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Natural-Product-Derived Transient Receptor Potential Melastatin 8 (TRPM8) Channel Modulators

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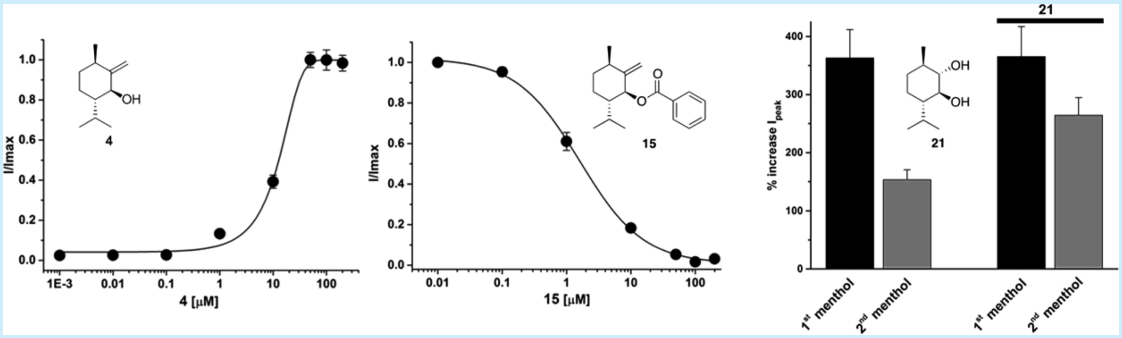
Supporting Information



ABSTRACT: A library of novel structural hybrids of menthol and cubebol was tested for each derivative's ability to interact with the transient receptor potential subfamily melastatin member 8 (TRPM8) channel. This structure–activity relationship study revealed three potent modulators of the TRPM8 ion channel: a novel agonist (**4**) with an EC_{50} value of 11 ± 1 μM, an antagonist (**15**) with an IC_{50} value of 2 ± 1 μM, and an allosteric modulator (**21**) that minimized channel desensitization toward menthol. Each of these novel exocyclic olefin analogues of menthol is readily accessible by synthesis and was tested using Ca^{2+} assays and electrophysiology.

Our group's interest in anti-inflammatory natural products led us to investigate the structure–activity relationships (SARs) of the clinically and fundamentally intriguing transient receptor potential (TRP) channel agonists.[1,2] The TRP family contains more than 30 cation channels, which are divided into seven main subfamilies, three of which are thermosensitive: the TRPV (vanilloid) family, the TRPM (melastatin) family, and the TRPA (ankyrin) family.[3,4] All three of these subfamilies display diverse cation selectivities and biophysical properties, but their expression within dorsal root and trigeminal ganglia make them viable targets for the modulation of nociception and pain.[4−6] TRPM8, which is expressed in nearly 15% of all somatosensory neurons, is of particular interest in terms of druggability, as it was also found in the epidermis, bladder, and prostate.[6−8] TRPM8 is selective for calcium and is activated by a range of low temperatures (8–28 °C) and by both natural and synthetic cooling compounds such as menthol (**1**), cubebol (**2**), and icilin (**3**) (Figure 1).[3−8] In addition to its role as the primary cold receptor of the somatosensory system, TRPM8 has been suggested as a potential target for cancer therapy because of the presence of TRPM8 mRNA in prostate tissue and at elevated levels in tumors of the prostate, breast, colon, lung, and skin.[9,10] TRPM8 has also been identified as a target for respiratory diseases and for cold hypersensitivity associated with inflammatory and neuropathic pain.[7,9−13] Discovering new natural product derivatives that are modulators of TRPM8 is a central goal of this research program because of their potential therapeutic applications in modulating TRPM8 in chronic diseases.

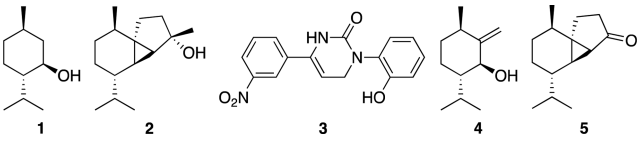


Figure 1. Natural and synthetic TRPM8 modulators menthol (**1**), cubebol (**2**), and icilin (**3**) and initial synthetic intermediates **4** and **5**.

