Organic LETTERS

Letter

pubs.acs.org/OrgLett

Two New Classes of T-Type Calcium Channel Inhibitors with New Chemical Scaffolds from *Ganoderma cochlear*

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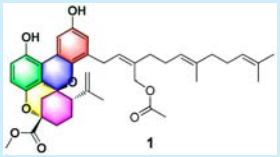
Supporting Information

ABSTRACT: T-type calcium channel (TTCC) inhibitors hold great potential for the treatment of a variety of neurological disorders. Cochleroids A–E (**1**–**5**), five pairs of dimeric meroterpenoid enantiomers, and cochlearines A (**6**) and B (**7**), two pairs of enantiomeric hybrid metabolites, were isolated and characterized from *Ganoderma cochlear*. Biological evaluation found that compounds (+)-**1**, (−)-**3**, and (±)-**6** significantly inhibited $Ca_v3.1$ TTCC and showed noticeable selectivity against $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, and $K_v11.1$ (hERG) channels.



T-type calcium channels (TTCCs) regulate neuronal excitability and rhythmic firing and are involved in various central nervous system (CNS) disorders such as absence epilepsy, insomnia, neuropathic pain, and Parkinson's disease.[1,2] Therefore, TTCCs have been identified as attractive therapeutic targets in modern drug development.[3] Many classic anti-CNS disease compounds act on TTCCs but generally have limited specificity.[3] In the past decade, novel inhibitors with high specificity for TTCCs have been successfully developed,[2,3] exhibiting efficacy in animal models of neurological disorders such as absence epilepsy,[4] neuropathic pain,[5] and sleep disorders.[6] However, most of these molecules are not yet on the market, since they are undergoing clinical trials and/or are covered by patents, limiting their utility in basic research toward understanding TTCC physiology and pathophysiology. Thus, discovery of a new generation of TTCC inhibitors is still in demand.

Natural products have not only greatly advanced our knowledge of basic biological processes in the CNS but also played a key role in the drug development for CNS diseases.[7] A few natural TTCC inhibitors have been discovered; however, these molecules mainly consist of ω-3-fatty acids,[8] cannabinoids,[9] and anandamide derivatives,[10] which have limited selectivity and structural complexity. Several *Ganoderma* species, *G. lucidum*, *G. sinense*, and *G. cochlear*, known as "Lingzhi", have been utilized as an adjuvant for CNS disorders in traditional Asian medicines since ancient time.[11] Modern pharmacological studies have revealed hypnotic, antiepileptic, and neuroprotective potentials of *Ganoderma* extracts.[12−14] During our in-depth investigation of the effect of *G. cochlear* on TTCCs, two new classes of TTCC inhibitors with novel chemical structures were isolated and identified. One class consists of cochleroids A–E (**1**–**5**), five pairs of polycyclic meroterpenoid enantiomers containing a unique methanobenzo[*c*]oxocino[2,3,4-*ij*]-isochromene scaffold (Figure 1). Another class consists of cochlearines A (**6**) and B (**7**), which have an unusual hybrid 6/6/6/5/5/6 ring system (Figure 1). Electrophysiological studies were conducted to uncover inhibition of the compounds toward TTCCs.

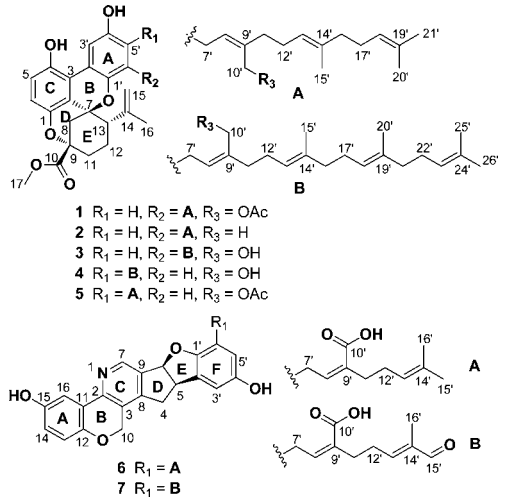


Figure 1. Structures of **1**–**7**.

