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Article

Zoanthamine alkaloids from the Zoantharian *Zoanthus* cf. *pulchellus* and their effect in neuroinflammation

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Abstract: Two new zoanthamine alkaloids, namely 3-acetoxynorzoanthamine (**1**) and 3-acetoxyzoanthamine (**2**), have been isolated from the zoantharian *Zoanthus* cf. *pulchellus* collected off the coast of the Peninsula of Santa Elena – Ecuador, together with three known alkaloids zoanthamine, norzoanthamine and 3-hydroxynorzoanthamine. The chemical structures of **1** and **2** were determined by interpretation of their 1D and 2D NMR data and comparison with literature data. This is the first report of zoanthamine-type alkaloids from *Zoanthus* cf. *pulchellus* collected in the Tropical Eastern Pacific. The neuroinflammatory activity of all the isolated compounds were evaluated in microglia BV-2 cells and high inhibitory effects were observed in ROS and NO generation.

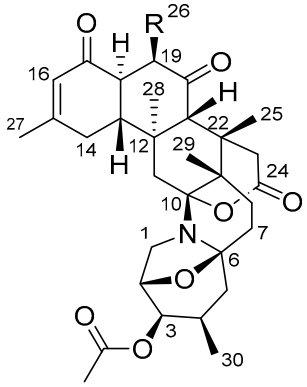
Keywords: Zoantharia; Tropical Eastern Pacific; *Zoanthus pulchellus*; zoanthamine; inflammation.

1. Introduction

Zoanthamines are known as a bioactive family of marine alkaloids featuring a unique chemical architecture of fused cycles culminating with an unusual azepane ring. They have been isolated essentially from marine zoantharians, and particularly from the genus *Zoanthus*. The first alkaloid of this group was isolated in 1984 from an unidentified species of *Zoanthus*, collected off the coast of India by Faulkner and co-workers [1]. Following this first description, several studies on the chemical diversity of species of the genus *Zoanthus* have disclosed additional zoanthamine-type alkaloids including zoanthenamine [2], zoanthenamide [2], norzoanthamine [3], oxyzoanthamine [3], norzoanthaminone [3], cyclozoanthamine [3], epinorzoanthamine [3], zoanthaminone [4], zoaramine [5], kuroshines [6], epioxyzoanthamine [7], zoanthenol [8], some hydroxylated zoanthamines and norzoanthamines [9] and two halogenated zoanthamines [10]. This interesting family of alkaloids has been structurally classified in two different groups based on the presence of a methyl at C-19 (Type I) or without the methyl (Type II) also called norzoanthamine [10]. Due to the structural complexity of these natural products, the first total synthesis of norzoanthamine was accomplished by Miyashita et al., later in 2004. Further studies in the synthesis of these complex compounds led the same research group to fully synthesize zoanthamine through a stereoselective introduction of the methyl at C-19

[11]. Up to date, 38 zoanthamine-type alkaloids have been reported from zoantharian species essentially inhabiting the central Indo-Pacific. Interestingly, these polycyclic alkaloids seem to be chemical markers of zoantharians from the genus *Zoanthus*. In addition, some members of this family displayed a wide range of biological activities against P388 murine leukemia cells [3], antiosteoporosis activity, anti-inflammatory, antibacterial, but also inhibitors of human platelet aggregation [9, 12]. The most promising therapeutic application is associated with norzoanthamine in the treatment of osteoporosis as it inhibits interleukin-6 which is a primary mediator of bone resorption [11, 13]. Furthermore, an interesting study by Tachibana and co-workers suggests that collagen strengthening is the principal function of norzoanthamine in *Zoanthus* sp. [14].

