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Article

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Development of the first Two-Pore Domain Potassium Channel TREK-1 (TWIK-Related K^+ Channel 1)-selective agonist possessing *in vivo* anti-nociceptive activity

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ABSTRACT : The TWIK-Related K^+ channel, TREK-1, has recently emerged as an attractive therapeutic target for the development of a novel class of analgesic drugs, suggesting that activation of TREK-1 could result in pain inhibition. Here we report the synthesis of a series of substituted acrylic acids (**1-54**) based on our previous work with caffeate esters. The analogues were evaluated for their ability to modulate TREK-1 channel by electrophysiology and for their *in vivo* antinociceptive activity (acetic acid-induced writhing and hotplate assays), leading to the identification of a series of novel molecules able to activate TREK-1 and displaying potent anti-nociceptive activity *in vivo*. The furyl analogue **36** is the most promising of these series.

INTRODUCTION

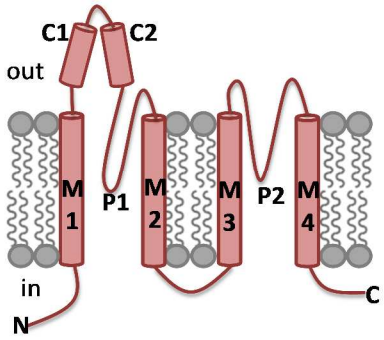
Pain represents a major public health issue worldwide. Acute or chronic, pain is responsible for 90% of medical consultations, with chronic pain affecting over 1.5 billion people worldwide.[1] Today, morphine remains the treatment of reference for severe acute or chronic pains. Its analgesic effect is often accompanied by adverse side-effects such as constipation, respiratory depression and dependence. This opioid drug produces its pharmacological action by interacting with the μ-opioid receptor (μOR). Recently Devilliers[2] reported that the beneficial and adverse effects of morphine could be dissociated by demonstrating that TREK-1 (TWIK-Related potassium channel-1, TWIK Tandem of pore domains in a Weak Inward rectifying K^+ channel) contributed to the analgesic effect of morphine but not to its adverse side effects. These observations suggest that direct activation of TREK-1 channel by acting downstream from μOR, might have a strong analgesic effect without opioid-ligand adverse effects.[3, 4]

The TREK-1 channel (KCNK2 gene),[5] first isolated from mouse brain in 1996,[6, 7] belongs to the two pore-domain potassium channels family (K2P), which are responsible of potassium (K^+) leak currents and are major contributors to resting membrane potential. Human TREK-1 (hTREK-1) is highly and broadly expressed in the central nervous system.[8, 9] Human and rodent TREK-1 is also expressed in

peripheral sensory neurons, particularly in small dorsal root ganglion neurons, that are associated with nociception. [3, 9-11] The functionality of TREK-1 results from its sensitivity to a variety of stimuli. TREK-1 is gated by membrane stretching (mechano-activation), pH, heat, polyunsaturated fatty acids (PUFA) such as arachidonic acid and some general volatile anesthetics (chloroform, diethyl ether, and nitrous oxide).[12] It can also be down-modulated by PKA / PKC phosphorylation pathways and tonically inhibited by the actin cytoskeleton.[13]

Structurally, TREK-1 channel (Figure 1) is a transmembrane (TM) protein with intracellular N- and C-terminal extremity.[14, 15] The channel pore results from the assembly of two dimers, containing each four helical transmembrane segments (M1-M4) arranged in tandem with two pore domains (P1-P2) and two extracellular cap helices (C1 and C2) following the M1 segment (Figure 1).

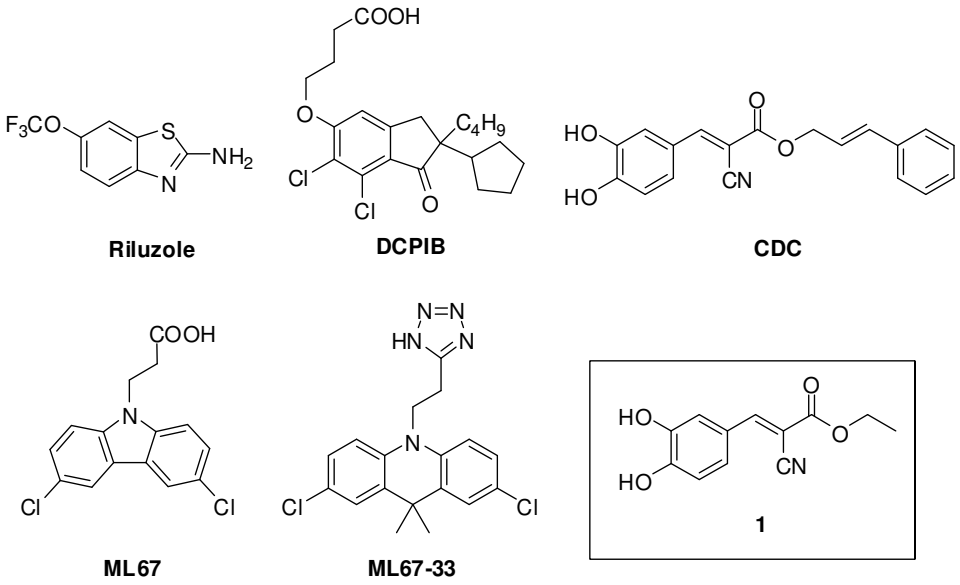
Figure 1. Topology of the TREK-1 subunit from the intracellular N-terminus to the intracellular C-terminus (including M1, C1, C2, P1, M2, M3, P2 and M4 domains).



The recent discovery of the involvement of TREK-1 in pain perception[3, 4] has prompted researchers to identify molecules capable to activate the pharmacologically orphan target.[5] The neuroprotective agent riluzole (Figure 2), used in the treatment of motor neuron diseases, was reported as a non-specific TREK-1 agonist.[16, 17] This activation was reported to be transient followed by an inhibition, process attributable to an increase in the intracellular cAMP concentration by riluzole that produces a PKA-dependent inhibition of TREK-1. The ethacrynic acid derivative DCPIB (Figure 2), a known inhibitor of

volume-regulated anion channels (VRAC), was reported to promote the activation of K^+ conductance mediated by K2P channel.[18] The DCPIB effect occluded that of arachidonic acid, which activates TREK-1incultured astrocytes. A high-throughput yeast screen allowed the discovery of a carbazole, ML67 (Figure 2), as a TREK-1 activator.[19] Optimization of this carbazole allowed the identification of an acridine analogue, ML67-33 (Figure 2) as a TREK-1 activator. Finally, cinnamyl 3,4-dihydroxyl-α-cyanocinnamate (CDC, Figure 2), derived from caffeic acid, was reported as a TREK-1 activator.[20] So far none of the reported TREK-1 activators has proven to be selective, since they modulate other K2P channels.

Figure 2. Reported TREK-1 activators



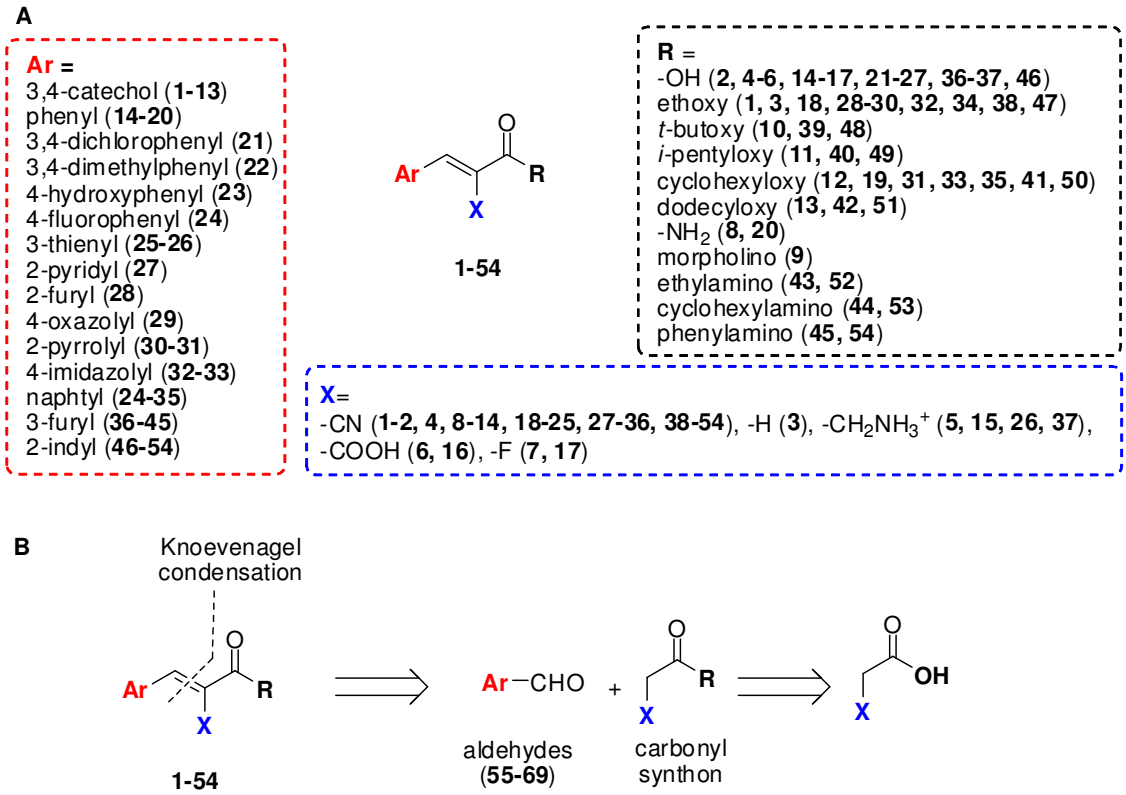
We have recently disclosed the synthesis of a small set of CDC analogues and identified compound **1** as a potent TREK-1 activator possessing antinociceptive activity *in vivo*.[21, 22] CDC and **1** displayed equipotent activity; hence in terms of ligand efficiency, we decided to focus our attention on shorter analogues. In the present study, we expanded our initial structure-activity relationship (SAR) study

around compound **1** and herein report the synthesis and pharmacological evaluation of 53 analogues of **1**.

CHEMISTRY

Considering that the skeleton of **1** is composed of three main moieties (Ar, R, X), we studied their influence on the pharmacological activity of the analogues (Figure 3A). The target molecules **1-54** were obtained by Knoevenagel-type condensation between the appropriately substituted aldehydes **55-69** and an α-substituted carbonyl synthon (Figure 3B).

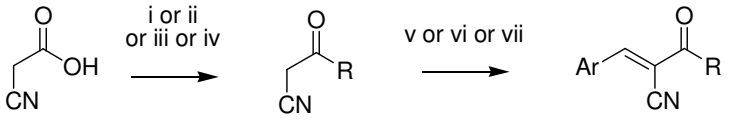
Figure 3. A. Structure of analogues 1-54. B. Strategy for the synthesis of analogues 1-54



The preparation of esters (R = ethoxy, *t*-butoxy, *i*-pentyloxy, cyclohexyloxy, docecyloxy), amides (R = morpholino, ethylamino, cyclohexylamino, phenylamino) and acids (R = OH) bearing a nitrile at the α-

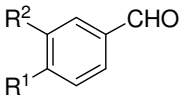
position (X = CN) is depicted on scheme 1. Esters (**1**, **10-13**, **18-19**, **28-35**, **38-42**, **47-51**) were prepared by reacting the appropriate aldehyde and the α-cyanoacetate esters **71-75**. Amides (**9**, **43-45**, **52-54**) were obtained by reaction of an aldehyde with the α-cyanoacetate amides **76-79**. Finally acids (**2**, **14**, **21-27**, **36**, **46**) were prepared by saponification of the *t*-butyl esters (**10**, **39**, **48**, **80-86**).

Scheme 1. Preparation of Esters **(1, 10-13, 18-19, 28-35, 38-42, 47-51),** Amides **(9, 43-45, 52-54)** and acids **(2, 14, 21-27, 36, 46)**

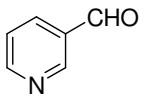


70

71, R = ethoxy (i. 66%)
72, R = *t*-butoxy (i. 45%)
73, R = *i*-pentyloxy (i. 48%)
74, R = cyclohexyloxy (i. 77%)
75, R = dodecyloxy (i. 87%)
76, R = ethylamino (ii. 30%)
77, R = cyclohexylamino (iii. 34%)
78, R = phenylamino (iv. 94%)
79, R = morpholino (i. >99%)



55, $R^1 = R^2 = OH$
56, $R^1 = R^2 = H$
57, $R^1 = R^2 = Cl$
58, $R^1 = R^2 = CH_3$
59, $R^1 = = OH$, $R^2 = H$
60, $R^1 = F$, $R^2 = H$



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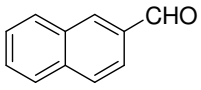
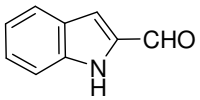
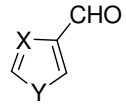


