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Isolation of Cholinesterase-Inhibiting Flavonoids from *Morus lhou*

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Supporting Information

ABSTRACT: Cholinesterases are key enzymes that play important roles in cholinergic transmission. Nine flavonoids displaying cholinesterase inhibitory activity were isolated from the root bark of *Morus lhou* L., a cultivated edible plant. The isolated compounds were identified as a new flavone (**1**), 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone (**2**), kuwanon U (**3**), kuwanon E (**4**), morusin (**5**), morusinol (**6**), cyclomorusin (**7**), neocyclomorusin (**8**), and kuwanon C (**9**). All compounds apart from compound **6** inhibited cholinesterase enzyme in a dose-dependent manner with K_i values ranging between 3.1 and 37.5 μM and between 1.7 and 19.1 μM against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes, respectively. The new compound was characterized as 5′-geranyl-4′-methoxy-5,7,2′-trihydroxyflavone (**1**). It showed the most potent inhibitory activity (K_i = 3.1 μM for AChE, K_i = 1.74 μM for BChE). Lineweaver−Burk and Dixon plots and their secondary replots indicated that flavones (**5**−**9**) with prenyl substitution on C-3 were noncompetitive inhibitors, whereas those unsubstituted (**1**−**4**) at C-3 were mixed inhibitors of both AChE and BChE. In conclusion, this is the first study to demonstrate that alkylated flavonoids of *M. lhou* have potent inhibitory activities against AChE and BChE.

KEYWORDS: Alzheimer's disease, acetylcholinesterase, butyrylcholinesterase, cholinesterase inhibitors, *Morus lhou*

INTRODUCTION

Alzheimer's disease (AD) accounts for 70−80% of all dementias and is one of the principal causes of disease and death in the elderly.[1] Consummate with its severity, research into treatments both curative and preventative has been avid. This has unveiled numerous important facets of this disease, including the hallmark β-amyloid plaques.[2] Many strategies aimed at preventing/curing this disease have targeted these plaques. Numerous enzymes have been investigated, including β- and γ-secretase enzymes.[3] In recent years there has been a burgeoning interest in cholinesterases in view of the role they play in the formation of β-amyloids during the early stages of Alzheimer's.[1,3] Acetylcholinesterase (AChE) serves as the key enzyme in cholinergic transmission, as it hydrolyzes the ester bond of the neurotransmitter acetylcholine in cholinergic synapses.[4] Its particular relevance to Alzheimer's disease can be traced to the fact that it is also known to accelerate the aggregation of β-amyloids during the early stages of AD.[5] In the case of butyrylcholinesterase (BChE), the physiological function is not clear, but it has a multitude of hydrolyzing activities. This is not confined to esterase activity but also includes acylamidase and peptidase (or protease) activities.[6] In particular, the peptidase activity of BChE is believed to be involved in the development and progress of AD as it is a causative factor in the production of β-amyloids.[7] Therefore, inhibitors of both AChE and BChE may be attractive targets for combating neurodegenerative disorders.

As part of our ongoing investigations aimed at the discovery of new cholinesterase inhibitors from natural sources, we have focused on the AChE and BChE inhibitory activity of extracts *Morus lhou*. This plant belongs to the genus *Morus* of the family Moracea and is widely cultivated throughout China.[8] Its roots, twigs, leaves, and fruits have all been used in traditional Chinese medicine.[9] This species continues to be a representative polyphenol-rich plant with a broad spectrum of biological activities including antioxidant,[10] hyperglycemic,[11] antinephritis,[12] and anti-inflammatory properties.[13] In recent years, several groups, including ourselves, have reported that polyphenols have strong tyrosinase inhibitory activity.[14−16]

In our present study, we have found that bioactivity-guided fractionation of methanol extracts of *M. lhou* elicited new flavone **1** together with eight known flavonoids (**2**−**9**). All isolated compounds were additionally evaluated for their inhibitory activities and kinetic modes toward both AChE and BChE.

MATERIALS AND METHODS

Plant Material. The roots of *M. lhou* were collected at Chojeon in Jinju, Korea, in September 2009. This plant was identified by Gyeongsangnam-do Agricultural Research and Extension Services in Korea.

General Apparatus and Chemicals. Thin-layer chromatography (TLC) (E. Merck Co., Darmstadt, Germany) was carried out using commercially available glass plates precoated with silica gel and visualized under UV at 254 and 366 nm or stained with 10% H_2SO_4. Column chromatography was carried out using silica gel (230−400 mesh, Kiesel

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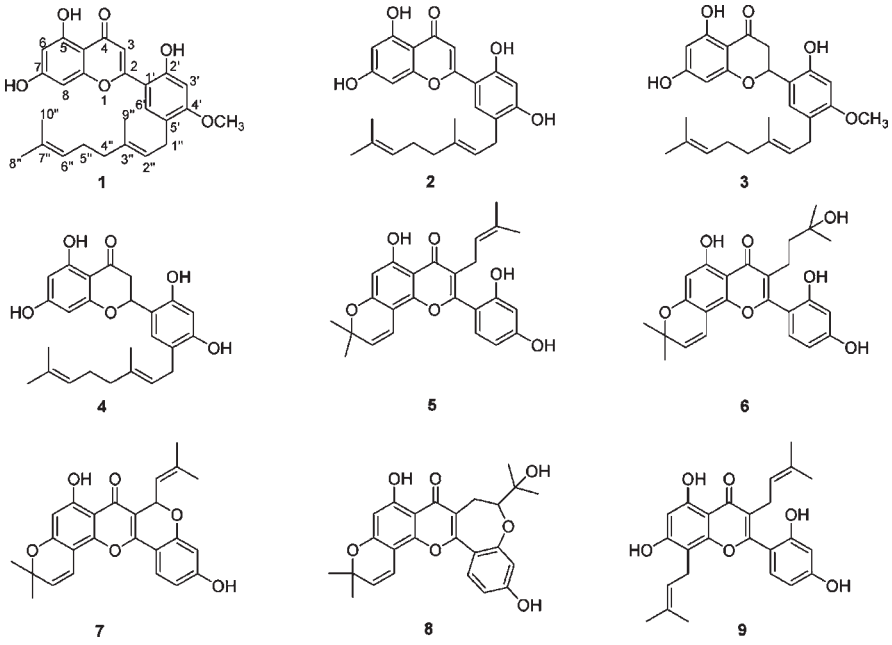


Figure 1. Structures of compounds **1**−**9** isolated from *Morus lhou*.

