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Discovery of novel (1*S*)-(−)-verbenone derivatives with anti-oxidant and anti-ischemic effects

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ABSTRACT

A series of novel (1*S*)-(−)-verbenone derivatives was synthesized bearing a 4-styryl scaffold. The synthesized compounds were tested for their anti-oxidant, anti-excitotoxic, and anti-ischemic activities. These derivatives significantly reduced oxygen–glucose deprivation-induced neuronal injury and *N*-methyl-D-aspartic acid-evoked excitotoxicity in cortical neurons. Furthermore, compound **3f** was identified as a potent anti-ischemic agent in an in vitro ischemic model, potentially due to the inhibition of *N*-methyl-D-aspartic acid-evoked excitotoxicity and oxidative/nitrosative stress.

Stroke is one of the leading causes of death and long-term disability worldwide. Despite its high mortality rate and social-economic burden, there are currently few treatment options available for acute stroke. Ischemic stroke, which accounts for 80% of all strokes, is caused by a sudden interruption in cerebral blood supply due to an embolus or a thrombus.[1] Rapid restoration of cerebral blood flow is critical to limit neuronal injury and consequent loss of brain function. Thus, therapeutic intervention for ischemic stroke generally entails prompt application of thrombolytic agents such as tissue plasminogen activator (tPA). Although tPA is the only thrombolytic agent approved by health authorities thus far, it has clinical limitations, such as a narrow therapeutic time window and a high risk of side effects.[2] Recent evidence indicates that neuronal injury in ischemic regions may progress even after reperfusion.[3] Therefore, the development of effective treatments against this progressive ischemic injury has been eagerly pursued to ensure high clinical efficacy.

The mechanisms underlying ischemic injury are diverse and may occur sequentially and even simultaneously.[3] Ischemic injury is initiated by energy failure due to deprivation of oxygen and glucose.[3] Intracellular acidification and mitochondrial electron leak induce the massive generation of reactive oxygen species (ROS).[4] The loss of ionic gradients and plasma membrane depolarization trigger intracellular accumulation of glutamate, resulting in excitotoxicity through over-activation of *N*-methyl-D-aspartate (NMDA) receptors. Subsequently, NMDA receptor-mediated calcium overload induces activation of neuronal nitric oxide synthase (*n*NOS) and other proteases, further increasing oxidative/nitrosative stress. Reperfusion is also reported to greatly increase production of free radicals. In addition, activated microglia and infiltrating peripheral leukocytes/monocytes produce ROS/reactive nitrogen species (RNS), exacerbating inflammatory responses and ischemic injury.[4] Neurons are especially susceptible to ischemic injury, expressing a higher density of glutamate receptors and possessing relatively limited defense mechanisms (e.g., low ATP/ionic buffering and anti-oxidant capacity).[5] Accordingly, an anti-ischemic agent alleviating both excitotoxicity and ROS/RNS-associated injury would possess considerable potential for synergistic benefits for ischemia.[6] Furthermore, the agents developed for this strategy may also have alternative therapeutic perspectives when employed in combination with a reperfusion remedy; either by promoting penumbral survival until later initiation of reperfusion therapy or by reducing secondary injury induced by reperfusion following treatment with thrombolytic agents.[7]

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(1*S*)-(−)-Verbenone is a naturally occurring anti-aggregation pheromone generated by bark beetles from a host tree resin precursor, α-pinene.[8] Essential oils containing (1*S*)-(−)-verbenone have been reported to have antibacterial, acaricidal, and anti-inflammatory properties,[9] but the anti-ischemic effects of (1*S*)-(−)-verbenone have not been elucidated yet. Verbenone is thought to be naturally generated as either a biotransformation[10] or an auto-oxidation product of verbenol,[8] which we previously identified as a lead compound with anti-ischemic and anti-inflammatory effects.[11] However, verbenol shows neither direct ROS/RNS scavenging ability nor the ability to directly inhibit NMDA receptor-coupled channel activity.[11] In the present study, we report synthesis and biological evaluation of novel verbenone derivatives bearing modified styryl moieties, which are often found in natural products with significant free radical scavenging and anti-inflammatory activity, such as curcumin, resveratrol, and ferulic acid.[12]

