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New class of acetylcholinesterase inhibitors from the stem bark of *Knema laurina* and their structural insights

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ABSTRACT

Bioassay-guided extraction of the stem bark of *Knema laurina* showed the acetylcholinesterase (AChE) inhibitory activity of DCM and hexane fractions. Further repeated column chromatography of hexane and DCM fractions resulted in the isolation and purification of five alkenyl phenol and salicylic acid derivatives. New compounds, (+)-2-hydroxy-6-(10′-hydroxypentadec-8′(*E*)-enyl)benzoic acid (**1**) and 3-pentadec-10′(*Z*)-enylphenol (**2**), along with known 3-heptadec-10′(*Z*)-enylphenol (**3**), 2-hydroxy-6-(pentadec-10′(*Z*)-enyl)benzoic acid (**4**), and 2-hydroxy-6-(10′(*Z*)-heptadecenyl)benzoic acid (**5**) were isolated from the stem bark of this plant. Compounds (**1–5**) were tested for their acetylcholinesterase inhibitory activity. The structures of these compounds were elucidated by the 1D and 2D NMR spectroscopy, mass spectrometry and chemical derivatizations. Compound **5** showed strong acetylcholinesterase inhibitory activity with IC_{50} of 0.573 ± 0.0260 μM. Docking studies of compound **5** indicated that the phenolic compound with an elongated side chain could possibly penetrate deep into the active site of the enzyme and arrange itself through π–π interaction, H-bonding, and hydrophobic contacts with some critical residues along the complex geometry of the active gorge.

Alzheimer's disease (AD) was first reported by a German scientist, Alois Alzheimer in 1907, and is the most common type a neurodegenerative disease characterized by the death of nerve cells. Neurodegenerative diseases are a group of disease conditions that result from chronic breakdown and progressive functional or structural loss of the neurons in the central nervous system (CNS). AD disease affects 25 million people worldwide in 2000 and it is expected to increase to 114 million by 2050.[1a] AD in the early stages is associated with a decline in cognitive function, particularly short term memory. As the disease progresses, patients face problems in reading, speaking and logical thinking, and long-term memory. Recent reports on medicinal plants showed that about 63% of the low molecular drugs developed from 1981 to 2006 are natural products. These reports suggested that natural products have strong potential to be developed into biologically active compounds with anti-AD activity. Only some natural sources such as *Ginkgo biloba* and *Huperzia serrata* have been studied extensively as natural therapeutic agents to treat AD patients.[1b]

Commercially available medicines such as tacrine, donepezil, and the natural product-based rivastigmine are used for the treatment of cognitive dysfunction and memory loss associated with AD.[1c] These drugs have been known for their adverse effects including gastrointestinal disturbances and problems associated with bioavailability.[1d,e] A variety of plants exhibited AChE inhibitory activity and are the potential candidate for further investigation towards the discovery of neurodegenerative disorders such as AD.[1f]

The genus *Knema* comprises approximately 60 Southeast Asian species.[2a] The methanolic extract of the stem bark of *Knema laurina* exhibited neuroprotective and anti-inflammatory effect in cell-based assay system of brain tissue.[2b] Some species of *Knema* are also used in cancer therapy.[2c] The extract of the stem bark of *Knema furfuraceae* Warb. is used in Thailand as a popular remedy for sores, pimples, and cancer.[2d,e] The genus *Knema* also contains a variety of natural compounds including alkyl and acyl resorcinol, substituted stilbenes, lignans, flavonoids, isoflavonoids, flavan,[2e,f]

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and phenylalkylphenol derivatives.[2f] Some species of *Knema* are also distributed in tropical Africa, Asia, and Australasia, and used in traditional medicine.[2g] (±)-Myristinins A and D, which were isolated from *Knema elegens*, exhibited the inhibition of DNA polymerase β and cleaved DNA.[2h,i] Phytochemical analysis of *K. laurina* revealed the presence of alkaloids, saponins, steroids, and triterpenes in the leaves and bark,[2j] while resorcinols as well as anacardic acids in its stem bark.[2e,k] Anacardic acid is also known to inhibit glyceraladehyde-3-phosphate dehydrogenase.[2l]

A preliminary cholinesterase inhibition assay using TLC bioautography method was conducted on different fractions of the stem bark of *K. laurina*.[3a–c] Based on the cholinesterase inhibiting activity of the fractions, hexane and DCM extracts were subjected to column chromatography, which led to the isolation and identification of compounds (**1–5**). The present article describes a new class of natural acetylcholinesterase inhibitors isolated from this plant.

The bioactive hexane and DCM fractions were subjected to silica gel column chromatography and high performance liquid chromatography allowed the purification of compounds (**1–5**). Compounds (**2–5**) were isolated from hexane, while **1** was from DCM soluble part of the stem bark of *K. laurina*. Compound **1** was obtained by the multiple column chromatography as a viscous liquid from the DCM fraction by using 50% ethyl acetate in hexane as mobile phase. The IR spectrum showed absorption bands at 3616–3420, 2920, 2860, 1648, 1455, 986, and 786 cm^{-1} suggesting the presence of hydroxyl, acidic carbonyl, aromatic, and aliphatic double bonds, respectively. The EI-MS showed the [M^+] at *m*/*z* 362. The high resolution electron impact mass spectrum (HREI-MS) of compound **1** showed the [M^+] at *m*/*z* 362.2667, suggesting the molecular formula as $C_{22}H_{34}O_4$ (calcd 362.2669), which was also supported by the ^{13}C and ^1H NMR spectra. Other important fragments were at *m*/*z* 55, 77, 82, 91, 108, 134, 161, 121, 207, 326, 344, and 362. The presence of base a peak at *m*/*z* 108 and prominent peak at *m*/*z* 91 resulting from the benzylic cleavage of aromatic moiety followed by the loss of CO_2 was suggestive of the presence of alkyl resorcinol.[4a–c] An important peak at *m*/*z* 344 was indicative of the loss of H_2O from the [M^+].

