ผลของอนุพันธ์เอซิลอะมิโนไพริดีน และ เอซิลอะนิลีน ต่อสมรรถนะของเอนไซม์โมโนเอมีนออกซิเดสของไมโตคอนเดรียที่แยกจากตับหนูขาว

นางสาว ตติยา ถนอมดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF ACYLAMINOPYRIDINE DERIVATIVES AND ACYLANILINE DERIVATIVES ON MONOAMINE OXIDASE ACTIVITY IN ISOLATED RAT LIVER MITOCHONDRIA

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จุดมุ่งหมายของการวิจัยนี้เป็นการศึกษาผลของอนุพันธ์เอซิลอะมิโนไพริดีนและเอซิลอะนิลีน (CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 และ CU-18-13) ต่อสมรรถนะของเอนไซม์โมโนเอมีนออกซิเดสในไมโตคอนเดรียที่แยกจากตับหนูขาว พบว่าสารทดสอบทั้งหมดสามารถออกฤทธิ์ยับยั้งเอนไซม์โมโนเอมีนออกซิเดสในความแรงค่อนข้างต่ำเมื่อเทียบกับ standard inhibitors และเป็น non selective inhibitor ยกเว้น CU-18-08 ซึ่งออกฤทธิ์เป็น selective MAO-B inhibitor CU-18-10 มีความแรงในการยับยั้งเอนไซม์โมโนเอมีนออกซิเดสชนิดบี และ CU-18-12 มีความแรงในการยับยั้งเอนไซม์โมโนเอมีนออกซิเดสชนิดเอ มากกว่าสารทดสอบตัวอื่นๆ ผลจากการศึกษาจลนศาสตร์ของการยับยั้งเอนไซม์โมโนเอมีนออกซิเดสทั้งชนิดเอและชนิดบีโดย CU-18-08 เมื่อใช้ double-reciprocal plots พบว่าเป็นการยับยั้งแบบผสมระหว่าง competitive และ non competitive สอดคล้องกับค่า kinetic parameters คือ ค่า Km มากขึ้น แต่ค่า Vmax ลดลง ส่วนการยับยั้งเอนไซม์โมโนเอมีนออกซิเดสชนิดเอและชนิดบีโดย CU-18-10 มีความแตกต่างกัน คือ ผลต่อเอนไซม์โมโนเอมีนออกซิเดสชนิดบีเป็นการยับยั้งแบบผสมระหว่าง uncompetitive และ non competitive ในขณะที่ผลต่อเอนไซม์โมโนเอมีนออกซิเดสชนิดเอเป็นการยับยั้งไม่แข่งขันแบบ uncompetitive ซึ่งผลต่อเอนไซม์โมโนเอมีนออกซิเดสทั้งชนิดเอและบีโดย CU-18-10 พบว่าค่า Km และ Vmax ลดลง ผลการทดลองทั้งหมดนี้ชี้แนะให้เห็นถึงความสัมพันธ์ของโครงสร้างของสารที่เหมาะสมต่อการออกฤทธิ์ยับยั้งเอนไซม์โมโนเอมีนออกซิเดส

ภาควิชา เภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

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TATIYA THANOMDEE : EFFECTS OF ACYLAMINOPYRIDINE DERIVATIVES AND ACYLANILINE DERIVATIVES ON MONOAMINE OXIDASE ACTIVITY IN ISOLATED RAT LIVER MITOCHONDRIA. THESIS ADVISOR : ASST. PROF. WITHAYA JANTHASOOT, 105 pp. ISBN 974-17-0232-9

The purpose of this study was to investigate the inhibitory effect and kinetics of inhibition of acylaminopyridine and acylaniline derivatives, CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13, on monoamine oxidase (MAO) activity of rat liver mitochondria. CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13 inhibited MAO-A and MAO-B activities with low potency. In all of tested compounds, the selective inhibitory potencies of CU-18-10 for MAO-B activity, and selectivity of CU-18-12 for MAO-A activity, were higher than those of the others. CU-18-08 showed selective inhibition towards MAO-B and CU-18-10 is a non-selective MAO inhibitor but showed preferential effect on type B of MAO. On the other hand, CU-18-07 , CU-18-09 , CU-18-11 CU-18-12 and CU-18-13 did not showed preferential effect on specific type of MAO. Kinetics of MAO inhibition of two interesting tested compounds were also performed. Double-reciprocal plots of MAO-A and MAO-B inhibition by CU-18-08 was reversibly mixed type of competitive and non-competitive inhibition by increased Michaelis constant, Km and decreased maximum velocity of the reaction, Vmax. Kinetic analyses of MAO by CU-18-10 revealed that the interaction with MAO-B was reversibly mixed type of non-competitive and uncompetitive inhibition but interaction with MAO-A was reversibly uncompetitive , by decreased both Km and Vmax values. Overall, this finding showed structure-activity relationship that may be applied to further examination of new potential and selective inhibitors of MAO.

Department PHARMACOLOGY Student 's signature……..…
Field of study PHARMACOLOGY Advisor 's signature…….
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Finally, I would like to thanks my family and my friends for their understanding and support

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List of Abbreviations

BSA	=	bovine resum albumin
°C	=	degree Celcius
conc.	=	concentration
DMSO	=	dimetyl sulfoxide
E	=	enzyme
EGTA	=	ethyleneglycol-bis-(β-aminocethyl ether) N,N,N,N-tetraacetic acid
FAD	=	flavin adenin dinucleotide
$FADH_2$	=	reduced flavin adenine dinucleotide
FMN	=	flavin mononucleotide
fl	=	oxidized flavin
g	=	centrifugal force unit (gravity)
gm	=	gram
H^+	=	proton
5-HT	=	5-hydroxytryptamine
IC_{50}	=	The concentration required for 50 % inhibition of enzymatic activity
Km	=	Michaelis constant
M	=	molar
mM	=	millimolar
ml	=	millilitre
mg	=	milligram
min	=	minute
MAO	=	monoamine oxidase
MAO-A	=	monoamine oxidase type A
MAO-B	=	monoamine oxidase type B

List of Abbreviations (continue)

MAOIs	=	monoamine oxidase inhibitors
M.W.	=	molecular weight
μM	=	micromolar
μg	=	microgram
μl	=	microlitre
N	=	normal
natoms	=	nanoatoms
O	=	oxygen
S	=	substrate
S.E.	=	standard error
Vmax	=	maximum velocity
V	=	initial velocity
v / v	=	volume by volume
w / v	=	weight by volume
[ ]	=	concentration
<	=	less than
>	=	more than
/	=	per
%	=	percent

CHAPTER I

INTRODUCTION

Monoamine oxidase is a flavin adenine dinucleotide (FAD) containing enzyme of the outermembrane of mitochondria existing as two isoenzymes , MAO-A and MAO-B , which differ in specificity for substrates , sensitivity to inhibitors and primary amino acid sequences. MAO-A and MAO-B catalyze the oxidative deamination of biologically important amines including neurotransmitters in the central nervous system and peripheral tissue by the production of hydrogen peroxide (Creasey , 1956 ; Alston , 1981 ; Shih , Chen and Ridd, 1999).

MAO-A has higher affinity for the substrates such as serotonin (5-HT) , norepinephrine (NE) , dopamine (DA) ,and the inhibitor, clorgyline. MAO-B has higher affinity for phenylethylamine (PEA) , benzylamine , and the inhibitor, deprenyl whereas tyramine was metabolized by both forms of enzyme (Lyles and Greenawalt , 1977 ; Murphy , 1978 ; Fowler , Mantle and Tipton, 1982).

MAO inhibitors (MAOIs) have been used into clinical practice primarily because of their mood elevating effect in depressed patients and had been used as antihypertensive drugs. Inhibition of MAO leads to an increase in intraneuronal store of catecholamines which plausibly accounts for other pharmacological effects (Alston , 1981 ; Baldessarini , 1990).

On the other hand, the ability of MAOIs to potentiate the actions of the amines is responsible for potentially serious side effects that severely limit the clinical usefulness of the MAO inhibitors. For instance, coadministration of MAOI with food rich in tyramine and other amines can produce hypertension which sometimes can be fatal. This phenomenon is sometimes referred to as the " cheese reaction " because of the high levels of tyramine in many cheeses (Blackwell , 1967).

The MAO activity is associated with various behavioral aberrations. Inhibitors of MAO-A are used to treat affective disorders, whereas MAO-B inhibitors are of

benefit for subjects with neurological disorders such as Parkinson 's disease (Shih et al.,1999).

Therefore, specificity of MAOIs which may govern their pharmacological activity should be studied extensively in order to provide lesser adverse effect and more effective treatments of Parkinson 's disease , depression and other disorders can be derived from the newer biochemical pharmacology of substrate – selective MAO inhibiting drugs.

N-acylaminopyridine derivatives (an old derivatives) were synthesized by Assist. Prof. Dr. C. Patarapanich and colleague. Several studies using isolated rat liver mitochondria demonstrated that N-acylaminopyridine derivatives, CU763-16-04 , CU763-18-01, CU763-18-02 and CU763-18-04 (Figure 13) which possessed MAOI activity and adversely suppressed respiration of mitochondria (Unchittha T., 2000). Preliminary studies showed that acylaminopyridine and acylaniline derivatives inhibited the contraction of smooth muscle of rabbit duodenum and inhibited MAO activity in mitochondria of rat liver (Witoon T. and Chaiporn B., 2000).

The main objective of the study was to investigate the inhibitory effect and kinetics of inhibition of new acylaminopyridine and acylaniline derivatives on activity of mitochondrial monoamine oxidase isolated from rat liver.

CHAPTER II

LITERATURE REVIEWS

In 1928, the first report on enzyme monoamine oxidase (MAO)(monoamine : O_2 oxidoreductase (deaminating) , EC.1.4.3.4.) was described. Hare (1928) named the enzyme she discovered in rabbit liver, tyramine oxidase, and noted that this enzyme did not oxidize adrenaline. Monoamine oxidase (MAO) catalyzes the oxidative deamination of a number of biogenic amines in brain and peripheral tissue by the production of hydrogen peroxide (H_2O_2) (Creasey , 1956 ; Shih et al., 1999).

$$R\text{-}CH_2\text{-}NH_2 + H_2O + O_2 \rightleftharpoons R\text{-}CHO + H_2O_2 + NH_3$$

The mitochondrial monoamine oxidase which are believed to be synthesized by the endoplasmic reticulum under nuclear DNA control, and later become attached to the outer mitochondrial membrane (Chau and Hackenbrock , 1975 ; Murphy , 1978). Enzyme in soluble form could be isolated by prolonged sonication and/or detergent treatment. The soluble form of enzyme has been showed to differ from membrane - bound enzyme in its sensitivity towards inhibitors and in the kinetic mechanism (Houslay and Tipton ,1975 ; 1976). It is now generally accepted that the enzyme contain 1 mole of flavin cofactor, identified as flavin adenine dinucleotide (FAD), covalently bound as a prosthetic group (Tipton 1968 ; Oreland ,1971 ; Youdim and Sourkes , 1971) and consist of a mononuclear Cu (II) ion (McGuirl and Dooley , 1999).

Multiple forms of monoamine oxidase

The existent of multiple forms of monoamine oxidase became a prominent field for investigation. The different forms of MAO are the products of two related but different genes. In human and other mammals the two genes are closely linked on the X – chromosome (Kochersperger et al., 1986). Sandler and Youdim (1972)

first used polyacrylamide gel electrophoresis to separate the different constituents of solubilized MAO preparation from rat liver and human placenta. Many studies suggested that the multiple forms of MAO may as a single enzyme protein with different amounts of attached phospholipid and that the phospholipid may govern the enzyme 's mobility during electrophoresis (Housley and Tipton , 1973 ; Neff and Yang ,1974).

Monoamine oxidase (MAO) is now understood to exist as at least two types : MAO-A and MAO-B which differ in substrate specificity and inhibitor sensitivity. The specificity must be reflected in the amino acid sequence and molecular structure of the active sites. The translated protein sequence of MAO-A and MAO-B cDNA showed that the two isoenzymes differed in size, the MAO-A cDNA coded for a protein of 527 amino acid residues (M.W. 59,700 Da.) and MAO-B cDNA coded for a protein of 520 residues (M.W. 58,800 Da.) (Bach et al., 1988 ; Powell , 1991). Comparison of the sequence of the different forms with from other species showed that there is a degree of similarity between the same form from different species than between different forms from the same species. For example, rat and human MAO-B have approximately 90 % of amino acid sequences identical (Powell , 1991).

As regard the active site and domain that confer the substrate and inhibitor specificity of MAO, in mammalian MAO isoenzyme and trout MAO there are 4 tightly conserved region (Figure 3). In MAO-B, these regions include (1) an ADP binding β-α-β unit (residues 6-43) ; (2) a putative substrate-binding domain (residues 178-221) ; (3) a site of flavin adenine dinucleotide (FAD) covalent attachment site (residues 380-458) ; and (4) a C terminus region (residues 491-511) predicted to form a transmembrane - associated α-helix (Powell ,1991 ; Chen , Wu and Shih, 1994). Each of these region has been studied to further elucidate the structure and function of MAO. Mutagenesis of highly conserve glutamic acid residue (Glu-34) and tyrosine residue (Tyr-44) located in the ADP-binding domain of human MAO-B drastically reduced catalytic activity (Zhou et al., 1995).

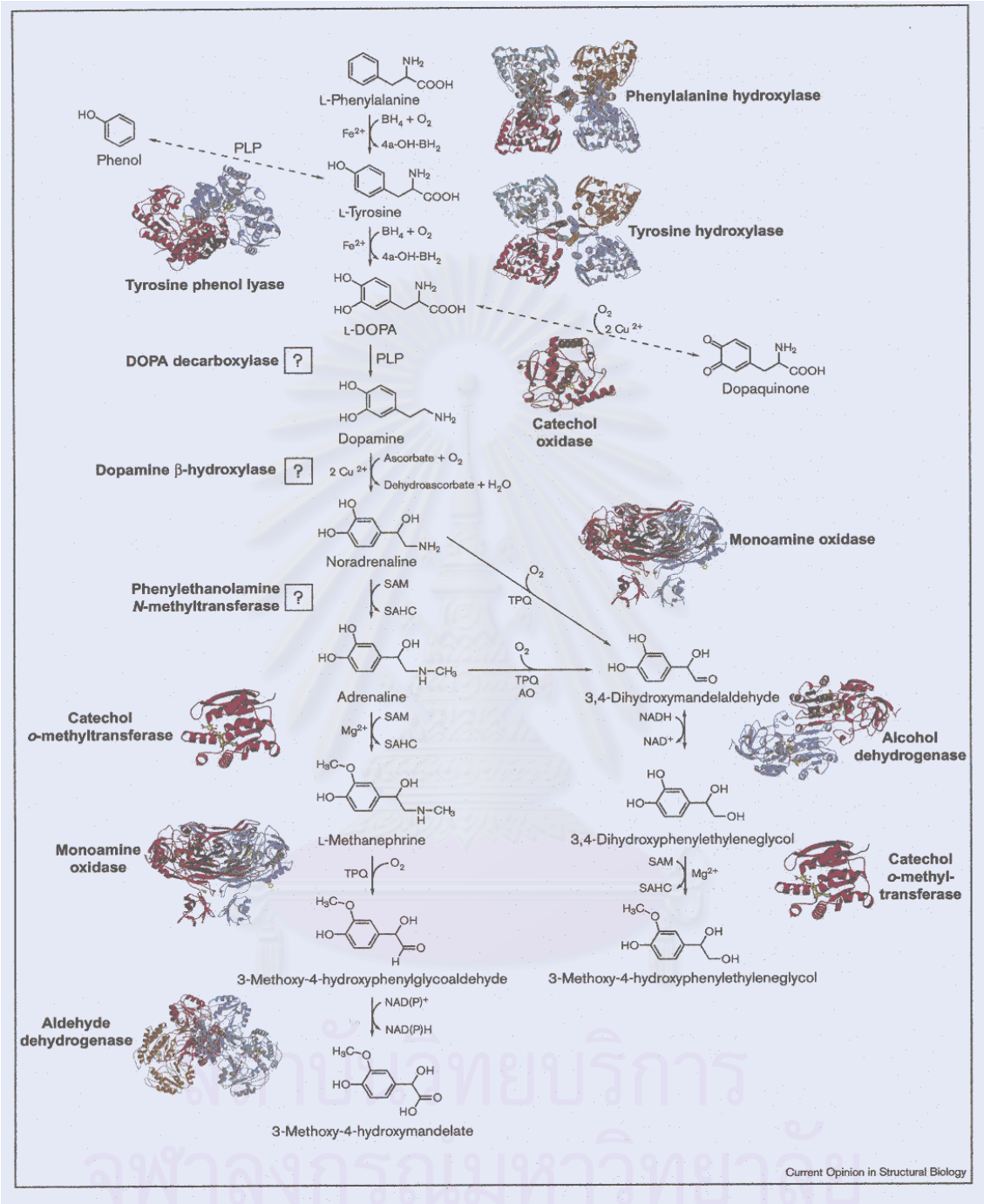


Figure 1 The catecholamine biosynthetic and degradation pathway (reaction depicted with dashed lines indicate step that do not occur in humans)

(Erlandsen , Abola and Stevens, 2000)

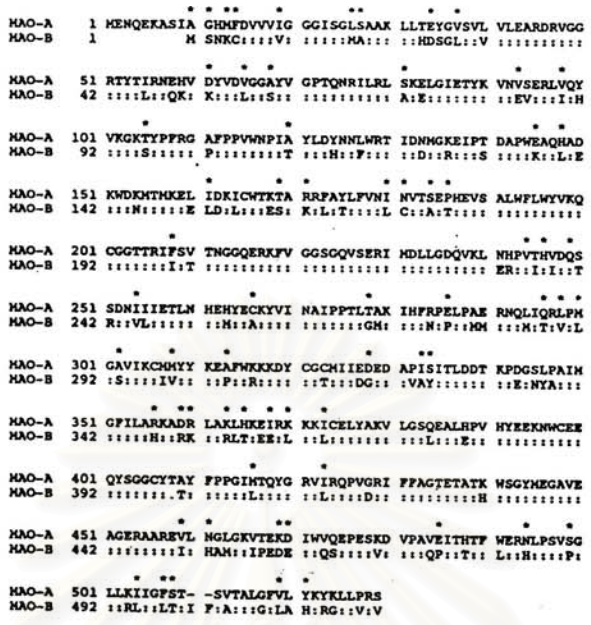


Figure 2 Comparison of amino acid sequence of human MAO-A and MAO-B. Sequences were deduced from cDNA cloned [2-4]. Colons indicate identity between MAO-A and MAO-B sequences, conserved amino acid residues differing between MAO-A and MAO-B are marked by asterisks. (Powell , 1991).

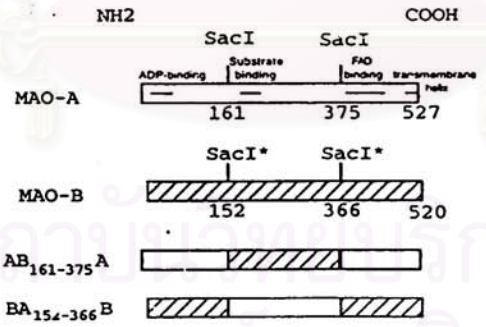


Figure 3 Schematic diagram showing wild-type and chimeric MAO forms. Open boxes represent MAO-A ; hatched boxes represent MAO-B. Four highly conserved regions are indicated. Restriction endonuclease sites used for construction of chimeric forms are shown. * SacI, introduced by PCR-mutagenesis. Number represent amino acid positions. (Grimsby et al., 1996)

Table 1 The distribution of the two major species of MAO activity in the rat (Housley et al., 1976)

Tissues	Species A activity (Percent of total)	Species B activity (Percent of total)
Liver	40	60
Liver parenchymal cells	50	50
Denervated liver	40	60
Kidney	70	30
Intestine	70	30
Intestinal mucosa	60-70	30-40
Spleen	95	5
Lung	50	50
Testis	90	10
Brain	55	45
Superior cervical ganglia	90	10
Pineal gland	15	85
Denervated pineal gland	5	95
Vas deferens	50	50
Denervated vas deferens	35	65

Mutagenesis of the cysteine residue to serine demonstrated that two cysteine (Cys-347 and -406) in MAO-A and three cysteine (Cys-156, -365 and -397) in MAO-B are important for catalytic activity (Wu et al., 1993). Hiro et al. (1996) suggested that the covalent attachment of FAD may function as a structural core for the active conformation in the membrane. The molecular structure of the active site of the two isoenzymes, except that both forms of MAO have identical sequences of amino acid immediately adjacent to the covalent bound flavin, Ser-Gly-Gly-Cys-(FAD)-Tyr (Dostert and Benedetti , 1991).

Localization and development of monoamine oxidase

Shih et al. (1999) summarized the localization of MAO in rodent , cat , primate ,and human by a variety of techniques. The distribution of MAO A and B in brain shows that MAO-A is predominantly found in catecholaminergic neurons, and MAO-B is the form most abundant in serotonergic and histaminergic neurons and glia cells. The distribution of MAO in the periphery varies within the same organism. Some tissue (human platelet and bovine liver and kidney) mainly contain MAO-B and others (human placenta and bovine thyroid gland) predominantly contain MAO-A. In human and rodents, MAO-A is exist before MAO-B in most tissue. MAO-A is almost at adult levels at birth whereas MAO-B activity increase several-fold with aging (Yang , Goridis and Neff, 1972 ; McEntire , Buchok and Parpermaster, 1979 ; Shih et al., 1999).

Goridis and Neff (1971) found that the ratio of MAO-A / MAO-B activity for brain was 6 / 4 while the ganglion it was 9 / 1 and they concluded that tyramine is deaminated by both MAO-A and MAO-B whereas noradrenaline (NE) is deaminated only by MAO-A, the enzyme which is most active in the ganglion. These observations are consistent with the hypothesis that a specific intraneuronal MAO plays an important role in the catabolism of NE in sympathetic nerves. In human blood platelet, only the B form of MAO is present (McEntire et al., 1979).

Substrate selectivity and inhibitor sensitivity

MAO has a wide range of substrate specificity, being able to oxidize biologically important amines, including neurotransmitters, trace amine, neurotoxins, and plays a central role in regulation of intracellular levels of these amine. The substrate specificity of the two forms of MAO vary from tissue to tissue (Table 2).

Several studies have shown that MAO-A preferentially oxidizes serotonin (5-HT) and noradrenaline (NE), and is irreversibly inactivated by low concentration of the acetylenic inhibitor, clorgyline whereas MAO-B preferentially oxidizes phenylethylamine (PEA) , benzylamine (BA) and is irreversibly inactivated by deprenyl (selegiline). Tyramine and tryptamine are substrates for both forms of MAO. In human, dopamine is oxidized by MAO-B and in rodent it is oxidized by MAO-A , in most species dopamine can be oxidized by both forms of the enzymes (Johnston , 1968 ; Neff and Yang , 1974 ; Green and Youdim , 1975 ; Glover et al., 1977 ; Grimsby , Zentner and Shih, 1996 ; Shih et al., 1999).

The mechanism of MAO - catalyzed reaction and inactivation

MAO catalyzes the oxidation of amines to the corresponding imines with reduction of the flavin. Silverman and Zieske (1985) proposed " one – electron mechanism " , the molecular mechanism of action for the enzyme reaction is shown in Figure 4. One electron is transferred from the amino group to the flavin to form the amine radical cation which can lose the proton to become the carbon radical. The carbon radical can be oxidized further either by second electron transfer or by radical combination with an active site radical (either the flavin radical just generated or an amino acid radical generated by hydrogen atom transfer from the amino acid to the flavin) followed by β-elimination to the immonium product.

Mechanism - based inactivators have been shown to be useful in the study of enzyme mechanisms and in the design of new classes of inactivators. Figure 5 shows the basis of previous studies on the mechanism of MAO and a chemical model study for the inactivation mechanism was proposed (Silverman , 1991).

Table 2 Substrate specificities and selective inhibitors of MAO-A and MAO-B (Neff and Yang , 1974)

	Monoamine oxidase	
	Type A	Type B
Preferred substrates	Serotonin Norepinephrine Normetanephrine	Benzylamine β-phenylethylamine
Specific inhibitor drugs	Clorgyline Harmaline	Deprenyl
Common substrates	Dopamine Tyramine Tryptamine	
Nonspecific inhibitor drugs	Pargyline* Isocarboxazid Tranylcypromine Pheniprazine Phenelzine Iproniazid Nialamide	

** May preferentially inhibit type B enzyme*

Monoamine oxidase inhibition

The discovery that inhibitors of mitochondrial MAO are antidepressant has resulted in the synthesis of large number of inhibitors. Four chemical types of " suicide substrate " of MAO have been described, namely ; substituted hydrazine , cyclopropyl amines , propargyl amines and allyl amines (Figures 6-9) (Alston, 1981). Patek and Hellerman (1974) found that the inactivation of MAO by hydrazines requires molecular oxygen. MAO catalyzed the oxidation of phenylhydrazine and aralkylhydrazine producing a highly unstable product, " diazine " (diimide) intermediate, which inactivated the enzyme irreversibly.

Johnston (1968) first reported that graphing the percentage inhibition of tyramine deamination which was produced by increasing concentrations of clorgyline yielded biphasic plot for rat brain (Figure 10), and postulated the existence of two MAO species. Serotonin and NE oxidation were completely inhibited at low clorgyline concentration ($<10^{-7}$ M) in rat tissue, while benzylamine and phenylethylamine oxidation were inhibited at higher clorgyline concentration ($>10^{-5}$ M). In contrast, two propargyl amine compounds, deprenyl and pargyline, which inhibit the oxidation of substrates, benzylamine and phenylethylamine at low drug concentrations (Ekstedt , 1976 ; Houslay , Tipton and Youdim, 1976 ; Murphy , 1978 ; Fowler et al., 1982).