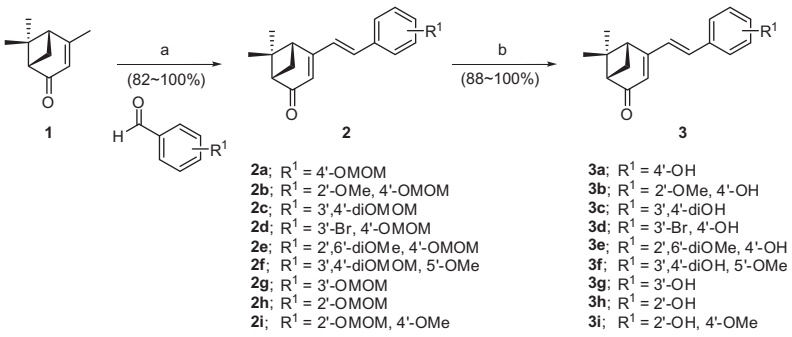
Commercially available (1*S*)-(−)-verbenone **1** was therefore coupled with various benzaldehydes bearing protected hydroxy moieties to afford verbenone derivatives **2a**–**i** in the *E*-configuration.[13] Methoxymethyl groups of compounds **2a**–**i** were then removed by acid to afford verbenone derivatives **3a**–**i** with phenolic functionality along with alkoxyl or bromo substitutions (Scheme 1).[14]

In order to assess the importance of the hydroxyl moieties on styryl scaffold, (1*S*)-(−)-verbenone **1** was then coupled with various benzaldehydes including methoxy and pyrrole groups in a similar condition to afford compounds **4a**–**h** (Scheme 2). It is noteworthy to mention that the pyrrole and methoxy-substituted phenyl groups may exhibit or modulate anti-oxidant activities alone or when conjugated with other groups.[15] In addition, a pyridine moiety is often found in the pharmacophores of anti-oxidant and anti-inflammatory reagents,[16] or some classes of NMDA receptor antagonists.[17] Thus, we further synthesized pyridine derivatives **5a**–**c** (Scheme 2).

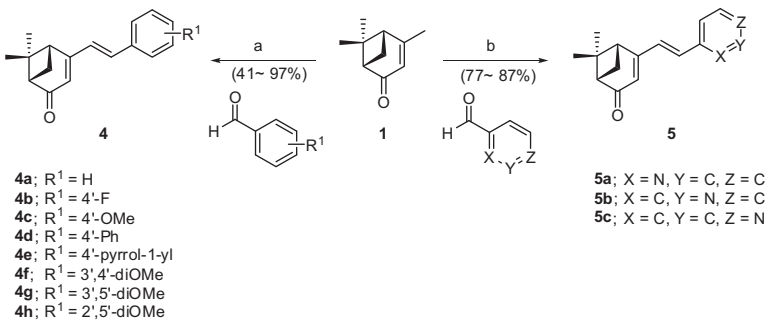
Anti-oxidant capacities of verbenone derivatives were determined using two different types of chemical reactions: 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and oxygen radical absorbance capacity (ORAC) assay (Table 1) with vitamin C and trolox as standards, respectively.

In the DPPH assay, most of the styryl derivatives with hydroxyl groups **3a**–**f** and **3h**–**i** showed direct scavenging activities against nitrogen free radicals (DPPH·) (Table 1). However, compound **3g** with a *metha*-hydroxyl group lacked DPPH radical scavenging activity. As expected, a lack of hydroxyl moieties in the phenyl ring such as compounds **4a**–**h** and **5a**–**c** resulted in no anti-oxidant activity in the DPPH assay. Among the phenolic compounds examined, **3c** and **3f** with 3,4-dihydroxy groups showed better scavenging activity than vitamin C. In the ORAC assay, all derivatives **3a**–**i** containing phenolic groups showed higher peroxyl radical scavenging activity than trolox, a well-known anti-oxidant (Table 1). Interestingly, compound **4e** with a *para*-pyrrole group exhibited significant peroxyl radical scavenging activity, even though it lacked the phenolic moiety (Table 1). A pyrrole group can act as a hydrogen atom transfer (HAT) agent alone and even better when connected to the electron-supplying aryl group through stabilization by resonance.[15] However, this was not the case for compound **4e**, since it is a pyrrole-1-yl derivative lacking a free H atom in the N–H group. Compound **4e** might demonstrate peroxyl radical scavenging activity in the ORAC assay as shown by other pyrrole *N*-conjugates with anti-oxidant activities.[15]

To evaluate anti-ischemic effects, we employed an in vitro ischemic model with oxygen–glucose deprivation/reoxygenation (OGD/R), which mimics a sudden disruption of blood supply and the energy depletion occurring during ischemia.[3] In cultured rat cortical neurons, OGD-induced cell injury and loss of membrane integrity increased the release of cytosolic lactate dehydrogenase (LDH) into the media. Although compounds **3a**–**i** showed significant free radical scavenging activities, only three derivatives with



Scheme 1. Synthesis of compounds **3a**–**i**. Reagents and conditions: (a) KOH, MeOH, 60 °C, 6 h; (b) 10% HCl, MeOH, rt, 24 h.