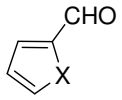
67



69



61, X = CH, Y = S
64, X = N, Y = O
66, X = N, Y = NH
68, X = CH, Y = O



63, X = O
65, X = NH

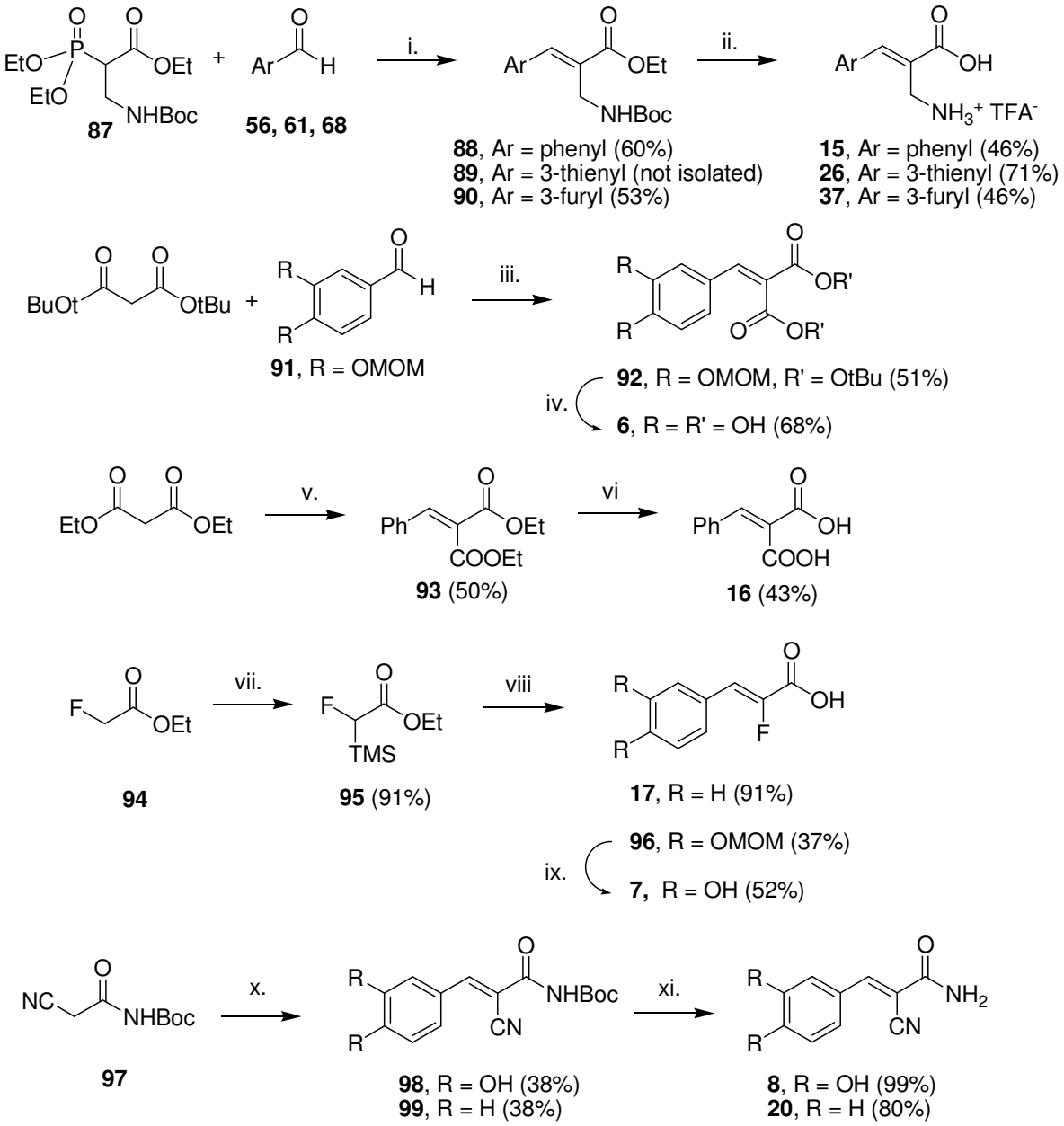
1, Ar = 3,4-catechol, R = ethoxy (v, 55%)
9, Ar = 3,4-catechol, R = morpholino(vi, 74%)
10, Ar = 3,4-catechol, R = *t*-butoxy (v, 52%)
11, Ar = 3,4-catechol, R = *i*-pentyloxy (v, 66%)
12, Ar = 3,4-catechol, R = cyclohexyloxy (v, 71%
13, Ar = 3,4-catechol, R = dodecyloxy (v, 76%)
18, Ar = phenyl**,** R = ethoxy (v, 84%)
19, Ar = phenyl, R = cyclohexyloxy (v, 73%)
28, Ar = 2-furyl**,** R = ethoxy (vii, 16%)
29, Ar = 4-oxazoyl**,** R = ethoxy (vii, 41%)
30, Ar = 2-pyrrolyl**,** R = ethoxy (v, 66%)
31, Ar = 2-pyrrolyl, R = cyclohexyloxy (v, 51%)
32, Ar = 4-imidazolyl**,** R = ethoxy (v, 77%)
33, Ar = 4-imidazolyl, R = cyclohexyloxy (v, 63%)
34, Ar = 2-naphtyl**,** R = ethoxy (v, 65%)
35, Ar = 2-naphtyl, R = cyclohexyloxy (v, 73%)
38, Ar = 3-furyl**,** R = ethoxy (v, 33%)
39, Ar = 3-furyl, R = *t*-butoxy (v, 42%)
40, Ar = 3-furyl, R = *i*-pentyloxy (v, 32%)
41, Ar = 3-furyl, R = cyclohexyloxy (v, 33%)
42, Ar = 3-furyl, R = dodecyloxy (v, 35%)
43, Ar = 3-furyl, R = ethylamino (v, 46%)
44, Ar = 3-furyl, R = cyclohexylamino (v, 43%)
45, Ar = 3-furyl, R = phenylamino (v, 11%)
47, Ar = 2-indyl**,** R = ethoxy (v, 61%)
48, Ar = 2-indyl, R = *t*-butoxy (v,42%)
49, Ar = 2-indyl, R = *i*-pentyloxy (v, 27%)
50, Ar = 2-indyl, R = cyclohexyloxy (v, 98%)
51, Ar = 2-indyl, R = dodecyloxy (v, 39%)
52, Ar = 2-indyl, R = ethylamino (v, 70%)
53, Ar = 2-indyl, R = cyclohexylamino (v, 57%)
54, Ar = 2-indyl, R = phenylamino (v, 74%)
80, Ar = phenyl, R = *t*-butoxy (v, 56%)
81, Ar = 3,4-dichlorophenyl, R = *t*-butoxy (v, 51%)
82, Ar = 3,4-dimethylphenyl, R = *t*-butoxy (v, 50%)
83, Ar = 4-hydroxyphenyl, R = *t*-butoxy (v, 71%)
84, Ar = 4-fluorophenyl, R = *t*-butoxy (v, 80%)
85, Ar = 3-thienyl, R = *t*-butoxy (v, 66%)
86, Ar = 3-pyridyl, R = *t*-butoxy (v, 58%)

10, 39, 48, 80-86

2, Ar = 3.4-catechol (90%)
14, Ar = phenyl (90%)
21, Ar = 3,4-dichlorophenyl (90%)
22, Ar = 3,4-dimethylphenyl 57%)
23, Ar = 4-hydroxyphenyl (35%)
24, Ar = 4-fluorophenyl (90%)
25, Ar = 3-thienyl (57%)
27, Ar = 3-pyridyl (80%)
36, Ar = 3-furyl (90%)
46, Ar = 2-indyl(78%)

Conditions. i. RH, DCC, DMAP, DCM, 0℃ to RT, 24h; **ii.** a. $(COCl)_2$, DCM/DMF, 0℃ to RT, 2h b. $EtNH_2$ (2M in THF), NEt_3, DCM, 0℃ to RT, 3h**. ii**i. cyclohexanamine, EtONa, EtOH, RT, 72h**. iv**. a. PCl_5, DCM, reflux, 30 min b. aniline, DCM, reflux, 12h. **v.** ArCHO **55-69**, piperidine, DCM, RT 24h. **vi**. ArCHO **55**, piperidine, AcOH, toluene reflux, 6h. **vii.** ArCHO **63-64**, H_2O, RT, 72h. **viii.** TFA, DCM, RT, 1h.

Scheme 2. Preparation of analogues **7-8, 15-17, 20, 26, 37.**



Conditions: **i.** NaH, THF, RT, 2 days. **ii**. a. LiOH 1M, MeOH, RT, 18h. b. TFA, DCM, RT, overnight**. ii**i. Piperidine, toluene, reflux, 2h. **iv.** TFA, DCM, RT, 12h. **v.** ArCHO **56**, piperidine, EtOH, reflux, 12h. **vi.** a. NaOH 1.5 M, MeOH, RT, 12h. b. H_2SO_4, Et_2O, RT, 4 days. **vii**. a. LDA, THF, -30 °C, 30 min. b. TMSCl, THF, -78 °C to RT over 4h. c. Tartaric acid, Et_2O, RT, 12h. **viii.** a. ArCHO (**56** or **91**), LDA, THF, -78 °C, 30 min. b. NaOH 1.5 M, MeOH, RT, 2h. **ix**. TFA, DCM, RT, 12h. **x**. a. EtOH, DCC, DCM, RT, 1h. b. ArCHO **55**-**56**, piperidine, DCM, RT, 12h. **xi.** TFA, DCM, RT, 1h.

Table 1. Biological evaluation of analogues **1-54**. Ar, R and X refer to the substitution of the scaffold molecule as presented in figure 3. (a) R_{TREK-1} is the ratio of currents measured after and before the application of the compounds (concentration in μM). TREK-1 current was measured in (b) HEK293 cells by whole-cell patch-clamp technique at +60mVor in (c) *Xenopus* oocytes by the double microelectrode technique at 0 mV. "% antinociceptive effect" refers to (d) the antinociceptive effect of compounds (10 mg/kg, s.c.) against acetic acid-induced abdominal writhing in CDI mice (n = 8) and (e) the antinociceptive effect of compounds (60 mg/kg, s.c.) on hotplate model in CDI mice (n=8), 30 min / 45 min after injection of modulators. T-test: *≤0.05, **≤0.01, ***≤0.001.

Cpds	Ar	R	X	R_{TREK-1} [a]	% antinociceptiveeffect	
					AcOH[d]	Hotplate[e]
Morphine (3 mg/kg)						185.0± 15.0% ***/ 159.7± 23.3%***
ML-67				8.56 ± 2.24 (100) [b]		77.4± 18.7%*** / 36.6± 13.9%
CDC	3,4-catechol	Cinnamyloxy-	-CN	10.30 ± 2.68 (100) [b] 2.64 ± 0.95 (20) [c]	50.8± 4.5%**	34.8± 14.5% / 30.5± 12.5%
1	3,4-catechol	-OEt	-CN	1.54 ± 0.1 (100) [b] 2.76 ± 0.72 (20) [c] 1.0 (40)[20]	57.8± 7.4 %*	34.6± 17.2% / 21.8± 11.3%
2	3,4-catechol	-OH	-CN	1.23 ± 0.08 (20) [c] 1.5 ± 0.2 (100) [b]	31.8± 14.5%[21]	21.4± 18.9 % / 59.4± 10.6 %**
3	3,4-catechol	-OH	-H	1.0 (40) [20]	34.6± 3.9%[23]*	ND
4	3,4-catechol	-OEt	-H	1.14 ± 0.12 (20) [c]	52.8 ± 7.9 %* 58.1± 4.4%%**[23]	ND
5	3,4-catechol	-OH	$-CH_2NH_3^+$	2.87 ± 0.32 (20) [c]	50.1± 5.4%***	ND

6	3,4-catechol	-OH	-COOH	1.76 ± 0.14 (20) [c]	-2.8± 11.0%	ND
7	3,4-catechol	-OH	-F	2.4 ± 0.2 (20) [c]	-5.8± 12.3%	ND
8	3,4-catechol	$-NH_2$	-CN	1.05 ± 0.08 (20) [c]	6.4± 21.0%	ND
9	3,4-catechol	Morpholino-	-CN	3.80 ± 0.86 (100) [b]	40.0± 5.8%**	28.7± 8.9%* / 16± 9.1 %
10	3,4-catechol	*t*Butoxy-	-CN	2.52 ± 0.54 (100) [b]	43.4± 10.4 %	ND
11	3,4-catechol	*i*Pentyloxy-	-CN	4.07 ± 0.57 (100) [b]	27.2± 17.1 %	ND
12	3,4-catechol	Cyclohexyloxy-	-CN	5.78 ± 0.64 (100) [b]	55.3± 4.5%**	25.5± 9.7 %* / 34.2± 12.1 %*
13	3,4-catechol	Dodecyloxy-	-CN	2.7 ± 0.37 (20) [b]	45.2± 12.5%	ND
14	Phenyl	-OH	-CN	1.29 ± 0.12 (20) [c]	43.1± 13.0%*	ND
15	Phenyl	-OH	$-CH_2NH_3^+$	0.95 ± 0.19 (20) [c]	-2.4± 16.8%	ND
16	Phenyl	-OH	-COOH	1.37 ± 0.18 (20) [c]	29.4± 15.8 %*	ND
17	Phenyl	-OH	-F	1.44 ± 0.12 (20) [c]	34.0± 5.9%***	ND
18	Phenyl	-OEt	-CN	1.80 ± 0.11 (100) [b]	30.8± 7.2%	55.7± 16.5%* / 45.1± 9.8%*
19	Phenyl	Cyclohexyloxy-	-CN	1.17 ± 0.22 (100) [b]	7.1± 8.7%	ND
20	Phenyl	$-NH_2$	-CN	1.7 ± 0.03 (20) [c]	18.0± 9.6%	ND
21	3,4-dichlorophenyl	-OH	-CN	1.62 ± 0.09 (20) [c]	7.5± 19.2 %	ND
22	3,4-dimethylphenyl	-OH	-CN	1.70 ± 0.05 (20) [c]	-6.1± 11.3%	ND
23	4-hydroxyphenyl	-OH	-CN	1.50 ± 0.13 (20) [c]	24.1± 23.1 %	ND
24	4-fluorophenyl	-OH	-CN	1.60 ± 0.22 (20) [c]	17.8± 15.2 %	ND
25	3-thienyl	-OH	-CN	1.20 ± 0.15 (20) [c]	1.8± 20.4 %	ND
26	3-thienyl	-OH	$-CH_2NH_3^+$	1.05 ± 0.06 (20) [c]	-13.5± 10.6%	ND
27	3-pyridyl	-OH	-CN	1.50 ± 0.27 (20) [c]	-39.8± 12.3% *	ND

