Identification of a Novel TRPM8 Agonist from Nutmeg: A Promising Cooling Compound

Tomohiro Shirai,[†] Kentaro Kumihashi,[†] Mitsuyoshi Sakasai,[‡] Hiroshi Kusuoku,[‡] Yusuke Shibuya,[‡] and Atsushi Ohuchi[†]

[†]Kansei Science Research and [‡]Biological Science Research, Kao Corporation, 2606 Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan

Supporting Information

ABSTRACT: The transient receptor potential melastatin 8 (TRPM8) ion channel is the primary receptor for innocuous cold stimuli (<28 °C) in humans. TRPM8 agonists such as *l*-(–)-menthol are widely used as flavors and additives to impart briskness, in addition to medicinal uses for inflammation and pain. Though various natural and synthetic agonists have been explored, only few natural compounds are known. We report herein the identification and characterization of the novel neolignan agonist *erythro*- and *threo*- Δ^8 -7-ethoxy-4-hydroxy-3,3',5'-trimethoxy-8-O-4'-neolignan (**1**) with an EC₅₀ of 0.332 μ M, which was isolated from a well-known spice, nutmeg (*Myristica fragrans* Houtt.). Structure activity relationships are also disclosed, showing that the 7-*d*-methoxy derivative is the most potent agonist (EC₅₀ = 11 nM). The combination of **1** and *l*-(–)-menthol has an additive effect, suggesting that neolignan compounds interact with TRPM8 at different sites from those of *l*-(–)-menthol.

KEYWORDS: TRPM8, Cooling compound, nutmeg, neolignan, menthol

Transient receptor potential (TRP) channels are one of the largest groups of ion channels, with at least 28 members in 6 families characterized in humans. Several TRP channels are expressed in sensory neurons and are receptors for various chemicals and a wide range of thermal stimuli.^{1,2} TRPM8, a member of the melastatin subfamily of TRP channels, is a primary receptor for innocuous cold stimuli (<28 °C) in humans.^{3,4} TRPM8 is also activated by “cooling compounds” such as menthol, icilin, their analogs, other synthetic compounds, and a few natural compounds.^{5–7} Recent findings have also shown that some pathological conditions, such as inflammation, pain, prostate cancer, asthma, and dry eye, are related to different states of TRPM8 expression and activation. Therefore, drug-like agonists and antagonists have been developed for clinical applications.⁵

Synthetic and natural *l*-(–)-menthol is the TRPM8 agonist with the most widespread use as an additive in food, drinks, tobacco, cosmetics, and medicinal products to impart a sense of cold and briskness or to relieve pain. However, high concentrations of *l*-(–)-menthol often create a burning sensation, unpleasant irritation, and a distinctive smell and taste, whereas low concentrations do not evoke enough of a cooling sensation or persist for a sufficient duration.⁸ Various natural and synthetic analogs have been explored to overcome such deficits; for example, synthetic menthancarboxamides are highly potent TRPM8 agonists, some of which are already in use.^{5–7} Other synthetic agonists with completely different structures and high potencies have also been discovered.^{9,10}

Despite these efforts, few natural agonists have been developed. Except for menthol and similar compounds, eugenol and some monoterpenes have been the only known natural agonists, though their potencies are fairly low.¹¹ Natural products provide diverse and complex structures that are often challenging to prepare via synthetic methods.¹² Novel natural agonists might expand the design of TRPM8 agonists and the field of study by unknown bioactivities.

Therefore, we explored novel TRPM8 activators from botanical and spice extracts by combination with *l*-(–)-menthol to find agonists that were not competitors with menthol. The TRPM8 activities were evaluated through calcium imaging of human TRPM8-expressing HEK293 cells with fluo-4. This screening revealed that the ethanol extract of dried nutmeg (*Myristica fragrans* Houtt.) seed showed menthol-enhancing activity and weak TRPM8 activity by itself. Nutmeg and its aril mace are common and unique spices, widely used in cooking. Their essential oils, oleoresins, and extracts are also contained in groceries and commodities as flavors and fragrances. Several medicinal uses have been studied and are traditionally known, but no reports have indicated TRPM8 activity or a cooling effect.¹³ Though eugenol is contained in the extract, the activity of the extract could not be explained by eugenol alone.

