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Fatty acyl amides of endogenous tetrahydroisoquinolines are active at the recombinant human TRPV1 receptor

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Abstract—The SAR of capsazepine revealed that tetrahydroisoquinoline (TIQ) moiety is a core pharmacophore of TRPV1 activity. This implied that conjugates of endogenous TIQs with fatty acids would be active at TRPV1 receptors. Six such compounds were synthesized and tested for calcium mobilization at recombinant TRPV1 receptors overexpressed in HEK293 cells. Three compounds showed partial TRPV1 agonism with EC_{50} values in the low micromolar range and maximal efficacies between 25% and 55% of capsaicin.

1. Introduction

The identification and pharmacological characterization of endogenous molecules involved in pain transmission is a topic of interest due to the potential impact on human suffering. A focus of our research has been the identification of endogenous lipids involved in pain transmission. To this end, *N*-arachidonoyl dopamine **1** a molecule bearing striking structural similarity to capsaicin **2**, the active component of chili peppers, was synthesized,[1] and shown to exhibit biological effects similar to those of endocannabinoids.[2] Subsequently, **2** was shown to be active at the capsaicin receptor or transient receptor potential vanilloid type 1 (TRPV1) cation channel and was identified as an endogenous compound in bovine brain by HPLC-ESI-LC/MS/MS.[3] In addition to exogenous and endogenous lipids, noxious thermal stimulation or protons activate TRPV1 channels, which leads to the influx of monovalent cations. Hence modulators of TRPV1 activity hold promise as therapeutic (Fig. 1) agents.[4]

A review of the literature on the development of the first TRPV1 antagonist, capsazepine **3**, revealed that the tetrahydroisoquinoline moiety is a core pharmacophore of TRPV1 antagonism.[5] While less potent than capsazepine **3**, compound **4** was a weak partial agonist at the TRPV1 receptor (Fig. 2).

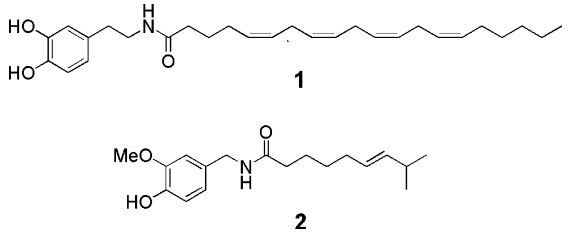

Figure 1. Structures of *N*-arachidonoyl dopamine **1** and capsaicin **2**.

A variety of tetrahydroisoquinolines (TIQs) occur in mammals including salsolinol **5**,[6] isosalsoline **6**,[7] norsalsolinol **7**,[8] and tetrahydropapaveroline (norlaudonosoline) **8**,[9] (Fig. 3). Salsolinol **5** has been found in human brain, although its function is unclear.[8]

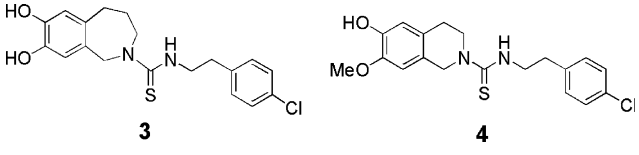

Figure 2. TRPV1 antagonists capsazepine **3** and compound **4** reported by Walpole et al.

Keywords: Fatty acyl amide; Tetrahydroisoquinoline; TRPV1; Lipid; Calcium; Capsaicin; Capsazepine; *N*-arachidonoyl dopamine; Salsolinol.
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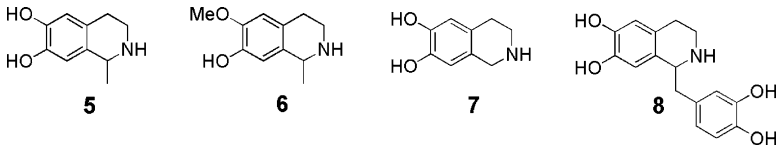


Figure 3. Selected naturally occurring tetrahydroisoquinolines.