28	2-furyl	-OEt	-CN	2.36 ± 0.65 (100) [b]	23.3± 11.4%	ND
29	4-oxazolyl	-OEt	-CN	2.56 ± 0.36 (100) [b]	28.9± 14.0%	ND
30	2-pyrrolyl	-OEt	-CN	1.89 ± 0.23 (100) [b]	25.8± 14.7%	ND
31	2-pyrrolyl	Cyclohexyloxy-	-CN	2.62 ± 0.47 (100) [b]	10.0± 13.6%	ND
32	4-imidazolyl	-OEt	-CN	1.80 ± 0.16 (100) [b]	33.0± 9.4%	145.2± 22.3%*** / 43.2± 13.0%**
33	4-imidazolyl	Cyclohexyloxy-	-CN	1.50 ± 0.21 (100) [b]	50.0± 13.9%**	27.0± 17.1 % / 62.4± 23.5%
34	Naphtyl	-OEt	-CN	1.59 ± 0.23 (100) [b]	44.7± 16.8%	93.2± 34.4%* / 67.5± 34.8%
35	Naphtyl	Cyclohexyloxy-	-CN	2.60 ±0.61 (100) [b]	30.8± 8.0%	70.3± 13.3%* / 50.9± 12.6%
36	3-furyl	-OH	-CN	4.53± 0.46 (100) [b] 1.43 ± 0.17 (20) [c]	30.7± 5.0%*	56.4± 14.4%* / 119.5± 21.9%**
37	3-furyl	-OH	$-CH_2NH_3^+$	1.49 ± 0.08 (20) [c]	-1.5 ± 16.8%	ND
38	3-furyl	-OEt	-CN	2.09 ± 0.4 (100) [b]	55.9± 7.5%***	28.4± 10.5% / 11.9± 10.6%
39	3-furyl	*t*Butoxy-	-CN	6.09 ± 1.24 (20) [b]	81.8± 1.8%***	20.8± 12.7% / -1.3± 4.0%
40	3-furyl	*i*Pentyloxy-	-CN	2.80 ± 0.41 (100) [b]	82.2± 9.1 %**	6.5± 6.9% / 25.5± 14.7%
41	3-furyl	Cyclohexyloxy-	-CN	1.79 ± 0.16 (100) [b]	91.2± 3.6%***	18.4± 14.2 % / 96.7± 20.8%***
42	3-furyl	Dodecyloxy-	-CN	2.33 ± 0.23 (100) [b]	53.8± 9.7%***	21.8± 15.1 % / 22.2± 13.6%
43	3-furyl	-NH-Ethyl	-CN	1.28 ± 0.13 (100) [b]	31.0± 17.7%	ND

44	3-furyl	-NH-Cyclohexyl	-CN	1.07 ±0.08 (100) [b]	0.0± 9.0%	ND
45	3-furyl	-NH-Phenyl	-CN	4.64 ± 0.61 (20) [b]	48.0± 15.8%*	107.7± 23.6%*** / 148.6± 41.3%**
46	2-indyl	-OH	-CN	1.18 ± 0.16 (100)[b] 2.44 ± 0.34 (20)[c]	25.8± 6.8%	ND
47	2-indyl	-OEt	-CN	4.71 ± 1.85 (20) [b]	88.4± 5.3 %**	33.3± 13.7%* / 32.7± 15.3%
48	2-indyl	*t*Butoxy-	-CN	4.07 ± 0.56 (20) [b]	51.3± 15.3%***	40.8± 11.4%* / 39.7± 16.5%*
49	2-indyl	*i*Pentyloxy-	-CN	4.01 ± 1.7 (20) [b]	46.6± 15.3%	21.8± 9.1% / 37.8± 15.9%*
50	2-indyl	Cyclohexyloxy-	-CN	1.98 ± 0.24 (10) [b]	83.6± 8.1 %***	65.7± 17.5%** / 52.6± 13.3%*
51	2-indyl	Dodecyloxy-	-CN	2.19 ± 0.24 (100) [b]	54.1± 11.6%***	23.2± 18.7% / 20.3± 13.7%
52	2-indyl	-NH-Ethyl	-CN	1.26 ± 0.10 (100) [b]	45.0± 7.5%*	ND
53	2-indyl	-NH-Cyclohexyl	-CN	1.52 ± 0.17 (100) [b]	45.0± 16.5%*	67.5± 14.1 %*** / 35.4± 13.9%
54	2-indyl	-NH-Phenyl	-CN	2.16 ±0.53 (10) [b]	52.0± 16.7%*	49.5± 18.6%* / 49.5± 29.7%

The preparation of analogues substituted at the α-position of the carbonyl function with various X groups [(ammonium salts (X = -CH_2-NH_3^+ **15**, **26**, **37**), malonic acids (R = -COOH **6**, **16**) and the α-fluoro acrylic acid (R = F **7**, **17**)] is presented in scheme 2. The ammonium salts were prepared as previously described[21]through a Wadsworth-Horner olefination between the appropriate benzaldehyde (**56**, **61** or **68**) and the phosphonate **87** followed by a deprotection in trifluoroacetic acid.

A Knoevenagel condensation between benzaldehydes (**56** and **91**) and malonate esters afforded, after deprotection, the desired 2-arylidenemalonic acids **6** and **16**. The α-fluoroacrylic acids (**7** and **17**) were obtained through a Peterson olefination between the benzaldehydes (**56** and **91)** and the fluorinated compound **95**.

Finally the two amides (R = NH_2, **8** and **20**) were obtained after deprotection of the carbamates **98-99**, the latter being formed through a Knoevenagel condensation between the benzaldehydes **55-56** and the 2-cyanoacetylcarbamate **97**.

BIOLOGICAL EVALUATION

The analogues (**1-54**) were first evaluated for their ability to enhance K^+ currents in TREK-1 transfectedHEK293 cells and/or in TREK-1 injected *Xenopus* oocytes using whole-cell patch-clamp technique and double microelectrodes technique respectively, in voltage-clamp configuration(Table 1).[6] In these electrophysiological experiments, ML-67 and CDC were assayed for comparative purposes. The results are expressed as the ratio of currents measured after and before the application of the compounds ($R_{TREK-1} = I_{max}/I_0$). Currents were measured at +60mV for HEK293 cells and at 0mV for oocytes.

The analogues (**1**-**54**) were then assessed for their *in vivo* antinociceptive activity using the acetic acid (AcOH by i.p.)-induced-writhing test in mice (Table 1).[24, 25] In this assay, the compounds or vehicle are administered into the subcutaneously (s.c.) to the animal 15 minutes before the AcOH injection into the

peritoneal cavity (i.p.). This activates nociceptors directly and/or produces inflamed viscera (sub-diaphragmatic organs) and subcutaneous (muscle wall) of tissus.[3] The number of induced abdominal writhes was determined during 20 min after the injection of acetic acid. The results are expressed as a percentage of antinociceptive effect (Table 1) determined according to the following equation:

$$\%\ \textbf{Antinociceptive effect}\ (\textbf{AcOH}) = \frac{(\text{nb writhes control} - \text{nb writhes test})}{\text{nb writhes control}} \text{x}100$$

Where "nb writhes control" is the mean number of writhes in the control group (n=8) and "nb writhes test" is the mean number of writhes in the test group (n=8).

For the compounds that showed an ability to activate TREK-1 ($R_{TREK\text{-}1}$>1.5 at 100 µM) and a percentage of analgesic activity in the AcOH writhing test higher than 30% (10 mg/kg), the *in vivo* analgesic activity was confirmed with a second screening, the hotplate assay. This model is more discriminating; it measures the response to a non-inflammatory acute nociceptive input and is used to study central antinociceptive activities. The withdrawal response latency evoked by exposing mice paws to a thermal stimulus (hotplate set to 52 °C) determines the antinociceptive reaction. Response was defined by the animal either licking the forepaws, hindpaws or flicking the hindpaws. To avoid tissue damage, animals were exposed to the hot plate (Ugo Basile model) for a maximum of 30 s (cutt-off time). Withdrawal latencies were measured before and 30 / 45 minutes after injection of vehicle, morphine (3 mg/kg) or analogues (60 mg/kg). The results are expressed as a percentage of antinociceptive effect (Table 1) determined according to the following equation:

$$\%\ \textbf{Antinociceptive effect}\ (\textbf{hotplate}) = \frac{(\text{postdrug latency} - \text{predrug latency})}{\text{predrug latency}} \text{x}100$$

Where "predrug latency" is the withdrawal latency before drug injection and "postdrug latency" is the withdrawal latency 30 and 45 min after drug injection

In order to confirm the role of TREK-1 in the analgesic response of the most potent compounds, their sensitivity to thermal stimuli (hotplate) was evaluated in TREK-$1^{-/-}$ mice (KO) and compared with the nociceptive behavior of wild type (WT) mice (Table 3).

Finally, the most promising drug candidates were evaluated for their selectivity to TREK-1 by testing them on others K2P channels (Table 4).

RESULTS AND DISCUSSION

Structurally diverse molecules were prepared sharing the same prop-2-en-1-one scaffold (Figure 3A) and bearing an aromatic moiety at the 3-position (Ar), a carboxy derivative (R), and various α-substituents (X). In each case, we chose groups that differed in nature: size/volume (Ar = Phenyl vs naphthyl), hydrophobicity (R = OC_2H_5 vs $OC_{12}H_{25}$), hydrogen bond ability (Ar = 3,4-catechol, 4-hydroxyphenyl, phenyl or X = F, COOH), electronic properties (Ar = 3.4-catechol, 3,4-dimethylphenyl, 3,4-dichlorophenyl). Since compounds featuring catechol moieties can be qualified as pan assays interference compounds (PAINS) since they can react with biomolecules and degrade or inactivate them, we aimed to prepare analogues bearing (hetero)aromatic moieties (Ar) that are not classified as PAINS such as Ar = phenyl, naphthyl, furyl, imidazolyl, pyrrolyl. Analogues (**1-54**) were prepared in two to four steps in moderate (20%) to excellent (85%) overall yields. Most of the compounds are new and have not been reported before.

The analogues (**1-54**) were evaluated for their ability to enhance K^+ currents in TREK-1 transfected HEK293 cells and/or *Xenopus* oocytes (Table 1). Most compounds were able to enhance TREK-1 current ($R_{TREK\text{-}1} > 1$) and the activation was rapidly reversed following washout of the tested compounds. The latter reveals that the compounds are not covalently bound to the target (reversible interaction), suggesting the molecules are not Michael acceptors. We note that the activities are ranging from inactive

to active compounds which comfort us in the fact that the activity of the compounds does not solely rely on the presence of the α,β–unsaturated carbonyl/nitrile. Compounds that can enhance the TREK-1 current by at least 2-fold ($R_{TREK-1} \geq 2$) can be considered as TREK-1 activators, which is the case for 24 compounds. Among these, eight analogues (**11-12**, **36**, **39**, **45**, **47-49**), all bearing a cyano- group at the α-position (X=CN), were particularly strong activators ($R_{TREK-1} \geq 4$). These compounds contained either the 3,4-catechol, the 3-furyl or the 2-indyl aromatic moieties (Ar), which is why we concentrated our efforts on the synthesis of several analogues in these families. Substitution of the carbonyl function to give carboxylic acids (R = OH), esters (R = alkoxy) or amides (R = amines) was well tolerated and the ability of these analogues to activate TREK-1 varied quadratically with the size/hydrophobicity of the R substituent, with an optimal R size (vertex) for the *t*-butyloxy group (R = OtBu). When we took into account both the aromatic ring (Ar) and the carboxyl substituent (R), we observed a trend suggesting that the volume of the molecule should be smaller than 230 $Å^3$ and the molecule should contain an aromatic ring with at least one hydrogen-bond donor group (N, O).

The *in vivo* antinociceptive activity of the analogues (**1**-**54**) was assessed in the acetic acid (AcOH)-induced-writhing test in mice (Table 1).

Overall, the influence of the α-substitution (X=CN, $CH_2NH_3^+$, COOH, F) was the most significant with a neat improvement in activity when the analogues bore an α-cyano substitution (15-30 times more active when X = CN versus X = F, COOH). Changing this substitution for an ammonium salt (X = $CH_2NH_3^+$) was unfavorable leading to compounds (**26** and **37**) which even caused hyperalgesia.

Variation of the carbonyl function to give carboxylic acids (R = OH), esters (R = alkoxy) or amides (R=amines) was well tolerated although it did not match the tendency observed *in vitro*. The best analgesic effects were observed for the esters bearing a cyclohexyloxy chain (**41** and **50**) able to inhibit 80-90% of AcOH-induced writhes. Presumably the esters are hydrolyzed *in vivo* and the parent carboxylic acids are exerting their activity. In order to confirm this hypothesis, we studied the metabolic

stability of compounds within the catechol / 3-furyl / 2-indyl series (R= OH **2/36/46**, R = ethoxy **1/38/47** and R = cyclohexyloxy **12/41/50**) in the presence of rat liver microsomes and the cofactor NADPH, hence simulating phase I metabolism. Under these conditions, the compounds were monitored over 24 hours by HPLC to determine their half-life ($t_{1/2}$). While the ethyl esters **1/38/47** were rapidly degraded into the corresponding carboxylic acids **2/36/46** ($t_{1/2}$ <4 min), the cyclohexyloxyanalogues **12/41/50** were more resistant to hydrolysis ($t_{1/2}$ = 25-45 min). Presumably the steric hindrance of the cyclohexyl ring protects the ester against rapid hydrolysis. This could explain why these compounds are potent both *in vitro* and *in vivo*.

We next examined the influence of the aromatic moiety (Ar) where the compounds bearing 3-furyl or 2-indyl series stood out with analgesic effects reaching the 80-90%.While the 3,4-catechol (**1-5** and **9-13**), 4-imidazoyl (**32-33**), naphthyl (**34-35**), 3-furyl (**36-45** except **37** and **44**) and 2-indyl (**46-54**) rings were well tolerated, the substituted phenyl (**15-24**), 3-thienyl (**25**), and 2-pyrrolyl (**30-31**) rings proved detrimental for the analgesic activity. We attempted to explain this observation by looking at the nature of the aryl moieties (size, hydrogen-bond acceptor/donor capacities, hydrophobicity) but none of the parameters seemed to correlate with the observed activity. We note that some heterocyclic rings even elicited hyperalgesia like the 3-thienyl bearing molecule **26** or more markedly the 3-pyridyl analogue **27**.

In order to validate the *in vivo* analgesic activity, the analgesic *hits* (% inhibition > 30% in writhing assay) were evaluated in the hotplate test (Table 1). While the first assay relied on chemically-induced nociception (AcOH), this assay relies on a thermal stimulus to induce pain. This assay is known to predict the clinical potency of opioid analgesics, such as morphine, but is usually not sensitive for non-opiate analgesics. To our delight, half of the hit molecules (16/32) caused a significant increase (>50%) in their action latencies (12-18 seconds compared to 8 seconds for the vehicle) against thermal stimuli. The most significant results were obtained for the 3-furyl analogues **36** (X = CN, R = OH) and **45** (X =

CN, R = anilino-) which presented higher antinociceptive activity at 45 minutes than at 30 minutes (lasting effect).

