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Synthesis and Evaluation of New Alkylamides Derived from α-Hydroxysanshool, the Pungent Molecule in Szechuan Pepper

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Szechuan pepper is widely used in Asia as a spice for its pleasant pungent and tingling sensations, produced by natural alkylamides called sanshools. α-Hydroxysanshool, the main alkylamide found in the pericarp of the fruit, stimulates sensory neurons innervating the mouth by targeting two chemosensitive members of the transient receptor potential (TRP) channels, TRPV1 and TRPA1. As it was previously found that configuration of the unsaturations in the α-hydroxysanshool alkyl chain is required for TRPA1 but not TRPV1 selectivity, this study aimed at obtaining more potent and selective TRPA1 agonists using α-hydroxysanshool as a starting material. This paper reports the preparation of new alkylamides derived from sanshool and their efficacy in stimulating TRPA1 and TRPV1 receptors. The data provide knowledge of the main sanshool chemical functionalities required for TRP channel activation, but they also evidence new selective and potent TRPA1 agonists based on α-hydroxysanshool.

KEYWORDS: TRPA1 and TRPV1 agonists; α-hydroxysanshool; synthesis; chemoreceptors

INTRODUCTION

Szechuan pepper (*Zanthoxylum piperitum*), which is commonly used in Asia as a spice and in traditional medicine, is particularly appreciated for its pleasant pungent and tingling sensations. Recent studies showed that α-hydroxysanshool, the major alkylamide in the pepper, is responsible for these sensations and activates TRPV1 and, to a lower extent, TRPA1 channels (*1*−*3*). TRPV1 receptors are involved in sensing a multitude of noxious stimuli, the most notable being capsaicin, the burning molecule in red hot chili pepper (*4*) and also common vanilloids such as zingerone and gingerol from ginger (*5*), eugenol from clove (*6*), or piperine contained in black and white pepper (*7*) (**Figure 1**). TRPA1 receptors are found in a subset of nociceptive sensory neurons, where they are coexpressed with TRPV1 (*8*) and respond specifically to noxious cold and pungent compounds such as isothiocyanate (mustard oil, wasabi, and horseradish) and cinnamaldehyde from cinnamon (*9*−*12*). Previous studies have highlighted the structure-activity relationship (SAR) between the agonist and TRP channels where chemical modification of the ligand modulates the receptor responsiveness, as observed in the capsaicin hydrophobic region on TRPV1 (*13*−*15*).

The main sanshools found in Szechuan pepper are α-, *β*-, *γ*-, and *δ*-sanshools and their analogues possessing one hydroxyl group on the amide moiety (**Figure 2**) (*16*−*20*). They differ from the configuration of one double bond (α- and *β*-sanshools, for instance) and the length of the polyenic system (12 carbons for α- and *β*-sanshools versus 14 carbons for *γ*- and *δ*-sanshools). Interestingly, sensory evaluations brought to the fore that α-hydroxysanshool produced a tingling sensation, whereas *δ*-, *γ*-, and α-sanshools were perceived as burning and the *β*-sanshools as rather numbing (*1, 21*). One common characteristic of all these natural compounds is their agonistic activity on TRPV1, consistent with their burning properties (*1*). Moreover, the sanshool pungent "sharp" and "biting" sensations may originate from TRPV1 and TRPA1 stimulation, but their contribution to its numbing effects has been controversial. Very recently, two-pore potassium channels sensing volatile anesthe-

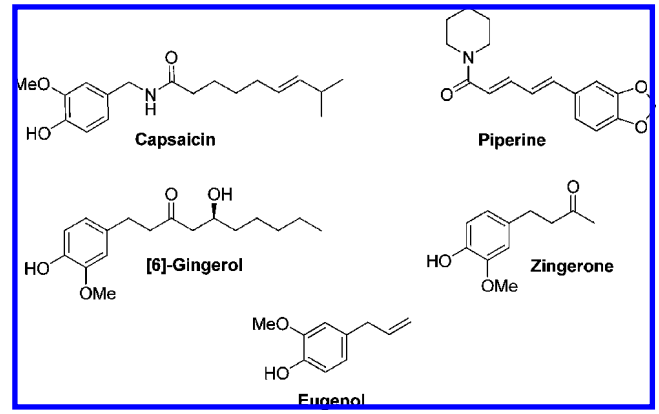

Figure 1. Examples of natural products activating TRPV1.

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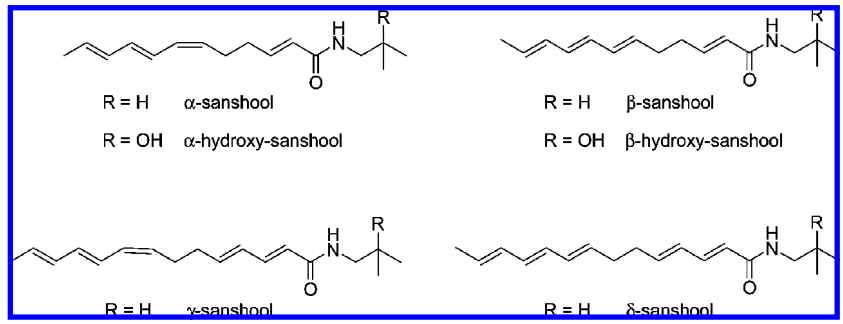


Figure 2. Structures of the main sanshools found in Szechuan pepper.

tics (*22*, *23*) were found to be stimulated by applications of α-hydroxysanshool, rationalizing some of the complex numbing and long-lasting perceptions associated with the spice (*3*). In our previous work (*24*), we observed that α-hydroxysanshool is likely to gate TRPA1 via covalent interaction on its reactive cysteines, whereas this mechanism is unlikely to occur for TRPV1, highlighting that the two channels behave differently in their responses. These results may account for dissociated sensory roles of the channels in their response to this compound. If TRPV1's crucial role in pungency mediated by burning molecules is well established (*4*, *25*), the physiological implication of TRPA1 in sensory perception remains so far partially elucidated (*26*). Therefore, to better understand the differences between TRPV1 and TRPA1 in sensing these compounds, we aimed at developing new synthetic molecules based on α-hydroxysanshool structure, which would possess a TRPA1 rather than a TRPV1 selectivity.

Here we report the elaboration of new alkylamides that are able to activate selectively TRPA1 receptors expressed in HEK 293 cells. We first focused on sanshool derivatives having variations in their alkyl chains. These analogues would be very useful to identify main chemical functionalities for the TRP channel activation. The second point was to evaluate the influence of the amide part, replacing the 2-methylpropan-2-ol moiety by various amino acids having different chemical properties. This study finally led to potentially new selective TRPA1 agonists easier to elaborate and more potent than the natural α-hydroxysanshool.