Scheme 2. Synthesis of compounds **4a**–**h** and **5a**–**c**. Reagents and conditions: (a) KOH, MeOH, 60 °C, 6 h; (b) NaOMe, MeOH, 60 °C, 6 h.

Table 1
Free radical scavenging activity of (1*S*)-(−)-verbenone derivatives

Compds	DPPH assay % inhibition[a]	ORAC assay fold change[b]	Compds	DPPH assay % inhibition[a]	ORAC assay fold change[b]
1	ND	1.19 ± 0.11	**4a**	ND	1.92 ± 0.05
3a	19.11 ± 0.67	10.66 ± 0.12	**4b**	ND	1.81 ± 0.09
3b	38.13 ± 5.62	7.37 ± 0.13	**4c**	ND	2.64 ± 0.14
3c	91.62 ± 3.27	9.09 ± 0.83	**4d**	ND	1.85 ± 0.09
3d	29.45 ± 2.41	11.64 ± 0.36	**4e**	ND	6.81 ± 0.03
3e	55.80 ± 7.52	11.51 ± 0.42	**4f**	ND	2.73 ± 0.12
3f	83.64 ± 6.19	6.86 ± 2.62	**4g**	ND	3.14 ± 0.04
3g	ND	5.11 ± 0.27	**4h**	ND	2.25 ± 0.11
3h	4.64 ± 0.60	6.84 ± 0.18	**5a**	ND	1.63 ± 0.05
3i	51.21 ± 3.83	9.48 ± 0.22	**5b**	ND	1.59 ± 0.04
			5c	ND	1.56 ± 0.02
Vit C	73.97 ± 8.35	—	Trolox	—	3.11 ± 0.07

Data were expressed as mean ± SEM.
[a] DPPH assay values were expressed as % inhibition in DPPH absorbance by derivatives at 80 μM, by comparison to the standard dose–response curves using from 0 to 500 μM vitamin C (Vit. C). *N* = 9. ND: Not detected.
[b] ORAC values were expressed as fold change of netAUC relative to vehicle control (14.44 ± 0.83). *N* = 12.

phenolic groups (**3a**, **3c**, and **3f**) significantly reduced neuronal injury (Fig. 1A), comparable to MK801, a well-known NMDA receptor channel blocker. The tested compounds did not cause cytotoxicity to cortical neurons (Fig. 1A).

In the H_2DCF-DA assay, **compounds 3c** and **3f**, but not **3a**, significantly decreased OGD-evoked intracellular oxidative stress (Fig. 1B and C), which might contribute to their anti-ischemic effect. Compounds **3c** and **3f**, compared to **3a**, showed better free radical scavenging activities against nitrogen radicals from DPPH (Table 1). Along with ROS, RNS is also elevated in ischemic regions and plays a critical role in ischemic injury cascades.[18] Inefficiency of **3a** in reducing intracellular oxidative stress might be partly