Considering the results obtained from the *in vitro* and *in vivo* assays, we grouped the molecules in different categories according to their *in vitro/in vivo* potencies (Table 2). The molecules possessing both a good ability to activate TREK-1 currents (R_{TREK-1}>4 at 100 µM or >2 at 20 µM) and good *in vivo* analgesic properties (> 30% inhibition in AcOH-induced writhing and >50% inhibition in hotplate assay) belonged to group **A** (3 molecules). Group **B** comprised 12molecules possessing a good ability to activate TREK-1 currents but no or little *in vivo* analgesic properties. Presumably these compounds are metabolized into inactive species that do not activate TREK-1 *in vivo* or are not bioavailable enough to reach their target. Group **C** included ten analgesic molecules which are not TREK-1 activators, hence may involve other pathways. Finally 29inactive molecules fitted into group **D**.

Table 2. Groups A-D. (a) TREK-1 activator if R_{TREK-1} > 4 at 100 µM or >2 at 20 µM. (b) not TREK-1 activator if R_{TREK-1} < 4 at 100 µM or <2 at 20 µM. (c) Analgesic if % antinociceptive effect AcOH > 30% and hotplate >50%. (d) Not analgesic if % antinociceptive effect AcOH< 30% or/and hotplate < 50%.

Groups	TREK-1 activator[a]	Not TREK-1 activator[b]
Analgesic[c]	**A** **36, 45, 54**	**C** **2, 18, 32-35, 41, 50, 52-53**
Not analgesic[d]	**B** **1, 5, 7, 9, 11-13, 39, 46-49**	**D** **3-4, 6, 8, 10, 14-17, 19-31, 37-38, 40, 42-44, 51**

In order to confirm the role of TREK-1 in the analgesic response of the compounds that belong to group **A**, the sensitivity to thermal stimuli (hotplate) of the most promising compounds (**36**, **45**, **54**) was evaluated in TREK-$1^{-/-}$ mice (KO) and compared with the nociceptive behavior of wild type (WT) mice. We also evaluated the naphthyl analogue **34** as a negative control since it does not activate TREK-1 (R_{TREK-1}< 2). The results (Table 3) show that compound **36** conserves its analgesic activity in WT mice, while KO mice showed an increase of sensitivity to heat, demonstrating that TREK-1 is a major effector of this compound's induced anti-nociceptive effect. On the other hand, compound **45**, as well as compound **54** and morphine, displayed less analgesic activity in this strain of mice (C57Bl/6J for Table 3 vs CD1 for Table 1). Strain differences in the analgesic effect of compounds such as morphine have previously been observed[26] and could be attributable to genetic differences in these animals. Future work will be carryout out to assess TREK-1 expression in different strains of mice and the specific pharmacological blockade of the TREK-1 channel to ascertain our conclusions. Finally compound **34** was expectedly inactive on both WT and KO mice, showing no dependence of TREK-1.

Finally the drug candidates **36**, **45** and **54** were evaluated for their selectivity against TREK-1 by testing them on other K2P channels (TREK-2, TRAAK and TASK-3).TREK-2 and TRAAK are a part from TREK subfamily and show high structure similarities with TREK-1 (78% homology with 63% identity for TREK-2 and 69% homology with 51% identity for TRAAK).[27] TASK-3 is a member of TASK subfamily and shares 50% of homology with TREK-1 with only 30% identity. The results (Table 4) show that only compound **36** is TREK-1-specific since its effects on other channels are weak (TREK-2) or absent (TRAAK, TASK-3). This difference in activity suggests that the compound is not PAINS. The other two compounds (**45** and **54**) show no selectivity between TREK-1 and TREK-2.

Table 3. Analgesic effect of compounds34, 36, 45, 54 (60 mg/kg, s.c.) on hotplate model in WT and KO black mice (n=7), 30 and 45 min after injection of modulators. % inhibition is the difference between the latency time observed after and before administration of compounds (60 mg/kg, s.c.). T-test: *≤0.05, **≤0.01, ***≤0.001.

Cpds	TREK-1$^{+/+}$	TREK-1$^{-/-}$	%Analgesic effect due to TREK-1
34	17.4± 8.8 %/31.1± 8.8%*	18.0± 7.9 %/34.4± 14.7 %	0% / 0%
36	121.2± 26.1%**/93.5± 19.4%**	78.1± 22.2%** /75.4± 21.1%**	36.6 ±25.1 % / 19.0 ± 25.7%
45	24.8± 10.8% */22.1± 6.4%	29.2± 11.5%/35.1± 14.4%	0 % ±/ 0%
54	27.9± 10.2%*/39.2± 13.4%**	18.7± 8.4%/51.2± 16.1 %*	33 ± 28.3% / 0%
Mor-phine	98.6± 22.5%/124.5± 15.6%	86.7± 27.7%/94.6± 18.5%	12.6 ± 17.9 %/24.3 ± 14.1%

Table 4. Modulation of K2P channels by group A compounds. R_{K2P} is the ratio of currents measured after and before the application of the compounds (concentration in μM). TREK-1, TREK-2, TRAAK and TASK-3 channels were expressed in HEK293 cells and currents were measured by whole-cell patch-clamp technique at +60 mV.

Cpds	TREK-1	TREK-2	TRAAK	TASK-3
36	4.5 (100)	1.8 (100)	1.1 (100)	1.0 (100)
45	4.6 (20)	3.0 (20)	1.0 (20)	0.5 (20)
54	2.2 (10)	1.6 (10)	0.9 (10)	1.2 (10)

CONCLUSION

We have conducted a focused SAR study to understand the influence of three main moieties (Ar, R, X), which allowed us to prepare 53 analogues of the lead compound **1**. In general, compounds bearing a cyano- group at the α-position (X = CN), either the 3-furyl or the 2-indyl as aromatic moieties (Ar), and acids (R = OH) displayed favorable activities both *in vitro* and *in vivo*. We also noted that the volume of the molecule should be below 230 Å^3. This SAR allowed us to identify the furyl analogue **36** for further evaluation due to potent *in vitro* and *in vivo* activities. Compound **36** demonstrates improved activities compared to the lead compound **1** and excellent selectivity for TREK-1 in the K2P channels panel.

Recent studies of the TREK-1 channel have already exposed its central role in pain perception,[3, 28, 29] and a number of pharmacologically active compounds have recently been reported to enhance the activity of TREK-1 channels.[5] However most, if not all, of these compounds are not selective for TREK-1 channels. We herein report the first TREK-1-selective agonist that displays analgesic activity *in vivo*. Thus, compound **36** represents a new tool for studying the effects of TREK-1 agonism *in vitro* and *in vivo* and should allow a better understanding of the implication of TREK-1 in the pain perception.

EXPERIMENTAL SECTION

Biological evaluation

Electrophysiology

Cell culture and transfection. HEK-293 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The coding sequence of the human TREK-1 (kcnk2) gene (GenBank Acc. No. NM_014217) was cloned into pIRES2-eGFP vector (Invitrogen, Cergy-Pontoise, France). In order to obtain a cell line that stably expresses the TREK-1 channel, the plasmid was transfected (25 ng) into HEK-293 cells using the Calcium Phosphate Method as described by the manufacturer protocol in the presence of 1.5 mg/mL Geneticin (G418) (Invitrogen, Cergy-Pontoise, France). After 20–30 days of culture in G418 selection medium, individual colonies of resistant cells were isolated by using cloning

cylinders (Sigma Aldrich, Saint-Quentin Fallavier, France).The human-TREK-1/HEK-293 (h-TREK-1/HEK) cells, which are a HEK-293 cells stably expressing the TREK-1 channel, were cultured in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Saint Aubin, France) supplemented with 10 % fetal calf serum (Hyclone, Thermo Fisher Scientific GMBH, Ulm, Germany), 1 % penicillin-streptomycin (Gibco, Life Technologies, Saint Aubin, France), and were grown in the presence of a h-TREK-1/HEK cell selection antibiotic (G418) (Gibco, Life Technologies, Saint Aubin, France) at 0.5 mg/mL, in a humidified incubator at 37 °C and 5 % CO2. Cells were passaged using 0.25 % trypsin-EDTA (Gibco, Life Technologies, Saint Aubin, France) after reaching a maximal confluence of 80 % and were used for up to 10 consecutive passages after thawing.

Patch-clamp recording. Electrophysiological experiments were performed on h-TREK-1/ HEK- cells plated on 35-mm dishes with a density of 30,000 cells/dish and used between 4 and 48 h after plating. Dishes were placed in the patch-clamp chamber and continuously perfused with the control bath solution containing (in millimol per litre): 140 NaCl, 10 TEA-Cl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and adjusted to pH 7.4 with NaOH. The pipette solution contained (in millimol per litre): 155 KCl, 3 $MgCl_2$, 5 EGTA, 10 HEPES and adjusted to pH 7.2 with KOH. All experiments were performed at room temperature (21–22 °C). TREK-1 current was observed using RK 400 patch clamp amplifier (Bio-Logic Science Instruments), low-pass filtered at 3 kHz and digitized at 10 kHz using a 12-bit analog-to-digital converter Digidata-1322 (Axon Instrument, Sunnyvale, CA, USA). For current visualization and stimulation protocol application, we used commercial software (Clampex 8.2). The patch pipettes were double-step-pulled from haematocrit-capillaries (Hirschmann Laborgeraete, Germany) using a vertical puller (PC-10, Narishige International, London, UK). Filled pipettes had resistances of 1–2 MΩ. Whole-cell patch-clamp configuration was obtained at a holding potential of −80 mV. Voltage ramp protocols consisted of a step to −100 mV (20 ms in duration) followed by a ramp going from −100 to +60 mV over 400 ms. Ramps were applied every 5 s. Current amplitudes were expressed as current densities (pA/pF, picoAmper per picoFarad) and results are presented as mean±standard error of the mean (SEM).

Current was recorded under control bath solution perfusion and under bath solution perfusion supplemented with different test molecules. The evaluation of the molecules effects was obtained by the ratio between currents after molecules application and currents under control perfusion. The ratio values were calculated at +60mV of the current/voltage (I/V) curves. Molecules were prepared as stock solutions in DMSO (Sigma Aldrich, Saint-Quentin Fallavier, France) (20 mM) and were used at a final concentration of 100µM.

Animals

Male CD1 mice (20-25 g) were purchased from Elevage Janvier. Homozygous TREK-1 (-/-) and wild-type (+/+) littermates were generated from crosses between heterozygous animals. The detailed experimental methodology used to generate and genotype TREK-1 knockout mice has been previously described.[30] Animals were housed under controlled environmental conditions (22 °C; 55% humidity) and kept under a 12/12 h light/dark cycle, with food and water *ad libitum* for a week before start the experiments to acclimatize.The behavioural experiments were performed blind to the treatment, in a quiet room, by the same experimenter taking great care to avoid or minimize discomfort of the animals. Animals were randomly divided in groups of 8 mice each and each animal was used only once per compound and euthanized. Animal care and experiments were performed in accordance with the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).All animal procedures were approved by the local Animal Ethics Committee of Auvergne (Comité Régional d'Ethique en Matière d'Expérimentation Animale Auvergne).

Behavioral testing in mice. *Acetic acid writhing assay.* Mice were pretreated with the compound (10 mg / kg, s.c.) or vehicle (5% Tween 80 in saline (0.9%)) 15 minutes before injection of acetic acid (0.6% solution, 10 mL/kg) into the peritoneal cavity of the animal. This activates nociceptors directly and / or produces inflamed viscera (sub-diaphragmatic organs) and subcutaneous (muscle wall) of tissues. The number of induced abdominal writhes was determined during 20 min after the injection of acetic acid. The inhibition of abdominal writhes (analgesic effect) is determined by determining the

percentage of the number of cramps induced in the presence of compound reported to the number of cramps induced in the presence of vehicle. *Hot-plate test.* After acclimatization for 5 min on a plate at room temperature, mice were placed on a plate set at 52 °C until they started licking their forepaws (Latencies) (cut-off time: 30 s). After having obtained two consecutive stable latencies, treatment effects were assessed after 30 and 45 min.

Microsomal stability. Aliquots contained 30 µL of rat liver microsomes (20 mg/mL), 237 µL Phosphate buffer (pH 7.4) and 30 µL of NADPH solution (40 mM) were prepared and mixed thoroughly. 3 µL drug solution (40 mM in DMSO) was added to each aliquot, the samples was vortexed and incubated at 37°C with stirring. Aliquots (300 µL) were stopped at specific time points (2, 5, 15, 30, 60 min and 24 hours), and protein were precipitated in equal volume of cold acetonitrile. The mixture was then filtered with microfilter (0.45µm) prior to HPLC analysis (wavelength: 254.4 nm).

Chemistry

General. Solvents were distilled prior to use. Other reagents were used as received. Products in organic solutions were dried over dry sodium sulphate prior to evaporation of the solvent under reduced pressure. Thin layer chromatography (TLC) was performed on pre-coated glass backed silica Duracil 25 UV 254 (Macherreynagel). Spots were visualized using UV light (254 nm) before using an ethanolic solution of phosphomolybdic acid (heating). Purifications by column chromatography were carried out on silica gel (70-230 mesh) or on C18 (30µm). Melting points were measured by a Reichert plate-heating microscope. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brücker Avance 400 spectrometer at 400.13 and 100.61 MHz respectively. Chemical shifts δ are reported in ppm relative to solvent residual signal (^{1}H, ^{13}C). The coupling constants *J* are given in Hertz (Hz). Electron Impact Mass Spectra (EI-MS) were obtained on a spectrometer Hewlett Packard 5989B at 70 eV. High Resolution Electro-spray Ionisation Mass Spectra (HR-ESI-MS) were obtained from the "Service de Spectrométrie de masse du service UBP-START, (Université Blaise Pascal)". GC/MS analysis conditions used for progress of the reaction; column: UB 1701 (14% cyanopropylphenyl)-

methylpolysiloxane, injector temperature : 250 °C; oven temperature : 50 °C for 2 min then heating 50 °C/min until 290 °C. High performance liquid chromatography (HPLC) analysis conditions used to determine purity of the product ; column: Poroschell 120 EC-C18 2.7 µm (3.0 x 50 mm), temperature column: 40 °C; injection: 10 µL, 1 mL/min.; solvents used A- water with 0.1% formic acid and B-methanol (95:5 to 5:95, method A) or A-water and B-acetonitrile (95:5 to 5:95, method B), Method UV using 254.4 nm and 280.5 nm). On the basis of LC-MS, all final compounds were >95% pure unless otherwise stated.

