CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200700960

Hopeahainol A: An Acetylcholinesterase Inhibitor from *Hopea hainanensis*

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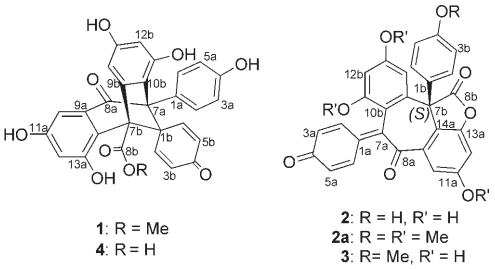
Abstract: A phytochemical study of *Hopea hainanensis* has led to the isolation of three new polyphenols and one known compound. The most important of these compounds are hopeahainols A (**2**) and B (**3**), which contain an unprecedented carbon skeleton. The structures were elucidated by analysis of the spectroscopic data including single-crystal X-ray spectroscopy and computational methods. Hopeahainol A was an acetylcholinesterase inhibitor with an IC_{50} value of 4.33 μM, which is comparable to that of huperzine A, a presently prescribed drug for the treatment of Alzheimer, while other similar structures were inactive. This observation was complemented by a 3D interaction model of the inhibitor with active sites.

Keywords: hopeahainol · inhibitor · natural products · polyphenols · structure elucidation

Introduction

A key hallmark for Alzheimer's disease (AD), which appears to grow rapidly worldwide among the elderly population, is the decreased level of acetylcholine, a neurotransmitter playing a decisive role in memory and learning.[1] Acetylcholinesterase (AChE), which degrades acetylcholine to its inactive metabolite choline, has emerged as a promising target for the management of Alzheimer disease, since inactivation of this enzyme leads to increased levels of acetylcholine.[2] In the light of the strategy, huperzine A was developed successfully as a plant-derived drug for the treatment of the disease.[3] In our investigations of novel and/or bioactive metabolites of *Hopea* species (Dipterocarpaceae),[4,5] a resveratrol oligomer-based AChE inhibitor, was characterized from the stem bark of *H. hainanensis*.[6] Re-assay of the combined mother liquors highlighted the presence of more AChE inhibitory compound(s) that may exist in "trace" amount(s) in the plant tissue. Subsequent bioassay-guided purification afforded hopeanol (**1**)[4] and three novel polyphenols, named hopeahainols A, B (**2**, **3**) and hopeanol B (**4**).

The mother liquors, which have been obtained from previous investigations on the *H. hainanensis* stem bark, were combined to give a residue from which compounds **1**--**4** (Scheme 1) were isolated after repeated column chromatography over silica gel and Sephadex LH-20.



Scheme 1. Structure of compounds isolated from *H. hainanensis*.

Results and Discussion

Hopeahainol A (**2**) has a molecular formula of $C_{28}H_{16}O_8$ that was evidenced from the $[M+H]^+$ and $[M+Na]^+$ ions at

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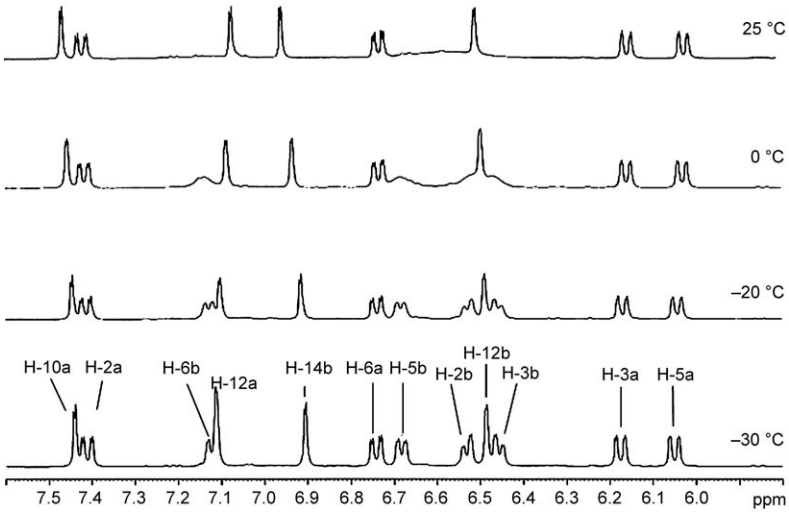