The formation of these heterocycles in vivo is usually accounted for by a Pictet–Spengler reaction of dopamine with aldehydes. This cyclization is particularly facile with electron rich aromatics and occurs under physiological conditions.[10] Since acetaldehyde, the primary oxidation product of ethanol, seems to be required for the formation of **5** a great deal of effort has focused upon **5** as an ethanol linked intoxicant.

Based on the aforementioned SAR of capsazepine, we hypothesized that fatty acyl amides of tetrahydroisoquinolines should possess the properties necessary for activity at TRPV1. Six of these compounds were synthesized and evaluated for both agonism and antagonism at human recombinant TRPV1 receptors expressed in HEK293 cells.

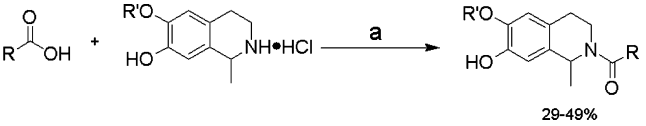
2. Results and discussion

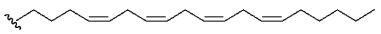
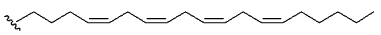
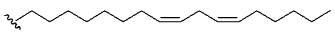
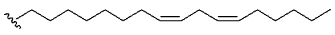
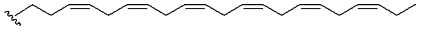
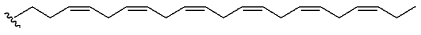
2.1. Chemistry

Utilizing the peptide coupling reagent TCTU under basic conditions, salsolinol and isosalsoline were conjugated to arachidonic, linoleic, and docosohexaenoic acid to give the amides **9**–**14** in moderate yield (29–49%) (Table 1). Also produced was a product arising from apparent O-acylation of the desired amide.

These compounds display complex ^{1}H and ^{13}C NMR spectra indicating that they exist as mixtures of interconverting *N*-acyl rotamers in solution. The rotamers can be observed on the NMR timescale as distinct species. Figure 4 shows the ^{1}H NMR spectrum of the low field region of compound **9** at both 25 °C and 100 °C.

Table 1. Synthesis of fatty *N*-acyl TIQs (**9**–**14**) used in this study



Compound	R	R′
9		H
10		CH_3
11		H
12		CH_3
13		H
14		CH_3

(a) 1-H-Benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-tetrafluoroborate(1)-3-oxide (TCTU) 1.3 equiv, Et_3N 5 equiv, CH_2Cl_2, rt.

