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Microbial transformation of (−)-isolongifolol and butyrylcholinesterase inhibitory activity of transformed products

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Abstract—The microbial transformation of (−)-isolongifolol (**1**) by using the standard two-stage fermentation technique with *Fusarium lini* afforded polar oxygenated metabolites: 10-oxoisolongifolol (**2**), 10α-hydroxyisolongifolol (**3**), and 9α-hydroxyisolongifolol (**4**). Metabolites **3** and **4** were also formed with the incubation of **1** with *Aspergillus niger*. All three metabolites were found to be new. Compounds **3** and **4** inhibited butyrylcholinesterase enzyme in a concentration-dependent manner with IC_{50} values 13.6 and 299.5 μM, respectively. Compound **3** showed un-competitive mode of inhibition against butyrylcholinesterase with K_i value 15.0 μM. The structures of metabolites **2**–**4** were deduced on the basis of spectroscopic techniques and single-crystal X-ray diffraction techniques.

1. Introduction

A large number of sesquiterpenes with a bridged tricyclic skeleton have been isolated from plants and fungi, some of them have exhibited interesting biological properties.[1] Very few reports about the fungal transformations of bridged tricyclic sesquiterpene skeleton have been reported.[2] In continuation of our investigations on the biotransformation of bioactive natural and synthetic products,[3] we have now studied the biotransformation of a bridged tricyclic sesquiterpene, (−)-isolongifolol (**1**), a derivative of longifolene, which is found in terpentine oil and produced commercially from the oleoresin of Himalayan Pine, *Pinus longifolia* Roxb.[4] Incubation of compound **1** with *Fusarium lini* and *Aspergillus niger*, afforded polar oxygenated metabolites **2**–**4**. Compounds **3** and **4** were obtained in good yields and subjected to different bioassays in order to evaluate their biological activities. Compound **3** showed potent butyrylcholinesterase inhibitory activity with an IC_{50} value of 13.6 μM. It has been found that butyrylcholinesterase inhibitors may act as potential lead in the discovery of clinically useful agents for the treatment of nervous system disorders, particularly by reducing memory deficiency in Alzheimer's patients by potentiating and affecting the cholinergic transmission process.

2. Results and discussion

2.1. Chemistry

Small scale screening experiments have shown that the *Fusarium lini* and *Aspergillus niger* were capable of converting compound **1** into polar metabolites. Preparative scale fermentation was therefore carried out to produce sufficient quantities of metabolites for structure elucidation. Compound **1** ($C_{15}H_{26}O$) was incubated with two-day old stage II cultures of *Fusarium lini* and *Aspergillus niger*, and two major (**3**–**4**) and one minor (**2**) products were detected by TLC analysis.

Compound **2** was obtained as a liquid, which showed the M^+ at *m*/*z* 236.7321 in the HREI MS, corresponded with the molecular formula $C_{15}H_{24}O_2$ (calcd. 236.7326), 14 amu higher than that of **1** and an additional

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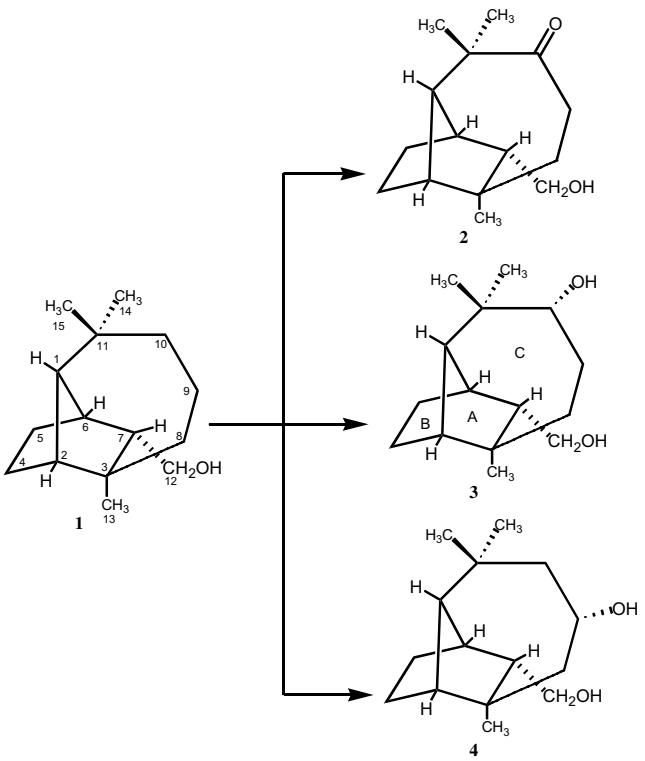
unsaturation than that of **1**. The IR spectrum of **2** showed absorptions for a hydroxyl (3407 cm^{-1}) and carbonyl groups (1731 cm^{-1}). These observation indicated an introduction of keto-functionality in the molecule (Scheme 1).