Inhibitors of MAO-A have been shown to be effective antidepressants, whereas MAO-B inhibitors appear to be of value in the treatment of neurological disorders such as Parkinson 's disease. In human , MAO-B inhibition associated with the enhance activity of dopamine as well as with the decreased production of hydrogen peroxide, a reactive oxygen species (Hauptmann et al., 1996 ; Cohen , Farooqui and Kesler, 1997).

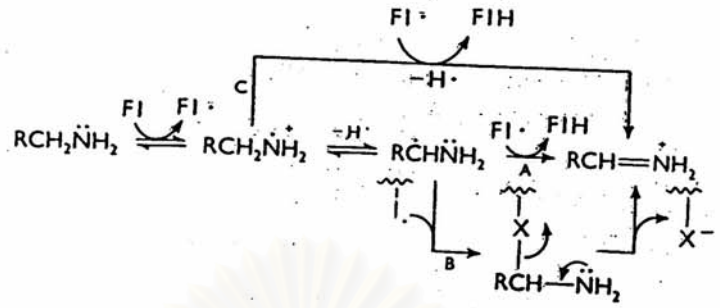


Figure 4 Proposed mechanisms for MAO-catalysed amine oxidations. (Silverman , 1991).

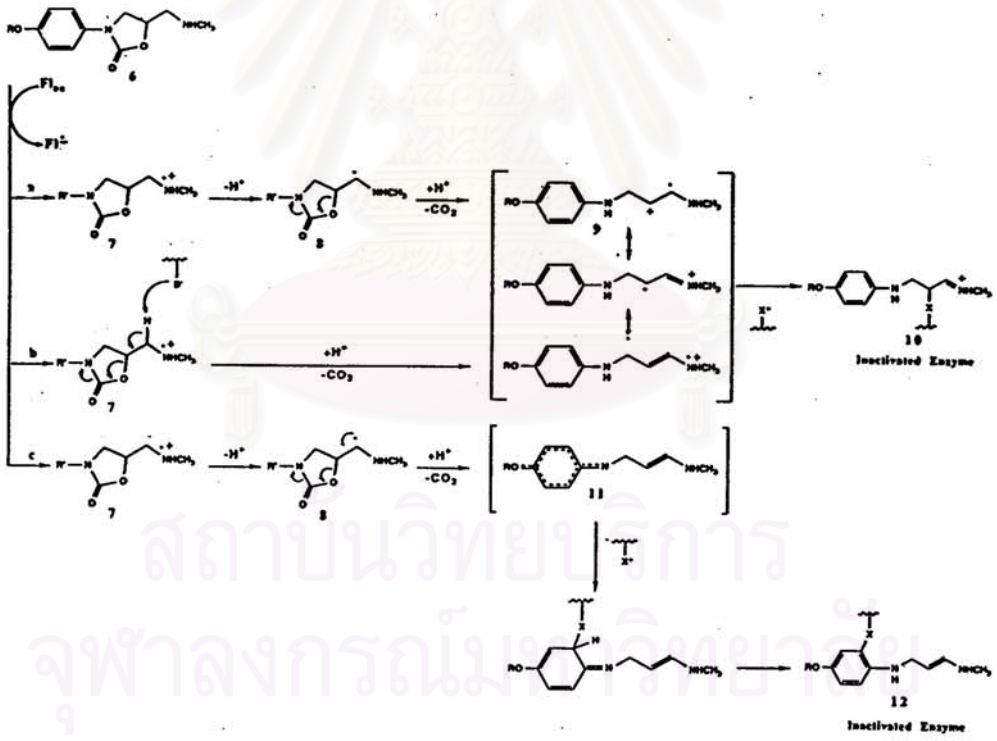


Figure 5 Initial proposal for the mechanism of inactivation of MAO by 5-(-aminomethyl)-3-aryl-2-oxazolidinones on the basis of model studies. (Silverman , 1991).

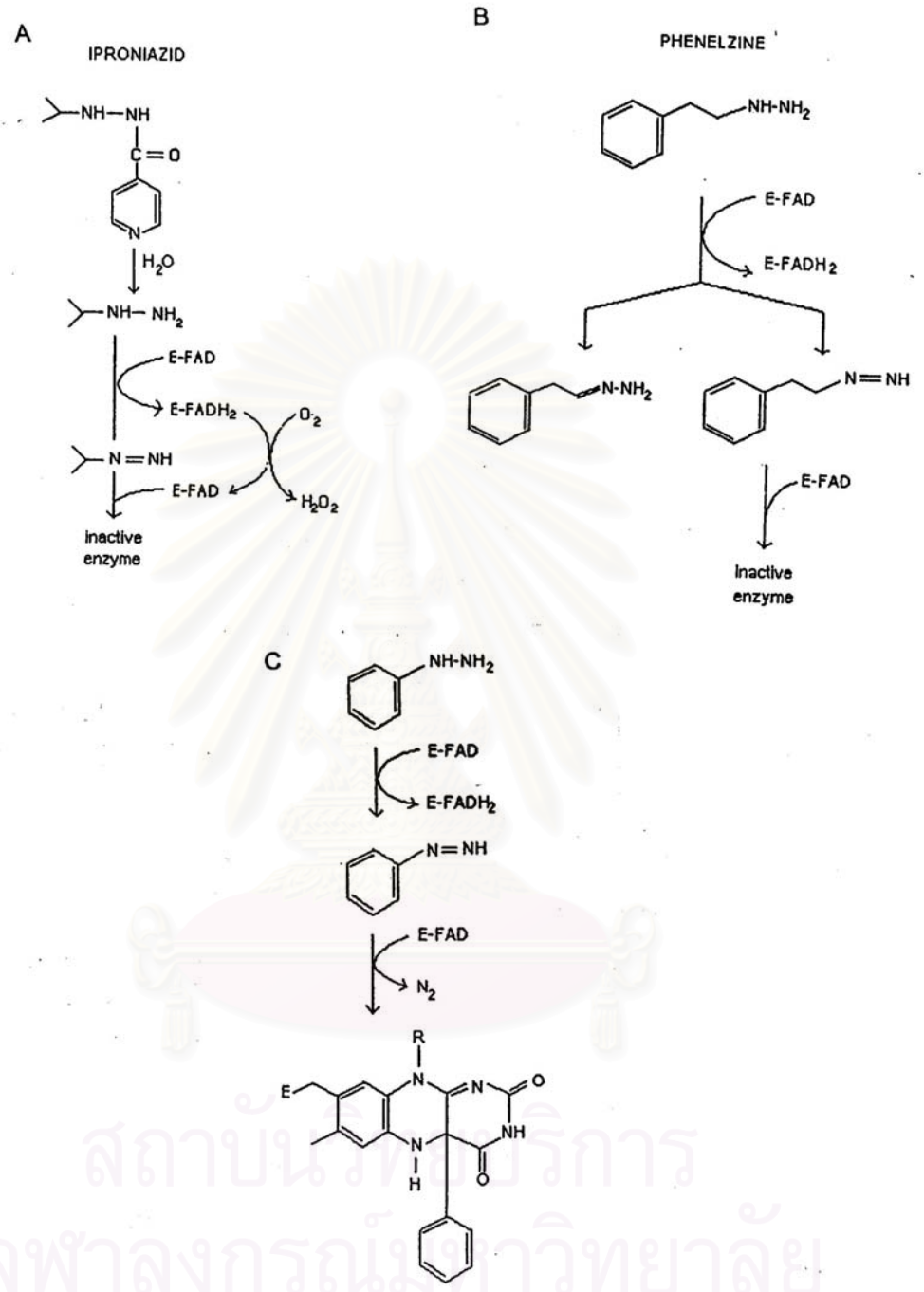


Figure 6 A : The hydrazide iproniazid hydrolyzes to isopropylhydrazine, which then inactivates MAO. B : The drug phenelzine is oxidized either to the inert hydrazone or the reactive diazine. C : Phenylhydrazine arylates the C(4a)-position of the flavin cofactor of amine oxidizing enzyme. (Alston , 1981)

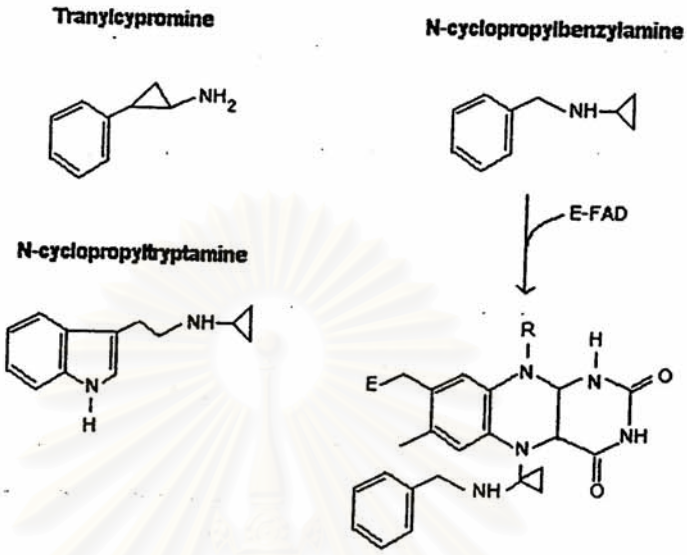


Figure 7 Cyclopropyl amines inactivate MAO include tranylcypromine, N-cyclopropyltryptamine, and N-cyclopropylbenzylamine. The flavin absorbance is bleached in the inactivate MAO, possibly secondary to meastable alkylation at the N (5)-position. (Alston , 1981)

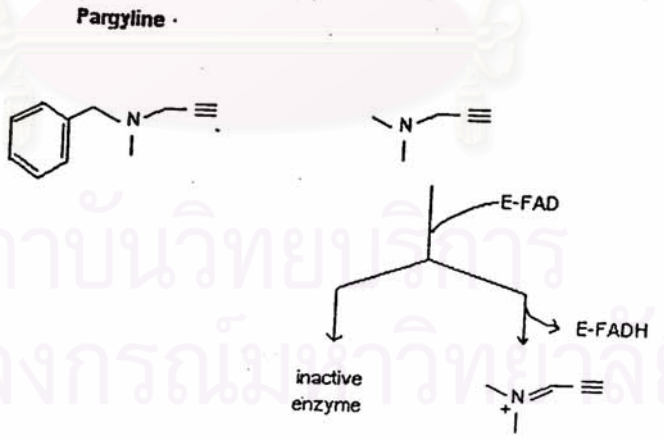


Figure 8 Pargyline inactivates . MAO whereas N,N-dimethylpropargylamine is sometimes oxidized to the reactive imine without inactivating MAO. (Alston , 1981)

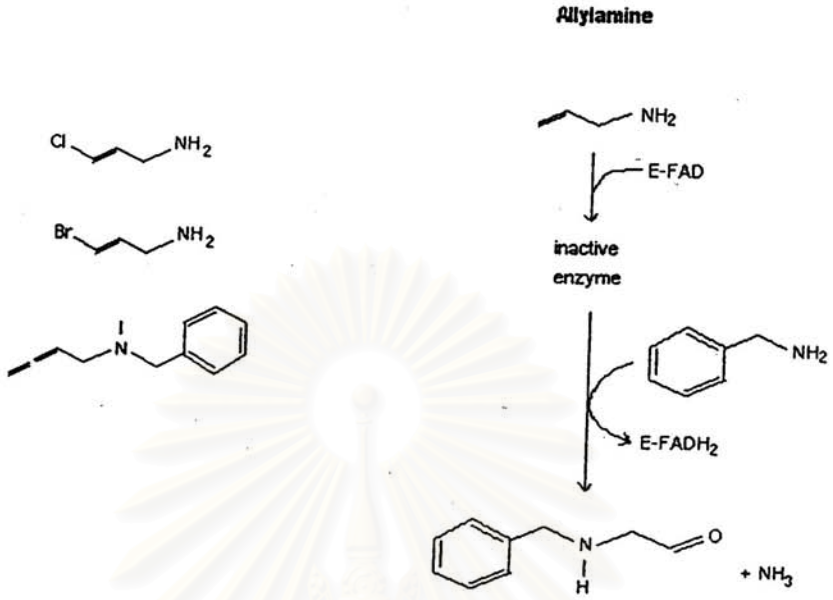


Figure 9 Left : Allenic amines and halogenated allyl amines are irreversible inactivators.

Right : The inactivation by allylamine itself is reversed during incubation with benzylamine. (Alston , 1981)

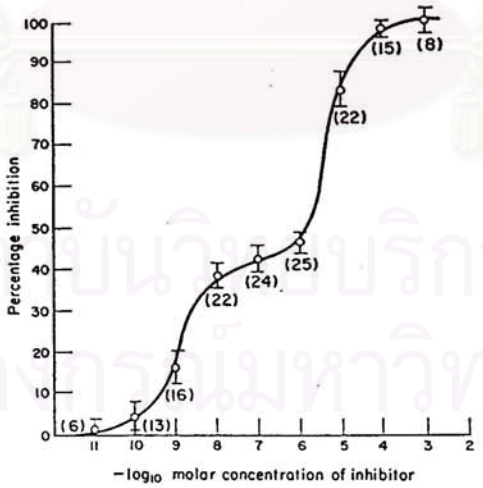


Figure 10 Inhibition curve of clorgyline against rat brain MAO in vitro using tyramine as a substrate. Points refer to the mean inhibition of tyramine oxidation and the vertical lines to the standard deviations. The figures in brackets indicate the number of experiments from which mean is calculated. (Johnston , 1968)

Irreversible monoamine oxidase inhibitors

The degree of inhibiting enzyme activity will be influenced by two factors, the affinity of the inhibitor for non-covalent binding to the enzyme and the rate of reaction within enzyme - inhibitor complex to form the irreversibly inhibited species. The interaction between MAO (E) and the mechanism-based inhibitor (I) can be represented by the following minimum reaction mechanism :

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \bullet I \overset{k_2}{\rightleftharpoons} E\text{-}I$$

where E • I represents the non-covalent complex analogous to the reversible complex formed between an enzyme and a competitive inhibitor or an enzyme – substrate complex, and E – I represents the covalent adduct between enzyme and inhibitor. Studies of the kinetics of inhibition by acetylenic amine derivatives; clorgyline , deprenyl and pargyline have shown that the degree of selectivity exhibited by these compounds to MAO-A or B can be derived either from a greater affinity for non-covalent binding to the enzyme or from a faster rate of reaction within the non-covalent complex to give the irreversibly inhibited species (Fowler et al., 1982 ; Tipton , 1994).

In other case, the formation of product and the mechanism-based inhibition of the enzyme will be competing reactions according to the general scheme :

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \bullet I \xrightarrow{k_2} E \bullet I^{*} \xrightarrow{k_4} E\text{-}I$$

$$\downarrow k_3$$

$$E + \text{products}$$

where $E \bullet I^{*}$ represents an activated complex. The inhibitor phenelzine has been shown to be substrate for the enzyme as mechanism-based inhibitors. Furthermore, 1-methyl–4 – phenyl - 1,2,3,6 - tetrahydropyridine (MPTP) and milacemide(schizophrenic

compound), which are both substrates for the enzyme and also time-dependent inhibitors of MAO-B (O'Brien et al., 1994 ; Tipton , 1994).

The MAO inhibitors in clinical use are site-directed, irreversible inhibitors. The first MAO inhibitors to be used in treatment of depression were derivatives of hydrazine. Phenelzine is the hydrazine analog of phenylethylamine (a substrate of MAO-B) ; isocarboxazid is a hydrazine derivative that probably must be converted to the corresponding hydrazine to produce long-lasting inhibition of MAO. Several of agents were structurally related to amphetamine. Cyclization of the side chain of amphetamine resulted in MAO inhibitor, tranylcypromine appears to involve the reaction of sulfhydryl group in the active center of the enzyme following the formation of an imine by the action of MAO (Baldessarini , 1990).

Selegiline ((-) Deprenyl) is an irreversible inhibitor of MAO – B, and now is applied for the treatment of Parkinson 's disease (PD) as an adjuvant to the L-DOPA therapy and more recently in depression. It is know that selegiline has been postulated to slow down the progression of PD and has potential neuroprotective actions. Selegiline is partially metabolized to (-) - amphetamine and (-)– metamphetamine, which in turn could even counteract potential neuroprotective action of deprenyl or lead to unwanted side effects. Thus, it appears to be important to develop agents MAO inhibitor, which are not metabolized to products with amphetamine-like action.

Rasagiline, (R(+) – N – propargyl – 1 – aminoindane, TVP 1012), is a restricted analogue of selegiline. Rasagiline is an irreversible MAO-B inhibitor and possesses the same propargyl moiety and high selectivity for human and rat brain MAO-B in vitro and in vivo and is 5-10 times more potent than selegiline with regard to MAO inhibition. However, it is devoid of either the hypertensive crisis and the sympathomimetic or neurotoxic amphetamine – like modes of action. Rasagiline is now attracting attention as an anti-Parkinson drug, because of its neuroprotective properties (Maruyama et al., 2000 ; Kupsch et al., 2001).

Figure 11 Structures of selegiline (A) and rasagiline (B) (Kupsch et al., 2001)

Figure 11 Structures of selegiline (A) and rasagiline (B) (Kupsch et al., 2001)

Reversible monoamine oxidase inhibitors

The affinity of MAO for reversible inhibitors is often higher than amine substrates. Thus, the recognition of reversible and competitive MAO inhibitors by the active site of MAO appears to be better than that of amine substrates. With several substrates, the substitution of hydrogen by methyl group at the carbon in the α - position with respect to the N atom has resulted in the formation of MAO-A selective reversible inhibitor (Mantle , Tipton and Garrett, 1976 ; Tipton , 1994).

Tipton (1994) suggested that the effect of reversible inhibitors are shorter duration than that of the irreversible type. The rate of recovery from the effects of a reversible inhibitor will be dependent on the rate at which it is eliminated from the tissue, since removal of the free drug will resulted in it dissociating from the enzyme. In contrast, the rate of recovery from the effects of an irreversible inhibitor will depend on the rate of turnover of the enzyme itself.

However, since the use of irreversible inhibitor of MAO appears to be of less adverse side - effect or dietary interaction than reversible inhibitor of MAO (Tipton, 1994).

Moclobemide is a reversible selective inhibitor of MAO-A that is widely prescribed for the treatment of depression. Metabolite of moclobemide, Ro 19627 (lazebemide) was found to be a potent and highly selective reversible inhibitor of MAO-B (Da Prada et al., 1990).

Monoamine oxidase and several disorders

Monoamine oxidase and Parkinson 's disease

Parkinson 's disease is a progressive neurodegenerative disorder that affects primarily the dopaminergic neurons projecting from the substantia nigra pars compacta to the putamen and caudate region of the brain (Cohen et al., 1997).

$$\text{Dopamine} + O_2 + H_2O \xrightarrow{\text{MAO-B}} \text{3,4 – dihidroxyphenylacetaldehyde} + H_2O_2 + NH_3$$

MAO - B activity increases with aging in human brain (Fowler et al., 1980). Increasing in oxidation of dopamine by MAO-B in elderly may be associated with the loss of dopaminergic neurons in the substantia nigra which underlies Parkinson 's disease and may produce levels of oxygen radicals that cause oxidative damage of nigrostriatal neurons. This is consistent with oxidative damage to mitochondrial DNA (mtDNA) by hydrogen peroxide generated during MAO – catalyzed oxidation of dopamine but tranylcypromine (an inhibitor to MAO-A and MAO-B) could abolished mtDNA oxidative damage (Hauptmann et al., 1996).

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been shown to induce Parkinsonism in man and non-human primate (Heikkila et al., 1984). The neurotoxic effects of MPTP depend on its conversion to 1-methyl–4-phenylpyridinium (MPP^+) and / or other neurotoxic metabolites generated by MAO-B (Singer and Ramsay, 1991). In addition, MAO-B has been reported to be implicated in MPTP-induced Parkinsonism, since selegiline (l -deprenyl) , an irreversible MAO-B inhibitor, prevent MPTP - induced neurotoxicity in mice (Heikkila et al., 1984). Thus, MAO-B appears to be involved in aging process in the brain either by exogenous / endogenous neurotoxin or by increasing the levels of toxic hydrogen peroxide.

Kupsch et al. (2001) summarized that treatment with either rasagiline (a more selective and potent inhibitor of MAO-B) or selegiline markedly attenuated the neurotoxic effect of MPTP at the behavioral , histological and at the biochemical level.

Monoamine oxidase and depression disorder

The neurotransmitter amines, norepinephrine and serotonin, are assumed to modulate mood and the antidepressant effect of the drugs that inhibit MAO may be the consequences of delaying the metabolism of these amines (Figure 12). These amines are deaminated by MAO-A and therefore, selective inhibitors of MAO-A usually are more effective in treating major depression than are type B inhibitors (Neff and Yang , 1974)

The first MAO inhibitors to be used in the treatment of depression were derivatives of hydrazine, a highly hepatotoxic substance. Subsequently, compound unrelated to hydrazine were found to be potent MAO inhibitors. Several of these agent were structurally related to amphetamine (tranylcypromine) were synthesized to enhance central stimulant properties. Short - acting selective MAO-A, moclobemide, has at least moderate antidepressant effect. For the irreversible action of clinically used MAO inhibitors up to two week, may be due to require to generate new MAO enzyme and restore amine metabolism to normal after discontinuation of the drugs. Selegiline also may have antidepressant effect particularly at high dose that also may inhibit MAO-A or yield amphetamine-like metabolites (Baldessarini , 2001).

Monoamine oxidase and hypertension

Hypertensive crisis is a most serious toxic effect of MAO inhibitors related to drug interaction and were noted to be associated with the ingestion of cheese in patients receiving MAO inhibitors. This effect has become known as the “cheese reaction”. Balckwell et al. (1967) suggested that certain cheese might contain a pressure amines or substances capable of liberating stored catecholamines. The average meal of natural or cheese contains enough tyramine to provoke a marked rise in blood pressure and other cardiovascular changes. As a result of inhibition of MAO, catabolism of tyramine and other monoamines are prevented. Tyramine can gives rise to strong hypertensive response by releasing NE from stores in the sympathetic nerve terminals (Baldessarini , 1990).

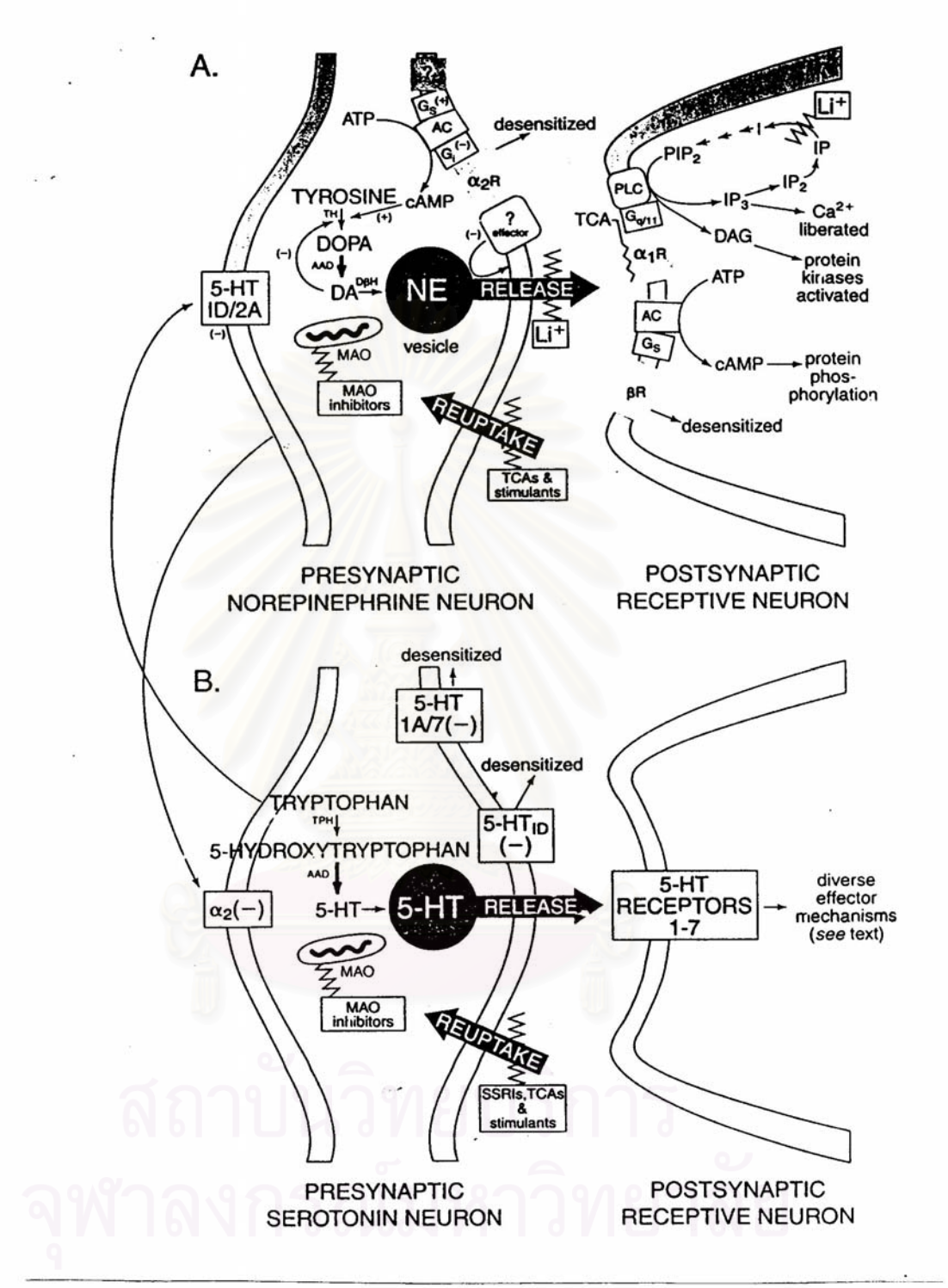


Figure 12 Sites of action antidepressants (Baldessarini , 2000)

A : Noradrenergic neurons , B : Serotonergic neurons

Protection against apoptosis by monoamine oxidase inhibitors

Apoptotic cell death is well known to be characterized by several subcellular alterations leading to a specific cell death program. In the first phase, the cell receives the death stimulus (chemical , exogenous physical etc.). In the subsequent , effector phase, several reactions can lead to cell death triggering and followed by the degradation phase which is irreversible and characterized by typical morphological and histochemical markers of apoptosis including DNA fragmentation (Tatton and Olanow , 1999).