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Therefore, we began to identify the TRPM8 agonists in the nutmeg extract through bioassay-guided purifications with silica gel column chromatography and semipreparative silica gel HPLC. Finally, the active fraction produced a single peak in silica gel HPLC and ODS-HPLC analyses. A wavelength of maximal absorption at 278 nm by the fraction indicated a conjugated aromatic moiety. A sodium adduct ion peak in positive MS mode was observed at m/z 425. However, over 39 carbon peaks were observed in the ^{13}C NMR spectrum measured in CDCl_3 , suggesting that there were two isomers of phenylpropanoid dimers that were not separated by HPLC. The 1D and 2D NMR spectra indicated that both isomers had 6-methoxyeugenol skeletons because 2 aromatic protons and 6 methoxy protons were obtained by integration, and substitution of the aromatic rings with allylic groups was observed in the both isomers. The spectra also revealed that both isomers had *n*-propyl skeletons that were substituted with ethoxy groups, guaiacol moieties, and 4-allyl-2,6-dimethoxyphenyl groups. From these results, we deduced that the isomers were *erythro*- and *threo*- $\Delta^{8',7}$ -ethoxy-4-hydroxy-3,3',5'-trimethoxy-8-O-4'-neolignan (**1**) (Figure 1).¹⁴ Though various 8-O-4'-neolignans

Figure 1. Structure of **1**.

have been identified from nutmeg, no 7-ethoxy analogs have been reported except for a suggestively semisynthetic compound.^{14,15} No spectroscopic data were available for **1**; therefore, the structures were elucidated by comparison with the spectroscopic data of synthetic **1**.

Diastereoselective synthesis of **1** was achieved by following the reported diastereoselective reduction of the ketone at C-7 in similar compounds (Scheme 1).¹⁶ The phenolic group of vanillin (**2**) was protected by a TBS group to give the TBS ether **3**. A Grignard reaction with ethyl magnesium bromide and oxidation of the secondary alcohol with manganese dioxide afforded the ketone **4**, and the α -position was brominated. In this bromination, the TBS group was partly eliminated, and the crude product was subsequently treated with hydrochloric acid to afford a completely deprotected α -bromoketone. Reprotection of the phenolic group by MOMCl gave the MOM ether **5**, which was condensed with 4-allyl-2,6-dimethoxyphenol (**6**) to afford the ketone **7**. To synthesize the *erythro* isomer, **7** was reduced by lithium aluminum hydride to give the *erythro*-secondary alcohol **8a** preferentially (77% yield compared with 10% yield of the *threo*-isomer **8b**). The relative configurations were deduced by the coupling constant between the C-7 and C-8 protons. **8a** had a smaller coupling constant (2.6 Hz) than **8b** (8.6 Hz), similar to other 8-O-4'-neolignans. Furthermore, the MOM group of **8a** was deprotected here to give the reported *erythro* compound to determine the relative configurations (see Supporting Information).¹⁵ Then, **8a** was alkylated with ethyl iodide to afford the *erythro*-ethyl ether **9a**, and subsequent deprotection of the MOM group with hydrochloric acid in THF gave the *erythro* isomer **1a**. Next, **7** was reduced by sodium borohydride with crown ether to afford **8b** preferentially (68% yield compared with 6% yield of **8a**). **8b** was alkylated with ethyl iodide to afford the *threo*-ethyl ether **9b**, which afforded the *threo* isomer **1b** following deprotection. The

Scheme 1. Diastereoselective Synthesis of **1**^a

^aReagents and conditions: (a) imidazole, TBSCl, DMF, 92%; (b) EtMgBr , THF, 0 °C; (c) MnO_2 , CH_2Cl_2 , 77% (for 2 steps); (d) Br_2 , CHCl_3 ; (e) 6 N HCl, MeOH; (f) MOMCl, DIPEA, CH_2Cl_2 , 67% (for 3 steps); (g) **6**, K_2CO_3 , acetone, 40 °C, 74%; (h) LiAlH_4 , THF, 77% (10% for **8b**); (i) NaH, EtI, THF, 0 °C to rt, 82% for **9a**, 66% for **9b**; (j) 6 N HCl, THF, 0 °C to rt, 61% for **1a**, 72% for **1b**; (k) NaBH_4 , 15-crown-5, *i*-PrOH/MeOH, 68% (6% for **8a**).