Received: May 8, 2015
Published: May 29, 2015

DOI: 10.1021/acs.orglett.5b01353
Org. Lett. 2015, 17, 3082−3085

The compounds were isolated through serial fractionation and purification, and their structures were determined by using spectroscopic and computational methods. The results of structural studies demonstrate that cochlearoid A (**1**) has a molecular formula $C_{40}H_{48}O_8$, shown by analysis of its HREIMS that contains a peak at *m/z* 656.3347 $[M]^+$ (calcd 656.3349) and its ^{13}C NMR and DEPT spectra, which show that it has 17 degrees of unsaturation. The ^1H NMR spectrum (Table S1, Supporting Information) of **1** contains typical resonances for *ortho* aromatic ring protons (6.87 ppm, d, J = 8.8 Hz, H-5; 6.72 ppm, d, J = 8.8 Hz, H-6) and a *meta* proton (6.52 ppm, d, J = 2.6 Hz, H-3′; 7.92 ppm, d, J = 2.6 Hz, H-5′), suggesting the presence of 1,2,3,4-tetrasubstituted and 1,3,4,5-tetrasubstituted benzene rings. Analysis of its ^{13}C NMR and DEPT spectra reveals that **1** has 40 carbons assignable to 6 methyls (1 methoxy at 52.8 ppm and 1 acetyl at 20.8 ppm), 9 sp^3 methylenes (one oxygenated at 62.2 ppm), 1 sp^2 methylene, 7 sp^2 methines, 1 sp^3 methine, and 16 quaternary carbons (four sp^2 oxygenated, two sp^3 oxygenated at 75.6 and 80.4 ppm, two carbonyls at 172.4 and 171.0 ppm).

When consideration is given to the previously reported properties of chizhines,[15] the above data suggest that **1** likely has a polymeric meroterpenoid structure. This conclusion is confirmed by the results of a careful interpretation of 2D NMR data (Figure S1). The $^1H-^1H$ COSY correlations of H-7′/H-8′ (5.58 ppm), H-11′/H-12′/H-13′ (5.15 ppm), and H-16′/H-17′/H-18′ (5.09 ppm) (bold lines) and the HMBC correlations of H_3-20′, H_3-21′/C-18′, C-19′, H-17′, H-18′/C-19′, H_3-15′/C-13′, C-14′, C-16′, H-13′, H-16′/C-14′, H-10′/C-8′, C-9′, C-11′, and H-8′, H-11′/C-9′ (arrows) in part A (drawn in black) suggest the presence of a side chain in **1** consisting of three isoprenyl groups. HMBC correlations of H-7′/C-1′ (145.4 ppm), C-5′, and C-6′ show that this side chain is positioned at C-6′. Additional HMBC correlations of H-10′ and a proton at 2.04 ppm with a carbon at 171.0 ppm indicate that an acetoxy group is located at C-10′. Meanwhile, HMBC correlations of H-7′ with C-1′ and C-5′, H-3′ and H-5′ with C-4′ (151.3 ppm) and C-1′, along with consideration of the quaternary carbon nature of C-2′ suggest that ring A in **1** has the substitution pattern shown. $^1H-^1H$ correlations designated by bold lines in Figure S1 for part B (blue lines) were observed in the spectrum of **1**. HMBC correlations of Hb-15 (4.28 ppm), H_3-16/C-13, C-14, and H-16/C-15 (113.7 ppm) indicate the presence of a propylene moiety connected to C-13. The presence of the E ring in this substance is supported by HMBC correlations of H-11/C-9, H-12/C-7, C-9, H-8/C-7, C-9, C-11, C-13, and H-13/C-7. Furthermore, HMBC correlations of H-8, H-11, an H_3-17/C-10 (172.4 ppm) suggest that C-9 is bonded to a methoxycarbonyl group. The *ortho* coupling pattern and HMBC correlations of H-5, H-6/C-1 (147.0 ppm), and C-4 (148.2 ppm) indicate that the C ring in **1** has the substitution pattern shown. Moreover, the presence of a linkage between rings C and E via C-2−C-7 is supported by HMBC correlations of H-6, H-8, H-13/C-2.

