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# Inhibition of Carbonic Anhydrase, Acetylcholinesterase and Butyrylcholinesterase by BisPMB, A Synthetic Analogue of Ajoene

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**Abstract:** BisPMB (1,8-(bis-*p*-methoxyphenyl)-2,3,7-trithiaocta-4-ene-7-oxide) is a synthetic analogue of the natural compound ajoene, which is a bioactive natural product obtained from the rearrangement of the unstable and reactive organosulfur compound allicin produced when garlic is freshly crushed. BisPMB has been shown to have superior cancer-cell cytotoxicity compared with ajoene and a modest selectivity towards cancer cells over non-cancerous ones. In this study, the inhibition effects of *E*/*Z*-bisPMB and *Z*-bisPMB against human carbonic anhydrase isozymes I and II (hCA I and II), acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were investigated in which *E*/*Z*-bisPMB and *Z*-bisPMB showed similar inhibition profiles against the four enzymes tested, with the $IC_{50}$values ranging from 10.9 to 439.7 nM and the $K_i$ values ranging from 5.4 to 195.4 nM. Furthermore, bisPMB was more potent at inhibiting CA I, CA II and AChE compared with commercially available inhibitors.

**Keywords:** Acetylcholinesterase, Ajoene, BisPMB, Butyrylcholinesterase, Carbonic Anhydrase.

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## INTRODUCTION

Garlic, *Allium sativum* L., has generated much interest throughout human history as both a food and therapeutic agent and is one of the most researched medicinal plants in which it has been used to treat various ailments including headaches, infections, colds, ulcers, worms, burns and wounds (1). In recent years garlic has been used to treat heart disease, atherosclerosis, coronary thrombosis and cancer, and the biological activity and health-promoting effects of garlic are attributed to the organosulfur compounds found in crushed garlic cloves, Figure 1 (1,2). Intact garlic cloves contain a cysteine allyl sulfoxide substrate called alliin separated from its enzyme alliinase, which upon crushing are brought together to promote a chemical reaction that produces the thiosulfinate allicin as the primary product. Allicin is unstable and with time degrades and rearranges to various other products including ajoene, a relatively stable compound.

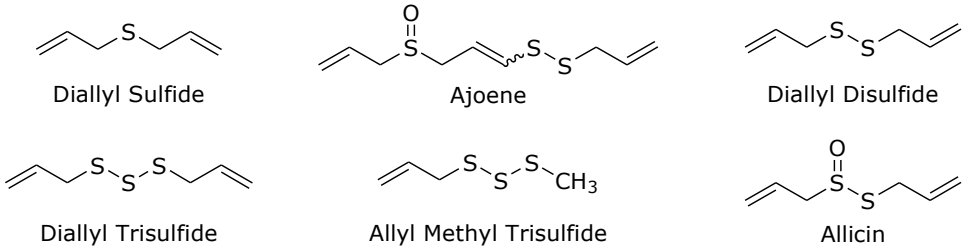



**Figure 1:** Some of the organosulfur compounds found in crushed garlic.

Ajoene possesses a wide range of biological activities which includes antifungal, antimicrobial, anti-obesity and anticancer, and more recently the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzyme inhibition activity of ajoene was reported (2–8). BisPMB (Figure 2) a synthetic analogue of ajoene has been shown to have superior cytotoxicity to ajoene as well as a modest selectivity for cancer cells over non-cancerous ones (9). In light of these results, it was decided to investigate the carbonic anhydrase (CA), AChE and BuChE inhibition potential of bisPMB.

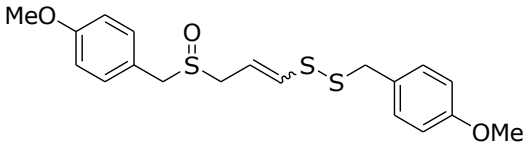



**Figure 2:** Chemical structure of the ajoene analogue *E*/*Z*-bisPMB.

Carbonic anhydrases (CAs) are ubiquitous metalloenzymes that are grouped into 7 distinct families: α-, β-, γ-, δ-, ζ-, η-, and θ-CAs (10–13). The α-CA family is found in mammals and has 16 isozymes, 5 cytosolic (CA I, CA II, CA III, CA VII, and CA XIII), 5 membrane-bound (CA IV, CA IX, CA XII, CA XIV, and CA XV), 2 mitochondrial (CA VA and CA VB), 1 secreted (CA VI), and 3 non-catalytic enzymes known as CA-related proteins (CA VIII, CA X, and CA XI) (12). CAs are essential for life in that they catalyze the most important physiological reaction as the hydration of carbon dioxide to bicarbonate and the dehydration of bicarbonate to carbon dioxide. Many physiological and pathological processes rely on this reaction, such as respiration, carbon dioxide and ion transport, pH regulation, carbon dioxide fixation, acid-base balance, bone resorption, calcification, tumorigenicity, biosynthetic reactions, electrolyte secretion in tissues and organs and body fluid generation (14–17). As a result, the isozymes essential for these processes serves as therapeutic targets for several diseases in which their inhibition has led to the treatment of glaucoma, altitude sickness, epileptic seizures, obesity and cancer, while their activation may enhance cognition and lead to treatment of Alzheimer's disease (17–19).