The ^1H NMR spectrum (in $CDCl_3$) of compound **1** exhibited a triplet at δ_H 0.90 (*J* = 7.5 Hz), which was assigned to the terminal methyl H-15′ protons. A broad multiplet appearing at δ_H 1.33–1.30, calculated for 8 protons (4 × CH_2) was assigned to the H-5′ to H-6′ and H-13′ to H-14′ protons. Another multiplet appearing at δ_H 1.43–1.40, which accounted for 6 methylene protons (3 × CH_2) was assigned to the H-3′ to H-4′ and H-12′ protons. These assignments were further securitized with the help of COSY, HSQC, HMBC, and ^{13}C NMR spectra, as presented in Table 1. A prominent signal at δ_H 2.99 (t, *J* = 7.5 Hz) was assigned to the benzylic (H-1′) proton, while two multiplets at δ_H 1.59 and 2.0 were assigned to the H-2′ and H-7′, respectively. A broad multiplet appearing at δ_H 4.15 was assigned to the hydroxymethine (H-10′) proton. This proton appeared as a sharp doublet at δ_H 4.47 ($J_{10',9'}$ = 4.5 Hz)[2d] when ^1H NMR was recorded in DMSO-d_6. The characteristic signals, which appeared as a doublet of triplet at δ_H 5.50 (dt, $J_{8',9'}$ = 14.5 Hz, $J'_{7,8'}$ = 6.5 Hz) and 5.35 ($J_{9',8'}$ = 14.5 Hz, $J_{9',10'}$ = 4.5 Hz) were assigned *trans* disposition for the H-8′ and H-9′ protons, respectively.[4d] The chemical shift of the allylic carbon (C-7′, δ_C 34.2) also supported the *trans* disposition of double bond.[4e] Two downfield doublets at δ_H 6.90 (d, *J* = 8.5 Hz) and 6.84 (d, *J* = 8.0 Hz) were attributed to the H-3 and H-5 protons, respectively. The most downfield signal at δ_H 7.32 (t, *J* = 8.0, 8.5 Hz) was assigned to the H-4 proton. The alkenyl side chain was comparable with those previously isolated carandols and anacardic acid from plants of same genus.[2k]

The H-8′ proton (δ_H 5.50) showed COSY correlation with H-9′ (δ_H 5.35), which was further extended to H-7′ (δ_H 2.01) and H-10′ (δ_H 4.15). The hydroxymethine proton H-10′ (δ_H 4.15) showed the HMBC correlations with C-9′ (δ_C 132.7) and C-8′ (δ_C 132.9) carbon atoms. These observations suggested that the double bond and hydroxyl group are adjacent to each other. The terminal methyl (H-15′) protons (δ_H 0.90) exhibited HMBC correlations with the C-14′ (δ_C 22.8) and C-13′ (δ_C 31.9) carbons atoms. The aromatic H-4 and H-5 (δ_H 7.32 and 6.84, respectively) showed the HMBC correlations with C-6 (δ_C 147.6) and C-1 (δ_C 11.2). Furthermore, the H-1′ proton (δ_H 2.99) showed strong HMBC correlations with the C-2′ (δ_C 34.4), C-6 (δ_C 147.6) and C-1 (δ_C 111.2) carbon atoms. The ^1H and ^{13}C NMR data are summarized in the Table 1 and selected COSY and HMBC correlations are shown in Figure 1.

To establish the position of double bond, compound **1** was treated with dimethyl disulfide (DMDS) and I_2.[4f,g] Analysis and the

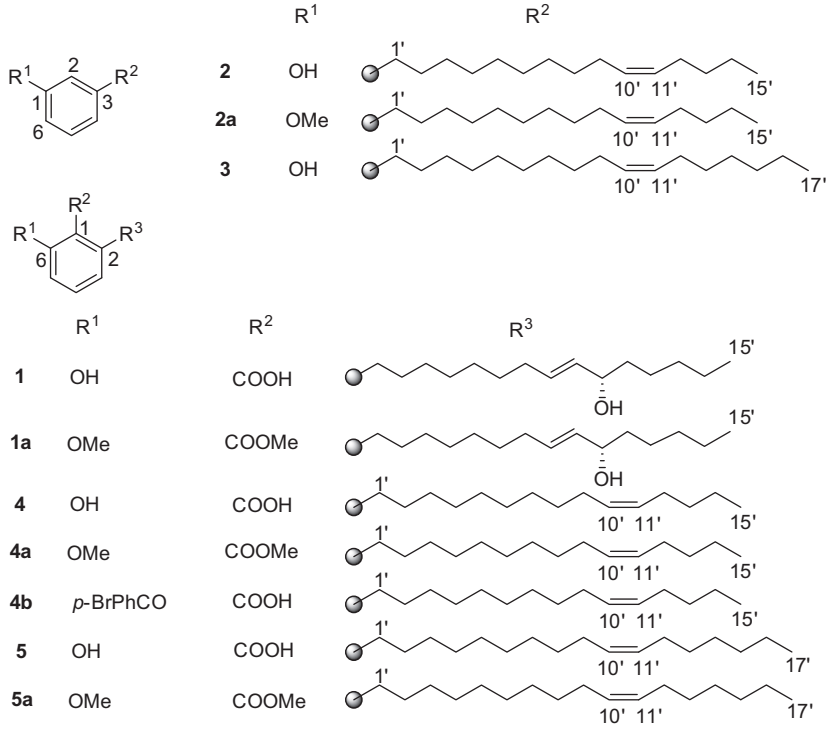
Table 1
^1H and ^{13}C NMR chemical shifts of compounds **1** and **2**