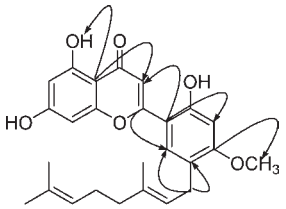
gel 60, Merck), RP-18 (ODS-A, 12 nm, S-150 mM, YMC), and Sephadex LH-20 (Amersham Biosciences). Melting points were measured on a Thomas Scientific capillary apparatus. IR spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (KBr). UV spectra were measured on a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA). ^{1}H and ^{13}C NMR as well as 2D NMR data were obtained on a Bruker AM 500 (^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz) spectrophotometer (Bruker, Karlsruhe, Germany) in CD_3OD, DMSO-d_6, $CDCl_3$, or acetone-d_6 with TMS as an internal standard. Qualitative analyses were made using a Perkin-Elmer HPLC S200 (Perkin-Elmer, Bridgeport, CT). Electron impact mass spectrometry (EIMS) was obtained on a JEOL JMS-700 mass spectrophotometer (JEOL, Tokyo, Japan). Reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Extraction and Isolation. The root bark (3 kg) of *M. lhou* was air-dried, chopped, and extracted with methanol (10 L × 3) for 7 days at room temperature. The combined methanol extract was concentrated in vacuo to yield a dark brown gum (120 g). The methanol extract was loaded onto Celite and fractionated by vacuum liquid chromatography (VLC), successively with hexane, ethyl acetate, and methanol (each 5 L), yielding a hexane extract (6 g), an ethyl acetate extract (43 g), and a methanol extract (67 g). The ethyl acetate extract was fractionated by column chromatography on silica gel (6 × 60 cm, 230−400 mesh, 300 g) using a chloroform to methanol gradient (150:1 → 1:1) to give six fractions (A−F). Fraction C (5.8 g) was purified by column chromatography using a glass column packed with silica gel (3 × 60 cm, 230−400 mesh, 130 g) and eluted using hexane to acetone (100:1 → 1:1) to afford six subfractions. Subfractions C4−C5 were purified by repeated silica gel column chromatography and eluted using hexane to ethyl acetate (120:1 → 1:1) to yield compound **1** (80 mg), hexane to acetone (100:1 → 1:1) to yield compound **5** (60 mg), and hexane to acetone (80:1 → 1:1) to yield compound **3** (10 mg) (see Figure 1 for structures). Fraction D was fractionated by silica gel column chromatography by employing a gradient of chloroform to methanol (200:1 → 1:1) resulting in seven subfractions (D1−D7), and then sub fractions D2−D4 were rechromatographed on Sephadex LH-20 using the same solvent gradient [elution methanol (1.5 × 60 cm)] to afford pure compounds **2** (45 mg), **6** (18 mg), and **9** (38 mg). Subfractions D5−D7 were subjected to silica gel column chromatography and eluted using chloroform to methanol (200:1 → 1:1) and then further purified by Sephadex LH-20 [elution (methanol (1.5 × 60 cm)] to yield pure compounds **4** (16 mg) and **7** (32 mg). Fraction E was subjected to silica gel column chromatography employing a gradient of chloroform to methanol (180:1 → 1:1), resulting in six subfractions (E1−E6). Subfraction E1 was rechromatographed and then further purified on sSephadex LH-20 [elution methanol (1.5 × 60 cm)] to afford pure compound **8** (15 mg).

*5′-Geranyl-4′-methoxy-5,7,2′-trihydroxyflavone (**1**):* amorphous yellow powder; mp 135−136 °C; EIMS *m*/*z* 436 $[M]^+$; HREIMS *m*/*z* 436.1890 (calcd for $C_{26}H_{28}O_6$, 436.1886); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.44 (3H, s, H-8″), 1.47 (3H, s, H-10″), 1.62 (3H, s, H-9″), 1.96 (2H, m, H-4″), 2.01 (2H, br t, H-5″), 3.17 (2H, m, H-1″), 3.74 (3H, s, OCH_3), 4.99 (1H, s, H-6″), 5.21 (1H, m, H-2″), 6.11 (1H, d, *J* = 1.9 Hz, H-6), 6.33 (1H, d, *J* = 2.0 Hz, H-8), 6.54 (1H, s, H-3′), 6.94 (1H, s, H-3), 7.59 (1H, s, H-6′), 13.01 (1H, s, 5-OH); ^{13}C NMR (125 MHz) δ 16.7 (C-9″), 18.2 (C-8″), 26.2 (C-10″), 27.9 (C-5), 28.6 (C-1″), 40.6 (C-4″), 56.4 (C4′-OCH_3), 94.9 (C-8), 99.8 (C-6), 100.8 (C-3′), 105.7 (C-4a), 109.3 (C-3), 110.9 (C-1′), 123.3 (C-5′), 123.8 (C-2″), 125.4 (C-6″), 100.8 (C-3′), 105.7 (C-4a), 109.3 (C-3), 110.9 (C-1′), 123.3 (C-5′), 123.8 (C-2″), 125.4 (C-6″), 129.9 (C-6′), 132.3 (C-7″), 137.2 (C-3″), 157.9 (C-2′), 159.2 (C-8a), 162.2 (C-4′), 163.2 (C-2), 163.7 (C-5), 165.1 (C-7), 183.8 (C-4).