The ^{1}H NMR spectrum of compound **2** showed C-11 geminal methyl groups resonated at δ 1.10 (s, Me-14) and 1.08 (s, Me-15), and C-9 methylene protons (at δ 2.78 and 2.40). The ^{13}C NMR spectrum of **2** exhibited resonances for 15 carbons; including three $-CH_3$, five $-CH_2$, four –CH, and three –C– carbons. An additional oxygenated quaternary carbon appeared at δ 216.4 (C-10). The HMBC spectrum of **2** showed two-bond correlation between 2H-9 (δ 2.78 and 2.40)/C-10 and three-bond correlations between 2H-8 (δ 1.92 and 1.55), 3H-14 (δ 1.10)/C-10, further supporting the structure as 10-oxoisolongifolol (**2**).

Compound **3** was obtained as a colorless crystalline compound. The HREI MS showed the M^+ at *m/z* 238.1611 in agreement with the formula $C_{15}H_{26}O_2$, (calcd. 238.1627). The IR spectrum of compound **3** showed an absorption at 3325 cm^{-1}, indicating the presence of hydroxyl groups. The ^{1}H NMR spectrum showed an additional oxygen-bearing methine proton signal at δ 3.92 (d, J = 11.3 Hz), indicating the introduction of a new hydroxyl group either at C-10 or C-8. The ^{13}C NMR of compound **3** also exhibited a signal for an oxymethine carbon at δ 75.7, which was found to be interacting with Me-14 (δ 1.07) and Me-15 (δ 0.97) in the HMBC spectrum, indicating the hydroxylation at C-10.



Scheme 1. Metabolism of Compound **1** by *Fusarium lini*.

The COSY 45° spectrum showed the homonuclear couplings between H-10 (δ 3.92) and H_2-9 (δ 1.98 and 1.53). The stereochemistry was investigated by NOESY experiment, which showed interaction of H-10 with βMe-15, indicating an α-orientation of the new hydroxyl group. Single-crystal X-ray crystallography technique was finally used to unambiguously assign the structure as 10α-hydroxyisolongifolol (**3**).

The structure of tricyclic triterpenoid **3** was deduced from X-ray diffraction studies. All the molecular dimensions, bond angles, and bond lengths were found to be within the normal range. The fused ring A (C-1, C-2, C-3, C-7, C-6) in the main frame of molecule adopts half chair conformation while the six membered ring B (C-2, C-3, C-7, C-6, C-5, C-4) was found to be in slightly distorted chair conformation. The seven membered ring found to have similar to a boat conformation. The three dimensional diagram of the compound **3** indicated an α-orientation of C-10 hydroxyl group with respect to C-7 hydroxy methylene substituent (see Fig. 1).