Carbon No.	^{13}C NMR (Ref.)	^{13}C NMR **1**	δ_H (multiplicity)	^{13}C NMR **2**	δ_H (multiplicity) **2**
1	110.5	111.2	—	155.7	—
2	163.6	163.7	—	115.5	6.67 (br, s)
3	115.8	115.9	6.90 (1H, d, *J* = 8.5 Hz)	145.2	—
4	135.3	135.0	7.32 (2H, t, *J* = 8.0, 8.5 Hz)	121.2	6.75 (d, *J* = 7.5 Hz)
5	122.7	122.6	6.84 (1H, d, *J* = 8.0 Hz)	129.6	7.13 (t, *J* = 8.0, 7.5 Hz)
6	147.6	147.6	—	112.7	6.65 (br d)
1′	35.5	36.7	2.99 (2H, t, *J* = 7.5, 4.5 Hz)	36.0	2.56 (t, *J* = 7.5, each)
2′	32.0	34.4	1.59 (2H, m)	32.0	1.62 m
3′	31.8	29.9	1.43 (2H, m)	31.5	1.28–1.33 (br m)
4′	30.9	29.4[a]	1.40 (2H, m)	29.7[a]	
3′	29.7	29.6[a]	1.30 (m)	29.5[a]	
6′	28.8	28.7[a]	1.30 (m)	29.5[a]	
7′	30.9	34.2	2.0–2.1 (2H, m)	28.5[a]	
8′	129.8	132.9	5.50 (1H, dt, $J_{8',9'}$ = 14.5 Hz, $J_{7',8'}$ = 6.5 Hz)	28.4[a]	
9′	129.8	132.7	5.35 (1H, dt, $J_{9',8'}$ = 14.5 Hz, $J_{9',10'}$ = 4.5 Hz)	27.2[b]	2.02–2.03 (m, 4H)
10′	29.7	74.4	4.15 (1H, dd, $J_{10',9'}$ = 7.5 Hz, $J_{10',11'}$ = 4.5 Hz)	130.2	5.37 (dt, *J* = 9.0,4.5 Hz)
11′	28.9	37.3	1.60 (2H, m)	130.1	5.35 (dt, 9.0, 4.5 Hz)
12′	28.9	32.2	1.43 (2H, m)	27.2[b]	2.03 (m, 4H)
13′	27.2!!	31.9	1.33 (2H, m)	27.4	1.28–1.33 (br m)
14′	22.8	22.8	1.30 (2H, m)	22.6	
15′	14.2	14.7	0.90 (dd, *J* = 7.5 Hz)	14.2	0.91 (t. 3H, *J* = 7.0 Hz)
COOH	175.4				

(Ref.) The data of has been taken from Ref. 2k. The data of compounds **1** and **2** are based on 1D and 2D NMR techniques.
[a] Assignments are interchangeable.
[b] Assignments are of overlapping signals.

mass spectrum of the DMDS derived product showed a molecular ion at *m*/*z* 412 along with important fragments ions at *m*/*z* 251 and 161, which confirmed the position of double at C-8′ and C-9′ as shown in Scheme 1. All the spectroscopic data indicated that the compound in hand is (+)-2-hydroxy-6-(10′-hydroxypentadec-8′(*E*)-enyl)benzoic acid (**1**).

Compound **2** was isolated from the hexane soluble fraction of the stem barks of *K. laurina* as a colorless oil. The mass spectrum showed a molecular ion peak at *m*/*z* 302, and prominent peaks at *m*/*z* 220, 300, 147, 134, and 108.[5a] The molecular formula $C_{21}H_{34}O$ was deduced from the HREI-MS at *m*/*z* 302.2604 (calcd 302.2610).



The ^{1}H NMR spectrum showed a triplet at δ_H 0.91 (J = 7.5 Hz), which was assigned to the terminal H-15′ protons. A broad singlet at δ_H 1.28–1.33, which accounted for 16 protons (8 × CH_2), were assigned to the H-3′ to H-8′ as well as to H-13′ to H-14′ methylene protons. Other methylene protons appearing at δ_H 1.62, and 2.56 were attributed to the H-2′ and H-1′, respectively.

A multiplet at δ_H 2.02–203 was attributed to the H-9′ and H-12′ allylic methylene protons, while a broad triplet at δ_H 5.37 ($J_{10',11'} = J_{11',10'} = 9.5$ Hz, $J_{9',10'} = J_{11',12'} = 4.5$ Hz) was assigned to the olefinic H-10′ and H-11′.[2f] A triplet at δ_H 2.56 (J = 7.5 Hz) was due the H-1′ proton. There were four signals in the aromatic region. Two broad singlets at δ_H 6.65 and 6.67 were assigned to the H-2 and H-6 protons, respectively, while a doublet at δ_H 6.75 (J = 7.5 Hz) and a triplets at δ_H 7.13 (J = 8.0 Hz, 7.5 Hz) were assigned to H-4 and H-5 protons, respectively. These assignments were supported by the HSQC, COSY, and HMBC correlations. The ^{13}C NMR downfield signal at δ_C 155.7 (C-1) indicated that only one phenolic carbon is present in compound **2**. Other ^{1}H and ^{13}C NMR data are shown in Table 1.

