Research Article

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Design, synthesis and pharmacological profile of (–)-verbenone hydrazones

https://doi.org/10.1515/chem-2020-0103
received May 01, 2020; accepted June 17, 2020

Abstract: A series of novel (–)-verbenone hydrazones was designed and synthesized via condensation of terpenoid with hydrazides derived from phenoxyacetic acid. The structure of target compounds was confirmed by ^{1}H-NMR and ^{13}C-NMR analysis, Raman and FT-IR spectroscopy, electrospray ionization method and fast atom bombardment (FAB) mass spectrometry. Thermal properties of (–)-verbenone hydrazones **3a**–**3e** were estimated by differential scanning calorimetry and their purity by HPLC coupled to mass spectrometry. Verbenone hydrazones were revealed to exist as *Z*/*E* geometrical isomers about C=N bond and *cis*/*trans* amide conformers. Verbenone derivatives were estimated as potential anticonvulsant agents after their oral administration against pentylenetetrazole and maximal electroshock-induced seizures in mice. Analgesic effect of hydrazones was studied by topical application on models of allyl isothiocyanate and capsaicin-induced pain. The present findings indicate that verbenone hydrazones contribute to seizure protection both at short (6 h) and long (24 h) time periods by blocking chemical- and electroshock-induced convulsions. Binding of compounds **3a**–**3e** to TRPA1/TRPV1 ion channels was suggested as a feasible mechanism explaining their significant analgesic activity.

Keywords: verbenone, hydrazones, phenoxyacetic acid, analgesic activity, anticonvulsant effect

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1 Introduction

Essential oils as well as their individual constituents possess both significant analgesic activity and effect on central nervous system-related disorders [1]. The results of investigation revealed the pronounced pain relief action of essential oils isolated from *Rosmarinus officinalis* and *Verbena officinalis* containing the dominant component bicyclic monoterpene verbenone [2–4]. Among other findings, a rodent study demonstrated the anticonvulsant activity of verbenone due to direct or indirect impact on the GABAergic system [5]. Moreover, terpenes were found to improve the delivery of drugs through the skin by co-administration with pharmaceutical compounds or through the use of microemulsions for the transdermal absorption of drugs [1,6]. Terpenes and terpenoids may also serve as a transmembrane carrier via their conjugation with hydrophilic compounds [7]. Consequently, terpenoids are unique scaffold that might be used for further chemical modification aimed at synthesizing the compounds simultaneously affecting the central and peripheral nervous systems.

In this context, substantial attention is concentrated on hydrazones comprising their structure azomethine group –NH–N=C– and undergoing hydrolytic reactions both *in vitro* and *in vivo* [8,9]. To date, hydrazones based on menthone and camphor were found to prevent seizures spread in the maximal electroshock (MES) test showing simultaneously low neurotoxicity [10,11].

In this study, bicyclic terpenoid (–)-verbenone was used as a base for hydrazone synthesis via its condensation with hydrazides of *para*-substituted phenoxyacetic acid. As known earlier, phenoxyacetic acid derivatives also possess the peripheral nociceptive action and anticonvulsant effect [12,13]. Taking into account the foregoing, a series of novel (–)-verbenone hydrazones were designed and synthesized, and their spectral characteristics have been described followed by their investigation as potential analgesic and anticonvulsant agents using different experimental models *in vivo*.

2 Materials and methods

2.1 General

The following chemicals were obtained from commercial sources and used without further purification: (−)-verbenone, 4-chlorophenol, 4-phenoxyphenol, 4-bromophenol, 4-*tert*-butylphenol and phenol (TCI, USA). Compounds **2a–2e** were synthesized in three steps starting from *para*-substituted phenols as previously reported [14]. The products were characterized by ^{1}H-NMR spectroscopy on AVANCE DRX 500 (500 MHz) instrument and by ^{13}C-NMR spectroscopy on Varian-Mercury 400 spectrometer with the use of DMSO-d_6 as a solvent and tetramethylsilane as an internal standard. FAB mass spectra were recorded on a VG 70-70EQ mass spectrometer equipped with Xe ion gun (8 kV); the samples were mixed with *m*-nitrobenzyl-alcohol matrix. High-resolution mass spectrometry (HRMS) was carried out on a 6530 Accurate Mass quadrupole time-of-flight (Q-TOF) spectrometer by means of electrospray ionization method (ESI) coupled to an Agilent 1260 Infinity HPLC system. Chromatographic separation was performed on Agilent ZORBAX Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) at 35°C. Elution was executed at a flow rate of 1.0 mL/min, and the injection volume was 1 µL. IR spectra were recorded on PerkinElmer Frontier FT-IR spectrometer with samples in KBr disks. Raman spectra were obtained on Thermo Fisher Scientific DXR Raman Microscope. Differential scanning calorimetry (DSC) was carried out on Q2000 Differential Scanning Calorimeter with the use of aluminum crucibles containing 2 mg of compounds **3a–3e** under dynamic nitrogen atmosphere and the heating rate of 1°C min^{-1} at temperature ranging from 20 to 200°C.