Compound **4** was obtained as a white crystalline solid. The HREI MS showed the M^+ at *m/z* 238.1639 ($C_{15}H_{26}O_2$, calcd. 238.1627). The IR spectrum showed an absorption at 3368 cm^{-1} (OH). The ^{1}H NMR spectrum showed a downfield signal at δ 3.99 (IH, m) that could be assigned to OH-bearing methine proton. The ^{13}C NMR spectra exhibited 15 carbon signals with three $-CH_3$, five $-CH_2$, five –CH, and two –C– atoms. An oxymethine carbon was appeared at δ 67.0, which further supported the hydroxylation of compound **1**. The 2D NMR techniques (COSY 45°, HMQC, and HMBC) further supported a hydroxyl group at C-9. The COSY 45° spectrum showed the interactions of H-9 (δ 3.99) with H-10 (δ 1.92 and 1.41) and H_a-8 (δ 2.01), while HMBC spectrum showed the interactions between H-9/C-11 (δ 38.1) and C-8 (δ 49.2); Me-15 (δ 0.99)/C-9(δ 67.0); H-10 (δ 1.92 and 1.41)/C-11 and C-9. The NOESY correlations between β Me-15 and H-9 and X-ray

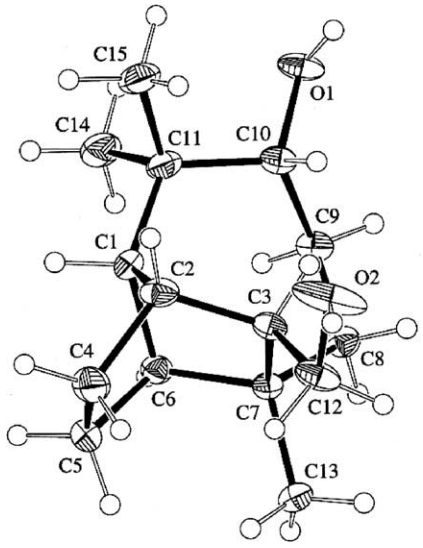


Figure 1. Computer-generated ORTEP diagram of the final X-ray model of compound **3**.

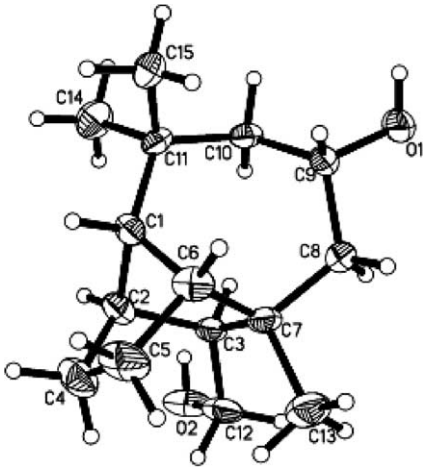


Figure 2. Computer-generated ORTEP diagram of the final X-ray model of compound **4**.

diffraction studies indicated an α-orientation of C-9 hydroxyl group.

The X-ray diffraction studies have been used to unambiguously establish the structure of compound **4**. All the bond angles and bond lengths were found to be in normal range. The newly formed hydroxyl group at C-9 position was found to be α-oriented with respect to C-7 hydroxymethylene substituent. The tricyclic fused rings A, B, and C in the main skeleton of the molecule adopt similar conformations as described for compound **3**. The only difference was a twisted chair form of six-membered ring B (C-2, C-3, C-7, C-6, C-5, C-4) (see Fig. 2).

The major metabolites **3** and **4**, showed inhibitory activity against butyrylcholinesterase with IC_{50} values 13.6 ± 0.5 and 299.5 ± 1.2 μM, respectively. Compound **1** was found to be inactive. Metabolite **2** cannot be screened because of the insufficient amount. Eserine, with an IC_{50} value 0.85 ± 0.001 μM, was used as a positive control in this assay. Kinetics study of compound **3** showed uncompetitive mode of inhibition against BChE with K_i value 15.0 ± 0.5 μM.

The higher inhibitory potential of compound **3** may be due to the presence of an OH group at C-10. The hydroxyl group at this position might be involved in the hydrogen bonding with the amino acid residues present in the active site of the enzyme.