These observations demonstrate the major architectural components of part B of the structure of **1**. Further key information comes from HMBC correlations of H-3′ and H-5/C-3, which indicate the connectivity of rings A and C. ROESY correlations of 4-OH (9.52 ppm)/H-5 and 4′-OH (8.67 ppm)/H-3′ and H-5′, the lack of signals for 1-OH and 1′-OH in the spectrum recorded using a DMSO-d_6 solution, the existence of two degrees of unsaturation, and the oxygenated nature of the quaternary carbons C-7 (75.6 ppm) and C-9 (80.4 ppm) all require that the two rings be bridged by oxygens.

This proposal leads to a question about whether the oxygen bridges are located at C-1−O−C-9 and C-1′−O−C-7 to form two six-membered rings or at C-1−O−C-7 and C-1′−O−C-9 to form a four-membered ring and an eight-membered ring. An attempt to use HMBC four-bond correlations (800 MHz and altering $^4J_{H,C}$ to 4 or 2 Hz) to construct the correct structure of **1** was unsuccessful. Fortunately, the observation of a key ROESY correlation of H-7′/Ha-15 (4.51 ppm) makes it impossible to conclude that a C-1−O−C-7 bridge is present in **1**. In addition, the structure in which rings B and D of **1** are eight- and four-membered, respectively, has a calculated ECD spectrum that is less well-matched to the experimental spectrum (data not shown) (Figure 2).

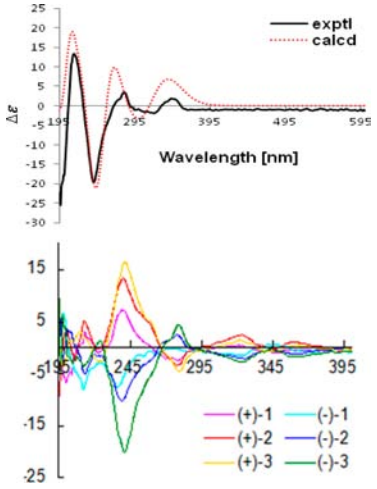


Figure 2. Calculated ECD spectrum for (7*R*,9*S*,13*S*)-**4** and experimental ECD spectrum of (−)-**4** (left). Experimental CD spectra of **1**−**3** (right).

The stereochemistry of the double bonds in the side chain of **1** is readily determined by analyzing correlations (double arrows in Figure S1) in the ROESY spectrum. The relative configuration of the stereogenic centers in **1** was determined by analyzing the results of ROESY and NOE irradiation experiments. The D and E rings cause **1** to have a rigid conformation, which then requires the C-2−C-7 and O−C-9 bonds to be axial. The ROESY correlation networks of H-13/Ha-8 (2.52 ppm), Hb-11 (1.99 ppm), and Ha-8/Hb-11 suggest that these protons are oriented on the same side of the ring E, which is only possible when ring E exists in a chair conformation with H-13 being axial. In addition, the doublet of doublets (J = 12.5, 2.0 Hz) coupling pattern of the signal for Hb-8 is a consequence of a long-range W coupling between Ha-11 and Hb-8, an observation that is consistent with the finding that no NOE enhancement is observed for Hb-11 when Hb-8 is irradiated (Figures S31−S33).

Notably, **1** was isolated as a racemic mixture. Separation by using chiral HPLC afforded (+)-**1** and (−)-**1**, whose absolute configurations were assigned as 7*R*,9*S*,13*S* for (+)-**1** by utilizing computational methods (Supporting Information).

Compounds **2**−**5** are structurally similar to **1**, but major differences exist in the side chain; for detailed structural elucidation, see the Supporting Information.