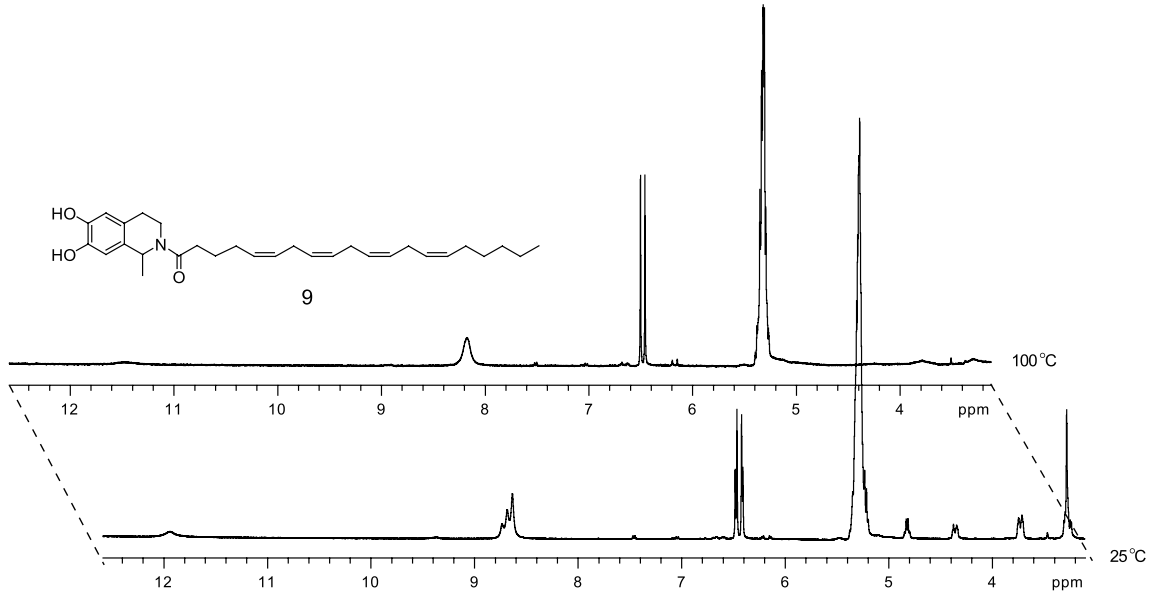


Figure 4. ^{1}H NMR of compound **9** in DMSO-d_6 at 25 °C and 100 °C, showing rotational isomerism about the amide bond.

At 25 °C, two distinct sets of signals for several protons occurred, including the phenolic protons near δ 12.0 and δ 8.8–8.6 as well as the aromatic protons at $\sim\delta$ 6.4 (four singlets). Upon heating to 100 °C the aromatic resonances at δ 6.4 have coalesced to two sharp singlets, and the phenolic proton signals partially coalesce. Moreover, upon cooling to 25 °C, the ^{1}H NMR spectrum was identical to that recorded prior to heating. The rotational isomerism of *N*-acyl tetrahydroisoquinolines about the amide bond is a well-known phenomenon.[11] All efforts at separation of the rotamers by column chromatography were unsuccessful.

2.2. Biology

The compounds **9**–**14** were assayed for activity in HEK293 cells stably expressing human TRPV1, by dual excitation ratio Ca^{2+} imaging with the commercial dye fura-2 AM[12] and compared with activity on non-transfected HEK 293 cells (Fig. 5).

Compounds **9**, **11**, and **13** were partial agonists at TRPV1 showing maximum effects of 55%, 36%, and 25% of that of the full agonist capsaicin (10 μM, Fig. 6). The N-acylated isosalsolines (**10**, **12**, and **14**, where R′ = Me) showed weak partial agonism with lower maximum efficacies of 18%, 11%, and 14%, respectively.

Partial agonists act as agonists when administered alone but exhibit antagonist properties in the presence of a full agonist. Indeed, capsaicin-induced calcium mobilization (10 nM–1 μM) following pretreatment with compounds **9**–**14** (100 nM–10 μM) resulted in reduced efficacy of capsaicin. For example, calcium mobilization in response to capsaicin (1 μM) following application of test compounds (1 μM) or vehicle yielded the following

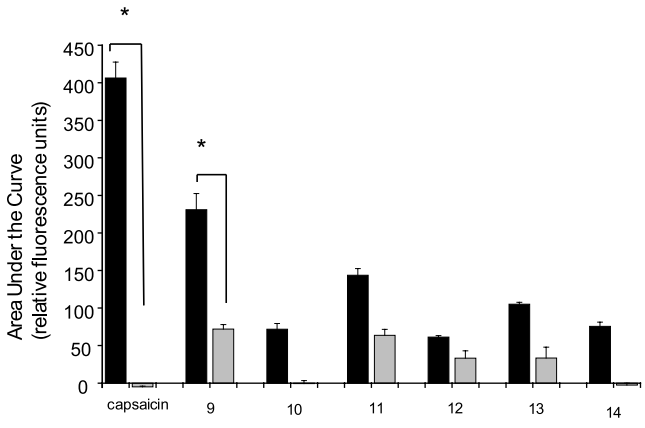


Figure 5. The effects of **9**–**14** or capsaicin on calcium mobilization in hTRPV1-transfected HEK 293 cells (black bars) or non-transfected HEK 293 cells (light bars). Data are presented as area under the curve in relative fluorescence units. All compounds were tested at 10 μM except for compound **14** (12 μM on non-transfected HEK 293 cells). Capsaicin and **9** evoked a significant calcium mobilization in hTRPV1 transfected cells compared with non-transfected controls (n = 3–28 per group, $^*P < 0.01$, analysis of variance, Bonferroni's post hoc). Error bars indicate standard error of the mean.