Figure 1. ^{1}H NMR spectra of hopeahainol A (**2**) measured at variable temperatures ([D$_6$]acetone, 500 MHz).

m/*z* 481.0920 (calcd for 481.0923) and 503.0749 (calcd for 503.0743) in the HRESI mass spectroscopical analysis. Methylation of **2** with MeI in the presence of K_2CO_3 afforded a tetramethyl ether (**2a**) which suggested that compound **2** has four phenolic hydroxyl groups. The ^{1}H NMR spectrum of **2** recorded at 25 °C was not informative owing to the poor resolution of some signals between δ_H 7.2 and 6.4 ppm, which was presumed to orginate from the free rotation of a 1,4-disubstituted benzene ring as indicated by comparing the spectra with those acquired at 0, −20 and −30 °C (Figure 1, Table 2).[7]

Scrutiny of its ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC spectra (all acquired at −30 °C) allowed the identification of an ester group (δ_C 174.7 ppm) and four substructures (Figure 2) including a 7,7-disubstituted 4-methylenecyclohexa-2,5-diene residue [ring A_1, giving characteristic signals at δ_H = 7.41 (H-2a, dd, J = 10.2, 2.4 Hz), 6.17 (H-3a, dd, J = 10.2, 1.3 Hz), 6.05 (H-5a, dd, J = 10.0, 1.3 Hz) and 6.74 ppm (H-6a, dd, J = 10.0, 2.4 Hz),[4] a 2,3,5-trisubstituted benzoyl group [ring A_2, observed at δ_H = 7.44 (H-10a, d, J = 2.1 Hz) and 7.11 ppm (H-12a, d, J = 2.1 Hz), a 4-substituted phenyl moiety [ring B_1, affording differentiated resonances at δ_H = 6.53 (H-2b, dd, J = 8.5, 1.9 Hz), 6.46 (H-3b, dd, J = 8.5, 2.1 Hz), 6.68 (H-5b, dd, J = 8.6, 2.1 Hz) and 7.12 ppm (H-6b, dd, J = 8.6, 1.9 Hz)], and a 1,2,3,5-tetrasubstituted benzene nucleus [ring B_2, resonating at δ_H = 6.49 (H-12b, d, J = 1.7 Hz) and 6.90 ppm (H-14b, d, J = 1.7 Hz)]. The HMBC correlation of C-7b (δ_C 59.1) with H-2b, H-6b and H-14b indicated that rings B_1 and B_2 were co-connected to the carbon. The attachment of the four hydroxyl groups was assigned by the NOESY and HMBC spectra of **2a**.

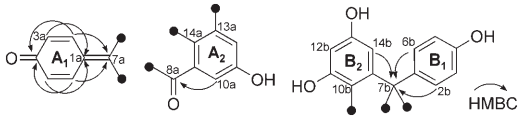
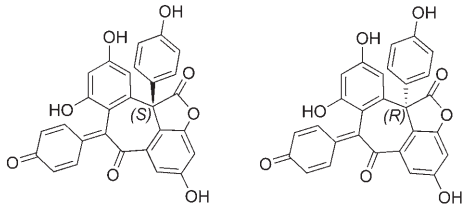


Figure 2. Substructures of **2** identified from NMR data.