While the discovery of compounds **2**−**5** is of great interest, their racemic nature as well as the presence of a methoxy or an acetoxy group in the structures motivated us to check whether these isolates are natural products or artifacts formed in protic solvents. Therefore, *G. cochlear* was extracted with $CHCl_3$ followed by gel filtration on Sephadex LH-20 ($CHCl_3$/

DOI: 10.1021/acs.orglett.5b01353
Org. Lett. 2015, 17, 3082−3085

Table 1. Inhibitory Potency of Compounds 1−7 on Different Ion Channels

	peak current inhibition ratio (%)					
compd	$Ca_v1.2^a$ (L-type)	$Ca_v2.1^a$ (P/Q-type)	$Ca_v2.2^a$ (N-type)	$Ca_v3.1^a$ (T-type)	$Ca_v3.1^b$ (T-type)	$K_v11.1^a$ (hERG)
(+)-**1**	9.6 ± 3.9	−2.4 ± 1.3	−6.8 ± 2.6	37.9 ± 2.9	61.5 ± 1.9	3.5 ± 2.3
(−)-**1**	7.3 ± 2.1	−5.6 ± 2.9	−8.2 ± 5.9	37.9 ± 2.9	54.3 ± 6.2	1.2 ± 1.3
(+)-**2**	–[d]	–	–	21.1 ± 2.6	–	
(−)-**2**	–	–	–	27.1 ± 4.1	–	
(+)-**3**	8.1 ± 7.5	−3.5 ± 1.4	1.3 ± 4.7	39.2 ± 5.8	64.8 ± 2.1	−1.1 ± 3.9
(−)-**3**	25.6 ± 2.4	3.9 ± 3.7	−9.6 ± 2.1	46.2 ± 2.1	65.9 ± 2.5	5.8[c]
(+)-**4**	–	–	–	32.7 ± 4.6	–	–
(−)-**4**	–	–	–	23.6 ± 7.4	–	–
(+)-**5**	–	–	–	32.6 ± 3.5	–	–
(−)-**5**	–	–	–	29.9 ± 4.2	–	–
(±)-**6**	18.9 ± 3.7	−4.3 ± 1.2	−8.7 ± 5.9	57.6 ± 0.6	79.5 ± 2.7	−8.7 ± 5.6
(+)-**7**	–	–	–	14.4 ± 2.3	–	–
(−)-**7**				13.4 ± 1.6		
mibefradil	–	–	–	47.7 ± 6.4	81.2 ± 2.8	–

[a]Peak current inhibition ratio of compounds at 10 μM. [b]Peak current inhibition ratio of compounds at 30 μM. [c]Data represent the mean of two cells. [d]–, not tested due to the limited amount of material. Data without superscript represent the mean ± SEM of three or four cells. Negative control: the current recorded without addition of individual compound.

Me_2CO, 1:1) to yield six fractions. We analyzed the fractions with LC−MS (Supporting Information), which clearly indicated the natural occurrence of **1** and **3**−**5**. In this study, compound **2** was not detected, which likely due to its low abundance.

Compounds **1**−**5** are a class of meroterpenoid dimers, and their structure novelty allows us to propose a plausible biosynthetic route taking ganocochlearin C, a meroterpenoid that has been isolated from *G. cochlear*, as a precursor; for details, see Scheme S1.[16]