MATERIALS AND METHODS

Chemistry. All commercially available reagents were obtained from Sigma Aldrich (Buchs, Switzerland) and used as received. Water- and air-sensitive reactions were carried out under argon atmosphere. Analytical thin layer chromatography (TLC) was carried out on silica 60 F254 (Merck) and RP-18 F254s (Merck) plates. The TLC plates were visualized by shortwave UV light with ceric ammonium molybdate stain. Flash chromatography was performed using a Biotage SP1 HPFC system and a FLASH cartridge (25+M and 40+M KP-SIL). ^{1}H NMR (360.13 MHz) and ^{13}C NMR (90.56 MHz) spectra were recorded on a Bruker DPX-360 spectrometer equipped with a broadband multinuclear *z*-gradient probehead. The chemical shifts (in ppm) were expressed with respect to an internal reference (TMS or TSP). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, bs = broad singlet. Melting points were recorded on Buchi B-545 melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P2000 polarimeter. Elementary analyses were performed at the University of Geneva (Service de microanalyse). HPLC of purified green Szechuan extract was carried out with a N-Nucleosil 100 column (Macherey-Nagel, 5 μm, C-18, 250 × 4 mm), with UV detection at 254 nm and a flow rate of 1 mL/min. Solvents used are A = 0.1% (g/vol) TFA in H_2O and B = Acetonitrile. HPLC/DAD/ESI-TOF-MS analyses were performed on an Agilent-1200 series Rapid Resolution LC System including binary pump SL, high-performance autosampler, diode array detector SL and thermostated column compartment SL with an Agilent 6210 time-of-flight mass spectrometer.

Purification of Green Szechuan Extract. 800 mg of Green Szechuan extract was purified by chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. Column *FLASH* 40+M was used on a Biotage HPFC purification system with a flow rate of 40 mL/min. The hydroxysanshools were detected at 275 nm and collected in 21 mL fractions. Fractions were concentrated under reduced pressure to give finally 200 mg of hydroxysanshools as a mixture of three isomers that are not separable by this technique of purification (25% yield by weight). The ratio after purification was established by HPLC and stayed unchanged (*m*/α/*β* 3.7:75.4:20.8); R_f = 0.35 (ethyl acetate).

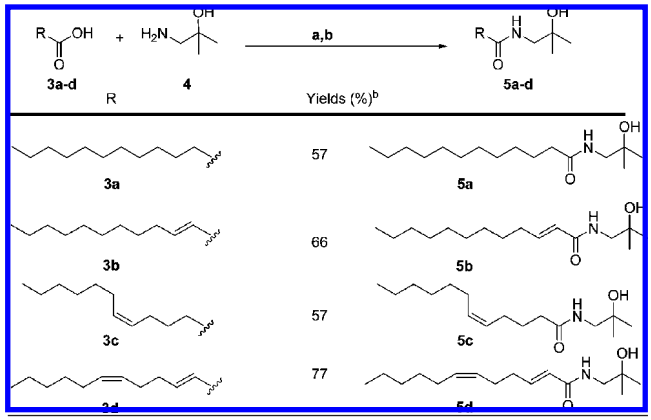
α-*Hydroxysanshool:* ^{1}H NMR (360 MHz, $CDCl_3$, TMS as reference) δ 6.88 (dt, J = 15.3, 6.7 Hz, 1H), 6.36−5.92 (m, 5H), 5.84 (dt, J = 15.3, 1.3 Hz, 1H), 5.74 (dt, J = 13.8, 6.5 Hz, 1H), 5.37 (dt, J = 10.8, 6.7 Hz, 1H), 3.32 (d, J = 6.1 Hz, 2H), 2.54 (s, 1H), 2.36−2.27 (m, 4H), 1.78 (d, J = 6.7 Hz, 3H), 1.23 (s, 6H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.95, 144.47, 133.51, 131.77, 130.2, 129.64, 129.53, 125.44, 123.70, 71.06, 50.41, 32.07, 27.34, 26.46, 18.34.

β-Hydroxysanshool: ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.93, 144.35, 132.03, 131.63, 131.57, 131.44, 130.07, 129.39, 123.71, 71.06, 50.42, 31.90, 31.37, 27.34, 18.29.

Ethyl (2E,6Z)-dodeca-2,6-dienoate ***2.*** To a solution of (ethoxycarbonylmethyl) triphenylphosphorane (9.40 g, 27 mmol, 2 equiv) in anhydrous THF (40 mL) was added dropwise 4-(Z)-decenal 1 (2.46 mL, 13.5 mmol, 1 equiv), and the mixture was stirred overnight at room temperature. The solution was then concentrated to 25 mL, and petroleum ether was added to precipitate the phosphonium salt. The white solid was filtered, and the filtrate was concentrated under reduced pressure. The resulting residue was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether (boiling range, 40−60 °C). Ethyl (2*E*,6*Z*)-dodeca-2,6-dienoate **2** was isolated as a colorless oil (1.65 g, 7.35 mmol, 54%); R_f = 0.62 (petroleum ether/ethyl acetate 95:5); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.96 (dt, J = 15.6, 6.6 Hz, 1H), 5.83 (dt, J = 15.6, 1.5 Hz, 1H), 5.45−5.30 (m, 2H), 4.18 (q, J = 7.0 Hz, 2H), 2.25−2.18 (m, 4H), 2.04−1.98 (m, 2H), 1.36−1.26 (m, 6H), 1.28 (t, J = 6.7 Hz, 3H), 0.88 (t, J = 6.7 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 166.67, 148.64, 131.64, 127.74, 121.57, 60.15, 32.30, 31.49, 29.27, 27.23, 25.75, 22.56, 14.28, 14.06.