Within our continuous interest for the bio- and chemo-diversity of marine invertebrates present in the understudied Marine Protected area El Pelado, Santa Elena, Ecuador located in the Tropical Eastern Pacific [15, 16], we came across a massive substrate cover of the intertidal region of this area by undescribed fluorescent green zoantharians. The first taxonomic assessment of these zoantharian species led to the identification of the major species being closely related to *Zoanthus* cf. *pulchellus*, previously described in the Caribbean [17]. No chemical study has been reported so far from this species and our first chemical screening by UHPLC-HRMS revealed unknown masses related to the zoanthamine family as major compounds of the extract. In this paper, we describe the isolation and structure elucidation of two new zoanthamine alkaloids namely 3-acetoxynorzoanthamine (**1**) and 3-aectoxyzoanthamine (**2**) along with the known zoanthamine [1], norzoanthamine [3] and 3-hydroxynorzoanthamine [18] from the Eastern Pacific Zoantharian *Zoanthus* cf. *pulchellus* as well as their biological activity in cellular pathways related with oxidative stress and neuroinflammation.



	R
3-Acetoxynorzoanthamine (**1**)	H
3-Acetoxyzoanthamine (**2**)	CH_3

Figure 1. Structures of 3-acetoxynorzoanthamine (**1**) and 3-acetoxyzoanthamine (**2**) isolated from *Zoanthus* cf. *pulchellus*

2. Results

Colonies of the zoantharian *Zoanthus* cf. *pulchellus* were collected by hand in the intertidal coast of San Pedro – Santa Elena. Sample was freeze-dried and extracted with a mixture of solvents CH_3OH:CH_2Cl_2 (v/v; 1:1). The extract was then fractioned through reverse phase C18 Vacuum Liquid Chromatography (VLC) using a mixture of solvents of decreasing polarity. The aqueous methanolic fractions were analyzed by UPLC-DAD-ELSD combined and then subjected to semipreparative RP-HPLC using a C18 column to yield two new zoanthamine-type alkaloids; 3-acetoxynorzoanthamine (**1**), and 3-acetoxyzoanthamine (**2**) along with the known zoanthamine [19], norzoanthamine [14] and 3-hydroxynorzoanthamine [18].

Compound **1** was obtained as a brown amorphous powder and (+)-HRESIMS analyses revealed a major molecular peak at *m/z* 540.2956 $[M+H]^+$, consistent with the molecular formula $C_{31}H_{41}NO_7$ for the neutral molecule. A preliminary inspection of the ^1H and ^{13}C NMR data revealed characteristic signals of the zoanthamine family as already speculated on the basis of the HRMS data: an olefinic

proton at δ_H 5.90 (H-16) along with four methyl singlets at δ_H 0.97 (H-28), 0.99 (H-25), 1.15 (H-29), 2.00 (H-27) and a doublet at δ_H 0.87 (H-30) together with two ketone signals at δ_C 198.5 (C-17) and δ_C 209.0 (C-20), one ester signal at δ_C 172.3 (C-24) and two olefinic carbons at δ_C 125.6 (C-16) and 160.0 (C-15) (Table 1). The absence of a second doublet of a methyl present in zoanthamines was indicative of a loss of the methyl CH_3-26 at C-19 and therefore the compound belonged to the norzoanthamine type. Unlike most studies on norzoanthamines and to make the NMR table more homogeneous, we decided to keep the numbering of the zoanthamines especially for the methyls 27, 28, 29 and 30. Comparing with analogues of this type we observed the presence of an additional methyl singlet signal at δ_H 2.11 corresponding to an acetyl moiety (Table 1). The presence of the acetyl group on the oxygen at C-3 was evidenced by the deshielding of the signal corresponding to the methine H-3 with δ_H 4.62 and key H-3/C-1′ and H_3-2′/C-1′ HMBC correlations.

Table 1. ^{1}H and ^{13}C NMR data in ppm for compounds **1** and **2** in $CDCl_3$ (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR data)