*5′-Geranyl-5,7,2′,4′-tetrahydroxyflavone (**2**):* amorphous yellow powder; mp 153−155 °C; EIMS *m*/*z* 422 $[M]^+$; HREIMS *m*/*z* 422.1728 (calcd for $C_{25}H_{26}O_6$, 422.1729); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.45 (3H, s, H-8″), 1.47 (3H, s, H-10″), 1.63 (3H, s, H-9″), 1.97 (2H, m, H-4″), 2.01 (2H, br t, H-5″), 3.18 (2H, d, *J* = 7.6 Hz, H-1″), 5.00 (1H, s, H-6″), 5.26 (1H, m, H-2″), 6.11 (1H, d, *J* = 1.8 Hz, H-6), 6.33 (1H, d, *J* = 2.0 Hz, H-8), 6.51 (1H, m, H-3′), 6.93 (1H, s, H-3), 7.58 (1H, s, H-6′), 13.03 (1H, s, 5-OH); ^{13}C NMR (125 MHz) δ 16.6 (C-9″), 18.1 (C-8″), 26.2 (C-10″), 27.9 (C-5″), 28.5 (C-1″), 40.9 (C-4″), 94.9 (C-8), 99.8 (C-6), 104.5 (C-3′), 105.7 (C-4a), 108.9 (C-3), 110.8 (C-1′), 121.9 (C-5′), 124.0 (C-2″), 125.4 (C-6″), 130.6 (C-6′), 132.2 (C-7″), 137.0 (C-3″), 158.1 (C-2′), 159.4 (C-8a), 160.2 (C-4′), 163.2 (C-2), 163.8 (C-5), 163.9 (C-7), 183.8 (C-4).

*Kuwanon U (**3**):* amorphous yellow powder; mp 136−137 °C; $[\alpha]_D$ −2.4° (CH_3OH, *c* 0.17); EIMS *m*/*z* 438 $[M]^+$; HREIMS *m*/*z* 438.2039



1

Figure 2. Important HMBC correlations of compound **1**.

(calcd for $C_{26}H_{30}O_6$, 438.2042); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.51 (3H, s, H-8″), 1.58 (3H, s, H-10″), 1.60 (3H, s, H-9″), 1.97 (3H, m, H-5″), 2.02 (2H, m, H-4″), 2.77−3.19 (2H, dd, J = 17.3, 3.0 Hz, H-3a, 3b), 3.19 (2H, m, H-1″), 3.73 (3H, s, OCH_3), 5.18 (1H, m, H-2″), 5.51 (1H, dd, J = 12.8, 2.9 Hz, H-2), 5.93 (1H, s, H-8), 5.94 (1H, s, H-6), 6.37 (1H, m, H-3′), 6.86 (1H, s, H-6′), 11.98 (1H, s, 5-OH); ^{13}C NMR (125 MHz) δ 16.4 (C-9″), 18.1 (C-8″), 26.1 (C-10″), 27.2 (C-4″), 28.0 (C-1″), 40.2 (C-5″), 42.2 (C-3), 55.9 (C4′-OCH_3), 78.3 (C-2), 96.0 (C-8), 97.6 (C-6), 100.6 (C-3′), 103.7 (C-4a), 115.1 (C-1′), 122.6 (C-5′), 123.1 (C-2″), 124.7 (C-6′), 131.8 (C-7″), 136.8 (C-3″), 153.7 (C-2′), 159.1 (C-4′), 162.9 (C-5), 164.8 (C-8a), 164.9 (C-7), 196.6 (C-4).

Kuwanon E (***4***)*:* amorphous yellow powder; mp 121−123 °C; $[\alpha]_D$ −0.25° (CH_3OH, *c* 0.29); EIMS *m*/*z* 424 $[M]^+$; HREIMS *m*/*z* 424.1889 (calcd for $C_{25}H_{28}O_6$, 424.1886); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.43 (3H, s, H-8″), 1.48 (3H, s, H-10″), 1.57 (3H, s, H-9″), 1.91 (3H, m, H-5″), 1.96 (2H, m, H-4″), 2.56 (2H, dd, J = 17.1, 3.0 Hz, H-3b), 3.06 (2H, dd, J = 17.1, 13.1 Hz, H-3a), 3.14 (2H, d, J = 7.2 Hz, H-1″), 5.22 (1H, m, H-2″), 5.57 (1H, dd, J = 13.0, 2.9 Hz, H-2), 5.81 (1H, s, H-8), 5.82 (1H, s, H-6), 6.37 (1H, m, H-3′), 7.07 (1H, s, H-6′); ^{13}C NMR (125 MHz) δ 16.7 (C-9″), 18.2 (C-8″), 26.3 (C-10″), 27.9 (C-4″), 28.6 (C-1″), 40.9 (C-5″), 43.1 (C-3a, 3b), 75.8 (C-2), 96.3 (C-8), 97.1 (C-6), 103.7 (C-3′), 103.9 (C-4a), 117.4 (C-1′), 120.8 (C-5′), 124.5 (C-2″), 125.6 (C-6″), 129.5 (C-6′), 132.1 (C-7″), 136.4 (C-3″), 154.6 (C-2′), 157.1 (C-4′), 165.3 (C-5), 165.7 (C-8a), 167.6 (C-7), 198.2 (C-4).

Morusin (***5***)*:* amorphous yellow powder; mp 182−184 °C; EIMS *m*/*z* 418 $[M]^+$; HREIMS *m*/*z* 418.1578 (calcd for $C_{25}H_{22}O_6$, 418.1411); ^{1}H NMR (500 MHz, CD_3OD) δ 1.32 (3H, s, H-5″), 1.33 (6H, m, H-4‴, 5‴), 1.48 (2H, m, H-4″), 3.01 (2H, d, J = 6.9 Hz, H-1″), 4.99 (1H, m, H-2″), 5.48 (1H, d, J = 10.0 Hz, H-2‴), 6.05 (1H, s, H-6), 6.29 (1H, dd, J = 8.3, 2.2 Hz, H-5′), 6.31 (1H, br s, H-3′), 6.50 (1H, d, J = 10.0 Hz, H-1‴), 7.01 (1H, d, J = 8.2 Hz, H-6′); ^{13}C NMR (125 MHz) δ 18.1 (C-5″), 25.3 (C-1″), 26.2 (C-4″), 28.8 (C-4″, 5″), 79.6 (C-3‴), 100.5 (C-6), 102.6 (C-8), 104.3 (C-5′), 106.3 (C-4a), 108.5 (C-3′), 113.5 (C-1′), 116.2 (C-1‴), 122.5 (C-3), 123.1 (C-2″), 128.6 (C-2‴), 132.8 (C-6′), 133.3 (C-3″), 154.2 (C-8a), 158.4 (C-4′), 161.0 (C-7), 162.5 (C-2′), 163.1 (C-5), 163.9 (C-2), 184.3 (C-4).

