ISOLATION AND SELECTIVE REDUCTION OF MITRAGYNINE, SYNTHESIS AND CHARACTERIZATION OF NEW INDOLE DERIVATIVES AND THEIR SELECTED BIOLOGICAL ACTIVITY STUDIES

GOH TEIK BENG

UNIVERSITI SAINS MALAYSIA

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BY

GOH TEIK BENG

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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To my parents,

For their boundless love……

ACKNOWLEDGEMENT

I would like to express my gratitude and thanks to everyone involved. This project could not accomplished without the guidance and help from all parties. First, I would like to express my heartiness gratitude and appreciation to my project supervisor, professor Sharif Mahsufi Mansor for his helpful advice, generous encouragement, kind attention and guidance throughout this project. My special thanks to my co-supervisor, associate professor doctor Mohammad Nizam Mordi for her support throughout the whole experiments.

I acknowledge with gratitude the help and support of the lecturers from The Drug Research Centre, Universiti Sains Malaysia. Not forgetting too, the help from Encik Razak, Dr. Jayant, Mr. Gowda, Mrs. Juwita, Mr. Hilman, Mr. Zamri, Mr. Rahim and Mr. Ashok

Last but not least, to my family, my friends and my beloved for their overwhelming encouragement, support and care given to me throughout this project. I would also like to acknowledge Universiti Sains Malaysia (RU grant : 1001 / CDADAH / 815020 and RUT Grant : 1001/CDADAH/855005) and Ministry of Science, Technology and Innovation (304 / CDADAH / 650384/I119) for providing research grant for the study carried out in this thesis.

Last but not least, thanks the support from all !

Goh Teik Beng
(DD0001/04)
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylCDR81zothiazoline-6-sulphonic acid)
BA	Betulinic acid
BHT	Butylhydroxytoluene
BZRs	Benzodiazepine receptors
CD	Circular dichroism
CDR80	Silane reduced mitragynine ((E)-methyl 2-((2S,3S,12bS)-3-ethyl-8-methoxy-1,2,3,4,6,7,7a,12,12a,12b-decahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxyacrylate).
CDR81	6- methoxy-1-phenyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole.
CDR82	6-methoxy-1-(4-methoxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole.
CDR83	6-methoxy-1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole.
CDR84	2-methoxy-4-(6-methoxy-2,3,4,9-tetrahydro-1H-pyrido[3,4- b]indol-1-l)phenol.
CHN	Carbon, hydrogen and nitrogen
CV	Coefficient of variation
DEPT	Distortionless enhancement by polarization transfer
DES	Diethylsilane
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
1D –NMR	One dimensional nuclear magnetic resonance spectroscopy
2D-NMR	Two dimensional nuclear magnetic resonance spectroscopy
1D NOE Diff	One dimensional nuclear overhauser effect difference
DPPH	2,2-diphenyl-1-picrylhydrazyl

DQF-COSY	Double quantum filter ^{1}H-^{1}H correlation spectroscopy
DSC	Differential scanning calorimetry
ESI-LCMS	Electrospray ionization liquid chromatography mass spectroscopy
ESI-TOF-HR-LC-MS/MS	Electrospray ionization tandem liquid chromatography mass spectrometry
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
5-Fu	5-Fluorouracil
GABA	Gamma-aminobutyric acid receptor
GCMS	Gas chromatography mass spectrometer
HMBC	Heteronuclear multiple-bond coherence
HPTLC	High performance thin layer chromatography
HR-MS	High resolution mass spectroscopy
HSQC	Heteronuclear single quantum coherence
5-HT	5-hydroxytryptamine receptor
IMR	Institute Medical Research
JRES	J-resolved couplings
HSQC	Heteronuclear single quantum coherence
IUPAC	International union of pure and applied chemistry
LLOQ	Lower limit of quantification
LOD	Limit of detection