2.2 General procedure to the synthesis of (−)-verbenone hydrazones (3a–3e)

To solution of (−)-verbenone (2.0 g, 13.3 mmol) in propanol (50 mL), the equimolar amount (13.3 mmol) of compounds **2a–2e** and 2–3 drops of CH_3COOH (glacial) were added. The reaction mixture was refluxed for 12 h, then cooled to room temperature and poured into the ice. The obtained precipitate was filtered and dried under vacuum followed by the recrystallization from methanol.

2.2.1 2-Phenoxy-*N′*-[4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]acetohydrazide (3a)

Yield 78%; white solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.83 (s, 3H, H-8), 1.42 (s, 3H, H-9), 1.60 (d, J = 8.8 Hz, 1H, H-7$_{endo}$), 1.91 (t, 3H, H-10), 2.27 (dd, J = 6.0 Hz, J = 1.8 Hz, 1H, H-5), 2.63–2.67 (m, 1H, H-1), 4.61 (s, 1H, CH_2), 4.96–4.98 (m, 1H, CH_2), 5.82 (d, J = 9.6 Hz, 1H, H-3), 6.85 (d, J = 7.8 Hz, 1H, Ar–H), 6.93 (t, 1H, Ar–H), 6.99 (d, J = 8.5 Hz, 1H, Ar–H), 7.28 (t, 2H, Ar–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 169.7; 167.3 (C=O), 158.7 (Ar–C), 157.5 (C-2), 155.4 (C-4), 130.0 (Ar–C), 129.8 (Ar–C), 121.7 (Ar–C), 115.0 (Ar–C), 114.9 (Ar–C), 110.5 (C-3), 66.5; 65.2 (CH_2), 48.8 (C-1), 47.8 (C-6), 43.6 (C-5), 42.9 (C-7), 26.3 (C-9), 23.2 (C-8), 22.5 (C-10). FT-IR (ν_{max}, cm^{-1}): 3,468 (N–H), 3197 (C–H, Ar), 2,970–2,934 (C–H), 1,697 (C=O), 1,661 (C=N), 1,599 (C–C, Ar), 838–688 (C–H, Ar). Raman (ν_{max}, cm^{-1}): 3,073 (N–H), 3,030 (C–H, Ar), 2,993–2,837 (C–H), 1,686 (C=O), 1,640 (C=N). MS (FAB) m/z: 299 [M + H]$^+$. HRMS (ESI-TOF) calculated for $C_{18}H_{22}N_2O_2$ [M]$^+$ 298.379, found 298.385. HPLC: t_r = 4.9 min; 5.3 min. M.p. (DSC) onset: 145.52°C, peak max: 149.22°C (first peak); onset: 154.91°C, peak max: 155.89°C (second peak).

2.2.2 2-(4-Bromophenoxy)-*N′*-[4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]acetohydrazide (3b)

Yield 82%; white solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.83 (s, 3H, H-8), 1.41 (s, 3H, H-9), 1.58 (d, J = 8.2 Hz, 1H, H-7$_{endo}$), 1.90 (t, 3H, H-10), 2.27 (dd, J = 6.0 Hz, J = 1.8 Hz, 1H, H-5), 2.64 (dd, J = 5.9 Hz, J = 1.7 Hz, 1H, H-1), 4.63 (d, J = 7.7 Hz, 1H, CH_2), 4.98 (d, J = 7.7 Hz, 1H, CH_2), 5.81 (d, J = 16.6 Hz, 1H, H-3), 6.84 (d, J = 8.2 Hz, 1H, Ar–H), 6.91–6.93 (m, 1H, Ar–H), 7.41 (d, J = 8.2 Hz, 1H, Ar–H), 7.44 (d, J = 7.7 Hz, 1H, Ar–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 169.1; 166.8 (C=O), 160.4 (Ar–C), 158.1 (C-2), 155.1 (C-4), 132.6 (Ar–C), 132.4 (Ar–C), 119.0 (Ar–C), 117.5 (Ar–C), 117.2 (Ar–C), 110.5 (C-3), 66.9; 65.5 (CH_2), 51.3 (C-1), 49.1 (C-6), 43.6 (C-5), 42.9 (C-7), 26.3 (C-9), 23.1 (C-8), 22.1 (C-10). FT-IR (ν_{max}, cm^{-1}): 3,418 (N–H), 3,204 (C–H, Ar), 2,977–2,937 (C–H), 1,698 (C=O), 1,669 (C=N), 1,489 (C–C, Ar), 817–606 (C–H, Ar). Raman (ν_{max}, cm^{-1}): 3,090 (N–H), 3,072 (C–H, Ar), 2,993–2,805 (C–H), 1,680 (C=O), 1,640 (C=N). MS (FAB) m/z: 377 [M]$^+$. HRMS (ESI-TOF) calculated for $C_{18}H_{21}BrN_2O_2$ [M]$^+$ 377.276, found 378.374. HPLC: t_r = 9.1 min; 9.9 min. M.p. (DSC) onset: 167.86°C, peak max: 170.91°C (first peak); onset: 174.64°C, peak max: 176.29°C (second peak).