	1		**2**	
No.	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C
1	3.24, t (7.0) 3.19, d (7.0)	45.3	3.24, t (7.5) 3.20, d (7.0)	45.5
2	4.58, br d (6.5)	75.6	4.59, d (7.0)	75.7
3	4.62, br t (3.0)	72.5	4.63, t (3.0)	72.6
4	2.44, br sext (5.5)	26.0	2.43, br sext (6.0)	26.1
5	1.92, dd (12.0, 6.0) 1.36, t (12.5)	40.3	1.95, dd (12.5, 6.0) 1.37, t (13.0)	40.4
6	-	90.1	-	90.2
7	1.88, dd (12.5, 4.5) 1.80, dt (12.5, 3.5)	29.8	1.90, dd (12.5, 4.5) 1.80, dt (12.5, 3.5)	29.9
8	1.66, td (13.5, 3.5) 1.57, dt (13.5, 4.0)	23.7	1.67, td (14.0, 3.5) 1.57, dt (14.0, 4.0)	23.8
9	-	40.0	-	40.5
10	-	100.9	-	101.0
11	2.08, d (13.0) 1.94, d (13.0)	41.8	2.11, d (13.0) 1.93, d (13.0)	42.0
12	-	39.9	-	39.8
13	2.20, td (12.0, 4.5)	53.1	2.41, td (12.0, 4.5)	48.1
14	2.26, br s 2.24, br s	32.0	2.24, br s 2.22, br s	30.7
15	-	160.0	-	160.1
16	5.90, s	125.6	5.92, s	127.0
17	-	198.5	-	197.3
18	2.69, td (12.0, 6.5)	46.4	2.66, dd (12.5, 6.5)	48.2
19	2.62, dd (14.5, 6.5) 2.50, dd (14.5, 12.0)	42.4	3.02, dq (7.0, 6.5)	45.9
20	-	209.0	-	212.2
21	2.83, s	59.1	3.23, s	53.9
22	-	36.5	-	40.3
23	3.65, d (20.0) 2.36, d (20.0)	35.9	3.68, d (20.0) 2.37, d (20.0)	36.1
24	-	172.3	-	172.4
25	0.99, s	21.1	0.98, s	20.8
26	-	-	**1.17, d (7.0)**	**13.9**
27	2.00, s	24.4	2.01, s	24.6
28	0.97, s	18.5	0.99, s	18.5
29	1.15, s	18.4	1.21, s	18.4
30	0.87, d (7.0)	16.3	0.89, d (7.0)	16.4
Ac	-	171.2	-	171.4
	2.11, s	21.1	2.14, s	21.2

We then addressed the question of the relative configurations of the different chiral centers. To the best of our knowledge, this is the first occurrence of an acetoxy group at position C-3 for zoanthamines but other oxygenated analogues at this position were already described. First, 3-hydroxynorzoanthamine was isolated from an undescribed species of *Zoanthus* from the Canary Islands in the Atlantic Ocean [18]. Later, kuroshines C and F as well as 3β-hydroxyzoanthenamide also possess an hydroxyl group at this position [6]. All these four derivatives were shown to have a hydroxyl group on the β side of the polycyclic compound and this position was deduced from nOes between H-3 and other protons of the azepane ring. In our case, and because both H-3/H-4a and H-3/H-4b coupling constant values were not fully conclusive, we relied on the key H-3/H-1b nOe

correlation to place H-3 on the opposite side of the bridged oxygen (α-side). Subsequently, the acetoxy group was located on the β-side like for the other four 3-hydroxylated analogues. The very low coupling constant values of H-3 with H-2 and H-4 were similar to those observed for all 3-hydroxylated compound and in perfect agreement with this relative configuration. Additionally, a previous study by Uemura and co-workers assigned the absolute configuration of norzoanthamine as 2*R*, 4*S*, 6*S*, 9*S*, 10*R*, 12*R*, 13*R*, 18*S*, 21*S*, and 22*S* and they suggest the same absolute configuration for all norzoanthamine-type alkaloids [20]. In our case, the positive specific rotation obtained for **1** is in accordance with the one obtained for 3-hydroxyzoanthamine and therefore comes as a confirmation of the same absolute configuration [18].