MeOH	Methanol
MTG	Mitragynine [(E)-methyl 2-(3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxyacrylate)]
5-M-TRP	5-methoxytryptamine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
***n*-BS**	*n*-Butylsilane
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
OH	Hydroxyl group
p-TsOH	Para toluene sulfonic acid
PBS	Phosphate buffered saline
PFTBA	Perfluorotributylamine
PMHS	Polydimethylhydroxysilane
hRf	Homologous retention factor
ROS	Reactive oxygen species
SAR	Structure-activity relationship
SD	Standard deviation
SEM	Standard error mean
SI	Selectivity index
SIM	Selected ion monitoring
TEAC	Trolox equivalent antioxidant capacity
TES	Triethylsilane

TFA	Trifluoroacetic acid
TOCSY	Total Correlation spectroscopy
UV	Ultra-violet spectroscopy

LIST OF SYMBOLS

Å	Angstrom
α	Alfa
ß	Beta
γ	Gamma
κ	Kappa
ca.	Approximately
d	Doublet
dd	Doublet doublet
m	Multiplet
q	Quartet
ddd	Doublet doublet doublet
m/z	Mass per charge
t	Triplets
s	Singlet
ºC	Degree Celsius
e.g.	For example
et al.	Co Workers
∆H	Enthalpy
h	Hours
Hz	Hertz

IC_{50}	Median inhibitory concentration
J	Coupling constant in hertz
Jg^{-1}	Joule per gram
$Kjmol^{-1}$	Kilojoule per molar
µL	Microlitre
µM	Micromolar
Mg	Miligram
mM	Milimolar
Min	Minutes
mL	Mililitre
eV	Electron volt
n	Number of replicates
pKa	Ionisation constant
ppm	Part per million
r	Correlation coefficient
s	Second
S	Sinister isomer
R	Rectus isomer
T_m	Melting temperature
v/v	Volume by volume
w/w	Weight by weight
<	Less Than

>	More than
=	Equal
±	Plus/minus
%	Percent
δ	NMR chemical shift in ppm

LIST OF PUBLICATIONS

1. Goh, T.B., Siddiqui, J., Razak, H., Mordi, M.N. & Mansor, S.M. (2011). A simple and cost effective isolation and purification protocol of MTG from *M. Speciosa* Korth leaves. *The Malaysian J. of Anal. Sci.*, **15 (1):** 54 – 60. (Scopus index).
2. Goh, T.B., Mordi, M.N., Mansor, S.M. & Fun, H.K. (2012a). Bis(6-methoxy-1-methyl- 2,3,4,9-tetrahydro-1H-β-carbolin-2-ium)tetrachloridozincate(II) dihydrate. *ActaCrystallographica. E*, **68**: 464–465, [doi:10.1107/S1600536812011130]. (IF=0.35)
3. Goh, T.B., Mordi, M.N., Mansor, S.M. & Fun, H.K. (2012b).6-Methoxy-1-(4-methoxyphenyl)-1,2,3,4-tetrahydro-9H-β-carbolin-2-ium acetate.ActaCrystallographica.E, **68**: o1483, [doi:10.1107/S1600536812016753].(IF = 0.35)
4. Goh T.B., Ramu,M., Mordi, M.N. & Mansor, S.M.(2013). Unexpected reduction of the indole double bond in MTG using *n*-butylsilane and catalytic tris(pentafluorophenyl)boranes. *Synth. Comm.* **44(9):** 1291-1295.(IF = 1.02) [doi: 10.1080/00397911.2013.853798].
5. Goh, T.B., Mordi, M.N. & Mansor, S.M. (2013). Demethylation of MTG picrate with BBr_3 and $C_{12}H_{25}$-SH/NaOMe: A new entry for ester derivatives g preparation based on position carbon 9. *Asian J. Research Chem*.,**6(9)**: 863-867.(Scopus index)
6. Goh, T.B., Mordi, M.N. & Mansor, S.M. (2015). NMR structural assignment of four new 6-methoxy- tetrahydro-β-carboline derivatives. *Magnetic Resonance in Chem*. *In press*. (IF = 1.55)
7. Goh, T.B., Mordi, M.N. & Mansor, S.M. (2014). Conformational stereoisomerism and absolute configuration of 6-methoxy-tetrahydro-β-carboline derivatives. *Magnetic Resonance in Chem. In Press.* (IF = 1.52).
8. Goh, T.B., Mordi, M.N. & Mansor, S.M. (2014). Mass Spectrometry (LCMS/MS) as a tool in the reaction optimisation and characterisation of new 6-methoxy-tetrahydro-β-carboline Derivatives. *Sains Malaysiana.* 44(1), 127-137. (IF = 0.40)
9. Goh, T.B., Mordi, M.N. & Mansor, S.M. (2014). Antioxidant value and antiproliferation efficacy of MTG and its silane reduced analogues. *Asian pacific journal of cancer prevention.* 15(14), 5659-5665. (IF = 1.60).
10. Goh, T.B., Mordi, M.N., Kho, R.Y., Yam, M.F., Azhar, M.E. & Mansor, S.M. (2015). 5-Methoxytryptamine reacts with natural food flavour to produce 6-methoxy scaffold ß-carbolines: In vitro investigation of their antioxidant and cytotoxicity properties. *Food Chemistry.* 183, 208-216. (IF = 3.30).
11 Goh, T.B., Mordi, M.N., Kho, R.Y., Yam, M.F., Azhar, M.E. & Mansor, S.M. (2014). 6-methoxy-tetrahydro-ß-carbolines induce cytotoxicity and apoptosis on colon and leukaemia cell lines Food Chemical Toxicology. In review. (IF = 3.20).