The cholinesterase enzymes AChE and BuChE are responsible for hydrolyzing the neurotransmitter acetylcholine (ACh), and reduced levels of ACh in the brain have been associated with Alzheimer's disease (AD) (20). At present, there is no effective treatment for AD and therefore new therapeutics are urgently needed. Inhibition of the cholinesterase enzymes is an effective mechanism for the treatment of AD, although the inhibitors in clinical use thus far only improve the symptoms of AD and for limited periods only.

In this communication the inhibitory potential of bisPMB against human CA (hCA I and II), AChE and BuChE were evaluated. Therapies and cures for various diseases are urgently needed and these results may assist the ongoing search for new leads in drug discovery.

## EXPERIMENTAL SECTION

### Chemicals and Enzymes

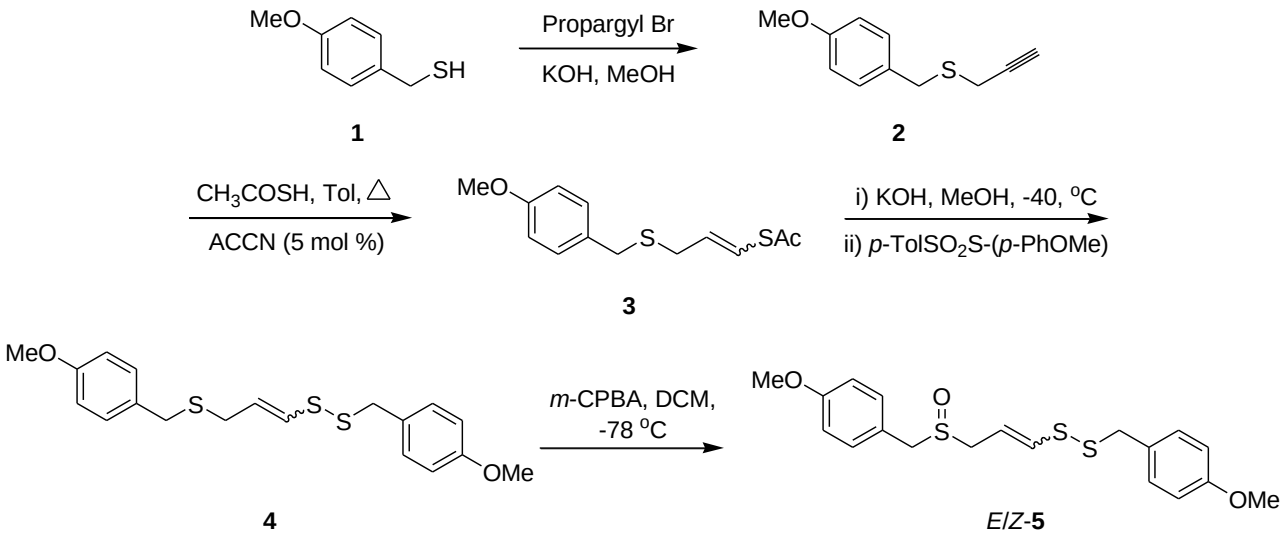
All chemicals and reagents for the synthesis and the CA, AChE and BuChE inhibition assays were purchased from Sigma Aldrich and used as received. AChE lyophilisate from the electric eel and BuChE lyophilisate from equine serum was obtained from Sigma-Aldrich and stored at -20 °C. All solvents were acquired from Merck. Distilled water was used for all experiments. The synthesized compounds were stored at -20 °C until needed.

### General Procedures for the Chemistry

The reactions were monitored by thin layer chromatography (TLC) using aluminum-backed plates covered with Merck silica-gel $F_{254}$ in an appropriate solvent system. TLC plates were inspected by UV light. Column chromatography was carried out using Fluka silica-gel 60 mesh with suitable solvent systems. Proton NMR spectra were recorded on a Varian Unity 400 spectrometer in deuterated chloroform ($CDCl_3$). Chemical shifts are quoted using residual chloroform (δ 7.24 in $^1H$ NMR) as an internal standard. Chemical shifts and coupling constants of the peaks are reported in ppm and Hz, respectively. Spectrophotometric analysis was performed on a Thermo Scientific Evolution 200 Series UV-Vis spectrophotometer.

