 Hz, 1H), 7.02 (s, 1H), 4.16 (t, J = 6.5 Hz, 2H), 1.81 (m, 2H), 1.48–1.33 (m, 4H), 0.90 (t, J = 7.3 Hz, 3H). LC–MS (APCI$^+$) m/z 276.3 (MH$^+$).

4.1.4.7. 5-(2-Hexyloxyphenyl)isoxazole-3-carboxylic acid (39). White solid (20%); mp 129 °C. ^{1}H NMR (DMSO) δ 12.78 (s, 1H), 7.88 (d, J = 7.6 Hz, 1H), 7.50 (t, J = 8.2 Hz, 1H), 7.21 (d, J = 8.5 Hz, 1H), 7.11 (t, J = 7.6 Hz, 1H), 7.05 (s, 1H), 4.15 (t, J = 6.4 Hz, 2H), 1.82 (m, 2H), 1.46 (m, 2H), 1.31–1.21 (m, 4H), 0.86 (t, J = 6.7 Hz, 3H). LC–MS (APCI$^+$) m/z 290.3 (MH$^+$).

4.1.4.8. 5-(2-Heptyloxyphenyl)isoxazole-3-carboxylic acid (40). White solid (55%); mp 113 °C. ^{1}H NMR (DMSO) δ 7.89 (d, J = 7.8 Hz, 1H), 7.50 (t, J = 7.9 Hz, 1H), 7.22 (d, J = 8.4 Hz, 1H), 7.10 (t, J = 7.6 Hz, 1H), 7.05 (s, 1H), 4.14 (t, J = 6.3 Hz, 2H), 1.80 (m, 2H), 1.49–1.39 (m, 2H), 1.38–1.21 (m, 6H), 0.84 (t, J = 6.6 Hz, 3H). LC–MS (APCI$^+$) m/z 304.2 (MH$^+$).

4.1.4.9. 5-(2-Octyloxyphenyl)isoxazole-3-carboxylic acid (41). Beige solid (55%); mp 95 °C. ^{1}H NMR (DMSO) δ 14.07 (s, 1H), 7.90 (d, J = 7.9 Hz, 1H), 7.50 (t, J = 7.9 Hz, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.11 (t, J = 7.6 Hz, 1H), 7.06 (s, 1H), 4.15 (t, J = 6.3 Hz, 2H), 1.81 (m, 2H), 1.50–1.39 (m, 2H), 1.34–1.22 (m, 8H), 0.83 (m, 3H). LC–MS (APCI$^+$) m/z 318.2 (MH$^+$).

4.1.4.10. 5-(3-Pentyloxyphenyl)isoxazole-3-carboxylic acid (42). White solid (60%); mp 107 °C. ^{1}H NMR (DMSO) δ 12.78 (s, 1H), 7.45 (m, 4H), 7.07 (d, J = 8.2 Hz, 1H), 4.04 (t, J = 6.4 Hz, 2H), 1.72 (m, 2H), 1.39–1.34 (m, 4H), 0.90 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$)m/z 276.2 (MH$^+$).

4.1.4.11. 5-(4-Pentyloxyphenyl)isoxazole-3-carboxylic acid (43). White solid (74%); mp 179 °C. ^{1}H NMR (DMSO) δ 12.79 (s, 1H), 7.85 (d, J = 8.7 Hz, 2H), 7.24 (s, 1H), 7.07 (d, J = 8.7 Hz, 2H), 4.03 (t, J = 6.4 Hz, 2H), 1.72 (m, 2H), 1.43–1.28 (m, 4H), 0.89 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$)m/z 276.1 (MH$^+$).

4.1.5. General procedure for the preparation of 5-arylisoxazole-3-carboxamides (44–70)

To a solution of carboxylic acid **33**–**43** in dry chloroform (20 mL) were added *N*,*N*-diisopropylethylamine (DIEA) (2 equiv) and 1-hydroxybenzotriazole (HOBt) (0.5 equiv), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (1.5 equiv). The resulting mixture was stirred at room temperature for 45 min. The appropriate amine (1.2 equiv) was then added, and the solution was stirred at room temperature for additional 24 h. The solution was filtered and washed with 0.5 N aqueous NaOH (20 mL), with 1 N aqueous HCl (20 mL), and water (20 mL). The organic layer was dried over $MgSO_4$ and evaporated under reduce pressure to give a brown oil. The crude material was purified by

TLC using the appropriate eluent (cyclohexane/EtOAc 7:3, v/v) and recrystallized in heptane to afford the desired compounds.

