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Dihydroagarofuranoid Sesquiterpenes as Acetylcholinesterase Inhibitors from Celastraceae Plants: *Maytenus disticha* and *Euonymus japonicus*

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ABSTRACT: Natural cholinesterase inhibitors have been found in many biological sources. Nine compounds with agarofuran (epoxyeudesmane) skeletons were isolated from seeds and aerial parts of *Maytenus disticha* and *Euonymus japonicus*. The identification and structural elucidation of compounds were based on spectroscopic data analyses. All compounds had inhibitory acetylcholinesterase (AChE) activity. These natural compounds, which possessed mixed or uncompetitive mechanisms of inhibitory activity against AChE, may be considered as models for the design and development of new naturally occurring drugs for management strategies for neurodegenerative diseases. This is the first report of these chemical structures for seeds of *M. disticha*.

KEYWORDS: *Celastraceae, Eudesmanolides, Maytenus disticha, Euonimus japonicus, dihydroagarofurans, acetylcholinesterase*

INTRODUCTION

Acetylcholinesterase (AChE), one of the most essential enzymes in the family of serine hydrolases, catalyzes the hydrolysis of the neurotransmitter acetylcholine, which plays a key role in memory and cognition.[1] Although the physiological role of AChE in neural transmission in humans or insects is well-known, it is still a significant focus of pharmaceutical research for treatments of myasthenia gravis, glaucoma, Alzheimer's disease (AD), Parkinson's disease (PD), and other neurodegenerative diseases (NDs) and also for the development of insecticides. Intensive research for drugs that can improve cholinergic transmission in NDs has produced only a few drugs useful for symptomatic treatment.[2] A marked variety of structural motifs occur in natural cholinesterase inhibitors isolated from botanical sources and microorganisms. These are a valuable source for novel AD, PD, and ND drug candidates.[3] Several inhibitors of cholinesterases of synthetic and natural origin are available in the drug market; however, side effects and relatively low bioavailability limit their uses in medicine, and there is still a great demand to discover new cholinesterase inhibitors.[4−8]

The family Celastraceae is indigenous to tropical and subtropical regions of the world including North Africa, South America, and many parts of East Asia, particularly China. The family consists of approximately 88 genera and 1300 species of plants. These plants generally grow as small trees, shrubs, or lianas that have resinous stems and leaves. The family is present in Chile as the endemic genus *Maytenus*, with four Chilean species *Maytenus disticha* (Hook. F.) Urban., *Maytenus boaria* Mol., *Maytenus chubutensis* (Speg.) Lourteig. O'Don. & Sleumer, and *Maytenus magellanica* (Lam.) Hook. F. Many papers describe diverse effects of *Maytenus* extracts on human health and on other animals. The leaves, aerial portions, seeds, and roots of plants of this family are used in traditional medicine.[9−11]

Plants belonging to the Celastraceae produce a variety of characteristic secondary metabolites including triterpenes, sesquiterpenes, and diterpenes. Dihydro-β-agarofurans have been considered "privileged structures" because many show cytotoxic, anti-HIV, immunosuppressive, and insecticidal activities. Because many species of the family Celastraceae contain dihydro-β-agarofurans, plants of this family have been intensively investigated. These types of compounds are typical and appear to be taxonomic markers for the family.[12,13] In Chile, the Celastraceae is represented exclusively by the four species of *Maytenus* mentioned above, although several species of the genus *Euonymus* have been introduced for ornamental purposes and are frequently found in parks and gardens.

We have studied the phytochemical constituents of the Chilean Celastraceae and have described the isolation procedure for sesquiterpene polyol esters with dihydro-β-agarofuran skeletons from aerial parts of *M. disticha* and seeds of *M. boaria*.[14−17]

Previously published information about members of this family and our field observations indicate these plants appear to be highly resistant to attack by insect pests. On the basis of this information, we have undertaken examination of published literature about members of this family. Our long-term goal is to correlate phytochemical composition and biological activity

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and, in particular, AChE inhibitors that will be valuable for medicine as well as for the discovery of biopesticides of botanical origin. Previously, we reported inhibition of AChE isolated from *Spodoptera frugiperda* by two agarofuran sesquiterpenes, one of them isolated from aerial parts of *M. disticha* and the other from seeds of *M. boaria*.[18] In a subsequent study we reported the inhibition of AChE from bovine erythrocytes by six agarofurans isolated from these species.[19] Herein, the isolation, structural characterization, and study of inhibition of AChE activity by five new dihydro-*β*-agarofuran sesquiterpenes from seeds of *M. disticha* are discussed.

MATERIALS AND METHODS

General Experimental Procedures. Analytical and preparative TLC was performed on silica gel 60 Merck plates, and the spots were visualized by spraying with a 10% solution of H_2SO_4, followed by heating at 110 °C. NMR spectra, including COSY, NOESY, HMBC, and HSQC experiments, were recorded on a Bruker spectrometer operating at 400 and 500 MHz (^{1}H) and 100 and 125 MHz (^{13}C), respectively, with chemical shifts given in parts per million (δ) using TMS as an internal standard.