Procedure A - Steglich esterification. Under an argon atmosphere, cyanoacetic acid **70** (1 to 1.1 equiv.) was dissolved in dry dichloromethane and cooled to 0 °C. At this temperature, DMAP (cat.) is added to the mixture, followed by a dropwise addition of the appropriate alcohol (1.0 to 1.3 equiv.). After 5 minutes of stirring, DCC (1.0 equiv.) is added in portion to the mixture. The mixture is allowed to reach room temperature and stirred overnight. The white precipitate is filtered and the resulting solution was concentrated *in vacuo*. Chromatography purification of the residue on silica gel with cyclohexane / ethyl acetate gave the desired ester.

Procedure B - Fischer esterification. To a solution of cyanoacetic acid 70 (1.0 equiv.) and alcohol (1.5 equiv.) in toluene were added a few drops of concentrated H_2SO_4. The mixture was stirred at reflux for 24 hours, using a Dean-Stark apparatus to remove water. The reaction mixture was concentrated to half its initial volume under reduced pressure, diluted with ethyl acetate (20 mL), and washed with saturated $NaHCO_3$ solution (3 x 8 mL), water and brine. The crude product was purified by flash chromatography (cyclohexane/ ethyl acetate) on silica gel to give the desired.

Procedure C - Knoevenagel reaction. To a solution of 3,4-dihydroxybenzaldehyde 55 (1.1 equiv.) in dry DCM (60 mL) under argon atmosphere were added 4-methylpentyl-2-cyanoacetate (1.0 equiv.) and piperidine (0.9 equiv.). The reaction was carried out at room temperature for 20 hours. The mixture was then quenched by the addition of a saturated aqueous solution of NH_4Cl (20 mL) and extracted with ethyl acetate (3 x 8 mL). The combined organic fractions were dried (Na_2SO_4). After removal of the

solvent under reduced pressure, flash chromatography purification of the residue on silica gel (cyclohexane - ethyl acetate: 1:0 to 0:1, v/v) gave the desired product.

Procedure D - Hydrolysis reaction with TFA. The *t*-butyl ester (1.0 equiv.) was dissolved in 3.6 mL of trifluoroacetic acid. The resulting solution was stirred for 1 h 30 at room temperature before removal of the TFA under reduced pressure. The residue was triturated with ether and the solvent was evaporated under reduce pressure. This work up was repeated three times to give the desired acid.

Procedure E - Horner-Watsworth-Emmons Reaction. A solution of phosphonate (1.3 eq) in dry THF was added dropwise to a stirred suspension of NaH (50% in oil, 1.4 eq) in dry THF under Ar atmosphere at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and a solution of aldehyde (1 eq) in dry THF was added. The reaction was carried out at room temperature until total consumption of aldehyde (TLC). The reaction mixture was quenched with water. The aqueous phase was extracted with EtOAc (3 x 20 mL) and the combined organic layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the purification of the residue by column chromatography on SiO_2 using cyclohexane/EtOAc as eluent gave the desired alkene.

(E)-2-Cyano-3-(3,4-dihydroxyphenyl)acrylic acid **2.** Scheme 1, step i : Tert-butyl 2-cyanoacetate **72** was prepared following procedure A using tert-butanol (1.86 mL, 19.40 mmol, 1.1 equiv.), cyanoacetic acid **70** (1.5 g, 17.63 mmol, 1.0 equiv.) and DCC (3.64 g, 17.63 mmol, 1.0 equiv.) in dry dichloromethane (20 mL). Chromatography purification of the residue on silica gel with 9:1, v/v, cyclohexane / ethyl acetate gave the desired product **72** as a pale liquid (1.13 g, 45%). Rf = 0.36 (SiO_2, ethyl acetate/ cyclohexane, 1:9, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.36 (s, 2H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ161.9, 113.6 (CN), 84.3, 27.8, 25.9. CAS Registry Number: 1116-98-9. Scheme 1, step v : (*E*)-tert-butyl-2-cyano-3-(3,4-dihydroxyphenyl)acrylate **10** was prepared following procedure C using 3,4-dihydroxybenzaldehyde **55** (1.11 g, 8.00 mmol, 1.0 equiv.), tert-butyl-2-cyanoacetate **72** (1.13 g, 8.00 mmol, 1.0 equiv.) and piperidine (710 µL, 7.20 mmol, 0.9 equiv.) in anhydrous DCM (150 mL). After removal of the solvent under reduced pressure, flash chromatography purification of the residue

on silica gel (cyclohexane - ethyl acetate: 1:0 to 0:1, v/v) gave the desired product **10**as a pale yellow solid (1.09 g, 52%). m.p: 211 °C. Rf = 0.20 (SiO_2, ethyl acetate/ cyclohexane, 4:6, v/v). ^{1}H NMR (400 MHz, MeOD-d4): δ 8.03 (s, 1H), 7.63 (d, J = 2.2 Hz, 1H), 7.36 (dd, J = 8.4, 2.2 Hz, 1H), 6.88 (d, J = 8.3 Hz, 1H),1.56 (s, 9H). ^{13}C NMR (101 MHz, MeOD-d4): δ 163.6, 155.6, 152.9, 147.1, 127.8, 125.1, 117.7, 117.5 (CN), 116.7, 100.2, 84.1, 28.3. HR-ESI-MS calcd for $C_{14}H_{15}NO_4$ ($M + Na^+$) = 284.0899; found 284.0905. HPLC: 254.4 nm: 97% (H_2O/MeOH) t_R = 18.681 min. Scheme 1, step viii : (*E*)-2-Cyano-3-(3,4-dihydroxyphenyl)acrylic acid **2** was prepared following procedure D using (E)-tert-butyl 2-cyano-3-(3,4-dihydroxyphenyl)acrylate **10** (75.5 mg, 0.29 mmol, 1.0 equiv.), TFA (1 mL) to give the desired product **2**as a yellow powder (53.5 mg, 90%). m.p: 261 °C. Rf = 0 (ethyl acetate/ cyclohexane, 2:8, v/v). ^{1}H NMR (400 MHz, MeOD-d4): δ 8.11 (s, 1H), 7.65 (s, 1H), 7.37 (2 x d, J = 8.0 Hz, 2H). 13C NMR (101 MHz, MeOD-d4): δ 166.3, 156.4, 153.1, 147.3, 128.0 - 116.9 (C-ar), 99.4, 85.2. HPLC: 254.4 nm: 100% (H_2O/MeOH), t_R = 12.422 min. (Method A). HR-ESI-MS calcd for $C_{10}H_7NO_4$ (M + Na+) = 228.0273; found 228.0263. CAS Registry Number: 122520-79-0.

2-(3,4-Dihydroxybenzylidene)malonic acid **6.** Scheme 2, step iii : To 3,4-dihydroxybenzaldehyde **55** (800 mg, 5.97 mmol, 1 eq) in anhydrous DMF (7 mL) was added a solution MOMCl (951.7 µL, 12.53 mmol, 1.1 eq), followed by i-PrNEt_2 (6.24 mL, 35.82 mL 6 eq). After stirring for 5 h at room temperature, a solution of 2 N NH_4OH was added slowly and the mixture was extracted with Et_2O (3 x 50 mL). The combined organic phases were washed with brine, dried (Na_2SO_4) and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, 20% EtOAc/cyclohexane) to obtain 3,4-bis(methoxymethoxy)-benzaldehyde **91** as a clear liquid obtained (917.4 mg, 68%). Rf = 0.55 (20% EtOAc/cyclohexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.85 (s, 1H, CHO), 7.66 (d, J = 2.0 Hz, 1H), 7.50 (dd, J = 8.0, 2.0 Hz, 1H), 7.27 (d, 1H, J = 8.0 Hz), 5.32 and 5.31 (2 x s, 4H), 3.55 and 3.54 (2 x s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 190.8 (CHO), 152.6, 147.5, 131.1, 126.3, 116.0, 115.3, 95.3, 95.0, 56.5, 56.3. CAS Registry Number: 6515-06-6. Scheme 2, step iv : A mixture of 3,4-bis(methoxymethoxy)benzaldehyde **91** (300 mg, 1.33 mmol, 1 eq), di-tert-butyl malonate (311.1 µL,

1.39 mmol, 1.05 eq), piperidine (22.3 µL, 0.23 mmol, 0.17 eq) and benzoic acid (18.0 mg, 0.15 mmol, 0.11 eq) in toluene (2 mL) was heated under reflux using a Dean-Stark trap. After 20 h, toluene was evaporated off and the reaction mixture was dissolved in EtOAc (10 mL). This solution was washed with H_2O (10 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by column chromatography (SiO_2, 20% EtOAc/cyclohexane) to lead to di-tert-butyl-2-(3,4-bis(methoxymethoxy)benzylidene)-malonate **92** as a clear oil (286.1 mg, 51%). Rf = 0.41 (20% EtOAc/cyclohexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.45 (s, 1H), 7.31 (d, J = 2.0 Hz, 1H), 7.19 (dd, J = 8.5, 2.0 Hz, 1H), 7.12 (d, J = 8.5 Hz, 1H), 5.25 (s, 2H), 5.21 (s, 2H), 3.50 (2 x s, 6H), 1.54 and 1.52 (2 x s, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.1, 163.8, 149.1, 146.9, 139.5, 127.6 - 115.9 (C-Ar), 95.6, 95.1, 82.2, 81.9, 56.3, 56.2, 28.1, 28.0. 2-(3,4-Dihydroxy-benzylidene)malonic acid **6** was prepared according to procedure D with TFA (4.5 mL) and di-tert-butyl 2-(3,4-bis(methoxymethoxy)-benzylidene)malonate **92** (286.1 mg, 0.67 mmol, 1.0 equiv.) in 4.5 mL of dry DCM. Acid **6** was obtained as a pale green powder (102.6 mg, 68%). m.p: 208 °C. Rf = 0 (ethyl acetate/ cyclohexane, 6:4, v/v). ^{1}H NMR (400 MHz, MeOD-d^4): δ 7.50 (s, 1H), 7.08 (d, J = 2.1 Hz, 1H), 6.97 (dd, J = 8.3, 2.1 Hz, 1H), 6.77 (d, J = 8.3 Hz, 1H). ^{13}C NMR (101 MHz, MeOD-d^4): δ 171.2, 167.5, 149.7, 146.5, 142.4, 125.7, 124.5, 124.4, 117.1, 116.2. HPLC: 254.4 nm: 98% (H_2O/MeOH), t_R = 0.879 min (Method A). HR–ESI-MS calcd for $C_{10}H_7O_6$ ($M + H^+$) = 223.0243; found 223.0256.

(*Z)-3-(3,4-Dihydroxyphenyl)-2-fluoroacrylic acid* **7**. Scheme 2, step vii : To a solution of freshly distilled of diisopropylamine (2.6 mL, 19.3 mmol, 4.1 equiv.) in dry THF (8 mL), was added 1.6 M of n-BuLi (11.7 mL, 18.84 mmol, 4.0 equiv.) at -30 °C. The solution was stirred for 30 min at -30 °C, then transferred into the prepared solution of ethyl 2-fluoroacetate **94** (500 mg, 4.71 mmol, 1.0 equiv.) and TMSCl (3.6 mL, 28.3 mmol, 6.0 equiv.) in 45 mL of dry THF at -78 °C. The reaction mixture was stirred then warmed to 0 °C over a period of 4 h. The reaction was quenched with a saturated aqueous solution of $NaHCO_3$ (100 mL) at 0 °C. The aqueous phase was extracted with ether (3 x 50 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated (20 mL). The organic phase was washed

with a solution of tartaric acid (50 mL) and was stirred overnight at room temperature. The aqueous layer was extracted twice with ether (3 x 20 mL). The organic layers were combined, dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by chromatography (Et_2O/pentane, 1:9, v/v) to lead to Ethyl 2-fluoro-2-(trimethylsilyl)acetate **95** as colourless oil (800 mg, 91%). R_f = 0.68 (ethyl acetate/ cyclohexane, 5:95, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.95 (d, J = 48.1 Hz, 1H), 4.20 (q, J = 7.0 Hz, 2H), 1.28 (t, J = 7.0 Hz, 3H), 0.18 (s, 9H).The ^{1}H RMN is agreement with the literature. CAS Registry Number: 128822-96-8. Scheme 2, step viii :To a solution of freshly distilled of diisopropylamine (436.8 µL, 3.37 mmol, 1.5 equiv.) in dry THF (13 mL), was added 1.6 M of n-BuLi (2.11 mL, 3.37 mmol, 1.5 equiv.) at -30 °C. The solution was stirred for 30 min at -30 °C and then allowed to cool to -78 °C. Ethyl 2-fluoro-2-(trimethylsilyl)acetate **95** (400 mg, 2.25 mmol, 1.0 equiv.) dissolved in dry THF (2 mL) was added to the LDA solution and stirred for 40 min at -78 °C, followed by addition of 3,4-bis(methoxymethoxy)-benzaldehyde **91** (523.2 mg, 2.31 mmol, 1.03 equiv.) in dry THF (2 mL). The reaction was carried out at -78 °C until total consumption of **95** by GC-MS. The reaction mixture was quenched with a saturated aqueous solution of NH_4Cl (30 mL) at 0 °C. The separated aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and evaporated. The residue was purified by silica gel chromatography (20% EtOAc/cyclohexane) giving (*Z*)-ethyl 3-(3,4-bis(methoxymethoxy)phenyl)-2-fluoroacrylate as a colourless oil (263.3 mg, 37%). R_f = 0.31 (ethyl acetate/ cyclohexane, 2:8, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38 (s, 1H), 7.19 – 6.80 (m, 2H), 6.79 (d, J = 35.1 Hz, 1H), 5.17 and 5.15 (2 x s, 4H), 4.24 (q, J = 7.2 Hz, 2H), 3.43 and 3.41 (2 x s, 6H), 1.28 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 161.5 (d, J = 34.0 Hz), 148.6, 147.6, 146.0 (d, J = 263.0 Hz), 125.5 (d, J = 7.1 Hz), 125.4 (d, J = 4 Hz), 118.7 (d, J = 8.5 Hz), 117.2 (d, J = 4.5 Hz), 116.2, 95.5, 62.1, 56.3, 14.7. ^{19}F NMR (376 MHz, $CDCl_3$): δ -127.6. A solution of (*Z*)-ethyl 3-(3,4-bis(methoxy-methoxy)phenyl)-2-fluoroacrylate (263.3 mg, 0.838 mmol, 1.0 equiv.) in MeOH (10 mL) and 1 M NaOH (1.68 mL, 1.68 mmol, 2.0 equiv.) was then stirred at room temperature for 2 h. After removing the solvent under reduced pressure, the residue was