Since some protons in the substructures A_{1-2} and B_{1-2} were separated by quaternary carbons over five bonds, the NMR data of hopeahainol A (**2**) failed to provide sufficient information required for accommodating the connection of those fragments to afford the entire structure of the compound. Moreover, compound **2** did not form single crystals that were necessary for the X-ray diffraction analysis. The frustration to clarify the structure of **2** was eventually overcome by the

Table 1. Calculated energies and optical rotations for **2** and (7b*R*)-**2**.



hopeahainol A (**2**) (7b*R*)-**2**

Configuration	**2**		(7b*R*)-**2**		Exp
conformation	(1*S*)	(2*S*)	(1*R*)	(2*R*)	
relative energy[a]	0.00	0.05	0.00	0.05	
$[\alpha]_D$[b]	+594.3	+636.5	−598.6	−639.5	+673.5 (MeOH, 0.090)
sum of $[\alpha]_D$[c]	+615.4		−619.1		

[a] Lowest energy conformation was used as the reference zero point, the geometries were obtained in the gas phase at the B3LYP/6-31G(d) level, the unit is kcal mol^{-1}. [b] The optical rotations in MeOH were evaluated at the same level. [c] The Boltzmann formula was used to produce the sum of two different conformational optical rotations.

single crystal X-ray crystallographic measurement of its permethylated derivative (**2a**) (Figure 3 and Supporting Information).

To allocate the absolute configuration and to rationalize the low resolution of some ^{1}H NMR signals (Figure 1), four conformations of **2** were obtained through calculations with the Gaussian 03 package at the B3LYP/6-31G(d) level.[8,9] The optical rotation was obtained at the same level with the gauge-including atomic orbital (GIAO) method.[10] The calculated optical rotation (+615.4) for **2**, which is very close to its experimental value (+673.5), suggested that C-7b had an *S* configuration (Table 1 and Supporting Information).

As evidenced from calculations, the continuous interconversion (namely, spin of ring B_2) between the two stable conformers (Figure 4) exists thermodynamically to allow the worse and better resolved signals of protons on ring B_2 at higher and lower temperatures, respectively (Figure 1). The most stable isomer was suggested to be Ca, which was close to the crystal structure of **2a**.

Hopeahainol B (**3**), a red amorphous powder, had a molecular formula of $C_{29}H_{18}O_8$ established by the $[M+H]^+$ ion at *m*/*z* 495.1075 in its HRESI MS analysis (calcd for $C_{29}H_{19}O_8$, 495.1080). The ^{1}H and ^{13}C NMR spectra (Table 2, acquired at −30 °C) of **3** were very similar to those of **2** except for the appearance of a methoxy group (δ_H 3.61, δ_C 55.2). This observation, along with the HMBC correlation of C-4b with OMe-4b, demonstrated that **3** is the 4b-*O*-methyl ether derivative of hopeahainol A (**2**).

Hopeanol B (**4**), which was obtained as a light yellow amorphous powder, has a molecular formula of $C_{28}H_{18}O_9$ [14 mass units less than for hopeanol (**1**)[4]] that was confirmed by the $[M+H]^+$ ion peak at *m*/*z* 499.1033 in its HRESIMS analysis (calcd for $C_{29}H_{19}O_9$, 499.1029). Its ^{1}H and ^{13}C NMR spectra (Table 2) were well comparable to those of **1** except for that the methoxyl ester signals (δ_H 3.52, δ_C 51.8), which was not observed for **4**.[4] Also reinforced by its 2D NMR data, compound **4** was identified as the 8b-*O*-demethyl derivative of hopeanol (**1**).

Table 2. ^{1}H and ^{13}C NMR assignments of **2**–**4**.