(2E,6Z)-Dodeca-2,6-dienoic acid ***3d.*** To a solution of ethyl (2*E*,6*Z*)-dodeca-2,6-dienoate **2** (800 mg, 3.56 mmol, 1 equiv) in THF/water (v/v 1/:1, 40 mL) was added LiOH (512 mg, 21.4 mmol, 6 equiv), and the mixture was stirred for 12 h at room temperature and for 4 h at 80 °C. The medium was then acidified with 1N HCl to pH 5. The organic was dried over sodium sulfate, filtered, and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. 420 mg of (2*E*,6*Z*)-dodeca-2,6-dienoic acid **3d** was isolated as a colorless oil (420 mg, 2.13 mmol, 60%); R_f = 0.35 (petroleum ether/ethyl acetate 2:1); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 7.07 (dt, J = 15.7, 6.4 Hz, 1H), 5.84 (dt, J = 15.6, 1.6 Hz, 1H), 5.46−5.29 (m, 2H), 2.31−2.20 (m, 4H), 2.04−1.96 (m, 2H), 1.38−1.21 (m, 6H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal

Table 1[a,b]



[a] Reagents and conditions: (a) (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h. [b] Isolated yields.

reference) δ 171.55, 151.75, 131.56, 127.49, 120.83, 32.49, 31.49, 29.26, 27.24, 25.60, 22.56, 14.20.

1-Amino-2-methylpropan-2-ol ***4.*** To a solution of 1,1-dimethylepoxide (4 mL, 44.98 mmol, 1 equiv) and benzylamine (5.39 mL, 49.42 mmol, 1.1 equiv) in 250 mL of water was added dropwise triethylamine (0.062 mL, 0.449 mmol, 0.01 equiv) at room temperature. The mixture was stirred overnight at the same temperature and then concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of methanol in dichloromethane. A colorless oil (7.95 g) was isolated as a mixture of 1-(benzylamino)-2-methylpropan-2-ol and benzylamine. The oil was dissolved in methanol (130 mL) and Pd/C (5%, 3.00 g) was added. The mixture was successively degassed and submitted to hydrogen atmosphere. The same operation was performed three times, and then the mixture was stirred for 1.5 h at room temperature under hydrogen atmosphere. The suspension was then filtered over Celite and concentrated under reduced pressure to give the 1-(amino)-2-methylpropan-2-ol **4** as a colorless oil (2.54 g, 28.49 mmol, 62% from the 1,1-dimethylepoxide): ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 2.60 (s, 2H), 2.04 (s, 2H), 1.16 (s, 6H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 69.76, 52.37, 26.74.

General Procedure for the Preparation of Sanshool Analogues 5a−d (Table 1). To a solution of acid **3a−d** (1 equiv), 1-amino-2-methylpropan-2-ol **4** (1 equiv), and di-isopropylethylamine (2 equiv) in anhydrous DMF (0.084 M) was added the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (1 equiv) at 0 °C. The solution was stirred for 12 h at room temperature and then diluted with water and ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether.

N-(2-Hydroxy-2-methylpropyl)-dodecanamide ***5a:*** 57% yield, white solid; R_f = 0.51 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.48 (s, 1H), 3.28 (d, J = 5.7 Hz, 2H), 3.17 (s, 1H), 2.28 (t, J = 7.5 Hz, 2H), 1.67−1.61 (m, 2H), 1.34−1.23 (m, 16H), 1.23 (s, 6H), 0.87 (t, J = 6.6 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 177.83, 73.71, 53.41, 39.28, 34.71, 32.41, 32.29, 32.14, 32.10, 30.05, 28.74, 25.49, 16.92; LC/ESI-TOF-MS, obsd m/z [M − 1]$^-$ 270.24. Anal. Calcd for $C_{16}H_{33}NO_2$: C, 70.80%; H, 12.29%; N, 5.12%. Found: C, 70.83%; H, 12.29%; N, 5.12%.

(2E)-N-(2-Hydroxy-2-methylpropyl)-dodec-2-enamide ***5b:*** 66% yield; pale orange oil; R_f = 0.60 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.79 (dt, J = 15.3, 7.0 Hz, 1H, H-10), 6.03 (s, 1H), 5.75 (dt, J = 15.2, 1.5 Hz, 1H), 3.26 (d, J = 6.0 Hz, 2H), 2.06 (qd, J = 7.7, 1.4 Hz, 2H), 1.40−1.34 (m, 2H), 1.24−1.17 (m, 12H), 1.16 (s, 6H), 0.81 (t, J = 6.7 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 167.35, 145.79, 123.23, 71.14, 50.57, 32.21, 32.00, 29.93, 29.55, 29.42, 29.33, 28.36, 27.42, 22.79, 14.23; LC/ESI-TOF-MS, obsd m/z [M − 1]$^-$ 268.23. Anal. Calcd for $C_{16}H_{31}NO_2$ + 0.2H_2O: C, 70.39%; H, 11.59%; N, 5.13%. Found: C, 70.36%; H, 11.47%; N, 5.08%.

(5Z)-N-(2-Hydroxy-2-methylpropyl)-dodec-5-enamide ***5c:*** 57% yield; pale orange oil; R_f = 0.60 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 5.87 (s, 1H), 5.37−5.22 (m, 2H), 3.19 (d, J = 6.0 Hz, 2H), 2.16 (t, J = 7.4 Hz, 2H), 2.01 (q, J = 6.9 Hz, 2H), 1.93 (q, J = 6.4 Hz, 2H), 1.64 (q, J = 7.5 Hz, 2H), 1.27−1.15 (m, 8H), 1.15 (s, 6H), 0.81 (t, J = 7.0 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 176.95, 133.96, 131.21, 73.73, 53.13, 39.94, 34.57, 32.48, 31.80, 30.10, 30.09, 29.48, 28.56, 25.45, 16.91; LC/ESI-TOF-MS, obsd m/z [M − 1]$^-$ 268.23. Anal. Calcd for $C_{16}H_{31}NO_2$ + 0.2H_2O: C, 70.39%; H, 11.59%; N, 5.13%. Found: C, 70.58%; H, 11.56%; N, 5.16%.

(2E,6Z)-N-(2-Hydroxy-2-methylpropyl)-dodeca-2,6-dienamide ***5d:*** 77% yield; [ratio (6*Z*,2*E*)/(6*E*,2*E*) 87.5/12.4, 77%]; colorless oil; R_f = 0.51 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.87 (dt, J = 15.3, 6.7 Hz, 1H), 5.95 (s, 1H), 5.83 (d, J = 15.4 Hz, 1H), 5.44−5.30 (m, 2H), 3.33 (d, J = 5.9 Hz, 2H), 2.59 (s, 1H), 2.27−2.15 (m, 4H), 2.04−1.97 (m, 2H), 1.36−1.21 (m, 6H), 1.23 (s, 6H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 166.99, 144.81, 131.21, 127.89, 123.34, 71.04, 50.41, 32.19, 31.47, 29.27, 27.31, 27.22, 25.91, 22.54, 14.05; LC/ESI-TOF-MS, obsd m/z [M − 1]$^-$ 266.18. Anal. Calcd for $C_{16}H_{29}NO_2$ + 0.3H_2O: C, 70.44%; H, 10.94%; N, 5.13%. Found: C, 70.53%; H, 10.97%; N, 5.10%.