3. Materials and methods

3.1. General

IR Spectra were recorded in $CHCl_3$ on FTIR-8900 spectrophotometer. MPs were determined on Büchi 535 melting point apparatus. Optical rotations were measured on Jasco DIP 360 digital polarimeter. UV spectra were recorded in MeOH on Hitachi U-3200 spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on Bruker Avance-500 NMR at 500 and 125 MHz, respectively, chemical shifts δ in ppm rel. to $SiMe_4$ as internal standard, coupling constants J in Hz. The EI MS and HREI MS were measured on Jeol JMS-600H mass spectrometer. TLC was performed with Si gel Merck precoated plates (PF_{254}, 20×20, 0.25 mm). Compound **1** was purchased from Sigma Aldrich.

3.2. Fungus and culture conditions

Stock cultures of the fungus, obtained from American Type Culture Collection (ATCC) and Northern Regional Research Laboratories (NRRL) were stored on Saboraud dextrose agar at 4 °C prior to use. The medium for *Aspergillus niger* (ATCC 10549) was prepared by adding the following chemicals into distilled H_2O (3.0 L): tartaric acid (105.0 g), sucrose (15.0 g), KH_2PO_4 (6.0 g), $MgSO_4{\cdot}2H_2O$ (3.0 g), NH_4NO_3 (6.0 g), and $Zn(OAc)_2$ (0.08 g), 0.04 N NaOH was used to increase the pH to 6.0. The medium for *Fusarium lini* (NRRL 68751) (3.0 L, distilled H_2O) contained: glucose (30.0 g), glycerol (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g). The fermentation medium thus obtained was distributed among 30 flasks of 250 mL capacity (100 mL in each) and autoclaved.

3.3. General fermentation and extraction conditions

The fermentation was carried out according to a standard two-stage protocol.[5] Compound **1** was dissolved in ethanol. The resulting clear solution was evenly distributed among 30 flasks (20 mg/0.5 mL in each flask) containing 24 h-old stage II cultures and fermentation was carried out for further additional time on a rotatory shaker (200 rpm) at 29 °C. During the fermentation time period, aliquots from one culture was taken daily and analyzed by TLC in order to determine the degree of transformation of the substrate. In all experiments, one control flask without biomass (for checking substrate stability) and one flask without exogenous substrate (for checking endogenous metabolite) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (1 L) and the filtrate extracted with CH_2Cl_2 (3×2 L). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressures, and analyzed by thin layer chromatography. Control flasks were also harvested and compared with test by TLC, to confirm the bio-transformation.

3.4. Fermentation of isolongifolol (1) with *Fusarium lini*

Compound **1** (600 mg) in ethanol (15 mL) was evenly distributed among 30 flasks containing stage-II cultures. The fermentation was allowed to process for 10 days after the final feed. The mycelial cells were filtered from the extraction of broth with CH_2Cl_2 obtaining a brown gum (1.23 g), which was purified using column chromatography. Elution with 19% ethyl acetate in petroleum ether afforded compound **2** (7.3 mg), and further elution yielded compounds **3** (98 mg, 33% ethyl acetate in

petroleum ether) and **4** (132.8 mg, 49% ethyl acetate in petroleum ether).

3.4.1. 10-Oxoisolongifolol (2). Colorless liquid. $[\alpha]_D^{25} = -221$ (*c* 0.23, $CHCl_3$). UV (MeOH): λ_{max} (log ∈) nm = 204 (3.2). IR ($CHCl_3$): ν_{max} = 3407, 2965, 2873, 1731, 1652 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): see Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 2. EI-MS: *m/z* = 236 (47, M^+), 218 (13, $[M-H_2O]^+$), 178 (51), 150 (71), 135 (70), 107 (35), 85 (100), 55 (42). HREI-MS: *m/z* = 236.7321 ($C_{15}H_{24}O_2$, calcd. 236.7326).

