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Screening of acetylcholinesterase inhibitors in marine organisms from the Caribbean Sea

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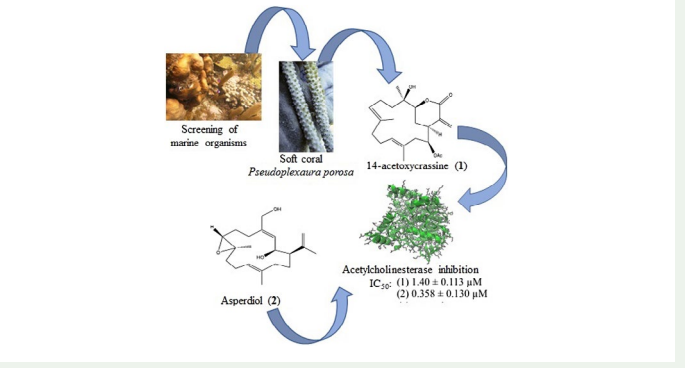
ABSTRACT

The acetylcholinesterase inhibitory activity of 89 organic extracts from marine organisms was evaluated through a TLC bioautography methodology. Extracts from soft corals (*Eunicea* and *Plexaura*) were the most active compared with extracts from sponges. The bioguided chemical study of the most active extract, obtained from *Pseudoplexaura porosa*, led to the isolation of a diterpene with spectroscopic properties consistent to those published to the cembrane Steylolide. However, further analysis by X-ray diffraction indicated that the compound was the 14-acetoxycrassine (**1**), correcting the structure reported to the Styelolide. Additionally, the acetylcholinesterase inhibitory activity of fourteen cembranoids (**2**–**15**) isolated from soft corals *Eunicea knighti* and *Pseudoplexaura flagellosa* was evaluated. Cembranoids **2**, **3** and **4** were the most active compounds in the TLC bioassay. Then, the most promising cembranoids, 14-acetoxycrassine (**1**) and asperdiol (**2**), were tested quantitatively and they exhibited IC_{50} values of 1.40 ± 0.113 and 0.358 ± 0.130 μM, respectively.



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1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a key enzyme responsible for the hydrolysis of acetylcholine (ACh) into choline and acetate during cholinergic synapses. AChE is involved in nerve transmission in many vertebrates and insects, where both the tridimensional structure and the amino acid active site sequence of the enzyme are highly conserved (Wiesner et al. 2007). Compounds with the capacity to inhibit AChE or acetylcholinesterase inhibitors (AChEIs) are also very attractive targets due to their importance in the treatment of several neurodegenerative diseases. Alzheimer disease (AD), a common form of dementia, is increasing its prevalence worldwide and is expected to be a considerable threat to human health during the next decades (Hickman et al. 2016). Although a cure has not been reached, the most promising results in AD treatments have been achieved by therapies based on AChEIs (Tabet 2006). Natural products constitute the best source of lead bioactive compounds, particularly for cancer therapy, where plants and microorganisms represent the principal source of these molecules (Newman and Cragg 2016). Although terrestrial organisms have been studied traditionally, marine organisms represent a remarkable source of bioactive compounds with cytotoxic, antibacterial, antifungal, and antitumoral activities (Blunt et al. 2016). Several AChEIs from marine organisms have been identified and some examples include diterpenes isolated from the soft coral *Lobophytum* sp. (Bonnard et al. 2010) and other compounds (Langjae et al. 2007; Nukoolkarn et al. 2008; Yoon et al. 2008; Ohlendorf et al. 2012). As the acetylcholinesterase inhibitory activity of marine organisms have not been studied exhaustively, extracts obtained from soft corals and sponges collected in the Colombian Caribbean sea were studied to identify compounds with acetylcholinesterase inhibitory activity.