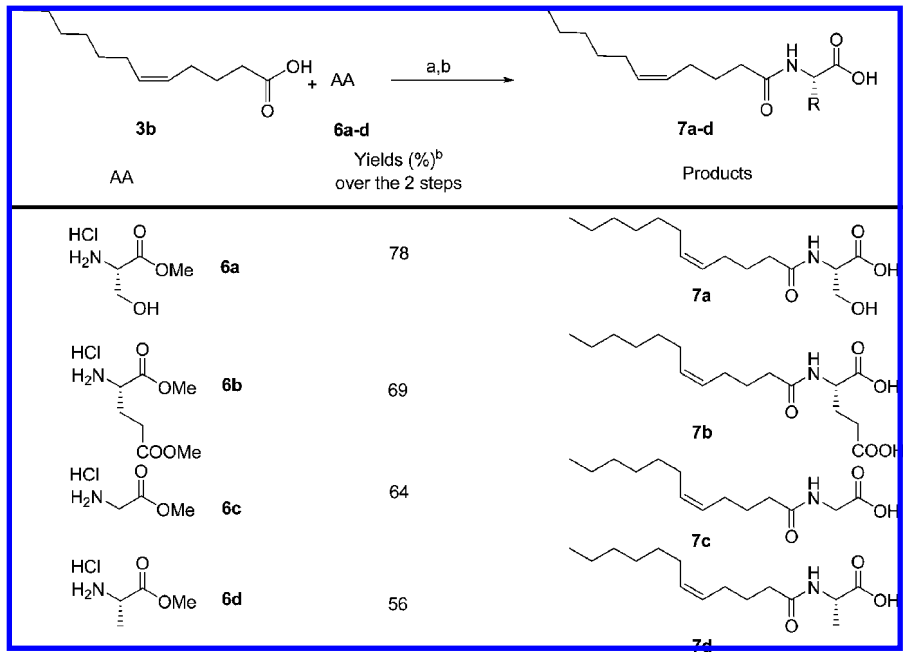
Typical Procedure for the Preparation of Compounds 7a−d and 8a−c (Tables 2 and 3). To a solution of carboxylic acid **3b−f** (1 equiv), amino acid methyl ester hydrochloride **6a−d** (1 equiv), and di-isopropylethylamine (2 equiv) in DMF (0.084 M) was added the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (1 equiv) at 0 °C. The solution was stirred for 12 h at room temperature and then diluted with water and ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. The resulting methyl ester (1 equiv) was dissolved in THF/H_2O (0.084 M, v/v, 3:1), and LiOH (3 equiv) was added at room temperature. The mixture was stirred for 2 h and then acidified with 0.5 N HCl to pH 6. Ethyl acetate was added, and the aqueous phase was concentrated under reduced pressure, purified by flash chromatography column on reverse phase using a gradient of methanol in water, and finally freeze-dried to give a white foam.

(S,Z)-2-Dodec-5-enamido-3-hydroxypropanoic acid 7a: 78% yield; white foam; R_f = 0.55 (methanol/water/AcOH 80:20:1); $[\alpha]^{24}_D$ +24.07° (*c* 1.08 mg/mL, MeOH); ^{1}H NMR (360 MHz, MeOD-d_4, TMS as internal reference) δ 5.45−5.34 (m, 2H), 4.31 (t, J = 4.8 Hz, 1H), 3.81 (d, J = 4.8 Hz, 2H), 2.29 (t, J = 7.1 Hz, 2H), 2.11 (q, J = 6.9 Hz, 2H), 2.06 (q, J = 6.3 Hz, 2H), 1.71 (dq, J = 7.9, 7.9 Hz, 2H), 1.36−1.30 (m, 8H), 0.91 (t, J = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d^4, TMS as internal reference) δ 178.16, 176.96, 133.11, 131.27, 65.75, 59.43, 38.28, 34.34, 32.24, 31.48, 29.60, 29.19, 28.34, 25.11, 15.83; LC/ESI-TOF-MS, obsd m/z [M − 1]$^-$ 284.16 (M − H). Anal. Calcd for $C_{14}H_{26}NO_4Li$ + 0.4H_2O: C, 60.35; H, 9.05; N, 4.69. Found: C, 60.22; H, 8.83; N, 4.62.

(S,Z)-2-Dodec-5-enamidopentanedioic acid 7b: 69% yield; white solid; R_f = 0.50 (methanol/water/AcOH 60:40:1); $[\alpha]^{24}_D$ −4.90° (*c* 1.02 mg/mL, MeOH); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as internal reference) δ 5.45−5.33 (m, 2H), 4.43 (dd, J = 8.9, 5.0 Hz, 1H), 2.40 (td, J = 7.9, 1.2 Hz, 2H), 2.27 (t, J = 7.4 Hz, 2H), 2.17−1.89 (m, 6H), 1.68 (dq, J = 7.3, 7.3 Hz, 2H), 1.35−1.30 (m, 8H), 0.91 (t, J = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d_4, TMS as internal reference) δ 177.88, 177.56, 176.74, 133.18, 131.20, 54.66, 37.79, 34.34, 32.79, 32.22, 31.46, 29.59, 29.46, 29.10, 28.38, 25.11, 15.83; LC/ESI-TOF-MS, obsd m/z [M + 1]$^+$ 345 (M + H_2O). Anal. Calcd for $C_{17}H_{29}NO_5$ + 0.3H_2O: C, 61.35; H, 8.96; N, 4.21. Found: C, 61.47; H, 8.81; N, 4.18.