4.1.5.1. *N*-(1-Adamantyl)-5-phenylisoxazole-3-carboxamide (44). White solid (52%); 157 °C. ^{1}H NMR (DMSO) δ 7.92–7.89 (m, 3H), 7.55–7.53 (m, 3H), 7.30 (s, 1H), 2.06 (m, 9H), 1.65 (m, 6H). LC–MS (APCI$^+$) *m*/*z* 323.2 (MH$^+$); Anal. Calcd for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.28; H, 6.65; N, 8.52.

4.1.5.2. *N*-(2-Adamantyl)-5-phenylisoxazole-3-carboxamide (45). White solid (51%); mp 158 °C. ^{1}H NMR (DMSO) δ 8.36 (d, *J* = 6.4 Hz, 1H), 8.27 (d, *J* = 7.6 Hz, 1H), 7.95 (t, *J* = 8.2 Hz, 1H), 7.53 (d, *J* = 8.2 Hz, 1H), 7.34 (t, *J* = 7.8 Hz, 1H), 7.29–7.26 (m, 2H), 4.09 (d, *J* = 5.3 Hz, 1H), 2.63 (m, 9H), 1.84 (m, 5H). LC–MS (APCI$^+$) *m*/*z* 323.1 (MH$^+$); Anal. Calcd for $C_{20}H_{22}N_2O_2$: C, 74.51; H, 6.88; N, 8.69. Found: C, 74.32; H, 6.72; N, 8.56.

4.1.5.3. *N*-(1-Adamantylmethyl)-5-phenylisoxazole-3-carboxamide (46). White solid (34%); mp 166 °C. ^{1}H NMR (DMSO) δ 8.60 (t, *J* = 6.1 Hz, 1H), 7.93–7.91 (m, 2H), 7.56–7.53 (m, 3H), 7.35 (s, 1H), 2.97 (d, *J* = 6.4 Hz, 2H), 1.92 (m, 3H), 1.68–1.49 (m, 12H). LC–MS (APCI$^+$) *m*/*z* 337.3 (MH$^+$); Anal. Calcd for $C_{21}H_{24}N_2O_2$: C, 74.97; H, 7.19; N, 8.33. Found: C, 74.84; H, 7.04; N, 8.19.

4.1.5.4. *N*-(1-Adamantyl)-5-(2-methoxyphenyl)isoxazole-3-carboxamide (47). White solid (13%); mp 102 °C. ^{1}H NMR (DMSO) δ 7.88–7.86 (m, 2H), 7.53 (t, *J* = 7.0 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 1H), 7.12 (t, *J* = 7.6 Hz, 1H), 7.04 (s, 1H), 3.96 (s, 3H), 2.06 (m, 9H), 1.65 (m, 6H). LC–MS (APCI$^+$) *m*/*z* 353.2 (MH$^+$); Anal. Calcd for $C_{21}H_{24}N_2O_3$: C, 71.57; H, 6.86; N, 7.95. Found: C, 71.52; H, 6.78; N, 7.92.

4.1.5.5. *N*-(1-Adamantyl)-5-(2-ethoxyphenyl)isoxazole-3-carboxamide (48). White solid (24%); mp 76 °C. ^{1}H NMR (DMSO) δ 7.96 (s, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.54 (t, *J* = 8.4 Hz, 1H), 7.25 (d, *J* = 8.4 Hz, 1H), 7.12 (t, *J* = 7.6 Hz, 1H), 7.06 (s, 1H), 4.28 (q, *J* = 6.5 Hz, 2H), 2.06 (m, 9H), 1.65 (m, 6H), 0.94 (t, *J* = 6.7 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 367.6 (MH$^+$); Anal. Calcd for $C_{22}H_{26}N_2O_3$: C, 72.11; H, 7.15; N, 7.64. Found: C, 72.06; H, 7.12; N, 7.59.

