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Inhibition of Various Cholinesterases with the Enantiomers of Malaoxon

O. P. Rodriguez, G. W. Muth, C. E. Berkman,* K. Kim, C. M. Thompson

Chemistry Department, University of Montana, Missoula, Montana 59812, USA

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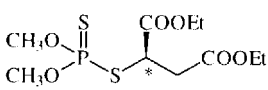
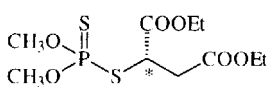
Malaoxon is the bioactivated form of malathion responsible for its anti-acetylcholinesterase (anti-AChE) or insecticidal activity. The species selectivity of malathion toward insects is primarily the result of a deficiency in detoxifying carboxylesterases by these organisms, which then allows malathion to undergo oxidation to form the highly toxic analog malaoxon (Cohen, 1984; Matolczy, 1988). The thionate (P=S) to oxon (P=O) conversion is the common mode of phosphorothionate insecticide activation. Whereas the phosphorothionate forms are generally not effective inhibitors of AChE, bioactivation to the oxon (P=O) analogues by monooxygenases in insects (Cohen, 1984) produces potent inhibitors of AChE (De Matteis, 1989). For example, malaoxon is about 1000-fold stronger an AChE inhibitor than malathion and nearly 80-fold more toxic *in vivo* to rats: malathion LD_{50} = 12500 mg/kg: malaoxon LD_{50} = 158 mg/kg (Ryan, 1985; Umetsu, 1977).

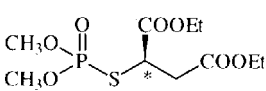
Enantiomers of both malathion and malaoxon exist due to an asymmetric carbon center (*) in the thiosuccinyl substituent. Enantiomers are expected to interact stereoselectively with enzymes such as AChE. The O,O-diethyl analogues of the malaoxon and malathion enantiomers have been previously prepared and examined for their anti-AChE and anti-carboxylesterase potency (Hassan and Dauterman, 1968). In that study, it was found that the (R)-O,O-diethyl malaoxon



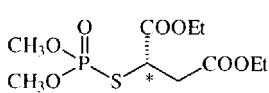
R_C-Malathion



S_C-Malathion



R_C-Malaoxon



S_C-Malaoxon

**Present address:* Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132
Correspondence to: C. M. Thompson

was a 4.4-fold stronger inhibitor of bovine erythrocyte AChE, and an 8.3-fold stronger inhibitor of rat liver carboxylesterase than its (S)-antipode. However, enantiomers of O,O-dimethyl malaoxon were not available until they were prepared in our group (Berkman, 1993a) and their preliminary anti-AChE activities tested against rat brain AChE (Berkman, 1993b). As part of our continued study of chiral phosphorothiolate cholinesterase inhibitors (e.g. isoparathion methyl and isomalathion), we report the cholinesterase inhibition by racemic and enantioenriched malaoxon. We previously showed that a P-chiral center plays a significant role in both the inhibition and reactivation rates of AChE following reaction with phosphorothiolates (Thompson, 1992). In this study we sought to quantify the specific effect of ligand carbon stereocenter (R_c or S_c) on the inhibitory potency.

MATERIALS AND METHODS

R_c-, S_c- and racemic malaoxon were synthesized as needed according to Berkman (1993a) using magnesium monoperoxyphthalate (MMPP) oxidation (Jackson, 1992) of the enantiomers of malathion. The enantiomers were greater than 95% chemically pure and greater than 99% optically pure. Acetylthiocholine (ATCh-I) was purchased from Aldrich Chemical Co., Milwaukee. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and the following enzymes: bovine erythrocytes (BEAChE), electric eel (EEAChE), human erythrocytes (HEAChE), and human serum (HSBuChE) cholinesterases were obtained from Sigma Chemical Co., St. Louis. Working solutions of the purchased enzymes were made by dissolving an appropriate amount (5- 10 mg) of the solid enzymes in phosphate buffer (pH 7.6) which gave hydrolysis rates of ATCh-I between 0.04-0.08 absorbance units/min. Rat brain acetylcholinesterase (RBAChE) stock solutions were prepared by the following procedure: male rats with body weights ranging from 175-250 g were sacrificed via decapitation and the brains excised, rinsed from excess blood, stored in 10.0 mM potassium phosphate buffer (pH 7.6), and chilled to 0°C if stored. Homogenized and solubilized rat brain acetylcholinesterase solutions were obtained as follows. The excised brains were homogenized at 0°C in phosphate buffer (pH 7.6) to give a final volume of 20 mL. For solubilized protein, Triton X-100 (10 mL: 1% solution) was added to a homogenized sample, mixed gently for 15 min and centrifugcd for 1 h at 100000g. The supernatant containing the solubilized AChE was removed and stored at 4 °C until ready for use. Working homogenized and solubilized rat brain AChE solutions were prepared by dilution of the corresponding stock preparations in phosphate buffer (pH 7.6) until the hydrolysis rate of ATCh-I reached 0.04-0.08 absorbance units/min.

