Inhibition of Acetylcholinesterase Activity by Monoterpenoids with a *p*-Menthane Skeleton

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Inhibition of acetylcholinesterase (AChE) activity by 17 kinds of monoterpenoids (hydrocarbons, alcohols, and ketones) with *p*-menthane skeletons was studied. Inhibition of AChE was measured by the colorimetric method. The terpene ketones showed stronger inhibition than the terpene alcohols. The terpene hydrocarbon compounds showed identical inhibitory activity with the terpene alcohols, but α-terpinene and (+)-*p*-menth-1-ene were equally strong inhibitors as the terpene ketones. Monoterpenoids used in this study were found to be competitive inhibitors.

Keywords: *Acetylcholinesterase; monoterpenoids; p-menthane skeleton; inhibition of enzyme activity; competitive inhibitor*

INTRODUCTION

Reversible inhibitors of cholinesterase are currently used in clinical trials examining the treatment of Alzheimer's disease. Anticholinesterase may interact with the central cholinergic system to improve memory and cognitive deficits of the patients by diminishing the breakdown of acetylcholine at the synaptic site in the brain. However, the therapeutic window is small, and testing of the inhibitory effect on acetylcholinesterase (AChE) in erythrocytes has been proposed as a guide to the efficacy and safety of putative therapies. Recently galanthamine, which is an alkaloid and is contained in Amarylidaceae, has been reported as the most effective treatment of Alzheimer's disease (Thomsen and Kewitz, 1990; Thomsen et al., 1991).

As part of our continuing program to search for the bioactive natural compounds, we investigated the inhibition of AChE activity by terpenoids. Terpenoids are contained in essential oils from plants. There are only three reports about anticholinesterase from the housefly and electric eel activity of monoterpenoids (eel, Gracza, 1985; Ryan et al., 1988; housefly and eel, Grundy et al., 1985). In these papers, pulegone (which has a *p*-menthane skeleton) was the most potent compound. Monoterpenoids with a *p*-menthane skeleton are contained in many kinds of mint oil, which is used as a flavor. However, there are no reports about inhibition of AChE of mammals by terpenoids. So, in the present paper, we report the inhibition of AChE from bovine erythrocytes by 17 kinds of monoterpenoids with the *p*-menthane skeleton. The structure−activity relationship of *p*-menthanes is also discussed.

MATERIALS AND METHODS

Materials. AChE from bovine erythrocytes was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Tokyo Kasei Kogyo Co., Ltd. (TCI) (Tokyo). Acetylthiocholine iodide (ATC) was purchased from Kanto Chemical Co., Inc. (Tokyo). Monoterpenoids (*p*-menthane skeleton) were purchased from TCI and Taiyo Perfume Co., Ltd. (Osaka).

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Table 1. Inhibition of AChE by Monoterpenoids with a *p*-Menthane Skeleton

compd	IC_{50} (mM)[a] or % inhibitory activity (1.2 mM)[b]	compd	IC_{50} (mM)[a] or % inhibitory activity (1.2 mM)[b]
	Hydrocarbons		
1	(39.8%)	**4**	1.64
2	1.0	**5**	(22.0%)
3	(22.6%)	**6**	(25.0%)
	Alcohols		
7	2.0	**10**	2.0
8	(38.5%)	**11**	(24.4%)
9	(28.8%)	**12**	(21.4%)
	Ketones		
13	0.89	**16**	1.42
14	1.85	**17**	1.57
15	1.38		

[a] Concentration of compound (treatment) required for 50% enzyme inhibition as calculated from the dose−response curve. [b] The percent AChE inhibition values (1.2 mM) were calculated as compared to control (without terpenoids) enzyme activity (assumed to be 0% inhibition).

Table 2. Inhibition Constants (K_i) for Monoterpene Alcohol and Ketone Inhibition of AChE

compd	K_i (mM)	compd	K_i (mM)
	Alcohols		
7	1.25	**10**	1.85
8	1.93	**11**	2.20
9	2.50	**12**	1.83
	Ketones		
13	0.58	**16**	1.20
14	1.60	**17**	0.95
15	1.12		

Preparatory Solutions. AChE (0.04 U/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 $NaHCO_3$. Monoterpenoids (*p*-menthane) 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 was assessed by the colorimetric method of Ellman (1961). Inhibitor solution (50 μL) and AChE (0.5 mL) were mixed in a test tube, and the tube was set on the incubator (25 °C). To the tube were added DTNB (100 μL) and buffer (2.4 mL). The tube was incubated at 25 °C for 5 min as preincubation. The reaction was started by adding ATC (40 μL), and the mixture