Malorni et al. (1998) concluded that the first evidence of a possible specificity of MAO-A inhibitors in partially prevent the mitochondrial triggered cell death program and in the maintenance of mitochondrial homeostasis.

Rasagiline (more selective and potent MAO-B inhibitor) was proven to increase the activity of anti - oxidative enzyme, superoxide dismutase and catalase, and protect dopamine cell from apoptosis in induced by peroxynitrite (reactive nitrogen species , RNS). From these results suggested that rasagiline might be used to treat or prevent neuronal cell death induced by oxidative stress in aging and neurodegenerative disorders, such as Parkinson 's disease (Maruyama et al., 2000).

Biochemical , behavioral , physiologic and neurodevelopmental changes in MAO - deficient mice

Holscheider et al. (2001) summarized some of the current knowledge of phenotypic finding in MAO-A deficient mice and contrasts these with those of MAO-B deficient mice. Differences are discussed in relation to the biochemical, behavioral, and physiologic changes investigated to date, as well as the role played by redundancy mechanism, adaptation response and alterations in neurodevelopment. Tables 3 and 4 summarize the changes of MAO-A knock-out mice (MAO-A KO) and MAO-B knock-out mice (MAO-B KO) compared to wild type mice.

Kim et al. (1997) found that mice deficient in MAO-A enzymatic activity have chronic elevated levels of monoamine (specifically NE and 5-HT) in brain and

display an enhancement in emotional (fear) learning. Furthermore, MAO-A KO mice have elevated brain levels of 5-HT and a distinct enhanced aggression (Cases et al., 1995). There was a report about the impulsive aggression in men from a Dutch family with a complete MAO-A deficiency, due to a point mutation in the gene encoding MAO (Brunner et al., 1993a).

Table 3 The phenotypic findings in MAO-A deficient mice (Holschneider et al., 2001)

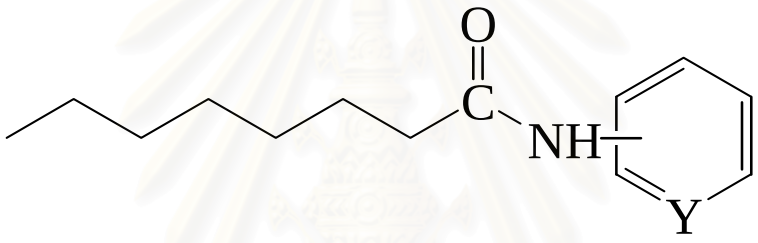
Changes of MAO A KO Mice Compared To Wild-type Mice
Biochemical
No enzymatic MAO A activity, protein or MAO A mRNA in brain and peripheral tissues
Normal levels of MAO B in brain and peripheral tissues
Increased levels of 5-HT, NE in whole brain, hippocampus, frontal cortex, and cerebellum
Decreased tissue levels of the 5-HT metabolite 5-HIAA
Downregulation of brain postsynaptic 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C} receptors and the vesicular monoamine transporter (VMAT2)
Neurodevelopmental
Abnormal development of somatosensory thalamocortical afferent fibers
Abnormal segregation of retinal afferents to the dorsal lateral geniculate nucleus, as well as to the superior colliculus
Atypical locations of 5-HT during embryonic and postnatal development
Abnormal activity and morphology of phrenic motoneurons in neonates
Transient delay of locomotor network maturation
Behavioral
Neonatal
Prolonged righting, trembling upon locomotion, and hunched posture
Frantic running and falling over, jumping, or prompt digging to hide under woodshavings in response to moderate sound and movement
Prolonged and stronger reactions to pinching
Propensity to bite the experimenter
Sleep accompanied by violent shaking and jumps
Adult
Normal nesting, nursing, and pup-retrieval behavior in females
Increased territorial aggression in the resident-intruder paradigm, increased bite wounds in male, group-housed mice
Decreased social investigative behavior in males in the resident-intruder paradigm with static, hunched, fluffed-fur posture after first olfactory stimulus.
Increased sensitivity to developing the serotonin syndrome with fenfluramine challenge
Behavioral syndrome characterized by restlessness, attentional deficits, disrupted social interaction, feeding and self-grooming after administration of the MAO B inhibitors L-deprenyl or lazabemide
Enhancement of classical fear conditioning and step-down inhibitory avoidance learning (emotional memory) but not of eyeblink conditioning (motor learning)
Increased mobility in the Porsolt Forced Swim Test
Increased time spent in the center in the Open Field test with much hesitation as to which direction to take
Abnormal walking on a balance-beam with adults grasping the lateral aspects of the beam with hindlimbs during movement
Altered courtship with increased grasping events in males and concurrent increased frequency and intensity of vocalization in females.
Physiologic
Decreased blood pressure and heart rate in the resting, restrained state
Exaggerated hyperthermic and tachycardic response to fenfluramine
Regional cerebral cortical blood flow: Higher in somatosensory and barrel field neocortex; lower in entorhinal and midline motor cortex
Decreased body weight (~ −10%)

Table 4 The phenotypic findings in MAO-B deficient mice (Holschneider et al., 2001)

Changes of MAO B KO Mice Compared To Wild-type Mice
Biochemical
No enzymatic MAO B activity, protein or MAO B mRNA in brain and peripheral tissues
Normal levels of MAO A in brain and peripheral tissues
Increased levels of PEA in whole brain
Increased urinary excretion of PEA
Normal levels of 5-HT, NE, DA, 5-HIAA, DOPAC, HVA in cortex, hippocampus, raphe nucleus, substantia nigra, thalamus
Normal levels of extracellular DA, DOPAC, 3-MT, HVA in striatum, but elevated DA levels after administration of high dose L-DOPA
Functional supersensitivity of D_1-like dopamine receptors in the nucleus accumbens
Normal density of D_1-like receptors and normal tyrosine hydroxylase immunoreactivity in striatum, nucleus accumbens, and olfactory tubercle
Up-regulation of the D_2-like dopamine receptors in the striatum and nucleus accumbers shell, but not in substantia nigra, and ventral tegmental area
Behavioral
Adult
No increase in aggressive behavior
Normal visual-spatial learning in adult and in aged animals in the Morris Water Maze
Intact working memory as tested in a Y-maze
Increased mobility in the Porsolt Forced Swim Test
Normal response in the Elevated Plus-Maze
Normal exploratory activity in the Open Field
Physiologic
Decreased blood pressure and heart rate in the resting, restrained state
Exaggerated hypertensive response to PEA
Regional cerebral cortical blood flow: Higher in midline motor cortex and medial portions of somatosensory and visual cortex; lower in piriform and anterolateral frontal cortex
No difference from wild-type mice in infarct volume or extent of brain edema following middle cerebral artery occlusion
Lack of MPTP toxicity in brain
Normal body weight

Acylaminopyridine and Acylaniline Derivatives

Acylaminopyridine and acylaniline derivatives (CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13) were synthesized by Witoon Thonge and Chaiporn Boonwan , in 2000 and modified the substitute group (Y) on benzene ring to increase relaxation effect and decrease mitochondrial toxicity. Preliminary studies suggested that these compounds inhibited the contraction of smooth muscle of rabbit duodenum and inhibited MAO activity in mitochondria of rat liver.



compound	Y	Position of substitutions	M.W.
CU-18-07	C-OCH_3	Para	235
CU-18-08	C-OCH_3	Meta	235
CU-18-09	C-NO_2	Para	250
CU-18-10	C-NO_2	Meta	250
CU-18-11	N	Meta	205
CU-18-12 (CU763-16-04)	N	Para	205
CU-18-13	CH	-	203

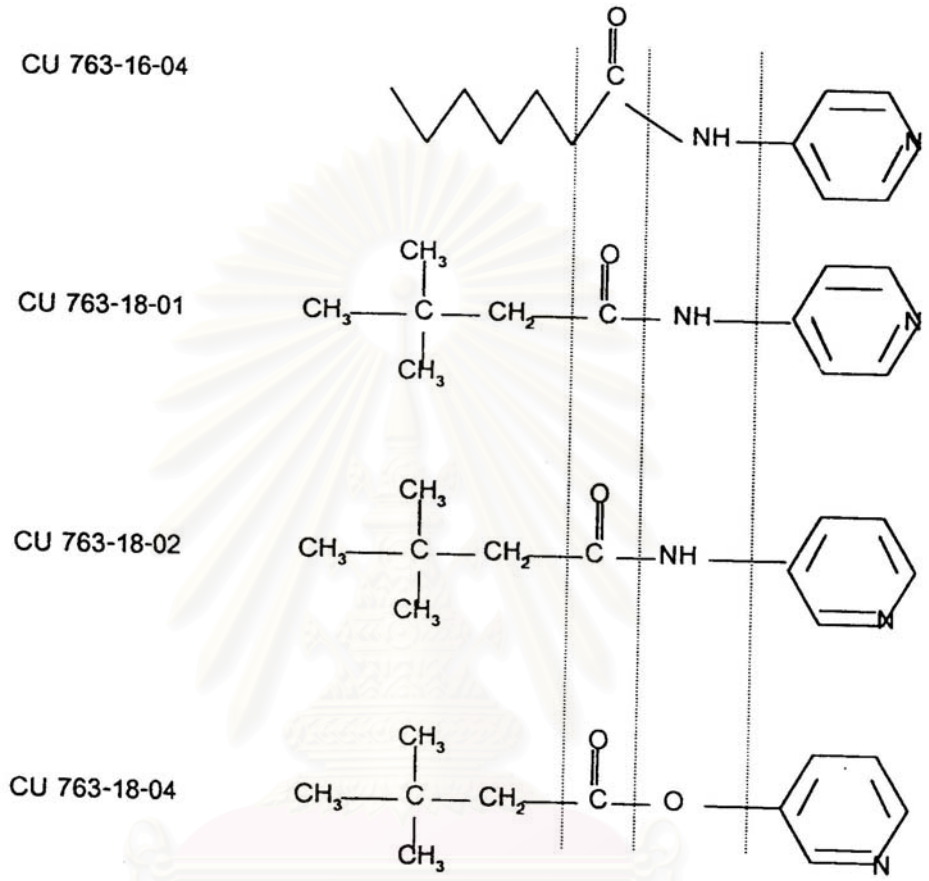
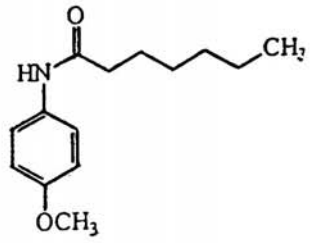
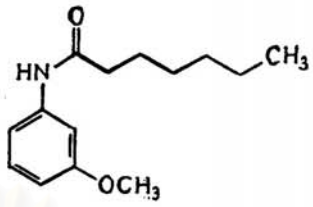


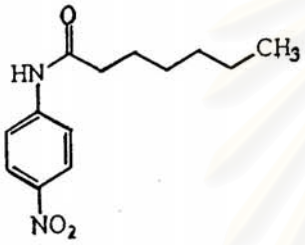
Figure 13 N-acylaminopyridine derivatives (old derivatives) : CU763-16-04 , CU763-18-01 , CU763-18-02 and CU763-18-04



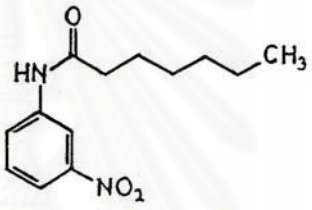
CU-18-07



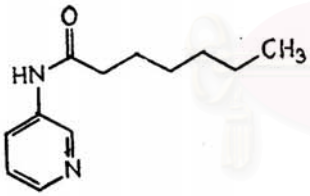
CU-18-08



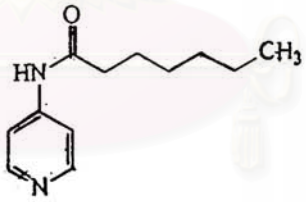
CU-18-09



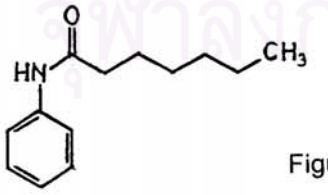
CU-18-10



CU-18-11



CU-18-12
(CU 763-16-04)



CU-18-13

Figure 14 Testing compounds : acylaminopyridine and acylaniline derivatives : CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13

Hypothesis

Acylaminopyridine and acylaniline derivatives may become a new compounds with potentially pharmacological actions for treatment of several disorders. If the tested compound can selectively inhibit MAO-A activity , these compounds could be used to treat depression disorder, whereas the treatment of Parkinson 's disease require the drug that selectively inhibit MAO-B. Furthermore, the treatment with the drug that have a selective type of MAO inhibition, will reduce the side effects for example , hypertensive crisis (this phenomenon is called " cheese reaction ").

Therefore, the MAO inhibitory effect of acylaminopyridine derivatives and acylaniline derivatives should be studied extensively.

Significance

1. Describe the inhibition of monoamine oxidase of acylaminopyridine and acylaniline derivatives.
2. Kinetics study of the inhibitory process can provide information on the potency and mode of interaction of some tested compounds with the enzyme.
3. Finding from this study may be used for further development of new potential inhibitor of monoamine oxidase.

CHAPTER III

MATERIALS AND METHODS

1. Experimental animals

Male albino rats of Wistar strain weighing about 200-250 g, supplied by the National Experimental Animal Center, were used throughout the study.

2. Instruments

2.1 Heidolph glass homogenizer (type SO203 RZR 2)

2.2 Hitachi high speed refrigerated centrifuge Himac SCR 20 B. Rotor model RP 18-3

2.3 Clark 's oxygen electrode

2.4 Oxygen monitor (YSI model 5300)

2.5 Strip chart recorder (Gilson model N2)

2.6 Spectrophotometer (Ultrospec II)

2.7 pH meter

2.8 Circulating temperature controlled water bath

3. Drugs and chemicals

The following agents were employed in the highest purity available without further purification and purchased from Sigma Chemical Co.. Most reagents were dissolved in ultra pure water and tested compounds were dissolved in dimethyl sulfoxide (DMSO).

3.1 Tested compounds obtained as gift from Assist. Prof. Dr. C. Patarapanich :
CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 , CU-18-13

3.2 Substrates for MAO enzymes :

5-Hydroxytryptamine creatinine sulfate complex (as a MAO-A substrate)

Benzylamine hydrochloride (as a MAO-B substrate)

Tyramine hydrochloride (as a MAO-A and B substrate)

3.3 Inhibitors of MAO :

Clorgyline hydrochloride (as a selective MAO-A inhibitor)

Selegiline ((-)-deprenyl hydrochloride) (as a selective MAO-B inhibitor)

Pargyline hydrochloride (as a non – selective MAO inhibitor)

3.4 Other :

Bovine serum albumin

Copper sulfate

Dimethyl sulfoxide (DMSO)

Ethylengylcol- bis-(β-aminocethyl ether) N,N,N,N – tetraacetic acid (EGTA)

Folin & Ciocalteu 's phenol reagent

Phosphate monobasic, sodium

Phosphate dibasic, sodium

Potassium hydroxide

Sodium hydroxide

Hydrochloric acid

Sodium carbonate

Sodium potassium tartrate

Sucrose

4. Reagents

4.1 Isolation medium

4.1.1 0.25 M sucrose + 1 mM EGTA

Dissolved of 42.7875 g sucrose with ultra pure water, then added with 2 ml of 0.25 M EGTA and the last, adjusted pH to 7.2 by 1 N hydrochloric acid and 0.3 N potassium hydroxide, and adjusted volume to 500 ml with ultra pure water.

4.1.2 0.25 M sucrose

Dissolved of 42.7875 g sucrose with ultra pure water, then adjusted pH to 7.2 by 1 N hydrochloric acid and 0.3 N potassium hydroxide, and adjusted volume to 500 ml with ultra pure water.

4.1.3 0.25 M EGTA stock solution

Dissolved of 4.755 g EGTA with ultra pure water and added potassium hydroxide to increase solubility. Adjusted pH to 7.2 by 1 N hydrochloric acid and 0.3 N potassium hydroxide, and adjusted volume to 50 ml with ultra pure water.

4.1.4 Sodium phosphate buffer , pH 7.4 (incubation medium)

Dissolved of 0.0300 g sodium phosphate monobasic and 0.0500 g sodium phosphate dibasic with ultra pure water. Mixed and adjusted pH to 7.4 by 1 N hydrochloric acid and 0.3 N potassium hydroxide. Adjusted volume to 100 ml with ultra pure water.

4.2 Reagents used for determination of mitochondrial protein were freshly prepared.

4.2.1 Alkaline copper reagent

Consisted of 1 part of 0.5 % copper sulphate in 1 % (w / v) potassium tartrate and 10 parts of 10 % sodium carbonate in 0.5 N sodium hydroxide.

4.2.2 Folin phenol reagent

Diluted of 1 part of Folin & Ciocalteu 's phenol reagent with 10 parts of ultra pure water.

5. Preparation of rat liver mitochondria

The method of Hogeboom (1955) modified by Myers and Slater (1957) was used to isolate rat liver mitochondria. The isolation media , homogenizer , centrifuge tubes and mitochondrial suspension were kept in an ice – bath through the preparation period. The temperature of the refrigerated centrifuge was set at 4 °C.

5.1 Preparation of liver homogenate

Rats with a body weigh of 180-250 g were cervical dislocated and the liver was removed into ice-cold isolation medium (0.25 M sucrose , 1 mM EGTA, pH 7.2). The tissue was washed frequently with ice-cold isolation medium to remove blood as much as possible. The rat liver was chopped finely with scissors and homogenized

in a glass homogenizer equipped with a motor – driven Teflon pestle (to break the cell membrane). The liver homogenate obtained was approximately 70 ml per rat.

5.2 Preparation of the liver mitochondrial suspension (Figure 15)

The liver homogenate was transferred to centrifuge tubes and centrifuged at 4 °C for 5 min. at 600 x g. The supernatant was collected and centrifuged at 4500 x g for 10 min.. The mitochondrial pellet was collected resuspended in 0.25 M sucrose and recentrifuged for 10 min. at 13000 x g. The isolated mitochondria was suspended in 0.25 M sucrose at concentration of 20-60 mg / ml of mitochondrial protein.

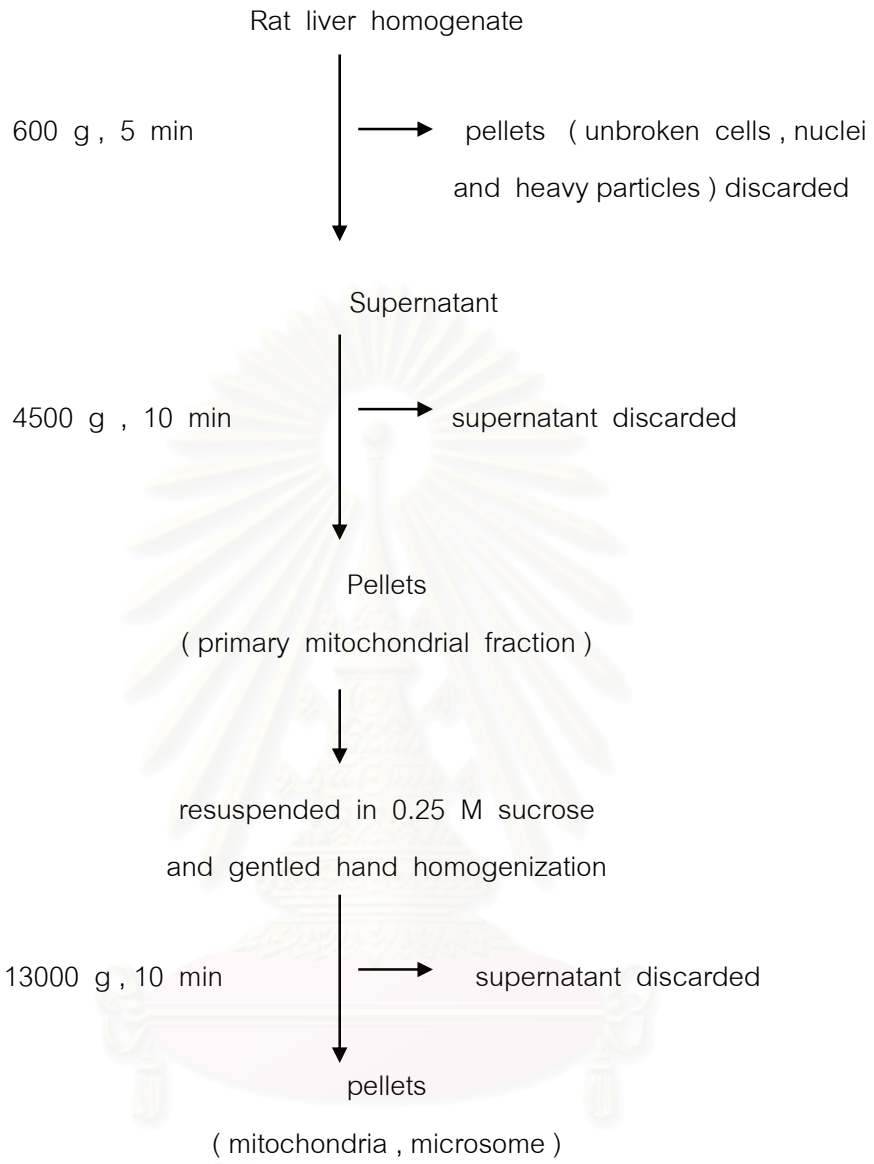


Figure 15 Schematic description of procedure for mitochondrial suspension from liver homogenate by differential centrifugation

6. Determination of monoamine oxidase activity by measurement of mitochondrial oxygen consumption rates

MAO activity is determined by oxygen consumption measured with the method of Tipton and Singer (1993). Oxygen consumption by mitochondria was measured polarographically with a Clark Oxygen Electrode in a Gilson reaction chamber (Figure 16). The chamber was about 2 ml and consisted of the water jacket encompassed the chamber and a hollow glass stopper through which the substrates and reagents were added into the chamber. Incubations were carried out at 37 °C controlled by circulating water bath and mixed by a small rotating magnetic stirrer. The electrode output was amplified with biological oxygen monitor (YSI model 5300) and recorded on strip – chart recorder. The tracings obtained were called "oxygraph" or "polarographic" tracings. The electrode was calibrated with purified water saturated with air at 37 °C.

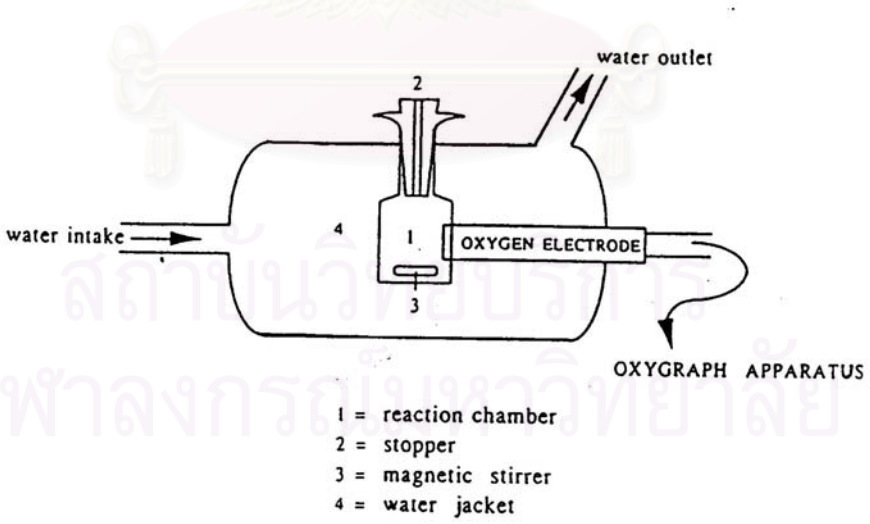


Figure 16 Gilson reaction chamber

7. Calculation of rates of oxygen consumption

Initial velocity of MAO catalyzes the oxidative of substrate was showed by oxygen consumption rate that can be calculated from oxygraph tracing as shown in figure 17.