4.1.5.6. *N*-(1-Adamantyl)-5-(2-propoxyphenyl)isoxazole-3-carboxamide (49). White solid (29%); mp 62 °C. ^{1}H NMR (DMSO) δ 7.88–7.86 (m, 2H), 7.50 (t, *J* = 8.5 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 1H), 7.11 (t, *J* = 7.6 Hz, 1H), 7.00 (s, 1H), 4.13 (t, *J* = 6.4 Hz, 2H), 2.06 (m, 9H), 1.81 (m, 2H), 1.65 (m, 6H), 1.04 (t, *J* = 7.3 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 381.1 (MH$^+$); Anal. Calcd for $C_{23}H_{28}N_2O_3$: C, 72.61; H, 7.42; N, 7.36. Found: C, 72.42; H, 7.25; N, 7.17.

4.1.5.7. *N*-(1-Adamantyl)-5-(2-butoxyphenyl)isoxazole-3-carboxamide (50). White solid (36%); mp 82 °C. ^{1}H NMR (DMSO) δ 7.88–7.86 (m, 2H), 7.50 (t, *J* = 8.5 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 1H), 7.10 (t, *J* = 7.6 Hz, 1H), 6.98 (s, 1H), 4.17 (t, *J* = 6.4 Hz, 2H), 2.06 (m, 9H), 1.81 (m, 2H), 1.65 (m, 6H), 1.48 (m, 2H), 0.96 (t, *J* = 7.3 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 395.4 (MH$^+$); Anal. Calcd for $C_{24}H_{30}N_2O_3$: C, 73.07; H, 7.66; N, 7.10. Found: C, 72.92; H, 7.58; N, 6.98.

4.1.5.8. *N*-(1-Adamantyl)-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (51). White solid (65%); mp 65 °C. ^{1}H NMR (DMSO) δ 7.88–7.86 (m, 2H), 7.49 (t, *J* = 8.5 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 7.10 (t, *J* = 7.6 Hz, 1H), 6.99 (s, 1H), 4.15 (t, *J* = 7.0 Hz, 2H), 2.05 (m, 9H), 1.82 (m, 2H), 1.77 (m, 6H), 1.68–1.21 (m, 4H), 0.90 (t, *J* = 7.0 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 409.4 (MH$^+$); Anal. Calcd for $C_{25}H_{32}N_2O_3$: C, 73.50; H, 7.90; N, 6.86. Found: C, 73.39; H, 7.66; N, 6.57.

4.1.5.9. *N*-(1-Adamantyl)-5-(2-hexyloxyphenyl)isoxazole-3-carboxamide (52). Uncolorless oil (38%). ^{1}H NMR (DMSO) δ 7.88–7.86 (m, 2H), 7.50 (t, *J* = 8.5 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 7.10 (t, *J* = 7.6 Hz, 1H), 6.99 (s, 1H), 4.15 (t, *J* = 5.2 Hz, 2H), 2.12 (m, 9H), 1.83 (m, 2H), 1.65 (m, 6H), 1.44 (m, 2H), 1.31 (m, 4H), 0.87 (t, *J* = 6.7 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 423.5 (MH$^+$); Anal. Calcd for $C_{26}H_{34}N_2O_3$: C, 73.90; H, 8.11; N, 6.63. Found: C, 73.77; H, 8.04; N, 6.54.

4.1.5.10. *N*-(1-Adamantyl)-5-(2-heptyloxyphenyl)isoxazole-3-carboxamide (53). White solid (26%); mp 88 °C. ^{1}H NMR (DMSO) δ 7.88 (s, 1H), 7.87 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.50 (t, *J* = 7.9 Hz, 1H), 7.23 (d, *J* = 8.5 Hz, 1H), 7.10 (t, *J* = 7.5 Hz, 1H), 6.99 (s, 1H), 4.15 (t, *J* = 6.4 Hz, 2H), 2.05 (m, 9H), 1.82 (m, 2H), 1.65 (s, 6H), 1.48–1.27 (m, 8H), 0.86 (t, *J* = 6.8 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 437.3 (MH$^+$); Anal. Calcd for $C_{27}H_{36}N_2O_3$: C, 74.28; H, 8.31; N, 6.42. Found: C, 74.17; H, 8.29; N, 6.31.

