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Inhibition of Acetylcholinesterase Activity by Bicyclic Monoterpenoids

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Inhibition of acetylcholinesterase (AChE) activity by 17 kinds of bicyclic monoterpenoids was investigated. Bicyclic monoterpenoids are contained in many kinds of essential oils. Inhibition of AChE was measured according to the colorimetric method. 3.1.1 and 4.1.0 bicyclic hydrocarbons with allylic methyl group showed strong inhibition. (+)- and (−)-α-pinene and (+)-3-carene were potent inhibitors of AChE. 3.1.1 and 2.2.1 bicyclic alcohols and ketones showed weak inhibition. 3.1.1 and 4.1.0 bicyclic hydrocarbons with allylic methyl group were found to be uncompetitive inhibitors.

KEYWORDS: Acetylcholinesterase; bicyclic monoterpenoids; inhibition of enzyme activity; uncompetitive inhibitor

INTRODUCTION

Reversible inhibitors of cholinesterase are currently used in clinical trials examining the treatment of Alzheimer's disease. The treatment of Alzheimer's disease is based on inhibition of acetylcholinesterase (AChE), which hydrolyzes acetylcholine, increasing acetylcholine available for transmission at the cholinergistic synapse. Some acetylcholinesterase inhibitors have been found naturally occurring in plants. Recently, galantamine, an amaryllidaceae alkaloid, has shown effective results for Alzheimer's disease and safety of treatment (*1*−*4*). As part of our continuing program to search for bioactive natural compounds, we investigated the inhibition of AChE activity by terpenoids. Terpenoids are contained in essential oils from plants. In a previous paper, inhibition of AChE activity from the house fly (*5*), electric eel (*5*−*7*), and rice weevil (*8*) was reported. The effects of *Salvia lavandulaefolia* Vhal (Spanish sage) essential oil and some of its constituent terpenoids on human erythrocyte acetylcholinesterase have been reported, too (*9*). In our previous study, monoterpenoids having a *p*-menthane skeleton (*10*), essential oils of *Mentha* species (*11*), volatile α,*β*-unsaturated ketones (*12*), and essential oil from *Citrus paradisi* (*13*) were reported.

Bicyclic monoterpenoids with a pinane skeleton, a carane skeleton, a fenchane skeleton, and a camphane skeleton are contained in many kinds of essential oils, which are used as fragrances. However, there are few studies on the inhibition of AChE of mammals by terpenoids. Therefore, in the present paper, we report the inhibition of AChE from bovine erythrocytes by 17 kinds of bicyclic monoterpenoids (**Table 1**; **Figure 1**). The structure−activity relationship is also discussed.

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Table 1. Inhibition of AChE Activity by Bicyclic Monoterpenoids

compound	IC_{50} (mM)[a] or % inhibitory activity (1.0 mM)[b]
3.1.1 bicyclic (pinane skeleton)	
(+)-α-pinene (**1**)	0.40
(−)-α-pinene (**2**)	0.44
(−)-*β*-pinene (**3**)	(48.5%)
(+)-*cis*-verbenol (**4**)	(17.7%)
(−)-myrtenol (**5**)	(15.0%)
(+)-*trans*-myrtanol (**6**)	(37.1%)
(−)-*trans*-myrtanol (**7**)	(37.4%)
(−)-verbenone (**8**)	(12.6%)
4.1.0 bicyclic (carane skeleton)	
(+)-2-carene (**9**)	0.90
(+)-3-carene (**10**)	0.20
2.2.1 bicyclic (fenchane skeleton)	
(+)-fenchol (**11**)	(37.7%)
(+)-fenchone (**12**)	(23.3%)
(−)-fenchone (**13**)	(28.2%)
2.2.1 bicyclic (camphane skeleton)	
(+)-borneol (**14**)	(22.2%)
(−)-borneol (**15**)	(22.6%)
(+)-camphor (**16**)	(26.4%)
(−)-camphor (**17**)	(21.2%)

[a] Concentration of compound (treatment) required for 50% enzyme inhibition as calculated from the dose–response curve. [b] The percent AChE inhibition values (1.0 mM) were calculated as compared to control (without terpenoids) enzyme activity (assumed to be 0% inhibition).

MATERIALS AND METHODS

Materials. AChE from bovine erythrocytes was purchased from Sigma Co., Ltd. (Tokyo, Japan).

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATC) were purchased from Tokyo Chemical Industry Co., Ltd. (TCI).

Terpenoids were purchased from TCI, Fluka Co. (Tokyo, Japan), and Wako Pure Chemical Co. (Osaka, Japan).