Rate of oxygen consumption before the addition of substrate (X)

$$= \quad Q \times S / P \quad \text{natoms oxygen / min.} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Rate of oxygen consumption after the addition of substrate (Y)

$$= \quad R \times S / P \quad \text{natoms oxygen / min.} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Oxygen consumption rate in the reaction $= Y - X$ natoms oxygen / min. .....(3)

Where, P , Q and R = the length of line P , Q and R, respectively

S = natoms oxygen initially dissolved in the reaction mixture

S value depends on volume of reaction mixture and temperature ; the larger the volume and the lower the temperature, the more oxygen dissolved in the reaction mixture. S value calculated by multiplying the amount of oxygen dissolved in 1 ml water (A) with total volume of the reaction mixture. The A value is calculated from the following formula :

$$A = s \times P \times N \times 10^{9} / V \times 100 \quad \text{natoms oxygen / ml} \ldots\ldots\ldots(4)$$

Where, A = natoms oxygen dissolved in 1 ml of water

s = absorption coefficient at 37 °C (volume of oxygen reduced to 0 °C and 760 mm, absorbed by one volume of water when the pressure of the gas itself amount to 760 mm) = 0.02373

P = percentage of oxygen in atmospheric air = 21 %

N = number of atoms in a molecule of oxygen = 2

V = volume of gas (at 0 °C and 760 mm) corresponding to 1 gm-mole = 22400 ml

Substituting these values in the above equation, the amount of oxygen

dissolved in 1 ml of water at 37 °C = 444.9 natoms oxygen / ml

The S value is calculated by multiplying the volume of reaction mixture (1.92 ml) with A value.

$$S = 1.92 \times A \text{ natoms oxygen} \quad \text{.....(5)}$$

Oxygen consumption rate in the reaction (natoms oxygen / min) must be divided by mitochondrial protein and the unit becomes natoms oxygen / mg protein / min.

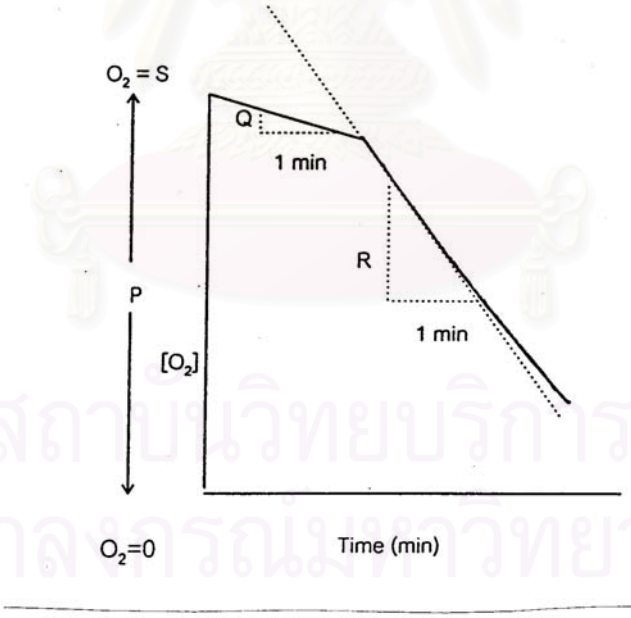


Figure 17 An oxygraph tracing illustrating the measurement of oxygen consumption rate

8. The study of the effects of tested compounds : acylaminopyridine and acylaniline derivatives on mitochondrial monoamine oxidase activity

The assay mixture composed of sodium phosphate buffer , pH 7.4 and mitochondria in a total volume of 1.92 ml. The reaction was started by the addition of substrate.

8.1 Determination of dose – dependent inhibitory effect MAO activity of tested compounds were assayed in the presence of MAO substrate : 100 μM tyramine (as a substrate for MAO-A and MAO-B) , 100 μM benzylamine (as a substrate for MAO-B) and 100 μM 5-HT (as a substrate for MAO-A). The inhibitory degree (IC_{50}) was measured under conditions of varying tested compound concentrations ; 10^{-3} M to 10^{-7} M.

8.2 Comparison of inhibitory effect on MAO activity by tested compounds with standard MAO inhibitors : clorgyline (selective MAO-A inhibitor) , selegiline (selective MAO-B inhibitor) and pargyline (non selective MAO inhibitor). The three substrates , 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT were used.

8.3 Determination of kinetics of enzyme inhibition and kinetic parameters, Km values (the Michaelis constant) and Vmax (maximum velocity) of reaction were obtained from double – reciprocal plots (or Lineweaver – Burk plots). The kinetic of enzyme inhibitions were determined under conditions of varying substrate concentrations and in the presence of fixed concentration of tested compound.

9. Protein determination

Protein content of rat liver mitochondria was determined by the method of Lowry et al (1951) as modified by Miller (1959).

9.1 Dilute of 10 μl mitochondrial suspension with 3 ml of purified water (1 : 300).

9.2 1 ml of alkaline copper reagent (0.5 % copper sulfate in 1 % potassium tartrate and 10 % sodium carbonate in 0.5 N sodium hydroxide) was added to 1 ml aliquot of the diluted mitochondrial suspension (from 1.) , mixed well and allowed to stand for 10 min at room temperature.

9.3 3 ml of diluted Folin phenol reagent (1 : 10) were added to 3 ml of the sample (from 2.). Mixed and incubated in water bath at 50 °C for 10 min. The mixtures were cooled to room temperature.

9.4 The standard curve plots obtained from the variation of Bovine Serum Albumin (BSA) concentrations : 0.05 , 0.10 , 0.15 , 0.20 , 0.25 and 0.30 mg/ml and purified water was used as the blank.

9.5 The samples were read optical density in a Ultrospec II spectrophotometer at 540 nm.

9.6 Protein content was calculated from a standard curve of BSA.

10. Data analysis

10.1 The MAO activity was analyzed by calculating the slope of the oxygraph tracing and expressed as % inhibition of MAO or natoms oxygen / min / mg protein. The value of inhibitory MAO activity on each concentration of tested compound and MAO inhibitors were obtained from at least 4 and 3 experiments.

10.2 Unpaired t – test was used to evaluate statistical significance of mean differences between control and tested compound – treated group.

10.3 The association between 1 / initial velocity (1 / V) and 1 / substrate concentration (1 / S) were analyzed using linear regression , $y = a + bx$.

CHAPTER IV

RESULTS

In these experiments, the rat liver mitochondrial preparation were suspended in an incubation medium containing phosphate buffer pH 7.4 , the temperature was maintained at 37 °C. The MAO activity was expressed as oxygen consumption rate which determined from the slope of oxygraph tracing and specific activity of MAO was calculated from differences in oxygen consumption rate at the time before and after substrate addition and represented in term nanoatoms of oxygen consumed per milligram of mitochondrial protein per one minute. Furthermore, the effect of tested compound on MAO activity could expressed as percent MAO activity inhibition, resulted from slope differences between control (the addition of MAO substrate only) and treated mitochondria (the addition of MAO inhibitor or tested compound and MAO substrate).

Part I : I*n vitro* effects of tested compounds : CU-18-07 , CU-18-08 , CU–18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13 on MAO activity in the rat liver mitochondrial preparation.

The inhibitory effect of tested compounds on MAO activity towards different MAO substrate by using 100 μM of tyramine (as a non - selective MAO substrate) , 100 μM of 5-HT (as a selective MAO-A substrate) and 100 μM of benzylamine (as a selective MAO-B substrate). The concentration of tested compound were varied from 10^{-7} M to 10^{-3} M. MAO inhibitory effect of tested compounds were determined by the addition of tested compound into reaction mixture (containing phosphate buffer and mitochondrial suspension) then followed by the addition of MAO substrate. The degree of inhibition of MAO substrate oxidative deamination was calculated from the slope of oxygraph tracing and expressed as percent MAO

activity inhibition (see Table 5-7). As shown in Figure 18-20, CU-18-10 inhibited the oxidative deamination of tyramine , benzylamine and 5-HT. The inhibitory effect of all tested compounds were exhibited to every substrate with the low potency and were concentration – dependent (Figures 21 – 27).

This study found that CU-18-10 (IC_{50} 3.09×10^{-5} M) was more potent to inhibit tyramine oxidative deamination than CU-18-07 , CU-18-08 , CU-18-09 , CU-18-12 and CU-18-13 (IC_{50} were 3.02×10^{-4} M , 1.54×10^{-4} M , 6.03×10^{-5} M , 6.92×10^{-5} M and 1.67×10^{-4} M, respectively) while the inhibitory effect of CU-18-11 (IC_{50} 5.62×10^{-4} M) was the lowest potency.

CU-18-12 (IC_{50} 6.52×10^{-5} M) was more potent to inhibit 5-HT oxidative deamination than CU-18-10 and CU-18-09 (IC_{50} were 9.02×10^{-5} M and 1.48×10^{-4} M, respectively) while CU-18-07 , CU-18-11 and CU-18-13 (IC_{50} were 9.27×10^{-4} M , 7.41×10^{-3} M and 6.17×10^{-4} M, respectively) were likely low inhibitory activity on 5-HT oxidative deamination and CU-18-08 (IC_{50} 0.0204 M) showed the lowest potency.

The oxidative deamination of benzylamine was inhibited approximately 50 % by low concentration of CU-18-10 (IC_{50} 8.81×10^{-6} M) while the other were inhibited at higher concentration (IC_{50} of CU-18-07 , CU-18-08 , CU-18-09 , CU-18-12 and CU-18-13 were 5.02×10^{-4} M , 3.85×10^{-5} M , 7.24×10^{-5}M , 4.74×10^{-4}M and 2.34×10^{-4} M, respectively) and the inhibitory effect of CU-18-11 (IC_{50} 1.45×10^{-3} M) was lowest.

Additional findings of interest were the effect of CU-18-08 and CU-18-10 showed a preferential inhibition on MAO-B activity by inhibiting benzylamine oxidative deamination at lower concentration than that required to inhibit 5-HT oxidative deamination. On the other hand, CU-18-07 , CU-18-09 , CU-18-11 , CU-18-12 and CU-18-13 not showed a preferential inhibition on type of MAO.

Part II : In vitro effects of pargyline (non – selective MAO inhibitor) , clorgyline (selective MAO-A inhibitor) and selegiline (or deprenyl ; selective MAO-B inhibitor) on MAO activity in the rat liver mitochondrial preparation.

Figures 28-30 showed that the inhibitory effect of pargyline , clorgyline and selegiline by using 100 μM tyramine , 100 μM 5-HT and 100 μM benzylamine as substrate were concentration-dependent. The inhibitory potency of pargyline , clorgyline and selegiline are shown in Table 8.

Figure 28 showed that the inhibitory effect of pargyline by varying concentrations. The oxidative deamination of tyramine , benzylamine and 5-HT were sensitive to inhibition with low concentration of pargyline. From the data obtained it was found that pargyline inhibited the oxidative deamination of 5-HT to a lesser than it inhibited the oxidative deamination of benzylamine.

Clorgyline showed a selective inhibition on MAO-A by inhibiting 5-HT oxidative deamination at lower concentration than that required to inhibit benzylamine and tyramine oxidative deamination (Figure 29). On the other hand, the effect of selegiline selectively inhibited MAO-B activity by benzylamine oxidative deamination was sensitive to inhibition by markedly lower concentration of selegiline than 5-HT and tyramine oxidative deamination (Figure 30).

Part III : Kinetics inhibition of rat liver mitochondrial monoamine oxidase by CU-18-08 and CU-18-10 in vitro.

The kinetics of MAO inhibition of CU-18-08 and CU-18-10 were assessed by determining MAO activity toward different MAO substrate concentrations in the presence of fixed concentration of CU-18-08 and CU-18-10. Kinetic parameters were calculated from double-reciprocal plots (Table 14).

Table 9 showed the substrate – activity relationship of MAO to oxidize tyramine in the presence of 1.5×10^{-4} M CU-18-08 and control (the addition of tyramine only). Tyramine concentration ranged from 0.05 mM to 1.2 mM. The initial velocities were significantly decreased at all concentration of tyramine in the presence of CU-18-08 compared to control (p value< 0.5). Double-reciprocal plots showed intercept effect with increased Michaelis constant, Km but decreased maximum velocity of the reaction, Vmax (Figure 31).

Table 10 showed the substrate – activity relationship of MAO to oxidize benzylamine in the presence of 4×10^{-5} M CU-18-08 and control. Benzylamine concentration ranged from 0.05 mM to 1.2 mM. It was found that the initial velocities were significantly decreased at concentration of 0.1 mM , 0.2 mM , 0.4 mM , 0.8 mM and 1.2 mM tyramine in the presence of CU-18-08 compared to control (p value < 0.5). Double-reciprocal plots showed intercept effect with increased Km but decreased Vmax (Figure 32).

Table 11 showed the substrate – activity relationship of MAO to oxidize tyramine in the presence of 3×10^{-5} M CU-18-10 and control. Tyramine concentration ranged from 0.05 mM to 1.2 mM. It was found that the initial velocities were significantly decreased at concentration of 0.2 mM , 0.4 mM and 0.8 mM tyramine in the presence of CU-18-10 compared to control (p value < 0.5). Double-reciprocal plots showed intercept effect with decreased both Km and Vmax (Figure 33).

Table 12 showed the substrate – activity relationship of MAO to oxidize benzylamine in the presence of 8×10^{-6} M CU-18-10 and control. Benzylamine

concentration ranged from 0.05 mM to 1.2 mM. The initial velocities were no significantly decreased in the presence of CU-18-10 compared to control. Double-reciprocal plots showed intercept effect with decreased both Km and Vmax (Figure 34).

Table 13 showed the substrate – activity relationship of MAO to oxidize 5-HT in the presence of 1×10^{-4} M CU-18-10 and control. 5-HT concentration ranged from 0.025 mM to 0.5 mM. The initial velocities were no significantly decreased in the presence of CU-18-10. Double-reciprocal plots not showed intercept effect. Km and Vmax values were decreased (Figure 35).

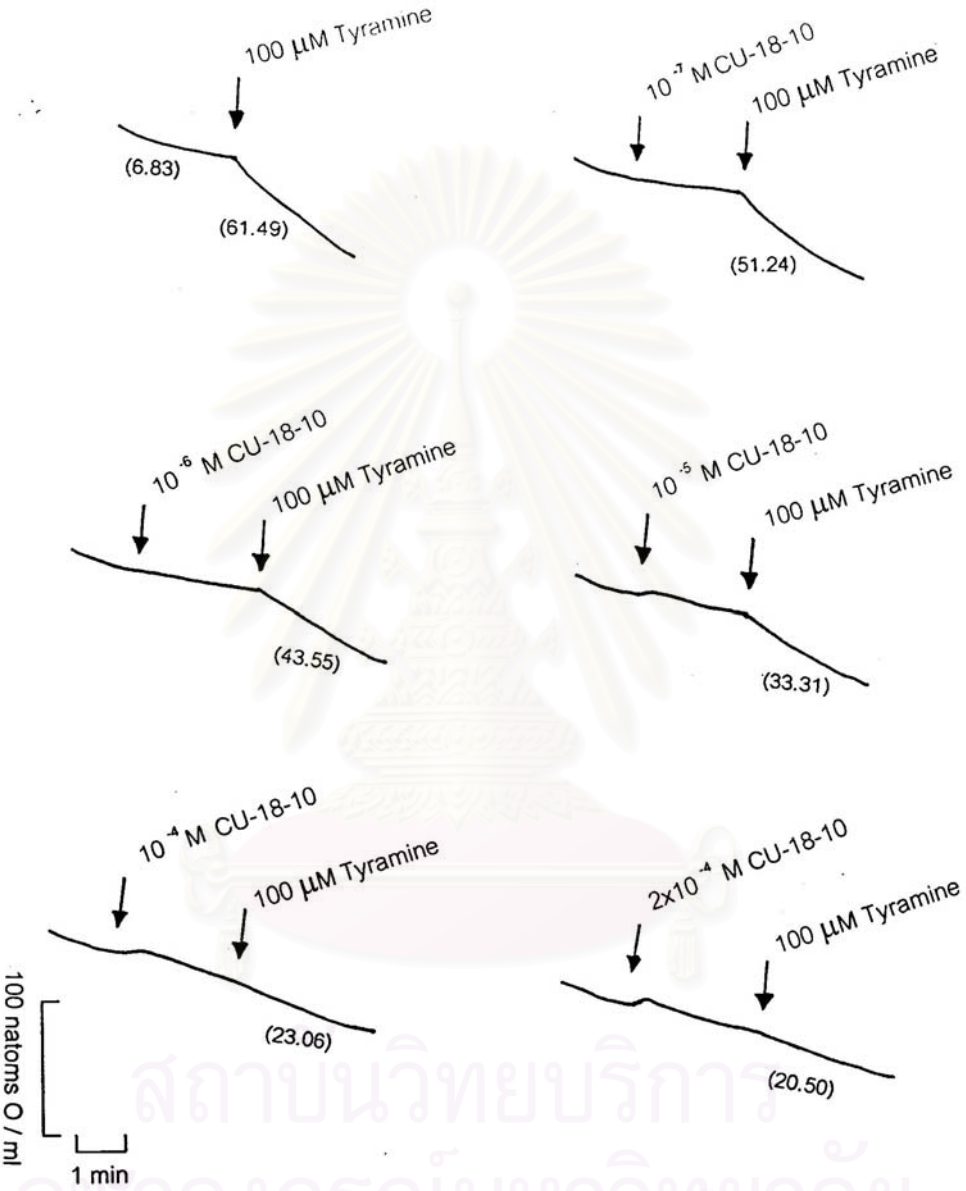


Figure 18 Oxygraph tracing for measuring oxygen consumption rate of rat liver mitochondria. CU-18-10 inhibition of MAO activity used 100μM tyramine as substrate. The reaction composed of 1.8 ml phosphate buffer, pH 7.4 and 100μl mtochondria suspension. The average protein concentration was 1.44 mg/ml. The figure in parentheses are rates of oxygen consumption in natoms O / ml / min.

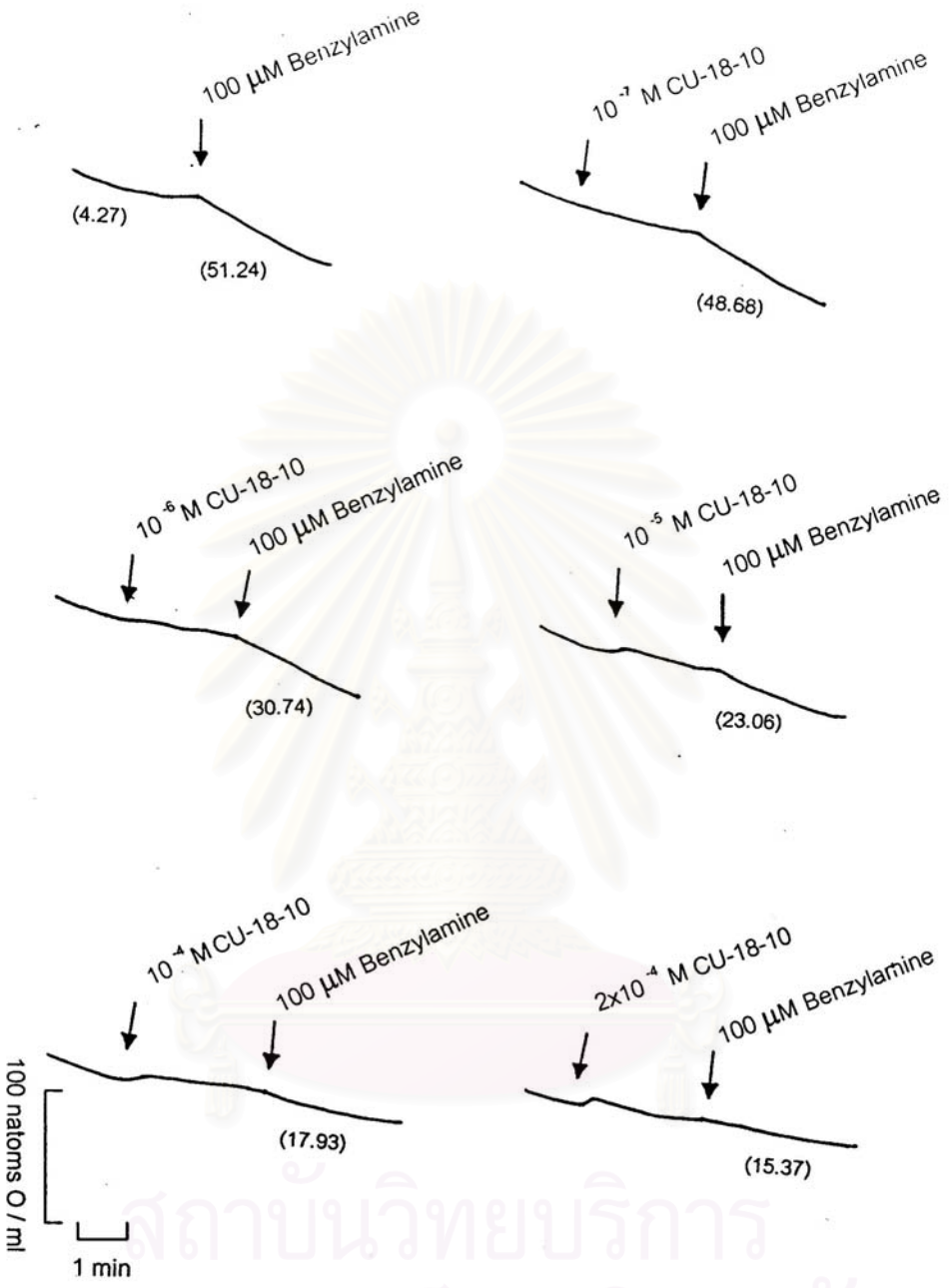


Figure 19 Oxygraph tracing for measuring oxygen consumption rate of rat liver mitochondria. CU-18-10 inhibition of MAO activity used 100 μM benzylamine as a substrate. The reaction mixture composed of 1.8 ml phosphate buffer, pH 7.4 and 100 μl of mitochondria suspension. The average protein concentration was 1.76 mg/ml. The figure in parentheses are rates of oxygen consumption in natoms O / ml / min.

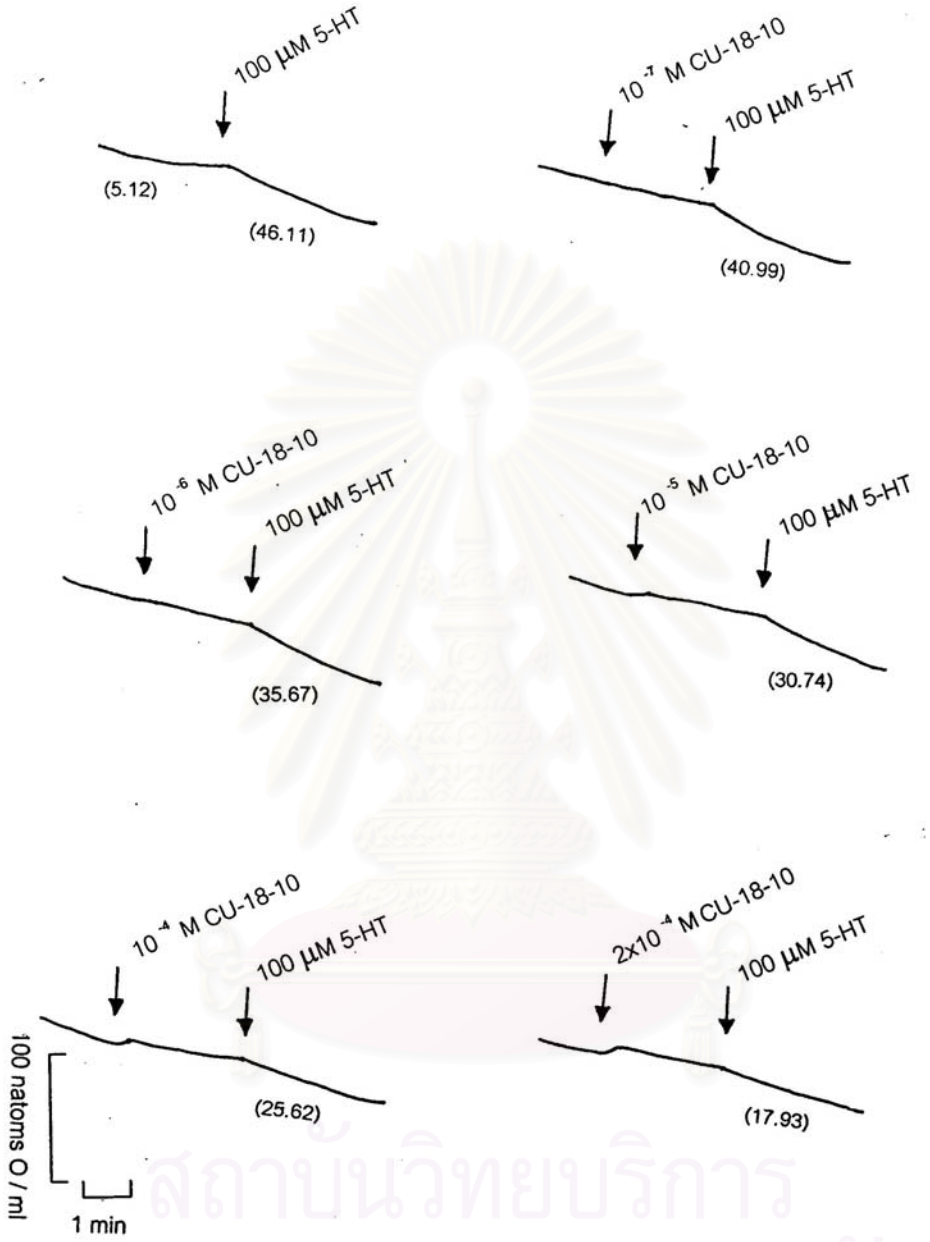


Figure 20 Oxygraph tracing for measuring oxygen consumption rate of rat liver mitochondria. CU-18-10 inhibition of MAO activity used 100 μM 5-HT as a substrate. The reaction mixture composed of 1.8 ml phosphate buffer, pH 7.4 and 100 μl of mitochondria suspension. The average protein concentration was 1.77 mg/ml. The figure in parentheses are rates of oxygen consumption in natoms O / ml / min.