2.2.3 2-(4-Chlorophenoxy)-*N'*-[4,6,6-trimethylbicyclo [3.1.1]hept-3-en-2-ylidene]acetohydrazide (3c)

Yield 84%; white solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.82 (s, 3H, H-8), 1.42 (s, 3H, H-9), 1.59 (d, J = 8.2 Hz, 1H, H-7$_{endo}$), 1.90 (t, 3H, H-10), 2.27 (dd, J = 6.0 Hz, J = 1.8 Hz, 1H, H-5), 2.65 (dd, J = 5.9 Hz, J = 1.7 Hz, 1H, H-1), 4.62 (d, J = 7.7 Hz, 1H, CH_2), 4.97 (d, J = 7.7 Hz, 1H, CH_2), 5.82 (d, J = 16.6 Hz, 1H, H-3), 6.85 (d, J = 8.2 Hz, 1H, Ar–H), 6.94–6.96 (m, 2H, Ar–H), 7.26 (d, J = 8.2 Hz, 1H, Ar–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 169.1; 167.8 (C=O), 160.8 (Ar–C), 158.6 (C-2), 155.3 (C-4), 132.9 (Ar–C), 132.4 (Ar–C), 119.0 (Ar–C), 117.7 (Ar–C), 117.2 (Ar–C), 110.5 (C-3), 66.9; 65.6 (CH_2), 51.3 (C-1), 49.1 (C-6), 43.6 (C-5), 42.9 (C-7), 26.3 (C-9), 23.1 (C-8), 22.1 (C-10). FT-IR (ν_{max}, cm^{-1}): 3,466–3,414 (N–H), 3,205 (C–H, Ar), 2,979–2,938 (C–H), 1,697 (C=O), 1,671 (C=N), 1,549 (C–C, Ar), 818–709 (C–H, Ar). Raman (ν_{max}, cm^{-1}): 3,070 (C–H, Ar), 2,977–2,828 (C–H), 1,679 (C=O), 1,642 (C=N). MS (FAB) m/z: 333 [M]$^+$. HRMS (ESI-TOF) calculated for $C_{18}H_{21}ClN_2O_2$ [M]$^+$ 332.824, found 332.038. HPLC: t_r = 8.0 min; 8.7 min. M.p. (DSC) onset: 117.35°C, peak max: 140.76°C (first peak); onset: 163.02°C, peak max: 171.38°C (second peak).

2.2.4 2-(4-*tert*-Butylphenoxy)-*N'*-[4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene] acetohydrazide (3d)