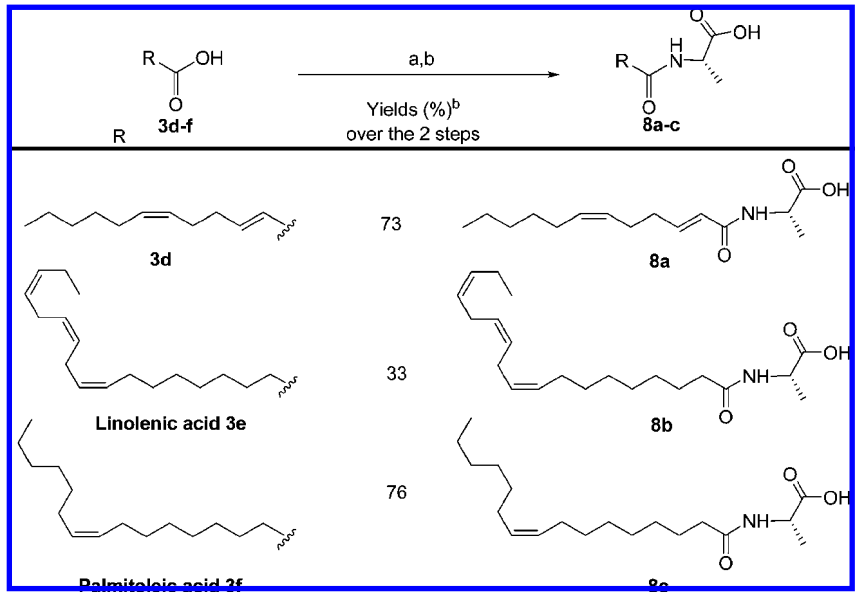
(Z)-2-Dodec-5-enamidoacetic acid 7c: 64% yield; white foam; R_f = 0.42 (methanol/water/AcOH 80:20:1); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as internal reference) δ 5.44−5.33 (m, 2H), 3.37 (s, 2H), 2.26 (t, J = 7.8 Hz, 2H), 2.10 (q, J = 7.0 Hz, 2H), 2.06 (q, J = 6.3 Hz, 2H), 1.64 (qd, J = 7.5, 7.5 Hz, 2H), 1.38−1.30 (m, 8H), 0.91 (t, J = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d_4, TMS as internal reference) δ 177.86, 177.03, 133.11, 131.26, 45.92, 38.06,

Table 2[a,b]



[a] Reagents and conditions: (a) (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h; (b) LiOH, THF/H_2O, rt, 2 h. [b] Isolated yields.

Table 3[a,b]



[a] Reagents and conditions: (a) alanine chloride methyl ester 6d, (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h; (b) LiOH, THF/H_2O, rt, 2 h. [b] Isolated yields.

34.34, 32.23, 31.48, 29.60, 29.17, 28.32, 25.11, 15.84; LC/ESI-TOF-MS, obsd *m*/*z* $[M - 1]^-$ 254.20 (M − H). Anal. Calcd for $C_{14}H_{24}NO_3Li + 0.35H_2O$: C, 62.42; H, 9.32; N, 5.20. Found: C, 62.28; H, 9.27; N, 5.19.

(S,Z)-2-Dodec-5-enamidopropanoic acid 7d: 56% yield; white foam; $R_f = 0.39$ (methanol/water/AcOH 80:20:1); $[\alpha]^{24}_D + 14.17°$ (*c* 1.20 mg/mL, MeOH); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as internal reference) δ 5.41−5.33 (m, 2H), 4.21 (q, $J = 7.1$ Hz, 1H), 2.22 (t, $J = 7.5$ Hz, 2H), 2.06 (q, $J = 6.8$ Hz, 2H), 2.02 (q, $J = 6.3$ Hz, 2H), 1.70−1.61 (m, 2H), 1.36−1.28 (m, 8H), 1.32 (d, $J = 7.1$ Hz, 3H), 0.91 (t, $J = 6.3$ Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d_4, TMS as internal reference) δ 179.87, 174.91, 131.71, 129.86, 51.83, 36.82, 32.94, 30.83, 30.08, 28.20, 27.73, 27.01, 23.71, 19.45, 15.84; LC/ESI-TOF-MS, obsd *m*/*z* $[M - 1]^-$ 268.20 (M − H). Anal. Calcd for $C_{15}H_{26}NO_3Li + 0.35H_2O$: C, 63.97; H, 9.56; N, 4.97. Found: C, 64.14; H, 9.48; N, 4.94.

(S,E,Z)-2-[Dodeca-2,6-dienamido]propanoic acid 8a: 73% yield; white solid, $R_f = 0.71$ (methanol/water 80:20); mp = 95−97 °C; $[\alpha]^{25}_D$ −7.84° (1.02 mg/mL, MeOH); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as reference) δ 6.78 (dt, $J = 15.4$, 6.5 Hz, 1H), 5.95 (d, $J = 15.3$ Hz, 1H), 5.44−5.34 (m, 2H), 4.40 (qd, $J = 7.2$, 7.2 Hz, 1H), 2.26−2.15 (m, 4H), 2.03 (q, $J = 6.9$ Hz, 2H), 1.39 (d, $J = 7.2$ Hz, 3H),1.39−1.30 (m, 6H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d_4, TMS as reference) δ 177.03, 168.04, 145.40, 132.01, 129.22, 124.85,

49.97, 33.26, 32.66, 29.27, 28.19, 27.13, 23.64, 18.23, 14.43; LC/ESI-TOF-MS, obsd *m*/*z* $[M - 1]^-$ 266.19. Anal. Calcd for $C_{15}H_{24}LiNO_3 + 0.5H_2O$: C, 63.82; H, 8.93; N, 4.96. Found: C, 63.93; H, 8.97; N, 4.81.

(Z,Z,Z)-2-[Octadeca-9,12,15-trienamido]propanoic acid 8b: 33% yield; white solid, $R_f = 0.24$ (methanol/water 80:20); mp = 100−102 °C; $[\alpha]^{25}_D$ +5.32° (0.94 mg/mL, MeOH); ^{1}H NMR (360 MHz, MeOD-d_4, TMS as reference) δ 7.27 (d, $J = 6.3$ Hz, 1H), 5.39−5.26 (m, 6H), 3.70 (qd, $J = 6.8$, 6.8 Hz, 1H), 2.77 (t, $J = 5.8$ Hz, 4H), 2.06−1.99 (m, 6H), 1.45 (qd, $J = 7.3$, 7.3 Hz, 2H), 1.32−1.20 (m, 8H), 1.12 (d, $J = 6.9$ Hz, 3H), 0.92 (t, $J = 7.5$ Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d_4, TMS as reference) δ 178.10, 174.64, 135.57, 133.99, 132.02, 131.98, 131.61, 131.06, 54.01, 36.48, 29.76, 29.46, 29.43, 29.32, 27.36, 26.12, 25.91, 25.82, 20.76, 20.17, 14.85; LC/ESI-TOF-MS, obsd *m*/*z* $[M - 1]^-$ 348.28. Anal. Calcd for $C_{21}H_{34}NO_3 + 0.6H_2O$: C, 68.87; H, 9.69; N, 3.82. Found: C, 69.02; H, 9.54; N, 3.74.