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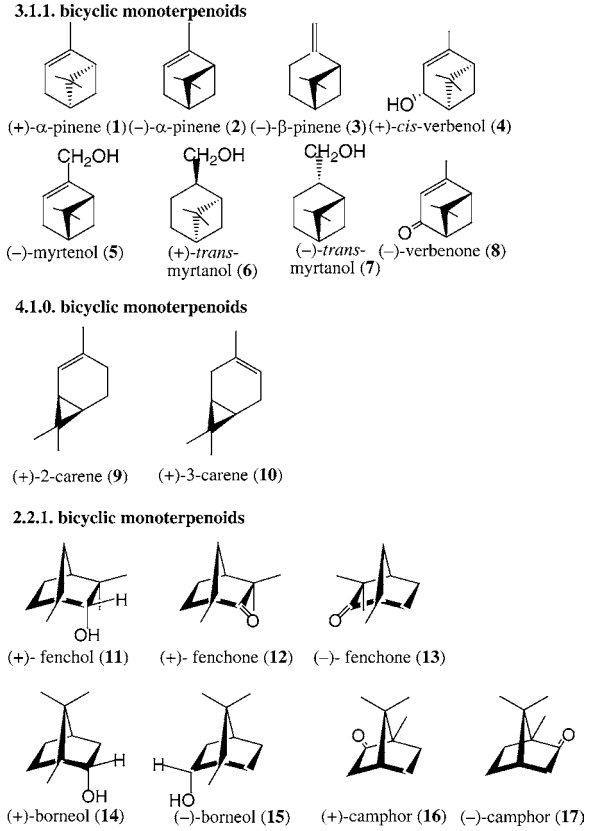

Figure 1. Structures of the 17 bicyclic monoterpenoids selected for investigation.

Preparatory Solutions. AChE (0.04 unit/mL) and ATC (75 mM) were dissolved in 0.1 M phosphate buffer (pH 8.0), respectively. DTNB (0.01 M) was made up in 10 mL of 0.1 M phosphate buffer (pH 7.0) containing 15 mg of $NaHCO_3$. Terpenoids were dissolved in ethanol. The final ethanol concentrations in all assays were maintained at 5% (v/v), including controls.

Inhibition of AChE Activity. Inhibition of AChE activity was assessed according to the colorimetric method of Ellman (*14*). Inhibitor solution (50 μL) and AChE (0.5 mL) were mixed in a test tube, and buffer (2.4 mL) was added to the tube. The tube was preincubated at 25 °C for 5 min. The reaction was started by adding ATC (40 μL), and the mixture was incubated at 25 °C for 20 min. The absorbance at 412 nm was measured spectrophotometrically (Spectronic 20D, Milton Roy Co., New York), and all test and control (without essential oil) assays were corrected by blanks for nonenzymic hydrolysis. Each assay was run in triplicate, at a minimum.

RESULTS AND DISCUSSION

Inhibition of AChE Activity by 3.1.1 Bicyclic Monoterpenoids (Pinane Skeleton). As shown in **Figure 2a**, (+)-α-pinene (**1**) and (−)-α-pinene (**2**) showed strong inhibition of AchE in the pinane skeleton. IC_{50} values obtained for **1** and **2** were 0.40 and 0.44 mM, respectively, whereas (−)-*β*-pinene (**3**) was 48.5% inhibited at 1.0 mM and the IC_{50} value was 1.1 mM. (+)-*cis*-Verbenol (**4**), (−)-myrtenol (**5**), (+)-*trans*-myrtanol (**6**), (−)-*trans*-myrtanol (**7**), and (−)-verbenone (**8**) were inhibited from 17.0 to 39.4%, and no IC_{50} values were obtained at concentrations of <2.0 mM.

Inhibition of AChE Activity by 4.1.0. Bicyclic Monoterpenoids (Carane Skeleton). As shown in **Figure 2b**, (+)-2-carene (**9**) and (+)-3-carene (**10**) were obtained with IC_{50} values at concentrations of <2.0 mM. Compound **10**, especially, showed potent inhibition with an IC_{50} value of 0.2 mM.

Table 2. Inhibition Constants (K_i) for Bicyclic Monoterpenoid Inhibition of AChE

compound	K_i (mM)	compound	K_i (mM)
1	0.15	**10**	0.03
2	0.17	**11**	1.92
3	1.10	**12**	2.03
9	0.38	**13**	1.99

Inhibition of AChE Activity by 2.2.1 Bicyclic Monoterpenoids (Fenchane and Camphane Skeletons). As shown in **Figure 2c**,**d**, all tested monoterpenoids with fenchane and camphane skeletons showed weak inhibition of AChE, and no IC_{50} values were obtained at concentrations of <2.0 mM. In the fenchane skeleton, (+)-fenchol (**11**), (+)-fenchone (**12**), and (−)-fenchone (**13**) inhibited from 36.6 to 42.1% activity at 2.0 mM. Monoterpenoids with the camphane skeleton showed identical inhibition (26.2−32.8%) of AChE activity.