Plant Material. Aerial parts and seeds of *M. disticha* and seeds of *Euonymus japonicus* were collected in November−December 2013 in Chillán, region VIII of Chile, and identified by Prof. Victor Finot, botanist. A voucher specimen is deposited in the Herbarium of the School of Agricultural Science, Universidad de Concepción (UDEC).

Extraction and Separation. The seeds (300 g) of *M. disticha* were separated from the fruit pericarp, pulverized, and extracted at room temperature with MeOH (2 × 1 L, 72 h each). The extract was solvent partitioned using $CHCl_3$, EtOAc, and H_2O. The $CHCl_3$ fraction was chromatographed on a silica gel column using *n*-hexane−ethyl acetate gradient mixtures resulting in the collection of 250 fractions of 50 mL each, which were combined on the basis of TLC analyses. Further purification of subfractions afforded compounds **1**−**4**, **6**, and **9**. The aerial part of the plant was extracted with MeOH at room temperature for a week. The resulting methanol extracted was concentrated at reduced pressure in rotatory evaporator at 40 °C and 250 mb to yield a syrupy methanol extract. The residue was partitioned with *n*-hexane, ethyl acetate, and water. The *n*-hexane fraction was chromatographed on a silica gel column, eluting with *n*-hexane−EtOAc. Fractiond 30−40 were combined and evaporated to dryness to yield compound **5**. Similarly, 720 g of seeds of *E. japonicus* was treated in the same manner to yield compounds **7** and **8**. The aerial portion of *M. disticha* was extracted with methanol. This crude extract was dissolved in water and washed with *n*-hexane and EtOAc. The *n*-hexane fraction was eluted by chromatography over silica gel with *n*-hexane−EtOAc mixtures containing increasing proportions of EtOAc.

Spectroscopic Data. *1α,6β,8α-Triacetoxy-9β-furoyloxy-β-agarofuran (**1**):* oil; ^{1}H NMR δ 5.33 (1H, dd, J = 4.0, 11.5 Hz, H-1), 1.84 (1H, m, H-2α), 1.58 (1H, m, H-2β), 1.44 (1H, m, H-3α), 2.15 (1H, m, H-3β), 2.24 (1H, m, H-4), 5.68 (1H, s, H-6), 2.46 (1H, br s, H-7), 5.21 (1H, d, J = 3.0 Hz, H-8), 4.91 (1H, s, H-9), 1.38 (3H, s, H-12), 1.45 (3H, s, H-13), 1.48 (3H, s, H-15), 1.00 (3H, d, J = 7.0 Hz, H-14), 1.71 (3H, s, OAc-1), 2.08 (3H, s, OAc-6), 2.18 (3H, s, OAc-8), 8.01 (1H, dd, J = 1.5, 1.0 Hz, OFu), 6.72 (1H, dd, J = 1.0, 2.0 Hz, OFu), 7.40 (1H, dd, J = 1.5, 2.0 Hz, OFu); ^{13}C NMR δ 73.3 (d, C-1), 21.3 (t, C-2), 26.6 (t, C-3), 33.7 (d, C-4), 90.3 (s, C-5), 76.4 (d, C-6), 52.9 (d, C-7), 76.0 (d, C-8), 75.7 (d, C-9), 49.7 (s, C-10), 81.7 (s, C-11), 30.8 (q, C-12), 25.5 (q, C-13), 18.6 (q, C-14), 17.3 (q, C-15).

*1α-Hydroxy-6β,8α-diacetoxy-9β-furoyloxy-β-agarofuran (**2**):* amorphous solid; ^{1}H NMR δ 6.03 (1H, s, H-6), 5.35 (1H, br s, H-9), 5.14 (1H, dd, J = 4.0,11.0 Hz, H-1), 4.32 (1H, br d, J = 3.5 Hz, H-8), 1.83 (1H, m, H-2α), 1.56 (1H, m, H-2β), 1.41 (1H, m, H-3α), 2.11 (1H, m, H-3β), 2.22 (1H, m, H-4), 2.46 (1H, br s, H-7), 1.42 (3H, s, H-15), 1.38 (3H, s, H-13), 1.31 (3H, s, H-12), 0.99 (3H, d, J = 7.0 Hz, H-14), 2.38 (3H, s, OAc-6), 8.07 (1H, dd, J = 1.5, 1.0 Hz, OFu), 6.75 (1H, dd, J = 1.0, 2.0 Hz, OFu), 7.45 (1H, dd, J = 1.5, 2.0 Hz, OFu); ^{13}C NMR δ 81.2, (s, C-11), 79.3 (s, C-5), 76.1 (d, C-9), 74.6 (d, C-6), 70.1 (d, C-1), 54.1 (d, C-7), 48.5 (s, C-10), 33.7 (d, C-4), 30.6 (q, C-12), 24.0 (t, C-3), 21.4 (t, C-2), 20.8 (q, C-13), 16.8 (q, C-14), 12.2 (q, C-15).