Compound **2** was isolated as an amorphous yellowish powder and the molecular formula $C_{32}H_{43}NO_7$ was deduced from HRESIMS revealing a major peak at *m*/*z* 554.3115 $[M+H]^+$ and therefore **2** is an homologue of **1**. A quick inspection of the ^1H NMR spectrum evidenced the presence of the acetoxy group at C-3 like for **1**. An additional methyl signal at δ_H 1.17 (d, J = 7.0 Hz, H_3-26) suggested that **2** was a member of the zoanthamine type alkaloids. The presence of the methyl at C-19 was confirmed by the key H-19/C-26 and H_3-26/C-18/C-19 HMBC correlations. The β position of the methyl 26 was then confirmed by the coupling constant value $J_{H\text{-}18/H\text{-}19}$ of 6.0 Hz reminiscent of an axial/equatorial coupling. Because H-18 is placed in an axial position, H-19 has to be placed in an equatorial position and therefore the methyl 26 occupies the corresponding axial β-position at C-19. The β position of the acetoxy at C-3 was inferred from the same coupling constant values from H-3 as for **1** and the absolute configuration was supposed to be the same as the one of **1** again due to similar positive specific rotations.

The compounds were tested for biological activity in BV-2 microglia cell line, a cellular model often used in neuroinflammation studies. The first step was to determine the effect of compounds over cells viability. Five concentrations (from 0.001 to 10 μM) were checked and after 24 h of incubation no effects in cell viability were observed which point to non-toxic compounds. Microglia-mediated inflammation is known to produce reactive oxygen species (ROS) and to release nitric oxide (NO) and, in this way to induce oxidative damage [21]. Therefore, zoanthamines were checked as modulators within these processes. BV-2 cells were activated with lipolysaccharide (LPS) to simulate neuroinflammatory conditions. As shown in Figure 2, when cells are pre-treated with the same concentrations of compounds for 1 h and then incubated for 24 h with LPS (500 ng/mL) a significant reduction in ROS production was observed. As expected, the stimulation of BV-2 cells with LPS have significantly increased the ROS production, 50 % ($p<0.001$), while the compounds alone did not induce any effect. However, when cells were pre-treated with norzoanthamine and **1**, a dose-dependent inhibitory effect was observed, while 3-hydroxynorzoanthamine, zoanthamine or **2** were effective at all concentrations tested, being **2** the most potent ROS inhibitor. From these results 0.1 and 1 μM were chosen to check the effect over NO release (Figure 3). Zoanthamine alkaloids alone did not produce any effect over NO production while LPS treatment increases it three times. In the presence of this family of compounds, NO release was significantly inhibited. The anti-inflammatory effect of zoanthamines was previously approached in neutrophils [10]. From our results in the BV-2 cellular model, zoanthamine and derivatives show effective properties as protective drugs in neuroinflammation processes