The configuration of the double bond was assigned as *cis* based on the diagnostic chemical shifts of the allylic methylene carbons at δ_C 27.2 and 28.1.[5b] The characteristic fragment, which appeared at *m*/*z* 108 (base peak) resulting from the benzylic cleavage indicated the presence of phenolic moiety in compound **2**.[4a,5a] Other

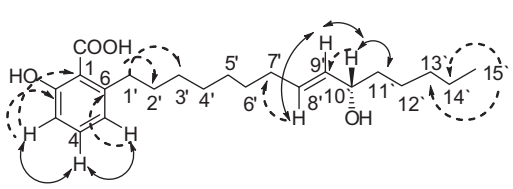
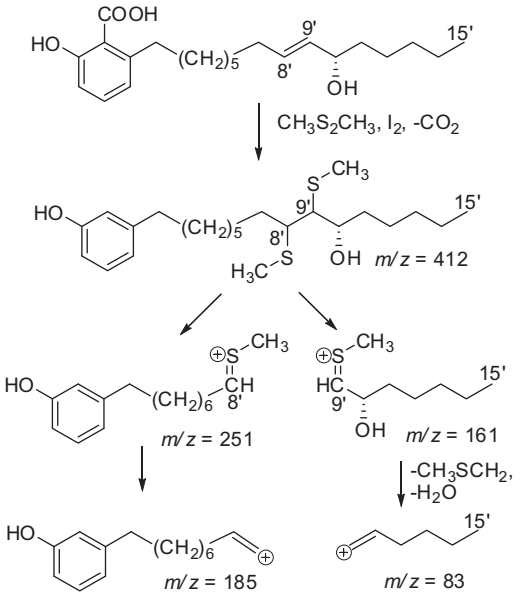


Figure 1. Important COSY (⟷) and HMBC (⋯→) correlations of compound **1**.

major fragment ion at *m*/*z* 206 and 41 were due to allylic cleavages of C-8′ and C-9′ and C-12′ and C-13 bonds (see Fig. 2).

The position of the double bond was further confirmed by the bisthiomethylation followed by MS analysis, which revealed the important fragments ions at *m*/*z* 396, 279, 117, 108, 107, 79, 67, and 55.[4g] The key fragments at *m*/*z* 279 and 117 confirmed the position of double at C-10′ and C-11′ as shown in Scheme 2. On the basis of these spectroscopic data and bisthiomethylation followed by MS analysis compound **2** is assigned as 3-(10′(*Z*)-pentadecenyl)phenol. Compound **2** was previously identified by GC–MS analysis as a mixture along with several cardanols.[5c] This Letter represents the first isolation of pure compound along with confirmation of double bond from *K. laurina*. Compound **4** was also treated with dimethyl disulfide (DMDS) and I_2,[4f,g] and respective fragmentation ions were also observed in its MS. Other known compounds were identified as 3-heptadec-10′(*Z*)-enylphenol (**3**),[5d] 2-hydroxy-6-pentadec-10′(*Z*)-enylbenzoic acid (**4**),[2k,5e] and 2-hydroxy-6-heptadec-10′(*Z*)-enylbenzoic acid (**5**),[5c] based on extensive MS and 2D NMR techniques, as well as by comparison with the previously published data.[5f] All compounds were tested for their AChE inhibitory activity in vitro. Compound **5** exhibited most potent AChE inhibitory activity with IC_{50} 0.573 ± 0.0260 μM (Table 2).



Scheme 1. The mass spectrometric fragmentations of bisthiomethylated product of compound **1**.

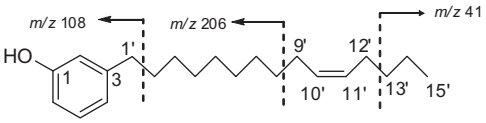
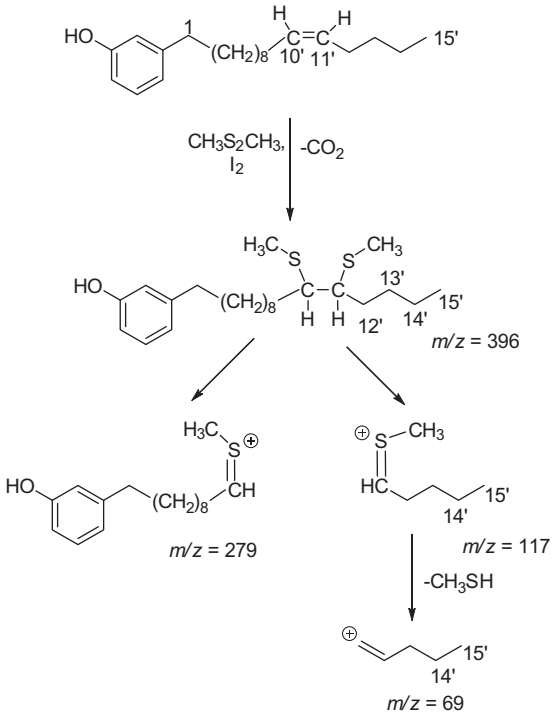


Figure 2. Main mass fragmentations of compound **2**.



Scheme 2. The MS fragmentations of bisthiomethylation adducts of compound **2**.

Compounds containing salicylic acid moiety seemed to demonstrate strong AChE inhibitory activity as represented by compounds **1**, **4**, and **5**, while compounds **2** and **3**, which lacked carboxyl moiety showed slightly a low activity (Table 2), thus suggesting that the acidic group is essential for good AChE activity. The activity dramatically decreased when the acidic and the phenolic OH groups were methylated as displayed by compounds **1a**, **2a**, **4a**, and **5a**, indicating the importance of the acidic and phenolic moieties (Table 2).[5g,h] This decrease of activity may be related to the loss of hydrophilicity of the aromatic terminal in this class of compounds. The brief data of compounds are presented in the References Section.[6a–i]

Table 2
Acetylcholinesterase inhibitory activity of compounds **1**–**5** and their IC_{50} values

Compounds	IC_{50} (μM)
1	3.182 ± 1.097
1a	342.06 ± 9.00
2	17.224 ± 2.231
2a	178.0 ± 3.226
3	13.114 ± 0.550
4	2.172 ± 2.025
4a	358.23 ± 5.441
4b	NA
5	0.573 ± 0.0260
5a	303.21 ± 2.77
Tacrine[a]	0.250 ± 0.001

The IC_{50} values are the mean ± standard deviations of three independent experiments. The inhibitory effects are represented as compounds concd (μM) giving 50% inhibition on AChE activity (IC_{50}).
[a] Tacrine was used as a positive control.