(Z)-2-[Hexadeca-9-enamido]propanoic acid 8c: 76% yield; white solid, $R_f = 0.24$ (methanol/eater 80:20); mp = 56−58 °C; $[\alpha]^{25}_D$ −14.42° (1.04 mg/mL, MeOH); ^{1}H NMR (360 MHz, DMSO-d_6, TMS as reference) δ 8.04 (d, $J = 7.2$ Hz, 1H), 5.32 (t, $J = 5.3$ Hz, 2H), 4.17 (qd, $J = 7.3$, 7.3 Hz, 1H), 2.07 (t, $J = 7.3$ Hz, 2H), 1.98−1.96 (m, 4H), 1.48−1.45 (m, 2H), 1.39−1.21 (m, 19H), 0.85 (t, $J = 6.2$ Hz, 3H); ^{13}C NMR (90 MHz, DMSO-d_6, TMS as reference) δ 174.52, 172.12, 129.82, 47.85, 35.17, 31.33, 29.30, 29.28, 28.88, 28.79, 28.74, 28.47, 26.79, 25.37, 22.28, 17.42, 14.12; LC/ESI-TOF-MS, obsd *m*/*z* $[M - 1]^-$ 324.29. Anal. Calcd for $C_{19}H_{35}NO_3 + 0.3H_2O$: C, 68.97; H, 10.84; N, 4.23. Found: C, 68.96; H, 10.74; N, 3.99.

Molecular Biology. *Cloning and Expression of Human TRPV1 and TRPA1 Receptors in HEK 293 Cells.* Cloning and expression of these receptors was performed following previously published protocols (*27*). Briefly, cloned human TRPV1 cDNA was obtained from RZPD (Germany) and hTRPA1 cDNA from OriGene (Rockville, MD). Genes were subcloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA) to generate stable cell lines using the Flp-In system (Invitrogen) after sequencing verification.

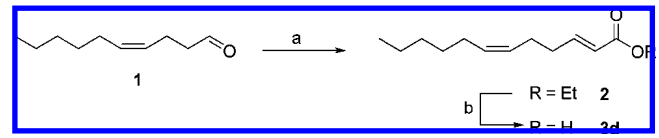
Measurement of Intracellular Calcium Levels $[Ca^{2+}]_i$ and Membrane Potential Variation in HEK 293 Cells Using a Fluorescent Plate Reader. Cell lines stably expressing TRP channels were seeded into 96-well plates previously coated with poly-D-lysine. Cells were incubated in Hank's Balanced Salt Solution (HBSS) supplemented with 2 mM $CaCl_2$ and 20 mM HEPES (pH 7.4), containing the cytoplasmic calcium indicator Fura-2/AM at 2 μM (Molecular Devices, Sunnyvale, CA) as previously described (*27*). Experiments were conducted at room temperature. $[Ca^{2+}]_i$ fluxes from a homogeneous cell population (approximately 100000 cells) were measured as changes in fluorescence intensity when stimulated with agonists using a FLEXstation (Molecular Devices). Cells were then challenged with the different sanshool derivatives. Mock cells transfected with pcDNA5/FRT were used as negative controls. Cell viability was checked after alkylamide stimulation using 100 μM ATP.

Data Analysis. Responses of molecules in HEK 293 cells were expressed as a percentage of maximum responses evoked by 150 μM cinnamaldehyde for TRPA1 and 1 μM capsaicin for TRPV1 (these concentrations were assessed independently to be saturating under these conditions). For all experiments, calcium fluxes were measured as changes in fluorescence intensity, before and after the addition of agonists. The peak response was taken to be the characteristic value and was obtained by subtracting the peak value from the baseline (value before injection). Dose-response curves were fitted using the Hill equation (GraphPad Prism Software, San Diego, CA) to obtain EC_{50} values. Data obtained from this study were expressed as mean ± SEM. Statistical analysis was performed using the unpaired Student *t* test.

RESULTS AND DISCUSSION

The syntheses of four α-hydroxysanshool derivatives **5a**−**d** with various alkyl chains were achieved starting from the corresponding carboxylic acids **3a**−**d** and the 1-amino-2-methylpropan-2-ol **4**. Carboxylic acids **3a**−**c** were commercially available, whereas the polyene **3d** was synthesized in a two-step sequence from the 4-(*Z*)-decenal **1** (**Scheme 1**). Wittig

Scheme 1[a]



[a] Reagents and conditions: (a) $Ph_3PCHCOOEt$, THF, rt, 12 h, 54%; (b) LiOH, THF/H_2O, rt, 12 h, and then 80 °C, 4 h, 60%.

olefination, using the (ethoxycarbonylmethyl) triphenylphosphorane in anhydrous THF, provided the diene **2** in 12 h at room temperature (54% yield). The ester function in **2** was then hydrolyzed under standard saponification conditions (LiOH, THF/H_2O) to afford the carboxylic acid **3d** in 60% yield. The coupling reaction of carboxylic acids **3a**−**d** and the 1-amino-2-methylpropan-2-ol **4**, activated by the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, led after 12 h at room temperature to the α-hydroxysanshool derivatives **5a**−**d**, respectively, in 57, 66, 57, and 77% yields (**Table 1**) (*28*). The alkylamide **5d** was isolated as a mixture of (6*Z*,2*E*) and (6*E*,2*E*) diastereoisomers due to the commercial 4-(*Z*)-decenal **1**, which contains 10% of (*E*)-isomer. The ratio (6*Z*,2*E*)/(6*E*,2*E*) was 88:12 and was established by HPLC.

The synthesis of alkylamides **7a**−**d** was achieved from the commercially available 5-(*Z*)-dodecenoic acid **3b** and various amino acid methyl esters **6a**−**d**. Polar (glycine, serine) and nonpolar (alanine) as well as acidic amino acids (glutamic acid) were used to prepare new components **7a**−**d**. The previous coupling reaction [(benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF] was followed by a saponification step under standard conditions (LiOH, THF/H_2O). Alkylamides **7a**−**d** were finally isolated, respectively, in 78, 69, 64, and 56% yields over the two steps (**Table 2**). Moreover, various carboxylic acids containing at least one *Z*-insaturation in the alkyl chain were coupled with alanine hydrochloride methyl ester **6d** using the same procedure as previously. The amidation reaction on (2*E*,6*Z*)-dodecadienoic **3d** and linolenic and palmitoleic acids **3e**−**f** followed by the hydrolysis of the methyl ester under basic conditions afforded the carboxylic acids **8a**−**c**, respectively, in 73, 33, and 76% yields over the two steps (**Table 3**).