Natural products including terpenes and alkaloids, such as resiniferatoxin and voacangine, respectively, have been a major source of ligands for the TRP channels.[10,14−16] The small-molecule natural products **1** and **2** are strong activators of the TRPM8 channel.[7,17] Given the structural similarities of **1** and **2**,

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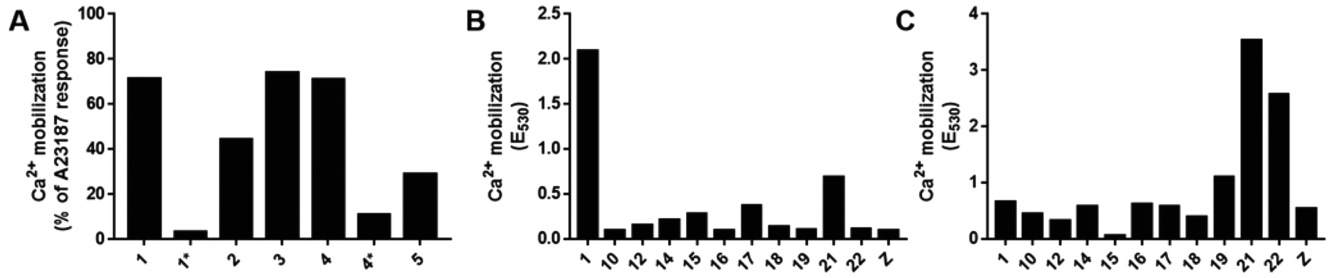


Figure 2. Evaluation of TRPM8 modulators by calcium measurements. Menthol (**1**), cubebol (**2**), icilin (**3**), and ethanol (**Z**) were used as controls. (A) Calcium mobilization in HEK cells transfected with TRPM8 after injection of analogues **1**, **2**, **4**, and **5** at 100 μM and **3** at 90 μM. * indicates nontransfected HEK cells used as a control. (B) Calcium mobilization in HEK cells transfected with TRPM8 after injection of analogues at 500 μM. (C) Calcium mobilization evoked by 100 μM menthol in HEK cells transfected with TRPM8 after stimulation with analogues at 500 μM.

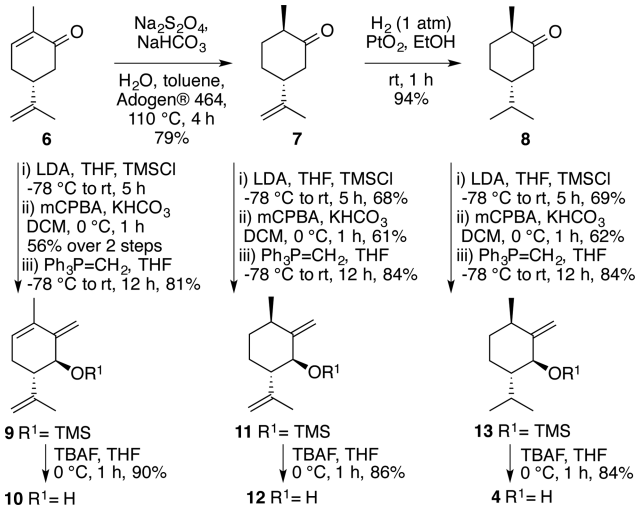
we recognized the value of a systematic study of synthetic analogues of these compounds with the goals of developing a better understanding of how these natural products are involved in regulating the cooling sensation mediated by the TRPM8 channel and of discovering selective modulators. Although high-resolution crystal structures of closely related TRP channels (TRPV1 and TRPA1) exist, no crystal structure of the TRPM8 channel has been reported to date.[18−20] A SAR study of natural product analogues and their interactions with TRPM8 is crucial to gain information on the therapeutically significant TRPM8 binding site.[20]

To efficiently prepare a new generation of small-molecule TRPM8 modulators, developing a synthetic route that would allow for the production of a variety of analogues was essential. On the basis of the brevity and reproducibility of the synthesis, the work of Hodgson et al.[21] was followed for the preparation of initial quantities of cubebol. Two intermediates of this route, allylic alcohol **4** and tricyclic ketone **5** (Figure 1), were evaluated for their impact on TRPM8. As an initial qualitative screen, fluorescent calcium imaging in TRPM8-transfected HEK cells was used to evaluate **2**, **4**, and **5** at high concentration (Figure 2A). Notably, **4** appeared to be an agonist of TRPM8 with a calcium mobilization greater than that of **2**, while **5** was not significantly active. Given that **4** clearly interacts with the TRPM8 receptor, we turned our attention to the development of a revised, low-cost synthesis of **4** from readily available (*R*)-(−)-carvone (**6**) instead of menthol as used in the Hodgson method.[21] This alternative route avoids the use of high-cost reagents to access the multigram quantities of **4** required for generating analogues.