Carbon	**2**[a]		**3**[a]		**4**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1a	135.0		135.1		124.6	
2a	136.8	7.41 (dd, 10.2, 2.4)	136.5	7.37 (dd, 10.2, 2.4)	131.9	7.00 (d, 8.5)
3a	129.2	6.17 (dd, 10.2, 1.3)	129.4	6.14 (dd, 10.2, 1.8	113.8	6.62 (d, 8.5)
4a	186.9		186.5		157.1	
5a	129.1	6.05 (dd, 10.0, 1.3)	129.4	5.99 (dd, 10.1, 1.8)	113.8	6.62 (d, 8.5)
6a	139.3	6.74 (dd, 10.0, 2.4)	139.0	6.66 (dd, 10.1, 2.4)	131.9	7.00 (d, 8.5)
7a	150.3		149.8		71.1	
8a	187.6		187.6		190.3	
9a	132.0		132.2		132.5	
10a	110.9	7.44 (d, 2.1)	110.9	7.43 (d, 1.5)	106.9	7.02 (d, 2.5)
11a	160.0		160.1		158.3	
12a	104.6	7.11 (d, 2.1)	104.6	7.11 (d, 1.5)	108.8	6.54 (d, 2.5
13a	153.9		154.0		154.4	
14a	123.4		123.2		121.1	
1b	130.7		131.5		66.8	
2b	127.1	6.53 (dd, 8.5, 1.9)	127.2	6.63 (dd, 8.7, 2.2)	148.0	7.36 (dd, 10.4, 2.8)
3b	114.8	6.46 (dd, 8.5, 2.1)	113.4	6.56 (dd, 8.7, 2.4)	133.6	6.27 (dd, 10.4, 2.0)
4b	157.8		159.8		185.3	
5b	117.5	6.68 (dd, 8.6, 2.1)	116.4	6.79 (dd, 8.8, 2.4)	131.2	5.79 (dd, 10.2, 2.0)
6b	130.3	7.12 (dd, 8.6, 1.9)	130.7	7.21 (dd, 8.8, 2.2)	150.4	6.98 (dd, 10.2, 2.8)
7b	59.1		59.0		63.7	
8b	174.7		174.7		171.2	
9b	142.0		141.8		152.7	
10b	109.9		109.8		112.4	
11b	158.3		158.3		160.2	
12b	102.1	6.49 (d, 1.7)	102.1	6.48 (d, 1.6)	102.8	6.20 (d, 2.0)
13b	160.0		160.1		157.2	
14b	106.0	6.90 (d, 1.7)	105.8	6.88 (d, 1.6)	107.7	7.01 (d, 2.0)
OMe			55.2	3.61 (s)		

[a] Data were recorded in [D6]-acetone at -30°C on BRUKER DRX-500 MHz spectrometers. All signals were assigned by the aid of 1D and 2D NMR spectra.

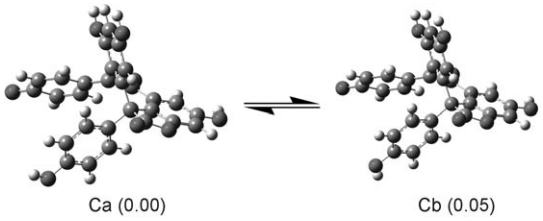


Figure 4. Two DFT-calculated minimum energy conformers (Ca and Cb) of its *S* configuration. Energies (in kcal mol^{-1}) relative to the stablest conformer **Ca** are given in parentheses.

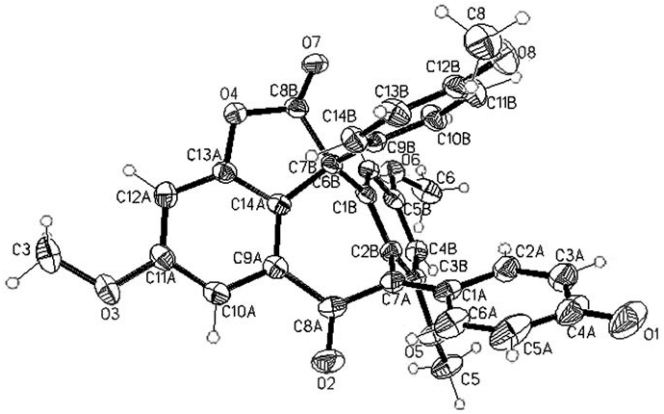


Figure 3. X-ray structure of **2a**.

Biosynthetically, hopeanol B (**4**) sharing the same biosynthetic pathway with that of hopeanol (**1**)[4] would be the common precursor of hopeahainols A (**2**) and B (**3**) via a presumable intermediate **4a** (Figure 5).