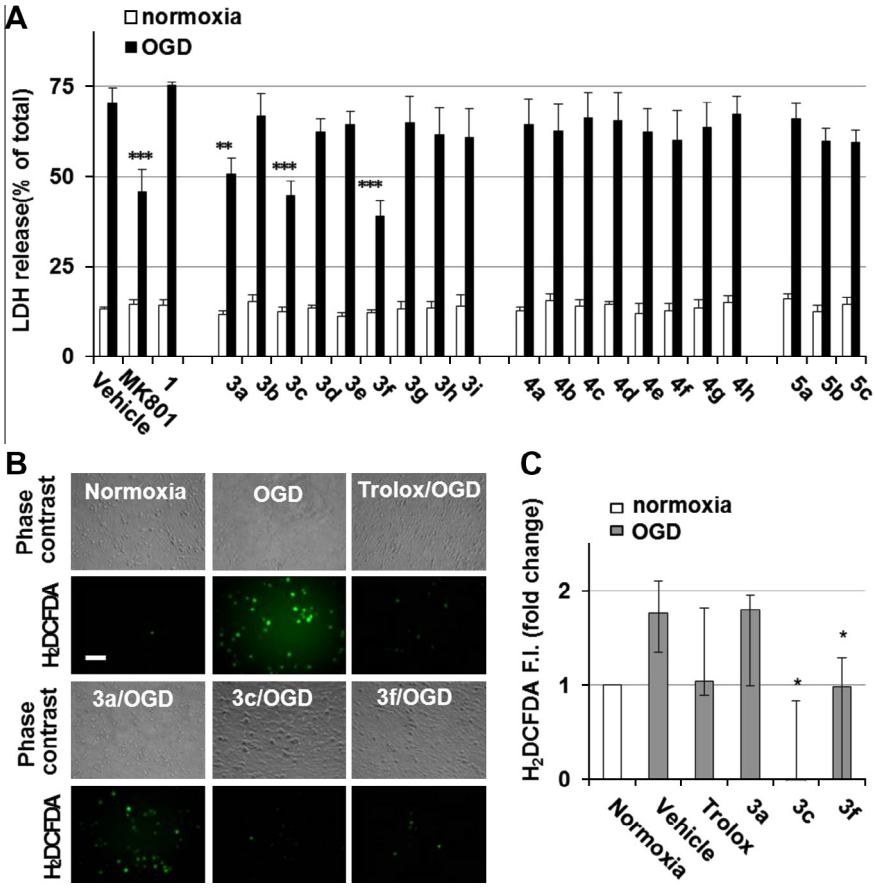


Figure 1. Neuroprotection by (1*S*)-(−)-verbenone derivatives against OGD/R-induced injury in an in vitro ischemic model. Cultured rat cortical neurons were exposed to OGD (90 min) and subsequent reoxygenation (R, 9 h). Derivatives, MK801, or trolox (10 μM or indicated concentrations of each) were pretreated for 30 min before OGD and maintained during OGD/R; (A) Neuronal injury was assessed by the LDH assay. Data represent the mean ± SEM. *N* = 24; (B, C) H_2DCFDA was loaded 1 h after reoxygenation and intracellular oxidative stress was measured by an increase in fluorescence intensity (F.I.) after 2 h. Representative images (B, scale bar = 50 μm) and quantitative data; (C) Data were expressed as the median (vertical column) and interquartile ranges from the first to the third quartile (vertical line). *N* = 9–18. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001: versus OGD/R group.