In Vitro Pharmacology and SAR. The natural compound α-hydroxysanshool provided the framework for establishing more potent and selective TRPA1 agonists. Despite the fact that the importance of the polyenic chain and particularly the olefin configuration in the activation of TRP channels has been already reported (*2*), the role of each specific double bond had never been clarified. Hence, the activation of the channels TRPV1 and TRPA1 by the four sanshool analogues **5a**−**d**, which possess variations in the α-hydroxysanshool alkyl chain (**Table 1**), was first evaluated (**Figure 3**). To assess TRPV1- and TRPA1-induced activities by our compounds, intracellular calcium $[Ca^{2+}]_i$ increases in HEK 293 cells were monitored using Fura-2-based calcium imaging. No $[Ca^{2+}]_i$ increase was monitored in mock-transfected cells when stimulated with the different compounds for the concentration range tested. Responses were expressed relative to the maximum responses evoked by 150 μM cinnamaldehyde for TRPA1 and 1 μM capsaicin for TRPV1 receptors (assessed independently to be saturating concentrations). Normalized maximal induced responses of compounds **5a**−**d** were compared with that of a mixture of natural hydroxysanshools SOH extracts. The latter was obtained by purification of Green Szechuan oil as described under Materials and Methods. The resulting purified extract contained α-,β-hydroxysanshool and a nonidentified isomer (*m*)

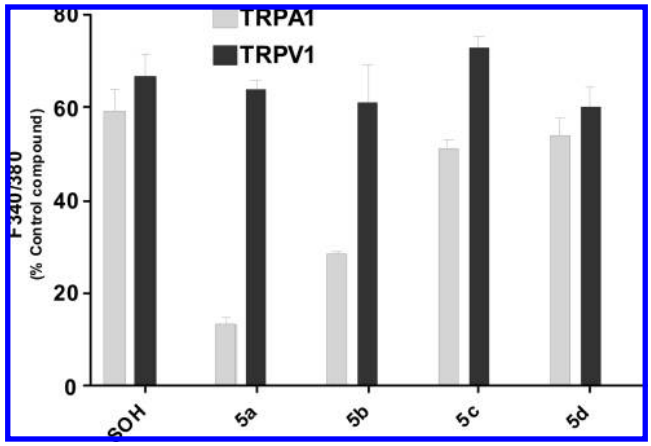


Figure 3. Normalized maximum induced responses of four sanshool analogues **5a**−**d**. Values are presented as means ± SEM, $n = 4$.

Table 4. EC_{50} Values of Selected TRPV1 and TRPA1 Ligands

EC_{50} (μM)	TRPA1	TRPV1
SOH	69.4	1.1
5a		7.0
5b		10.2
5c	100.1	3.5
5d	125.2	5.0
7a	271	250.4
7b	539.6	350.9
7c	259.2	283.7
7d	263.1	-
8a	66.8	27.5
8b	20.3	-
8c	25	115.1

with a α/β/*m* ratio of 76:21:3 (*24*). Because previous studies demonstrated that only α-hydroxysanshool was able to excite sensory neurons (*2*), the response elicited from the mixture containing predominately the α-hydroxysanshool was assimilated to that induced by the α-hydroxysanshool itself. As shown in **Figure 3** and **Table 4**, maximal activation of TRPV1 channels by all four sanshool analogues **5a**−**d** and half-maximum activation concentrations (EC_{50}) were quite similar to those induced by natural SOH. These alkylamides elicited saturating responses on TRPV1 of about 80% of capsaicin response and EC_{50} values of ≤10 μM. Consequently, modification of the alkyl system seemed to have very low influence on the TRPV1 activation, meaning that this channel does not exhibit selectivity relative to the sanshool unsaturations. On the contrary, TRPA1 activation by 500 μM sanshool analogues **5a**−**d** was dependent on the alkyl moiety (**Figure 3**). We observed weak responses induced by the fully saturated compound **5a** and the α,β-unsaturated component **5b**, whereas analogues **5c**,**d**, possessing the *Z*-olefin, were active on TRPA1 and evoked increases of the intracellular calcium in HEK 293 cells similar to those of the SOH with EC_{50} values of ≈100 μM and maximal responses reaching 60% of cinnamaldehyde response. These results point toward the crucial role of olefins in the alkyl chain and particularly that of the *Z*-olefin in the activation of expressed TRPA1 receptors.

With the alkyl moiety fixed as being 5-(*Z*)-undecenyl, a similar SAR study was initiated by modifying the amide part. Thus, the 2-methylpropan-2-ol was replaced by various amino acids to develop more selective and potent TRPA1 agonists. Normalized maximal activation of TRPA1 and TRPV1 channels by new alkylamides **7a**−**d** was evaluated and compared with activation induced by compound **5c** (**Figure 4**). It is seen that

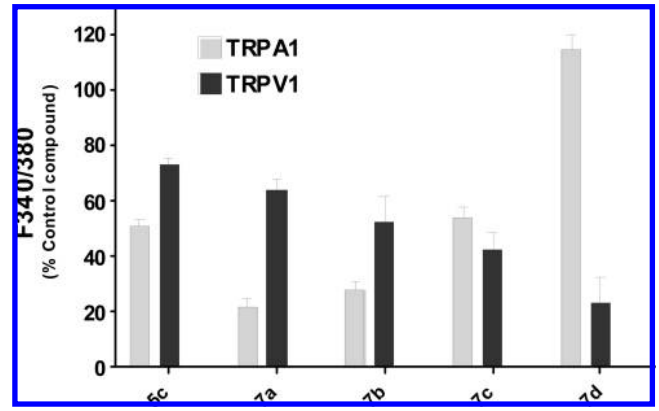


Figure 4. Normalized maximum induced responses of alkylamides **5c** and **7a**−**d**. Values are presented as means ± SEM, $n = 4$.

changing the 2-methylpropan-2-ol part of **5c** by serine or glutamic acid moieties (compounds **7a** and **7b**) resulted in a significant decrease of TRPA1 activation ($p < 0.0001$). Interactions between these molecules and the TRPA1 receptors could be disfavored either by steric hindrance or by an electronic repulsion due to the lateral chain present in serine (hydroxymethanyl) and glutamic acid (propanoic acid group). Maximal responses of expressed TRPV1 receptors induced by 500 μM monoacid **7a**, diacid **7b**, and glycinyl containing **7c** were slightly lower compared with that evoked by a 500 μM concentration of the alkylamide **5c**. However, a drastic change superior to 100-fold is observed in the transition of EC_{50} values from **5c** to **7a**−**c**, as a consequence of hampered hydrophobicity of the amide moiety. With regard to TRPA1 activation, component **7c** was a more potent agonist than **7a** and **7b** on TRPA1 channels but was still a partial agonist inducing ≈60% of cinnamaldehyde response. In addition, acid **7c** was not able to discriminate significantly the TRPA1 and TRPV1 receptors ($p > 0.05$). Interestingly, when glycine was replaced with alanine, alkylamide **7d** exhibited a stronger TRPA1 activation than that caused by sanshool analogue **5c** and SOH. Furthermore, component **7d** exhibited specificity toward TRPA1 as it was a full agonist at TRPA1 receptors and had only a low effect on TRPV1 channels. However, its activity at TRPA1 was monitored in the high micromolar range (**Table 4**).