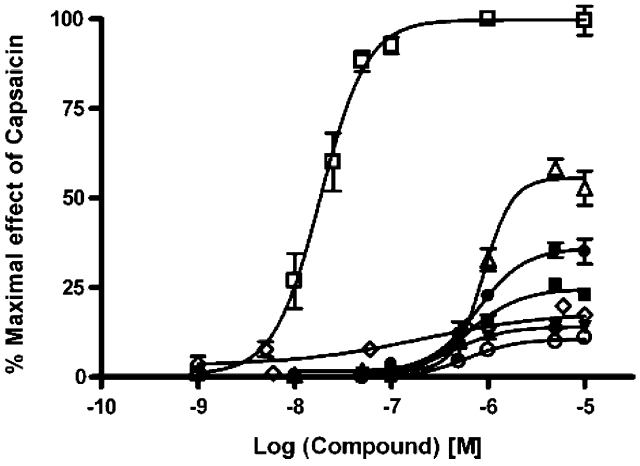


Figure 6. The effects of test compounds **9**–**14** (□ = capsaicin, Δ = **9**, ◇ = **10**, ● = **11**, ○ = **12**, ■ = **13**, ▼ = **14**) and capsaicin on calcium mobilization in hTRPV1-transfected HEK293 cells. Data are presented as a percent of the maximal effect of capsaicin using the formula (response-background fluorescence)/(maximal response to capsaicin-background fluorescence).

reductions in the maximal effect of capsaicin: **9** = 40%, **10** = 35%, **11** = 35%, **12** = 25%, **13** = 45%, **14** = 5%. All test compounds reduced the maximal effect of capsaicin without affecting its potency. These observations are consistent with a partial agonist action although receptor desensitization due to pre-application of the test compounds may account for some fraction of the inhibition produced by the test compounds.

In the 1970s salsolinol **5** was postulated to play a role in the etiology of alcoholism, due to its apparent formation by condensation of dopamine with acetaldehyde, the primary oxidation product of ethanol. This so-called biogenic amine hypothesis of alcoholism is controversial and has been largely abandoned.[13] However, the identification of endogenous acylation products of tetrahydroisoquinolines (**9**–**14**) would add a new dimension to such claims. The veracity of such hypotheses requires further study, and current efforts are underway to identify these compounds in tissue samples. Recently, the in vivo transamidation of acetaminophen with arachidonic acid has been reported to occur to yield the potent TRPV1 agonist AM404.[14] Additionally, it would be unsurprising for **9**–**14** to be active at GPCRs that respond to lipid ligands, given the cross reactivity of other lipoamides such as **1**, anandamide, and AM404 at CB1[3,15] and TRPV1.[3,16]

3. Conclusions

N-Acyl tetrahydroisoquinolines are prepared by acylation of the corresponding tetrahydroisoquinoline with a fatty acid. These compounds exist as mixtures of interconvertible rotamers observable on the NMR timescale. The behavior of these compounds toward the human TRPV1 receptor overexpressed in HEK293 cells was examined by Ca^{2+} influx. All of the compounds (**9**–**14**) exhibited a degree of partial agonism.

4. Experimental

4.1. General

All fatty acids (except linoleic acid) were purchased from Nu-Chek Prep (Elysian, MN). TCTU was purchased from Matrix Innovation (Montreal, QC) and (±)-isosalsoline HCl was a gift from NIMH (Dr. George Brine). Solvents used for reactions were of the highest commercial grade and used without distillation, however they were degassed prior to use and all manipulations performed under nitrogen or argon using Schlenk techniques. Mass spectra were obtained on a Thermo Finnigan Mat 95 XP magnetic sector instrument. Infrared spectra were recorded on an Avator 3600 FT-IR. All NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a 400 MHz Varian Inova NMR spectrometer and referenced to residual solvent protons. Flash chromatography was conducted using silica gel 60 (40–63 mm) and analytical thin-layer chromatography (TLC) was performed on precoated (250 mm thick) glass-backed silica gel (F_{254}) plates both of which were purchased from Silicycle (Quebec City, QC). Reactions were monitored by TLC and conveniently visualized by development with Schlittler's stain (amides give a transient white color).[17]