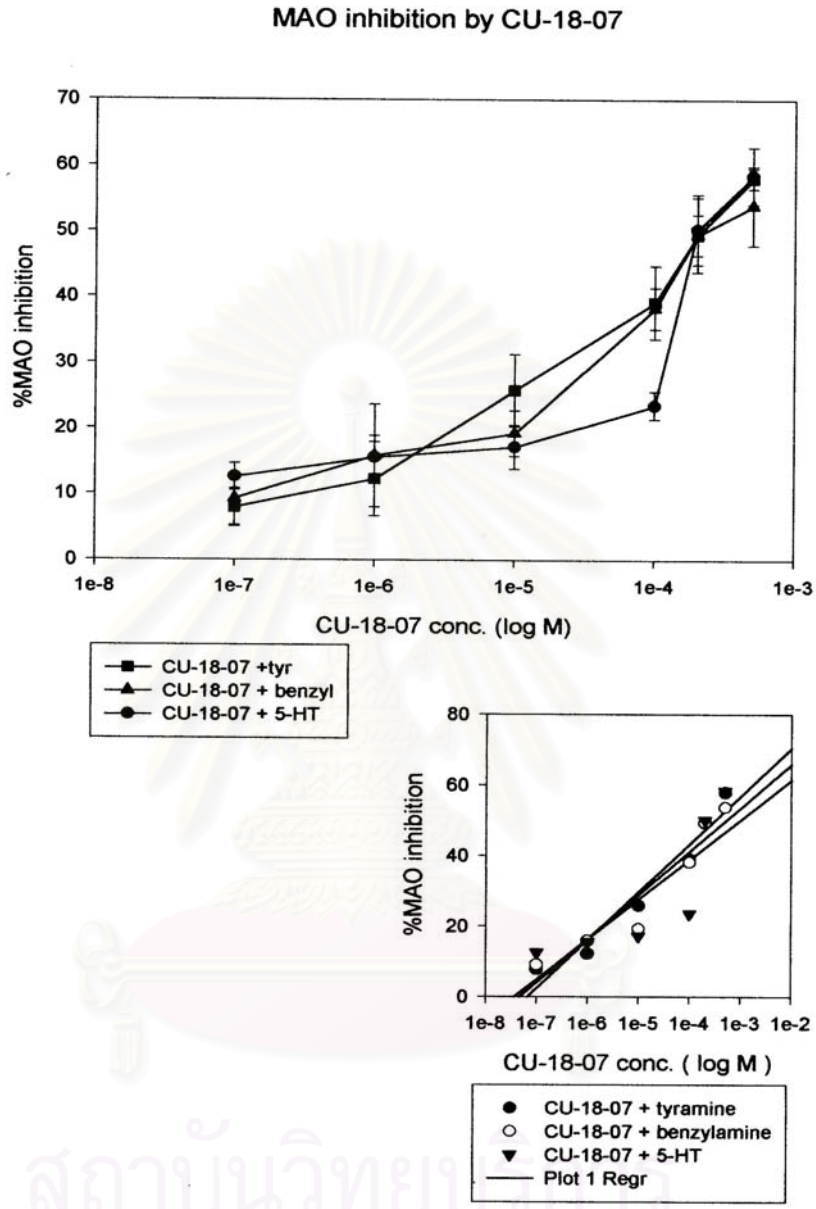


Figure 21 The inhibition of rat liver mitochondrial MAO by CU-18-07 towards 100μM tyramine , 100 μM benzylamine and 100μM 5-HT as substrate. The reaction composed of 1.8 ml phosphate buffer, pH 7.4 and 100 μl mitochondria suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.68 ml/mg , 1.47 mg/ml and 1.88 mg/ml, respectively. Each point is the mean±SE (n=4).

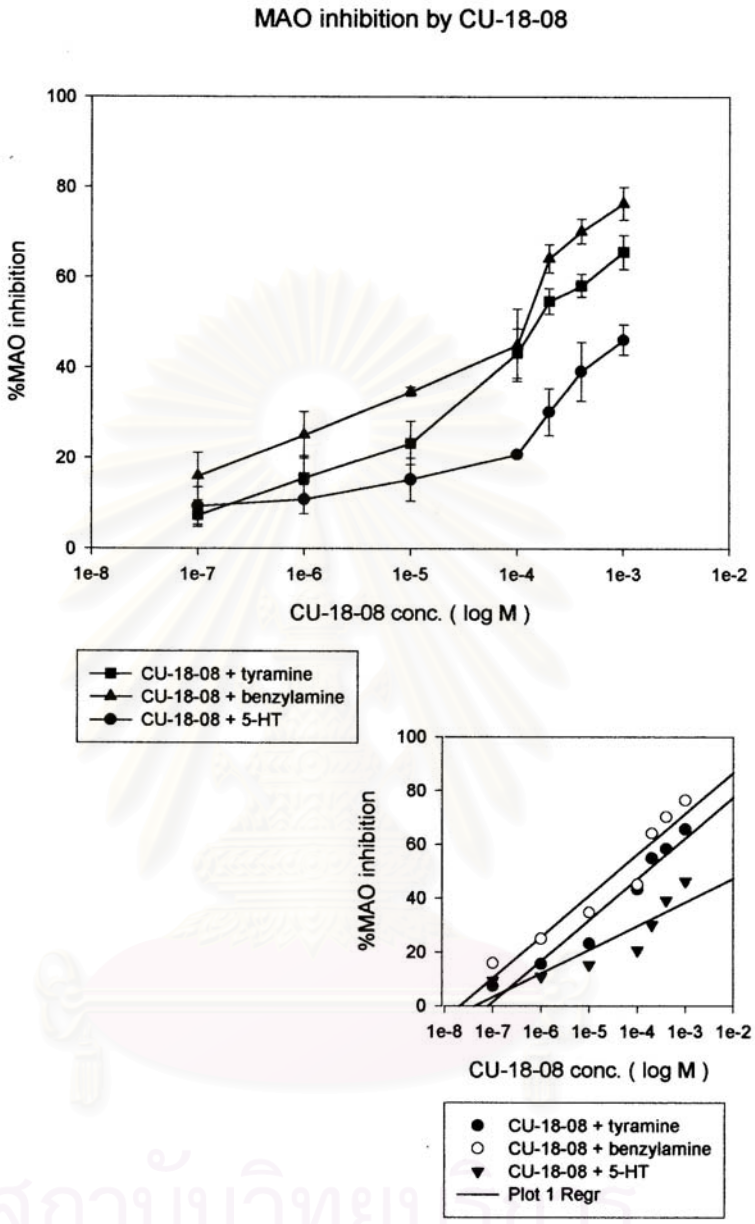


Figure 22 The inhibition of rat liver mitochondrial MAO by CU-18-08 towards 100μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml phosphate buffer, pH 7.4 and 100 μl of mitochondria suspension, and maintained at 37 C. The average protein concentration of reaction using tyramine , benzylamine and 5-HT were 1.30 mg/ml , 1.54 mg/ml and 1.70 mg/ml, respectively. Each point is the mean ±SE (n=4).

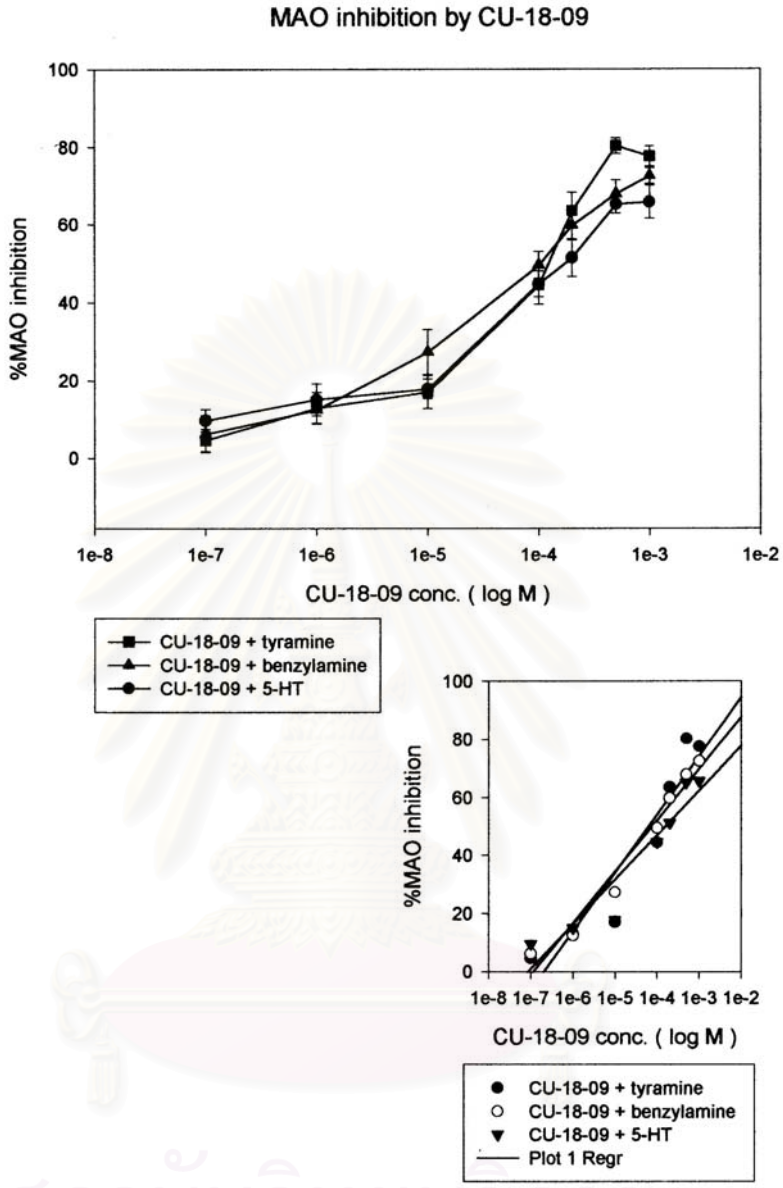


Figure 23 The inhibition of rat liver mitochondrial MAO by CU-18-09 towards 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer, pH 7.4 and 100 μl of mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-Ht were 1.38 mg/ml , 1.49 mg/ml and 1.56 mg /ml, respectively. Each point is the mean ±SE (n=4).

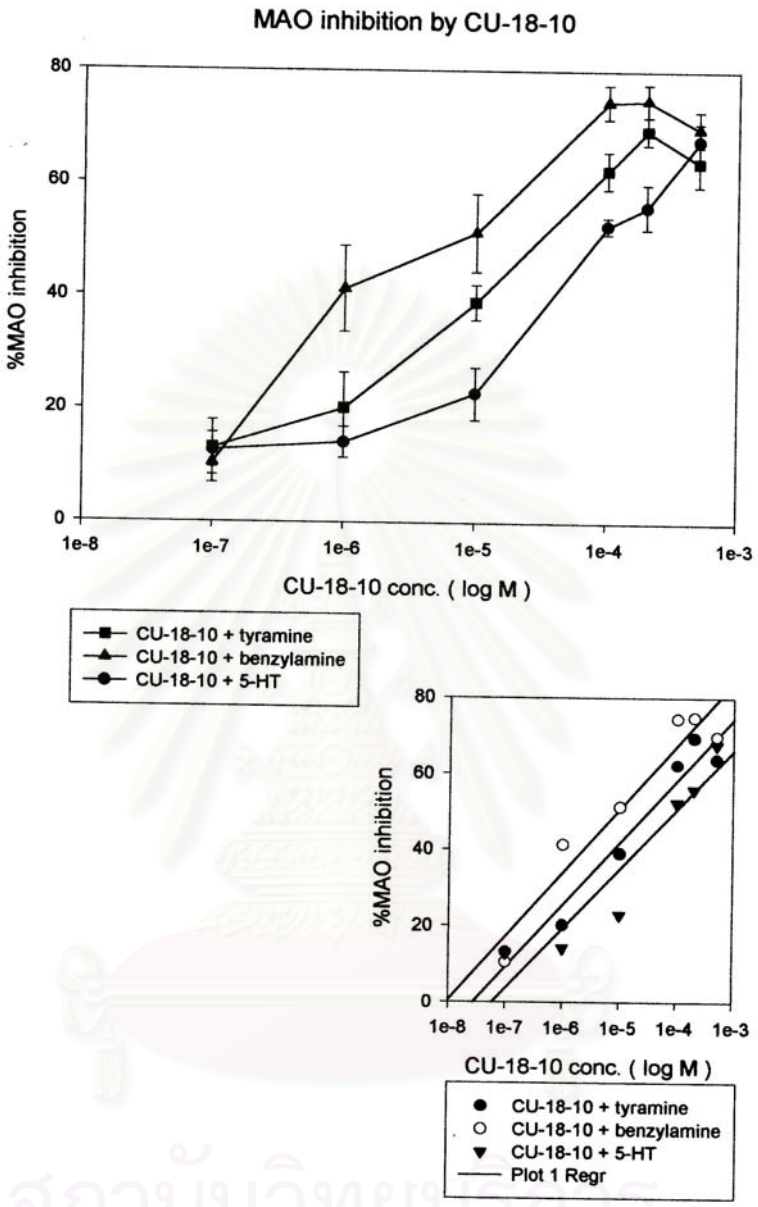


Figure 24 The inhibition of rat liver mitochondrial MAO by CU-18-10 towards 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer, pH 7.4 and 100μl of mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.44 mg/ml , 1.76 mg/ml and 1.77 mg/ml, respectively. Each point is the mean ±SE (n=4).

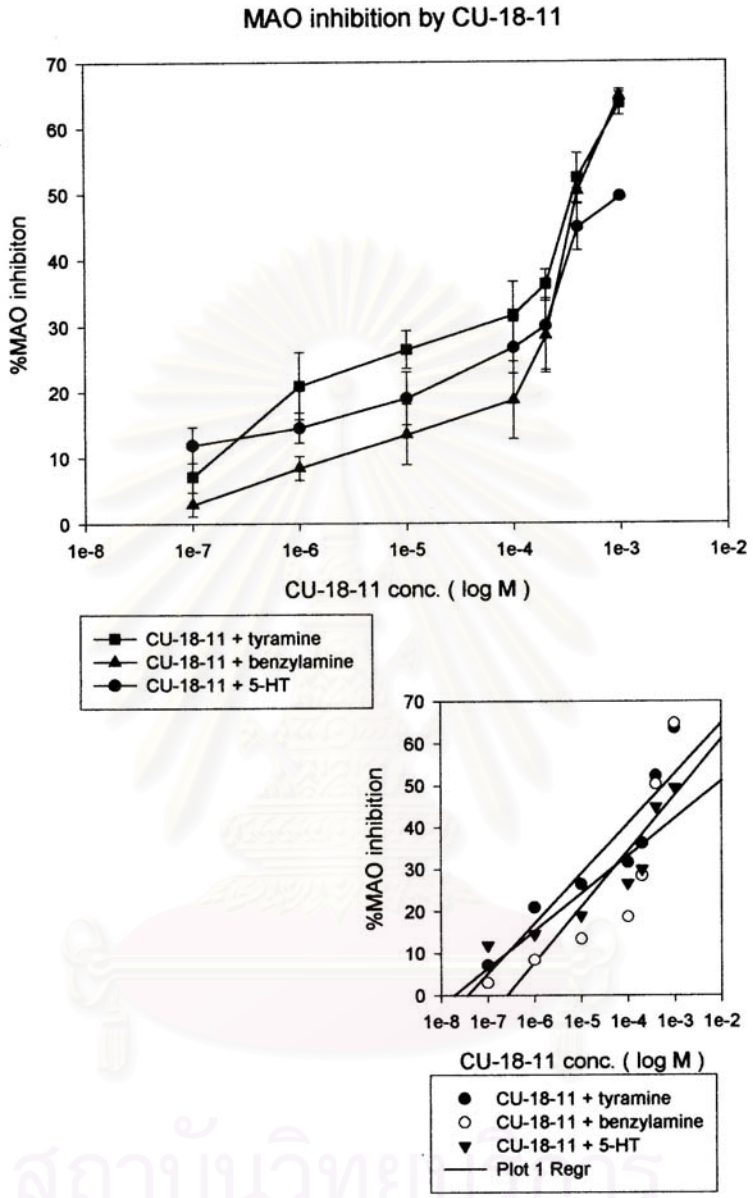


Figure 25 The inhibition of rat liver mitochondrial MAO by CU-18-11 towards 100μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer, pH 7.4 and 100 μl mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.54 mg/ml , 1.84 mg/ml and 2.01 mg/ml, respectively. Each point is the mean ±SE (n=4).

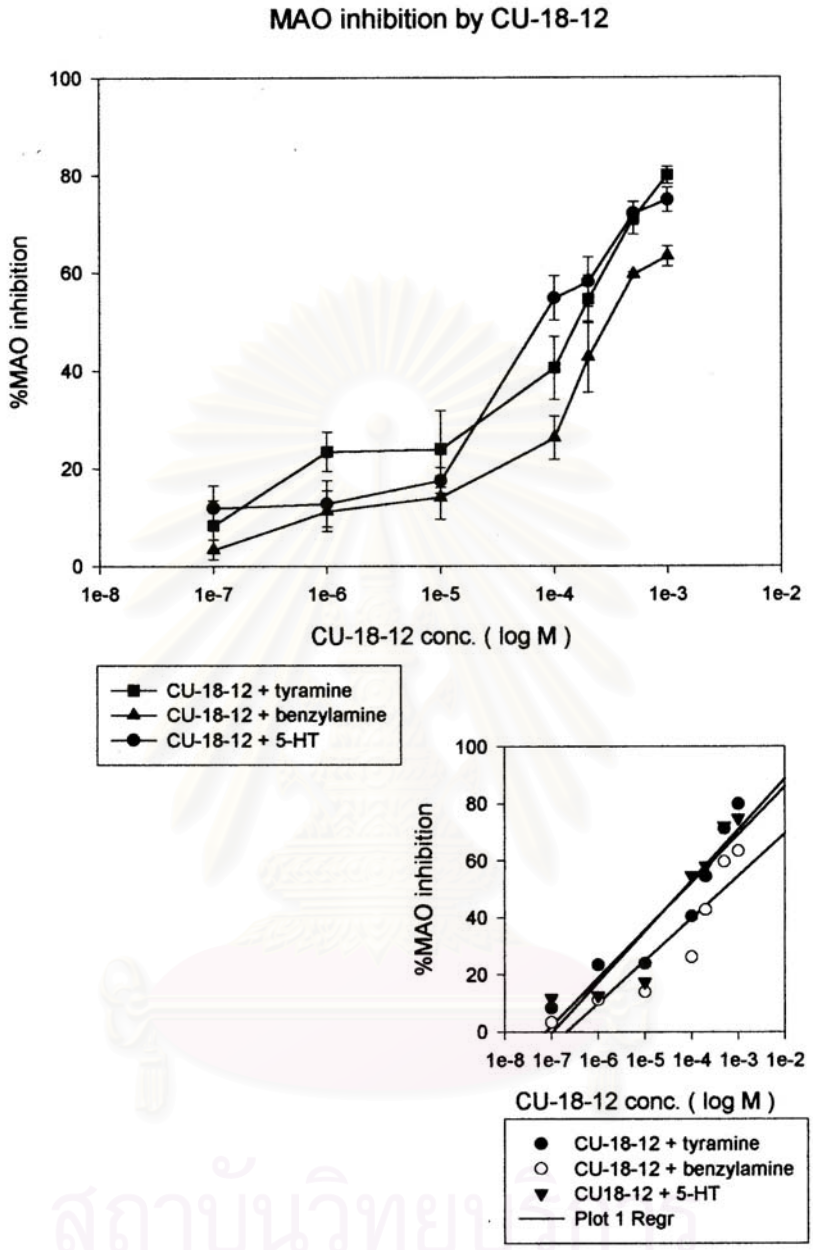


Figure 26 The inhibition of rat liver mitochondrial MAO by CU-18-12 towards 100μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer, pH 7.4 and 100 μl of mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine, benzylamine and 5-HT were 1.59 mg/ml , 1.83 mg/ml and 1.68 mg/ml, respectively. Each point is the mean ±SE (n=4).

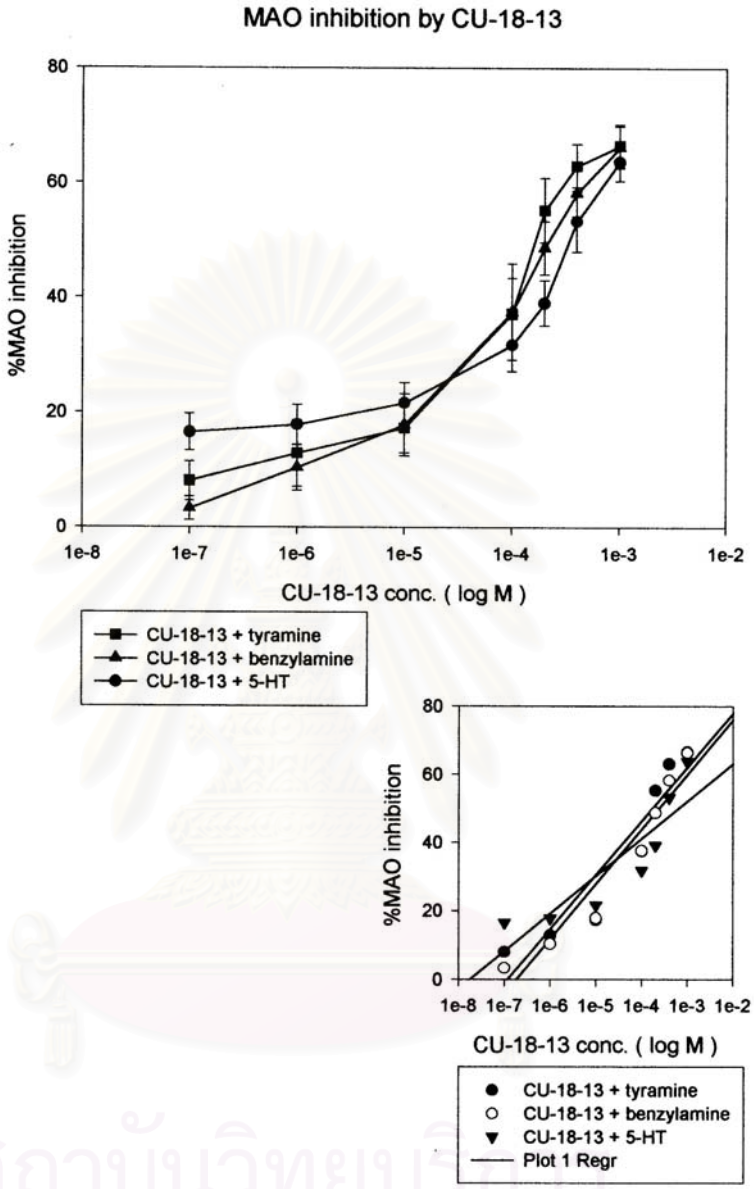


Figure 27 The inhibition of rat liver mitochondrial MAO by CU-118-13 towards 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reactioncomposed of 1.8 ml of phosphate buffer, pH 7.4 and 100 μl mitochondrial suspension , and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.36 mg/ml , 1.79 mg/ml and 1.59 mg/ml, respectively. Each point is the mean ± SE (n=4).