*1α,6β-Diacetoxy-8α-hydroxy-9β-furoyloxy-β-agarofuran (**3**):* amorphous solid; ^{1}H NMR δ 5.35 (1H, dd, J = 4.0,12.0 Hz, H-1), 1.60 (1H, dddd, J = 3.5, 11.0, 12.0, 13.0 Hz, H-2α), 1.83 (1H, m, H-2β), 1.43 (1H, m, H-3α), 2.14 (1H, m, H-3β), 2.25 (1H, m, H-4), 5.87 (1H, br s, H-6), 2.40 (1H, br d, J = 3.5 Hz, H-7), 4.27 (1H, br d, J = 3.5 Hz, H-8), 4.77 (1H, br s, H-9), 1.34 (3H, s, H-12), 1.41 (3H, s, H-13), 1.45 (3H, s, H-15), 1.01 (3H, d, J = 7.0 Hz, H-14); ^{13}C NMR δ 73.5 (d, C-1), 21.3 (t, C-2), 26.7 (t, C-3), 33.8 (d, C-4), 90.6 (s, C-5), 75.4 (d, C-6), 55.4 (d, C-7), 74.5 (d, C-8), 80.0 (d, C-9), 49.3 (s, C-10), 81.5 (s, C-11), 25.5 (q, C-12), 31.0 (q, C-13), 19.0 (q, C-14), 17.3 (q, C-15).

*1α-Acetoxy-6β,8α-dihydroxy-9β-furoyloxy-β-agarofuran (**4**):* oil; ^{1}H NMR δ 5.35 (1H, dd, J = 4.0, 11.0 Hz, H-1), 1.60 (1H, m, H-2α), 1.83 (1H, m, H-2β), 1.43 (1H, m, H-3α), 2.15 (1H, m, H-3β), 2.25 (1H, m, H-4), 4.90 (1H, br s, H-6), 2.45 (1H, br d, J = 3.0 Hz, H-7), 4.25 (1H, br d, J = 3.0 Hz, H-8), 4.75 (1H, br s, H-9), 1.35 (3H, s, H-12), 1.40 (3H, s, H-13), 1.55 (3H, s, H-15), 1.15 (3H, d, J = 7.0 Hz, H-14), 1.70 (3H, s, OAc-1), 8.10 (1H, dd, J = 1.5, 1.0 Hz, OFu), 6.75 (1H, dd, J = 1.2 Hz, OFu), 7.45 (1H, dd, J = 1.5, 2.0 Hz, OFu); ^{13}C NMR δ 73.2 (d, C-1), 21.5 (t, C-2), 26.8 (t, C-3), 33.5 (d, C-4), 91.8 (s, C-5), 73.2 (d, C-6), 57.1 (d, C-7), 74.7 (d, C-8), 81.1 (d, C-9), 48.9 (s, C-10), 81.6 (s, C-11), 25.7 (q, C-12), 31.6 (q, C-13), 19.3 (q, C-14), 17.8 (q, C-15).

*1α,2α,6β,8α,15-Pentaacetoxy-9β-benzoyloxy-β-agarofuran (**5**):* crystalline solid; mp 250−251 °C; ^{1}H NMR δ 5.71 (1H, d, J = 3.5 Hz, H-1), 5.59 (1H, dd, J = 2.5, 3.5 Hz, H-2), 1.77 (1H, ddd, J = 1.2, 2.5, 15.0 Hz, H-3α), 2.49 (1H, ddd, J = 4.0, 6.5, 15.0 Hz, H-3β), 2.39 (1H, ddq, J = 1.2, 6.5, 7.5 Hz, H-4), 6.38 (1H, d, J = 1.0 Hz, H-6), 2.38 (1H, brd, J = 3.0 Hz, H-7), 5.27 (1H, d, J = 3.0 Hz, H-8), 5.52 (1H, s, H-9), 1.56 (3H, s, H-12), 1.43 (3H, s, H-13), 1.16 (3H, d, J = 7.5 Hz, H-14), 5.10 (1H, d, J = 12.5 Hz, H-15), 4.53 (1H, d, J = 12.5 Hz, H-15).

*1α,2α,3β,15-Tetraacetoxy-6β,9β-dibenzoyl-8-oxo-β-agarofuran (**6**):* amorphous solid; ^{1}H NMR δ 6.23 (1H, d, J = 3.5 Hz, H-1), 5.46 (1H, ddd, J = 3.5, 3.0, 1.5 Hz, H-2), 4.90 (1H, dd, J = 3.0, 1.5 Hz, H-3), 2.72 (1H, ddq, J = 1.5, 1.5, 8.0 Hz, H-4), 6.33 (1H, d, J = 1.0 Hz, H-6), 3.03 (1H, d, J = 1.0 Hz, H-7), 5.68 (1H, s, H-9), 1.46 (3H, s, H-12), 1.46 (3H, s, H-13), 5.11 (1H, d, J = 13.0 Hz, H-15′), 4.53 (1H, d, J = 13.0 Hz, H-15), 1.30 (3H, d, J = 8 Hz, H-14), 2.15 (3H, s, OAc-2), 2.15 (3H, s, OAc-3), 2.15 (3H, s, OAc-6), 1.54 (3H, s, OAc-9), 2.01 (3H, s, OAc-14), 7.96 AA′, 7.46 BB′, 7.59 C; ^{13}C NMR δ 72.4 (d, C-1), 80.2 (d, C-2), 73.3 (d, C-3), 37.2 (d, C-4), 90.7 (s, C-5), 75.4 (d, C-6), 64.0 (d, C-7), 197.7 (s, C-8), 79.2 (d, C-9), 51.6 (s, C-10), 83.6 (s, C-11), 25.0 (q, C-12), 30.5 (q, C-13), 59.8 (t, C-14), 15.3 (q, C-15).