3.4.2. 10α-Hydroxyisolongifolol (3). Colorless crystalline solid. Mp 144–145 °C. $[\alpha]_D^{25} = -172$ (*c* 0.5, $CHCl_3$). UV (MeOH): λ_{max} (log ∈) nm = 201 (3.5). IR ($CHCl_3$): ν_{max} = 3417, 3325, 2965, 2891 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): see Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 2. EI-MS: *m/z* = 238 (11, M^+), 220 (27, $[M-H_2O]^+$), 174 (61), 144 (25), 133 (51), 109 (35), 85 (100), 55 (65). HREI-MS: *m/z* = 238.1611 ($C_{15}H_{26}O_2$, calcd. 238.1627).

3.4.3. 9α-Hydroxyisolongifolol (4). White crystalline solid. Mp 151–152 °C. $[\alpha]_D^{25} = -139$ (*c* 0.41, $CHCl_3$). UV (MeOH): λ_{max} (log ∈) nm = 203 (2.6). IR ($CHCl_3$): ν_{max} = 3368, 2965, 2881 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): see Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 2. EI-MS: *m/z* = 238 (47, M^+), 218 (13, $[M-H_2O]^+$), 178 (51), 150 (71), 137 (62), 109 (24), 85 (100), 55 (39). HREI-MS: *m/z* = 238.1639 ($C_{15}H_{26}O_2$, calcd. 238.1627).

Table 1. The ^1H NMR data for compounds **2**–**4** (500 MHz; $CDCl_3$)

H	2	3	4
1	1.67, m	1.41, br s	1.46, m
2	1.97, m	1.94, m	1.90, m
4	1.47, m, 1.35, m	1.28, m, 1.15, m	1.41, m, 1.32, m
5	1.65, m, 1.42, m	1.59, m, 1.37, m	1.61, m, 1.22, m
6	2.32, d, (3.9)	2.27, m	2.22, m
7	2.28, (2.9)	2.23, d (2.7)	2.14, m
8	1.92, br t (14.5); 1.55, m	1.57, m, 1.38, m	2.01, m, 1.53, m
9	2.78, dt, (14.1) 2.40, dd, (6.45, 1.87)	1.98, m, 1.53, m	3.99, m
10	—	3.92, d, (11.3)	1.92, m, 1.41, m
12	3.72, dd, (10.8, 7.2) 3.60, dd, (10.7, 7.3)	3.64, 2H, m	3.67, 2H, m,
13	1.03[a], s	0.83, s	0.88, s
14	1.10, s	1.07, s	1.08, s
15	1.08[a], s	0.97, s	0.99, s

^1H–^1H COSY, ^{13}C–^1H HMQC, and ^{13}C–^1H HMBC experiments were recorded for complete assignments of all protons and carbons.
[a] These values can be interchanged.

Table 2. The ^{13}C NMR data of new metabolites **2**–**4** (125 MHz; $CDCl_3$)

Carbon	2	3	4
1	62.9, d	62.6, d	61.4, d
2	45.4, d	45.3, d	45.6, d
3	29.7, s	29.0, s	31.7, s
4	21.5, t	21.1, t	20.9, t
5	26.4, t	25.5, t	25.8, t
6	40.7, d	40.1, d	40.4, d
7	46.4 d	47.2 d	50.4 d
8	39.7, t	41.9, t	49.2, t
9	45.6, t	39.0, t	67.0, d
10	216.4, s	75.7, d	54.1, t
11	33.5, s	33.5, s	38.1, s
12	60.4, t	61.2, t	60.8, t
13	15.2, q	15.1, q	22.9, q
14	30.9[a], q	18.4, q	32.3, q
15	30.2[a], q	22.1, q	30.6, q

[a] Values are interchangeable. Carbon multiplicities were determined by DEPT experiments; s = quaternary, d = methine, t = methylene, q = methyl.

3.5. Fermentation of isolongifolol (1) with *Aspergillus niger*

The procedure was similar to that described above from *Fusarium lini*. From 600 mg of compound **1**, EtOAc extract (1.96 g) was obtained after 8 days, which was subjected to column chromatography on silica gel with gradient fraction of ethyl acetate–petroleum ether to obtain compounds **3** (71 mg, petroleum ether–EtOAc, 69:31) and **4** (64 mg, petroleum ether–EtOAc, 54:46) Table 3.