acidified to pH 2 with 0.5 M HCl. The aqueous phase was extracted with EtOAc (3 x 20 mL) and the combined organic layer was dried with $MgSO_4$. Evaporation of the solvent under reduced pressure gave (*Z*)-3-(3,4-bis(methoxymethoxy)phenyl)-2-fluoro-acrylic acid **96** as a pale yellow oil (219 mg, 91%). R_f = 0.30 (ethyl acetate/ cyclohexane, 4:6, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.80 (s, 1H), 7.51 (s, 1H), 7.24 (m, 2H), 6.95 (d, *J* = 35 Hz, 1H), 5.28 and 5.26 (2 x s, 4H), 3.53 (br s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.2 (d, *J* = 34.8 Hz), 149.2, 146.6, 145.6 (d, *J* = 261.6 Hz), 126.1 (d, *J* = 8.3 Hz), 125.3 (d, *J* = 4.0 Hz), 119.86 (d, *J* = 3.8 Hz), 119.2 (d, *J* = 8.4 Hz), 116.4, 95.5, 56.5. ^{19}F NMR (376 MHz, $CDCl_3$): δ -128.8. Scheme 2, step ix : TFA (4 mL) was then added to a solution of (*Z*)-3-(3,4-bis(methoxymethoxy)-phenyl)-2-fluoroacrylic acid **96** (219 mg, 0.765 mmol, 1.0 equiv.) in 4 mL of dry DCM. The reaction mixture was stirred overnight at room temperature. The solvent and TFA were removed under reduced pressure. The residue was triturated with Et_2O and evaporated under reduced pressure (3 times) and was purified by chromatography (C_{18}, H_2O/MeOH) to give the acid **7** as a pale grey powder (86.4 mg, 57%). m.p: 172 °C. R_f = 0 (ethyl acetate/ cyclohexane, 2:8, v/v). ^{1}H NMR (400 MHz, MeOD-*d*$_4$): δ 7.19 (s, 1H), 6.97 (d, *J* = 8.2 Hz, 1H), 6.79 (d, *J* = 36.1 Hz, 1H), 6.78 (d, *J* = 8.3 Hz, 1H). ^{13}C NMR (101 MHz, MeOD-*d*$_4$): δ 165.1 (d, *J* = 34.8 Hz), 146.9, 146.1, 145.6 (d, *J* = 261.6 Hz), 124.2 (d, *J* = 4.0 Hz), 123.7 (d, *J* = 7.0 Hz), 117.5 (d, *J* = 8.2 Hz), 116.9, 116.4. ^{19}F NMR (376 MHz, MeOD-*d*$_4$): δ -129.3. HPLC: 254.4 nm: 95% (H_2O/MeOH), t_R = 7.206 min (Method A). HR –ESI-MS calcd for $C_9H_6O_2F$ (M - H^+) = 197.0250 found 197.0249.

(E)-2-Cyano-3-(3,4-dihydroxyphenyl)acrylamide **8**. Scheme 2, step x : (E)-tert-butyl-2-cyano-3-(3,4-dihydroxyphenyl)acryloyl-carbamat*e* **98** was prepared following procedure A using *tert*-butyl 2-cyanoacetylcarbamate **97** (40.1 mg, 0.218 mmol, 1.0 equiv.), 3,4-dihydroxybenzaldehyde **55** (31.6 mg, 0.229, 1.05 equiv.), piperidine (cat), dry DCM (2.0 mL) to give the desired product**98**as a orange powder (25 mg, 38%). R_f = 0.35 (ethyl acetate/ cyclohexane, 2:8, v/v). ^{1}H NMR (400 MHz, MeOD-*d*$_4$) δ 7.99 (s, 1H), 7.64 (d, *J* = 2.2 Hz, 1H), 7.39 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.89 (d, *J* = 8.3 Hz, 1H), 1.54 (br s, 9H). ^{13}C NMR (101 MHz, MeOD-*d*$_4$): δ 163.3, 158.0, 156.0, 148.2, 146.4, 136.8- 129.9, 110.0, 83.8,

28.0. Scheme 2, step xi :(*E*)-2-Cyano-3-(3,4-dihydroxyphenyl)acrylamide **8** was prepared following procedure D with TFA (2 mL), (E)-tert-butyl 2-cyano-3-(3,4-dihydroxyphenyl)acryloylcarbamate**98** (70 mg, 0.23 mmol, 1.0 equiv.) in 2 mL of dry DCM. The reaction mixture was stirred for 1 h at room temperature. The amide **8** was obtained as a pale grey (50 mg, 99%). m.p: 252 °C. Rf = 0 (ethyl acetate/ cyclohexane, 4:6, v/v). ^{1}H NMR (400 MHz, DMSO-d6): δ 10.13 and 9.58 (2 x br s, 2H), 7.95 (s, 1H), 7.73 and 7.60 (2 x br s, 2H), 7.53 (d, J = 2.5 Hz, 1H), 7.28 (dt, J = 8.0, 2.5 Hz, 1H), 6.88 (d, J = 8.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d^{6}): δ 163.4, 150.6, 145.7, 125.1 - 116.1 (C-ar), 115.9, 101.1. HPLC: 254.4 nm: 100 % (H_2O/MeOH), t_R = 3.288 min (Method A). HR –ESI-MS calcd for $C_{10}H_8N_2O_3$ (M + H^+) = 204.9697; found 205.0535. CAS Registry Number: 122520-85-8.

(E)-3-(3,4-Dihydroxyphenyl)-2-(morpholine-4-carbonyl)-acrylonitrile **9**. Scheme 1, step i :To a mixture of morpholine (1 mL, 11.8 mmol, 1.0 equiv.) in 4 mL dichloromethane was added dropwise a solution of cyanoacetic acid **70** (1.0 g, 11.8 mmol, 1.0 equiv. in 8 mL dichloromethane) at room temperature. After the addition, a solution of DCC (2.4 g, 11.8 mmol, 1.0 equiv.) in 5 mL of dichloromethane was added to the mixture. Then the mixture was heated to reflux for 24 hours. After cooling down, the precipitate was filtrated and the filtrate was concentrated under vacuum to give pure product 3-morpholino-3-oxopropanenitrile **79** (beige solid, 1.8 g, <99%). Rf = 0.08 (SiO_2, ethyl acetate/ cyclohexane, 4:6, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.75-3.65 (m, 4H); 3.65-3.59 (m, 2H); 3.49 (s, 2H, H-2); 3.48-3.42 (m, 2H). ^{13}C NMR (101 MHz, CDCl3): δ 160.4, 113.9 (CN), 66.6, 66., 46.8, 42.9, 24.8. CAS Registry Number: 15029-32-0. Scheme 1, step vi :To a mixture of 3-morpholino-3-oxopropanenitrile **79** (500 mg, 3.24 mmol, 1.1 equiv.), 3,4-dihydroxybenzaldehyde **55** (407 mg, 2.95 mmol, 1.0 equiv.) and 15 mL toluene were added 0.10 mL piperidine (1.03 mmol, 0.35 equiv.) and 0.40 mL acetic acid (6.79 mmol, 2.3 equiv.). Then the mixture was heated to reflux until the TLC test shows the reaction is complete. Cooled the mixture to room temperature, then the precipitate was filtered, washed to afford **9** as yellow solid (600 mg, 74%). Rf = 0.14 (ethyl acetate/ cyclohexane, 7:3, v/v). ^{1}H NMR (400 MHz, MeOD-d^{4}): δ 7.56 (m, 2H); 7.28 (dd, J = 8.4, 1.9 Hz, 1H); 6.85 (d, J = 8.4 Hz, 1H);

3.77-3.64 (m, 8H, H-morph). ^{13}C NMR (101 MHz, MeOD-d^4): δ 166.1, 153.4, 151.9, 147.0, 126.3, 116.9, 116.6, 125.6, 117.7, 101.3, 67.6. HR –ESI-MS calcd for $C_{14}H_{15}N_2O_4$ (M + H$^+$) = 275.1032; found 275.1019. HPLC: 254.4 nm: 95% (H_2O/CH_3CN) t_R = 13.697 min. (Method B). 280.4 nm: 95% (H_2O/CH_3CN) t_R = 13.697 min. (Method B).

(E)-2-Carboxy-3-phenylprop-2-en-1-aminium 2,2,2-trifluoroacetate **15**. Scheme 2, step i : *(E)*-Ethyl 2-((tert-butoxycarbonylamino)methyl)-3-phenylacrylate **88** was prepared following procedure E using ethyl 3-*tert*-butoxycarbonylamino-2-diethoxyphosphoryl-3-propionate **87** (500 mg, 1.41 mmol, 1.3 equiv.), benzaldehyde **56** (110.7 µL, 1.08 mmol, 1.0 equiv.), NaH (50% in oil, 76.6 mg, 1.51 mmol, 1.4 equiv.) in dry THF (4 + 4 + 1.5 mL) to give the desired product **88** as a colourless oil (201 mg, 53%). R_f(*E*) = 0.43 (ethyl acetate/ cyclohexane, 1:9, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.78 (s, 1H), 7.53 – 7.31 (m, 5H), 5.07 (br s, 1H), 4.30 (q, *J* = 7.1 Hz, 2H), 4.22 (d, *J* = 5.7 Hz, 2H), 1.44 (br s, 9H), 1.36 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): *δ* 167.8, 155.5, 141.8, 134.4, 129.6 -128.8, 128.7, 79.3, 61.2, 37.8, 28.4, 14.3. Scheme 2, step ii :A solution of (*E*)-ethyl 2-((*tert*-butoxycarbonyl-amino)methyl)-3-phenylacrylate **88** (201.0 mg, 0.658 mmol, 1.0 equiv.) in MeOH (15 mL) and 1 M of LiOH (1.97 mL, 1.97 mmol, 3.0 equiv.) was stirred at room temperature for 18 h. After removing the solvent under reduced pressure, the residue was acidified to pH 2 with 0.5 M HCl. The aqueous phase was extracted with EtOAc (3 x 20 mL) and the combined organic phase was dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave (*E*)-2-((*tert*-butoxycarbonylamino)methyl)-3-phenylacrylic acid as a pale yellow oil (186.2 mg, 99%). R_f = 0 (ethyl acetate/ cyclohexane, 1:9, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.84 (s, 1H), 7.33 (m, 5H), 5.10 (br s, 1H), 4.19 (d, *J* = 4.5 Hz, 2H), 1.40 (br s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): *δ* 170.9, 154.7, 144.2, 129.8, 80.5, 38.1, 28.4. CAS Registry Number: 889855-42-9. *(E)*-2-Carboxy-3-phenylprop-2-en-1-aminium 2,2,2-trifluoroacetate**15** was then prepared according to procedure D using (*E*)- 2-((*tert*-butoxycarbonylamino)methyl)-3-phenylacrylic acid (186.2 mg, 0.67 mmol, 1.0 equiv.) and TFA (4 mL) in dry DCM (4 mL). The reaction mixture was stirred overnight at room temperature. The ammonium salt **15** was obtained as a pale green powder (152 mg, 87%). R_f = 0

(ethyl acetate/ cyclohexane, 5:5, v/v). m.p. 160 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.39 (br s, 1H), 8.12 (br s, 3H), 7.92 (s, 1H), 7.49 (m, 5H), 3.80 (br s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 167.5, 143.8, 133.5-125.6, 35.4. HPLC: 254.4 nm: 98% (H_2O/MeOH), t_R = 1.421 min (Method A). HR –ESI-MS cald for $C_{10}H_{12}NO_2$ ($M + H^+$) = 178.0868; found 178.0876.

2-Benzylidenemalonic acid **16**. Scheme 2, step v :Under an atmosphere of argon, piperidine (cat) was added to a solution of benzaldehyde **56** (478.9 µL, 4.71 mmol, 1.0 equiv.) and diethyl malonate (715.1 µL, 4.71 mmol, 1.0 equiv.) in absolute EtOH (10 mL). The reaction mixture was heated under reflux overnight. After cooling, the solvent was removed under vacuum, the residue was dissolved in EtOAc (20 mL), and extracted with H_2O (3 x 20 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by column chromatography (ethyl acetate/ cyclohexane, 5:95, v/v) to obtain diethyl 2-benzylidenemalonate **93** as a clear oil (874 mg, 75%). R_f = 0.5 (ethyl acetate/ cyclohexane, 5:95, v/v). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (s, 1H), 7.60 – 7.32 (m, 5H), 4.46 – 4.25 (m, 4H), 1.43 – 1.21 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.7, 164.1, 142.1, 132.9, 130.5, 129.4, 128.8 126.4, 61.7, 61.6, 14.1, 13.9. CAS Registry Number: 5292-53-5. Scheme 2, step vi : To a solution of diethyl 2-benzylidenemalonate **93** (874 mg, 3.52 mmol, 1.0 equiv.) in MeOH (60 mL) was added aqueous solution a 3 M KOH (3.0 mL, 10.56 mmol, 3.0 equiv.) and the mixture was stirred at room temperature for 12 h. After removing the solvent under reduced pressure, the residue was acidified to pH 2 with 0.5 M HCl. The organic layer was extracted with EtOAc (3 x 10 mL), dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave a mixture (clear oil and white powder). The residue was dissolved in 20 mL of ether and 3 drops of sulphuric acid were added. The mixture was stirred for 4 days at room temperature. A 1M aqueous solution of HCl (5 mL) was added; the organic phase was extracted with Et_2O (3 x 10 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The residue was purified by column chromatography (C_{18}, H_2O/MeOH) to lead to the corresponding acid **16** as a white powder (293.7 mg, 43%). m.p. 218 °C. ^{1}H NMR (400 MHz, MeOD-d_4): δ 7.57 (s, 1H), 7.50 – 7.29 (m, 5H). ^{13}C NMR (101 MHz, MeOD-d_4): δ 170.6, 165.6, 141.8 - 129.9,

128.8. HPLC: 254.4 nm: 98% (H_2O/MeOH), t_R = 7.668 min (Method A). HR –ESI-MS calcd for $C_{10}H_7O_4$ ($M + H^+$) = 191.0344; found 191.0344. CAS Registry Number: 584-45-2.