4.2. General procedure for the synthesis of *N*-acyl tetrahydroisoquinolines (9–14)

To a stirred suspension of the requisite fatty acid (0.746 mmol), salsolinol or isosalsoline HCl (0.82 mmol, 1.1 equiv), and TCTU (318 mg, 0.895 mmol, 1.2 equiv) in CH_2Cl_2 (20 mL) was added Et_3N (520 μL, 3.73 mmol, 5 equiv) dropwise over 5 min. The reaction was monitored by TLC for the disappearance of the fatty acid and upon completion (24–48 h) diluted with CH_2Cl_2 (30 mL) and washed sequentially with 0.1 M HCl (3× 20 mL), satd $NaHCO_3$ (2× 20 mL), brine (1× 20 mL), and H_2O (20 mL). The organic solution was dried with $MgSO_4$ and concentrated in-vacuo to give an oil that was purified by flash chromatography with a gradient elution of 55% Et_2O/hexanes to pure Et_2O to give the product as an oil.

4.2.1. 1-(6,7-Dihydroxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-icosa-5,8,11,14-tetraen-1-one (9). (Oil, 32%); IR (film) 3358 (br), 3007, 2957, 2926 (s), 2848, 1606, 1451, 1268 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 8.06 (br s, 1H), 6.71 (s, 1H), 6.60 (s, 1H), 5.98 (br s, 1H), 5.54 (q, major, J = 6.64 Hz, 0.85H), 5.43–5.28 (m, 8H), 4.82 (q, minor, J = 6.45 Hz, 0.15H), 4.59 (m, minor, 0.15H), 3.77 (td, major, J = 12.69, 4.5 Hz, 0.85H), 3.47 (m, major, 0.85H), 2.97 (m, minor, 0.15H), 2.86–2.50 (m, 9H), 2.47–2.30 (m, 2H), 2.11 (apparent pentet, J = 6.06 Hz, 2H), 2.01 (q, J = 6.83 Hz, 2H), 1.72 (m, 2H), 1.45 (d, minor, J = 6.83 Hz, 0.46H), 1.38–1.68 (m, 9.54H), 0.86 (t, J = 6.84 Hz, 3H); ^{13}C NMR (100.57 MHz, DMSO-d_6) δ 174.9, 170.6, 144.4, 130.6, 130.2, 129.8, 129.1, 128.9, 128.8, 128.7, 128.6, 128.5, 128.3, 128.2, 124.8, 124.6, 115.6, 114.3 (2), 47.7, 33.7, 32.7, 32.3, 31.5, 29.4, 28.9, 28.1, 27.3, 27.0, 25.9, 25.4, 25.1, 23.5, 22.6, 22.1, 14.6; HR-MS (CI) m/z 465.3239 (100, M^+, $C_{30}H_{43}NO_3$ requires 465.3237), 450 (35), 206 (47), 178 (44), 164 (70).

4.2.2. 1-(7-Hydroxy-6-methoxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-icosa-5,8,11,14-tetraen-1-one (10). (Oil, 40%); IR (film) 3281 (br), 3003, 2927 (s), 2850, 1630, 1511, 1423, 1260 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.68 (s, major, 0.56 H), 6.63 (s, minor, 0.44H), 6.55 (s, minor, 0.44H), 6.53 (s, major, 0.56H), 5.94–5.64 (br s, 1H), 5.51 (q, J = 6.64 Hz, major, 0.56H), 5.41–5.24 (m, 8H), 4.84 (q, J = 6.64 Hz, minor, 0.44H), 4.67 (ddd, J = 13.06, 5.28, 1.50 Hz, minor, 0.44H), 3.82 (s, 3H), 3.76 (m, major, 0.56H), 3.38 (m, major, 0.56H), 2.92 (m, minor, 0.44H), 2.86–2.53 (m, 9H), 2.48–2.25 (m, 2H), 2.12 (apparent pentet, J = 6.26 Hz, 2H), 2.04 (q, J = 7.03 Hz, 2H), 1.72 (apparent pentet, J = 6.26 Hz, 2H), 1.48–1.20 (m, 10H), 0.86 (t, J = 7.04 Hz, 3H); ^{13}C NMR (100.57 MHz, $CDCl_3$) δ 171.4, 171.2, 145.7, 145.5, 144.6, 144.4, 131.6, 130.6, 130.4, 129.6, 128.9, 128.8, 128.4, 128.3, 128.1, 127.7, 125.9, 124.6, 113.2, 112.5, 111.1, 110.7, 56.1, 51.6, 48.2, 39.8, 35.1, 33.3, 32.8, 31.7, 29.5, 29.4, 28.5, 27.4, 27.0, 25.8, 25.4, 25.3, 23.0, 22.7, 21.7, 14.3; HR-MS (CI) m/z 480.3467 (100, MH^+, $C_{28}H_{44}NO_3$ requires 480.3472), 220 (35), 194 (62), 192 (75), 178 (80).