Our synthetic route began with **6**, which was transformed into (*R*)-dihydrocarvone (**7**) via stereoselective reduction with sodium dithionite under phase-transfer conditions (Scheme 1).[22,23] These conditions were chosen to selectively reduce the conjugated olefin, and the minor diastereomer that is formed can be easily separated from the product using silica gel column chromatography. Hydrogenation over PtO_2 in ethanol was employed to prepare (*R,R*)-tetrahydrocarvone (**8**) from **7**. PtO_2 was found to be more selective than Pd/C, which resulted in the formation of a substantial amount (ca. 20%) of another diastereomer resulting from double-bond migration. The same sequence of four steps (silyl enol ether formation, Rubottom oxidation, Wittig olefination, and silyl removal) was used to transform **6**, **7**, and **8** into the corresponding allylic alcohols **10**, **12**, and **4** (Scheme 1).

From allylic alcohol **4**, a variety of analogues were prepared via alcohol functionalization. These compounds were synthesized using established synthetic methods. To examine the

Scheme 1. (*R*)-(−)-Carvone-Based Synthesis of Allyl Alcohols



effect of ester substituents on the binding of allylic alcohol **4** with TRPM8, the syntheses of the acetate (**14**) and the benzoate (**15**) were carried out (Scheme 2). In order to elucidate the effect of the exocyclic double bond, **16**, **17**, **18**, and **19** were synthesized as analogues of Frescolat ML,[24] Coolact 5,[25] (−)-menthyl succinate,[26] and *N,N*-dimethyl (−)-menthyl succinamide,[27] respectively (Scheme 2).

To boost the yield of the sequential silyl enol ether formation and Rubottom oxidation steps and to prepare a number of derivatives without the terminal alkene, triethylchlorosilane (TESCl) was used instead of trimethylsilyl chloride (TMSCl) in the first step (**20**, Scheme 2). This change of the hydroxyl protecting group resulted in a noticeable increase in yield for both the silyl enol ether preparation step (from 69 to 78%) and the Rubottom oxidation step (from 62 to 66%). These improved results can be attributed to the stability of the TES protecting group, which is 64 times more stable toward acid and 10−100 times more stable toward base than the TMS moiety.[28] TES-protected hydroxy ketone **20** was then used for the preparation of various derivatives, including diol **21** via reduction and deprotection, amino alcohol **22** via reductive amination and deprotection, and TES-protected allylic alcohol **23** via Wittig olefination (Scheme 2).

The qualitative evaluation of each derivative's ability to activate TRPM8 was again conducted using a fluorescence-based calcium assay in TRPM8-transfected HEK cells.[29] On the basis of the high concentration of compounds used for the

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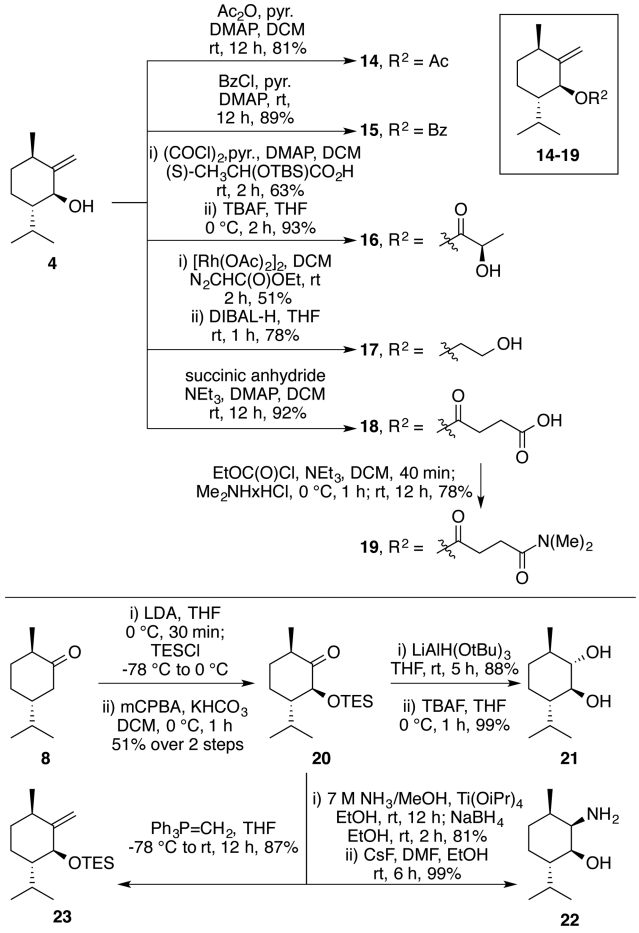
Scheme 2. Syntheses of Unsaturated Menthol Analogues 14−19, Diol 21, and Amino Alcohol 22