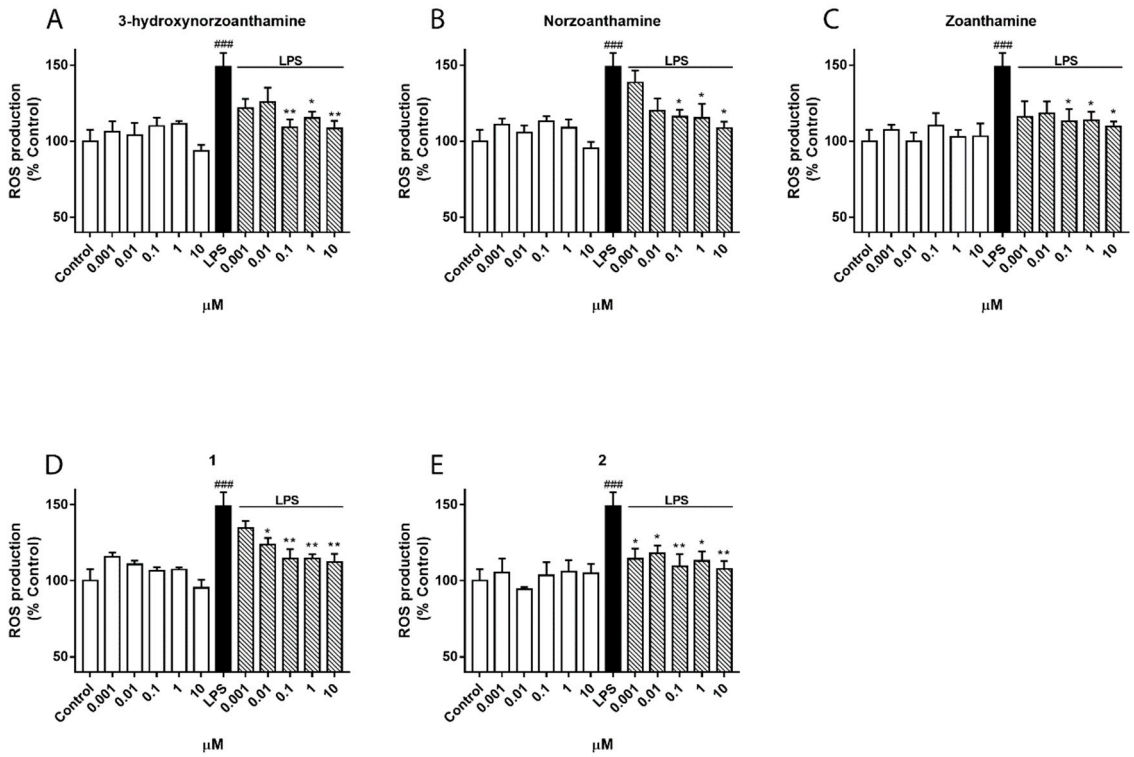


Figure 2. Effects of the five zoanthamine derivatives isolated from *Z.* cf. *pulchellus* on the ROS production in BV-2 microglia cell line

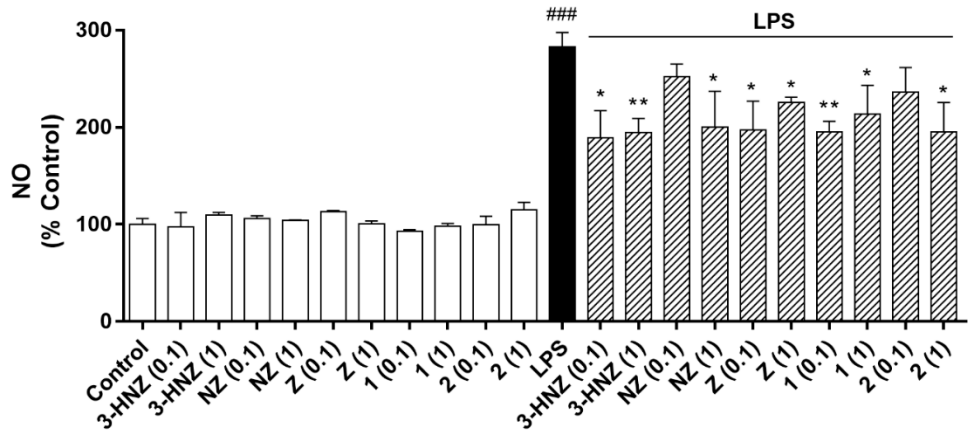


Figure 3. Effects of the five zoanthamine derivatives isolated from *Z.* cf. *pulchellus* on the NO production in BV-2 microglia cell line

3. Discussion

The isolation of 3-acetoxy derivatives zoanthamine and norzoanthamine in *Z.* cf. *pulchellus* strengthens the hypothesis of zoanthamines being markers of the genus *Zoanthus*. However, we need to point out here that another species of *Zoanthus* identified as *Z.* cf. *sociatus* found in the same area did not present any zoanthamine derivatives [17]. Even if these compounds should therefore not be considered as taxonomic markers of the genus *Zoanthus* they are clear and characteristic features of some species of *Zoanthus* that could help for a more precise classification of this group.

Interestingly we first run the NMR analyses in a different solvent CD_3OD and we observed clear changes for the signals surrounding the nitrogen atom of **1**. Especially the signals corresponding to H-11 were not observed anymore. This observation reinforced the conclusions given on zoanthamine analogues by the group of Norte [18]. In a highly polar and protic solvent, the opening of the lactone ring would give rise to an iminium ion at C-11 in equilibrium with its enamine base that can be trapped by exchangeable deuterium atoms provided by the protic deuterated solvent. This behavior points out the high reactivity of this family of compounds.