Morusinol (***6***)*:* amorphous yellow powder; mp 213-214 °C; EIMS *m*/*z* 438 $[M]^+$; HREIMS *m*/*z* 438.1678 (calcd for $C_{25}H_{26}O_7$, 438.1679); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.01 (6H, s, H-4″, 5″), 1.46 (6H, m, H-4‴, 5‴), 1.66 (2H, m, H-2″), 2.51 (2H, m, H-1″), 5.66 (1H, d, J = 10.0 Hz, H-2‴), 6.16 (1H, s, H-6), 6.54 (1H, d, J = 8.0 Hz, H-5′), 6.58 (1H, s, H-5′), 6.61 (1H, d, J = 8.3 Hz, H-1‴), 7.30 (1H, d, J = 8.2 Hz, H-2′); ^{13}C NMR (125 MHz) δ 21.5 (C-1″), 28.7 (C-4‴, 5‴), 31.0 (C-4″, 5″), 43.4 (C-2″), 70.6 (C-3″), 79.2 (C-3‴), 100.1 (C-6), 101.9 (C-8), 104.5 (C-3′), 106.0 (C-4a), 108.6 (C-6′), 160.3 (C-5′), 161.9 (C-8a), 162.7 (C-1′), 163.2 (C-2), 183.9 (C-4).

Cyclomorusin (***7***)*:* amorphous yellow powder; mp 246−248 °C; EIMS *m*/*z* 418 $[M]^+$; HREIMS *m*/*z* 418.1411 (calcd for $C_{25}H_{22}O_6$, 438.1416); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.45 (3H, s, H-5‴), 1.47 (3H, s, H-4‴), 1.68 (3H, d, J = 1.1 Hz, H-5″), 1.92 (3H, d, J = 1.2 Hz, H-4″), 5.47 (1H, m, H-2″), 5.72 (1H, d, J = 10.0 Hz, H-2‴), 6.12 (1H, s, H-6), 6.15 (1H, d, J = 9.5 Hz, H-1″), 6.41 (1H, s, H-3′), 6.61 (1H, d, J = 7.5 Hz, H-5′), 6.85 (1H, d, J = 9.9 Hz, H-1‴), 7.73 (1H, d, J = 8.5 Hz, H-6′), 12.93 (1H, s, 5-OH); ^{13}C NMR (125 MHz) δ 17.8 (C-4″), 24.9 (C-5″), 27.4 (C-5‴), 27.5 (C-4‴), 69.4 (C-1″), 77.9 (C-3‴), 99.5 (C-6), 101.3 (C-4a), 103.9 (C-3′), 105.1 (C-8), 107.5 (C-3), 109.0 (C-1′), 109.9 (C-5′), 114.5 (C-1‴), 121.2 (C-2″), 125.4 (C-6′), 127.7 (C-2‴), 137.9 (C-3‴), 151.0 (C-5), 155.5 (C-2′), 158.2 (C-2), 158.9 (C-8a), 163.2 (C-4′), 161.8 (C-7), 178.3 (C-4).

Table 1. ^{1}H and ^{13}C NMR Data of New Compound 1 in Acetone-d_6[a]

position	δ_C	δ_H (mult,[b] J)	HMBC
2	163.3 (s)		H-3, H-6′
3	109.3 (d)	6.94 (1H, s, H-3)	
4	183.8 (s)		
4a	105.7 (s)		H-3, H-6, H-8
5	163.8 (s)		
6	99.8 (d)	6.11 (1H, d, J = 1.9 Hz, H-6)	H-8
7	165.1 (s)		
8	94.9 (d)	6.33 (1H, d, J = 2.0 Hz, H-8)	
8a	159.2 (s)		
1′	110.9 (s)		H-3, H-3′
2′	157.9 (s)		
3′	100.8 (d)	6.54 (1H, s, H-3′)	
4′	162.2 (s)		H4′-OCH_3, H-6′, H-1″
5′	123.3 (s)		
6′	129.9 (d)	7.59 (1H, s, H-6′)	
1″	28.6 (t)	3.17 (2H, m, H-1″)	H-4′, H-5′, H-6′
2″	123.8 (d)	5.21 (1H, m, H-2″)	
3″	137.2 (s)		H-1″
4″	40.6 (t)	2.01 (2H, br t, H-4″)	
5″	27.9 (t)	1.96 (2H, m, H-5″)	
6″	125.4 (d)	4.99 (1H, m, H-6″)	H-4″, H-8″
7″	132.3 (s)		
8″	18.2 (q)	1.44 (3H, s, H-8″)	H-6″, H-10″
9″	16.7 (q)	1.62 (3H, s, H-9″)	
10″	26.2 (q)	1.47 (3H, s, H-10″)	
4′-OCH_3	56.4 (s)	3.74 (3H, s, OCH_3)	H-4′

[a] Assignments were made by ^{1}H−^{1}H COSY, HMQC, and HMBC data. [b] Multiplicity was established from DEPT data.