*1α,6β,15-Triacetoxy-9-benzoyloxy-β-agarofuran (**7**):* oil; ^{1}H NMR δ 5.95 (1H, br s, H-6), 5.55 (1H, dd, J = 12.5, 5.0 Hz,H-1), 5.39 (1H, d, J = 7.5 Hz, H-9), 4.68 (1H, d, J = 12.0 Hz, H-15), 4.46 (1H, d, J = 12.0 Hz, H-15), 2.6 (1H, d, J = 1.0 Hz, H-7), 2.23 (3H, s, OAc-6), 2.09 (3H, s, OAc-14), 1.52 (3H, s, OAc-1), 1.44 (3H, s, H-13), 1.39 (3H, s, H-12), 0.96 (3H, d, J = 8.0 Hz, H-15), 8.05; ^{13}C NMR δ 89.5 (s, C-11), 82.4 (s, C-5), 78.1 (d,C-1), 73.4, 69.9, 65.3 (t, C-14), 53.0 (s, C-10), 48.7 (d, C-7), 34.9 (t, C-8), 33.4 (d, C-5), 26.31 (t, C-2), 22.1 (t, C-3), 16.5 (q, C-15).

*2α,3β,6β,8α,15-Pentaacetoxy-1α,9β-benzoyloxy-β-agarofuran (**8**):* oil; ^{1}H NMR δ 5.60 (1H, dd, J = 5.0, 12.5 Hz, H-1), 5.93 (1H, br s, H-6), 2.42 (1H, d, J = 2.0 Hz, H-7), 5.38 (1H, d, J = 7.5 Hz, H-9), 4.43 (1H, d, J = 12.0 Hz, H-15′), 4.67 (1H, d, J = 12.0 Hz, H-15), 1.40 (3H, s, H-12), 1.44 (3H, s, H-13), 0.98 (3H, d, J = 7.0 Hz, H-14), 1.55 (3H, s, OAc-1), 2.09 (3H, s, OAc-14), 2.25 (3H, s, OAc-6), 7.50 (3H, m, OBz), 8.10 (2H, m, OBz).

*1α-Acetoxy-6β,9β-difuroyloxy-4β-hydroxy-β-agarofuran (**9**):* amorphous solid; ^{1}H NMR δ 5.27 (1H, dd, J = 4.0, 12.0 Hz, H-1), 1.20 (1H, m, H-2α), 1.50 (1H, m, H-2β), 1.90 (1H, m, H-3α), 1.70 (1H, m, H-3β), 5.53 (1H, s, H-6), 2.32 (1H, dd, J = 3.0, 3.0 Hz, H-7),

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

2.51 (1H, m, H-8α), 2.21 (1H, d, J = 7.0 Hz, H-8β), 4.98 (1H, d, J = 7.0 Hz, H-9), 1.51 (3H, s, H-12), 1.53 (3H, s, H-13), 1.40 (3H, s, H-14), 1.34 (3H, s, H-15).

Microplate Assay To Inhibit Cholinesterase. To assess AChE/BChE inhibition, an adapted version of the Ellmann et al. assay[20] in 96-well plates was used. The enzyme hydrolyzes the substrate ATCh or BuTCh, resulting in thiocholine, which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which are detected at 405 nm. In the 96-well plates, 50 μL of each sample, dissolved in phosphate buffer (8 mmol/L K_2HPO_4, 2.3 mmol/L NaH_2PO_4, 150 mmol/L NaCl, and 0.05% Tween 20 at pH 7.6), was added, as well as 50 μL of the AChE/BChE solution (0.25 U/mL) from *Electroporus electrics* and equine serum, respectively. The assay solutions without substrate were pre-incubated with the enzyme for 30 min at room temperature. After pre-incubation, the substrate was added. The solution substrate consisted of Na_2HPO_4 (40 mmol/L), AChE/BChE (0.24 mmol/L), and 5,5′-dithiobis(2-nitrobenzoic acid) (0.2 mmol/L, DTNB). Absorbance of the yellow anion product—due to the spontaneous substrate hydrolysis—was measured at 405 nm for 5 min on a microtiter plate reader (Multiskan EX, Thermo, Finland). The AChE/BChE inhibition was determined for each extract. The enzyme activity was calculated as a percentage compared with a control using only the buffer and enzyme solution. The extracts were assayed in a dilution interval of 500−15 μg/mL. Galanthamine was used as a positive control. Each assay was run in triplicate, and each reaction was repeated at least three times independently. The IC_{50} values were calculated via regression analysis.

Kinetic Characterization of AChE Inhibition. Kinetic characterization of AChE was performed using a reported method. Five different concentrations of AChE and inhibitors were mixed in the assay buffer (pH 8.0), containing 40 μM 5,5′-dithiobis(2-nitrobenzoic acid), 0.036 units/mL AChE, and 550 μM acetylthiocholine chloride. Test compounds were added into the assay solution and pre-incubated with the enzyme a 37 °C for 15 min, followed by the addition of substrate. Kinetic characterization of the hydrolysis of acetylcholine catalyzed by AChE was done spectrometrically at 412 nm. A parallel control with no inhibitor in the mixture allowed adjusting activities to be measured at various times.