Among the salicylic acid group, **5** displayed the strongest AChE inhibition activity. This compound is fourfold stronger than compound **4**, which has shorter alkenyl side chain by two carbons. Thus, a longer alkenyl side chain in the salicylic acid analogues seemed to increase the activity. The presence of polar hydroxyl group slightly decreased the activity, as shown by compound **1**, thus suggesting that hydrophilicity of this moiety may play an important role binding. Similar trend was also observed in the phenol group. Compound **3**, which contains C_{17} alkenyl side chain exhibited stronger activity than **2**, (with C_{15} alkenyl side chain), although only slightly. Smaller difference in activity between the two analogues may be due to lower potency of this group of compounds. Acetylcholinesterase inhibition was performed using Ellman's protocol.[8a]

Structural insights: The catalytic site of *Torpedo californica* acetylcholinesterase (TcAChE) comprises five important regions, which include the peripheral anionic site (PAS), the esteractic site (CT), the oxyanion hole (OH), the acyl pocket and the anionic subsite (AS).[7a] PAS is located at the surface of the gorge, and consists of Tyr70, Asp72, Tyr121, Tyr334, and Trp279 residues. Several studies revealed that the binding of ligand in PAS region could possibly change the conformation of the active center allosterically,[7b] while recent discovery showed that thioflavin T could act as substrate inhibitor by blocking the entry of acetylcholine (ACh) or departure of any products.[7c] Deep bottom into the narrow gorge, where the active site of the enzyme resides, there are four interesting sites including the CT (Ser200, His440, and Glu327), OH (Gly118, Gly119, and Ala201), acyl pocket (Phe288 and Phe290) and AS (Trp84, Phe330, and Glu199), which are responsible for the hydrolysis of ACh into acetate and choline. The docking methodology as mentioned in Ref. [9a–c].

This conserved residue of Trp84 in the anionic subsite showed some interesting details, where it formed a π–π stacking interaction between the acridine and the inhibitor. The positively charged quarternary group of decamethonium was opposed to the indole group of the residue. The studies revealed that Trp84 served as

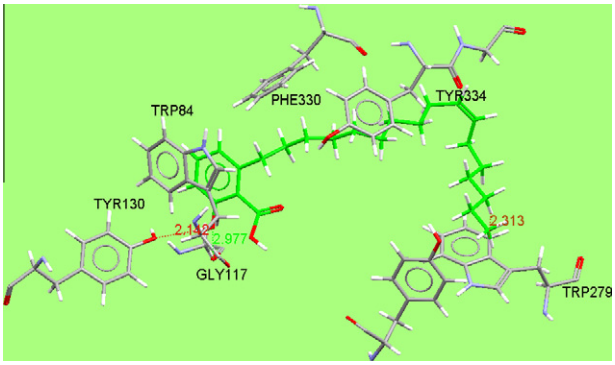


Figure 3. Binding interactions of compound **5** in the catalytic site of TcAChE (green stick = compound **5**). The most important residues involved in the interactions with **5** including the peripheral anionic site (PAS) and anionic subsite (choline-binding subsite).

an important molecular recognition especially for the discovery of new inhibitors.

Based on docking studies of compound **5** (Fig. 3), a phenolic compound with an elongated side chain could possibly penetrate deep into the active site and arrange itself along the complex geometry of the active gorge. Early insight reveals the flexibility of the long alkyl chain could demonstrate a similar arrangement as decamethonium. The alkyl linker is well placed along the narrow aromatic gorge starting from the surface until the anionic active site where Trp84 residue is located (Fig. 4). According to the biological results, compounds (**1**–**5**) showed potential inhibition against AChE, which could be due to the presence of hydroxyl group at C-2 in the phenyl ring. This is further strengthened by the docking results, the importance of this hydroxyl group which binds to the carbonyl oxygen of Gly117 through hydrogen bonding at the distance of 2.977 Å and could help to position the phenyl ring to involve in a π–π stacking interaction with Trp84. This type of interaction is common in some of the AChE inhibitors along with

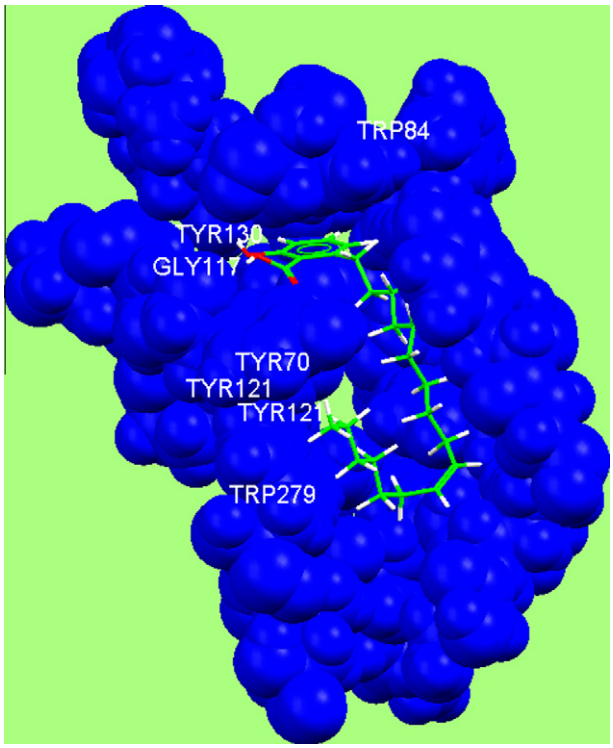


Figure 4. Molecular interactions of compound **5** with the residues in the gorge of the catalytic site of TcAChE (in green space filled structure).