Neocyclomorusin (***8***)*:* amorphous yellow powder; mp 261−263 °C; $[\alpha]_D$ −0.55° (CH_3OH, *c* 0.103); EIMS *m*/*z* 436 $[M]^+$; HREIMS *m*/*z* 436.1520 (calcd for $C_{25}H_{24}O_7$, 436.1522); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29 (3H, s, H-5″), 1.30 (3H, s, H-4″), 1.40 (3H, s, H-4‴, 5‴), 2.61 (1H, dd, J = 16.6, 9.3 Hz, H-1″b), 3.22 (1H, dd, J = 16.6, 2.5 Hz, H-1″a), 5.52 (1H, d, J = 10.0 Hz, H-2‴), 6.19 (1H, s, H-6), 6.52 (1H, d, J = 2.1 Hz, H-3′), 6.65 (1H, dd, J = 8.8, 2.1 Hz, H-3′), 6.68 (1H, d, J = 9.9 Hz, H-1‴), 7.78 (1H, d, J = 8.8 Hz, H-6′); ^{13}C NMR (125 MHz) δ 25.1 (C-4′), 25.4 (C-1″a, 1″b), 25.9 (C-4″, 5″), 28.5 (C-4‴, 5‴), 73.1 (C-3″), 78.4 (C-3‴), 90.9 (C-2″), 100.2 (C-6), 101.4 (C-8), 104.4, (C-4a), 108.3 (C-5′), 112.0 (C-3′), 114.7 (C-3), 115.2 (C-1‴), 116.6 (C-1′), 127.7 (C-2‴), 130.2 (C-6′), 137.9 (C-3″), 152.1 (C-8a), 158.7 (C-2), 159.8 (C-7), 160.2 (C-2′), 161.5 (C-5), 181.5 (C-4).

Kuwanon C (***9***)*:* amorphous yellow powder; mp 148−150 °C; EIMS *m*/*z* 422 $[M]^+$; HREIMS *m*/*z* 436.1726 (calcd for $C_{25}H_{24}O_7$, 432.1729); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.44 (3H, s, H-5‴), 1.58 (3H, s, H-4‴), 1.59 (6H, s, H-4″, 5″), 3.14 (2H, d, J = 6.6 Hz, H-1″), 3.38 (2H, d, J = 6.9 Hz, H-1‴), 5.15 (1H, m, H-2″), 5.21 (1H, m, H-2‴), 6.33 (1H, s, H-6), 6.54 (1H, dd, J = 8.2, 2.0 Hz, H-5′), 6.58 (1H, d, J = 1.9 Hz, H-3′), 7.23 (1H, d, J = 8.3 Hz, H-6′) 13.10 (1H, s, 5-OH);

Table 2. Inhibitory Activity of Compounds 1−9) on Cholinesterase

	acetylcholinesterase		butyrylcholinesterase	
compound	IC_{50}[a] (μM)	type of inhibition K_i[b] (μM)	IC_{50} (μM)	type of inhibition K_i (μM)
1	10.95	mixed (3.14)	3.43	mixed (1.74)
2	16.21	mixed (10.3)	7.93	mixed (4.49)
3	19.69	mixed (6.48)	10.11	mixed (9.59)
4	23.79	mixed (10.39)	16.21	mixed (10.3)
5	36.4	noncompetitive (22.86)	24.08	noncompetitive (19.08)
6	173.49	nt[c]	76.32	nt
7	31.69	noncompetitive (37.5)	21.48	noncompetitive (17.51)
8	26.69	noncompetitive (33.62)	15.35	noncompetitive (10.61)
9	25.06	noncompetitive (18.37)	14.86	noncompetitive (10.26)
eserin	0.52	nt	4.72	nt

[a] All compounds were examined in a set of experiments repeated three times; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss. [b] Values of inhibition constant. [c] nt, not tested.

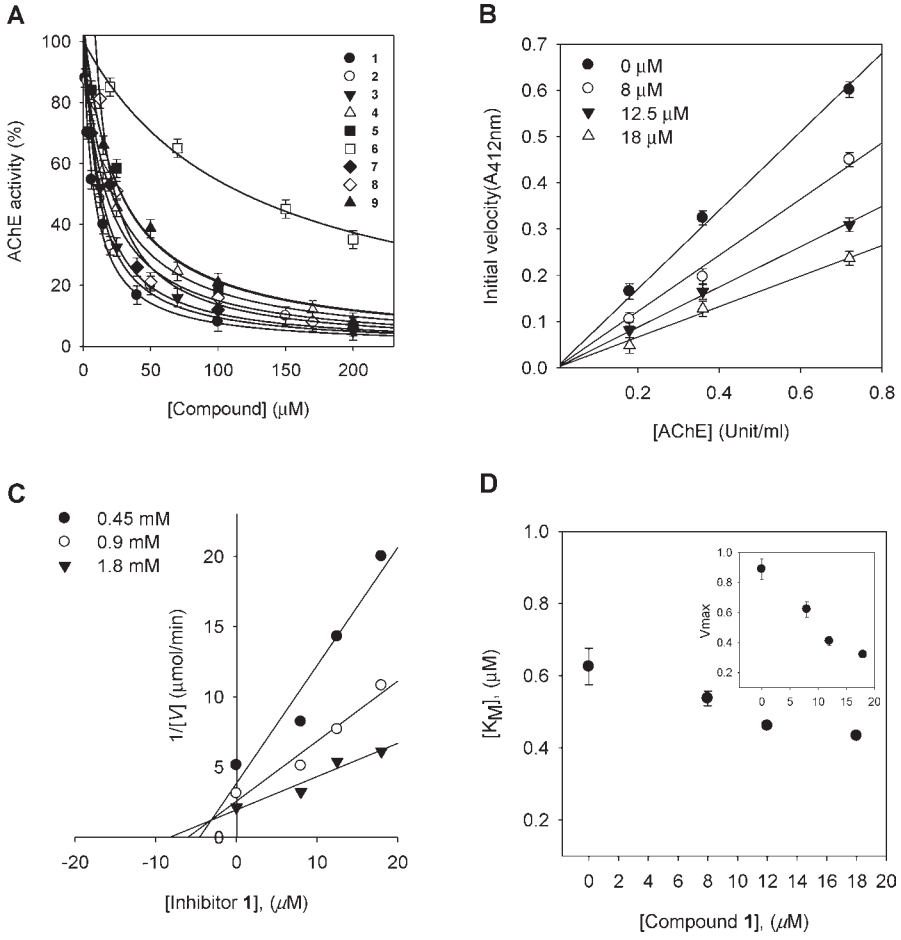


Figure 3. (A) Effect of compounds **1**−**9** on the activity of acetylcholinesterase for the hydrolysis of acetylthiocholine iodide. (B) Hydrolytic activity of acetylcholinesterase as a function of enzyme concentration at different concentrations of compound **1**. Concentrations of compound **1** for curves from top to bottom were 0, 8, 12.5, and 18 μM, respectively. (C) Dixon plot for the inhibition of compound **1** on the hydrolysis activity of acetylcholinesterase in the presence of different concentrations of substrate for lines from bottom to top: (●) 1.8 mM; (○) 0.9 mM; (▼) 0.45 mM. (D) K_m values as a function of the concentrations of the geranylated flavones **1**. (Inset) Dependence of the values of V_{max} on the concentration of geranylated flavone **1**.