3.6. In vitro butyrylcholinesterase inhibition assay

Horse-serum butyrylcholinesterase (E.C 3.1.1.8), butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), and eserine were purchased from Sigma (St. Louis, MO, USA). Buffer and other chemicals were of analytical grade. Butyrylcholinesterase inhibition activity was measured by the spectrophotometric method developed by Ellman et al.[6] The reaction mixture contained 150 μL of (100 mM) sodium phosphate buffer (pH 8.0), 10 μL of DTNB, 10 μL of test-compound solution and 20 μL of butyrylcholinesterase solution, mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μL butyrylthiocholine (substrate). The hydrolysis of butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of butyrylthiocholine at a wavelength of 412 nm (15 min). Test compounds and the control were dissolved in EtOH. All the reactions were performed in triplicate in 96-well micro-plate on *SpectraMax 340* (Molecular Devices, USA). The percentage (%) inhibition was calculated as $(E - S)/E \times 100$, where E is the activity of the enzyme without test compound and S is the activity of enzyme with test compound.

Table 3. In vitro quantitative inhibition of butyrylcholinesterase by compounds **3** and **4**

Compounds	IC_{50} ± SEM[a]	K_i[b] ± SEM	Type of inhibition
3	13.6 ± 0.5	15.0 ± 0.5	Uncompetitive
4	299.5 ± 1.2	—	—
Eserine[c]	0.85 ± 0.0001	0.92 ± 0.01	Noncompetitive

[a] Standard mean error of 3–5 assays.
[b] K_i is the mean of three values calculated by using the Dixon and Lineweaver–Burk secondary plots.
[c] Standard Inhibitor of butyrylcholinesterase.

3.6.1. Determination of kinetic parameters. The concentrations of test compounds that inhibited the hydrolysis of the substrate (butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (*Perrella Scientific Inc., Amherst*, USA). Dissociation constant (K_i) were determined by the interpretation of Dixon plot,[7] Lineweaver–Burk plot[8] and its secondary replots, using initial velocities obtained over a substrate concentrations between 0.05–0.2 mM for BTCh (Fig. 3).

3.6.2. Statistical analysis. Graphs were plotted using GraFit program.[9] Values of the correlation coefficients, slopes, intercepts and their standard errors were obtained from the linear regression analysis using the same program. The correlation for all the lines of all graphs was found to >0.99. Each point in the constructed graphs represents the mean of three experiments.