4.2.3. 1-(6,7-Dihydroxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-octadeca-9,12-dien-1-one (11). (Oil, 29%); IR (film) 3210 (br), 3008, 2921 (s), 2845, 1603, 1527, 1451, 1266, 1189, 716 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.95 (s, minor, 0.25H), 6.68 (s, major, 0.75H), 6.62 (s, minor, 0.25H), 6.60 (s, major, 0.75H), 5.51 (q, J = 6.84 Hz, major, 0.75H), 5.40–5.25 (m, 4H), 4.83 (q, J = 6.64 Hz, minor, 0.25H), 4.58 (m, minor, 0.25H), 3.77 (td, J = 12.9, 4.4 Hz, major, 0.75H), 3.46 (m, major, 0.75H), 2.95 (m, minor, 0.25H), 2.83–2.62 (m, 2H), 2.53 (m, 2H), 2.47–2.44 (m, 2H), 2.01 (apparent q, J = 6.84 Hz, 2H), 1.62 (m, 2H), 1.45 (d, J = 6.84 Hz, minor, 0.75H), 1.43–1.12 (m, 16.25H), 0.86 (t, J = 6.84 Hz, 3H); ^{13}C NMR (100.57 MHz, $CDCl_3$) δ 174.1, 172.5, 143.9, 143.6, 143.5, 143.4, 140.0, 130.4, 130.26, 129.8, 129.2, 128.3, 128.1, 125.9, 125.7, 124.6, 115.5, 114.6, 113.8, 113.3, 52.1, 48.8, 40.3, 37.7, 35.8, 35.7, 34.2, 34.0, 33.6, 31.7, 30.5, 29.9, 29.8, 29.6 (m), 29.5, 29.4, 28.9, 28.1, 27.4, 25.8, 25.7, 25.5, 25.4, 23.1, 22.8, 21.8, 14.3; HR-MS (CI) m/z 442.3297 (100, MH^+, $C_{28}H_{44}NO_3$ requires 442.3316), 441.3217 (70, M^+, $C_{28}H_{43}NO_3$ requires 442.3237), 426 (50), 178 (25), 164 (63).

4.2.4. 1-(7-Hydroxy-6-methoxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-octadeca-9,12-dien-1-one (12). (Oil, 49%); IR (film) 3299 (br), 3008, 2923 (s), 2852, 1626, 1513, 1455, 1435, 1264, 1194, 1120 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 6.68 (s, major, 0.61H), 6.63 (s, minor, 0.39H), 6.55 (s, minor, 0.39H), 6.53 (s, major, 0.61 H), 5.85 (br s, major, 0.61H), 5.73 (br s, minor, 0.39H), 5.51 (q, J = 6.84 Hz, major, 0.61H), 5.40–5.25 (m, 4H), 4.85 (q, J = 6.64 Hz, minor, 0.39H), 4.58 (ddd, J = 12.99, 5.47, 1.56 Hz, minor, 0.39H), 3.83 (s, minor, 1.17H), 3.82 (s, major, 1.83H), 3.78 (m, major, 0.61H), 3.41 (m, major, 0.61H), 2.94 (m, minor, 0.39H), 2.84–2.56 (m, 4H), 2.44–2.24 (m, 2H), 2.01