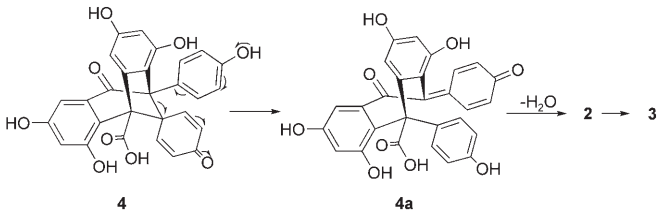


Figure 5. Plausible biogenetic pathway for **2** and **3**.

Compounds **1**--**4** and **2a** were subjected to in vitro AChE inhibitory evaluations. Surprisingly, only hopeahainol A (**2**) was found to be significantly active with an IC_{50} value of 4.33 ± 0.17 μM ($n = 3$) whereas the other isolated compounds were all inactive. The magnitude of **2** in the enzyme inhibition was well comparable to that of the positive reference, such as (±)-huperzine A ($IC_{50} = 1.6$ μM).[11]

To understand the structure–activity relationships, a three-dimensional interaction model of the inhibitor with the active site(s) was generated by InsightII (Figure 6). The main binding sites of **2** with AChE were realized through the direct hydrogen bonding of the 4b-OH with the N atom in Trp286, and of the two O atoms in the ester group with H in Ser293. This may explain why only compound **2** was inhibitory on the enzyme.

The inhibition of **2** on AChE was very fast and time independent since the IC_{50} value without incubation was not significantly ($P > 0.05$) different from that observed for up to 30 min incubation. Thus, hopeahainol A (**2**) was a reversible inhibitor of AChE. Reciprocal Lineweaver–Burk plots describing the inhibition pattern of **2** gave increasing slopes and growing *y*-axis intercepts with higher inhibitor concentrations (Figure 7a). This demonstrated a mixed typed inhibition, resulting from the significant co-interaction of **2** with the free and the acetylated forms of the enzyme. Replots of the slope versus the inhibitor concentration allowed the estimation of the K_i value of 21.85 ± 1.25 μM ($n = 3$) (Figure 7b).

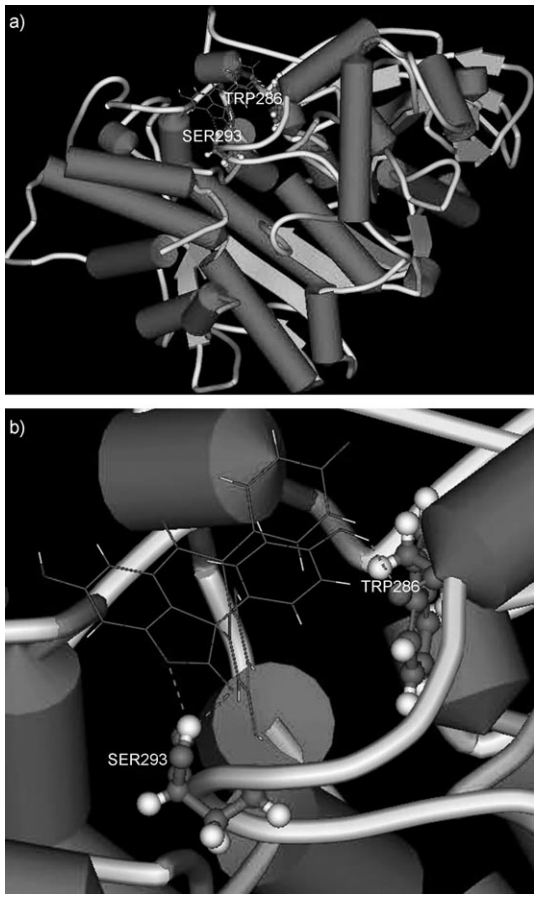


Figure 6. Complex of hopeahainol A (**2**) and *Electrophorus electricus* acetylcholinesterase (PDB code: 1C2B) obtained with InsightII.