Table 5 : The percent MAO activity inhibition by tested compounds : CU-18-07 to CU-18-13 by using 100 μM tyramine as a substrate. Each value is the mean ± S.E. (n = 4).

compound	% MAO inhibition / Tested compounds concentration (M)							
	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	$2x10^{-4}$M	$4x10^{-4}$M	$5x10^{-4}$M	10^{-3}M
CU-18-07	7.96%± 2.87	12.31% ±5.61	25.86% ±5.48	39.19% ±5.55	49.45% ±3.10	-	58.02% ±1.52	-
CU-18-08	7.38%± 2.53	15.56% ±4.85	23.31% ±4.84	43.26% ±5.46	54.79% ±2.82	58.28% ±2.55	-	65.64% ±3.77
CU-18-09	4.64%± 2.82	12.94% ±4.04	17.07% ±4.27	44.49% ±4.97	63.61% ±4.71	-	80.29% ±1.98	77.61% ±2.57
CU-18-10	13.15% ±4.85	20.24% ±6.28	39.05% ±3.04	62.34% ±3.31	69.41% ±2.45	-	63.82% ±4.22	-
CU-18-11	7.18%± 4.60	20.95% ±5.11	26.44% ±2.85	31.65% ±4.99	36.24% ±2.16	52.35% ±3.75	-	63.68% ±1.76
CU-18-12	8.39%± 5.05	23.38% ±4.00	23.92% ±7.87	40.52% ±6.41	54.53% ±4.86	-	71.18% ±3.40	79.96% ±1.78
CU-18-13	8.09%± 3.42	12.90% ±5.78	17.36% ±4.34	37.39% ±6.05	55.27% ±5.64	63.08% ±3.77	-	66.56% ±3.68

Table 6 : The percent MAO activity inhibition by tested compounds : CU-18-07 to CU-18-13 by using 100 μM benzylamine as a substrate. Each value is the mean ± S.E. (n = 4).

compound	% MAO inhibition / Tested compounds concentration (M)							
	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	$2x10^{-4}$M	$4x10^{-4}$M	$5x10^{-4}$M	10^{-3}M
CU-18-07	9.24%± 3.93	15.87% ±7.89	19.30% ±3.49	38.29% ±3.13	49.48% ±5.68	-	53.88% ±6.01	-
CU-18-08	15.96% ±5.23	25.09% ±5.13	34.73% ±0.98	45.06% ±7.97	64.18% ±3.07	70.21% ±2.75	-	76.38% ±3.64
CU-18-09	6.25%± 4.73	12.46% ±3.36	27.30% ±5.79	49.57% ±3.42	59.81% ±3.87	-	67.95% ±3.48	72.59% ±2.00
CU-18-10	10.51% ±3.57	41.31% ±7.57	51.25% ±6.92	74.52% ±3.01	74.84% ±2.90	-	69.79% ±3.12	-
CU-18-11	2.99%± 1.82	8.46%± 1.81	13.51% ±4.58	18.69% ±5.87	28.51% ±5.18	50.36% ±0.36	-	64.75% ±1.12
CU-18-12	3.33%± 2.04	11.25% ±4.15	14.05% ±4.41	26.19% ±4.43	42.76% ±7.24	-	59.58% ±0.42	63.30% ±2.06
CU-18-13	3.33%± 2.04	10.42% ±3.99	17.89% ±5.40	37.62% ±8.38	48.72% ±4.62	58.28% ±4.04	-	66.31% ±3.59

Table 7 : The percent MAO activity inhibition by tested compounds : CU-18-07 to CU-18-13 by using 100 μM 5-HT as a substrate. Each value is the mean ± S.E. (n = 4).

compound	% MAO inhibition / Tested compounds concentration (M)							
	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	$2x10^{-4}$M	$4x10^{-4}$M	$5x10^{-4}$M	10^{-3}M
CU-18-07	12.63% ±2.08	15.62% ±3.29	17.22% ±3.31	23.58% ±2.13	50.29% ±5.32	-	58.46% ±4.36	-
CU-18-08	9.41%± 4.15	10.90% ±3.19	15.26% ±4.72	20.82% ±0.53	30.26% ±5.19	39.24% ±6.52	-	46.27% ±3.38
CU-18-09	9.72%± 2.85	15.10% ±4.11	17.88% ±2.50	44.74% ±3.33	51.40% ±4.80	-	65.22% ±2.38	65.81% ±4.24
CU-18-10	12.72% ±3.06	14.18% ±2.73	22.91% ±4.65	52.53% ±1.47	55.92% ±3.97	-	67.68% ±3.10	-
CU-18-11	11.98% ±3.74	14.58% ±2.28	19.02% ±4.04	26.70% ±3.93	30.00% ±7.07	44.86% ±3.54	-	49.52% ±5.64
CU-18-12	11.91% ±4.51	12.78% ±4.75	17.47% ±2.66	54.77% ±4.49	58.10% ±5.04	-	72.15% ±2.34	74.92% ±2.47
CU-18-13	16.57% ±3.21	17.88% ±3.49	21.70% ±3.57	31.82% ±4.53	39.16% ±3.94	53.30% ±5.30	-	63.77% ±3.42

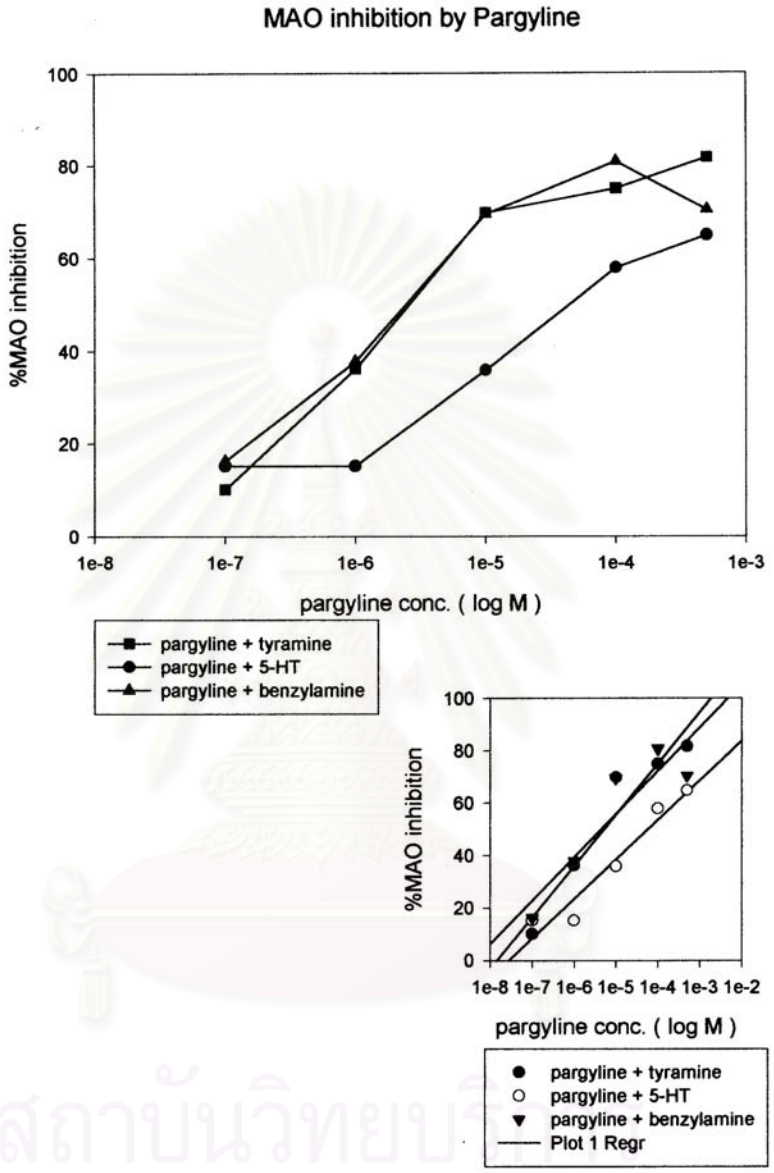


Figure 28 The inhibition of rat liver mitochondrial MAO by pargyline towards 100 μM tyramine , 100 μM benzylamine and 100μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer, pH 7.4 and 100 μL mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.00 mg/ml , 1.04 mg/ml and 1.01 mg/ml, respectively. Each point is the mean ±SE (n=3).

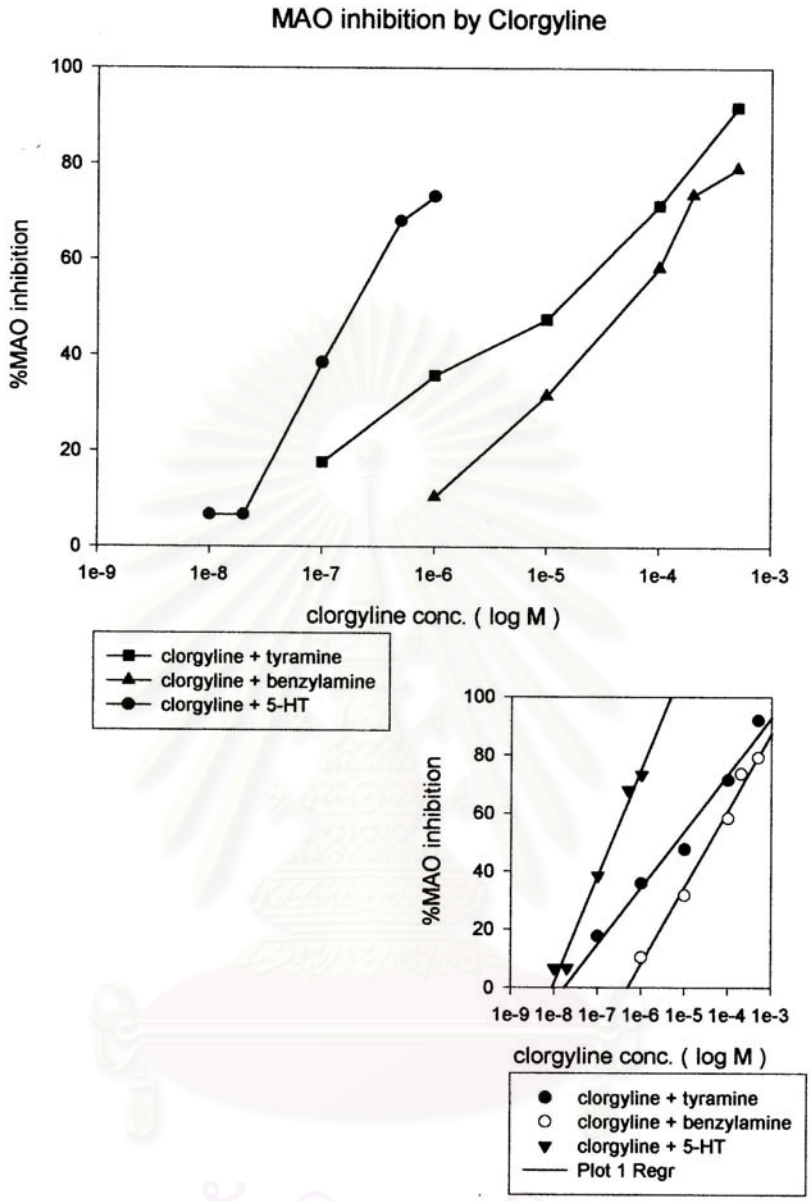


Figure 29 The inhibition of rat liver mitochondrial MAO by clorgyline towards 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT as sbustrate. The reaction composed of 1.8 ml of phosphate buffer, pH7.4 and 100 μl of mitochondrial suspension, and maintained at 37 C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 0.93 mg/ml , 0.91 mg/ml and 0.91mg/ml, respectively. Each point is the mean ±SE (n=3).

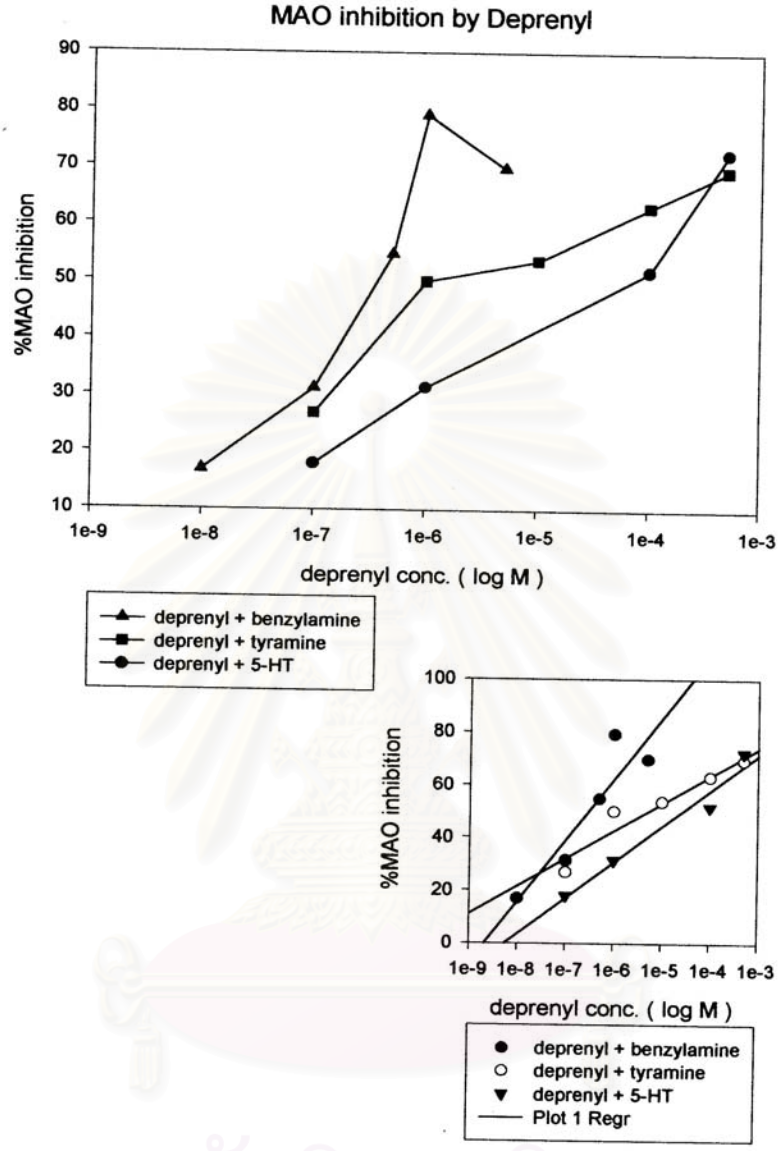


Figure 30 The inhibition of rat liver mitochondria MAO by selegiline towards 100 μM tyramine , 100 μM benzylamine and 100 μM 5-HT as substrate. The reaction composed of 1.8 ml of phosphate buffer , pH 7.4 and 100 μl of mitochondrial suspension, and maintained at 37 ° C. The average protein concentration of the reaction using tyramine , benzylamine and 5-HT were 1.21 mg/ml , 1.08 mg/ml and 1.21 mg/ml , respectively. Each point is the mean± S.E. (n=3).

Table 8 : A comparison of MAO inhibitory activity of MAOIs (pargyline as a non selective MAO inhibitor , clorgyline as a selective MAO-A inhibitor and selegiline as a selective MAO-B inhibitor) and their corresponding tested compounds (CU-18-07 to CU-18-13) when used 100 μM of tyramine (as a substrate of MAO-A and -B) , 100 μM of 5-HT (as a substrate of MAO-A) and 100 μM of benzylamine (as a substrate of MAO-B).

Tested compounds and MAOIs	tyramine as substrate IC_{50} (M)	5-HT as substrate IC_{50} (M)	benzylamine as substrate IC_{50} (M)	Ratio $\frac{\text{MAO-A } (IC_{50})}{\text{MAO-B } (IC_{50})}$
CU-18-07	3.02×10^{-4}	9.27×10^{-4}	5.02×10^{-4}	1.85
CU-18-08	1.54×10^{-4}	$2.04 \times 10^{--2}$	3.85×10^{-5}	529
CU-18-09	6.03×10^{-5}	1.48×10^{-4}	7.24×10^{-5}	2.04
CU-18-10	3.09×10^{-5}	9.02×10^{-5}	8.81×10^{-6}	10.24
CU-18-11	5.62×10^{-4}	7.41×10^{-3}	1.45×10^{-3}	5.11
CU-18-12	6.92×10^{-5}	6.52×10^{-5}	4.74×10^{-4}	0.14
CU-18-13	1.67×10^{-4}	6.17×10^{-4}	2.34×10^{-4}	2.64
Pargyline	5.07×10^{-6}	5.56×10^{-5}	4.36×10^{-6}	12.75
Clorgyline	6.17×10^{-6}	2.04×10^{-7}	3.80×10^{-5}	0.0054
Selegiline	4.90×10^{-6}	2.57×10^{-5}	2.88×10^{-7}	89.24

Results from the determine of MAOIs and tested compounds are the average of at least 3 and 4 independent experiment for each compound and expressed as mean IC_{50} values.

Table 9 : Substrate-activity relationship of MAO to tyramine in the presence of 0.15 mM CU-18-08 and absence of CU-18-08 (control). The varying concentration of tyramine ranged from 0.05 mM – 1.2 mM. The average protein concentration was 1.53 mg / ml. Each value is the mean ± S.E. (n = 4).

Tyramine concentration (mM)	Initial velocities (nanoatoms of oxygen / mg protein / min)	
	control	CU-18-08 (0.15 mM)
0.05	13.38 ± 1.71	5.84 ± 0.67*
0.1	16.32 ± 1.94	6.95 ± 0.62*
0.2	19.72 ± 2.06	8.10 ± 0.56*
0.4	21.40 ± 2.25	10.34 ± 2.27*
0.8	23.17 ± 2.48	11.95 ± 1.82*
1.2	24.85 ± 2.59	14.56 ± 1.07*

(* = p value < 0.05 compared to control)

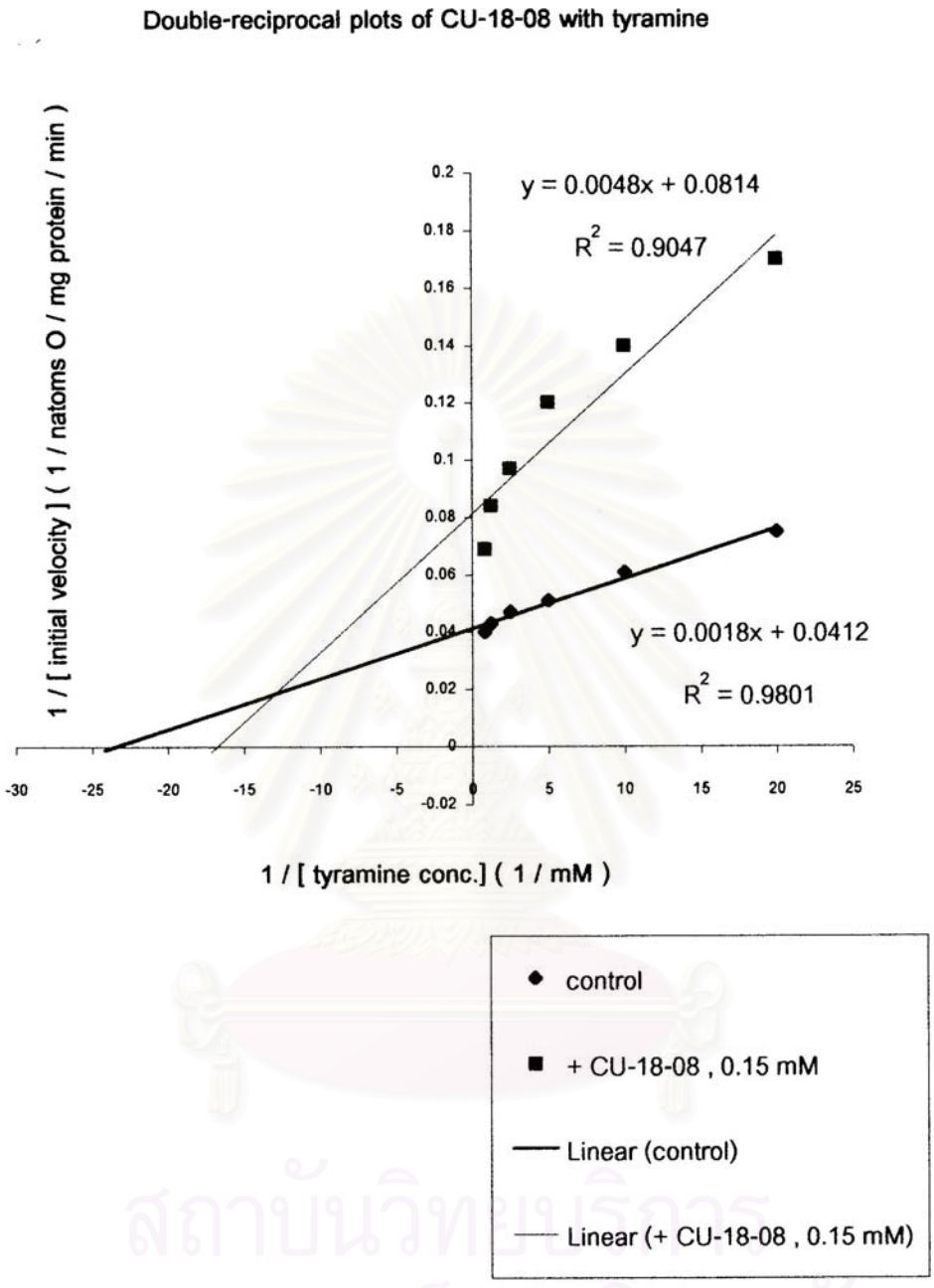


Figure 31 Double-reciprocal plots of the oxidative deamination of tyramine by 0.15 mM CU-18-08. Data plotted as 1 / [initial velocity] against 1 / [tyramine concentration]. The average protein concentration was 1.53 mg/ml. Each point is the mean (n=4).

Table 10 : Substrate-activity relationship of MAO to benzylamine in the presence of 0.04 mM CU-18-08 and absence of CU-18-08 (control). The varying concentration of benzylamine ranged from 0.05 mM – 1.2 mM. The average protein concentration was 1.49 mg / ml. Each value is the mean ± S.E. (n = 4).

Benzylamine concentration (mM)	Initial velocities (nanoatoms of oxygen / mg protein / min)	
	control	CU-18-08 (0.04 mM)
0.05	6.90 ± 1.39	3.45 ± 0.69
0.1	9.82 ± 1.26	5.25 ± 1.13*
0.2	11.74 ± 1.51	7.18 ± 1.08*
0.4	14.70 ± 0.80	7.99 ± 1.52*
0.8	16.52 ± 0.64	11.93 ± 1.07*
1.2	17.73 ± 0.43	13.14 ± 0.75*

(* = p value < 0.05 compared to control)

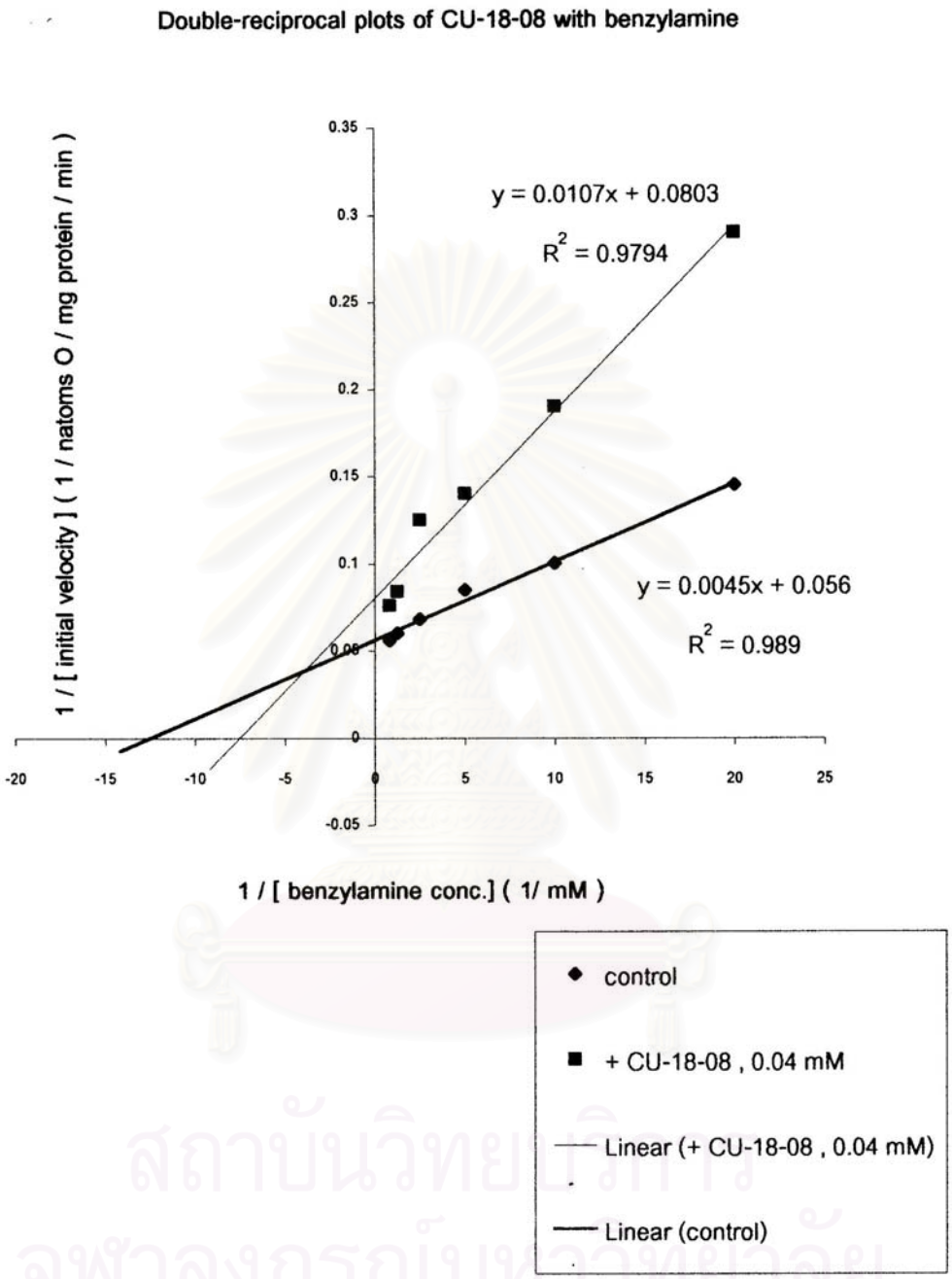


Figure 32 Double-reciprocal plots of the oxidative deamination of benzylamine by 0.04 mM CU-18-08. Data plotted as 1 / [initial velocity] against 1 / [benzylamine concentration]. The average protein concentration was 1.49 mg/ml. Each point is the mean (n=4).

Table 11 : Substrate-activity relationship of MAO to tyramine in the presence of 0.03 mM CU-18-10 and absence of CU-18-10 (control). The varying concentration of tyramine ranged from 0.05 mM – 1.2 mM. The average protein concentration was 1.30 mg / ml. Each value is the mean ± S.E. (n = 4).