^{13}C NMR (125 MHz) δ 18.1 (C-5‴), 18.2 (C-4‴), 22.5 (C-1‴), 25.0 (C-1″), 26.3 (C-4″, 5″), 99.2 (C-6), 104.3 (C-3′), 105.7 (C-4a), 107.3 (C-8), 108.5 (C-5′), 113.5 (C-1′), 121.5 (C-2′), 161.2 (C-5), 161.8 (C-4′), 162.0 (C-7), 162.7 (C-2), 183.8 (C-4).

Enzyme Assays. *Acetylcholinesterase and Butyrylcholinesterase Activity.* Acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) activities were assayed according to the spectrophotometric methods developed by Ellman et al. (with minor modifications).[17] The

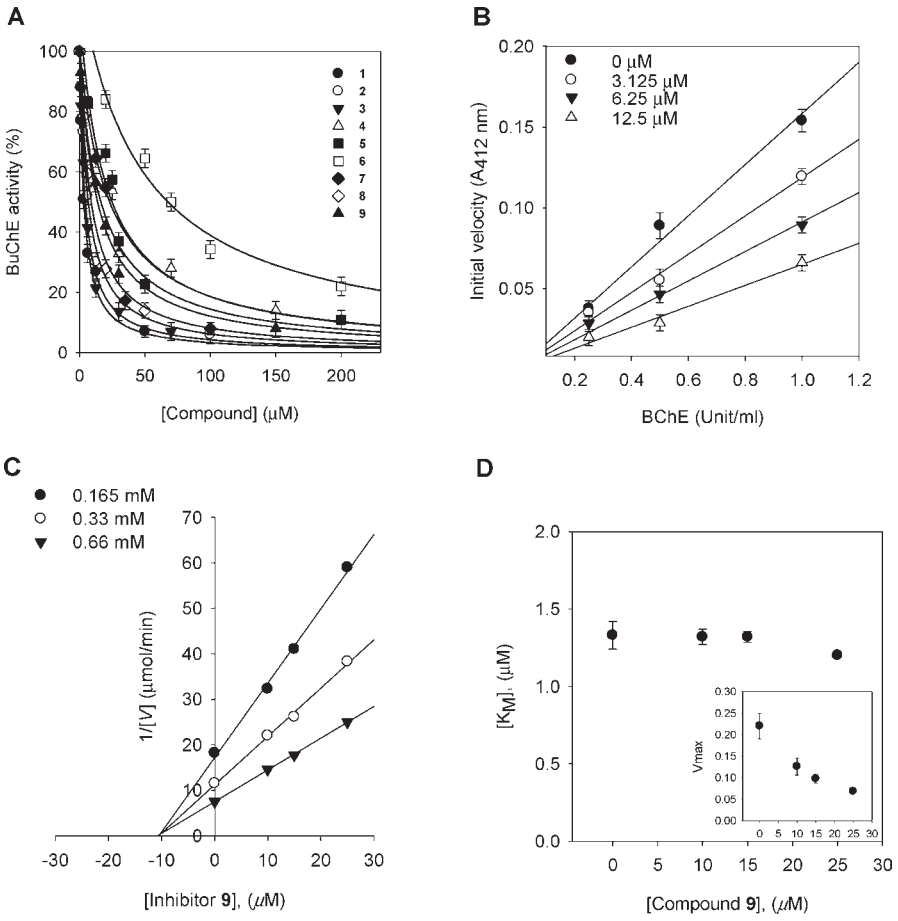


Figure 4. (A) Effect of compounds **1**−**9** on the activity of butyrylcholinesterase for the hydrolysis of butyrylthiocholine chloride. (B) Hydrolytic activity of butyrylcholinesterase as a function of enzyme concentrations at different concentrations of compound **9**. Concentrations of compound **9** for curves from top to bottom were 0, 3.125, 6.25, and 12.5 μM. (C) Dixon plot for the inhibition of compound **9** on the hydrolysis activity of butyrylcholinesterase in the presence of different concentrations of substrate for lines from bottom to top: (●) 0.66 mM; (○) 0.33 mM; (▼) 0.165 mM. (D) K_m values as a function of the concentrations of the prenylated flavone **9**. (Inset) Dependence of the values of V_{max} on the concentration of prenylated flavone **9**.

reaction mixture contained 140 μL of 100 mM sodium phosphate buffer (pH 8.0), the enzyme solution (either 0.36 unit of AChE, 20 μL; or 0.5 unit of BChE, 20 μL), and test sample in methanol (20 μL). After incubation for 15 min at room temperature, the reaction was initiated with the addition of substrate (0.9 mM acetylthiocholine iodide, 10 μL; or 0.33 mM butyrylthiocholine chloride, 10 μL) and 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (10 μL). The hydrolysis of acetylthiocholine or butyrylthiocholine was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm. The concentration of inhibitor required to achieve a half-maximal degree of inhibition is referred to as the IC_{50} (for inhibitor concentration giving 50% inhibition). To determine the IC_{50} value, a protocol in which initial velocity was recorded over a range of inhibitor concentrations was used. These data were analyzed using a nonlinear regression program [Sigma Plot (SPCC Inc.)]. The inhibitor concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to eq 1:[18]

$$\text{activity } (\%) = 100[1/(1 + ([I]/IC_{50}))] \quad (1)$$

A number of kinetic measures have also been described to test the exclusivity of inhibitor interactions with the target enzyme. The effects of inhibitors on the velocity of an enzymatic reaction can be generally described by the reciprocal relationship. Parameters were determined using the Lineweaver−Burk double-reciprocal-plot and Dixon plot.[19]