Structure−Activity Relationship of Bicyclic Monoterpenoids. *3.1.1 Bicyclic Monoterpenoids.* The hydrocarbon compounds showed stronger inhibition than alcohol and ketones. The presence of the oxygenated functional group decreased the strength of inhibition of AChE. The hydrocarbon compounds, **1** and **2**, were more potent inhibitors than **3**. The position of C═C double bonds is related to the strength of inhibition of AChE. The compounds with an allylic methyl group show more potent inhibitory activity. In other words, the presence of a terminal olefin decreases the strength of inhibition of AChE. (−)-Verbenol (**4**) and (−)-verbenone (**8**) showed weaker inhibition than **1** and **2**, although **4** and **8** have an allylic methyl group. As mentioned above, the presence of the oxygenated functional group decreases the inhibitory strength of **4** and **8**. As shown by some of the comparative results, the inhibition of AChE activity of (−)-*trans*-myrtanol (**7**) was stronger than that of (−)-myrtenol (**5**). The degree of saturation does affect the inhibitory activity of AChE.

4.1.0 Bicyclic Monoterpenoids. Both **9** and **10** were strong inhibitors of AChE activity and have allylic methyl groups. (+)-3-Carene (**10**), especially, showed more potent inhibition than **9**. The position of C═C double bonds is related to the strength of inhibition of AChE.

2.2.1 Bicyclic Monoterpenoids. The oxygenated compounds of this structural type were weak inhibitors of AChE. The compounds with a camphane skeleton showed identical inhibitory activity. (+)- and (−)-fenchone (**12** and **13**) and the corresponding ketone to (+)-fenchol (**11**) showed weaker inhibition than **11**, showing that the ketone group is not as effective as the alcohol functional group.

As shown by some of the comparative results in enantiomers, **13** [(−)-form] was a slightly more potent inhibitor than **12** [(+)-form]. **16** [(+)-form] was a slightly more potent inhibitor than **17** [(−)-form]. Each of the sets of enantiomers (**1** and **2**, **6** and **7**, **14** and **15**) showed little difference in their inhibition of AChE.

AChE Inhibition Kinetics. The Dixon plot of (+)-3-carene (**10**) on inhibition of AChE is shown in **Figure 3**. The plots of (+)-α-pinene (**1**), (−)-α-pinene (**2**), and (+)-2-carene (**9**) were similar to that of **10**. These compounds were uncompetitive inhibitors, as indicated by decreasing inhibition associated with decreasing substrate concentrations and by the parallelism of the Dixon plot. These compounds bind not to the enzyme but to the enzyme−substrate complex, thus preventing product formation. On the other hand, the Dixon plot of (−)-*β*-pinene (**3**) on the inhibition of AChE is shown in **Figure 4**. The plots