Yield 70%; white solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.82 (s, 3H, H-8), 1.24 (s, 9H, C(CH$_3$)$_3$), 1.41 (s, 3H, H-9), 1.59 (d, J = 8.3 Hz, 1H, H-7$_{endo}$), 1.91 (t, 3H, H-10), 2.27 (dd, J = 6.0 Hz, J = 1.8 Hz, 1H, H-5), 2.64 (dd, J = 5.9 Hz, J = 1.7 Hz, 1H, H-1), 4.58 (d, J = 7.2 Hz, 1H, CH_2), 4.92 (d, J = 6.5 Hz, 1H, CH_2), 5.81 (d, J = 10.9 Hz, 1H, H-3), 6.76–6.86 (m, 2H, Ar–H), 7.25–7.28 (m, 2H, Ar–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 169.5; 164.4 (C=O), 160.3 (Ar–C), 156.5 (C-2), 154.9 (C-4), 143.2 (Ar–C), 126.5 (Ar–C), 126.4 (Ar–C), 114.4 (Ar–C), 114.3 (Ar–C), 110.5 (C-3), 66.4; 65.2 (CH_2), 51.3 (C-1), 49.1 (C-6), 43.5 (C-5), 42.8 (C-7), 34.2 (C(CH$_3$)$_3$), 31.8 (CH$_3$), 26.3 (C-9), 23.1 (C-8), 22.1 (C-10). FT-IR (ν_{max}, cm^{-1}): 3,552–3,414 (N–H), 3,232 (C–H, Ar), 2,961–2,863 (C–H), 1,702 (C=O), 1,638 (C=N), 1,616–1,596 (C–C, Ar), 828–611 (C–H, Ar). Raman (ν_{max}, cm^{-1}): 3,075 (N–H), 3,038 (C–H, Ar), 2,990–2,832 (C–H), 1,677 (C=O), 1,642 (C=N). MS (FAB) m/z: 355 [M + H]$^+$. HRMS (ESI-TOF) calculated for $C_{22}H_{30}N_2O_2$ [M]$^+$ 354.486, found 354.456. HPLC: t_r = 21.3 min; 23.3 min. M.p. (DSC) onset: 166.36°C, peak max: 170.14°C (first peak); onset: 172.50°C, peak max: 173.62°C (second peak).

2.2.5 2-(4-Phenoxyphenoxy)-*N'*-[4,6,6-trimethylbicyclo [3.1.1]hept-3-en-2-ylidene]acetohydrazide (3e)

Yield 74%; white solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.83 (s, 3H, H-8), 1.41 (s, 3H, H-9), 1.60 (s, 1H, H-7$_{endo}$), 1.90 (t, 3H, H-10), 2.26 (m, 1H, H-5), 2.65 (m, 1H, H-1), 4.62 (s, 1H, CH_2), 4.96 (s, 1H, CH_2), 5.82 (d, J = 12.0 Hz, 1H, H-3), 6.91 (d, J = 6.8 Hz, 2H, Ar–H), 6.99 (s, 4H, Ar–H), 7.06 (t, 1H, Ar–H), 7.34 (t, 2H, Ar–H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 166.5; 164.2 (C=O), 158.4 (Ar–C), 155.0 (Ar–C), 149.9 (C-2), 144.9 (Ar–C), 135.4 (C-4), 130.4 (Ar–C), 123.1 (Ar–C), 121.1 (Ar–C), 117.8 (Ar–C), 116.3 (Ar–C), 110.5 (C-3), 66.9; 65.8 (CH_2), 51.3 (C-1), 49.1 (C-6), 43.4 (C-5), 42.9 (C-7), 26.3 (C-9), 24.1 (C-8), 22.5 (C-10). FT-IR (ν_{max}, cm^{-1}): 3,552–3,415 (N–H), 3,231–3,194 (C–H, Ar), 2,953–2,867 (C–H), 1,681 (C=O), 1,638 (C=N), 1,616 (C–C, Ar), 836 (C–H, Ar). Raman (ν_{max}, cm^{-1}): 3,060 (C–H, Ar), 2,990–2,828 (C–H), 1,685 (C=O), 1,644 (C=N). MS (FAB) m/z: 391 [M + H]$^+$. HRMS (ESI-TOF) calculated for $C_{24}H_{26}N_2O_3$ [M]$^+$ 390.474, found 389.950. HPLC: t_r = 15.8 min; 17.3 min. M.p. (DSC) onset: 87.15°C, peak max: 92.29°C (first peak); onset: 122.70°C, peak max: 132.31°C (second peak).