Cochlearine A (**6**) has the molecular formula $C_{31}H_{29}NO_6$ (18 degrees of unsaturation) as determined by HREIMS and ^{13}C NMR and DEPT spectra. The ^{13}C NMR and DEPT spectra of **6** contain signals for 31 carbons classified as 2 methyls, 5 methylenes (one oxygenated), 10 methines (eight sp^2), and 14 quaternary carbons (all sp^2, including one carbonyl, four oxygenated, one nitrogenated). The ^1H NMR spectrum of **6** contains a resonance for a typical ABX coupling system (6.83 ppm, d, J = 8.7 Hz, H-13; 6.84 ppm, dd, J = 8.7, 2.4 Hz, H-14; 7.45 ppm, d, J = 2.4 Hz, H-16), a *meta* coupling pattern (6.61 ppm, d, J = 2.1 Hz, H-3′; 6.43 ppm, d, J = 2.1 Hz, H-5′), and a singlet at 8.68 ppm. Careful inspection of NMR spectral patterns indicates that **6** has a structure that is quite similar to that of sinensine E, an alkaloid isolated from *G. sinense*.[17] This conclusion gains support from a detailed analysis of its 2D NMR (Figure S2). In addition, NMR data corresponding to the side chain region of **6** are similar to those of chizhine D, a meroterpenoid isolated from *G. lucidum*.[15] HMBC correlations of H_2-7′/C-1′, C-5′, C-6′, C-8′, C-9′, and H-8′ (5.85 ppm)/C-6′, C-9′, C-10′ (171.4 ppm) (Figure S2) show that the 7′-oxo group of chizhine D is replaced by a $\Delta^{8'}$ double bond in **6**. The presence of the furan E ring in **6** is supported by the observed HMBC correlations of H-5, H-6, H-7′/C-1′, H-5, H-6/C-2′, and H-5/C-3′. Moreover, the *Z*-configuration of the $\Delta^{8'}$ double bond is demonstrated by the ROESY correlation of H-8′/H-11, and the 8.2 Hz coupling constant between H-5 and H-6 suggest that a *cis*-ring fusion exists in **6**.[18] Finally, **6** is also racemic. Chiral HPLC separation afforded two enantiomers, and the absolute configurations at the two stereogenic centers in (−)-**6** were determined to be 5*R*,6*S* by using computational methods (Figure S12, Supporting Information).

The structure of compound **7** is highly similar to that of **6**, with some differences in the side chain; for detailed structural elucidation, see the Supporting Information.

The hypnotic, antiepileptic, and neuroprotective effects of *Ganoderma* extracts[12−14] led us to assume that some of these effects may be mediated, at least partly, through inhibition of TTCCs and to test the effect of compounds **1**−**7** on $Ca_v3.1$, one of three subtypes of TTCCs that is highly expressed in the brain and is involved in absence epilepsy and sleep disorders.[1] All compounds inhibited $Ca_v3.1$ (expressed in *Xenopus* oocytes) at 10 μM, with 13.4−57.6% reduction of peak current (Table 1). None of the compounds obviously affected the current kinetics (Figures 3A−C). Dose−response relationships were obtained

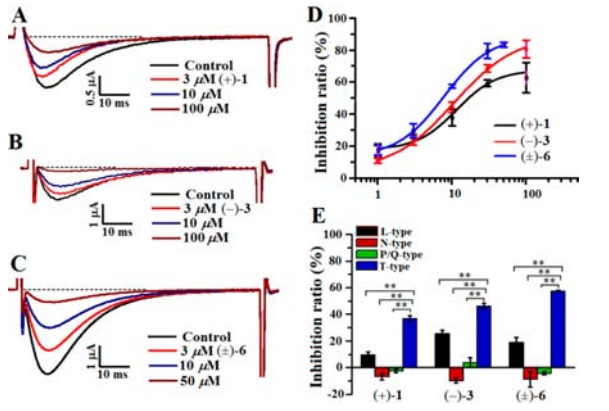


Figure 3. Inhibitory effects of compounds (+)-**1**, (−)-**3**, and (±)-**6** on $Ca_v3.1$ and their selectivity against other voltage-gated Ca^{2+} channels. (A−C) Representative $Ca_v3.1$ current traces evoked by 50 ms depolarizations to −10 mV at 3 s intervals from a holding potential (HP) of −80 mV in the absence and presence of the indicated compounds. $Ca_v3.1$ was expressed in *Xenopus* oocytes. (D) Dose−response relationships of the indicated compounds for $Ca_v3.1$ at HP of −80 mV. Data points represent mean ± SEM of three measurements. Solid curves represent fits to the Hill equation. (E) Effect of compounds (+)-**1**, (−)-**3**, and (±)-**6** on the indicated voltage-gated Ca^{2+} channels. The effect on the peak current was determined for each compound at 10 μM. Data represent mean ± SEM (n = 3). $^{**}P < 0.01$ according to two-tailed student's t test.