Because these compounds were isolated after a purification step involving acetic acid in the eluent of the HPLC, we then wanted to ascertain the presence of these compounds in the collected specimen. For this purpose, we inspected the chemical profiles obtained before any contact with acetic acid and we were able to observe the masses corresponding to the new compounds **1** and **2** which therefore rules out the possibility of a transformation during the purification process.

Finally, the activity observed for all compounds highlights the potential of zoanthamine derivatives as new ROS and NO modulators in neuronal processes and we will continue our efforts onto the study of their mode of action over neuroinflammatory related diseases.

4. Materials and Methods

4.1 General Experimental Procedures. Optical rotation measurements were obtained at the sodium D line (589.3 nm) with a 10-cm cell at 20 °C on a UniPol L1000 polarimeter (Schmidt + Haensch, Berlin, Germany). The UV measurements were obtained by the extraction of the Diode Array Detector (DAD) signal of the Ultra-High-Pressure Liquid Chromatography (UHPLC) Dionex Ultimate 3000 (Thermo Scientific, Waltham, MA, USA). NMR spectra were recorded on a Variant 500 MHz spectrometer (500 and 125 MHz for ^{1}H and ^{13}C, respectively), and signals were referenced in ppm to the residual solvent signals ($CDCl_3$, at δ_H 7.26 and δ_C 77.16 ppm). HRESIMS data were obtained with a UHPLC-qTOF Agilent 6540 mass spectrometer. Purification was carried out on a JASCO HPLC equipped with a PU4087 pump and a UV4070 UV/Vis detector.

4.2 Biological Material. Specimens of *Zoanthus* cf. *pulchellus* was collected by hand on rocks of the shoreline of San Pedro located in the Peninsula of Santa Elena, Ecuador. A sample with a voucher 161125SP-01 is held at CENAIM-ESPOL (San Pedro, Santa Elena, Ecuador). This species has been previously identified with morephological and molecular data [17].

4.3 Extraction and Isolation. The freeze-dried sample of *Z.* cf. *pulchellus* (200 g) was extracted with a mixture of solvents DCM/MeOH (1:1) three times (500 mL) at room temperature. The collected extract was concentrated under reduced pressure to obtain the crude extract (10 g). The crude extract was subjected to C18 reversed phase vacuum liquid chromatography (LiChroprep® RP-18, 40–63 µm) using a mixture of solvents of decreasing polarity (1). H_2O, (2). H_2O/MeOH (1:1), (3). H_2O/MeOH (1:3), (4). MeOH, (5). MeOH/DCM (3:1), (6). MeOH/DCM (1:1), and (7). DCM using 500 mL of each solvent. The aqueous-methanolic fractions F3 was purified by reversed-phase HPLC (Ultra AQ C18, 10 x 250 mm, 5 µm) using an isocratic method CH_3CN:H_2O:Acetic acid (30:70:0.1) as a mobile phase with a flow rate of 3 mL/min with detection at λ 254 nm for 20 min yielding compound **1** (52.7 mg) and the known compounds norzoanthamine (6.3 mg) [22] and zoanthamine (6.6 mg) [19]. The methanolic fraction F4 was purified by reversed-phase HPLC (Ultra AQ C18, 10 x 250 mm, 5 µm) using the following mobile phases: A) CH_3CN/acetic acid 0.1 %, B) H_2O/acetic acid 0.1%; starting with an isocratic 0-25 min with A 22, B 78; linear gradient for 25-30 until A 100; then isocratic for 30-60 min at a flow rate of 3 mL/min with UV detection at λ 254 nm to yield compound **2** (12.3 mg) and the known 3-hydrozynorzoanthamine (2.7 mg)[18].