### Experimental Procedures

BisPMB was prepared according to our published synthetic route to obtain ajoene analogues as outlined in Scheme 1 (9). All NMR spectra agree with those published in the literature (9). *E*/*Z*-bisPMB was obtained as a mixture of isomers via column chromatography while *Z*-bisPMB was obtained via fractional recrystallization of the *E*/*Z*-mixture using dichloromethane and hexane. Pure *E*-bisPMB could not be obtained.




**Scheme 1:** Synthesis of the ajoene analogue *E*/*Z*-bisPMB.

***E*/*Z*-BisPMB (1,8-(Bis-*p*-methoxyphenyl)-2,3,7-trithiaocta-4-ene-7-oxide)** *E*/*Z*-**(5)**
$^1$H-NMR (400 MHz, $CDCl_3$,$\delta_H$, ppm): 3.22 (1H, ddd, *J* = 1.0, 8.0, 13.2 Hz, *E*-$CH_2$-$H_a$), 3.34 (1H, ddd, *J* = 1.0, 8.0, 13.2 Hz, *E*-$CH_2$-$H_b$), 3.37 (1H, ddd, *J* = 1.0, 8.0, 13.4 Hz, *Z*-$CH_2$-$H_a$), 3.46 (1H, ddd, *J* = 1.0, 7.7, 13.4 Hz, *Z*-$CH_2$-$H_b$), 3.74 (3H, s, *E*-$OCH_3$), 3.76 (3H, s, *Z*-$OCH_3$), 3.78 (3H, s, *Z*-$OCH_3$), 3.79 (3H, s, *E*-$OCH_3$), 3.86 (2H, s,*Z*-$CH_2$), 3.86 (2H, s, *E*-$CH_2$), 3.88 (2H, s, *Z*-$CH_2$), 3.88 (2H, s, *E*-$CH_2$), 5.63 (1H, dt, *J* = 7.7, 9.5 Hz, *Z*-CH), 5.80 (1H, dt, *J* = 7.6, 14.8 Hz, *E*-CH), 6.15 (1H, dt, *J* = 1.0, 14.8 Hz, *E*-CH), 6.26 (1H, dt, *J* = 1.0, 9.5 Hz, *Z*-CH), 6.81 (2H, d, *J* = 8.6 Hz, *Z*-ArH), 6.81 (2H, d, *J* = 8.5 Hz, *E*-ArH), 6.87 (2H, d, *J*= 8.7 Hz, *Z*-ArH), 6.88 (2H, d, *J*= 8.6 Hz, *E*-ArH), 7.18 (4H, m, *Z*-ArH), 7.18 (4H, m, *E*-ArH).

***Z*-BisPMB (1,8-(Bis-*p*-methoxyphenyl)-2,3,7-trithiaocta-4-ene-7-oxide)** *Z*-**(5)**
Obtained via fractional recrystallization of the *E*/*Z*-mixture using dichloromethane and hexane. $^1$H-NMR (400 MHz, $CDCl_3$,$\delta_H$, ppm): 3.37 (1H, ddd, *J* = 1.0, 8.0, 13.4 Hz, $CH_2$-$H_a$), 3.46 (1H, ddd, *J* = 1.0, 7.7, 13.4 Hz, $CH_2$-$H_b$), 3.77 (3H, s, OCH3), 3.78 (3H, s, OCH3), 3.86 (1H, s, $CH_2$-$H_a$), 3.87 (1H, s, $CH_2$-$H_b$), 3.88 (2H, s, $CH_2$), 5.64 (1H, dt, *J* = 7.7, 9.5 Hz, CH), 6.26 (1H, dt, *J* = 1.0, 9.4 Hz, CH), 6.81 (2H, d, *J* = 8.8 Hz, ArH), 6.87 (2H, d, *J*= 8.8 Hz, ArH), 7.18 (4H, m, ArH).

**Inhibition Assay of CA I and CA II**
Human blood was supplied by the Blood Center at Ataturk University Research Hospital. The blood samples were centrifuged (15,000 rpm, 15 min) and the white blood cells and plasma were isolated. The red blood cells were rinsed a few times with 0.9% saline solution and then they were hemolyzed with ice-cold deionized water. The hemolysate was isolated by centrifugation (20,000 rpm, 30 min, 4 °C) and solid Tris was added until pH 8.7. The enzymes were purified on a Sepharose-4B-aniline-sulfanylamide affinity column (21). Prior to addition of the hemosylate and 400 mL flushing solution (25 mM Tris-HCl/22 mM $Na_2SO_4$, ph:8.7), the column was equilibrated (25 mM Tris-HCl/0.1 M $Na_2SO_4$, pH:8.7). Elution of hCA I was achieved with 1 M NaCl/25 mM $Na_2HPO_4$, pH:6.3. Elution of hCA II was achieved with 0.1 M $CH_3COONa$/0.5 M $NaClO_4$, pH:5.6.