PEMENCILAN DAN PENURUNAN SELEKTIF MITRAGININA, SINTESIS DAN PENCIRIAN TERBITAN INDOL BARU DAN KAJIAN KEAKTIFAN BIOLOGI TERPILIH MEREKA

ABSTRAK

Mitraginina (MGT) memiliki sifat analgesik yang poten tetapi ketoksikan MGT telah juga dilaporkan. Objektif utama tesis ini adalah untuk memencilkan MGT bagi pengubahsuaian lanjutan struktur dan juga untuk mensintesis terbitan indolnya bagi menilai aktiviti antiproliferatif, antioksida dan antinosiseptif masing-masing. MGT telah dipencilkan daripada daun *M. speciosa* dengan menggunakan prosedur penulenan yang ringkas dan berkesan. Pelbagai silana telah digunakan untuk menurunkan kumpulan karbonil dan ikatan dubel indol bagi MGT tetapi hanya ikatan berkembar indol telah berjaya diturunkan kepada indolina [CDR80 ((E)-metil 2-((2*S*,3*S*,12b*S*)-3-etil-8-metoksi-1,2,3,4,6,7,7a,12,12a,12b-dekahidroindolo[2,3-a]quinolizin-2-il)-3-metoksiakrilat)]. Tindak balas Pictet-Spengler telah diubahsuai dengan menggunakan 5-metoksitriptamina dan asid trifluoroasetik dalam medium akueus untuk mensintesis terbitan indol yang baru yang telah dicirikan dan dikenalpastikan sebagai CDR81 (6- metoksi-1-fenil-2,3,4,9-tetrahidro-1H-pyrido[3,4-b]indol) , CDR83 (6-metoksi-1-metil-2,3,4,9-tetrahidro-1H-pirido[3,4-b]indol), CDR82 (6-metoksi-1-(4-metoksifenil)-2,3,4,9-tetrahidro-1H-pirido[3,4-b]indol) dan CDR84 (2-metoksi-4-(6-metoksi-2,3,4,9-tetrahidro-1H-pirido[3,4-b]indol-1-l) fenol). Konfigurasi mutlak C-1 telah dikenalpastikan sebagai *S* dengan menggunakan analisis NMR, analisis CD (circular dichroism) spektra dan analisis data kristal tunggal dengan merujuk kepada peraturan keutamaan Cahn-Ingold. Empat terbitan indol baru iaitu CDR81, CDR82, CDR83 dan CDR84 telah dinilai menggunakan pelbagai panel sel kanser manusia: HT 29, K 562,HCT 116, MCF -7, CEM SS, HEPG2, SH - 5YSY, MDA -MB- 231 dan Hela. Sebagai tambahan, CDR80 dan MTG juga telah diuji terhadap barisan sel kanser K 562 dan HCT 116. CDR82 telah didapati paling aktif terhadap barisan sel kanser K 562 , HCT 116 dan HT29 dengan