DOI: 10.1021/acs.orglett.5b01353
Org. Lett. 2015, 17, 3082−3085

for (+)-**1**, (−)-**3**, and (±)-**6**, which exhibited notable inhibition at 10 μM (Table 1) and had a sufficient amount of material (Figure 3D).[19] The IC_{50} values of these compounds were 11.4, 10.4, and 7.8 μM, respectively, with Hill coefficients of 1.6, 1.0, and 1.4, respectively. Mibefradil, a classic TTCC inhibitor[20] that was clinically used for the treatment of hypertension but was later withdrawn from the market due to potential deadly drug interactions, inhibited $Ca_v3.1$ with an IC_{50} value of 10.4 μM and a Hill coefficient of 1.6 under our experimental conditions (Figure S16). Thus, compounds (+)-**1**, (−)-**3**, and (±)-**6** have a potency on $Ca_v3.1$ similar to that of mibefradil. Compounds (−)-**1** and (+)-**3**, at 10 and 30 μM, produced an amount of inhibition of $Ca_v3.1$ similar to that of their enantiomers (Table 1).

It is worth noting that (+)-**1** inhibited $Ca_v3.1$ by only 62.9 ± 9.3% at a near saturation concentration of 100 μM (Figure 3D). This observation suggests that (+)-**1** modulates channel gating rather than blocks channel conduction. We examined the specificity of (+)-**1**, (−)-**1**, (+)-**3**, (−)-**3**, and (±)-**6**, the most potent compounds on $Ca_v3.1$, by testing their effects on $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$, and $K_v11.1$, which represent, respectively, L-, P/Q- and N-type high-voltage-gated calcium channels and hERG potassium channel (Table 1, Figure 3E). At 10 μM, (+)-**1**, (−)-**3** and (±)-**6** inhibited $Ca_v1.2$, but the inhibition was significantly weaker than that against $Ca_v3.1$. All these compounds potentiated $Ca_v2.2$, but only slightly (<10%). Finally, all these compounds had negligible effects on $Ca_v2.1$ and $K_v11.1$. It is notable that (−)-**1** and (+)-**3** in general has a stronger selectivity against $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$ and $K_v11.1$ (Table 1, Figure S15). These results, collectively, indicate that aforementioned compounds have a clear preference for $Ca_v3.1$. It remains to be determined whether these compounds show specificity among TTCC isoforms ($Ca_v3.1\backslash3.2\backslash3.3$).[21] It is notable that cannabinoids, a class of compounds that act on cannabinoid receptors and repress neurotransmitter release in the brain, and cochleariods both share a common *6H*-benzo[*c*]chromene motif. That whether this cyclic core is pivotal for neurological activity needs further investigation.

In summary, we describe in this work the isolation and evaluation of two new classes of molecules that preferably inhibit $Ca_v3.1$ TTCC. These molecules have novel chemical structures and differ markedly from known $Ca_v3.1$ inhibitors. They provide exciting and challenging opportunities for future chemical synthesis and modification to generate more potent and more specific TTCC inhibitors, especially TTCC isoform-specific inhibitors. In addition, despite previous study on *G. cochlear*,[16] the present findings provide fundamental evidence for *G. cochlear* in the treatment of neurological disorders.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01353.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the NSFC-Joint Foundation of Yunnan Province (U1202222 to Y.-X.C.), the National Natural Science Foundation of China (21472199 to Y.-X.C. and 31370821 to J.Y.), the Top Talents Program of Yunnan Province (2011HA012 to J.Y.), and High-Level Overseas Talents of Yunnan Province (J.Y.).

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(19) The amount of (+)-**1** and (−)-**3** was sufficient for constructing dose−response relationships of inhibition of $Ca_v3.1$. Compounds (−)-**6** and (+)-**6** were mixed together to yield enough material for this purpose, justified by the observation that each enantiomer produced the same degree of inhibition of $Ca_v3.1$ at 10 μM. Because of the limited amount of material, dose−response relationships were not obtained for the other compounds.

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(21) Because of the limited amount of material, the effects of (±)-**1**, (±)-**3**, and (±)-**6** on $Ca_v3.2$ and $Ca_v3.3$ were not tested.

DOI: 10.1021/acs.orglett.5b01353
Org. Lett. 2015, 17, 3082−3085