2. Results and discussion

Marine natural products have not been explored systematically as source of AChEIs. As part of our continuous research of bioactive compounds from marine organisms (Tello et al. 2009; Pardo-Vargas et al. 2014), eighty-nine organic extracts obtained from different soft corals and sponge species were tested in an AChE inhibitory bioassay employing a bioautography methodology. The extracts were separated by TLC under three chromatographic conditions, including low, medium and high polarity eluents to evaluate compounds from all polarities in these organic extracts. Thirty extracts from soft corals and three extracts from sponges showed inhibition of AChE (Table S1 contains all the results). In general, the most active extracts were afforded by *Eunicea* soft corals, followed by *Pseudoplexaura* and *Plexaura* whereas the extracts obtained from *Antillogorgia* species showed less bioactivity. On the other side, among the studied sponges, only the extract obtained from *Xestospongia proxima* exhibited an inhibition similar to that from soft corals. In general, soft corals are recognized as source of diterpenes such as briaranes, cembranes, dolabellanes, among others (Lei 2016). Then, the organic extract from *Pseudoplexaura porosa* was selected for chemical study because it was one of the most active extracts (Table S1) and its inhibitory activity was concentrated on a single spot. The extract was submitted to repetitive column chromatography over SiO_2 to isolate compound **1** in a bioguided fractionation scheme employing the TLC bioautography assay. Compound **1** was isolated as a white solid with a $C_{22}H_{32}O_5$ molecular formula based on HRESIMS analysis and its ^{1}H and ^{13}C NMR properties are indicated in the supporting material (Table S3). COSY

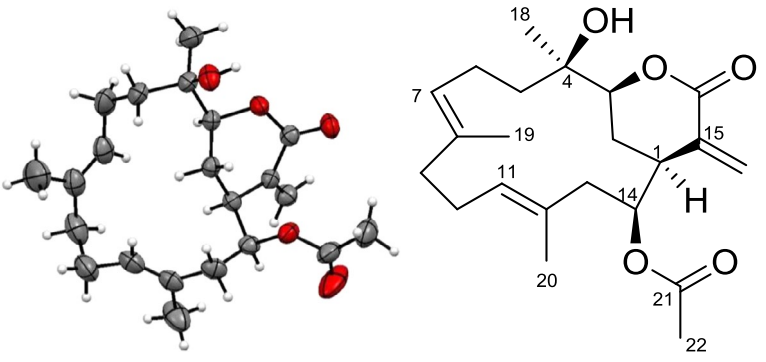

Figure 1. ORTEP diagram and structure of 14-acetoxycrassine (**1**).

and HSQC experiments were used to establish four different spin systems and the HMBC experiment allowed connecting those systems according to the observed correlations (Figure S1). The proposed structure was consistent to that reported for Styelolide (**1a**), a cembrane diterpene previously isolated from the tunicate *Styela plicata* collected off the coast of Florida (USA) (Wasylyk and Alam 1989). However, the structure reported to the cembrane diterpene 14-acetoxycrassine (**1**) also was consistent with the observed 2D NMR correlations (Figure S1). Then, to determine unambiguously the structure of the isolated compound, it was analysed by X-ray diffraction as the ORTEP diagram shows in the Figure 1. This information showed that the structure corresponds to that published for 14-acetoxycrassine (**1**) (Rice et al. 1970). Additionally, the results allowed us describing unambiguously the absolute configuration of 14-acetoxycrassine (**1**) (Flack parameter = 0.02(3)) as (1*S*,3*S*,4*R*,14*S*). In this way, we correct the proposed structure for Styelolide (**1a**) (Wasylyk and Alam 1989) and establish that together with 14-acetoxycrassine (**1**) are the same compound. As compound **1** showed a promising activity in the TLC bioautography assay and this encouraged us to test the AChE inhibitory activity of other cembranoids previously isolated from *Eunicea* and *Pseudoplexaura* soft corals (Tello et al. 2009, 2011). Therefore, fourteen cembranoid diterpenes **2–15** (Figure 2) were tested employing the same TLC bioautography methodology and the results are shown in Table S2.