4.1.5.11. *N*-(1-Adamantyl)-5-(2-octyloxyphenyl)isoxazole-3-carboxamide (54). White solid (14%); mp 88 °C. ^{1}H NMR (DMSO) δ 7.87 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.85 (s, 1H), 7.50 (t, *J* = 7.9 Hz, 1H), 7.23 (d, *J* = 8.5 Hz, 1H), 7.10 (t, *J* = 7.5 Hz, 1H), 6.99 (s, 1H), 4.15 (t, *J* = 6.4 Hz, 2H), 2.05 (m, 9H), 1.82 (m, 2H), 1.65 (s, 6H), 1.48–1.25 (m, 10H), 0.85 (t, *J* = 7.0 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 451.3 (MH$^+$); Anal. Calcd for $C_{28}H_{38}N_2O_3$: C, 74.63; H, 8.50; N, 6.22. Found: C, 74.47; H, 8.19; N, 6.08.

4.1.5.12. *N*-(1-Adamantyl)-5-(3-pentyloxyphenyl)isoxazole-3-carboxamide (55). White solid (59%); mp 109 °C. ^{1}H NMR (DMSO) δ 7.87 (s, 1H), 7.44 (m, 3H), 7.34 (s, 1H), 7.08 (d, *J* = 8.2 Hz, 1H), 4.04 (t, *J* = 6.4 Hz, 2H), 2.05 (m, 9H), 1.73 (m, 2H), 1.65 (m, 6H), 1.42–1.32 (m, 4H), 0.90 (t, *J* = 6.7 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 409.4 (MH$^+$); Anal. Calcd for $C_{25}H_{32}N_2O_3$: C, 73.50; H, 7.90; N, 6.86. Found: C, 73.42; H, 7.80; N, 6.71.

4.1.5.13. *N*-(1-Adamantyl)-5-(4-pentyloxyphenyl)isoxazole-3-carboxamide (56). White solid (47%); mp 156 °C. ^{1}H NMR (DMSO) δ 7.83 (s, 1H), 7.80 (d, *J* = 8.6 Hz, 2H), 7.12 (s, 1H), 7.07 (d, *J* = 9.0 Hz, 2H), 4.03 (t, *J* = 6.4 Hz, 2H), 2.05 (m, 9H), 1.73 (m, 2H), 1.65 (m, 6H), 1.39–1.33 (m, 4H), 0.89 (t, *J* = 7.0 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 409.1 (MH$^+$); Anal. Calcd for $C_{25}H_{32}N_2O_3$: C, 73.50; H, 7.90; N, 6.86. Found: C, 73.34; H, 7.71; N, 6.69.

4.1.5.14. *N*-(2-Adamantyl)-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (57). White solid (32%); mp 68 °C. ^{1}H NMR ($CDCl_3$) δ 7.97 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.20 (s, 1H), 7.19 (s, 1H), 7.08–6.99 (m, 2H), 4.26 (m, 1H), 4.12 (t, *J* = 6.7 Hz, 2H), 2.07 (s, 2H), 1.95–1.90 (m, 10H), 1.78 (s, 2H), 1.72 (s, 1H), 1.68 (s, 1H), 1.52–1.38 (m, 4H), 0.95 (t, *J* = 7.0 Hz, 3H). LC–MS (APCI$^+$) *m*/*z* 409.2 (MH$^+$); Anal. Calcd for $C_{25}H_{32}N_2O_3$: C, 73.50; H, 7.90; N, 6.86. Found: C, 73.27; H, 7.72; N, 6.74.

4.1.5.15. *N*-(3-Noradamantyl)-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (58). White solid (56%); mp 101 °C. ^{1}H NMR ($CDCl_3$) δ 7.97 (d, *J* = 7.9 Hz, 1H), 7.41 (t, *J* = 7.9 Hz, 1H), 7.19 (s, 1H), 7.04 (m, 3H), 4.11 (t, *J* = 7.0 Hz, 2H), 2.60 (t, *J* = 6.8 Hz, 1H), 2.34 (s, 2H), 2.22 (m, 2H), 2.14–2.01 (m, 4H), 1.93 (quint, *J* = 7.0 Hz, 2H), 1.70–1.59 (m, 4H), 1.49–1.35 (m, 4H), 0.95 (t, *J* = 7.0 Hz, 3H). HRMS (FTMS + p ESI Full ms) *m*/*z* 395.2316 (MH$^+$); Anal. Calcd for $C_{24}H_{30}N_2O_3$: C, 73.07; H, 7.66; N, 7.10. Found: C, 72.93; H, 7.48; N, 6.87.