*4.4 3-Acetoxynorzoanthamine (**1**):* amorphous yellow powder; $[\alpha]_D^{20}$ +10 (*c* 0.45, CH_3OH); UV (DAD) λ_{max} 240 nm; ^{1}H NMR and ^{13}C NMR data see Table 1; HRESIMS (+) *m/z* $[M + H]^+$ 540.2956 (calc. for $C_{31}H_{42}NO_7$ 540.2956 Δ +0.0 ppm).

*4.5 3-Acetoxyzoanthamine (**2**):* amorphous yellowish powder; $[\alpha]_D^{20}$ + 6.7 (*c* 0.12, CH_3OH); UV (DAD) λ_{max} 238 nm; ^{1}H NMR and ^{13}C NMR data see Table 1; HRESIMS (+) *m/z* $[M + H]^+$ 554.3115 (calc. for $C_{32}H_{44}NO_7$ 554.3112 Δ +0.5 ppm).

4.6 Biological Assays.

4.6.1 Cell Culture. Microglia BV-2 cell line was obtained from InterLab Cell Line Collection (ICLC), number ATL03001. Cells were maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and 100 µg/mL streptomycin at 37 ºC in a humidified atmosphere of 5% CO_2 and 95% air. Cells were dissociated twice a week using 0.05% trypsin/EDTA.

4.6.2 Cell viability. The MTT assay was used to analyzed cell viability as previously described [23]. Briefly, microglia BV-2 cell line was grown in 96 well plate at a density of 4 x 10^4 cells per well. Cells were exposed to different compounds concentration (0.001, 0.01, 0.1, 1 and 10 µM) for 24 h. Then, the cells were rinsed and incubated with MTT (500 µg/mL) diluted in a saline buffer for 1 h at 37 ºC. The resulting formazan crystals were dissolved with 5% sodium dodecyl sulfate (SDS) and the absorbance values were obtaining using a spectrophotometer plate reader (595 nm). Saponin was used as a cellular death control and its absorbance was substrate from the other data.

4.6.3 Measurement of intracellular ROS production. The intracellular ROS levels in microglia activation were performed using 7′,2′-dichlorofluorescein diacetate (DCFH-DA), as previously described [24]. Cells were pre-treated with different compounds concentration (0.001, 0.01, 0.1, 1 and 10 µM) 1 h prior to the stimulation with LPS (500 ng/mL) for 24 h. Afterwards, cells were rinsed twice with saline solution and incubated 1 h at 37 ºC with 20 μM DCFH-DA. Then, cells were washed and keep with saline solution for 30 min at 37 ºC. Intracellular production of ROS was measured by fluorescence detection of dichlorofluorescein (DCF) as the oxidized product of DCFH-DA on a spectrophotometer plate reader (495 nm excitation and 527 nm emission).

4.6.4 NO determination. The NO concentration in the culture media was established by measuring nitrite formed by the oxidation of NO, using the Griess reagent kit, according to manufacturer's instructions. The detection limit of this method is 1 µM. Briefly, microglia cells were seeded in 12-well plate at a density of 1 x 10^6 cells per well and pre-incubated with compounds (0.1 and 1 µM) 1 h and then were stimulated with LPS (500 ng/mL) for 24 h. Thereafter, in a microplate were mixed: 150 µL of cells supernatant, 130 µL of deionized water and 20 µL of Griess Reagent and was incubated for 30 min at room temperature. The absorbance was measured on a spectrophotometer plate reader at a wavelength of 548 nm.

4.6.5 Statistical analysis. Results were expressed as mean ± SEM of a minimum of three experiments and were performed by duplicate or triplicate. Comparisons were analysed using Student´s t-test or one-way ANOVA with Dunnett´s *post hoc* analysis. P values < 0.05 were considered statistically significant.

Supplementary Materials: The following are available online: HRMS and NMR data for compounds **1** and **2**.

Author Contributions: Methodology and Formal Analysis, P.G., S.G., K.J., K.C.; Validation, E.A., A.A., K.C.; Writing-Original Draft Preparation, P.G.; Writing-Review & Editing, A.A., O.T.; Supervision, E.A., O.T.; Project Administration, J.R., O.T.; Funding Acquisition, J.R., L.B., O.T.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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