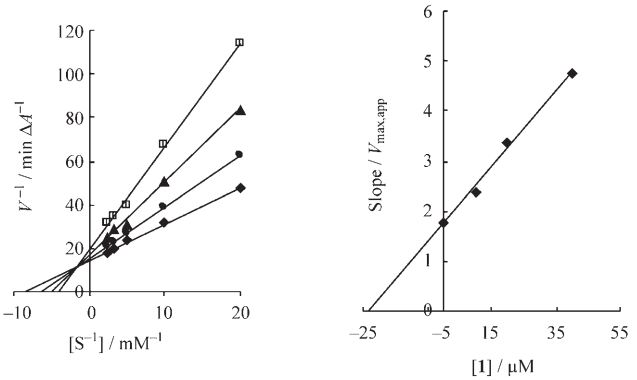


Figure 7. Steady-state inhibiotion of AChE by **2**. a) Lineweaver–Burk plot of reciprocal of initial velocities versus reciprocal of five fixed acetylthiocholine iodide (ATCh) concentrations in the absence (◆) and presence of 10 μM (●), 20μM (▲) and 40 μM (■) of **2**. b) Secondary plots of the Lineweaver–Burk plot, slope versus various concentrations of **2**. (*x* axis intercept represents the K_i of the inhibitor).

Conclusion

Three new and a known polyphenols were isolated from the stem bark of *H. hainanensis*. Compounds **2**--**4** were unambiguously assigned on the basis of the NMR correlations, X-ray analysis and computational methods. The former two share an unprecedented carbon skeleton. Hopeahainol A (**2**) showed an AChE inhibitory activity with its magnitude comparable to that of huperzine A, a presently prescribed AD-treating drug, while other similar structures were inactive. This phenomenon was elucidated by a 3D interaction model of the inhibitor with active site(s) in Insight II. The reason for the poor resolution of some ^{1}H NMR signals at room temperature, which was reported in the literature,[7] was discussed for the first time at theoretical level through computational methods.

Experimental Section

General methods: Melting points were measured on an XT-4 apparatus. Optical rotations were determined in MeOH on a WXG-4 disc polarimeter, and IR spectra in KBr disks on a Nexus 870 FT-IR spectrometer. The UV spectra were recorded on a Hitachi U-3000 spectrophotometer. ESI and HR-ESI mass spectra were obtained on a Mariner Mass 5304 instrument. All NMR experiments were performed on a Bruker DRX-500 NMR spectrometer and using solvent signal ([D_6]acetone, δ_H: 2.05 ppm) as an internal standard. Silica gel (200–300 mesh) for CC and GF254 (10–20 μm) for TLC were produced by Qingdao Marine Chemical Company, China. Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden. Electric-eel AChE (EC 3.1.1.7), acetylthiocholine iodide (ATCh), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade.

Plant material: *Hopea hainaninesis* was collected on July 21, 2003 from the Botanical Garden of South China University of Tropical Agriculture with the specimen identified by Professor X. Q. Zheng (South China University of Tropical Agriculture). A voucher specimen (no. IFB030721) was deposited at Institute of Functional Biomolecules, Nanjing University, Nanjing, China.

Extraction and isolation: All mother liquors left over by previous isolations[6] were combined to give, after evaporation of solvent, a residue (98.8 g) that was subsequently purified by chromatography over silica gel column and eluted with $CHCl_3$/MeOH gradients of growing polarity to give eight fractions. And the second fraction (2.8 g) with bioactivity, afforded by elution with $CHCl_3$/MeOH 20:1, was separated on silica gel with $CHCl_3$/MeOH 50:1, 30:1, 20:1, 10:1, 5:1, 0:100 to give six fractions (fractions 1–6). Fraction 2 (110 mg) was further subjected to Sephadex LH-20 column chromatography (MeOH) to give **1** (31 mg). Fraction 3 (530 mg) was further purified on a silica gel column eluted with petroleum ether/acetone 3:1, 2:1, 1:1, 0:1 to give four fractions (fractions 3a–3d). Compound **3** (54 mg) and **2** (73 mg) were obtained from fractions 3-b and 3-d, respectively, after purification by Sephadex LH-20 CC (MeOH). Compound **4** (17 mg) was obtained from fraction 5 after purification by PTLC (EtOAc/$CHCl_3$/MeOH/H_2O 5:10:1:0.1) and Sephadex LH-20 CC (MeOH).