^1H and ^{13}C NMR chemical shifts of synthetic **1a** and **1b** and the purified fraction of nutmeg extract were identical to each other (see Supporting Information). We determined that the TRPM8-active compounds in the nutmeg extract were an approximately 1:1 diastereomixture of **1**.

We further investigated the enantiomeric ratio of the isolated compound **1**. Analysis on a Daicel Chiralpak IC column revealed four peaks, suggesting four stereoisomers in the almost equal proportions caused by two chiral centers at C-7 and C-8. To determine the absolute configurations of each peak, we prepared the four stereoisomers from optically pure intermediates as follows (Scheme 2). (*S*)- and (*R*)-MTPA esterification of the synthetic *erythro* intermediate **8a** and

Scheme 2. Preparation of the Four Stereoisomers of **1**^a

^aReagents and conditions: (a) (*S*)-(+)-MTPACl or (*R*)-(–)-MTPACl, Et_3N , DMAP, CH_2Cl_2 , 78–79% as a diastereomixture; (b) 50 w/v% NaOH aq, MeOH, 93–100%.

threo intermediate **8b** enabled the separation of the enantiomers and determination of the absolute configurations by a modified Mosher method (see [Supporting Information](#)).¹⁷ Hydrolysis of the (S)-MTPA ester **10aa-s**, which had a (7*R*,8*S*) configuration, gave the (7*R*,8*S*)-secondary alcohol **8aa**. The (7*S*,8*R*) isomer **8ab** was synthesized in the same way, and the optically pure *threo* isomers **8ba**, which has a (7*R*,8*R*) configuration, and **8bb**, which has a (7*S*,8*S*) configuration, were also obtained using the same method as for synthetic **8b**. Each isomer was alkylated with ethyl iodide and deprotected as indicated before to afford four stereoisomers of **1**. By comparing the retention times in the chiral column of **1** from the nutmeg extract and the synthetic standards, the absolute configurations of all peaks were determined (see [Supporting Information](#)).

From this result, it was suspected that the identified **1** was an artifact because most identified 8-*O*-4'-neolignans in nutmeg had *erythro* configurations and ethanol was used as an extraction solvent.¹⁵ We identified the *erythro*-7-hydroxy analog named myrislignan **11a** and the (7*S*,8*R*)-7-acetoxy analog **12ab** and observed that the amount of **1** in the nutmeg extract was increased in ethanol, suggesting that **11a** and/or **12ab** were gradually converted to **1**. Thus, the majority of identified **1** should be an artifact. On the other hand, LC/MS/MS analysis of nutmeg extract purified without using ethanol contained a trace amount of **1** (see [Supporting Information](#)). Further experiments including biosynthetic studies are required to determine the actual naturality of **1**; however, the existence of natural **1** is suggested.

The activities of TRPM8 and other TRP channels were measured for **1** and its stereoisomers ([Figure 2](#)). A mixture of

Figure 2. Dose–response curve of **1** and *l*-menthol: (a) for TRPM8; (b) for TRPA1. *N* = 6 (mean $\pm$ SEM).

the four stereoisomers of **1** had an EC_{50} of 0.332 μ M, which is the lowest of the natural products with known TRPM8 activity. Additionally, **1bb**, which has a (7*S*,8*S*) configuration, produced the lowest EC_{50} value (0.087 μ M) compared with the other isomers, which had EC_{50} values of 0.583–1.15 μ M ([Table 1](#)).

1 also exhibited weak TRPA1 activity (EC_{50} = 19.1 μ M) like *l*-menthol (EC_{50} (M8) = 10.1 μ M, EC_{50} (A1) = 96.0 μ M). TRPA1 activity often causes irritation, and higher selectivity for TRPM8 is preferable for use as a cooling additive. The submicromolar TRPM8 EC_{50} value of **1** presumably enables display of the cooling effect *in vivo* with relatively lower TRPA1 activity. Therefore, **1** is a promising novel cooling compound. **1**