Molecular Docking Studies on Acetylcholinesterase. Molecular docking is a computational process that searches for a ligand able to fit both geometrically and energetically the binding site of a protein. In this manner, five ligand structures (four molecules of agarofuran and one of galanthamine) were constructed using the Gaussview 5.0 program. Then, docking studies were performed using Glide. Docking uses a series of hierarchical filters to find the best possible ligand binding locations in a protein grid space previously built. The filters include a systematic searching approach, which samples the positional, conformational, and orientational space of the ligand before evaluating the energy interactions of the ligand with the protein. The Glide program is contained in the Maestro 9.0 suite (New York, NY, USA). Therefore, the protein coordinates were extracted from the X-ray crystal structure of AChE from the *E. electricus* (PDB ID 1EEA, 1C20, and 1C2B) and *T. californica* (PDB ID 1EVE) organisms acquired from the Protein Data Bank. The experiments were carried out using the default parameters and the following protocol: Initially, the standard precision (SP) level of accuracy was used for the generation and scoring of 10 poses for each ligand.[21,22]

RESULTS AND DISCUSSION

Plant extract of the Celastraceae have been used for centuries throughout South America and China as insect repellents and insecticides in traditional agriculture and also for the treatment of diseases such as rheumatoid arthritis and cancer. Much of the medicinal interest in these plants has been attributed to a family of highly oxygenated sesquiterpenoids based on a tricyclic dihydroagarofuran skeleton[13]

Chemical Study. Chemical study of the fractions obtained from CC and preparative TLC resulted in isolation of nine compounds. Their NMR spectra showed signals characteristic of dihydro-β-agarofurans. Sesquiterpene **1** was isolated by conventional chromatographic methods from an *n*-hexane fraction obtained from seeds of *M. disticha*. The ^{1}H NMR spectrum of this compound showed the typical signals of a β-substituted furan ring (δ 6.72, H-3′; 7.40, H-4′; 8.01, H-5′). Furthermore, typical signals were present for a eudesmane with an angular methyl group (H-15) at 1.48 (s), a secondary methyl group (H-14) at 1.00 (d), and singlets at 1.38 (H-12) and 1.45 (H-13). Signals at 2.18 (s), 2.08 (s), and 1.71 (s) corresponded to protons of acetate groups. Spin decoupling allowed assignment of all signals. Together with the ^{13}C NMR spectrum, the presence of a eudesmane system with oxygen functions at C-1, C-5, C-6, C-8, C-9, and C-11 was established. Furthermore, inspection of a model indicated that the furan moiety was responsible for the observed shielding of the 1-acetoxy methyl. The HMBC correlation confirms the assigned chemical structure for **1** (Figure 1). The structures of

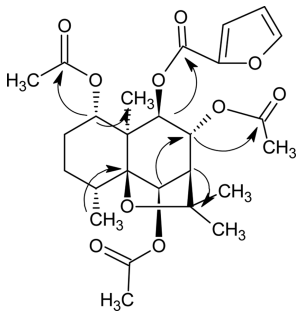

Figure 1. Selected HMBC correlations of agarofuran (**1**).

sesquiterpenes **2**, **3**, and **4** followed immediately from comparison of their spectral data with those of sesquiterpene **1**. The ^{1}H and ^{13}C NMR data for sesquiterpenes **2** and **3** indicated the presence of an agarofuran with two acetates, a furanoate, and a hydroxyl group, respectively. On the other hand, the spectral data of sesquiterpene **4** indicated the presence of an agarofuran with an acetate, a furanoate, and two hydroxyl groups. The protonated sites in the ^{13}C NMR spectrum were assigned with the aid of an HMQC experiment, whereas quaternary carbons and the relative position of the ester groups followed from the results of an HMBC experiment. Compound **5** showed signals for five protons at 8.00, 7.44, and 7.58 ppm and five singlets of three protons each that appear at 1.46, 2.07, 2.10, 2.19, and 2.26 ppm. The AB system at 4.53 and 5.10 (J = 12.5 Hz) corresponds to the protons of a methylene attached to an ester group. The ^{13}C NMR spectrum of sesquiterpene **6** displayed, in addition to 15 signals for the sesquiterpene part (including a keto group), signals for 4 acetates and 2 benzoates. In the ^{1}H NMR spectrum, all signals for the A-ring were assigned by spin decoupling. Thus, the keto group had to be placed in the B ring. The placement at C-8 was already indicated by a downfield shift of H-7 (if compared with the spectral data with above-described agarofurans), confirmed by the results of an HMBC experiment. The signals at 5.68 ppm must be assigned to H-9 as it shows three bond correlations with C-1, C-14, and the acetate carbonyl at C-9 and two bond correlations with C-8 and C-10. From the other side, H-7 correlates with C-5 and C-9 (^3J) and C-6 and C-8 (^2J). From these results, the relative

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

position of the ester groups was deduced as well as the assignment of all quaternary carbons. In particular, the correlation between H-1 and H-2′/H-6′ of the benzoate, both with the benzoate carbonyl, placed the only remaining ester group at the correct position.