A modified Ellman method (1961) was used to determine the enzyme activities as follows. All solution volumes in this procedure were measured with Eppendorf pipettes. To each of six cuvettes was added 20 µL ATCh-I solution (75 mM ATCh-I; phosphate buffer pH 7.6) and 2.50 mL of DTNB solution (0.33 mM DTNB, 0.59 mM $NaHCO_3$; phosphate buffer pH 7.6). These cuvettes were placed

in a Beckman DU-7500 diode-array spectrophotometer and were maintained at 25°C or 37°C. A 500 µL aliquot of the appropriate working solution of the enzyme was placed in a test tube and maintained at 25 °C. A 20 µL aliquot of this enzyme solution was drawn and added to cuvette 1 to serve as control (A_0). To the remaining 480 µL of the enzyme solution in the test tube, 20 µL of the inhibitor solution was added and gently mixed (the timer was started). At 2, 4, 6, 8, and 10 min, 20 µL of the enzyme-inhibitor solution was added to cuvettes 2, 3, 4, 5, and 6 respectively. Blanks were run by adding 20 µL of 95% EtOH to the enzyme solution instead of the inhibitor and running the assay as before. The rate of hydrolysis of ATCh-I was monitored at 412 mm at 60 sec intervals for 30 min following the addition of the enzyme. The bimolecular inhibition constants (k_i) were determined as the average of 10-16 runs by plotting the $\ln(A_0/A_t)$ against incubation time. The resulting slopes were analyzed (the linear portion of the curves were used, all r's >0.97) by linear regression based on the equation $\ln(A_0/A_t) = [i]k_it$ (Aldridge, 1950). A_0 represents the activity of the uninhibited enzyme (t = 0, cuvette 1) and A_t is the depressed enzyme activity at time = t following addition of the inhibitor. The inhibitor concentrations [i] in the enzyme-inhibitor reaction tube ranged from 0.38 mM to 0.094 µM depending on the particular inhibitor and enzyme used. The final concentration of the other reactants during enzyme assay were: 0.33 mM DTNB, 0.59 mM ATCh-I, and 0.58 mM $NaHCO_3$. Statistical analyses of the results were carried out using ANOVA. A statistical comparison using the Student's t-test was performed on the k_i means and ratios and evaluated at the 99% confidence level.

RESULTS AND DISCUSSION

The bimolecular reaction constants for the inhibition of five different cholinesterases by the malaoxon stereoisomers and the racemate are shown in Table 1. Results reported by Hassan and Dauterman with bovine erythrocytes (1968) are included for comparison. Malaoxon was found to be an excellent inhibitor of rat brain and electric eel AChE, a good inhibitor of bovine and human erythrocyte AChE, and a modest inhibitor of human serum butyrylcholinesterase with a 100-fold range in inhibitory potency. In all instances, the R-malaoxon isomer was found to be a more potent inhibitor than S-malaoxon regardless of the sensitivity of the particular enzyme to the inhibitor. The R/S inhibition potency ratios ranged from 3.4 for HSBuChE to 22.5 for BEAChE and, as expected, racemic malaoxon showed near intermediate anti-cholinesterase activity.

Inhibition of rat brain acetylcholinesterase was conducted with two forms; homogenized and solubilized. These sources differ in that the homogenized RBAChE is an estimation of the inhibitor-enzyme interaction while the enzyme is still membrane-bound. The solubilized RBAChE form has been freed from the membrane by treatment with Triton-X. The higher stereoselectivity shown by the solubilized enzyme may reflect this subtle difference.

Table 1. Bimolecular reaction constants (k_i; $M^{-1}min^{-1}$)[a] for the inhibition of cholinesterases by stereoisomeric malaoxon.