The esterase activity was measured by the method described by Verpoorte and co-workers (22). The test compound (1 mg) was dissolved in dimethylsulfoxide (1 mL) and diluted with deionized water to six various concentrations. In a cuvette the following were added: 100 µL of buffer (0.5 M, pH 7.4: Tris-$SO_4$ buffer), 350 µL of 4-nitrophenylacetate (3 mM), 540 µL of water and 10 µL of enzyme solution. The test compound was added in 10 µL increments (10-60 µL). A control (no inhibitor) and blank (no enzyme) measurement was recorded. The $IC_{50}$ values (the concentration of the inhibitor needed to reduce the enzyme activity by half) was calculated from activity (%) vs [inhibitor] graphs while the inhibition constants ($K_i$ values) were obtained from the Cheng-Prussoff equation (23).

**Inhibition Assay of AChE and BuChE**

AChE and BuChE inhibitory activities of the synthesized compound were measured spectrophotometrically using a modified method of Ellman and co-workers (24). In a cuvette the following were added: 200 µL of buffer (AChE assay: 1 M Tris-HCL buffer, pH:8.0; BuChE assay: 1 M phosphate buffer, pH:8.0), 100 µL of 0.5 mM 5-mercapto-2-nitrobenzoic acid (DTNB), 100 µL of 10 mM acetylthiocholine or butyrylthiocholine (substrate), 590 µL of water and 10 µL of enzyme. The test compound was added in 10 µL increments (10-60 µL). A yellow color obtained from the reaction of thiocholine (acquired from the enzymatic hydrolysis of the substrates) and DTNB was measured at 412 nm. Data from concentration-inhibition experiments were integrated through linear regression analysis

using Microsoft Excel 2000, producing $IC_{50}$ values from which the $K_i$ values were calculated.

## RESULTS AND DISCUSSION

The inhibitory activities of *E*/*Z*-bisPMB (*E*/*Z*-**5**) and *Z*-bisPMB (*Z*-**5**) against CA, AChE and BuChE were evaluated using commercial acetazolamide (CA inhibitor) and rivastigmine (AChE and BuChE inhibitor) as reference standards. The $IC_{50}$ values were derived as an average of three independent experiments and are summarized in Table 1 and Table 2. The literature reveals that the *Z*-isomer of ajoene and ajoene analogues are more potent at inhibiting compared with the *E*-isomers for a range of diseases (9,25), and thus it was decided to test the inhibition activity of pure *Z*-bisPMB and compare it to the *E*,*Z*-mixture. However, the difference among the values of these two test compounds against the tested enzymes turned out to be statistically insignificant.

### Inhibitory activity of bisPMB against hCA I and hCA II

**Table 1:** Inhibition of hCA I and hCA II by bisPMB and standard compound acetazolamide.

| Compound | $IC_{50}$ (nM) hCA I | $IC_{50}$ (nM) hCA II | $K_i$(nM) hCA I | $K_i$(nM) hCA II |
|---|---|---|---|---|
| *E*/*Z*-**5** | 12.5 | 11.5 | 6.5 | 5.4 |
| *Z*-**5** | 10.9 | 13.2 | 5.7 | 6.2 |
| Acetazolamide* | 32.1 | 51.0 | 16.2 | 24.1 |

Results are reported as means of three independent experiments.
Errors are in the range of ± 2% of the reported values.
*Commercially available carbonic anhydrase inhibitor.