4.1.5.16. *N*-Cyclopropyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (59). White solid (18%); mp 95 °C. ^{1}H NMR (DMSO) δ 8.86 (d, *J* = 4.3 Hz, 1H), 7.88 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.50 (t, *J* = 7.9 Hz, 1H), 7.23 (d, *J* = 8.5 Hz, 1H), 7.10 (t, *J* = 7.5 Hz, 1H), 7.04

(s, 1H), 4.16 (t, J = 6.4 Hz, 2H), 2.86 (m, 1H), 1.83 (m, 2H), 1.50–1.31 (m, 4H), 0.91 (t, J = 7.0 Hz, 3H), 0.73–0.59 (m, 4H). LC–MS (APCI$^+$) m/z 315.1 (MH$^+$); Anal. Calcd for $C_{18}H_{22}N_2O_3$: C, 68.77; H, 7.05; N, 8.91. Found: C, 68.59; H, 7.14; N, 9.12.

4.1.5.17. *N*-Cyclobutyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (60). White solid (77%); mp 131 °C. ^{1}H NMR ($CDCl_3$) δ 7.97 (dd, J = 7.9, 1.7 Hz, 1H), 7.41 (t, J = 7.9 Hz, 1H), 7.19 (s, 1H), 7.08–6.96 (m, 3H), 4.59 (quint, J = 8.0 Hz, 1H), 4.11 (t, J = 6.9 Hz, 2H), 2.43 (m, 2H), 2.07–1.75 (m, 6H), 1.50–1.40 (m, 4H), 0.95 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 329.2 (MH$^+$); Anal. Calcd for $C_{19}H_{24}N_2O_3$: C, 69.49; H, 7.37; N, 8.53. Found: C, 69.62; H, 7.49; N, 8.39.

4.1.5.18. *N*-Cyclopentyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (61). White solid (41%); mp 101 °C. ^{1}H NMR (DMSO) δ 8.78 (d, J = 7.6 Hz, 1H), 7.95 (dd, J = 7.9, 1.7 Hz, 1H), 7.57 (t, J = 7.9 Hz, 1H), 7.31 (d, J = 8.5 Hz, 1H), 7.18 (t, J = 7.6 Hz, 1H), 7.11 (s, 1H), 4.32–4.26 (m, 1H), 4.23 (t, J = 6.4 Hz, 2H), 2.00–1.86 (m, 4H), 1.79–1.71 (m, 2H), 1.69–1.57 (m, 4H), 1.55–1.41 (m, 4H), 0.98 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 343.2 (MH$^+$); Anal. Calcd for $C_{20}H_{26}N_2O_3$: C, 70.15; H, 7.65; N, 8.18. Found: C, 70.23; H, 7.36; N, 8.47.

4.1.5.19. *N*-Cyclohexyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (62). White solid (21%); mp 69 °C. ^{1}H NMR (DMSO) δ 8.61 (d, J = 5.7 Hz, 1H), 7.90 (d, J = 7.3 Hz, 1H), 7.50 (t, J = 8.4 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 7.11 (t, J = 7.3 Hz, 1H), 7.04 (s, 1H), 4.16 (t, J = 6.4 Hz, 2H), 2.72 (m, 1H), 2.07 (m, 2H), 1.80–1.64 (m, 5H), 1.61–1.55 (m, 4H), 1.34–1.19 (m, 5H), 0.90 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 357.3 (MH$^+$); Anal. Calcd for $C_{21}H_{28}N_2O_3$: C, 70.76; H, 7.92; N, 7.86. Found: C, 70.91; H, 8.73; N, 7.64.