Enzyme	Compound			
	R_C-Malaoxon	S_C-Malaoxon	R/S ratio	Racemic Malaoxon
RBAChE, 25°C[b] (Homogenized)	1.52×10^5	3.00×10^4	5	1.01×10^5
RBAChE, 37°C (Solubilized)	7.31×10^5 (± 7 %)	8.50×10^4 (± 9 %)	8.5	1.41×10^5 (± 9 %)
BEAChE, 5°C[c]	2.80×10^4	6.30×10^3	4.4	1.49×10^4
BEAChE, 25°C	1.96×10^5 (± 13 %)	8.70×10^3 (± 11 %)	22.5	1.54×10^5 (± 11 %)
EEAChE, 25°C	9.39×10^5 (± 4 %)	5.79×10^4 (± 4 %)	16.2	4.74×10^5 (± 6 %)
HEAChE, 25°C	1.27×10^5 (± 3 %)	1.09×10^4 (± 3 %)	11.6	6.50×10^4 (± 1 %)
HSBuChE, 25°C[d]	1.46×10^4 (± 8 %)	4.24×10^3 (± 3 %)	3.4	7.78×10^3 (± 4 %)

[a] k_i's are the average of 10-16 determinations; coefficient of variation ($s/\bar{x}$) in parentheses. Comparison of means by the Student t-test showed that all the results were significantly different at the 99% confidence level. [b]data from Berkman (1994); coefficients of variation were not reported. [c]data with O,O-diethyl malaoxon (Hassan and Dauterman, 1968); coefficients of variation were not reported for these data. [d]HSBuChE is a butyryl cholinesterase.

The results presented in Table 1 are in general agreement with those found by Hassan and Dauterman (1968). In their study, R-malaoxon was a 4.4-better inhibitor although the O,O-diethyl analogs of malaoxon did not show as near as high a degree of stereoselectivity as the O,O-dimethyl compounds did against BEAChE in this study (22.5-fold difference). It should also be noted that the Hassan and Dauterman study was conducted at low temperature (5 °C) where rate differences should be greater for stereoisomers.

Malaoxon is the putative active anticholinesterase agent formed from malathion and therefore, likely to be responsible for the intoxication of both target and non-target organisms. Intoxication by malaoxon occurs either through bioactivation of

malathion or through direct exposure as an impurity in technical malathion formulations. Moreover, malaoxon indirectly aids the intoxication of warm-blooded mammals by inhibition of carboxylesterases that detoxify malathion via hydrolysis of the carboxyester groups.

Many OP compounds have been shown to be stereoselective inhibitors of cholinesterase and carboxylesterase enzymes, and in certain instances, the stereochemical differences play a critical role in the mechanism of action. Regardless of whether malaoxon is present in existing formulations or formed *in vivo* the material is racemic; therefore, it is important to analyze the individual stereoisomers to assess differences in their interaction with target enzymes.

In this study we have shown that the stereoisomers of malaoxon are significantly different in their ability to inhibit cholinesterase enzymes. Most surprising in this study was the finding that R-malaoxon showed more than a 22-fold greater inhibitory potency than S-malaoxon for BEAChE. In previous work with isoparathion methyl (Thompson, 1992), which has only a P-chiral center, rat brain AChE best discriminated the stereoisomers. The malaoxon result would suggest that BEAChE may be more sensitive to ligand stereochemistry and alignment than rat brain AChE. However, rat brain AChE is able to better discriminate a P-chiral center (Thompson, 1992). In contrast, the low stereoselectivity found for the malaoxon stereoisomers against human serum butyrylcholinesterase (3.4-fold) may reflect the larger active site domain for butyrylcholinesterases. Using site-specific mutagenesis, Taylor (Radic, 1993) showed that there are three distinct domains that confer selectivity for the butyryl- and acetyl-cholinesterase inhibitors. Based on this model, a lack of a P-chiral center [to force a specific alignment] and the non-aromatic and uncharged nature of leaving group, malaoxon stereoisomers are less likely to be discriminated by butyrylcholinesterase than acetylcholinesterase leading to a lower differentiation between the isomers.

The stereoisomers of malaoxon have been shown to be potent, stereoselective inhibitors of various cholinesterases. It is important to note that certain inhibition results were obtained with commercial enzymes that may exist as multiple forms or be partially degraded. Although the magnitude of the inhibitory potency and stereoisomer ratio may vary slightly with purified protein, R_c-malaoxon is the more potent inhibitor. With the data presented in this paper, we can now better assess the individual contribution of a C-chiral ligand group on the anti-cholinesterase potency and use this information to study reactivation kinetics and the influence of dual stereocenters on phosphorothiolate mode of action (e.g., isomalathion; Berkman, 1993).

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