(q, J = 6.84 Hz, 4H), 1.63 (apparent pentet, J = 7.23 Hz, 2H), 1.45 (d, minor, J = 6.65 Hz, 1.17H), 1.37–1.18 m, 15.83H), 0.86 (t, J = 6.84 Hz, 3H); ^{13}C NMR (100.57 MHz, $CDCl_3$) δ 171.7, 171.6, 145.7, 145.5, 144.6, 144.4, 131.7, 130.4, 130.5, 130.4, 130.3, 128.2, 128.1, 125.9, 124.6, 113.2, 112.5, 111.1, 110.7, 110.1, 56.1, 51.7, 48.2, 39.9, 35.1, 34.1, 33.6, 31.7, 30.5, 29.8, 29.7, 29.6, 29.5, 29.4, 28.5, 27.4, 25.8, 25.7, 25.6, 23.0, 22.8, 21.7, 14.3; HR-MS (CI) m/z 456.3461 (35, MH^+, $C_{29}H_{46}NO_3$ requires 456.3478), 455.3402 (53, M^+, $C_{29}H_{45}NO_3$ requires 455.3394), 440 (45), 192(33), 178 (100).

4.2.5. 1-(6,7-Dihydroxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-docosa-4,7,10,13,16,19-hexaen-1-one (13). (oil, 45%); IR (film) 3291 (br), 3008 (s), 2960, 2932, 1618, 1524, 1451, 1268, 1186, 711 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20–7.80 (br s, 2H), 6.67 (s, major, 0.75H), 6.62 (s, minor, 0.25H), 6.60 (s, major, 0.75H), 6.59 (s, minor, 0.25H), 5.50 (q, J = 6.80 Hz, major, 0.75H), 5.42–5.25 (m, 12H), 4.83 (q, J = 6.80 Hz, minor, 0.25H), 4.67 (ddd, J = 13.2, 5.6, 2.4 Hz, minor 0.25H), 3.77 (td, J = 12.8, 4.8 Hz, major, 0.75H), 3.42 (m, major, 0.75H), 2.96 (m, minor, 0.25H), 2.86–2.77 (m, 11H), 2.67 (m, major, 0.75H) 2.54 (m, minor, 0.25H), 2.50–2.32 (m, 4H), 2.04 (apparent pentet, J = 7.6 Hz, 2H), 1.44 (d, J = 6.80 Hz, minor, 0.75H), 1.33 (d, J = 6.80 Hz, major, 2.25 H), 0.94 (overlapping t's, J = 7.60 Hz, 3H); ^{13}C NMR (100.57 MHz, DMSO-d_6) δ 171.9, 170.2, 144.6, 144.5, 144.4, 132.2, 129.9 (2), 129.5, 129.1, 128.8, 128.5 (3), 128.4, 128.4, 127.6, 124.8, 124.6, 115.7, 115.6, 114.3, 51.0, 47.8, 37.3, 35.4, 35.2, 33.2, 32.9, 32.8, 31.0, 28.9, 28.1, 25.9, 25.8, 23.5, 23.4, 23.2, 22.1, 20.7, 14.7; HR-MS (CI) m/z 490.3303 (100, MH^+, $C_{32}H_{44}NO_3$ requires 490.3316), 489.3235 (51, M^+, $C_{32}H_{43}NO_3$ requires 489.3237), 356 (35), 178 (37), 164(50).