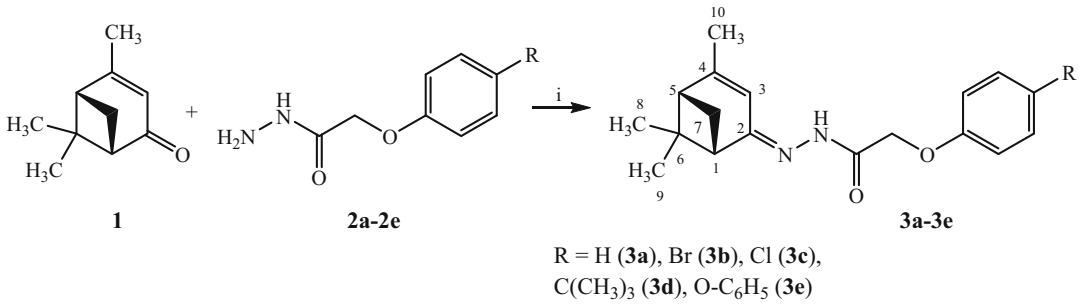
2.3 Anticonvulsant activity

2.3.1 Pentylenetetrazole (PTZ)-induced seizures

The anticonvulsant effect of hydrazones **3a–3e** was evaluated by intravenous injection of 1% aqueous solution of PTZ into a tail vein as described in Ref. [15]. Pure (−)-verbenone at a dose of 50 mg/kg in Tween 80/water emulsion has been administered to mice orally. Hydrazones **3a–3e** were used in equimolar to verbenone doses, and Tween 80/water emulsion served as a vehicle control and valproic acid (VPA, 400 mg/kg) – as a reference drug [16]. PTZ doses that induced clonic–tonic convulsions (DCTC) and tonic extension (DTE) in experimental animals were calculated with respect to control. The anticonvulsant effect of compounds was assessed at different time points (6 and 24 h) from the increase of PTZ minimum effective dose (MED) in comparison to the control group. MED (in %) was determined according to the following formula:

$$\mathrm{MED} = V/m \times 10^4,$$

taking into account the volume of PTZ solution (in mL) and the weight of each animal (in g); 10^4 – conversion factor for animal weight (g to kg, 10^3) and dose of PTZ (mL of PTZ solution to mg of PTZ, 10).



Scheme 1: Synthesis of (–)-verbenone hydrazones. Reagents and conditions: (i) AcOH (glacial), *n*-PrOH, reflux, 12 h.

2.3.2 MES-induced seizures

In this method, corneal electrodes (50 mA, 50 Hz) have been applied to induce seizures in experimental animals (mice) pretreated with compounds **3a–3e** as described in Section 2.3.1. The percent of mortality was considered as an index of seizure protection.

2.4 Analgesic action

The analgesic activity of verbenone hydrazones **3a–3e** was estimated by their topical application as 2% w/w ointments. Pain in experimental animals was induced by chemical stimuli, that is, by subcutaneous injection of TRPV1 and TRPA1 selective agonists –capsaicin and allyl isothiocyanate (AITC) with subsequent identification of pain index as described in our previous study [17]. The study was approved by the Animal Ethics Committee (agreement no. 04/2020) of Odessa National Polytechnic University (Ukraine).

2.5 Statistical analysis

All results are expressed as mean ± standard error mean (SEM). One-way analysis of variance was used to determine the statistical significance of the results followed by Tukey's *post hoc* comparison. $p < 0.05$ was considered as statistically significant.

3 Results and discussion

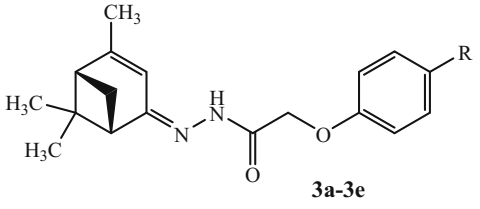
3.1 Chemistry

The synthesis of (–)-verbenone hydrazones **3a–3e** was performed via condensation of 4,6,6-trimethylbicyclo [3.1.1]hept-3-en-2-one **1** with hydrazides of 4-*R*-phenoxyacetic acid (**2a–2e**) using a catalytic amount of CH_3COOH (glacial), according to Scheme 1.

Here, we have to notice that condensation of bicyclic terpenoid verbenone with hydrazides requires more harsh reaction conditions (reflux for 12 h in *n*-PrOH), whereas monocyclic terpenoids (such as menthone or carvone [18,19]) react under mild conditions (reflux for 4 h in MeOH).

Hydrazones **3a–3e** were isolated in 70–84% yield after recrystallization from methanol as white solids soluble in organic solvents such as acetonitrile and chloroform (Figure 1).

The formation of verbenone derivatives was elucidated by mass spectrometry – molecular ion peaks of target compounds **3a–3e** correspond to their molecular formulas (FAB and ESI). The FT-IR spectra of verbenone hydrazones exhibit absorption bands of C=O and C=N groups, N–H bonds and alkyl and aromatic C–H. In addition, the formation of C=N bond of hydrazones was confirmed by Raman spectroscopy as intense peak at 1,640–1,644 cm^{-1}. Chemical shift, multiplicity and coupling constants observed in ^{1}H-NMR and ^{13}C NMR spectra prove the proposed structure of compounds **3a–3e**. Phase transitions (melting points) of derivatives **3a–3e** have been studied by DSC. The purity of (–)-verbenone derivatives was estimated by HPLC analysis using a mixture of acetonitrile and formic acid aqueous solution



R = H (**3a**), Br (**3b**), Cl (**3c**), $C(CH_3)_3$ (**3d**), O-C_6H_5 (**3e**)

Figure 1: Structures of (–)-verbenone hydrazones **3a–3e**.