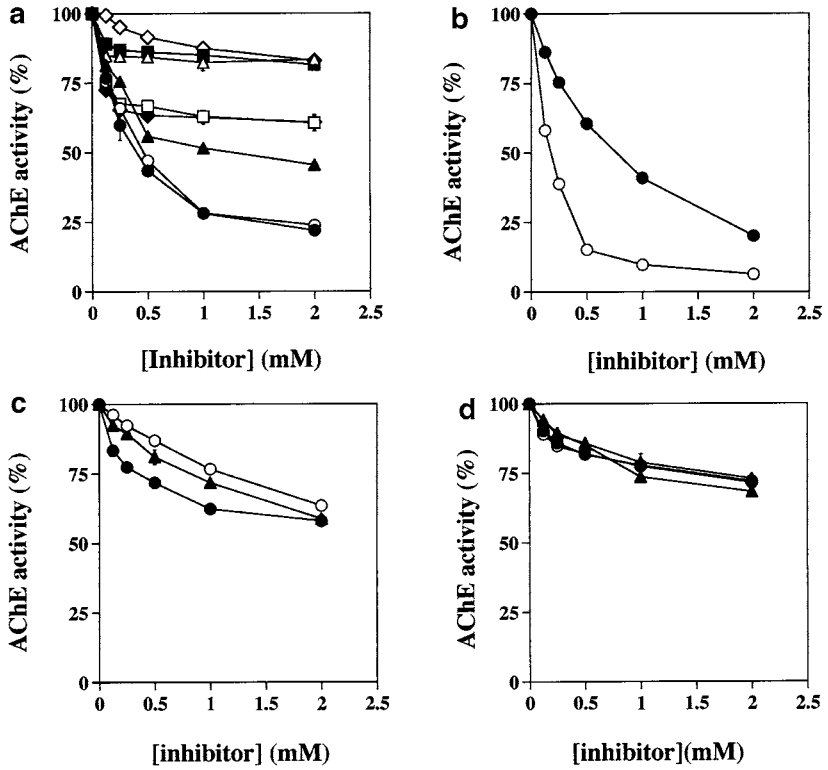

Figure 2. (**a**) Effect of 3.1.1 bicyclic monoterpenoids (pinane skeleton) on AChE activity. The percentage enzyme activity values for the inhibitors were calculated as compared to the control activity, assumed to be 100%: (●) (+)-α-pinene (**1**); (○) (–)-α-pinene (**2**); (▲) (–)-β-pinene (**3**); (△) (+)-*cis*-verbenol (**4**); (■) (–)-myrtenol (**5**); (□) (+)-*trans*-myrtanol (**6**); (◆) (–)-*trans*-myrtanol (**7**); (◇) (–)-verbenone (**8**). (**b**) Effect of 4.1.0 bicyclic monoterpenoids (carane skeleton) on AChE activity. The percentage enzyme activity values for the inhibitors were calculated as compared to the control activity, assumed to be 100%: (●) (+)-2-carene (**9**); (○) (+)-3-carene (**10**). (**c**) Effect of 2.2.1 bicyclic monoterpenoids (fenchane skeleton) on AChE activity. The percentage enzyme activity values for the inhibitors were calculated as compared to the control activity, assumed to be 100%: (●) (+)-fenchol (**11**); (○) (+)-fenchone (**12**); (▲) (–)-fenchone (**13**). (**d**) Effect of 2.2.1 bicyclic monoterpenoids (camphane skeleton) on AChE activity. The percentage enzyme activity values for the inhibitors were calculated as compared to the control activity, assumed to be 100%: (●) (+)-borneol (**14**); (○) (–)-borneol (**15**); (▲) (+)-camphor (**16**); (△) (–)-camphor (**17**).

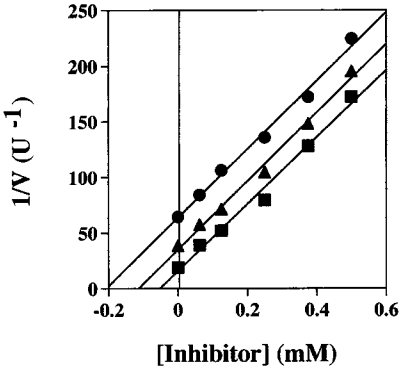

Figure 3. Dixon plots derived from the inhibition of AChE by (+)-3-carene (**10**). Concentrations of (+)-3-carene were (●) 0.163, (▲) 0.325, and (■) 0.650 mM.

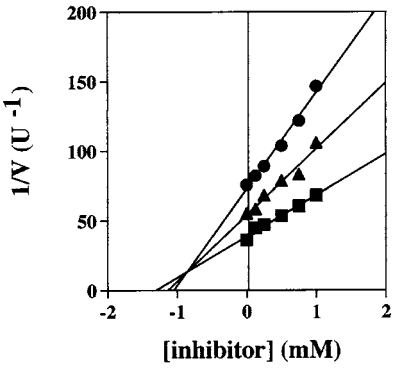

Figure 4. Dixon plots derived from the inhibition of AChE by (–)-β-pinene (**3**). Concentrations of (–)-β-pinene were (●) 0.163, (▲) 0.325, and (■) 0.650 mM.

of (+)-fenchol (**11**), (+)-fenchone (**12**), and (−)-fenchone (**13**) were similar to that of **3**. These compounds were found to be competitive inhibitors, as indicated by increasing inhibition associated with decreasing substrate concentration and by the intersections on the Dixon plots. These compounds compete with the substrate for its active site on the enzyme. The K_i values determined by the intersections on the Dixon plots are shown in **Table 2**. However, alcohols and ketones with pinane and camphane skeletons did not give reproducible Dixon plots.

In this study, bicyclic hydrocarbons with an allylic methyl group have a potent inhibition of AChE. Bicyclic monoterpenoids are contained in many kinds of essential oils. Inhibition of AChE activity by bicyclic monoterpenoids is reported to have insecticidal effect. In vitro inhibition of human erythrocyte AChE by some of the bicyclic monoterpenoids is reported. However, no report of bicyclic monoterpenoids inhibiting bovine erythrocyte AChE has appeared. The plant terpenoids may be available as AChE antagonists.

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