4.1.5.20. *N*-(Piperidin-1-yl)-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (63). White solid (27%); mp 74 °C. ^{1}H NMR (DMSO) δ 9.78 (s, 1H), 7.87 (d, J = 7.6 Hz, 1H), 7.48 (t, J = 7.3 Hz, 1H), 7.21 (d, J = 8.5 Hz, 1H), 7.11 (t, J = 7.6 Hz, 1H), 7.03 (s, 1H), 4.16 (t, J = 6.4 Hz, 2H), 2.80 (m, 4H), 1.87 (m, 2H), 1.58–1.56 (m, 4H), 1.40–1.37 (m, 6H), 0.90 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 358.2 (MH$^+$); Anal. Calcd for $C_{20}H_{27}N_2O_3$: C, 67.20; H, 7.61; N, 11.76. Found: C, 67.03; H, 7.57; N, 11.87.

4.1.5.21. *N*-Cycloheptyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (64). White solid (32%); mp 109 °C. ^{1}H NMR ($CDCl_3$) δ 7.96 (dd, J = 7.9, 1.7 Hz, 1H), 7.40 (t, J = 7.9 Hz, 1H), 7.19 (s, 1H), 7.04 (m, 2H), 6.80 (s, 1H), 4.17 (s, 1H), 4.11 (t, J = 6.9 Hz, 2H), 2.04 (m, 2H), 1.92 (quint, J = 7.0 Hz, 2H), 1.71–1.58 (m, 8H), 1.55–1.37 (m, 6H), 0.95 (t, J = 7.0 Hz, 3H). HRMS (FTMS + p ESI Full ms) m/z 371.2322 (MH$^+$); Anal. Calcd for $C_{22}H_{30}N_2O_3$: C, 71.32; H, 8.16; N, 7.56. Found: C, 71.45; H, 8.43; N, 7.29.

4.1.5.22. *N*-Cyclooctyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (65). White solid (43%); mp 104 °C. ^{1}H NMR ($CDCl_3$) δ 7.97 (dd, J = 7.9, 1.7 Hz, 1H), 7.41 (t, J = 7.9 Hz, 1H), 7.20 (s, 1H), 7.06 (t, J = 7.5 Hz, 1H), 7.01 (d, J = 8.5 Hz, 1H), 6.82 (d, J = 7.8 Hz, 1H), 4.25–4.17 (m, 1H), 4.12 (t, J = 6.7 Hz, 2H), 2.00–1.88 (m, 4H), 1.75–1.59 (m, 12H), 1.52–1.38 (m, 4H), 0.96 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 385.6 (MH$^+$); Anal. Calcd for $C_{23}H_{32}N_2O_3$: C, 71.84; H, 8.39; N, 7.29. Found: C, 71.96; H, 8.64; N, 7.57.

4.1.5.23. *N*-Phenyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (66). White solid (19%); mp 95 °C. ^{1}H NMR ($CDCl_3$) δ 8.60 (s, 1H), 8.00 (dd, J = 7.9, 1.7 Hz, 1H), 7.71 (d, J = 8.1 Hz, 2H), 7.42 (m, 3H), 7.31 (s, 1H), 7.22–7.02 (m, 3H), 4.15 (t, J = 6.7 Hz, 2H), 1.96 (quint, J = 7.2 Hz, 2H), 1.53–1.40 (m, 4H), 0.97 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 351.2 (MH$^+$); Anal. Calcd for $C_{21}H_{22}N_2O_3$: C, 71.98; H, 6.33; N, 7.99. Found: C, 72.16; H, 6.51; N, 7.71.

4.1.5.24. *N*-Benzyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (67). White solid (73%); mp 118 °C. ^{1}H NMR ($CDCl_3$) δ 7.96 (dd, J = 7.9, 1.7 Hz, 1H), 7.44–7.30 (m, 6H), 7.24 (s, 1H), 7.17 (s, 1H), 7.04 (m, 2H), 4.66 (d, J = 6.0 Hz, 2H), 4.12 (t, J = 6.9 Hz, 2H), 1.93 (quint, J = 7.0 Hz, 2H), 1.52–1.38 (m, 4H), 0.96 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 365.2 (MH$^+$); Anal. Calcd for $C_{22}H_{24}N_2O_3$: C, 72.51; H, 6.64; N, 7.69. Found: C, 72.73; H, 6.81; N, 7.42.