preliminary screen, all of the synthetic analogues of **4** were essentially inactive as agonists (Figure 2B). Suspecting that the analogues could potentially interact with TRPM8 but were unable to induce calcium mobilization, we evaluated the analogues as antagonists by pretreating cells with a test compound followed by addition of menthol (Figure 2C). Benzoate derivative **15** gave a reduced fluorescence response consistent with an antagonistic modulator. Unexpectedly, diol **21** gave a pronounced enhancement in fluorescence. With these results in hand, compounds **4**, **15**, and **21** were evaluated for TRPM8-mediated calcium mobilization via electrophysiological measurements using the conventional whole-cell patch clamp method (see the Supporting Information (SI) for more details).

When applied at a concentration of 100 μM at room temperature, compound **4** and menthol (**1**) both evoked a TRPM8 current in transfected HEK cells. Upon exposure to **4** and **1**, the TRPM8 current density elicited by depolarization steps from a holding potential of 0 mV to +100 mV showed similar increases of 3.6 times (from 48 ± 13 to 170 ± 18 pA/pF, n = 5) and 4.1 times (from 34 ± 9 to 140 ± 16 pA/pF, n = 5) from the basal current, respectively (see the SI for more details). It was found that **4** and **1** both evoke a TRPM8 current in a dose-dependent manner with half-maximal effective concentrations (EC_{50}) of 11 ± 1 and 33 ± 3 μM respectively, demonstrating the potency of allylic alcohol **4** to activate the TRPM8 channel.

Through the preliminary screen of the derivatives by fluorescent calcium imaging, benzoate derivative **15** was suspected to have antagonist activity on menthol-induced TRPM8 currents. Indeed, pretreatment with **15** (50 μM) completely blocked the TRPM8 current induced by menthol (100 μM, n = 6). At +100 mV under control conditions, the peak current density was 13 ± 2 pA/pF compared with 140 ± 20 pA/pF after the application of menthol and 17 ± 3 pA/pF after the application of **15** followed by menthol (see the SI for more details). Benzoate derivative **15** exhibited dose-dependent inhibition of the TRPM8 currents with a half-maximal inhibitory concentration (IC_{50}) of 2 ± 1 μM.

The desensitization of the TRPM8 channels that occurs after long-term or repeated stimulation by cold or menthol is a well-known phenomenon and is dependent on the presence of extracellular Ca^{2+}.[17] The peak of the menthol-evoked currents at +100 mV was reduced (56 ± 3% decrease, n = 4) between consecutive applications of menthol. When the TRPM8-transfected HEK cells were pretreated with diol **21** (100 μM) between applications of menthol, the current density of the second stimulation was reduced by only 27 ± 2% (n = 6). These results indicate that **21** is an allosteric modulator of TRPM8 that significantly prevents menthol-induced channel desensitization.

The growing amount of data on TRPM8 accumulated since its discovery provides evidence that this channel has roles more diverse than simply being a cold sensor. TRPM8 has been shown to be involved in the cold hypersensitivity and hyperalgesia that appear in neuropathic conditions (e.g., complex regional pain syndrome, trigeminal neuralgia, peripheral nerve injury, or chemotherapy-induced neuropathy) and are often accompanied by cold hypoesthesia.[5,6,13,30] Furthermore, TRPM8 has been implicated in certain types of cancer as well as chronic inflammatory diseases including inflammatory bowel disease.[9,10,31,32] Novel, potent modulators of TRPM8 could provide pharmacological tools in the treatment of various pathological conditions, as indicated by the recent finding that TRPM8 antagonists are viable agents for the treatment of both cold hypersensitivity and hyperalgesia.[5,13] The current SAR study of several menthol derivatives and their interactions with the TRPM8 channel has revealed three potent modulators. The in vitro assays conducted demonstrate the agonistic activity of allylic alcohol **4**, the antagonistic activity of benzoate derivative **15**, and allosteric modulation of the TRPM8 channel by diol **21**. To further investigate the implications of modulating this channel, a second library of menthol derivatives is being assembled on the basis of these results. With a second generation of menthol derivatives in hand, in vivo evaluations of the best candidates from each library (including derivatives **4**, **15**, and **21**) will be conducted to develop a better understanding of the implications of modulating the TRPM8 channel using small-molecule natural product analogues.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01222.

Experimental procedure, physical characterization of the products, calcium assay methods, and electrophysiology methods and figures (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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