Tyramine concentration (mM)	Initial velocities (nanoatoms of oxygen / mg protein / min)	
	control	CU-18-10 (0.03 mM)
0.05	5.73 ± 0.59	4.28 ± 0.46
0.1	8.10 ± 1.35	5.24 ± 0.51
0.2	10.48 ± 0.73	6.62 ± 0.94*
0.4	12.49 ± 0.78	8.22 ± 1.49*
0.8	14.15 ± 0.75	9.06 ± 1.80*
1.2	14.78 ± 1.01	11.39 ± 1.18

(* = p value < 0.05 compared to control)

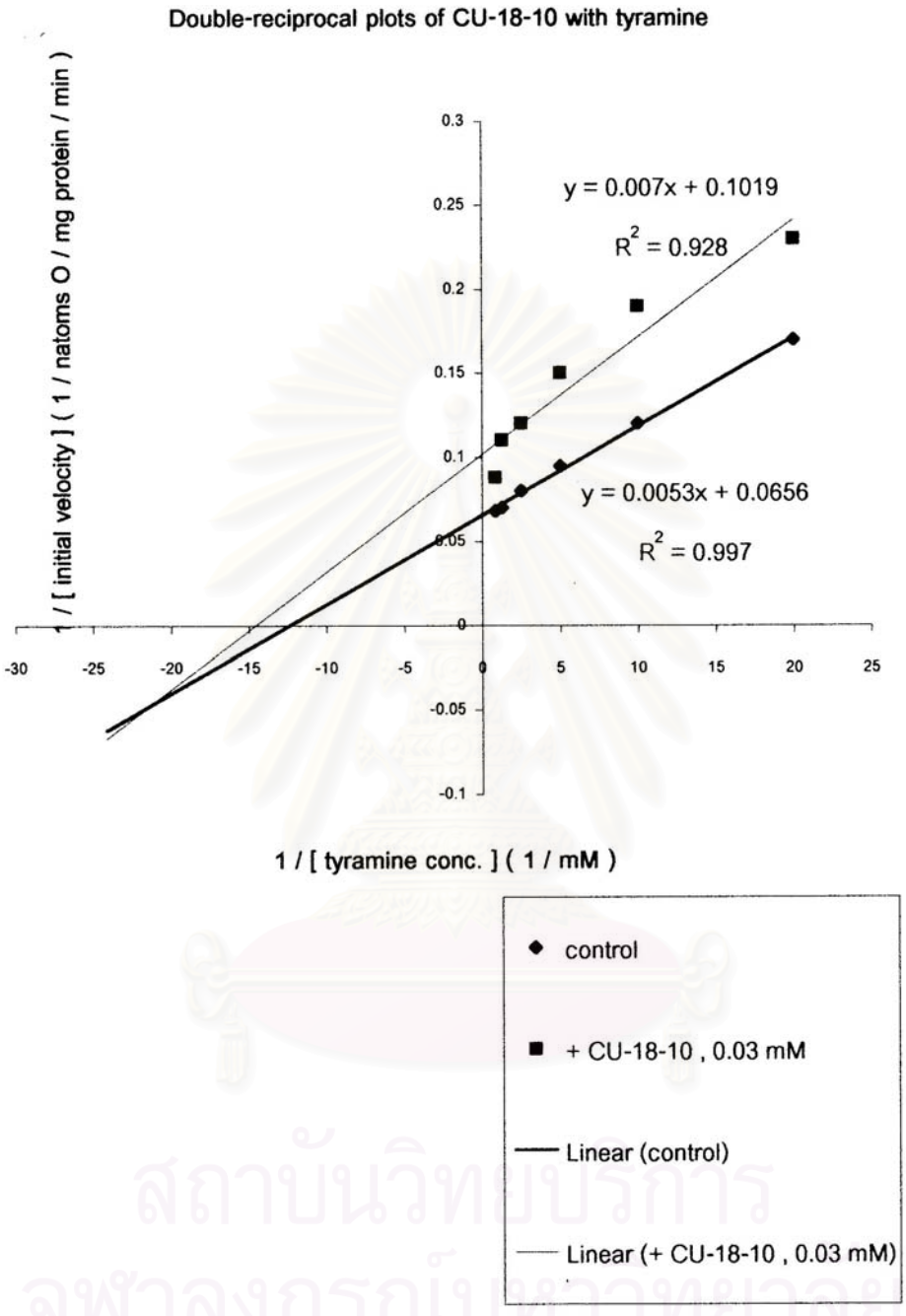


Figure 33 Double-reciprocal plots of the oxidative deamination of tyramine by 0.03 mM CU-18-10. Data plotted as 1 / [initial velocity] against 1 / [tyramine concentration]. The average protein concentration was 1.30 mg/ml. Each point is the mean (n=4).

Table 12 : Substrate-activity relationship of MAO to benzylamine in the presence of 0.008 mM CU-18-10 and absence of CU-18-10 (control). The varying concentration of benzylamine ranged from 0.05 mM – 1.2 mM. The average protein concentration was 1.21 mg / ml. Each value is the mean ± S.E. (n = 4).

Benzylamine concentration (mM)	Initial velocities (nanoatoms of oxygen / mg protein / ml)	
	control	CU-18-10 (0.008 mM)
0.05	4.66 ± 0.79	3.47 ± 0.99
0.1	7.85 ± 1.72	4.22 ± 0.75
0.2	14.08 ± 6.00	4.86 ± 0.90
0.4	15.40 ± 5.57	7.44 ± 1.98
0.8	16.38 ± 5.25	10.33 ± 3.16
1.2	17.15 ± 5.01	11.93 ± 4.68

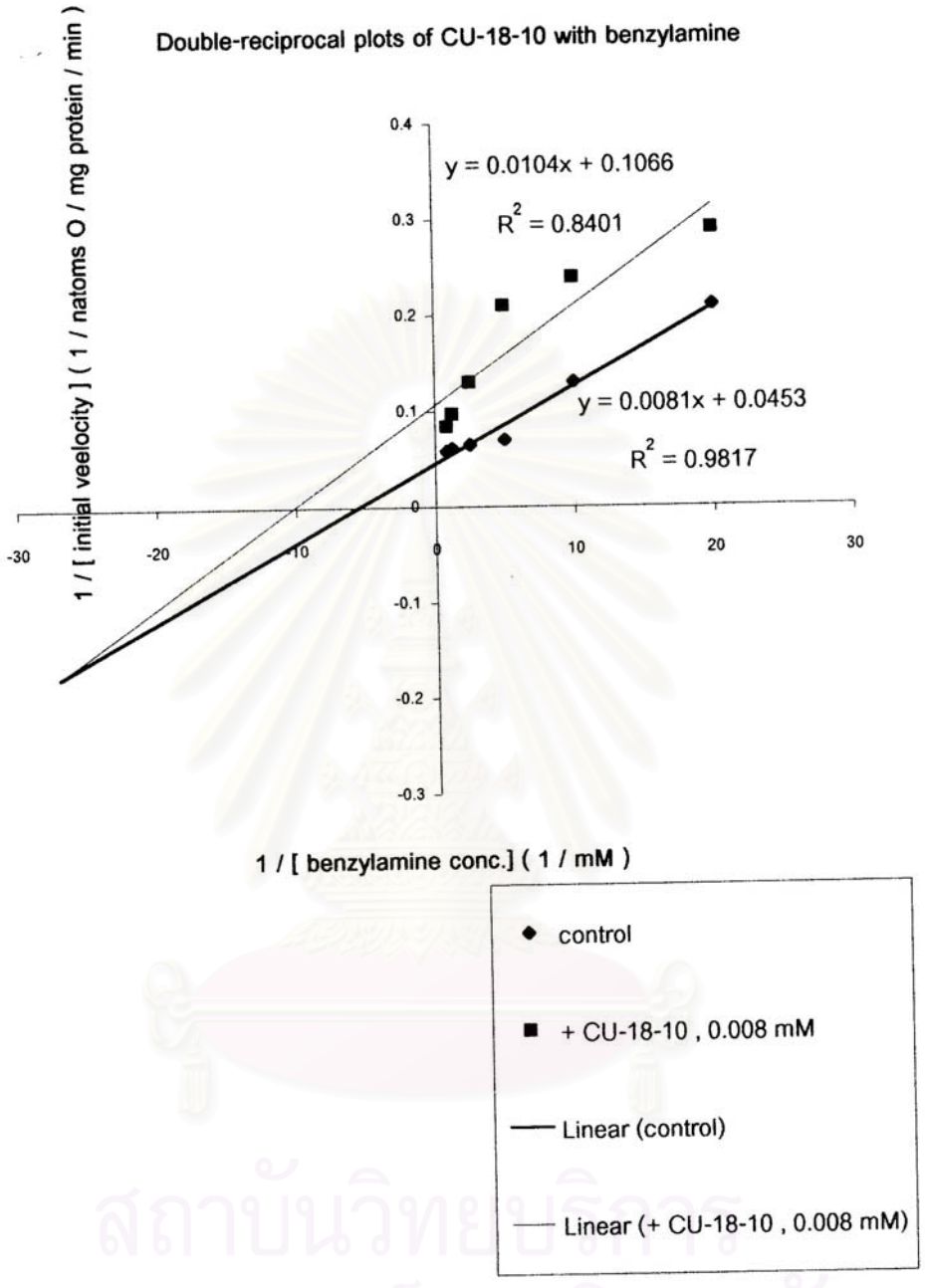


Figure 34 Double-reciprocal plots of the oxidative deamination of benzylamine by 0.008 mM CU-18-10. Data plotted as 1 / [initial velocity] against 1 / [benzylamine concentration]. The average protein concentration was 1.21 mg/ml. Each point is the mean (n=4).

Table 13 : Substrate-activity relationship of MAO to 5-HT in the presence of 0.1 mM CU-18-10 and absence of CU-18-10 (control). The varying concentration of 5-HT ranged from 0.025 mM – 0.5 mM. The average protein concentration was 1.07 mg / ml. Each value is the mean ± S.E. (n = 4).

5-HT concentration (mM)	Initial velocities (nanoatoms of oxygen / mg protein / min)	
	control	CU-18-10 (0.1 mM)
0.025	7.9 ± 3.87	4.41 ± 2.59
0.050	10.43 ± 5.13	4.89 ± 2.64
0.10	12.53 ± 6.55	5.10 ± 2.66
0.25	15.16 ± 7.79	5.36 ± 2.66
0.50	16.17 ± 7.44	7.65 ± 4.00

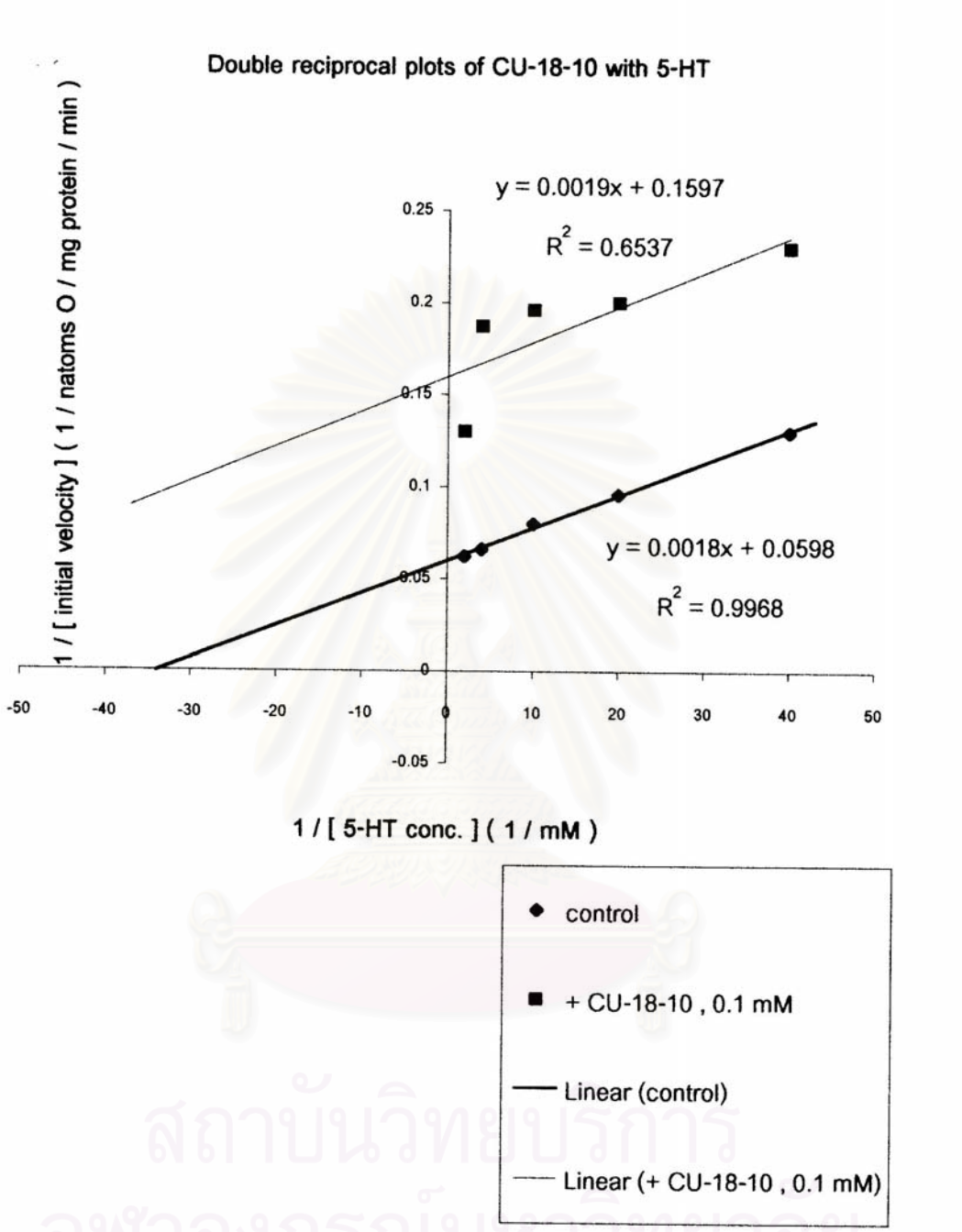


Figure 35 Double-reciprocal plots of the oxidative deamination of 5-HT by 0.1 mM CU-18-10. Data plotted as 1 / [initial velocity] against 1 / [tyramine concentration]. The average protein concentration was 1.07 mg/ml. Each point is the mean (n=4).

Table 14 : Kinetic parameters of rat liver MAO by CU-18-08 and CU-18-10 were obtained from double – reciprocal plots towards MAO substrate. Results were plotted as 1 / [initial velocity] against 1 / [substrate concentration] to calculate Km and Vmax values from each plot by linear regression analysis. All values represent the means for determinations in 4 mitochondrial preparations assayed at pH 7.2 and 37 °C.

Substrate	Condition	Kinetic parameters	
		Km (mM)	Vmax (n atom O_2 / mg protein / min)
Tyramine	Control	0.044	24.27
	+ CU-18-08 , 0.15 mM	0.059	12.28
Benzylamine	Control	0.080	17.86
	+ CU-18-08 , 0.04 mM	0.130	12.45
Tyramine	Control	0.081	15.24
	+ CU-18-10 , 0.03 mM	0.069	9.81
Benzylamine	Control	0.179	22.07
	+ CU-18-10 , 0.008 mM	0.098	9.38
5-HT	Control	0.030	16.72
	+ CU-18-10 , 0.1 mM	0.012	6.26

CHAPTER V

DISCUSSION

The aims of this research were to study the MAO inhibitory effect of acylaminopyridine derivatives (CU-18-11 and CU-18-12) and acylaniline derivatives (CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 and CU-18-13) in rat liver mitochondria. The experiments were divided into two major parts.

The first part demonstrated about the inhibitory potencies of these derivatives on MAO activity to oxidize different kinds of substrate. There are at least two types of MAO isoenzyme, MAO-A and MAO-B (Johnston , 1968) MAO-A and MAO-B oxidized tyramine whereas 5-HT and benzylamine are selectively oxidized by MAO-A and MAO-B, respectively (Neff and Yang , 1974). Furthermore, the inhibitory potency of tested compounds was also compared to standard MAO inhibitors such as pargyline (a non-selective MAO inhibitor) , clorgyline (a selective MAO-A inhibitor) and selegiline (a selective MAO-B inhibitor).

Results showed that acylaminopyridine and acylaniline derivatives inhibited MAO activity of rat liver mitochondria but in slightly high concentration ($>10^{-6}$ M). In all of tested compounds we found that the inhibitory potency of CU-18-10 on MAO-B activity and inhibition of MAO-A activity by CU-18-12 were higher than the others.

The inhibition of MAO activity by standard MAO inhibitors demonstrated that pargyline as a non-selective MAO inhibitor, inhibited both 5-HT and benzylamine oxidative deamination. In agreement with Fuller and Hemrick (1978) , this study found that pargyline preferentially inhibited MAO-B activity, about 10 times more potent than the inhibition of type A. For selective MAO-A inhibitor, clorgyline selectively inhibited 5-HT oxidative deamination while benzylamine oxidative deamination was inhibited to a lesser extent. The inhibitory potency of clorgyline

towards MAO-A is 100 times more higher than that towards MAO-B. For selective MAO-B inhibition, benzylamine oxidative deamination was inhibited approximately 50 percent by low concentration (10^{-7} M) of selegiline. The inhibitory potency of selegiline towards MAO-B is approximately 100 times higher than that towards MAO-A (Table 8).

Interestingly, CU-18-08 showed a more selective inhibition on MAO-B by inhibiting benzylamine oxidative deamination at lower concentration than that required to inhibit 5-HT oxidative deamination and the inhibitory potency towards MAO-B is about 500 times higher than towards MAO-A. Furthermore, the inhibitory effect on MAO-B of CU-18-08 was of low potency when compared to selegiline (Figure 22 and Table 8).

On the other hand, CU-18-07 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13 were non-selective MAO inhibitors. It is interesting that the inhibition of benzylamine oxidative deamination occurred at a markedly lower concentration of CU-18-10 than the inhibition of 5-HT oxidative deamination (Figure 24). This finding suggested that CU-18-10 acted as a non-selective MAO inhibitor which preferentially inhibited MAO-B. The IC_{50} values of CU-18-10 to MAO-B was about 10 times more potent than MAO-A (Table 8). It is assumed that CU-18-10 exhibited similar effect to pargyline that apparently inhibited the MAO-B activity to a greater extent than MAO-A.

The second part focused on the kinetics inhibition of some interesting tested compounds. CU-18-08 and CU-18-10 were selected to examine since the results from preliminary study found that CU-18-08 and CU-18-10 inhibited mitochondria respiration less than that of CU-18-07 , CU-18-09 , CU-18-11 , CU-18-12 and CU-18-13. In addition, CU-18-08 and CU-18-10 clearly showed to preferentially inhibit on type B of MAO more than the others.

From kinetic analysis, CU-18-08 and CU-18-10 decreased initial velocity of MAO-A and MAO-B. Double-reciprocal plots of CU-18-08 were obtained with

tyramine and benzylamine as substrate and resulted in interception effect (Figure 31 , 32). Considering the kinetic parameters, it was found that Km (the Michaelis constant) increased while Vmax decreased in the presence of CU-18-08. This finding indicate type of MAO inhibition. CU-18-08 displayed a reversibly mixed inhibition between competitive and non-competitive inhibition (see appendix). The inhibition of the MAO-A and MAO-B by CU-18-08 could be explained by CU-18-08 bound to the enzyme thereby, the opportunity of MAO substrate binding to the enzyme was reduced. This caused a decreasing in velocity rate of reaction while raising concentration of MAO substrate could increase the substrate competitive binding to MAO.

Kinetic studies in the presence of CU-18-10 showed that double-reciprocal plots exhibited intercept effect when used tyramine and benzylamine as substrate (Figure 33 , 34). In contrast, in the presence of CU-18-10 with 5-HT as a substrate resulted in no interception effect, as shown by slopes of control and of CU-18-10 treated were roughly equal (Figure 35). From these results indicated that there was differences in interaction with MAO-A and MAO-B by CU-18-10. The kinetic parameters, Km and Vmax obtained when using tyramine , benzylamine and 5-HT were decreased. The assumption of the kinetic inhibition in the presence of CU-18-10 on MAO-A indicated a reversibly uncompetitive inhibition whereas, on MAO-B it was a reversibly mixed inhibition between uncompetitive and non-competitive inhibition (see appendix).

For the mechanism of interaction between MAO-A and CU-18-10, it may bind to binding site which is not the same active site of MAO substrate, to form enzyme-substrate-inhibitor complex. The reduction of MAO inhibitory effect could not occurred by increasing concentration of 5-HT. However, the mechanism of the binding between CU-18-08 and MAO-A or MAO-B , and between CU-18-10 and MAO-B are unknown and need to further investigation.

In comparison to previous studies, N - acylaminopyridine derivatives, CU763-16-04 , CU763-18-01 , CU763-18-02 and CU763-18-04 (Figure 13) exhibited the MAO inhibitory potency, to a lesser degree than the new derivatives, acylaminopyridine and acylaniline derivatives (Unchittha T., 2000).

Regarding to structure-activity relationship, the molecular structure of CU-18-08 and CU-18-10 were different in the groups substituted on the benzene ring. CU-18-08 is N-heptanoyl,-3-methoxyaniline and CU-18-10 is N-heptanoyl,-3-nitroaniline. The MAO inhibitory effect of CU-18-10 was more potent than CU-18-08 but in the selectivity inhibitory effect on type of MAO, CU-18-08 was more clear than CU-18-10. This findings suggested that the selectivity on type of MAO may be related to the hydrophobic region in chemical structure. The results of the present study offer support for the belief that MAO-B may differ from MAO-A by the presence of a hydrophobic or nucleophillic site near the active center complex (Dostert and Benedetti , 1991 ; Yu and Davis , 1999). Therefore, the selective and potential inhibition on MAO-A and MAO-B activity of acylaminopyridine and acylaniline derivatives are corresponding with the idea that lipophilicity and/or electrophilicity of the microenvironment of the enzyme is an important factor for the selectivity of MAO.

CHAPTER VI

CONCLUSION

1. Acylaminopyridine and acylaniline derivatives, CU-18-07 , CU-18-08 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13, inhibited MAO-A and MAO-B activity in the rat liver mitochondria with the low potency when compared to standard MAO inhibitors.

2. CU-18-08 was a more selective MAO-B inhibitor whereas CU-18-07 , CU-18-09 , CU-18-10 , CU-18-11 , CU-18-12 and CU-18-13 were non selective MAO inhibitor but it is interesting that CU-18-10 showed preferential inhibitory effect on type B of MAO.

3. For enzyme kinetic studies, CU-18-08 and CU-18-10 were selected to study for kinetics analysis. This findings suggested that CU-18-08 inhibited MAO-A and MAO-B by display a reversibly mixed inhibition between competitive and non-competitive inhibition with increased Km but decreased Vmax. Kinetic analyses of CU-18-10 revealed that the interaction with MAO-A was reversibly uncompetitive inhibition while the interaction with MAO-B was a reversibly mixed inhibition between non-competitive and uncompetitive inhibition. Both Km and Vmax were decreased.

4. This findings showed the structure-activity relationship that may be used to modify and develop a new selective MAO-B inhibitors which have low mitochondrial toxicity.

5. The assay of MAO activity was investigated by measuring oxygen consumption rate. There was the advantage that these assay can directly measure the MAO

activity. However, consumed oxygen may be used in other reaction such as in mitochondrial respiration and other oxidation. To solve these problem by the addition of mitochondrial respiratory inhibitor, rotenone and should be careful in the procedure of mitochondrial preparation to decrease interference from the reaction of other enzyme which mainly come from microsome.

6. Further research is necessary to investigate the effect of acylaminopyridine and acylaniline derivatives on central nervous system (CNS) both in vitro and in vivo.

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APPENDIX

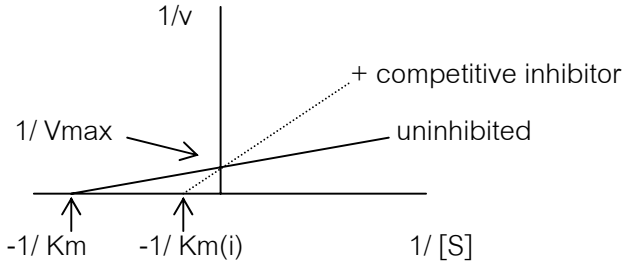
Enzyme inhibition

Inhibitors are substance which tend to decrease the rate of an enzyme-catalyzed reaction. Although some act on a substrate or cofactor. Reversible inhibitors bind to an enzyme in a reversible fashion and can be removed by dialysis (or simply dilution) to restore full enzymic activity whereas irreversible inhibitors cannot be remove from an enzyme by dialysis. Sometimes it may be possible to remove an irreversible inhibitors from an enzyme by introducing another component to the reaction mixture, but this would not affect the classification of the original interaction (Palmer , 1995).

1. Reversible inhibition

1.1 Competitive inhibition

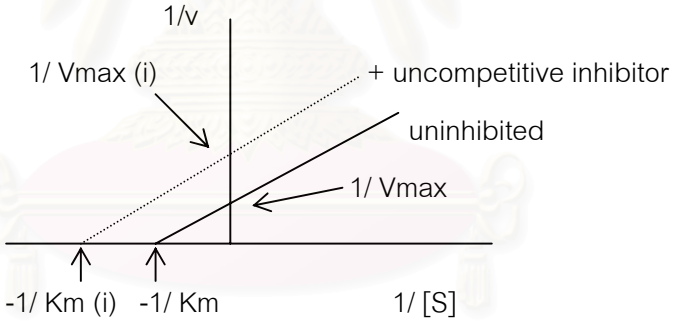
Competitive inhibitors often closely resemble in some respects the substrates whose reactions they inhibited, and because of this structural similarity they may compete for the same binding-site on the enzyme. The enzyme- bound inhibitor then either lacks the appropriate reactive group or it is held in an unsuitable position with respect to the catalytic-site of the enzyme or to other potential substrate for a reaction to take place. In general at a particular inhibitor and enzyme concentration, if the substrate concentration is low, the inhibitor will compete favorably with the substrate for the binding site on the enzyme and the degree of inhibition will be great. However if, at this same inhibitor and enzyme concentration, substrate concentration is high, then the inhibitor will be much less successful in competing with the substrate for the available binding sites and the degree of inhibition will be less marked. At very high substrate concentration, molecules of substrate will greatly outnumber molecules of inhibitor and the effect of the inhibitor will be negligible. Hence Vmax for the reaction is unchanged. However the apparent Km is clearly increase as a result of the inhibition.