In general, cembranes such as asperdiol and its related compounds isolated from *Eunicea knighti* were more active than plexaurolones from *Pseudoplexaura flagellosa*. Asperdiol (**2**), asperdiol diacetate (**3**) and 8*R*-dihydroplexaurolone (**4**) were the most active compounds. On the other hand, 8*S*-plexaurolone (**12**) and 8*R*-plexaurolone (**13**) did not show representative activity. 8*R*-dihydroplexaurolone (**4**) was more active compared to 8*S*-dihydroplexaurolone (**8**), indicating that the 8*R* configuration is favourable for the bioactivity. The remaining compounds were mildly active. Asperdiol (**2**) and its related compounds include more polar substituents compared with plexaurolones. This observation suggests that the presence of acetates and hydroxyl groups influences the AChE inhibitory activity of cembranoids. Therefore, 14-acetoxycrassine (**1**) was treated with acetic anhydride in presence of catalytic quantities of 4-dimethylaminopyridine (4-DMAP) to obtain the acetylated derivate (**16**). The presence of an acetate group was confirmed by the signals at δ_H 2.11 (s, 3H), δ_C 170.3 and 20.7 in its ^{1}H and ^{13}C NMR spectra, assigned to the acetate carbonyl and a methyl group, respectively. The evaluation of AChE inhibitory activity for compound **16** indicated that was

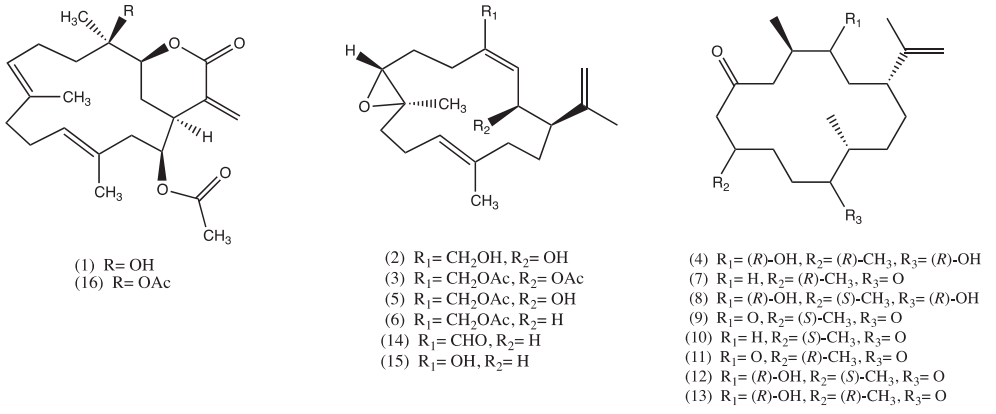

Figure 2. Cembranoids isolated from soft corals that were tested in the AChE inhibitory assay.

not active, supporting the role of the hydroxyl group in the bioactivity of this compound. As AChE inhibitory activity has only been reported for the cembrane diterpene crassumolide E (Bonnard et al. 2010), the obtained results extend the range of biological activities for cembranes and contributes to the discovery of AChEIs from marine sources. Once a preliminar screening was obtained in the AChE inhibition TLC bioautography assay, a quantitative 96-well colorimetric assay based in the Ellman method was developed (Di Giovanni et al. 2008). The compounds 14-acetoxycrassine (**1**) and asperdiol (**2**) were analysed due to their remarkable activity in the qualitative assay and compatibility needed for the Ellman assay. 14-acetoxycrassine (**1**) and Asperdiol (**2**) show a dose-dependent inhibition of the AChE activity (Figures S11 and S12, Supplementary material), exhibiting IC_{50} values of 1.40 ± 0.113 µM and 0.358 ± 0.130 µM, respectively. In comparison, both compounds were less active than galantamine (IC_{50} = 0.118 ± 0.008 µM). The last result was not expected based on the TLC bioautography assay where compounds **1** and **2** were more active than galantamine. However, the obtained IC_{50} values exhibit similar magnitudes and the quantitative result could be explained in terms of the differences of solubility. Cembranoid diterpenes are less soluble than the alkaloid galantamine at the conditions of the quantitative bioassay.

3. Experimental section

3.1. General information

UV spectrum was recorded using a Genesys^{TM} 10S UV-vis Spectrophotometer in a 1 cm path-length cell. IR spectrum was obtained on a Thermo Scientific Nicolet iS10 Spectrometer. NMR spectra were recorded with a Bruker 400 MHz spectrometer using $CDCl_3$ (δ: 7.26 ppm, 77.0 ppm). The analyses by LC-MS were obtained using a Shimadzu LC-10A coupled to a selective mass detector (LCMS-2010EV). Electrospray ion source ESI-MS spectra were acquired in positive mode. The single-crystal X-ray diffraction data for compound **1** were obtained on a Bruker D8 Venture diffractometer using radiation CuKα (λ = 1.5418 Å). The structure was solved by direct methods and refined by full-matrix least squares on F^2 with SHELX package (Sheldrick 2015). The positions of hydrogen atoms were generated geometrically and refined according to a riding model. All non-hydrogen atoms were refined anisotropically.