(*Z)-2-Fluoro-3-phenylacrylic acid* **17**. Scheme 2, step viii : To a solution of freshly distilled of diisopropylamine (343.6 µL, 2.57 mmol, 1.15 equiv.) in dry THF (13 mL), was added 1.6 M of n-BuLi (1.6 mL, 2.57 mmol, 1.15 equiv.) at -30 °C. The solution was stirred for 30 min at -30 °C, then allowed to cool to -78 °C. Ethyl 2-fluoro-2-(trimethylsilyl)acetate **95** (400 mg, 2.25 mmol, 1.0 equiv.) dissolved in dry THF (2 mL) was added LDA solution and stirred for 40 min at -78 °C, followed by addition of benzaldehyde **56** (235.1 µg, 2.31 mmol, 1.03 equiv.) in dry THF (2 mL). The reaction was carried out at -78 °C until total consumption of ethyl 2-fluoro-2-(trimethylsilyl)acetate **95** by GC-MS. The reaction mixture was quenched with a saturated aqueous solution of NH_4Cl (30 mL) at 0 °C. The aqueous layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were dried ($MgSO_4$), filtered and evaporated under reduced pressure. The residue was purified by silica gel chromatography (5% EtOAc/cyclohexane) giving (Z)-ethyl 2-fluoro-3-phenylacrylateas a colourless oil (216.7mg, 50%). Rf = 0.4 (ethyl acetate/ cyclohexane, 2:8, v/v). 1H NMR (400 MHz, $CDCl_3$): δ 7.65 - 7.31 (m 5H), 6.92 (d, J = 35.2 Hz, 1H), 4.36 (q, J = 7 Hz, 2H), 1.40 (t, J = 7 Hz, 3H). 13C NMR (101 MHz, $CDCl_3$): δ 161.5 (d, J = 34.2 Hz), 147.0 (d, J = 267.4 Hz), 131.2 (d, J = 4.0 Hz), 130.3 (d, J = 8.1 Hz), 129.7, 128.8, 117.5 (d, J = 4.7 Hz), 61.9, 14.2. ^{19}F NMR (376 MHz, $CDCl_3$): δ -126.8. CAS Registry Number: 20397-59-5. To a solution of (Z)-ethyl 2-fluoro-3-phenylacrylate (216.7 mg, 1.12 mmol, 1.0 equiv.) in MeOH (10 mL) was added 1 M NaOH (2.23 mL, 2.23 mmol, 2 equiv.) and was stirred at room temperature for 2 h. After removing the solvent under reduced pressure, the residue was acidified to pH 2 with 0.5 M HCl. The organic phase was extracted with EtOAc (3 x 20mL), dried with MgSO4. Evaporation of the solvent under reduced pressure gave**17** as a white powder (169.6 mg, 91%). m.p. 155 °C. Rf = 0 (ethyl acetate/ cyclohexane, 2:8, v/v). ^1H NMR (400 MHz, $CDCl_3$): δ 8.75 (br s, 1H), 7.67-7.38 (m, 5H), 7.07 (d, J = 34.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.2 (d, J = 35.0 Hz), 145.9 (d, J = 264.2 Hz), 131.0, 130. 7, 130.4, 128.9, 120.1 (d, J = 4.0 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): δ -126.8. HPLC: 254.4 nm:

95% (H_2O/MeOH), t_R = 7.206 min (Method A). HR–ESI-MS calcd for $C_9H_6O_2F$ (M - H+) = 165.0352; found 165.0341. CAS Registry Number: 20397-61-9.

(E)-Ethyl 2-cyano-3-(furan-2-yl)acrylate **28**. Scheme 1, step vii : To a solution of furan-2-carbaldehyde **63** (450 µL, 5.20 mmol, 1.0 equiv.) in water (20 mL) was added ethyl-2-cyanoacetate **71** (590 mg, 5.20 mmol, 1.0 equiv.). The reaction was carried out at room temperature for 72 hours. The solid produced was isolated by filtration and dried to give the desired product **28**as a pale yellow solid (160 mg, 16%). m.p. 91 – 92 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.22 (dd, J = 1.1 Hz, 0.6 Hz, 1H), 8.14 (s, 1H), 7.52 (d, J = 3.4, 1H), 6.87 (dd, J = 3.6 Hz, 1.7 Hz, 1H), 4.28 (q, J = 7.1 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ 162.2, 150.1, 148.2, 139.2, 124.7, 115.4, 114.4, 96.9, 62.2, 14.0. HR – ESI-MS calcd for $C_{10}H_{10}NO_3$ (M + H^+) = 192.0661 found 192.0654. HPLC: 254.4 nm: 99% (H_2O/CH_3CN) t_R = 15.603 min. (Method B), 280.4 nm: 99% (H_2O/CH_3CN) t_R = 15.603 min. (Method B).

(E)-2-cyano-3-(furan-3-yl)acrylic acid **36**. (*E*)-*tert*-butyl 2-cyano-3-(furan-3-yl)acrylate **39** was prepared following procedure A using *tert*-butyl 2-cyanoacetate **72** (300 mg, 2.12 mmol, 1.0 equiv.), 3-furaldéhyde **68** (202.5 µL, 2.34 mmol, 1.1 equiv.), piperidine (cat), dry DCM (15 mL) to give the desired product as a white powder (205.5 mg, 45%). m.p. 124 °C. R_f = 0.40 (ethyl acetate/ cyclohexane, 5:95, v/v). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.28 (s, 1H, H-3), 8.18 (s, 1H, H-ar), 7.75 – 7.70 (m, 1H, H-ar), 7.27 - 7.23 (m, 1H, H-ar), 1.56 (br s, 9H, 3 x CH_3). ^{13}C NMR (101 MHz, MeOD-d_4): δ 162.7 (C-1), 152.3, 146.9, 146.2 (CH-ar and C-3), 122.2 (C-ar), 116.8 (CN), 109.9 (CH-ar), 104.2 (C-2), 84.5 (C-4), 28.2 (3 x CH_3). Acid **36** was prepared following procedure D using (*E*)-*tert*-butyl 2-cyano-3-(furan-3-yl)acrylate **39** (75.5 mg, 0.29 mmol, 1 equiv.), TFA (1 mL) to give the desired product as a pale yellow powder (53.5 mg, 90%). m.p. 218 °C. R_f = 0 (ethyl acetate/ cyclohexane, 1:9 v/v). ^{1}H NMR (400 MHz, MeOD-d_4): δ 8.28 (s, 1H, H-4'), 8.26 (s, 1H, H-3), 7.73 (m, 1H, H-3'), 7.27 (d, J = 1.9 Hz, 1H, H-2'). ^{13}C NMR (101 MHz, MeOD-d_4): δ 165.2 (C-1), 152.2 (C-2' or C-4'), 147.0 (C-3'), 146.8 (C-3),

122.3 (C-1'), 117.0 (CN), 109.0 (C-2' or C-4'), 103.3 (C-2). HPLC: 254.4 nm: 100% (H_2O/MeOH), t_R = 9.249 min (Method A). HR –ESI-MS calcd for $C_8H_5NO_3$ (M + Na^+) = 186.0167; found 186.0170.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

Procedures for the synthesis and characterization of compounds **11-14, 18-27, 29-54, 73-75, 79, 81, 83-86, 90, 97, 99**

Molecular formula strings for final compounds **1-54**

^{13}C and ^{1}H NMR spectra for new compounds

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Author Contributions

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ABBREVIATIONS

cAMP, cyclic adenosine-3',5'-monophosphate ; CDC, Cinnamyl 1-3,4-dihydroxy-alpha-cyanocinnamate ; DCPIB, 4-(2-Butyl-6,7-Dichlor-2-CycloPentyl-Indan-1-on-5-yl) oxybutyric acid ; HEK, Human Embryonic Kidney cells ; K2P, Two-Pore Domain Potassium channel ; KO, Knock-Out ; PKA, Protein kinase A ; PKC, Protein kinase C ; PUFA, PolyUnsaturated Fatty Acid ; TRAAK, TWIK-related arachidonic acid-stimulated K^+ channel ; TREK, TWIK-RElated K^+ channels ; TWIK, Tandem of pore domains in a Weak Inward rectifying K^+ channel ; VRAC, volume-regulated anion channels.

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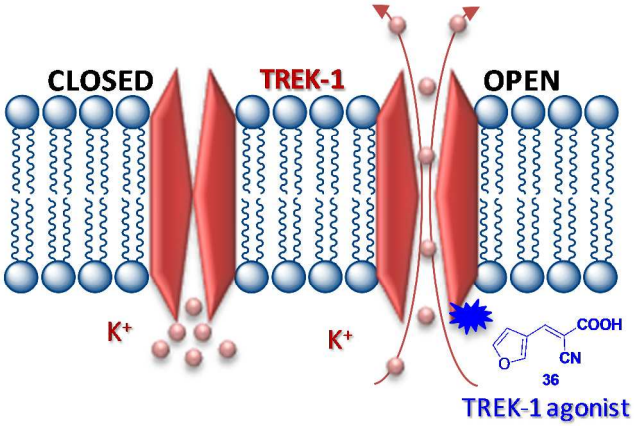

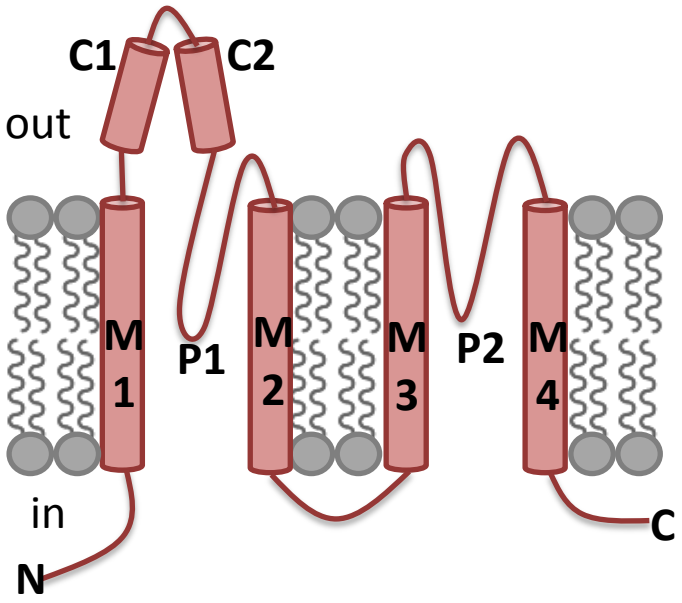
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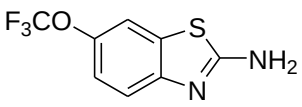

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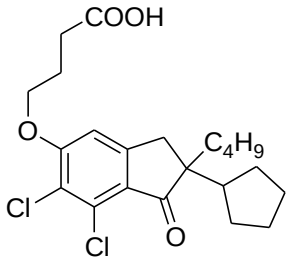

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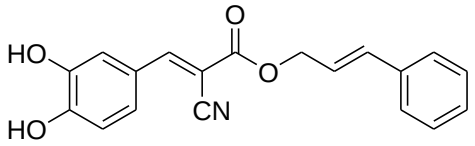

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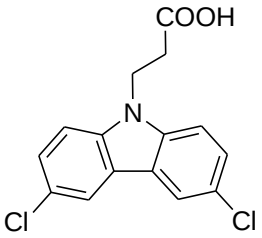

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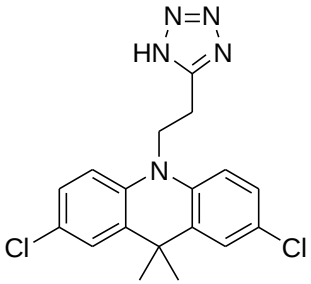

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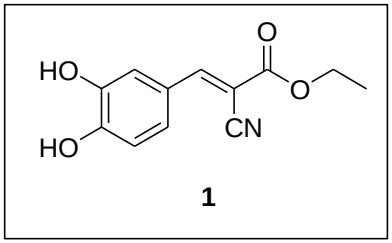

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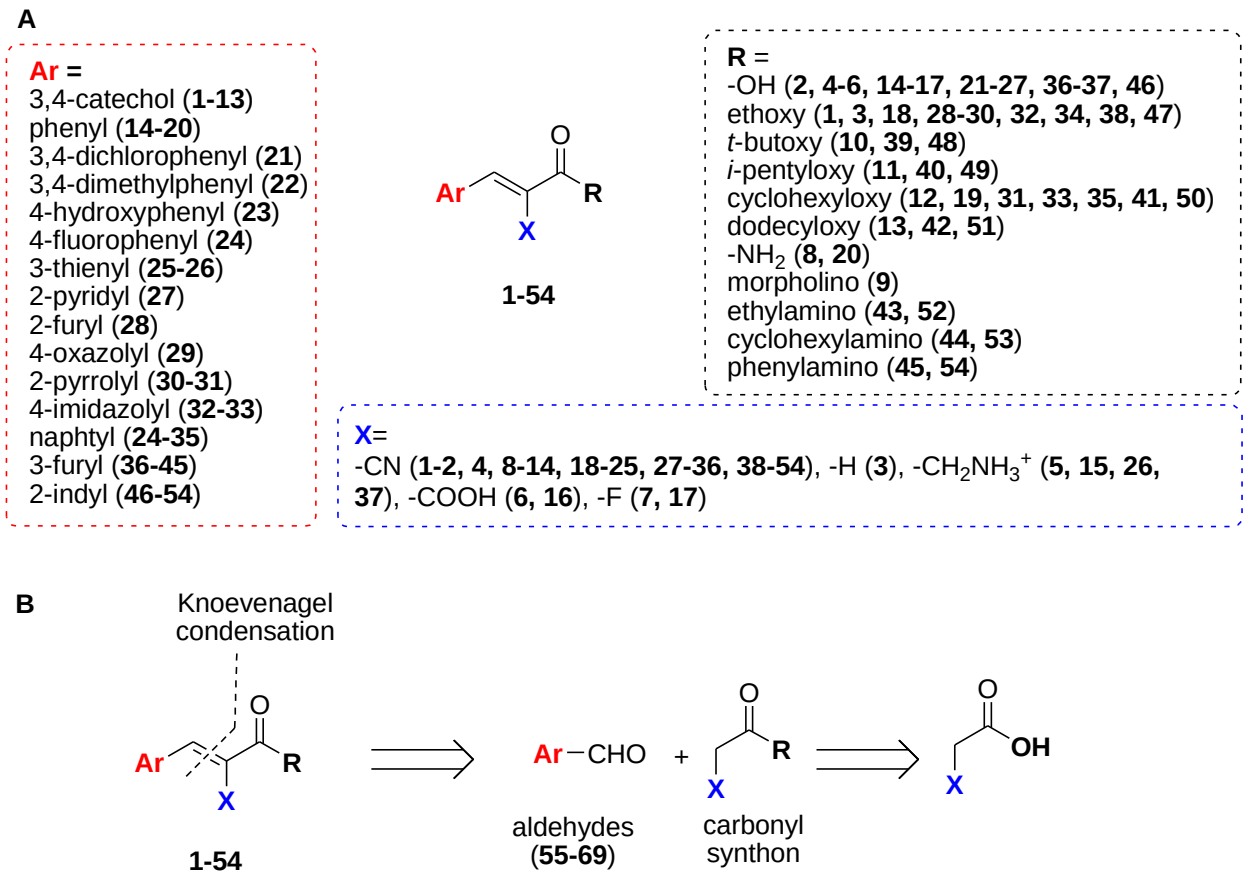
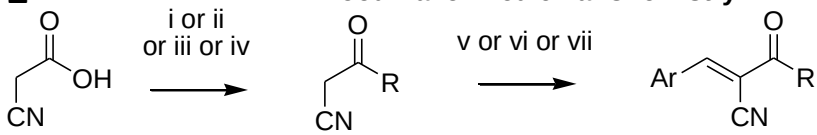
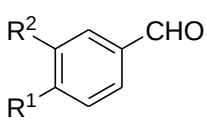