Competitive inhibition

1.2 Uncompetitive inhibition

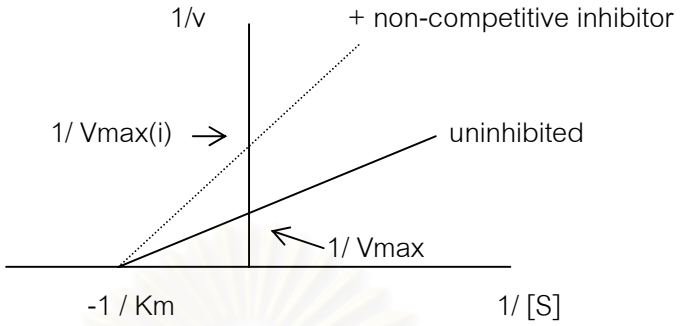
Uncompetitive inhibitors bind only to the enzyme-substrate complex and not to the free enzyme. Substrate binding could cause a conformational change to take place in the enzyme and reveal an inhibitor- binding site, or the inhibitor could bind directly to the enzyme–bound substrate. In neither case does the inhibitor compete with the substrate for the same binding site, so the inhibition can not be overcome by increasing the substrate concentration. Both Km and Vmax are altered.



Uncompetitive inhibition

1.3 Non-competitive inhibition

A non-competitive inhibitor can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not. Hence the inhibitor must bind at a different site from the substrate. The total enzyme concentration is effectively reduced by inhibitor, decreasing the value of Vmax but not altering Km , since neither inhibitor nor substrate affects the binding of the other.



Non-competitive inhibition

1.4 Mixed inhibition

There are two process by which inhibitor may bind to the enzyme :

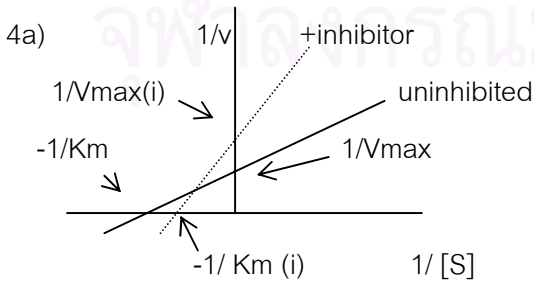
$$E + I \rightleftharpoons EI \quad (\text{inhibitor constant}, K_i)$$

and

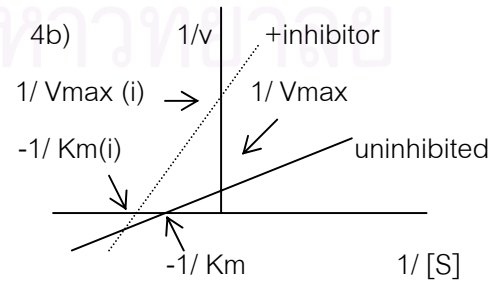
$$ES + I \rightleftharpoons ESI \quad (\text{inhibitor constant}, K_i)$$

In the situation where $K_i > K_i$, the plot cross to the left of the 1 / V axis but above the 1 / S axis. This situation has been termed *competitive-non-competitive inhibition*, because the pattern observed list between those for competitive and non-competitive inhibition (Figure 4a).

In the situation where $K_i < K_i$, the plot cross to the left of the 1 / V axis and below the 1 / S axis. This form of mixed inhibition has been termed *non-competitive-uncompetitive inhibition* because the pattern is intermediate between those for non-competitive and uncompetitive inhibition (Figure 4b).



competitive-non-competitive inhibition



non-competitive-uncompetitive inhibition

2. Irreversible inhibition

An irreversible inhibitor bind to the active site of the enzyme by an irreversible reaction :

$$E + I \longrightarrow EI$$

and hence cannot subsequently dissociate from it. A covalent bond is usually formed between inhibitor and enzyme. The inhibitor may act by preventing substrate-binding or it may destroy some component of the catalytic site. Compounds which irreversibly denature the enzyme protein or cause non-specific inactivation of the active site are not usually regarded as irreversible inhibitors.

Irreversible inhibitors effectively reduce the concentration of enzyme present. If a substrate is introduced after the reaction between inhibitor and enzyme has gone to completion, a system which obeys the Michaelis-Menten equation in the absence of inhibitor will still do so. The Km value will be the same as for the uninhibited reaction, but Vmax will be reduced.

CU-18-07 + 100 μM tyramine

N =4	CU-18-07 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	2.85%	7.14%	20%	34.48%	51.35%	58.33%
2	5.71%	8.33%	13.33%	25.86%	42.86%	53.85%
3	7.14%	4.76%	34.62%	46.43%	46.43%	58.82%
4	16.13%	29%	35.48%	50%	57.14%	61.11%
Mean	7.95%	12.31%	25.86%	39.19%	49.44%	58.03%
S.E.	2.87	5.61	5.48	5.54	3.10	1.52

CU-18-07 + 100 μM benzylamine

N=4	CU-18-07 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	7.41%	9.09%	16.67%	30%	42.11%	44.44%
2	4.17%	11.11%	22.17%	44.74%	60%	66.67%
3	20.83%	4.17%%	11.11%	37.5%	37.5%	61.54%
4	4.55%	39.13%	27.27%	40.91%	58.33%	42.86%
Mean	9.24%	15.87%	19.30%	38.29%	49.48%	53.88%
S.E.	3.93	15.87	19.30	38.29	49.48	53.88

CU-18-07 +100 μM 5-HT

N=4	CU-18-07 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	10%	15%	20%	18.52%	40%	60%
2	10%	10%	13.89%	22.22%	46.15%	53.85%
3	11.76%	12.5%	10%	25%	50%	50%
4	18.75%	25%	25%	28.57%	65%	70%
Mean	12.63%	15.62%	17.22%	23.58%	50.29%	58.46%
S.E.	2.08	3.29	3.31	2.12	5.32	4.36

CU-18-08 + 100 μM tyramine

N=4	CU-18-08 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	11.42%	14.29%	13.33%	52.%	56.76%	53.85%	68.18%
2	9.52%	8.33%	20%	27.59%	46.43%	64.71%	72.73%
3	8.57%	29.63%	36.36%	45.83%	58.82%	60%	66.67%
4	0%	10%	23.53%	46.67%	57.14%	54.55%	55%
Mean	7.38%	15.56%	23.30%	46.26%	54.79%	58.28%	65.64%
S.E.	2.53	4.85	4.84	5.46	2.82	2.54	3.78

CU-18-08 + 100 μM benzylamine

N=4	CU-18-08 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	29.17%	14.81%	37.5%	26.32%	68.42%	66.67%	81.25%
2	3.7%	25%	34.78%	45.83%	62.5%	66.67%	75%
3	16.67%	39.13%	33.33%	65.22%	56.25%	69.23%	66.67%
4	14.29%	21.43%	33.33%	42.86%	69.57%	78.26%	82.61%
Mean	15.96%	25.09%	34.73%	45.06%	64.18%	70.21%	76.38%
S.E.	5.23	5.13	0.98	7.97	3.06	2.75	3.64

CU-18-08 +100 μM 5-HT

N=4	CU-18-08 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	15%	20%	11.11%	22.22%	22.22%	38.89%	42.86%
2	17.65%	8.33%	29.41%	20.%	30%	55%	40%
3	5%	10%	10%	20%	23.81%	40%	55.56%
4	0%	5.26%	10.53%	21.05%	45%	23.08%	46.67%
Mean	9.41%	10.90%	15.26%	20.82%	30.26%	39.24%	46.27%
S.E.	4.15	3.19	4.72	0.53	5.19	6.52	3.38

CU-18-09 + 100 μM tyramine

N=4	CU-18-09 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	0%	22%	20%	34.48%	56.76%	75%	76.67%
2	30.7%7	3.57%	11.43%	50%	57.14%	84.62%	76.92%
3	11.42%	16.67%	9.09%	37.93%	63.64%	80.77%	72.22%
4	0%	9.52%	27.78%	55.56%	76.92%	80.77%	84.62%
Mean	4.64%	12.94%	17.075%	44.49%	63.615%	80.29%	77.61%
S.E.	2.82	4.04	4.27	4.97	4.71	1.98	2.57

CU-18-09 + 100 μM benzylamine

N=4	CU-18-09 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	20%	20%	37.5%	44.74%	50%	75%	73.68%
2	12.5%	3.7%	11.11%	50%	62.5%	69.23%	75%
3	0%	12.5%	33.33%	44.44%	68.42%	69.23%	75%
4	0%	13.64%	27.27%	59.1%	58.33%	58.33%	66.67%
Mean	6.25%	12.46%	27.30%	49.57%	59.81%	67.95%	72.59%
S.E.	4.73	3.35	5.79	3.42	3.87	3.48	2.00

CU-18-09 + 100 μM 5-HT

N=4	CU-18-09 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	10%	25%	13.88%	36.11%	50%	69.23%	73.9%
2	5%	8.33%	17.65%	50%	38.46%	66.67%	57.14%
3	17.65%	8.33%	15%	50%	57.14%	58.33%	60%
4	6.25%	18.75%	25%	42.86%	60%	66.67%	72.22%
Mean	9.72%	15.1%	17.88%	44.74%	51.40%	65.22%	65.81%
S.E.	2.85	4.11	2.50	3.33	4.79	2.37	4.24

CU-18-10 + 100 μM tyramine

N=4	CU-18-10 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	17.14%	3.57%	38.1%	53.33%	67.57%	53.82%
2	4.76%	25%	47.62%	61.9%	71.43%	60%
3	5.71%	19.05%	37.14%	65.38%	63.64%	69.23%
4	25%	33.33%	33.33%	68.75%	75%	72.22%
Mean	13.15%	20.24%	39.05%	62.34%	69.41%	63.82%
S.E.	4.84	6.28	3.04	3.31	2.45	4.22

CU-18-10 + 100 μM benzylamine

N=4	CU-18-10 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	5%	25%	33.33%	71.43%	77.78%	62.5%
2	16.67%	59.09%	50%	70%	66.67%	66.67%
3	3.7%	47.83%	55%	73.33%	79.92%	75%
4	16.67%	33.33%	66.67%	83.33%	75%	75%
Mean	10.51%	41.31%	51.25%	74.52%	74.84%	69.79%
S.E.	3.57	7.57	6.91	3.01	2.90	3.12

CU-18-10 + 100 μM 5-HT

N=4	CU-18-10 concentration (M)					
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	20%	20%	29.41%	50%	66.67%	60%
2	5.88%	9.09%	10%	54.55%	53.85%	69%
3	10%	17.65%	22.22%	50%	47.62%	66.67%
4	15%	10%	30%	55.56%	55.56%	75%
Mean	12.72%	14.18%	22.91%	52.53%	55.92%	67.67%
S.E.	3.06	2.72	4.65	1.47	3.96	3.10

CU-18-11 +100 μM tyramine

N=4	CU-18-11 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	9.52%	6%	28.57%	23.33%	40.54%	53.57%	63.33%
2	19.23%	23.8%	27.78%	28.57%	39.29%	46.67%	61.54%
3	0%	25%	18.18%	46.15%	31.82%	46.67%	61.11%
4	0%	29%	31.25%	28.57%	33.33%	62.5%	68.75%
Mean	7.19%	20.95%	26.44%	31.66%	36.24%	52.35%	63.68%
S.E.	4.59	5.12	2.85	4.98	2.15	3.75	1.76

CU-18-11 + 100 μM benzylamine

N=4	CU-18-11 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	7.4%	8.57%	7.4%	4.76%	41.67%	50%	63.16%
2	0%	3.7%	16.66%	20%	31.58%	50%	62.5%
3	4.55%	12.5%	5%	16.67%	18.57%	50%	66.67%
4	0%	9.09%	25%	33.33%	22.22%	51.43%	66.67%
Mean	2.98%	8.46%	13.51%	18.69%	28.51%	50.35%	64.75%
S.E.	1.82	1.81	4.57	5.87	5.17	0.35	1.12

CU-18-11 + 100 μM 5-HT

N=4	CU-18-11 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	15%	20%	16.67%	35%	20%	55%	65.22%
2	17.65%	16.67%	29.41%	31.82%	20%	40%	42.86%
3	10%	11.11%	10%	20%	30%	44.44%	50%
4	5.26%	10.53%	20%	20%	50%	40%	40%
Mean	11.98%	14.58%	19.02%	26.07%	30%	44.86%	49.52%
S.E.	2.74	2.27	4.04	3.92	7.07	3.54	5.64

CU-18-12 + 100 μM tyramine

N=4	CU-18-12 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	0%	14.29%	14.29%	43.33%	54.05%	70%	76.67%
2	4.76%	30.77%	23.81%	22.86%	42.86%	69.23%	80.77%
3	5.71%	19.05%	11.43%	42.31%	54.55%	64.71%	77.78%
4	23.08%	29.41%	46.15%	53.57%	66.67%	80.77%	84.62%
Mean	8.39%	23.38%	23.92%	40.51%	54.53%	71.18%	79.96%
S.E.	5.05	4.00	7.87	6.41	4.86	3.40	1.78

CU-18-12 + 100 μM benzylamine

N=4	CU-18-12 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	5%	12.5%	16.67%	38.1%	21.05%	60%	58.33%
2	0%	0%	10%	25%	50%	60%	66.67%
3	0%	12.5%	4.55%	16.67%	50%	60%	61.54%
4	8.33%	20%	25%	25%	50%	58.33%	66.67%
Mean	3.33%	11.25%	14.05%	26.19%	42.76%	59.58%	63.3%
S.E.	2.04	4.15	4.41	4.43	7.24	0.42	2.05

CU-18-12 + 100 μM 5-HT

N=4	CU-18-12 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$	10^{-3}
1	10%	20%	20%	50%	46.67%	70%	78.26%
2	17.64%	20%	10%	59.1%	53.85%	76.92%	71.43%
3	0%	0%	17.65%	45%	61.9%	66.67%	70%
4	20%	11.1%1	22.22%	65%	70%	75%	80%
Mean	11.91%	12.78%	17.47%	54.77%	58.1%	72.15%	74.92%
S.E.	4.51	4.75	2.66	4.49	5.04	2.34	2.47

CU-18-13 +100 μM tyramine

N=4	CU-18-13 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	0%	14.29%	19.05%	46.67%	59.46%	53.85%	66.67%
2	8.57%%	0%	9.1%	20%	42.86%	60%	61.54%
3	7.14%	9.52%	12.5%	38.46%	50%	69.23%	61.11%
4	16.67%	27.78%	28.81%	44.44%	68.75%	69.23%	76.92%
Mean	8.09%	12.90%	17.36%	37.39%	55.26%	63.08%	66.56%
S.E.	3.41	5.78	4.33	6.05	5.63	3.76	3.67

CU-18-13 + 100 μM benzylamine

N=4	CU-18-13 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	5%	16.67%	7.41%	57.14%	47.37%	55.56%	63.16%
2	0%	0%	29.17%	35%	50%	58.33%	68.75%
3	0%	8.33%	10%	16.67%	37.5%	69.23%	75%
4	8.33%	16.67%	25%	41.67%	60%	50%	58.33%
Mean	3.33%	10.42%	17.89%	37.62%	48.72%	58.28%	66.31%
S.E.	2.04	3.99	5.39	8.38	4.62	4.04	3.59

CU-18-13 + 100 μM 5-HT

N=4	CU-18-13 concentration (M)						
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$4x10^{-4}$	10^{-3}
1	25%	20%	20%	30%	40%	38.46%	65.22%
2	17.64%	25%	20%	20%	33.33%	55.56%	57.14%
3	10%	8.33%	15%	36.37%	33.33%	55.56%	60%
4	13.64%	18.18%	31.82%	40.91%	50%	63.64%	72.73%
Mean	16.57%	17.88%	21.70%	31.82%	39.16%	53.30%	63.77%
S.E.	3.21	3.49	3.57	4.53	3.94	5.30	3.42

Pargyline + 100 μM tyramine

N=3	Pargyline concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$5x10^{-4}$
1	25%	33.33%	62.5%	66.67%	83.33%
2	5.56%	44.44%	77.78%	77.78%	80%
3	0%	30.77%	69.23%	80.77%	81.82%
Mean	10.19%	36.18%	69.84%	75.07%	81.72%
S.E.	6.56	3.63	3.83	3.71	0.83

Pargyline + 100 μM benzylamine

N=3	Pargyline concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$5x10^{-4}$
1	29.41%	52.94%	57.14%	76.47%	80%
2	12.5%	25%	75%	87.5%	81.25%
3	7.14%	35.71%	76.47%	78.57%	50%
Mean	13.65%	37.88%	69.54%	80.85%	70.41%
S.E.	5.81	7.05	5.38	2.92	8.84

Pargyline + 100 μM 5-HT

N=3	Pargyline concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$5x10^{-4}$
1	10%	10%	35%	58.82%	50%
2	18.18%	18.18%	37.5%	50%	84.62%
3	17.65%	17.65%	35.29%	65%	60%
Mean	15.27%	15.27%	35.93%	57.94%	64.87%
S.E.	2.28	2.28	0.68	3.77	8.91

Clorgyline + 100 μM tyramine

N=3	Clorgyline concentration (M)					
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$5x10^{-4}$
1	4.17%	29.17%	50%	43.75%	79.17%	91.67%
2	0%	5%	20%	45%	60%	90.91%
3	6.25%	18.75%	37.5%	54.17%	75%	93.33%
Mean	3.47%	17.64%	35.83%	47.64%	71.39%	91.97%
S.E.	1.62	6.06	7.53	2.84	5.41	0.61

Clorgyline + 100 μM benzylamine

N=3	Clorgyline concentration (M)				
	10^{-6}	10^{-5}	10^{-4}	$2x10^{-4}$	$5x10^{-4}$
1	23.53%	47.06%	53.85%	76.47%	90%
2	0%	25%	62.5%	75%	87.5%
3	7.69%	23.08%	58.82%	69.23%	60%
Mean	10.41%	31.71%	58.39%	73.57%	79.17%
S.E.	5.99	6.66	2.17	1.91	8.32

Clorgyline + 100 μM 5-HT

N=3	Clorgyline concentration (M)				
	10^{-8}	$2x10^{-8}$	10^{-7}	$5x10^{-7}$	10^{-6}
1	11.11%	11.11%	55.56%	72.73%	76.92%
2	0%	0%	23.53%	64.71%	60%
3	9.09%	9.09%	36.36%	66.67%	82.61%
Mean	6.73%	6.73%	38.48%	68.03%	73.18%
S.E.	2.59	2.59	8.06	2.09	5.88

Selegiline +100 μM tyramine

N=3	Selegiline concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	$5x10^{-4}$
1	22.22%	55%	50%	66.67%	83.33%
2	38.89%	55%	61.11%	60%	66.67%
3	20%	40%	50%	62.5%	58.33%
Mean	26.96%	50%	53.70%	63.06%	69.44%
S.E.	5.18	8.66	3.21	1.68	6.36

Selegiline + 100 μM benzylamine

N=3	Selegiline concentration (M)				
	10^{-8}	10^{-7}	$5x10^{-7}$	10^{-6}	$5x10^{-6}$
1	23.08%	38.46%	58.33%	84.62%	78.95%
2	11.11%	22.22%	44.44%	77.78%	60%
3	16.67%	33.33%	61.54%	75%	71.43%
Mean	16.95%	31.37%	54.79%	79.13%	69.79%
S.E.	2.99	4.15	4.55	2.47	4.54

Selegiline + 100 μM 5-HT

N=3	Selegiline concentration (M)			
	10^{-7}	10^{-6}	10^{-4}	$5x10^{-4}$
1	12.5%	25%	55.56%	70%
2	8.33%	25%	50%	69.23%
3	33.33%	44.44%	50%	78.26%
Mean	18.05%	31.48%	51.85%	72.5%
S.E,	6.70	5.61	1.60	2.50

Initial velocities (natoms O / mg protein / min) : control (tyramine only) and in the presence of 0.15 mM CU-18-08

N=4	Tyramine concentration (mM)					
	0.05	0.1	0.2	0.4	0.8	1.2
N1, control	14.47	14.47	20.83	24.71	26.01	26.01
N1, + 08	7.2	7.2	9.00	6.54	10.41	11.7
N2, control	16.77	18.45	20.12	19.1	23.15	28.8
N2, + 08	6.71	8.39	8.39	13.02	14.47	14.4
N3, control	13.66	20.48	23.9	25.61	27.31	27.31
N3, +08	5.12	6.38	8.54	15.36	15.36	15.36
N4, control	8.64	11.88	14.04	16.20	16.20	17.28
N4, + 08	4.32	5.4	6.48	6.48	7.56	16.79
Mean±SE, control	13.38±1.71	16.32±1.94	19.72±2.06	21.4±2.25	23.17±2.48	24.85±2.59
Mean±SE, +08	5.84±0.67	6.95±0.62	8.1±0.56	10.34±2.27	11.951.82	14.56±1.07

Initial velocities (natoms O / mg protein / min) : control (benzylamine only) and in the presence of 0.04 mM CU-18-08

N=4	benzylamine concentration (mM)					
	0.05	0.1	0.2	0.4	0.8	1.2
N1, control	10.8	12.6	14.4	16.2	18	18
N1, + 08	5.4	7.2	9	10.8	14.4	14.4
N2, control	5.79	10.13	11.57	13.02	15.92	17.36
N2, + 08	2.9	7.23	8.68	10.13	13.02	14.47
N3, control	6.71	10.06	13.42	15.91	15.09	16.77
N3, +08	3.35	3.35	6.71	6.71	10.06	11.74
N4, control	4.32	6.48	13.66	13.66	17.07	18.78
N4, + 08	2.16	3.24	4.32	4.32	10.24	11.95
Mean±SE, control	6.9±1.39	9.82±1.26	11.74±1.51	14.7±0.8	16.52±0.64	17.73±0.43
Mean±SE, +08	3.45±0.69	5.25±1.13	7.18±1.08	7.99±1.52	11.93±1.07	13.14±0.75

Initial velocities (natoms O / mg protein / min) : control (tyramine only) and in the presence of 0.03 mM CU-18-10

N=4	Tyramine concentration (mM)					
	0.05	0.1	0.2	0.4	0.8	1.2
N1, control	4.58	6.76	10.32	12.45	14.59	14.82
N1, + 10	4.58	4.58	6.76	11.03	11.03	12.1
N2, control	5.87	9.23	10.91	13.43	15.53	16.79
N2, + 10	5.45	6.71	9.23	10.50	13.01	14.27
N3, control	5.15	5.15	8.59	10.3	12.02	12.02
N3, +10	3.43	5.15	5.15	5.15	5.15	10.46
N4, control	7.32	11.26	12.1	13.79	15.48	15.48
N4, + 10	3.66	4.51	5.35	6.19	7.04	8.72
Mean±SE, control	5.73±0.59	8.1±1.35	10.48±0.73	12.49±0.78	14.15±0.75	14.78±1.01
Mean±SE, +10	4.28±0.46	5.24±0.51	6.62±0.94	8.22±1.49	9.06±1.8	11.39±1.18

Initial velocities (natoms O / mg protein / min) : control (benzylamine only) and in the presence of 0.008 mM CU-18-10

N=4	benzylamine concentration (mM)					
	0.05	0.1	0.2	0.4	0.8	1.2
N1, control	4.61	5.87	9.23	11.75	13.01	14.27
N1, + 10	2.94	2.94	5.45	6.71	9.23	9.23
N2, control	6.39	12.78	31.95	31.95	31.95	31.95
N2, + 10	6.39	6.39	6.39	12.78	19.17	25.56
N3, control	5.07	7.60	9.01	9.85	11.54	12.39
N3, +10	1.97	3.66	5.35	7.04	8.73	8.73
N4, control	2.58	5.16	6.13	8.06	9.03	10.00
N4, + 10	2.60	3.91	2.26	3.22	4.19	4.19
Mean±SE, control	4.66±0.79	7.85± 1.72	14.08±6.00	15.40±5.57	16.38± 5.25	17.15± 5.01
Mean±SE, +10	3.47±0.99	4.22 ±0.75	4.86 ±0.9	7.44±1.98	10.33± 3.16	11.93 ±4.68

Initial velocities (natoms O / mg protein / min) : control (5-HT only) and in the presence of 0.0.1 mM CU-18-10

N=4	5-HT concentration (mM)				
	0.025	0.05	0.1	0.25	0.5
N1, control	6.73	7.79	8.85	9.92	10.98
N1, + 10	2.48	2.48	4.60	5.67	6.73
N2, control	3.43	5.15	5.15	7.24	8.59
N2, + 10	1.72	1.72	1.72	1.72	3.43
N3, control	19.17	25.56	31.95	38.34	38.34
N3, +10	12.14	12.78	12.78	12.78	19.17
N4, control	2.26	3.22	4.19	5.16	6.77
N4, + 10	1.30	2.60	1.29	1.29	1.29
Mean±SE, control	7.9±3.87	10.43±5.13	12.53± 6.55	15.16±7.79	16.17±7.44
Mean±SE, +10	4.41±2.59	4.89 ±2.64	5.10±2.66	5.36±2.66	7.65±4.00

Curriculum Vitae

Miss Tatiya Thanomdee was born in Bangkok on February 13, 1974. She received her Bachelor Degree in Pharmacy from Faculty of Pharmaceutical Science, Mahidol University in 1997 and has been a pharmacist in Phraputthabath Hospital. In 2000, she came to study for Master 's degree in Pharmacy at Chulalongkorn University.