Table 1. EC_{50} Values of **1** and Its Enantiomers

Compounds	Configuration	EC_{50} (M8)(μ M) ^a	EC_{50} (A1)(μ M) ^a
1	4 isomers	0.332 $\pm$ 0.046	19.1 $\pm$ 0.7
/-Menthol		10.1 $\pm$ 1.0	96.0 $\pm$ 5.1
1aa	7 <i>R</i> ,8 <i>S</i>	1.13 $\pm$ 0.55	N.D.
1ab	7 <i>S</i> ,8 <i>R</i>	1.15 $\pm$ 0.44	N.D.
1ba	7 <i>R</i> ,8 <i>R</i>	0.583 $\pm$ 0.276	N.D.
1bb	7 <i>S</i> ,8 <i>S</i>	0.087 $\pm$ 0.017	N.D.

^a*N* = 3–5 (mean $\pm$ SEM). N.D. = not determined.

did not activate the TRPV1, TRPV3, or TRPV4 channels (data not shown).

The TRPM8 activity of other 8-*O*-4'-neolignans from nutmeg extract and their analogs were also investigated. In addition to **11a** and **12ab**, the (7*S*,8*R*)-4-methoxy-7-acetoxy analog **13ab** and (7*S*,8*R*)-4-methoxy analog **14ab** were obtained by purification and derivatization. Interestingly, natural **12ab** and **13ab** were optically active, whereas **11a** was racemic (see [Supporting Information](#)). The substitution of various alkoxy groups at C-7 was carried out using acids and alcohols from natural **11a**, which is the main nonvolatile component in nutmeg, through a quinone methide intermediate ([Scheme 3](#)).¹⁸ This biomimetic alternation was

Scheme 3. Acid-Induced Conversion of the Substituents at C-7 with a Configurational Randomization through a Quinone Methide Intermediate

possible with primary and secondary alcohols using hydrochloric acid, whereas it produced a low yield with *tert*-butanol because of the bulkiness. This is a fairly convenient method to prepare various analogs, though the configuration at C-7 is epimerized. The same reaction was applied for **12ab**. We prepared 10 analogs **15–24** from **11a** using the following 10 alcohols: methanol, *n*-hexanol, *n*-octanol, *n*-decanol, 2-butoxyethanol, *iso*-propanol, *sec*-butanol, cyclohexanol, *l*-menthol, and *d*-menthol. The TRPM8 activities of these analogs were measured, and their EC_{50} values were calculated ([Table 2](#)). The 4-methoxy analogs **13ab** and **14ab** completely lost the activity, suggesting that the 4-hydroxy group is essential for the activity. The 7-hydroxy analog **11a** and 7-acetoxy analog **12ab** also showed low activity, suggesting that the alkoxy groups at C7 are also important for the activity. Longer alkoxy chains resulted in smaller EC_{50} values (**18** < **17** < **16** $\leq$ **1** < **15**), and bulkier alkoxy groups resulted in smaller EC_{50} values as well (**24** < **23** < **22** < **20** < **21** < **1**). The introduction of an ether bond did not affect the activity significantly (**19** versus **16** and **17**). The *d*-menthoxy analog **24** had the lowest EC_{50} of 11 nM, which was smaller than that of the synthetic menthanecarboxamide WS-12 (EC_{50} = 66 nM), a representative cooling compound. Though

Table 2. TRPM8 Potency SAR of 8-*O*-4'-Neolignan Derivatives

$Ar =$

1: EC₅₀ = 332 ± 46 nM (R₁ = H, R₂ = Et, *ery:thr* = 50:50)
1bb: EC₅₀ = 87 ± 17 nM (R₁ = H, R₂ = Et, (7*S*,8*R*))

No.	R ₁	R ₂	<i>dr</i> ^a (<i>ery:thr</i>)	EC ₅₀ (nM) ^b	No.	R ₁	R ₂	<i>dr</i> ^a (<i>ery:thr</i>)	EC ₅₀ (nM) ^b
11a	H	H	100:0 (racemi)	>10000	18	H		62:38	66 ± 7
12ab	H	Ac	100:0 (7 <i>S</i> ,8 <i>R</i>)	>10000	19	H		64:36	153 ± 2
13ab	Me	Ac	100:0 (7 <i>S</i> ,8 <i>R</i>)	>10000	20	H		60:40	82 ± 4
14ab	Me	H	100:0 (7 <i>S</i> ,8 <i>R</i>)	>10000	21	H		60:40	138 ± 26
15	H	Me	59:41	3450 ± 314	22	H		62:38	54 ± 7
16	H		60:40	329 ± 49	23	H		N.D. ^c	28 ± 3
17	H		61:39	86 ± 10	24	H		N.D. ^c	11 ± 3