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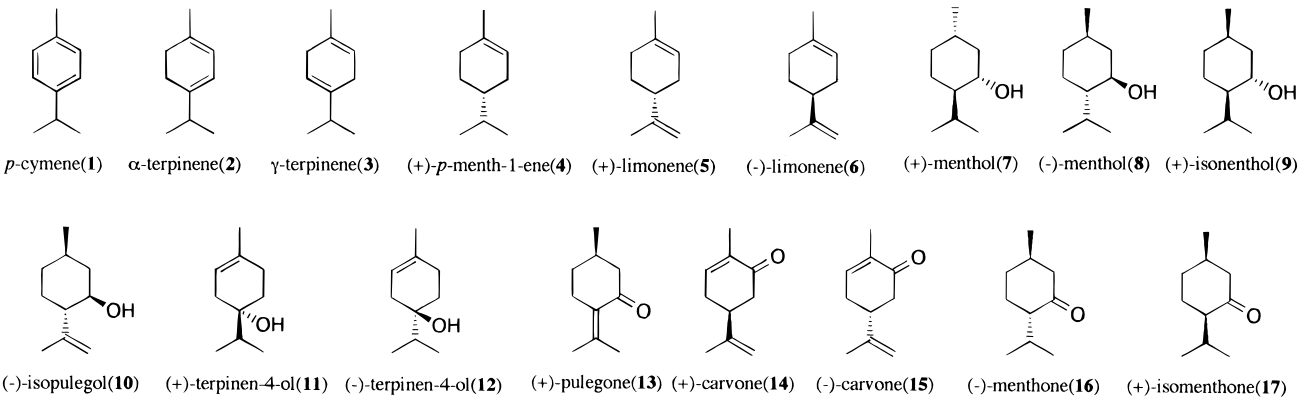

Figure 1. Structures of the 17 monoterpenoids with a *p*-menthane skeleton selected for investigation.

was incubated at 25 °C for 20 min. The absorbance at 412 nm was measured spectrophotometrically (SPECTRONIC 20D, Milton Roy Co., NY), and all test and control (without terpenoid) assays were corrected by blanks for nonenzymic hydrolysis. Each assay was run in triplicate, at a minimum.

RESULTS AND DISCUSSION

The dose dependent inhibition of AChE activity following 17 kinds of monoterpenoid (Figure 1) treatments, is shown in Figure 2. The percentage of AChE activity values for the inhibitors were calculated as compared to the control (without terpenoids) AChE activity, assumed to be 100%. Table 1 shows 50% enzyme inhibition (IC_{50}) or percentage of inhibition of AChE activity (%) at 1.2 mM calculated from the inhibition dose−response curve.

Inhibition of AChE Activity by Monoterpene Hydrocarbons. As shown in Figure 2a, α-terpinene (**2**) and (+)-*p*-menth-1-ene (**4**) showed strong inhibition of AChE in hydrocarbons. **2** and **4** demonstrated 64% and 60% inhibition at 2.0 mM, and IC_{50} values were 1.0 and 1.64 mM, respectively. *p*-Cymene (**1**), γ-terpinene (**3**), (+)-limonene (**5**), and (−)-limonene (**6**) inhibited from 30% to 40%, and no IC_{50} values were obtained at concentrations < 2 mM.

Inhibition of AChE Activity by Alcohols. As shown in Figure 2b, (+)-menthol (**7**), (−)-menthol (**8**), and (−)-isopulegol (**10**) showed similar percentages of inhibition of AChE activity at 2.0 mM. However, **10** had sharper dose−response curves than **7** and **8**; for example, **7**, **8**, and **10** showed 45%, 38.5%, and 35% inhibition of AChE at 1.2 mM, respectively. (+)-Isomenthol (**9**) inhibition was not changed more than 1.0 mM, and **9** showed approximately 30% inhibition of AChE.

Inhibition of AChE Activity by Ketones. As shown in Figure 2c, all monoterpene ketones could be obtained with IC_{50} values at concentrations < 2.0 mM (Table 1). (+)-Pulegone (**13**) was the most potent inhibitor of AChE in ketones. The other ketones showed identical inhibition of AChE activity.

Relationship of the Structure and Activity of Monoterpenoids with the *p*-Menthane Skeleton. As shown in Table 1 and Figure 2, **13** was a strong inhibitor of AChE activity. The monoterpene ketones showed stronger inhibition than the alcohols. The hydrocarbon compounds showed identical inhibitory activity with the terpene alcohols, except for **2** and **4** that showed equally strong inhibition as the terpene ketones. Specifically, **2** showed the strongest inhibition next to **13**. The presence of conjugated double bonds is related to the strength of inhibition of AChE. The inhibition of AChE activity of **4** was stronger than that of **5** or **6**. Also **7** and **8** were more potent inhibitors of AChE than **10** between 0.5 and 1.5 mM as shown in Figure 2b. The compounds with isopropyl groups show more potent inhibitory activity. In other words, the