(0.01%) in 50:50 ratio as the eluent system. According to the HPLC analysis, two chromatographic peaks were revealed identifying the presence of *Z*/*E* geometrical isomers of verbenone hydrazones about C=N bond that was additionally supported by the MS data. Retention time recorded for these peaks ranges from 4.9 to 21.3 min (first peak) and from 5.3 to 23.3 min (second peak).

Along with *Z*/*E* geometrical isomers, hydrazones were proven to exist in solution as a mixture of *cis*/*trans* conformers caused by hindered rotation on the C–N amide bond [20]. The existence of verbenone hydrazones as *cis*/*trans* conformers was reliably corroborated by ^{1}H NMR analysis – the protons of methylene group (CH_2) appear as two sets of signals. As described, in DMSO-d_6 solutions of hydrazones, both *cis* and *trans* amide conformers are formed; nevertheless, *cis* form is dominated due to the formation of dimers that are stabilized by hydrogen bonds between CO and NH groups [20,21]. The upfield peak of the methylene group corresponds to *trans* conformer, while downfield peak corresponds to *cis* form [20]. We have to emphasize that ^{1}H NMR spectral data of compounds **3a–3e** displayed the similar pattern – two singlets for protons of methylene group (4.58–4.63 and 4.92–4.98 ppm). ^{13}C NMR spectra of hydrazones **3a–3e** contain also two resonance signals of methylene carbons (66.4–66.9 and 65.2–65.8 ppm) along with two signals of carbonyl carbons (166.5–169.7 and 164.2–167.8 ppm) (Table 1).

In this case, upfield signals of carbonyl carbons and downfield lines of methylene carbons refer to amide *trans* conformer, while downfield lines of carbonyl carbons and upfield signals of methylene carbons are assigned to *cis* conformer [22]. Thus, we may conclude that according to ^{1}H NMR and ^{13}C NMR analysis target verbenone derivatives **3a–3e** present in DMSO-d_6 solution both in *cis* and *trans* form.

3.2 Anticonvulsant activity

Considering the antiseizure effect of pure terpenoids and taking into account the high reactivity of pharmacophore group –CO–NH–N=CH–, significant interest is focused on hydrazones based on terpenoids as scaffold [8,23].

Antiseizure action of verbenone hydrazones **3a–3e** was elucidated both on models of chemically and electrically induced seizures at 6 and 24 h after their single oral administration. As illustrated in Figure 2, starting terpenoid along with derivatives **3a–3e** demonstrated protective effect against PTZ-induced seizures at 6 h after administration as corroborated by increasing of DCTC and DTE values compared with control (100%).

At this time point, compound **3b** (with bromine atom at the *para* position of benzene ring) was shown to possess maximal anticonvulsant activity with the average values: 278% for DCTC and 303% for DTE. Interestingly, reference anticonvulsant drug VPA (400 mg/kg, p.o.) is inferior in action to hydrazones **3a–3d** ($p < 0.01$).

Bearing in mind the enzymatic cleavage of C=N double bond in hydrazone molecules [24] and, consequently, their possible prolonged action, anticonvulsant estimation was additionally performed at 24 h after administration (Figure 3).

In the PTZ-induced convulsion model, antiseizure action was manifested in experimental animal groups treated with verbenone hydrazones **3a–3e** at 24 h after oral administration demonstrating DCTC and DTE values on average 212% and 214%, accordingly, in comparison with the control data (100%) that point out to a significant prolonged action. Furthermore, compounds **3a–3e** exhibited higher anticonvulsant potency at long time period versus VPA ($p < 0.01$) that might be interpreted by enzymatic cleavage of labile bonds (N–N or C–N amide bond) in hydrazone molecules followed by

Table 1: ^{1}H and ^{13}C NMR data of *cis*/*trans* conformers of compounds **3a–3e** in DMSO-d_6

Compound	Conformer	^{1}H NMR –CH_2– (δ, ppm)	^{13}C NMR –CH_2– (δ, ppm)	^{13}C NMR –C=O (δ, ppm)
3a	*cis*	4.97	65.2	169.7
	trans	4.61	66.5	167.3
3b	*cis*	4.98	65.5	169.1
	trans	4.63	66.9	166.8
3c	*cis*	4.97	65.6	169.1
	trans	4.62	66.9	167.8
3d	*cis*	4.92	65.2	169.5
	trans	4.58	66.4	164.4
3e	*cis*	4.96	65.8	166.5
	trans	4.62	66.9	164.2