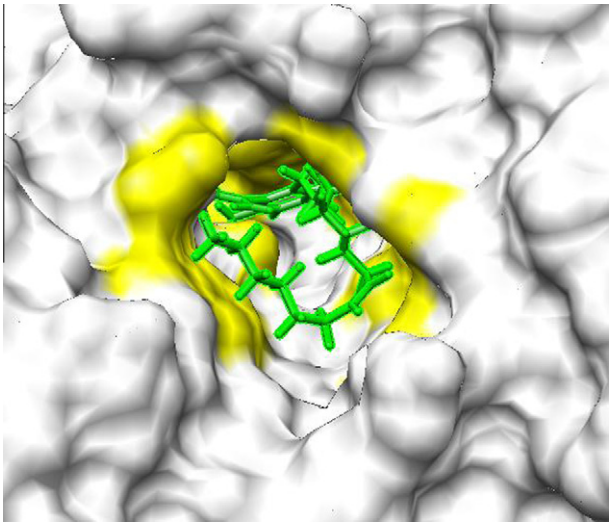


Figure 5. Interatomic contacts (yellow) of the ligand with the residues in the active site based on Van der Waals radii generated by Chimera software.

the interactions between quaternary nitrogen of endrophonium[7d] and decamethonium and the stacking of acridine with indole of Trp84. Interestingly the hydroxyl group is also involved in a short contact with Tyr130 at the distance of 2.142 Å. In another example, their inhibition are totally diminished once the hydroxyl group is methylated, preventing the free hydrogen to participate in the above hydrogen bonding. However, none of the 10 best poses generated by docking of compound **5**, make any important binding in the active site of CT, OH and acyl pocket possibly due to the bulkiness of the whole structure. Compound **5** could not be considered as a bivalent ligand due to its inability to occupy the ES region.[7e] The occupancy of alkyl group of **5** in the peripheral site could act as an anchor while permitting the penetration of the alkyl chain and the aromatic residues in the gorge. These hydrophobic interactions between C15′, 16′, and 17′ of **5** with PAS residues Trp279, Trp70 and Tyr121 are made accessible due the presence of *cis* (*Z*) configured double bond moiety at C-10′. This double bond could be directing the alkyl group to twist towards PAS at the surface of the gorge. The whole molecule eventually appears to be in L-shaped (Fig. 5). Short contact is also observed between H-15 and the aromatic Trp 279 at distance of 2.313 Å. Overall, the mechanism of inhibition for compound **5** is based on its capability of blocking the path leading to the active site while docking studies showed that compound **5** interacted on the basis of π–π interaction, H-bonding, and hydrophobic contacts with some critical residues in the active site. Failure to occupy the esteractic site explains its incapability to match the inhibition activity demonstrated by tacrine. However, the potential of this compound should not be undermined especially since it could serve as a potential candidate in combating AChE related diseases.

In conclusion, a new potential class of AChE inhibitor has been isolated from a plant, *K. laurina*, and identified based spectrometric techniques. Molecular docking experiment suggested a unique structural alignment of the substrate to suit the active gorge site. Alkenylsalicylic acid may provide a new template for further structural modification into a new drug.