4.2.6. 1-(7-Hydroxy-6-methoxy-1-methyl-3,4-dihydro-1H-isoquinolin-2-yl)-docosa-4,7,10,13,16,19-hexaen-1-one (14). (Oil, 42%); IR (film) 3297 (br), 3009, 2965, 2965, 2921, 1631, 1511, 1434, 1266, 1130, 710 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.67 (s, major, 0.56H), 6.62 (s, minor, 0.44H), 6.55 (s, minor, 0.44H), 6.53 (s, major, 0.56H), 5.90–5.70 (br s, 1H), 5.51 (q, J = 6.64 Hz, major, 0.56H), 5.41–5.26 (m, 12H), 4.84 (q, J = 6.84 Hz, minor, 0.44H), 4.67 (dd, J = 12.9, 3.9 Hz, 0.44H), 3.84 (s, minor, 1.32 H), 3.82 (s, major, 1.68H), 3.78 (m, major, 0.56H), 3.42 (m, major, 0.56H), 2.96 (dt, J = 12.7, 3.7 Hz, minor, 0.44H), 2.86–2.77 (m, 11H), 2.68 (td, J = 15.63, 1.3 Hz, major, 0.56H) 2.58 (td, J = 14.85, 1.3 Hz, minor, 0.44H), 2.50–2.33 (m, 4H), 2.04 (apparent pentet, J = 7.62 Hz, 2H), 1.45 (d, J = 6.84 Hz, minor, 1.32H), 1.36 (d, J = 6.84 Hz, major, 1.68H), 0.94 (t, J = 7.62 Hz, 3H); ^{13}C NMR (100.57 MHz, $CDCl_3$) δ 170.9, 170.7, 145.8, 145.6, 144.7, 144.4, 132.2, 131.6, 130.4, 129.1, 128.9, 128.8, 128.4 (3), 128.3, 128.1, 127.2, 125.9, 124.6, 113.1, 112.6, 111.1, 110.7, 56.2, 51.6, 48.3, 39.9, 35.2, 33.8, 33.4, 30.5, 29.4, 28.5, 25.8, 25.7, 23.4, 23.3, 22.9, 21.7, 20.8, 14.5; HR-MS (CI) m/z 504.3483 (100, MH^+, $C_{33}H_{46}NO_3$ requires 504.3472), 503.3396 (30, M^+, $C_{33}H_{45}NO_3$ requires 503.3394), 194 (19).

4.3. Cell culture

HEK293 cells expressing human TRPV1 were a kind gift of Merck Research Laboratories (Whitehouse Station, NJ). The HEK293 cell line (passages 11–25) was cultured in minimal essential medium, Eagle, modified with non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1.5 g/L Sodium bicarbonate (Cat # 30-2005, ATCC, Mabassas, VA), containing 1% Penicillin–streptomycin (Gibco, Cat # 15140-122) and 10% fetal bovine serum. Cells were passaged three times a week using Trypsin-EDTA 1× (Gibco Cat # 25200). Cells were grown under 5% CO_2 at 37 °C.

4.4. Ca^{2+} mobilization assay

Ca^{2+} mobilization was measured using a 96-well plate reader (Flexstation II, Molecular Devices, Union City, CA). HEK293 cells stably expressing human TRPV1 receptors were plated 24–48 h before imaging on CellBIND 96-well flat clear bottom black polystyrene microplates (Corning, Corning NY). Cells were loaded with 3 μM of the ratiometric dye Fura 2 AM (Molecular probes, Eugene, OR) in 0.05% W/V Pluronic 127 (molecular probes, Eugene, OR) for 70 min in HEPES TYRODE buffer, pH 7.4 (25 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, and 5 mM glucose). The cells were washed twice, resuspended, and left to de-esterify for 20 m before imaging. The Flexstation II was programmed with the following settings: excitation wavelengths: 340 nm and 380 nm, emission wavelength: 510 nm. The initial volume per well was 175 μL of buffer that was recorded for 40 s to establish a baseline fluorescence. Following the baseline recording, 75 μL buffer containing drug or vehicle was added using the automated fluidic module of Flexstation II at a rate of 1 (26 μL/s) per well. The total run time was 200 s with an interval of 3.95 s. All test compounds were dissolved in DMSO and diluted in buffer before application. Ca^{2+} mobilization per well was calculated as area under the curve using Softmax Pro (Molecular Devices, Union City, CA) integration functions. Further statistical analysis including EC_{50} and the Hill slope of the test compounds were calculated using non-linear regression curve fit (GraphPad Prism, San Diego, CA). Each experimental group consisted of 3–8 single wells from at least two independent 96-well plates.

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