RESULTS AND DISCUSSION

We conducted phytochemical investigations to isolate bioactive compounds from the MeOH extract of the roots of the target plant. This was achieved via repeated column chromatography over silica gel and Sephadex LH-20. These efforts led to the isolation of nine compounds. The structure identification of these compounds was carried out by spectroscopic analyses and by comparisons with published data.[16,20−22] Compounds **1**−**9** were identified as 5′-geranyl-4′-methoxy-5,7,2′-trihydroxyflavone (**1**), 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone (**2**), Kkuwanon U (**3**), kuwanon E (**4**), morusin (**5**), morusinol (**6**), cyclomorusin (**7**), neocyclomorusin (**8**), and kuwanon C (**9**). The structural elucidation of new compound **1** is detailed as follows: the molecular formula was determined to be $C_{26}H_{28}O_6$. It had 13 degrees of unsaturation by HREIMS ($[M]^+$ at m/z 436.1886) data. The ^1H and ^{13}C NMR data including DEPT experiments showed the presence of 26 carbon atoms: 3 methylenes (sp^3), 7 methines (sp^2), 4 methyls, and 12 quaternary carbons. ^{13}C NMR data enabled carbons corresponding to 10 C−C double bonds to be identified, thus accounting for 10 of 13 degrees of unsaturation. The remaining 3 degrees of unsaturation were ascribed to cyclic systems. The ^1H NMR spectrum displayed a peak at 13.0 (1H, s) indicating a hydroxyl group, and

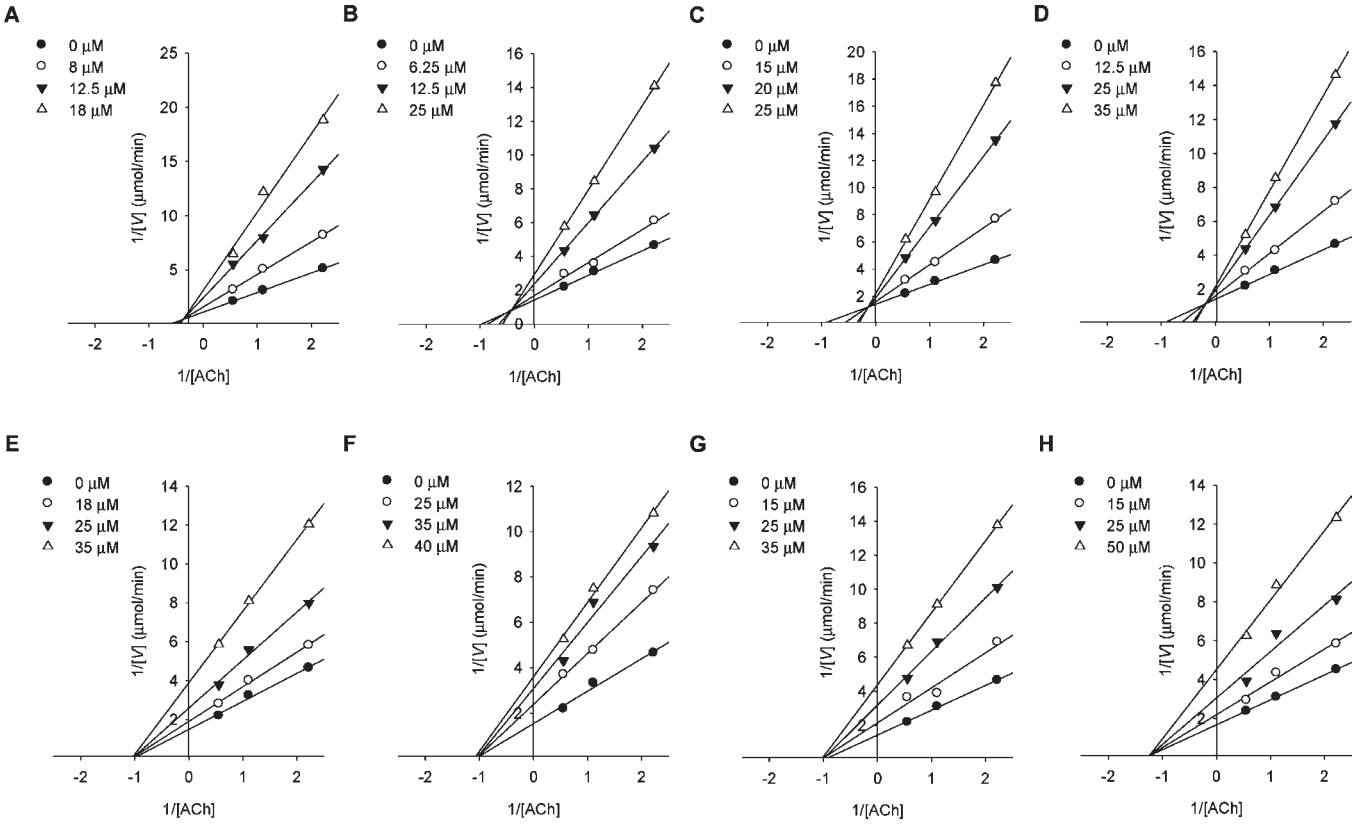


Figure 5. Lineweaver–Burk plots for the inhibition of compounds **1**–**9** on the hydrolysis activity of acetylcholinesterase: (A) for compound **1** of 0, 8, 12.5, and 18 μM; (B) for compound **2** of 0, 6.25, 12.5, and 25 μM; (C) for compound **3** of 0, 15, 20, and 25 μM; (D) for compound **4** of 0, 12.5, 25, and 35 μM; (E) for compound **5** of 0, 25, 35, 40 μM; (F) for compound **7** of 0, 15, 25, and 40 μM; (G) for compound **8** of 0, 15, 25, and 35 μM; (H) for compound **9** of 0, 15, 25, and 50 μM.