Acetylcholinesterase (EC. 3.1.1.7) enzyme from electric eel *Electrophorus electricus* (type VI-S 500 A), bovine serum albumin (BSA), Tris (hydroxymethyl)-aminomethane (Tris base), 1-naphthyl acetate and fast blue salt (Sigma-Aldrich). Galantamine was used as positive control. Thin layer chromatography was developed on aluminum plates precoated with silica gel $60F_{254}$ from Merck. Silica gel (0.043 – 0.069 mm, Merck) was employed for column chromatography. Separations and solvents of analytical quality were employed (all Merck).

3.2. Preparation of extracts from marine organisms and tested compounds

Samples of soft corals and sponges were collected at various sites of the Colombian Caribbean Sea by SCUBA diving, December 2011. Samples were identified by Dr Sven Zea and Dr Monica Puyana. Vouchers of each one of the studied species were deposited at collections in Instituto de Ciencias Naturales-Universidad Nacional de Colombia (ICN-UNAL) and Invemar. Soft corals (1.0–2.0 g) were cut and extracted three times with DCM:MeOH (1:1) (15 mL each time). The extracts were combined, filtered, dried under vacuum and finally weighted. The soft corals *E. knighti and P. flagellosa* afforded the pure compounds 2–15 as reported previously (Tello et al. 2009, 2011).

3.3. Isolation of 14-acetoxycrassine (1) from P. porosa

The soft coral *P. porosa* was collected at Santa Marta Bay, Colombian Caribbean, in December 2011 (Voucher ICN-CO-0108 was deposited at the ICN-UNAL). The material (290 g) was cut in small pieces, and extracted three times with a MeOH-CH_2Cl_2 (1:1 v/v) mixture. The extracts were filtered and concentrated under vacuum to obtain a dark green oil (7.6 g). The oil was fractioned over silica gel vacuum column chromatography eluting with mobile phases (500 mL) of increasing polarity (Hexane, EtOAc, MeOH) to yield 8 fractions (F1-F8). All the obtained fractions were tested for AChE inhibitory activity and fraction F4 resulted the most active. Fraction 4 (1009 mg), eluted with Hexane-EtOAc (80:20 v/v) and a small amount (290 mg) was further subjected to silica gel column chromatography eluting with a mixture of CH_2Cl_2-EtOAc 70:30. Fraction F4.2 was active in the AChE inhibition assay. The fraction was further analysed by NMR and mass spectrometry, corresponding to pure 14-acetoxycrassine (**1**) (113 mg).

14-acetoxycrassine (**1**): White solid, UV (MeOH) λ_{max} (log ε): 214.0 nm (3.61); IR (KBr) ν_{max} 3494, 2930, 1739, 1640, 1619; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) (Table S3); HRESIMS *m/z* 377.2153 $[M + H]^+$ (calcd for $C_{22}H_{33}O_5$, 377.2323).

3.4. Acetylation of 14-acetoxycrassine (1) to obtain compound 16

14-acetoxycrassine (**1**) (40 mg, 0.106 mmol) was dissolved in triethylamine (5.0 mL) and acetic anhydride (400 µL) and of 4-dimethylaminopyridine (1.0 mg) were added. The reaction mixture was stirred at room temperature for 72 h and after that, water was added. The mixture was extracted once with DCM (50 mL) and the organic phases were washed with saturated solutions of $NaHCO_3$, NaCl and dried with sodium sulfate. The mixture was concentrated under vacuum to obtain a residue (73 mg) that was separated through column chromatography over silica gel eluting with Hexane:EtOAc (9:1) to obtain compound **16** (7.1 mg, 20.0%).