The ajoene analogue bisPMB (*E*/*Z*-**5** and *Z*-**5**) exhibited strong inhibition of hCA I and hCA II (Table 1). However, no distinct difference was observed between the inhibitory activity of *E*/*Z*-**5** and *Z*-**5** against these two cytosolic enzymes. $IC_{50}$ values of 12.5 and 10.9 nM were achieved for *E*/*Z*-**5** and *Z*-**5**, respectively against hCA I. They were threefold more active compared with the standard acetazolamide ($IC_{50}$ = 32.1 nM). The ubiquitous hCA II isozyme was inhibited by *E*/*Z*-**5** and *Z*-**5** with $IC_{50}$ values of 11.5 and 13.2 nM, respectively. In this case a fourfold better activity was observed compared with the standard ($IC_{50}$ = 51.0 nM). $K_i$ values in the range 5.4 and 6.5 nM were obtained for the CA isozymes. Inhibiting CA could lead to the treatment of many diseases since the various isozymes have been associated to diseases such as glaucoma, osteoporosis, edema, altitude sickness, and epilepsy (16,17,26). Although many carbonic anhydrase inhibitors (CAIs) are commercially available they are often accompanied by a wide range of undesirable side effects. These side effects arise because these inhibitors act systemically and are non-isozyme selective. It is therefore of paramount importance to find inhibitors that are isozyme selective with increased CA inhibition activity. The results obtained for bisPMB shows that its activity is more potent compared with acetazolamide and this exciting discovery of bisPMB as a lead opens up a programme of investigation into mode of action in chemical biology studies towards medicinal chemistry development.

### Inhibitory activity of bisPMB against AChE and BuChE

**Table 2:** Inhibition of AChE and BuChE by bisPMB and standard compound rivastigmine.

| Compound | $IC_{50}$ (nM) AChE | $IC_{50}$ (nM) BuChE | $K_i$(nM) AChE | $K_i$(nM) BuChE |
|---|---|---|---|---|
| *E*/*Z*-**5** | 46.9 | 439.7 | 22.5 | 195.4 |
| *Z*-**5** | 35.9 | 373.3 | 17.2 | 165.9 |
| Rivastigmine* | 60.2 | 14.0 | 29.0 | 6.1 |

Results are reported as means of three independent experiments.
Errors are in the range of ± 2% of the reported values.
*Commercially available cholinesterase inhibitor.

An altered level of AChE and BuChE is a characteristic of AD. Imbalances of these enzymes cause a decrease in the levels of the neurotransmitter ACh and results in a loss of communication between the nerve cells and thus a loss of brain function. Developing inhibitors that suppress the cholinesterase enzymes from hydrolyzing ACh is an effective management strategy of AD, and despite years of AD research there are very few drugs on the market available for the treatment of AD. Furthermore, these drugs only relieve the symptoms of the disease, for limited periods and cause many side effects. Studies have suggested that the number of AD cases will rise exponentially and by the year 2050 131.5 million people may be affected by dementia (27), which points towards an urgent need for new drugs, particularly with less or no side effects. In light of this, it was decided to investigate the cholinesterase inhibitory potential

of bisPMB in which, pleasingly, *E*/*Z*-**5** and *Z*-**5** showed strong AChE inhibitory activity with $IC_{50}$ values of 46.9 and 35.9 nM, respectively (Table 2). These inhibitory concentrations were 1.3 (*E*/*Z*-**5**) and 1.7 (*Z*-**5**) fold better than the cholinesterase inhibitor rivastigmine ($IC_{50}$ = 60.2 nM). BuChE was, however, significantly less inhibited ($IC_{50}$ *E*/*Z*-**5**= 439.7 nM and $IC_{50}$ *Z*-**5**= 373.3 nM) and was not as effective as the standard ($IC_{50}$ = 14.0 nM). $K_i$ values for *E*/*Z*-**5** and *Z*-**5** against AChE were 22.5 and 17.2 nM, respectively. Against BuChE *E*/*Z*-**5** and *Z*-**5** gave $K_i$ values of 195.4 and 165.9 nM, respectively. A recent study showed that ajoene inhibits AChE with an $IC_{50}$ value of 2.34 μM and BuChE with an $IC_{50}$ value of 2.09 μM (8). BisPMB, however, exhibited nanomolar $IC_{50}$ values for both cholinesterases. BisPMB was roughly 58-fold and 5.2-fold more potent at inhibiting AChE and BuChE, respectively, compared with ajoene. It is therefore significant that the ajoene analogue bisPMB turns out to be more potent than the parent ajoene, but with these enzymes the increase in inhibition potency is far more pronounced than in the cancer cell case.

## CONCLUSION

In this study, bisPMB was synthesized via our reported four-step method for preparing ajoene analogues, and *Z*-bisPMB and *E*/*Z*-bisPMB samples obtained. These were separately evaluated against four enzymes (hCA I, hCA II, AChE and BuChE) in which both samples behaved in the same manner, exhibiting very similar $IC_{50}$ values. Compared to known inhibitors against these enzymes, bisPMB was more effective than acetazolamide against the CA isozymes and more effective than rivastigmine against AChE. BisPMB, however, showed weaker activity against BuChE. These results are highly significant since low $K_i$ values were also obtained. According to these results, bisPMB merits further investigation as a lead in the pursuit of a therapeutic. Further studies will address aspects of a mode of action in chemical biology studies.

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