Acknowledgments

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References and notes

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6. (a) The stem bark of *K. laurina* (6.2 kg) was collected from the Pasir Raja Forest Reserve, Trengganu, Malaysia, in May 2008. The voucher specimen SK 1540/08 was deposited at the Herbarium of the Institute of Bioscience (IBS), University Putra Malaysia. The plant was identified by the Resident Botanist of IBS. The stem barks were cleaned, cut into small pieces and left under the shade for 5 days at room temperature. The dry pieces were ground to powder and then steeped in distilled methanol (10 L) at room temperature for 2 days. The methanol extract was then filtered off and the filtrate was concentrated under reduced pressure. This procedure was repeated three times and the extracts were combined to yield 90.1 g of crude methanolic extract. The combined extract was then fractionated between water and organic solvents (sequentially: hexane; dichloromethane; ethyl acetate and finally butanol) to yield the hexane (20.2 g), DCM (3.64 g), EtOAc (7.41 g), BuOH (6.90 g) and aqueous (45.8 g) soluble fractions. The hexane soluble fraction was subjected to silica gel column chromatography (230–400 mesh, ASTM, Merck) using the gradient solvent system hexane/EtOAc (8:2 to 6.5:3.5) to yield seven sub-fractions (kl-1 to kl-7). Subfractions kl-3 (1.20 g) and kl-4 (0.09 g) were separately subjected to repeated column chromatography (230–400 mesh) by eluting it gradiently using hexane/EtOAc (9:5 to 5.5:4.5) as solvent system, to yield compounds **2** (45.5 mg) and **3** (57.0 mg), respectively. Using the same elution system, subfraction kl-5 (1.80 g) was subjected to repeated silica gel column chromatography (230–400 mesh) to obtained compounds **4** (90.4 mg) and **5** (21.0 mg). The semi pure compounds (**2**–**5**) were purified by HPLC [mobile phase: ACN/H_2O (40:60 to 45:55; UV: 254 nm; flow rate: 4.00 ml/min; column Xterra® Prep MS C_{18} OBD™ (19 × 150 mm, 5 μm)], which led to the purification of **5** (t_R: 11.2 min), **4** (t_R: 11.7 min), **3** (t_R: 16.3 min) and **2** (t_R: 16.8 min).; (b) *Physical and spectroscopic data of compounds: (a) (+)-2-hydroxy-6-(10′-hydroxypentadec-8′(E)-enyl)benzoic acid* (**1**): colorless oil, $[\alpha]_D^{25}$ +23.2, $CHCl_3$). $UV_{\lambda_{max}}^{MeOH}$ nm (log ε): 306 (3.9), 288 (3.3), 272 (2.3), 227 (1.8). IR ν_{max} ($CHCl_3$) cm^{-1}; 3616–3420, 2920, 2860, 1648, 1455, 786. El-MS *m*/*z* (rel. int.): 362 [M^+], HREI-MS: 362.2667 (362.2669 calcd for $C_{22}H_{34}O_4$), 344, 326 (29), 330 (15), (22), 302 (34), 285 (10), 257 (10), 227 (11), 175 (20), 161 (24), 152 (50), 147 (42), 134 (37), 120 (10), 108 (50), 91 (100), 82 (19), 55 (37). ^1H and ^{13}C NMR data (Table 2).; (c) *(+)-2-Methoxy-6-(10′-hydroxypentadec-8′(E)-enyl)benzoic acid methyl ester* (**1a**): In a 25 ml round bottomed flask, compound **1** (5.0 mg) was dissolved in $CHCl_3$ and few drops of diazomethane were added. After drying compound **1a** was obtained. Colorless oil, El-MS *m*/*z* (rel. int.): 390 [M^+], HREI-MS, 390.3254 (calcd 390.3248 for $C_{24}H_{38}O_4$), 362, 344, 326, 330, 245, 175, 161, 121, 91, 81, 55. IR ν_{max} ($CHCl_3$) cm^{-1}; 3205, 2824, 1650, 1580, 1450. ^1H NMR (500 MHz, DMSO-d_6): δ 7.30 (t, *J* = 8.0 Hz, 8.5Hz, H-4), 6.90 (d, *J* = 8.5 Hz, H-3), 6.87 (d, *J* = 8.0 Hz, H-5), 5.50 (dt, $J_{8',9'}$ = 14.5, 6.5 Hz, H-8′), 5.36 (dt, $J_{9',8'}$ = 14.5, 4.5 Hz, H-9′), 4.47 (d, $J_{10',9'}$ = 4.5 Hz), 3.79 (s, 3H, OMe), 3.72 (s, 3H, COOMe), 2.56 (t, *J* = 7.5 Hz, C-1′), 1.99 (m, 2H, C-7′), 1.50 (m, 2H, C-11′), 1.42–1.40 (br m, 6H, 3 × CH_2, C-3′ to C-4′ and C-12′), 1.31–129 (br m, 8H, 4 × CH_2, C-5 to C-6′ and C-13′ to C-14′), 0.89 (t, *J* = 7.5 Hz, 3H, C-15′).; (d) *Bisthiomethylation of compound* **1**: Compound **1** (3.0 mg) was dissolved in dimethyl disulfide (3 ml) in a 25 ml single necked round bottom flask and iodine (10 mg) was added. The reaction mixture was kept at 50–60 °C for overnight. The reaction mixture was diluted with 5 ml diethyl ether and excess iodine was decomposed with 5% sodium thiosulfate solution. The diethyl ether layer was separated and dried over sodium sulfate anhydrous and subjected to GC–MS analysis, which showed the peaks at *m*/*z* = 412, 396, 394, 251, 185, 161, 108, and 83 (Scheme 1).; (e) *3-(pentadecen-10′(Z)-yl)phenol* (**2**): Colorless oil; $UV_{\lambda_{max}}^{MeOH}$ nm (log ε): 277 (2.8), 272 (2.6): 222 (3.0); IR ν_{max} ($CHCl_3$) cm^{-1}, 3418–3220, 2928, 2809, 1610, 1455, 890. EI-MS *m*/*z* (rel. int.): 302 [M^+], HREI-MS, *m*/*z* 302.2604 (calcd 302.2610 for $C_{21}H_{34}O$), 302, 276, 220, 206, 175, 161, 147, 133, 121, 108 (100%), 77, 43. ^1H and ^{13}C NMR $CDCl_3$ (125 MHz) (Table 2).; (f) *3-Methoxypentadec-(10′(Z)-enyl)-benzene* (**2a**): Compound **2** (10 mg) was dissolved in diethyl ether at room temperature, and into it few drops of diazomethane was added. After drying the sample in a fume hood the compound **2b** was obtained as colorless oil. El-MS *m*/*z* (rel. int.): 316 [M^+], 302, 276, 220, 206, 175, 161, 147, 133, 123, 121, 108 (100%), 77, 55. IR ν_{max} ($CHCl_3$) cm^{-1}: 3252–3160, 2930–2936, 1620, 1456, 1289, 780. ^1H NMR (500 MHz, $CDCl_3$): δ 7.13 (t, *J* = 8.0, 7.5 Hz, H-5), (d, 6.66 (br d, IH, C-2), 6.75 (d, *J* = 7.5 Hz, H-4), 6.65, (br d, 1H, H-6), 3.73 (s, 3H, OMe), 2.55 (t, 7.5, each, H-1′), 2.02 (m, 4H, H-9′ and H-12′), 1.62 (m, 2H, H-2′), 1.27–1.30 (br m, 16H, 8 × CH_2, H-3′ to C-8′ and H-13′ to H-14′), 0.90 (t, 3H, *J* = 7.5, H-15′).; (g) *Bisthiomethylation of compound* **2**: Dimethyl disulfide (DMDS) adduct was prepared by taking 5 mg of compound **2** in a 25 ml round bottle flask containing 10 ml hexane. About 1.5 ml dimethyl disulfide was added into it followed by 20 mg of iodine. The reaction mixture was stirred at 50–60 °C for overnight. The diethyl ether layer was separated and dried over sodium sulfate anhydrous and subjected to GC–MS analysis. Fragmentation peaks observed include *m*/*z* = 396, 279, 117, 108, 107, 69, and 55.; (h) *2-Hydroxy-6-(pentadec-10′(Z)-enyl)benzoic acid* (**4**): Colorless oil, IR ν_{max} ($CHCl_3$) cm^{-1}; 3510–3228, 2920, 2860, 1650, 1609, 1580, 1450. El-MS *m*/*z* (rel. int.): 346 [M^+], $C_{22}H_{34}O_3$, (55), 328 (29), 320 (15), 310 (22), 302 (34), 285 (10), 257 (10), 227 (11), 175 (20), 161 (24), 152 (50), 147 (42), 134 (37), 120 (10), 108 (50), 91 (100); ^1H NMR (500 MHz, $CDCl_3$): δ 7.36 (t, *J* = 8.0, 8.5 Hz, each, H-4), 6.87 (dd, *J* = 8.0 Hz, H-3), 6.76 (dd, *J* = 8.5 Hz, H-5), 5.35 (centre of AB system of H-10′ and H-11′ $J_{10',11'} = J_{11',10'}$ = 9.0 Hz, $J_{9',10'} = J_{11',12'}$ = 6.5 Hz), 2.98 (d, *J* = 7.5 Hz, C-1′), 2.01 (m, 4H, C-9′ and C-12′), 1.60 (m, 2H, C-2′), 1.41–1.40 (br m, 8H, 4 × CH_2, C-3′ to H-4′ and C-13′ to C-14′), 1.30–1.29 (br m, 8H, 4 × CH_2, C-5′ to C-8′), 0.91 (t, *J* = 7.5 Hz, 3H, C-15′).; (i) *2-Hydroxy-6-(pentadec-10′(Z)-enyl)benzoic acid methyl ester* (**4a**): Compound **4** (10 mg) was treated with ethereal solution of diazomethane and methylated compound **4a** was purified by evaporation of solution and passing through silica gel funnel. The EI-MS showed *m*/*z* 374.2 [M^+], $C_{24}H_{38}O_3$, [M^+], (55), 328 (29), 320 (15), 310 (22), 302 (34), 285 (10), 257 (10), 227 (11), 175 (20), 161 (24), 152 (50), 147 (42), 134 (37), 121 (10). ^1H NMR (500 MHz $CDC1_3$): δ 7.36 (t, *J* = 8.0, 8.5 Hz, H-4), 6.87 (dd, *J* = 8.5 Hz, H-3), 6.76 (dd, *J* = 8.0 Hz, H-5), 5.35 (centre of AB system of H-10′, $J_{10',11'} = J_{11',10'}$ = 9.0 Hz, $J_{9',10'} = J_{11',12'}$ = 6.5 Hz, H-10′ and H-11′), 3.80 (s, 3H, OMe), 3.74 (s, 3H, COOMe), 2.98 (d, *J* = 7.5 Hz, 2H, C-1′), 2.01 (m, 4H, C-9′ and C-12′), 1.60 (m, 2H, C-2′), 1.41–1.39 (br m, 8H, 4 × CH_2, C-3′ to H-4′, C-13′ to C-14′), 1.31–1.29 (br m, 8H, 4 × CH_2, C-5′ to C-8′), 0.90 (t, *J* = 7.5 Hz, 3H, C-15′).
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8. *In vitro cholinesterase inhibition assay*: Acetylcholinesterase inhibiting activity was measured by the spectrophotometric method of Ellman et al.[8a] Electric eel AChE (type VI-S, electric eel, Sigma Chemical Co. code: C2888), were used as sources of the cholinesterases. Acetylthiocholine iodide (Sigma Chemical Co. [ATCI] code: A5751) were used as substrates of the reaction, and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) (Sigma Chemical Co. Code: D21200, 99%) were used for the measurement of the cholinesterase activity. In this procedure; 200 μl of 0.15 mM sodium phosphate buffer pH 7.4 with 10 μl of DTNB (0.15 mM), 20 μl of test compound solution, and 20 μl of acetylcholinesterase (final concentration 0.037 U/ml in 0.1 M phosphate buffer solution) were mixed and incubated for 10 minutes at 25 °C. The reaction was then started by the addition of 20 μl of acetylthiocholine iodide (0.25 mM, substrate). The final volume of the assay in each well was 270 y monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine released by the enzymatic hydrolysis of acetylthiocholine at a wavelength of 412 nm at 25 °C monitored for 3 min. Test compounds were dissolved in analytical grade DMSO. The same volume 270 μl was used for negative control by the addition of 20 μl of buffer instead of sample. All reactions were carried out in triplicate. The concentration used in μM, percentage inhibition was calculated in Microsoft excel and IC_{50} were calculated by using graph Pad software. (a) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.