Sesquiterpenes **7** and **8** were isolated from seeds of *E. japonicus*. Sesquiterpene 7 was obtained previously by acetylation of the natural sesquiterpene isolated from aerial parts of *M. disticha*.[23,24] The ^{1}H NMR spectrum confirmed the presence of a benzoate and acetate groups as well as five aromatic protons that were observed between δ 7.30 and 8.10, and methyl groups for three acetates appeared at δ 2.23, 2.09, and 1.52 as singlets together with the geminal protons for these groups as a doublet centered at δ 5.38 (J = 7.5 Hz) and a broad singlet at δ 5.93 and a double doublet at δ 5.60 (J_1 = 12.5, J_2 = 5.0 Hz), respectively. Two protons of a hydroxymethylene group were seen as doublets of an AB system with signals centered at δ 4.67 and 4.43 (J = 12 Hz). The structure of **9** followed from the ^{1}H NMR spectra, and was close to that of compound **1**. In the spectrum of **9** a second furan ester group was visible, whereas the methyl doublet (H-14) was replaced by a downfield-shifted methyl singlet at δ 1.40. The downfield shift at δ 5.53 of H-6 indicated the relative position of the furan ester. Comparison of the spectrum of **1** with that of **9** clearly showed that the 8-acetoxy group was missing. Compounds **1**−**4** and **9** are new in the phytochemical composition of seeds of *M. disticha*.

Inhibition of Acetylcholinesterase Activity. As part of our ongoing research program dealing with the use of natural compounds for pest control in ecological agriculture,[17,24,25] we began evaluating the ability of sesquiterpenes to inhibit AChE. The history of drug discovery showed that plants are rich sources in the search for new active compounds, and they have become a challenge to modern pharmaceutical industry. Many synthetic drugs owe their origin to plant-based complementary medicine. Because AD, one of the most common causes of death worldwide, has become a threat to public health, new treatment strategies based on medicinal plants have come into focus.[4,26,27]

A large number of plant extracts and compounds have been found to inhibit AChE. Studies of 183 compounds obtained from plants and fungi were alkaloids, such as physostigmine, neostigmine, and coronaridine, and showed strong inhibition of AChE.[3] In addition, other studies found 260 chemically defined natural molecules reported in the literature that have been evaluated for acetylcholinesterase inhibition. The compounds isolated and identified belong to numerous classes of compounds including alkaloids, monoterpenes, coumarins, triterpenes, flavonoids, benzenoids, diterpenes, oxygen heterocycles, sesquiterpenes, stilbenes, lignans, sulfur compounds, proteids, polycyclic, quinonoids, benzoxazinones, carotenoids, and alicyclics.[28] This information demonstrates that much more information is needed, especially for molecules as little studied as sesquiterpenes.

Inhibitory activities for the rate of hydrolysis of acetylthiocholine (ATCh) and butyrylthiocholine (BuTCh) in comparison to the reference compound galanthamine were determined for agarofuran compounds **1**−**9** (Figure 2) isolated from *M. disticha* and *E. japonicus* using the method of Ellman et al.[20] The results are listed in Table 1 as IC_{50} values. All data are presented as the mean ± SE of three independent experiments. On the basis of the IC_{50} values for AChE inhibition, most of the compounds exhibited satisfactory inhibitory activity in the range of submicromolar concentrations (IC_{50} values 0.097−0.363 mg/mL) compared with galanthamine as the reference drug. As can be seen in Table 1, compounds **1**, **4**, and **5** were the most potent anti-AChE agents.

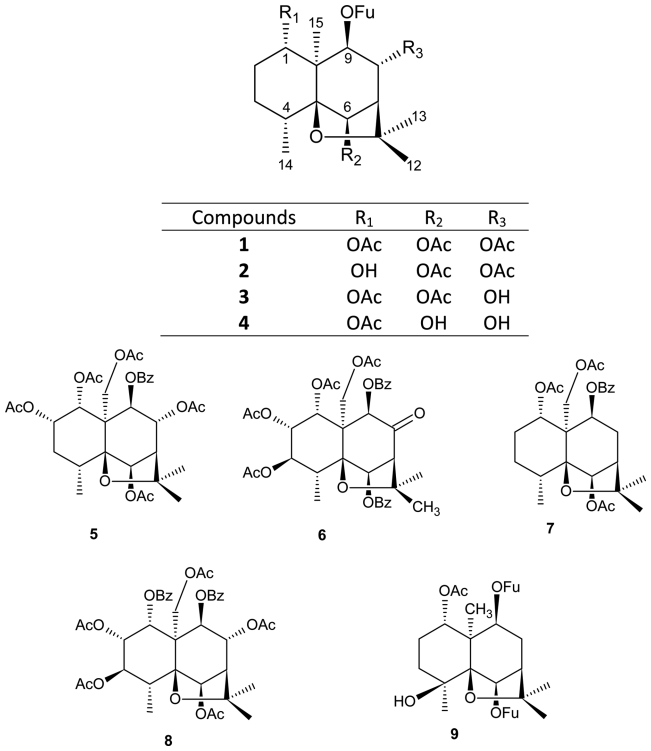

Figure 2. Chemical structures of dihydro-β-agarofuran compounds.