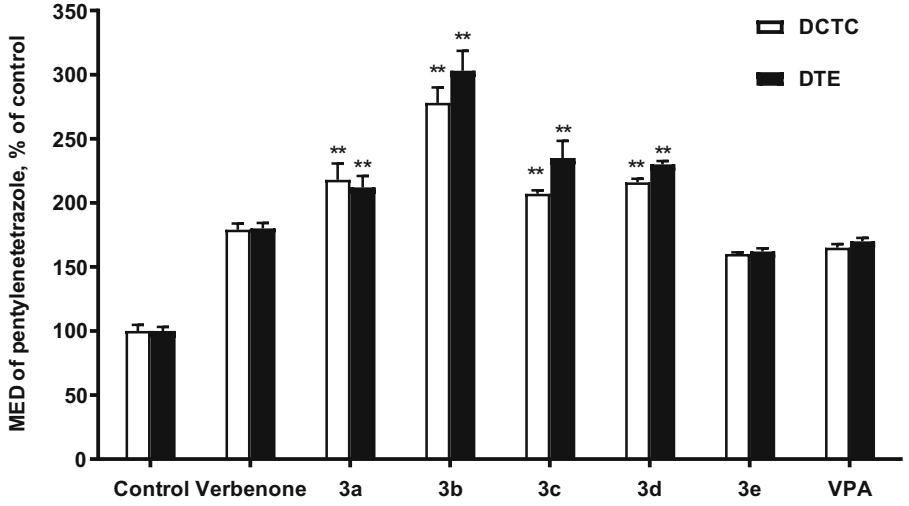


Figure 2: Anticonvulsant activity of compounds **3a–3e** at 6 h after oral administration. Values are given as mean ± SEM, $n = 5$ mice; for all groups $p < 0.01$ compared with control; $^{**}p < 0.01$ compared with VPA.

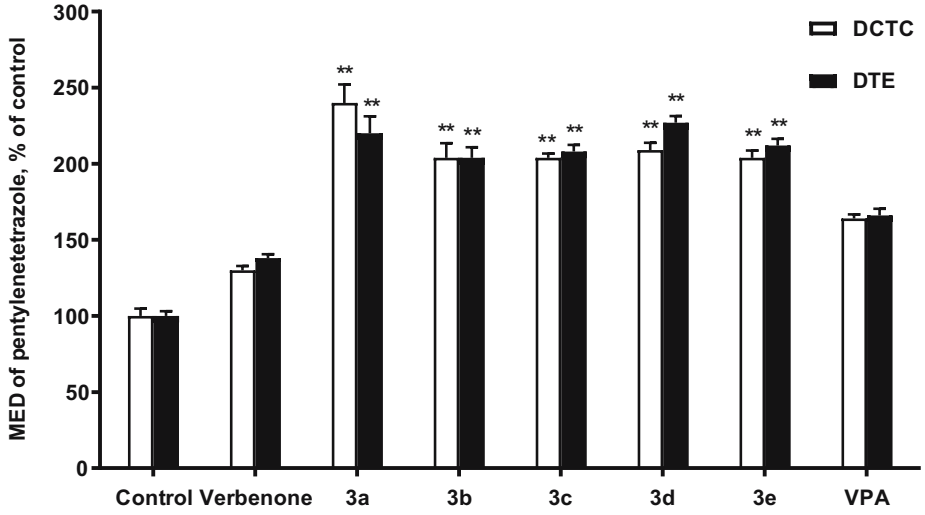


Figure 3: Anticonvulsant activity of compounds **3a–3e** at 24 h after oral administration. Values are given as mean ± SEM, $n = 5$ mice; for all groups $p < 0.01$ compared with control; $^{**}p < 0.01$ compared with VPA.

gradual release of active ingredients. Moreover, the prolonged presence of verbenone derivatives in the body may also be realized due to their lipophilicity (log *P* for **3a–3e** ranges from 3.66 to 5.35, log *P* for VPA is 2.72; ACD/Labs software). Low activity of pure verbenone (log *P* 1.97) at 24 h after administration (130% for DCTC and 138 for DTE) is explained, in turn, by its excretion at this time point.

Anticonvulsant potency of verbenone hydrazones was additionally assessed in the MES test that is considered as generalized tonic–clonic seizure model and reflects the ability of compounds to prevent seizure spread throughout the brain [25]. According to the data of the MES test, electrical stimuli induced the rigid extension of the hind limbs in 100% of control animals (Table 2).