9. *Computational study*: Protein crystal structure with PDB code 1ACL[9a] was obtained from the protein data bank followed by docking studies using GOLD4.14.[9b] The ligands were constructed and energy minimized using MOPAC program with MMFF94 force field calculation. The binding site was defined based on the cavity detection by GOLD centered on the reference ligand and the atoms selection was restricted to solvent-accessible surface. All other parameters were set up according to the standard protocols of GOLD. The standard docking procedure includes the use of genetic algorithm[9c] to explore the full range of ligand conformational flexibility with the partial flexibility of protein while the scoring function includes the terms for hydrogen-bonding, vdW, and intramolecular energies. Validity of the docking was justified from the redocking of the crystal ligand (decamethonium) into the active site. Finally, the ligand was superimposed on the reference crystal structure with a RMSD of 1.8 Å (good pose <2.0 Å) which justified the ability of the software to produce correct solution. Ten top ranked poses results were saved and evaluated. The highest GOLD fitness score was chosen as a resultant coordinate. (a) Harel, M.; Schalk, I.; Ehret-Sabatier, L.; Bouet, F.; Goeldner, M.; Hirth, C.; Axelsen, P. H.; Silman, I.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9031; (b) Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Taylor, R. D. *Proteins* **2003**, *52*, 609; (c) Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. *J. Mol. Biol.* **1997**, *267*, 727.