AChE inhibition assay: The AChE inhibitory activities were measured by a spectrophotometric method developed by Ellman et al.[12] The reaction was run at 25 °C in a final volume of 200 μL of a 0.1 M phosphate buffer pH 8.0, 333 μM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.035 unit per mL AChE and 530 μM of acetylthiocholine iodide in 96-well microplates. Test compounds were added to the assay solution and followed at 412 nm for 5 min with a plate reader (Sunrise, Tecan, Austria). Inhibition curves were performed in triplicate by incubating with at least 10 concentrations of each test compound. One triplicate sample without inhibitor was always present to yield 100% of AChE activity. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. IC_{50} (concentration of drug producing 50% of enzyme–activity inhibition) values were determined graphically from log concentration–inhibition curves.

Molecular docking of hopeahainol A into *Electrophorus electricus* acetylcholinesterase: Molecular docking was performed on a Silicon Graphics Iris O2 (SGI Inc, Silicon, CA, USA) workstation using the DOCK modules of the commercial software packages InsightII 2000 (MSI, St Louis, MI, USA). Three high-resolution X-ray crystal structures of *Electrophorus electricus* acetylcholinesterase (PDB code: 1C2B),[13] mouse acetylcholinesterase (PDB code: 2 JGE)[14] and *Torpedo californica* acetylcholinesterase (PDB code: 1E3Q)[15] were downloaded from protein data bank. The results of sequence alignment by BLAST show there is higher homologous property between *Electrophorus electricus* acetylcholinesterase and mouse acetylcholinesterase or *Torpedo californica* acetylcholinesterase, the former identity up 100% _i540/540_jand the latter similarity nearly 87.2% (471/540) (Table S3, Supporting Information). There are three active sites (Tyr72, Tyr124, and Trp286 (underlined in Table S3, Supporting Information) in mouse acetylcholinesterase in the complex with organophosphorus compounds and four inhibitor binding sites (Tyr70, Trp84, Trp279, and Phe330 underlined in Table S3, Supporting Information) in a complex of *Torpedo californica* acetylcholinesterase with its inhibitor Bw284c51. By superimposition of the three crystal structures, the results reveal the common sites maybe exist in *Electrophorus electricus* acetylcholinesterase, namely Tyr72 and Trp286. And three other sites, Trp86, Tyr124 and Tyr 337, also play a role during interaction between acetylcholinesterase and its inhibitor. Thus, hopeahainol A (**2**) was docked in a pocket surrounding by these sites mentioned above and the interactional energy (including whole energy, electrostatic energy and steric energy) was calculated and optimized. The docked conformations (Figure 6) were then used to analyze the binding interactions.

Enzyme kinetic studies:[16] Kinetic studies were performed using AChE from an electric eel. Enzyme actvities were determined at 25 °C using five concentrations of substrate (50, 100, 200, 300 and 400 μM) in the presence or absence of three concentrations of **2** (10, 20 and 40 μM) against AChE. Data were plotted by the method of Lineweaver–Burk to reveal the mechanism of inhibition. Plots of the slopes versus the inhibitor concentrations gave estimates of K_i, the dissociation constant for inhibitor binding to AChE.