^a*dr* was calculated from ¹H NMR data. ^b*N* = 3 (mean ± SEM).
^cMixture of four stereoisomers. *dr* = 12:26:26:36 for **23** and **24**.
 Calculated from ¹H NMR data.

it was challenging to separate the stereoisomers of **24**, the (7*S*,8*S*) isomer **24bb** is expected to be a more potent agonist.

Intriguingly, **24** had a smaller EC₅₀ than the *l*-menthoxy analog **23** (EC₅₀ = 28 nM), though *l*-menthol is more potent than *d*-menthol. It was speculated that the interaction sites of these neolignans are different from those of menthols because of the entirely novel skeleton compared to the reported TRPM8 agonists. Previous reports have noted that one of the key residues in the interaction between *l*-menthol and TRPM8 is Tyr745, located in the middle of putative transmembrane segment 2.¹⁹ Therefore, we prepared Y745H-TRPM8 through a point mutation at this residue, and the activity of **1** was measured (Figure 3). Though the activity of *l*-menthol significantly disappeared with the mutated channel, the activity of **1** was not affected at all. Additionally, **1** did not evoke a competitive effect with *l*-menthol but an additive effect, suggesting that the interaction sites of **1** are different from those of *l*-menthol (Figure 3). Future studies exploring the actual interaction sites will be directed toward designing fresh agonists and understanding the molecular dynamics of TRPM8.

Lastly, the cooling effect of **1** was evaluated by a simple mouth washing test. **1** or *l*-menthol (0.02%) was dissolved in water with a surfactant (EMANON CH-40, 0.6%), and the cooling effect was scored for 30 min after washing (score: 0–5, in increments of 0.5) (Figure 4). *l*-Menthol evoked a cooling sensation soon after washing, whereas **1** evoked this sensation a few minutes later. This difference was partly due to the difference in absorption properties because of the high molecular weight of **1** (402 Da) and the low molecular weight of *l*-menthol (156 Da). However, further investigation of the kinetic differences in TRPM8 activity is required to discuss the

Figure 3. (a) Effect of a 745Y mutation in TRPM8. *N* = 3 (mean ± SEM). (b) Additive effect of TRPM8 activity with **1** and *l*-menthol. *N* = 4 (mean ± SEM).

Figure 4. Cooling effect of **1** and *l*-menthol by a simple mouth washing test. *N* = 6 (mean ± SEM).

precise kinetic differences. This absorption discrepancy probably caused a difference in the duration of the cooling sensation as well; thus, **1** exhibited a long-lasting cooling effect, which is unique and industrially valuable. Therefore, **1** is a promising cooling compound and clearly exhibits TRPM8 activity *in vivo*.

In summary, we identified a novel TRPM8 agonist **1** that is the most potent agonist discovered from natural sources to date. 8-*O*-4'-Neolignans are major nonvolatile components in nutmeg, and the safety of **1** might be ensured experientially. **1** did not compete with *l*-menthol but showed an additive effect. The high molecular weight of **1** resulted in a cooling effect with a long duration time. These features make **1** an attractive cooling compound. Additionally, we demonstrated the easy alternation of the alkoxy group at C-7 under acidic conditions, and more potent derivatives were discovered. This reaction can be utilized in industrial processes, and various structural modifications would be useful for the preparation of labeled derivatives as chemical tools for studying the TRPM8 channel at the molecular level.

■ ASSOCIATED CONTENT

Supporting Information

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Purification procedures, synthetic procedures, compound characterization, and experimental protocols for the assays (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: shirai.tomohiro@kao.co.jp.

ORCID

Tomohiro Shirai: 0000-0001-6278-4021

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ABBREVIATIONS

HEK, human embryonic kidney; ODS, octadecyl silica; TBS, *tert*-butyldimethylsilyl; MOM, methoxymethyl; MTPA, α -methoxy- α -(trifluoromethyl)phenylacetic

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