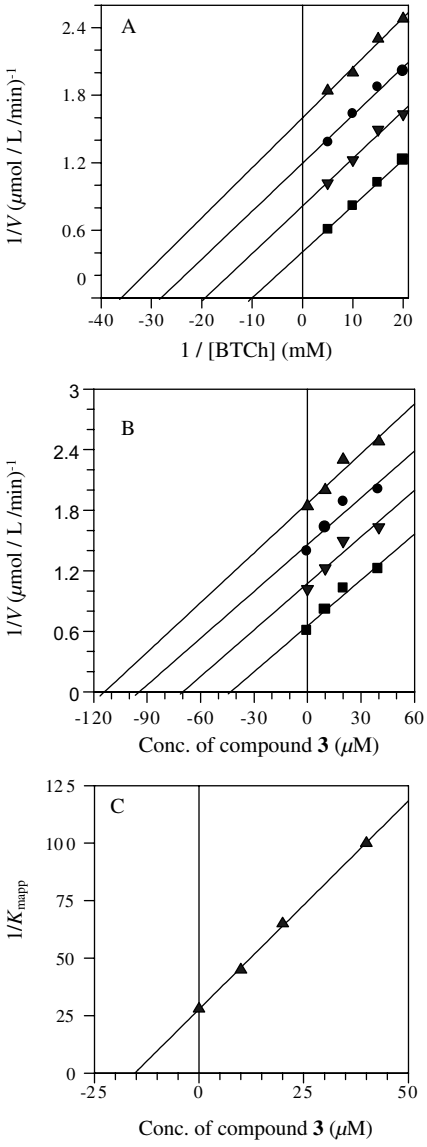


Figure 3. Steady-state inhibition of BChE by compound **3**. (A) is the Lineweaver–Burk plot in absence (■) and presence of 10.0 μM (▼), 20.0 μM (●), 40.0 μM (▲) of **3** (B) is the Dixon plot at fixed BTCh concentrations, (■) 0.2 mM, (▼) 0.1 mM, (●) 0.066 mM, and (▲) 0.05 mM. (C) is the secondary replots of the Lineweaver–Burk plot, 1/K_{mapp} or slope versus various concentrations of **3**.

3.7. Crystallographic data of compound 3

The structure of compound **3** was unambiguously determined by single-crystal X-ray diffraction technique. A suitable crystal of compound **3** was obtained by recrystallization from petroleum ether–CH_2Cl_2–MeOH (2:2:1). A colorless crystal with dimensions $0.5 \times 0.20 \times 0.18$ mm^3 was selected for the crystallographic measurements. $C_{15}H_{26}O_2$: M_r 238.36; monoclinic, $a = 7.1560$ (2), $b = 21.9220$ (6), $c = 8.6580$ (2) Å, $\beta = 91.5880$ (10)°, $V = 1357.69$ (6) Å^3, space group = P_1 $Z = 4$, $D_{calc} = 1.166$ mg/m^3, F (0 0 0) = 528, Mo-K$\lambda = 0.71073$ Å. Unit cell dimensions were determined by least squares fit of 1715 reflections measured at 293° (2) K using Mo-Kα radiations on a Nonius KappaCCD diffractometer. The intensity data within (θ) range of 2.3–27.5° were collected at 173° (2) K. A total of 5588 reflections were collected, of which 3178 reflections were judged observed on the basis of $I > 2\,\sigma(I)$. The structure was solved by the direct methods and expanded using Fourier transformation techniques.[10] The structure was refined by a full-matrix least-square calculation on F^2 with the aid of program SHELXL97.[11] The final R and R_w factors were measured as 0.041 and 0.103, respectively. The figures were plotted with the aid of ORTEPII program.[12] Crystallographic data for compound **3** has been deposited in Cambridge Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

3.8. Crystallographic data of compound 4

The structure of compound **4** was unambiguously determined by single-crystal X-ray diffraction technique. Compound **4** was recrystallized from diethyl ether–CH_2Cl_2–MeOH (3:1:1). A colorless crystal with dimensions $0.5 \times 0.4 \times 0.1$ mm^3 was selected for the crystallographic measurements. $C_{15}H_{26}O_2$: M_r 238.36; monoclinic, $a = 9.2590$ (10), $b = 14.0229$ (17), $c = 21.613$ (2) Å, $\beta = 90.0$ (10)°, $V = 2800.2$ (6) Å^3, space group = C 222 (1), $Z = 4$, $D_{calc} = 1.128$ mg/m^3, F (0 0 0) = 1056, Mo-K$\lambda = 0.71073$ Å. Unit cell dimensions were determined by least squares fit of 2466 reflections measured at 293 (2) K using Mo-Kα radiations on a SMART APEX CCD diffractometer (Erauf-Nonius Bruker). The intensity data within (θ) range of 1.88–25.0° were collected at 173° (2) K. A total of 7031 reflections were collected, of which 2466 reflections were judged observed on the basis of $I > 2\sigma(I)$. The structure was solved by the direct methods and expanded using Fourier transformation techniques.[10] The structure was refined by a full-matrix least-square calculation on F^2 with the aid of program SHELXL97.[11] The final R and R_w factors were measured as 0.1029 and 0.2285, respectively. The diagrams were plotted with the aid of ORTEPII program.[11] Crystallographic data for compound **4** has been deposited in the Cambridge

Crystallographic Data Center, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.01.015.

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