Table 1. AChE Inhibitory Activity of the Compounds Isolated from Seeds of *M. disticha* and *E. japonicus*[a]

	AChE		BuChE
compound	IC_{50} ± SE (mg/mL)	IC_{50} ± SE (mM)	IC_{50} ± SE (mg/mL)
1	0.098 ± 0.012	0.248 ± 0.030	>0.500
2	0.341 ± 0.016	0.738 ± 0.045	
3	0.102 ± 0.004	0.161 ± 0.006	
4	0.097 ± 0.006	0.312 ± 0.019	
5	0.070 ± 0.002	0.122 ± 0.003	
6	0.299 ± 0.015	(0.463 ± 2.0) × 10^{-5}	
7	0.359 ± 0.006	(0.695 ± 1.16) × 10^{-5}	
8	0.363 ± 0.009	(0.482 ± 1.19) × 10^{-5}	
9	0.381 ± 0.007	0.738 ± 0.0007	
galanthamine	0.001	0.100	

[a]IC_{50} is the concentration producing 50% of AChE inhibition calculated as the mean ± standard error.

Nine compounds were used, and they cluster as celapanol (**1**−**5**), 3-hydroxy-8-oxo-alatol (**6**), 14-hydroxycelorbicol (**7**), and 3-hydroxyalatol (**8**). Additionally, the results show that all compounds were less potent in inhibitory activity against BuChE in comparison with AChE, indicating that these compounds can serve as selective inhibition agents for AChE over BuChE. On the basis of these data and previously published results, our findings confirm that the polyhydroxyagarofuran nucleus is responsible for the biological activity.[19]

We further examined the kinetics of inhibition of AChE compounds **2**, **5**, and **7**−**9**, to understand the nature of AChE inhibition. Double-reciprocal plots of the inhibition kinetics of

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX

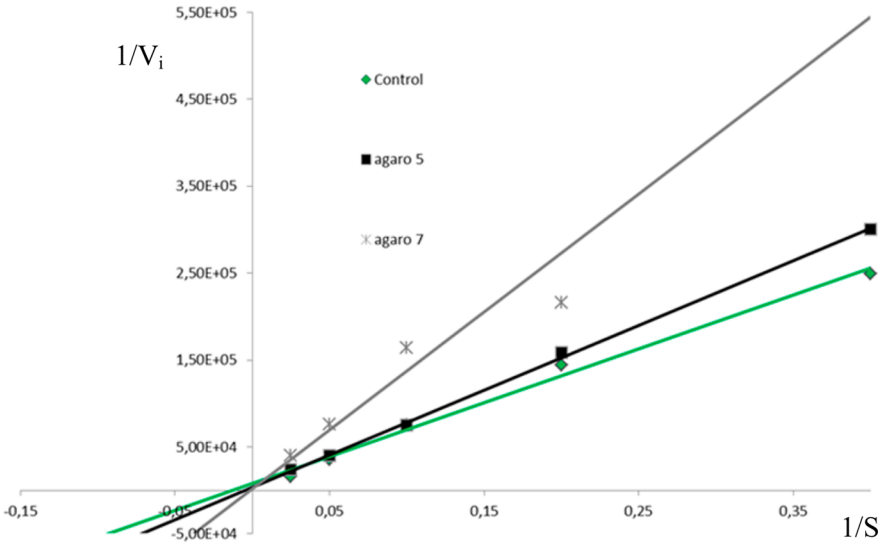

Figure 3. Lineweaver–Burk plot for inhibition of AChE by agarofurans **5** and **7**.

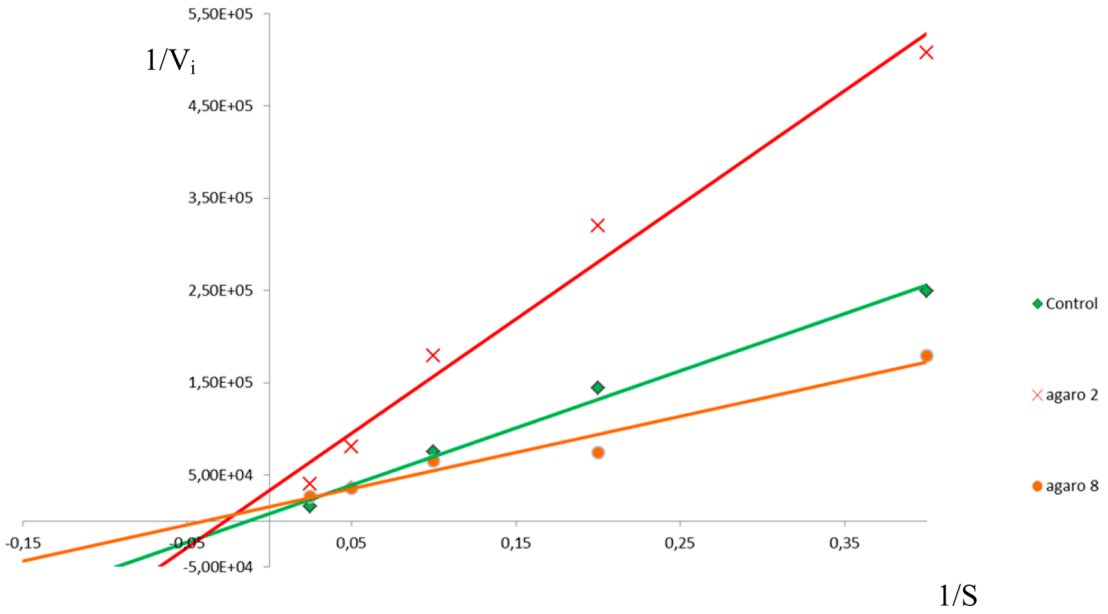

Figure 4. Lineweaver–Burk plot for inhibition of AChE by agarofurans **2** and **8**.