Having identified alanine as an optimal amide substituent, an additional SAR study was carried out with alkylamides **8a**−**c** containing at least one *Z*-insaturation and various lengths of alkyl chain. As shown in **Figure 5A**, TRPA1-expressing cells responded to all four alkylamides **7d** and **8a**−**c** in a dose-dependent manner. Interestingly, the gain in activity and specificity obtained from **5c** to **7d**, when the 2-methylpropan-2-ol was replaced by the alanine, was not observed in the alkylamides **5d** to **8a**. Therefore, the presence of the α,β-unsaturation does not significantly modify the TRP activity induced by the α-hydroxysanshool analogue **5d** compared to that obtained with **5c** (**Figure 3**). However, this unsaturation negatively affects the transition from the alanine conjugate **7d** (**Figure 4**) and **8a** (**Figure 5A**). These effects underlie the fact that both the alkyl chain tail and the polar amide head of the components play a role in TRPA1 activation. The best synergy between the head and the tail of the molecule to produce maximal responses at TRPA1 was obtained with **7d**. From these results, it is difficult to weigh for the TRPA1 activation, first, the importance of each part of the molecule (tail versus head) and, second, the importance of the intrinsic molecular shape versus the self-aggregation of these components in solution. On the basis of these findings, we selected mono- and pluri-Z

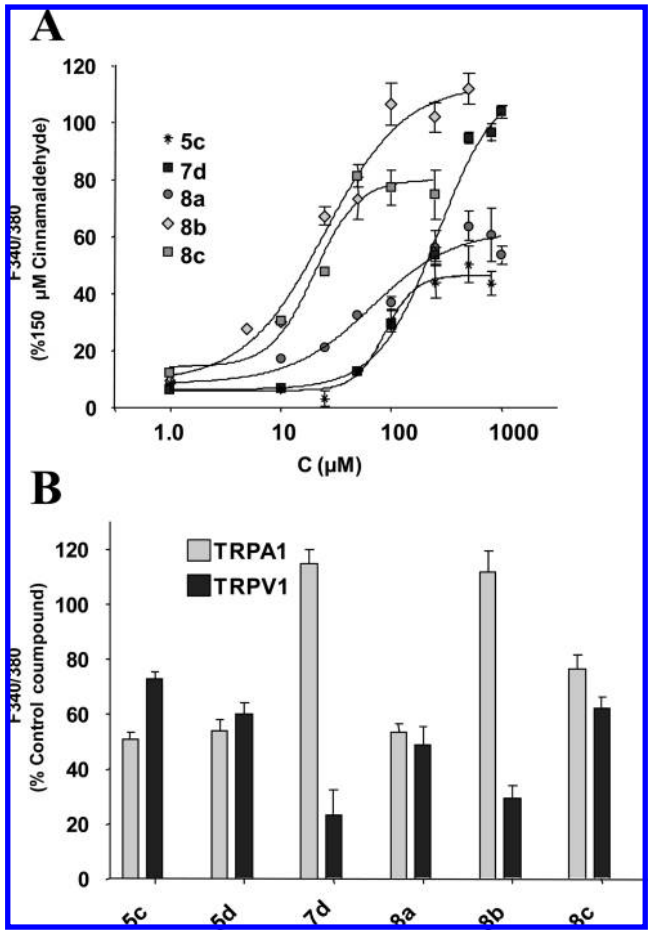


Figure 5. (**A**) Alkylamides **7d** and **8a**−**c** induced dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPA1 expression in HEK 293 cells. Dose−responses were monitored until saturation concentrations were reached and were as follows: **7d**, **8a** (0.001−1 mM), **8b** (1−250 μM), **8c** (1−500 μM). (**B**) Normalized maximum induced responses of alkylamides **5c**, **5d**, **7d**, and **8a**−**c**. Values are presented as means ± SEM, $n = 4$.

unsaturated moieties to be coupled to alanine being compounds **8b** derived from linolenic acid and **8c** derived from palmitoleic acid. Consistent with the results obtained with **7d**, we observed that the monounsaturated acid **8c** induced a relatively high TRPA1 activation but still exhibited an important TRPV1 response (**Figure 5**). In addition, the obtained half-maximal activation concentration EC_{50} (25 μM) was greatly superior to that obtained with **7d** (263 μM). Finally, the best results were observed with the alkylamide **8b**, which contained three *Z*-olefins in its C18 carbon chain. Actually, 100 μM **8b** induced potent and selective TRPA1 activation, meaning its effect on TRPV1 was relatively low. From all tested molecules, compound **8b** is the most potent and selective TRPA1 agonist. Normalized maximum value was similar to that evoked by 500 μM compound **7d**, but its EC_{50} was smaller than **8a** and **7d**, with only 20.3 versus 25 and 263 μM for **8a** and **7d**, respectively. Because components **8a** and **8b**, which differ only in the number of insaturations in their alkyl system (1*Z* for **8a** vs 3*Z* olefins in **8b**), had different reactivities toward TRPA1 channels, this result reinforced the importance of *Z*-olefins to interact strongly with TRPA1 receptors.

New alkylamides were designed from α-hydroxysanshool, the molecule responsible for the burning and tingling perceptions of Szechuan pepper. Their evaluation on TRPA1 and TRPV1 channels, two TRPs involved in the trigeminal perception, led to the elaboration of new selective and more potent TRPA1 agonists **7d** and **8b**. In particular, it has been shown that unsaturations in the alkyl chain and the amide moiety modulate differently TRPA1 and TRPV1 channels. In the future, the alkylamide **8b**, which is easily accessible from the natural linolenic acid, would be prepared in food grade quality to evaluate its sensory characteristics and correlate them with its TRPA1 activity.

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