another at 6.94 (1H, s), which is characteristic of flavones (C-3). The spectrum also displayed isolated aromatic protons at δ_H 7.59 (1H, s), 6.54 (1H, s), and 6.33 (1H, d), which were assignable to a steppogenin[23] moiety bearing an alkyl substituent. The presence of a geranyl group was deduced from the successive connectivity from H-1″ (δ_H 3.17, m) to H-9″ (δ_H 1.62, s) and from H-4″ (δ_H 2.01, m) to H-6″ (δ_H 4.99, m) in the COSY spectrum and correlation of H-6″ with two carbons, C-8″ (δ_C 18.2) and C-10″ (δ_C 26.2). The above-mentioned geranyl group was positioned at C-5′ on the B-ring. This is because of a HMBC correlation between H-1″ and C-4′, C-5′, and C-6′ (Figure 2 and Table 1). The position of the OCH_3 group was confirmed by a strong correlation between OCH_3 (δ_H 3.74, s) and C-4′ (δ_C 162.2). Thus, compound **1** was identified as 5′-geranyl-4′-methoxy-5,7,2′-trihydroxyflavone.

The isolated flavonoids (**1**–**9**) were screened for their in vitro AChE and BChE inhibitory activities at different concentrations using a UV assay developed by Ellman.[17] All compounds were found to inhibit both enzymes with IC_{50} values ranging between 11.0 and 174.5 μM against AChE and between 3.4 and 76.3 μM against BChE (Table 2). Both enzymes were inhibited dose-dependently by all tested compounds (Figures 3A and 4A). The most potent compound identified in this study was the new flavone, **1**, which had K_i values of 3.1 μM (AChE) and 1.7 μM (BChE). Compound **1**, a representative AChE inhibitor with a mixed inhibition mechanism, and compound **9**, a representative BChE inhibitor with a noncompetitive mechanism, were reversible because plots of the initial velocity versus enzyme concentration in the presence of different concentrations of compounds gave a family of straight lines, all of which passed through the origin. Taken as an ensemble, the following general features of the SAR can be deduced from these data. The presence of a free hydroxyl group at C-7, a component of the 5,7-dihydroxyphenol motif at the A ring, is important for activity. A comparison of the two most potent inhibitors, **1** and **2**, unveils methylation at the C4′–OH has little effect on the inhibitory potency (IC_{50} = 10.95 vs 16.21 μM, respectively). A similar pattern was observed for compounds **3** and **4**. Perhaps most pointedly, hydration of the prenyl group at C-3 diminished inhibition potency significantly; for example, **5** (IC_{50} = 36.4 μM for AChE and IC_{50} = 24.1 μM for BChE) versus **6** (IC_{50} = 173.0 μM for AChE and IC_{50} = 76.3 μM for BChE). This implies hydrophobicity is important for inhibition. These inhibitory potencies indicated alkylated flavonoids from *M. lhou* were better inhibitors of cholinesterases than representative flavonoid-derived inhibitor quercetin.[24] Furthermore, as the flavones we have isolated are alkylated, their lipophilicity may have potential benefits in facilitating crossing the blood–brain barrier.[25]

To further study the mode of inhibition, we used both Lineweaver–Burk and Dixon plots. The kinetic and inhibition constants obtained are listed in Table 2. As a result of this kinetic analysis, we found that *M. lhou*-derived cholinesterase inhibitors could be divided into two classes based upon their mode of inhibition. These classes could also be divided on the basis of their alkyl substitution patterns. Compounds that were prenylated (addition of a five-carbon unit) at the C-3 position of the flavonoid skeleton (**5**–**9**) showed noncompetitive inhibition

kinetics. The only exception to this rule was compound **6**. Otherwise, the inhibition by flavonoids (**1**−**4**) on both AChE and BChE was mixed. Detailed kinetic studies were as follows (Figure 5).

As illustrated in Figure 3C, the inhibition kinetics analyzed by Dixon plots shows that compound **1** has a mixed inhibition mechanism because increasing substrates resulted in a family of lines which intersected above the [inhibitor] axis. Analysis of compound **1** using Lineweaver−Burk plots also showed characteristics of mixed inhibition (see Supporting Information S26). The equilibrium constant for inhibitor binding, K_i, was obtained from the −[inhibitor] value at the intersection of three lines. To verify mixed inhibition kinetics, we estimated values of K_m and V_{max} as a function of compound **1** concentration. As shown in Figure 3D, increasing concentrations of inhibitor resulted in increased K_m values and diminished V_{max}.

In the case of prenyl-substituted flavonoids (**5**−**9**), the Dixon plot of [inhibitor] versus $1/V$ resulted in a family of straight lines with a common x-axis intercept. This is illustrated for the three substrate concentrations in Figure 4C using prenylated flavone **5** as an example. This indicates that prenylated flavones (**5**−**9**) exhibit noncompetitive inhibition characteristics toward cholinesterases because V_{max} decreased without a change in K_m value in the presence of increasing concentrations of inhibitors (Figure 4D).

In conclusion, we have shown that extracts of *M. lhou* have significant and reasonably broad range cholinesterase inhibitory activities. The latter is particularly important as both enzymes we studied have been implicated in the onset of AD. We demonstrated activity by isolating nine inhibitors of both AChE and BChE from this plant. Many of these inhibitors had low micromolar IC_{50} values for each enzyme. Our detailed kinetic analysis revealed that the inhibition modes of these inhibitors varied between mixed and noncompetitive in a predictable way. We believe that that this study validates *M. lhou* as a source of cholinesterase inhibitors. We also hope that these compounds may prove to be useful leads for the discovery of new compounds targeting cholinesterase activity.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

IC_{50}, inhibitor concentration leading to 50% activity loss; K_i, inhibition constant; K_m, Michaelis−Menten constant.

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