A
Ar =
3,4-catechol (1-13)
phenyl (14-20)
3,4-dichlorophenyl (21)
3,4-dimethylphenyl (22)
4-hydroxyphenyl (23)
4-fluorophenyl (24)
3-thienyl (25-26)
2-pyridyl (27)
2-furyl (28)
4-oxazolyl (29)
2-pyrrolyl (30-31)
4-imidazolyl (32-33)
naphtyl (24-35)
3-furyl (36-45)
2-indyl (46-54)
Ar
X
R
1-54
R =
-OH (2, 4-6, 14-17, 21-27, 36-37, 46)
ethoxy (1, 3, 18, 28-30, 32, 34, 38, 47)
t-butoxy (10, 39, 48)
i-pentyloxy (11, 40, 49)
cyclohexyloxy (12, 19, 31, 33, 35, 41, 50)
dodecyloxy (13, 42, 51)
-NH2 (8, 20)
morpholino (9)
ethylamino (43, 52)
cyclohexylamino (44, 53)
phenylamino (45, 54)
X=
-CN (1-2, 4, 8-14, 18-25, 27-36, 38-54), -H (3), -CH2NH3+ (5, 15, 26, 37), -COOH (6, 16), -F (7, 17)
B
Knoevenagel condensation
Ar
X
R
1-54
Ar−CHO
aldehydes (55-69)
carbonyl synthon
OH



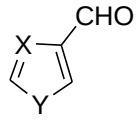
70

71, R = ethoxy (i. 66%)
72, R = *t*-butoxy (i. 45%)
73, R = *i*-pentyloxy (i. 48%)
74, R = cyclohexyloxy (i. 77%)
75, R = dodecyloxy (i. 87%)
76, R = ethylamino (ii. 30%)
77, R = cyclohexylamino (iii. 34%)
78, R = phenylamino (iv. 94%)
79, R = morpholino (i. >99%)

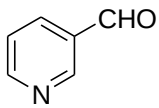
1, Ar = 3,4-catechol, R = ethoxy (v, 55%)
9, Ar = 3,4-catechol, R = morpholino(vi, 74%)
10, Ar = 3,4-catechol, R = *t*-butoxy (v, 52%)
11, Ar = 3,4-catechol, R = *i*-pentyloxy (v, 66%)
12, Ar = 3,4-catechol, R = cyclohexyloxy (v, 71%
13, Ar = 3,4-catechol, R = dodecyloxy (v, 76%)
18, Ar = phenyl**,** R = ethoxy (v, 84%)
19, Ar = phenyl, R = cyclohexyloxy (v, 73%)
28, Ar = 2-furyl**,** R = ethoxy (vii, 16%)
29, Ar = 4-oxazoyl**,** R = ethoxy (vii, 41%)
30, Ar = 2-pyrrolyl**,** R = ethoxy (v, 66%)
31, Ar = 2-pyrrolyl, R = cyclohexyloxy (v, 51%)
32, Ar = 4-imidazolyl**,** R = ethoxy (v, 77%)
33, Ar = 4-imidazolyl, R = cyclohexyloxy (v, 63%)
34, Ar = 2-naphtyl**,** R = ethoxy (v, 65%)
35, Ar = 2-naphtyl, R = cyclohexyloxy (v, 73%)
38, Ar = 3-furyl**,** R = ethoxy (v, 33%)
39, Ar = 3-furyl, R = *t*-butoxy (v, 42%)
40, Ar = 3-furyl, R = *i*-pentyloxy (v, 32%)
41, Ar = 3-furyl, R = cyclohexyloxy (v, 33%)
42, Ar = 3-furyl, R = dodecyloxy (v, 35%)
43, Ar = 3-furyl, R = ethylamino (v, 46%)
44, Ar = 3-furyl, R = cyclohexylamino (v, 43%)
45, Ar = 3-furyl, R = phenylamino (v, 11%)
47, Ar = 2-indyl**,** R = ethoxy (v, 61%)
48, Ar = 2-indyl, R = *t*-butoxy (v,42%)
49, Ar = 2-indyl, R = *i*-pentyloxy (v, 27%)
50, Ar = 2-indyl, R = cyclohexyloxy (v, 98%)
51, Ar = 2-indyl, R = dodecyloxy (v, 39%)
52, Ar = 2-indyl, R = ethylamino (v, 70%)
53, Ar = 2-indyl, R = cyclohexylamino (v, 57%)
54, Ar = 2-indyl, R = phenylamino (v, 74%)
80, Ar = phenyl, R = *t*-butoxy (v, 56%)
81, Ar = 3,4-dichlorophenyl, R = *t*-butoxy (v, 51%)
82, Ar = 3,4-dimethylphenyl, R = *t*-butoxy (v, 50%)
83, Ar = 4-hydroxyphenyl, R = *t*-butoxy (v, 71%)
84, Ar = 4-fluorophenyl, R = *t*-butoxy (v, 80%)
85, Ar = 3-thienyl, R = *t*-butoxy (v, 66%)
86, Ar = 3-pyridyl, R = *t*-butoxy (v, 58%)



55, $R^1 = R^2 = OH$
56, $R^1 = R^2 = H$
57, $R^1 = R^2 = Cl$
58, $R^1 = R^2 = CH_3$
59, $R^1 = = OH$, $R^2 = H$
60, $R^1 = F$, $R^2 = H$



61, X = CH, Y = S
64, X = N, Y = O
66, X = N, Y = NH
68, X = CH, Y = O



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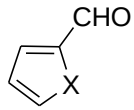
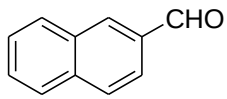


63, X = O
65, X = NH



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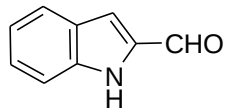


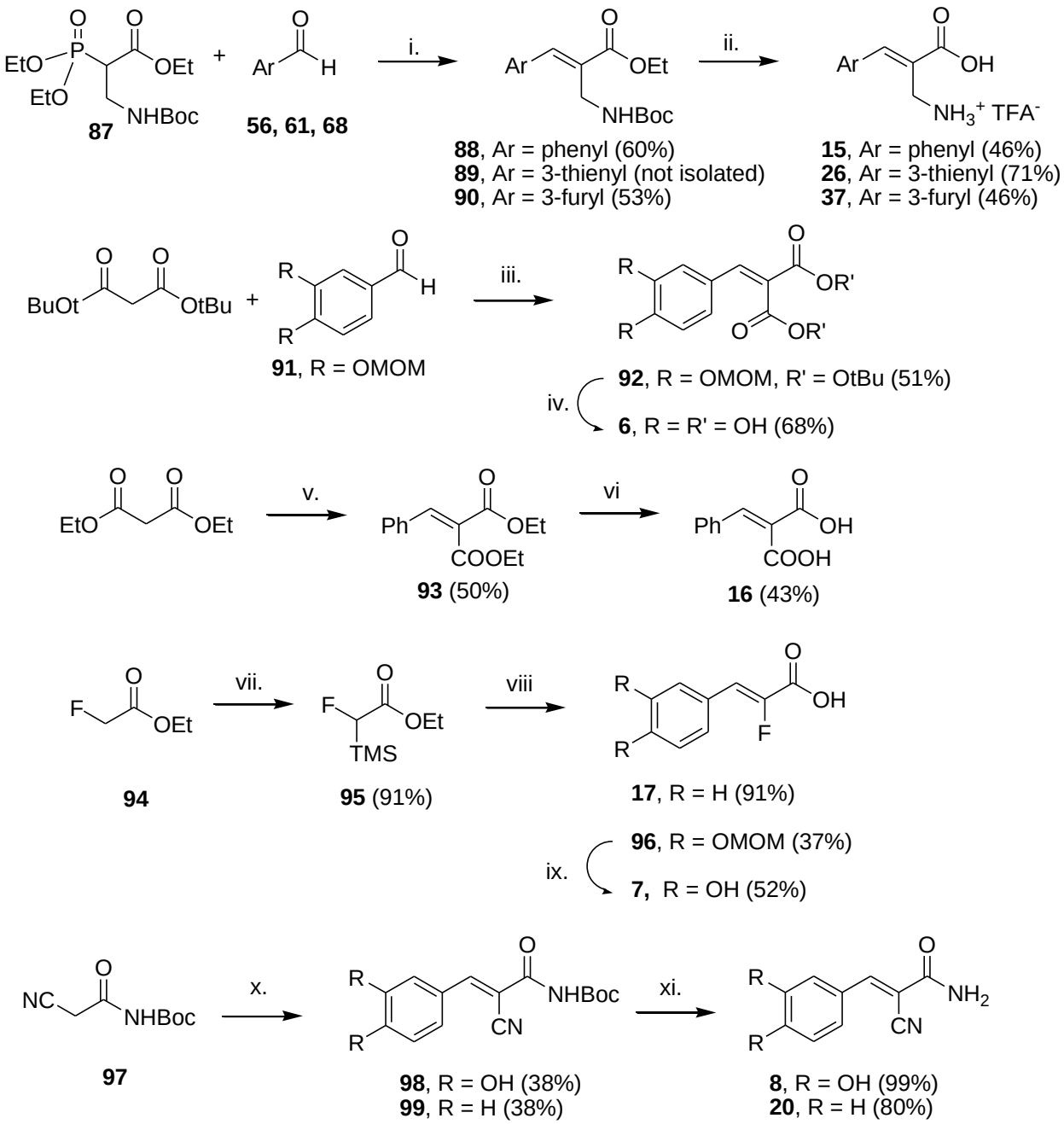
69

10, 39, 48, 80-86

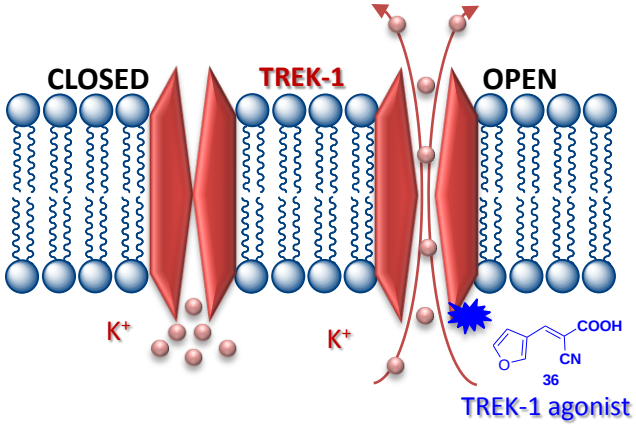
2, Ar = 3.4-catechol (90%)
14, Ar = phenyl (90%)
21, Ar = 3,4-dichlorophenyl (90%)
22, Ar = 3,4-dimethylphenyl 57%)
23, Ar = 4-hydroxyphenyl (35%)
24, Ar = 4-fluorophenyl (90%)
25, Ar = 3-thienyl (57%)
27, Ar = 3-pyridyl (80%)
36, Ar = 3-furyl (90%)
46, Ar = 2-indyl(78%)

Conditions. i. RH, DCC, DMAP, DCM, 0°C to RT, 24h; **ii.** a. $(COCl)_2$, DCM/DMF, 0°C to RT, 2h b. $EtNH_2$ (2M in THF), NEt_3, DCM, 0°C to RT, 3h. **iii.** cyclohexanamine, EtONa, EtOH, RT, 72h**. i**v. a. PCl_5, DCM, reflux, 30 min b. aniline, DCM, reflux, 12h. **v.** ArCHO **55-69**, piperidine, DCM, RT 24h. **vi**. ArCHO **55**, piperidine, AcOH, toluene reflux, 6h. **vii.** ArCHO **63-64**, H_2O, RT, 72h. **viii.** TFA, DCM, RT, 1h.

Scheme 2



Conditions: **i.** NaH, THF, RT, 2 days. **ii**. a. LiOH 1M, MeOH, RT, 18h. b. TFA, DCM, RT, overnight**. iii**. Piperidine, toluene, reflux, 2h. **iv.** TFA, DCM, RT, 12h. **v.** ArCHO **56**, piperidine, EtOH, reflux, 12h. **vi.** a. NaOH 1.5 M, MeOH, RT, 12h. b. H_2SO_4, Et_2O, RT, 4 days. **vii**. a. LDA, THF, -30 °C, 30 min. b. TMSCl, THF, -78 °C to RT over 4h. c. Tartaric acid, Et_2O, RT, 12h. **viii.** a. ArCHO (**56** or **91**), LDA, THF, -78 °C, 30 min. b. NaOH 1.5 M, MeOH, RT, 2h. **ix**. TFA, DCM, RT, 12h. **x**. a. EtOH, DCC, DCM, RT, 1h. b. ArCHO **55**-**56**, piperidine, DCM, RT, 12h. **xi.** TFA, DCM, RT, 1h.


CLOSED
TREK-1
OPEN
K+
K+
COOH
CN
O
36
TREK-1 agonist