Hopeahainol A (2): Red amorphous powder, m.p. 216–217 °C; $[\alpha]_D^{20}$ = +673.5° (c = 0.090, MeOH); UV/Vis (MeOH): λ_{max}(log ε) = 218 (4.41), 307 (3.91), 450 nm (3.63); IR (KBr): $\tilde{\nu}_{max}$ = 3195, 2975, 1796, 1695, 1633, 1592, 1508, 1448, 1335, 1261, 1162 1076 cm^{-1}; NMR: see Table S1, Supporting Information; positive ESIMS: m/z: 481 $[M+H]^+$, 503 $[M+Na]^+$; HR-ESIMS: m/z: calcd for $C_{28}H_{17}O_8$: 481.0923; found: 481.0920 $[M+H]^+$, 503.0749 $[M+Na]^+$.

Hopeahainol B (3): Red amorphous powder, m.p. 227–228 °C; $[\alpha]_D^{20}$ = +1105.6° (c = 0.024, MeOH); UV/Vis (MeOH): λ_{max} (log ε) = 214 (4.32), 306 nm (3.88); IR (KBr): $\tilde{\nu}_{max}$ = 3382, 3160, 2969, 2258, 1797, 1698, 1654, 1633, 1606, 1507, 1448, 1333, 1252, 1163 1076, 1003 cm^{-1}; NMR: see Table S1, Supporting Information; positive ESIMS: m/z: 495 $[M+H]+$, 517 $[M+Na]^+$; HR-ESIMS: m/z: calcd for $C_{29}H_{19}O_8$: 495.1080; found: 495.1075 $[M+H]^+$.

Hopeanol B (4): Light yellow amorphous powder, m.p. 198–199 °C; $[\alpha]_D^{20}$ = +146.0° (c = 0.373, MeOH); UV/Vis (MeOH): λ_{max} (log ε) = 207 (4.91), 225 (4.77), 3011 (3.32), 355 nm (3.41); IR (KBr): $\tilde{\nu}_{max}$ = 3200, 2923, 2882, 1718, 1660, 1609, 1608, 1516, 1461, 1336, 1263, 1160, 1113, 1082, 1056, 1017 cm^{-1}; NMR: see Table S1, Supporting Information; positive ESIMS: m/z: 499 $[M+H]^+$, 521 $[M+Na]^+$; HR-ESIMS: m/z: calcd for $C_{29}H_{19}O_9$: 495.1029; found: 499.1033 $[M+H]^+$.

Methylation of 2: Hopeahainol A (**2**) (20 mg) was allowed to react with K_2CO_3 (500 mg) and MeI (200 mg) in dry acetone under reflux for 6 h at 65 °C. The reaction was treated in the usual manner and the crude product 25 mg was purified by Sephadex LH-20 CC ($CHCl_3$/MeOH 1:1) to give **2a** (16 mg). A yellow rhombic crystal; positive ion ESIMS: m/z: 537 $[M+H]^-$; ^{1}H NMR (500 MHz, [D_6]acetone, −30 °C): δ = 7.46 (d, J = 2.2 Hz, H-10a), 7.35 (dd, J = 10.3, 2.4 Hz, H-2a), 7.31 (d, J = 2.2 Hz, H-12a), 7.21 (dd, J = 8.7, 1.7 Hz, H-6b), 7.04 (d, J = 2.3 Hz, H-14b), 6.77 (dd, J = 8.7, 2.1 Hz, H-5b), 6.75 (d, J = 2.3 Hz, H-12b), 6.60 (dd, J = 8.6, 1.9 Hz, H-2b), 6.53 (dd, J = 8.6, 2.1 Hz, H-3b), 6.52 (dd, J = 10.1, 2.4 Hz, H-6a), 6.15 (dd, J = 10.3, 1.8 Hz, H-3a), 5.95 (dd, J = 10.1, 1.8 Hz, H-5a), 3.97 (s, OMe), 3.93 (s, OMe), 3.74 (s, OMe), 3.54 ppm (s, OMe).

Acknowledgements

The work was co-financed by the key projects from the National Natural Science Foundation (20432030 and 20533060) and the Ministry of Education of China (104195).

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Received: June 22, 2007
Published online: October 17, 2007