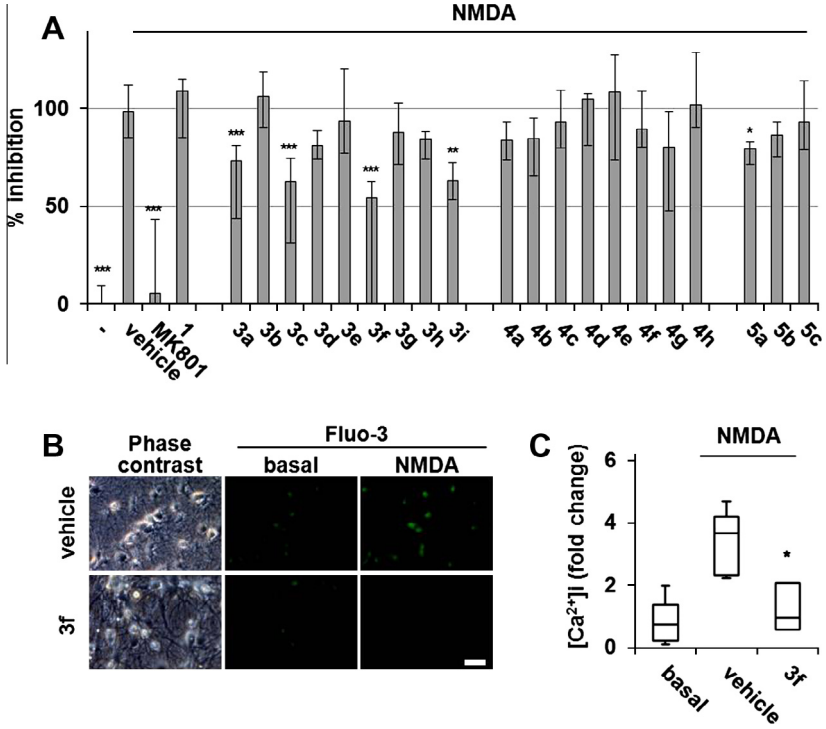


Figure 2. The effect of (1*S*)-(−)-verbenone derivatives on NMDA-evoked excitotoxicity. (A) Cortical neurons were treated with 100 μM NMDA for 15 min and then incubated for 9 h. Derivatives or MK801 (10 μM of each) administered 30 min before and maintained during NMDA treatment. Neuronal injury was assessed as % inhibition of LDH release and expressed as the median (vertical column) and interquartile ranges (Q1–Q3, vertical line). $N = 9–18$; (B, C) Cells were loaded with Fluo-3 AM for 30 min in the absence or presence of 10 μM of **3f**. 50 s after NMDA treatment, intracellular free calcium levels ($[Ca^{2+}]_i$) were assessed as the fold change in integrated fluorescence density of Ca^{2+}-bound Fluo-3 relative to basal levels. Representative images (B, scale bar = 50 μm) and quantitative data; (C) Data were expressed as the median (vertical bar), interquartile ranges (box), and min–max (whisker plots). $N = 4–5$. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001: versus NMDA-treated group.

due to its limited scavenging activity against nitrogen radicals, although further experiments are needed to confirm this observation.

In addition to oxidative stress, excitotoxicity is a major injury mechanism occurring during OGD and cerebral ischemia.[19] Although none of these derivatives demonstrated anti-excitotoxic activities comparable to MK801, derivatives **3a**, **3c**, **3f**, **3i**, and **5a** significantly reduced NMDA-evoked neuronal injury (Fig. 2A). Moreover, treatment with **3f** inhibited NMDA-induced intracellular calcium uptake (Fig. 2B and C).

In the present study, we performed the synthesis and biological evaluation of (1*S*)-(−)-verbenone derivatives bearing a styryl scaffold, in which additional anti-oxidant and anti-excitotoxic moieties were introduced into this pharmacophore. We identified compound **3f** as a potent anti-ischemic agent in an in vitro ischemic model, potentially due to the inhibition of NMDA-evoked excitotoxicity and oxidative/nitrosative stress. The results of these in vitro studies encourage further in vivo evaluation of these compounds, in particular compound **3f**, as promising anti-ischemic agent in the treatment of cerebral ischemia. Hence, this chemical structure will provide not only a useful drug template but also a novel strategy to develop anti-ischemic drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.07.038.

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14. Procedure for the synthesis of (1*S*,5*R*)-4-(3,4-dihydroxy-5-methoxystyryl)-6,6-dimethylbicyclo[3.1.1]hept-3-en-2-one (**3f**). For the synthesis of diene from (1*S*)-(−)-verbenone by aldol condensation, (1*S*)-(−)-verbenone **1** (200 mg, 1.33 mmol) was reacted with 3-methoxy-4,5-bis(methoxymethoxy) benzaldehyde (332 mg, 2.00 mmol) while stirring in MeOH (7 mL) and treated with KOH (149 mg, 2.66 mmol). The mixture was stirred at 60 °C for 6 h and cooled to room temperature. After a few drops of H_2O were added, the mixture was allowed to stand for 24 h at ambient temperature. The mixture was concentrated in vacuo and purified by column chromatography toafford **2f** as a yellow syrup (465 mg, 90%). To a stirred solution of **2f** (200 mg, 0.51 mmol) in MeOH (3 mL), 10% HCl was added dropwise. The reaction mixture was then stirred overnight until the completion of reaction. After addition of saturated $NaHCO_3$, the mixture was extracted with ethyl acetate and dried over anhydrous $MgSO_4$. The final compound was separated by column chromatography on a silica gel to give **3f** as a yellow solid (142 mg, 92%): mp 168–170 °C; $[\alpha]_D^{20}$ −158.8° (*c* 1.0, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) *d* 6.82 (d, *J* = 16.50 Hz, 1H), 6.79 (d, *J* = 1.71 Hz, 1H), 6.79 (d, *J* = 16.50 Hz, 1H), 6.65 (d, *J* = 1.71 Hz, 1H), 5.91 (s, 1H), 5.71 (br s, 2H), 3.93 (s, 3H), 3.09 (t, *J* = 5.75 Hz, 1H), 2.91 (td, *J* = 5.59, 9.35 Hz, 1H), 2.73 (dt, *J* = 1.71, 5.75 Hz, 1H), 2.12 (d, *J* = 9.54 Hz, 1H), 1.58 (s, 3H), 1.02 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) *d* 204.92, 165.07, 147.23, 144.24, 135.54, 134.13, 128.07, 125.50, 121.62, 108.63, 58.08, 56.25, 53.12, 43.75, 40.18, 26.78, 22.16; HRMS calculated for $C_{18}H_{20}O_4$ (M+H) 301.1440, found 301.1453; HPLC analysis: flow rate of 0.2 mL/min, 90% acetonitrile in water in 20 min) 100% (t_R = 3.46 min).
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