AChE (Figures 3– 5) demonstrated that the mechanism of AChE inhibition of these compounds corresponds to mixed-type or acompetitive inhibition, whereas that of galanthamine–HBr exhibited competitive inhibition.

Docking Studies on Acetylcholinesterase. The structural change in AChE toward a more favorable conformation for catalysis would depend on molecular size and shape, functional group, lipid solubility, or a combination of all. To clarify these parameters we did docking studies of our molecules.

Docking experiments with compounds **1**, **5**, **6**, **8** and galanthamine in silico were conducted. These experiments did not result in the determination of the active site of AChE. Several attempts were made involving molecular coupling between agarofuran molecules with different crystals indicated in the methodology with no positive result. When these same experiments were conducted with galanthamine, one characteristic pose as described by other researchers was obtained.[28]

Concluding Remarks. In the present study, cholinesterase enzyme inhibitory activities of bioactive chemical constituents of *M. disticha* and *E. japonicus* were investigated. The majority of previous studies have been focused on anticholinesterase alkaloids, such as physostigmine and galanthamine.[29] Until now, more than 35 alkaloids have been reported to have AChE inhibitory activity.[29] Other classes of compounds reported with anticholinesterase activity are terpenoids, glycosides, and coumarins.[30] Our results show that compounds with agarofuran (epoxyeudesmane) skeletons provide an interesting group of molecules capable of inhibiting the enzyme acetylcholinesterase.[31,32] This activity is dependent on the type and degree of ring substitution, as was demonstrated by docking and kinetic studies.

Nevertheless, our compounds with an agarofuran (epoxyeudesmane) skeleton have shown good activity. The kinetics of compounds assayed display mixed types of inhibition similar to that of tacrine.[33] Compound **9** showed noncompetitive

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J. Agric. Food Chem. XXXX, XXX, XXX–XXX

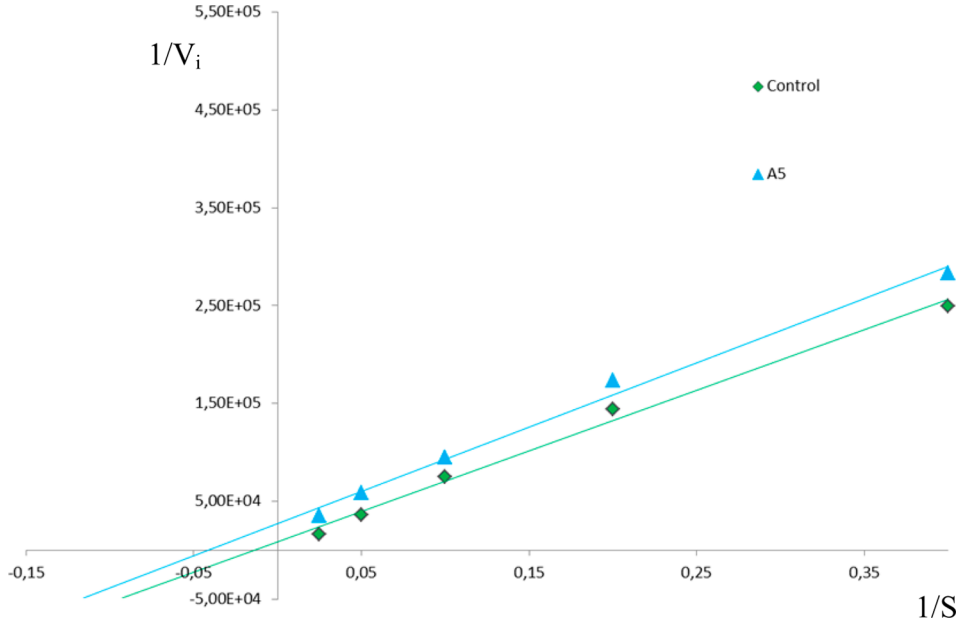

Figure 5. Lineweaver–Burk plot for inhibition of AChE by agarofuran **9**.

binding similar to that of propidium[33] In summary, our study suggests that agarofuran compounds might serve as lead compounds to design new potential AChE inhibitors with high selectivity, low toxicity, and additional positive pharmaceutical effects. Inestrosa et al.[34] reported that AChE accelerates amyloid formation from amyloid-β-peptide, which alone produces few amyloid-like fibrils, and that the formation of amyloid induced by the association of AChE with amyloid-β-peptide can be inhibited by peripheral anionic site ligands such as propidium and decamethonium. Presently, we are developing the synthesis and evaluation of agarofuran-type compounds as acetylcholinesterase inhibitors under a QSAR program.

Finally, this paper reports a new phytochemical composition in seeds of *M. disticha*.

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Notes

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