As mentioned earlier, hydrazones **3a–3d** substantially prevented the mortality of animals at 6 h after administration and showed 80–100% protection, which is comparable to the effect of VPA (80%), while moderate antiseizure action was observed for compound **3e** and starting verbenone (60%). Interestingly, the activity of the aforementioned hydrazones is maintained for a long time period (24 h) with 60–100% of mortality protection. In turn, pure verbenone administered 24 h prior to the test produced weak action in mice with only 20% of protection. We have to emphasize that seizures in the MES test were characterized by Straub tail when the mice

Table 2: Anticonvulsant effect of compounds **3a–3e** against maximal electroshock (MES)-induced seizures in mice

Compound	3a	3b	3c	3d	3e	Verbenone	VPA	Control
6 h after single oral administration								
% Mortality protection	80	80	80	100	60	60	80	0
24 h after single oral administration								
% Mortality protection	80	100	60	60	80	20	60	0

were orally pretreated with compounds **3a–3e**. This phenomenon consisted of the mouse tail becoming rigid and erected across the back of the animal in an S-shaped curve [26]. Straub reaction in mice is described as one of the techniques for indicating analgesic and addicting properties of novel compounds [27]. Although Straub tail response is thought to be mediated by activation of the opioid receptor mechanism, this reaction can also be caused by dopaminergic, α-2 noradrenergic and nicotinic acetylcholine receptor agonists [28,29].

3.3 Analgesic action

Bearing in mind the aforementioned Straub reaction and molecular targets of terpenoids (transient receptor potential [TRP] channels), verbenone hydrazones **3a–3e** were examined as potential analgesic agents by their topical application using agonists of TRP channels to induce the pain. Analgesic activity of compounds **3a–3e** was comparable with the standard drug benzocaine (BZC) used as a topical pain reliever that activates both TRPV1 and TRPA1 channels [30]. As presented in Table 3, after the treatment with the ointment comprising either verbenone or its derivatives **3a–3e**, the reaction time recorded in the capsaicin test statistically differed from the control data ($p < 0.01$ vs control animals). Positive control (BZC) was also manifested to decrease the licking time to 9 ± 1 s, affirming its significant effect on TRPV1 channel. Despite the fact that synthesized compounds **3a–3e** along with pure verbenone reduced pain sensitivity threshold, they were inferior to BZC when topically applied.

The intraplantar injection of TRPA1 channel antagonist (AITC) after the application of ointment base led to the occurrence of pain sensations with licking time duration of 63 ± 2 s, whereas for reference drug (BZC), this value was 38 ± 4 s. When treated with verbenone or its hydrazones **3a–3e**, the reaction time ranged from 31 to 46 s, reflecting that these derivatives significantly attenuate AITC-induced acute pain and possess an analgesic action comparable with that in BZC treatment. Given the results of analgesic trials, TRPA1 and TRPV1 ion channels might be proposed as one of the molecular targets for compounds **3a–3e**.

Table 3: Analgesic activity of hydrazones **3a–3e** on capsaicin- and AITC-induced acute pain in mice (2% w/w ointment)

Compound	Licking time (in s)	
	Capsaicin test	AITC test
Control	54 ± 3	63 ± 2
Benzocaine	9 ± 1	38 ± 4
Verbenone	32 ± 2	45 ± 6
3a	26 ± 5	31 ± 5
3b	21 ± 1	34 ± 3
3c	27 ± 6	32 ± 7
3d	23 ± 2	46 ± 7
3e	43 ± 1	46 ± 3

All values are expressed as mean ± SEM; $n = 5$; for all groups, $p < 0.01$ compared with control group.

4 Conclusion

Hydrazones based on bicyclic terpenoid (−)-verbenone were synthesized as promising compounds simultaneously affecting both the central and peripheral nervous system. Verbenone derivatives **3a–3e** have been demonstrated to protect against electroshock- and PTZ-induced seizures at different time points – 6 and 24 h after oral administration. Binding of verbenone hydrazones to TRPA1/TRPV1 ion channels has been suggested as a feasible mechanism for explanation of significant analgesic activity of obtained compounds.

Abbreviations

AITC	allyl isothiocyanate
BZC	benzocaine
DCTC	dose inducing clonic–tonic convulsions
DSC	differential scanning calorimetry
DTE	dose inducing tonic extension
ESI	electrospray ionization method
GABA	gamma-aminobutyric acid
HRMS	high-resolution mass spectrometry
MED	minimum effective dose

MES	maximal electroshock
PTZ	pentylenetetrazol
SEM	standard error mean
TOF	time-of-flight
TRP	transient receptor potential
VPA	valproic acid

Conflict of interest: No potential conflict of interest was reported by the authors.

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