4.1.5.25. *N*-(5-Methyl-1,3,4-thiadiazol-2-yl)-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (68). White solid (23%); mp 73 °C. ^{1}H NMR ($CDCl_3$) δ 10.20 (s, 1H), 8.02 (d, J = 8.0 Hz, 1H), 7.46 (t, J = 7.9 Hz, 1H), 7.33 (s, 1H), 7.10 (t, J = 7.6 Hz, 1H), 7.04 (d, J = 8.4 Hz, 1H), 4.15 (t, J = 6.7 Hz, 2H), 2.77 (s, 3H), 1.95 (quint, J = 7.1 Hz, 2H), 1.54–1.38 (m, 4H), 0.97 (t, J = 7.0 Hz, 3H). LC–MS (APCI$^+$) m/z 373.1 (MH$^+$); Anal. Calcd for $C_{18}H_{20}N_4O_3$: C, 58.05; H, 5.41; N, 15.04. Found: C, 58.21; H, 5.31; N, 15.17.

4.1.5.26. *N*-Naphtyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (69). Purple solid (25%); mp 89.6 °C. ^{1}H NMR (DMSO) δ 10.88 (s, 1H), 8.02–7.95 (m, 3H), 7.90 (d, J = 8.1 Hz, 1H), 7.65 (d, J = 7.1 Hz, 1H), 7.60–7.49 (m, 4H), 7.25–7.19 (m, 2H), 7.14 (t, J = 7.6 Hz, 1H), 4.16 (t, J = 6.4 Hz, 2H), 1.83 (quint, J = 7.1 Hz, 2H), 1.50–1.29 (m, 4H), 0.98 (t, J = 7.1 Hz, 3H). LC–MS (APCI$^+$) m/z 401.1 (MH$^+$); Anal. Calcd for $C_{25}H_{24}N_2O_3$: C, 74.98; H, 6.04; N, 6.99. Found: C, 74.67; H, 5.91; N, 7.03.

4.1.5.27. *N,N*-Diisobutyl-5-(2-pentyloxyphenyl)isoxazole-3-carboxamide (70). Yellow oil (25%). ^{1}H NMR ($CDCl_3$) δ 7.99 (dd, J = 7.9, 1.8 Hz, 1H), 7.39 (t, J = 7.9 Hz, 1H), 7.05 (t, J = 7.6 Hz, 1H), 7.01–6.98 (m, 2H), 4.10 (t, J = 7.0 Hz, 2H), 3.50 (d, J = 7.6 Hz, 2H), 3.40 (d, J = 7.6 Hz, 2H), 2.16 (m, J = 6.8 Hz, 1H), 1.90 (m, 3H), 1.51–1.35 (m, 4H), 0.99 (d, J = 6.7 Hz, 6H), 0.93 (t, J = 7.0 Hz, 3H), 0.85 (d, J = 6.7 Hz, 6H). LC–MS (APCI$^+$) m/z 387.2 (MH$^+$); Anal. Calcd for $C_{23}H_{34}N_2O_3$: C, 71.47; H, 8.87; N, 7.25. Found: C, 71.79; H, 9.02; N, 7.14.

4.2. Competition binding assay

Stock solutions of the compounds were prepared in DMSO and further diluted with the binding buffer to the desired concentration. Final DMSO concentrations in the assay were less than 0.1%. The competitive binding experiments were performed as described in an earlier publication. Briefly, [^{3}H]-CP-55,940 (0.5 nM) as a radioligand for the human CB_1 and CB_2 cannabinoid receptors was added to 6 μg of membranes resuspended in 550 μL (final volume) binding buffer (20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA, 0.3% bovine serum albumine, pH 7.4). After 1 h at 30 °C, the incubation was stopped and the solutions were rapidly filtered over Unifilter-96 GF/C glass fiber pre-soaked in binding buffer on a Filtermate Unifilter 96-Harvester (Perkin–Elmer), and washed 20 times with of ice-cold binding buffer without serum albumin. The radioactivity on the filters was measured using TopCount NXT™ Microplate Scintillation Counter (Perkin–Elmer) using 60 μL of MicroScint™ 40 (Perkin–Elmer) after 30 min resting. Assays were performed at least in duplicate. The nonspecific binding was determined in the presence of 5 μM (R)-(+)-WIN 55,212-2 (Sigma).