Compound **16**: White solid, $[\alpha]_D^{20} = 3.9$ (0.001; $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz): δ_H 2.67 (ddd, $J = 11.5, 5.7, 2.6$ Hz, 1H, H-1), 2.15 (m, 1H, H-2a), 1.82 (m, 1H, H-2b), 3.92 (d, $J = 10.8$ Hz, 1H, H-3), 1.90 (m, 1H, H-5a), 1.61 (m, 1H, H-5b), 2.31 (m, 1H, H-6a), 2.09 (m, 1H, H-6b), 5.09 (t, $J = 7.9$ Hz, 1H, H-7), 2.24 (m, 1H, H-9a), 1.93 (m, 1H, H-9b), 2.39 (m, 1H, H-10a), 1.72 (m, 1H, H-10b), 5.30 (t, $J = 7.4$ Hz, 1H, H-11), 2.50 (m, 1H, H-13a), 2.14 (m, 1H, H-13b), 5.43 (ddd, $J = 11.5, 5.1, 2.6$ Hz, 1H, H-14), 6.51 (d, $J = 2.4$ Hz, 1H, H-17a), 5.70 (d, $J = 2.4$ Hz, 1H, H-17b), 1.75 (s, 3H, H-18), 1.59 (s, 3H, H-19), 1.72 (s, 3H, H-20), 1.99 (s, 3H, H-22), 2.04 (s, 3H, H-24); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 35.4 (C-1), 21.1 (C-2), 82.4 (C-3), 84.9 (C-4), 35.4 (C-5), 24.4 (C-6), 125.8 (C-7), 136.3 (C-8), 40.3 (C-9), 22.9 (C-10), 128.6 (C-11), 130.6 (C-12), 42.0 (C-13), 73.0 (C-14), 136.7 (C-15), 166.9 (C-16), 128.3 (C-17), 20.9 (C-18), 15.1 (C-19), 15.2 (C-20), 170.6 (C-21), 21.3 (C-22), 170.9 (C-23), 22.9 (C-24); ESIMS: *m/z* 441.15 $[M + Na]^+$ (calcd for $C_{24}H_{34}O_6Na$, 441.23).

3.5. Acetylcholinesterase enzyme inhibition test

The AChE inhibitory activity was tested according to the method described by (Marston et al. 2002). All extracts were dissolved in dichloromethane and applied on a TLC plate for the AChE inhibition test. Galantamine was used as positive control at 0.1 mg/spot. TLC plates were eluted in three different solvent systems (mixtures of n-hexane:EtOAc in 8:2, 1:1 and 0:1 ratios); then, dried and sprayed with the AChE solution, leaving to stand for 5 min at room temperature (20 °C). Finally, the plates were sprayed (mixture 1:4 of naphthyl acetate:Fast Blue B salt solutions) until a purple coloration was observed (1–2 min) and the presence of white spots was associated with inhibition zones. Crude extracts were assayed in the TLC bioautography assay at 0.1 mg/spot approximately (Table S1) and pure compounds were tested dissolving 0.1 mg of each compound in DCM and applying them on the TLC plates.

3.6. Colorimetric AChE inhibition assay

A microplate assay was carried out to determine the inhibitory activity of pure compounds following the method described by (Di Giovanni et al. 2008). The compounds assayed were diluted in DMSO to avoid interferences. DMSO did not present interference, the final concentration was 1.08% in well plates. Solvent concentrations were maintained below 1.08% in each well. The concentration range was from 1 to 1000 µg/mL for all compounds. IC_{50} values were obtained through nonlinear regressions of percentage of inhibition versus Inhibitor concentration. Data were measured in triplicate. A statistical analysis by one-way analysis of variance (ANOVA) with a value of $p < 0.0001$ was carried out with Prism 6 Version 6.01 GraphPad Software (San Diego, CA).

4. Conclusions

The screening of acetylcholinesterase inhibitors in the studied marine organisms showed that the extracts from soft corals are a promising source of AChEIs. Particularly, the extracts obtained from *Eunicea* and *Pseudoplexaura* genera resulted the most active. The most active extract, from the soft coral *P. porosa*, was submitted to chemical study and the 14-acetoxycrassine (**1**) was identified as an AChE inhibitor. Some other cembranoids previously isolated also exhibited AChE inhibitory activity in a TLC bioautography assay. A colorimetric inhibition assay showed that Asperdiol (**2**) is more active than 14-acetoxycrassine (**1**), but they were

less active compared with the positive control galantamine. The obtained results contribute to increase the range of biological activities reported for cembranoid diterpenes and encourage us to continue studying bioactive compounds from marine sources.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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