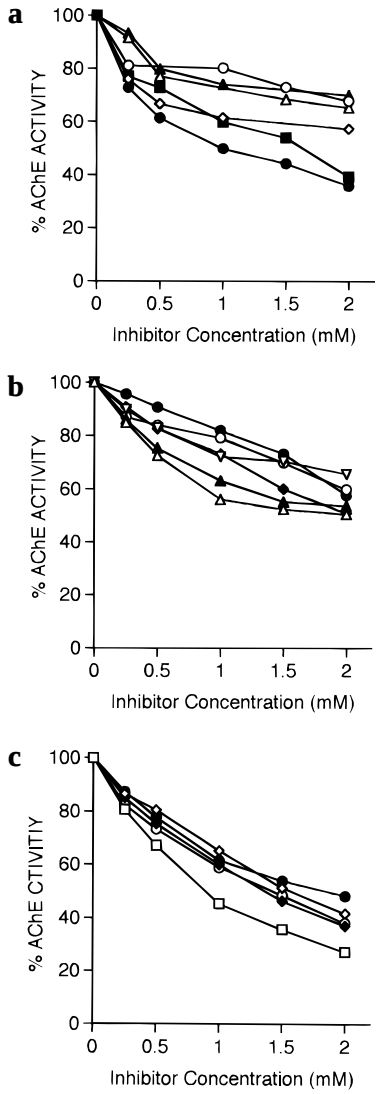

Figure 2. Effect of monoterpenoids with *p*-menthane skeleton on AChE activity. The percentage of enzyme activity values for the inhibitors was calculated as compared to the control activity, assumed to be 100%. (a) Hydrocarbons: (◇) *p*-cymene (**1**), (●) α-terpinene (**2**), (○) γ-terpinene (**3**), (■) (+)-*p*-menth-1-ene (**4**), (△) (+)-limonene (**5**), and (▲) (−)-limonene (**6**). (b) Alcohols: (△) (+)-menthol (**7**), (▲) (−)-menthol (**8**), (▽) (+)-isomenthol (**9**), (◆) (−)-isopulegol (**10**), (○) (+)-terpinen-4-ol (**11**), and (●) (−)-terpinen-4-ol (**12**). (c) Ketones: (□) (+)-pulegone (**13**), (◇) (+)-carvone (**14**), (◆) (−)-carvone (**15**), (●) (−)-menthone (**1**) and (○) (+)-isomenthone (**17**).

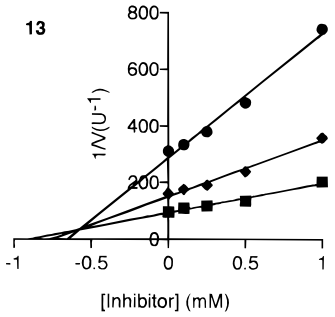

Figure 3. Dixon plots derived from the inhibition of AChE by (+)-pulegone. In each plot the concentrations of ATC are (●) 0.0656 mM, (◆) 0.197 mM, and (■) 0.98 mM.

presence of an isopropenyl group decreases the strength of inhibition of AChE. Carvone (**14**, **15**) showed slightly weaker inhibition than **13**, although **14** and **15** have conjugated double bonds in the molecule. As mentioned above, the presence of an isopropenyl group decreases the inhibitory strength of **14** and **15**.

As shown in some of the comparative results between enantiomers, **15** ((−)-form) was a slightly more potent inhibitor than **14** ((+)-form). The other enantiomers (**5** and **6**, **7** and **8**, **11** and **12**) showed little difference in their inhibition of AChE.

AChE Inhibition Kinetics. The Dixon plot of **13** on inhibition of AChE is shown in Figure 3. The plots of terpene alcohols and ketones were similar to that of **13**. These compounds were competitive inhibitors, as indicated by increasing inhibition associated with decreasing substrate concentration and by the intersections in the Dixon plots. The K_i values determined by replotting these plots are shown in Table 2. However hydrocarbon compounds cannot reproduce the Dixon plots because of their low solubility.

In this study, ketones have a potent inhibition of AChE in *p*-menthanes. Monoterpenoid ketones with the *p*-menthane skeleton are contained in many essential oils. These are reported as bioactivities, such as antimicrobial activity (Kurita et al., 1982) and spasmolytic activity (Gamez et al., 1990). These essential oils can be expected in new applications. Recently some new bioactivities of *p*-menthanes have been reported. For example, limonene showed antimutagenic activity (Crowell et al., 1992), menthol and limonene showed antiacne effects (Kubo et al., 1994), and menthone and menthol showed antiallergic effects (Arakawa et al., 1992). Inhibition of AChE (electric eel and housefly) activities by monoterpenoids were reported on related insecticidal effects (Gracza, 1985; Grundy et al., 1985; Ryan et al., 1988). However, no reports of monoterpenoid-inhibited bovine erythrocytes AChE have appeared. The plant terpenes may be available as an AChE antagonist.

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