4.3. [^{35}S]-GTPγS assays

The binding experiments were performed at 30 °C in tubes containing 10 μg of protein in 0.5 mL (final volume) binding buffer (20 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4) supplemented with 30 μM GDP. The assay was initiated by the addition of [^{35}S]-GTPγS (0.1 nM, final concentra-

tion). After 1 h at 30 °C, the incubation was stopped and the solutions were rapidly filtered over Unifilter-96 GF/B glass fiber and washed 20 times with ice-cold binding buffer. The radioactivity on the filters was counted as above. Assays were performed in duplicate. Nonspecific binding was measured in the presence of 100 μM Gpp(NH)p. Results were expressed as EC_{50} (nM) and E_{max} (%). Basal constitutive activity of the receptor was set at a value of 100%; reported E_{max} values above 100% indicated that the compound behaves as an agonist (either partial or full), while values lower than 100% indicated inverse agonist properties. K_i and EC_{50} values were determined by nonlinear regression analysis performed using the GraphPad prism 5.0 program (GraphPad Software, San Diego).

4.4. Cell culture and Cell proliferation assay

Colon cancer cells (HT29) were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in DMEM + Glutamax-I (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL). In the cell proliferation assay, cells were plated in triplicate on 96-well plates (3000 cells/well) and incubated for 24 h. The cells were then incubated in culture medium that contained a 10 μM concentration of tested compounds, each dissolved in less than 0.1% DMSO. After 72 h, cell growth was estimated by the colorimetric MTS test.

4.5. Animals

Seven weeks old C57BL6 male mice were purchased from JANVIER Laboratory (Le Genest St. Isle, France). Animals were maintained in specific pathogen free conditions. They had access to standard tap water and chow diet ad libitum. All animal experiments were approved by local animal care program and were in accordance with European convention on research animal protection.

4.6. Induction and scoring of acute colitis

Acute colitis was induced with 2.5% (w/v) DSS (molecular mass 35–50 kDa, TdB consultancy) dissolved in sterile, distilled water ad libitum for 7 days. The DSS solutions were made fresh every 2 days. Body weight was determined regularly. At day 7, disease activity index (DAI) was assessed by adding body weight variation, stool consistency and rectal bleeding scores according to Supplementary Table 1. Rectal bleeding was assessed with ColoScreen III Lab Pack (Elitech). The DAI ranged from 0 (healthy) to 18 (greatest activity of colitis).

4.7. Histopathological analysis

Formalin-preserved colon sections were processed and embedded in paraffin by standard techniques. Longitudinal sections of 4 μM thick were stained with May Grünwald Giemsa (MGG) and examined blindly. Histological lesions were assessed using a score quantifying the intensity of the inflammatory cell infiltrate (score 0–3) and the tissue damage (score 0–3). Briefly, the presence of occasional inflammatory cells in the lamina propria was scored as 0, increased numbers of inflammatory cells in the lamina propria as 1, confluence of inflammatory cells extending into the submucosa as 2, and transmural extension of the infiltrate as 3. For tissue damage, scores were: 0, no mucosal damage; 1, lymphoepithelial lesions; 2, surface mucosal erosion or focal ulceration; 3, extensive mucosal damage and extension into deeper structures of the bowel wall. The combined histological score ranged from 0 (no changes) to 6 (extensive infiltration and tissue damage).

4.8. RNA extraction and real-time qPCR

Total RNA was extracted from colonic samples with NucleoSpin RNAII kit (Macherey-Nagel). cDNA was prepared with the High Capacity cDNA Archive kit and RT-qPCR was performed with SyBr-Green (Applied Biosystems). Polymerase RNA II (PolR2A) was used as a reference gene and primer sequences are listed in Supplementary Table 2.

4.9. Statistial analyses

Statistical analyses were made by Mann-Whitney U tests except for body weight variation for which Two-way RM ANOVA test was used.

Acknowledgments

The authors thank Ms. Perrine Six and Frederique Klupsch for the LC–MS analyses. This work was financially supported by the 'Conseil Régional Nord Pas De Calais'.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.06.010.

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Please cite this article in press as: Tourteau, A.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.06.010