selektiviti yang baik berbanding dengan barisan sel kawalan iaitu NIH/3T3 , CCD18 -Co dan B98 -5. Di samping itu, CDR82 menunjukkan aktiviti dan selekviti yang lebih baik daripada ubat-ubatan antikanser piawai iaitu cisplatin , asid betulinik dan 5 -fluorourasil bagi barisan sel kanser HT29 (SI = 3.89), K 526 (SI = 8.71) dan HCT116 (SI = 5.68) masing-masing. CDR81 merupakan terbitan yang kedua paling aktif, CDR83 menunjukkan aktiviti yang lemah dan CDR84 tiada mempunyai aktiviti antiproliferatif. Terbitan indol ini telah didapati mempamerkan ciri antioksida yang sederhana berdasarkan gabungan cerakin DPPH, ABTS dan kuasa penurunan. CDR84 mempamerkan aktiviti antioksida yang paling aktif disebabkan kehadiran kedua-dua kumpulan indol dan fenolik pada strukturnya sedangkan terbitan β-karbolina yang lain tidak mempunyai kumpulan fenolik tersebut. Akan tetapi tiada satu pun daripada keempat-empat terbitan ini menunjukkan aktiviti analgesik pusat seperti yang dipamerkan oleh mitraginina.

ISOLATION AND SELECTIVE REDUCTION OF MITRAGYNINE, SYNTHESIS AND CHARACTERIZATION OF NEW INDOLE DERIVATIVES AND THEIR SELECTED BIOLOGICAL ACTIVITY STUDIES

ABSTRACT

Mitragynine (MTG) possesses potent analgesic properties but the toxic effects of MTG have also been reported. The main objective of this thesis is to isolate MTG for further structural modification and also to synthesize its indole derivatives and evaluate their antiproliferative, antioxidant and antinociceptive activities. MTG was isolated from *M. speciosa* leaves using a simple and effective purification procedure. Various silanes were used to reduce the carbonyl and indole double bonds of MTG but only the indole double bond was successfully reduced to indoline [CDR80 ((E)-methyl 2-((2*S*,3*S*,12b*S*)-3-ethyl-8-methoxy-1,2,3,4,6,7,7a,12,12a,12b-decahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxy acryl ate)]. The Pictet-Spengler reaction was modified using 5-methoxytryptamine and trifluoroacetic acid in an aqueous medium to synthesize new indole derivatives which were identified and characterized as CDR81 (6- methoxy-1-phenyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole), CDR82 (6-methoxy-1-(4-methoxyphenyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole), CDR83 (6-methoxy-1-methyl-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole) and CDR84 (2-methoxy-4-(6-methoxy-2,3,4,9-tetrahydro-1H-pyrido[3,4- b]indol-1-l)phenol). The absolute configuration of C-1 was determined as *S* using NMR analysis, CD (circular dichroism) spectra analysis and single crystal data analysis with reference to Cahn-Ingold priority rule. Four new indole derivatives, namely CDR81, CDR82, CDR83 and CDR84 were evaluated on a panel of human cancer cell line namely HT 29, K 562, HCT 116, MCF-7, CEM SS, HEPG2, SH-5YSY, MDA-MB-231 and HELA. In addition, CDR80 and MTG were tested against K 562 and HCT 116 cell lines. CDR82 was found to be the most active derivative, with good selectivity on the K 562, HCT 116 and HT29 cancer cell lines as

compared to the non-tumorous cell lines of NIH/3T3, CCD18-Co and B98-5. In addition, CDR82 showed better activity and selectivity than the standard anticancer drugs cisplatin, betulinic acid and 5-fluorouracil on the HT29 (SI=3.89), K 526 (SI=8.71) and HCT116 (SI=5.68) cancer cell lines respectively. CDR81 was the second most active, CDR83 showed only mild activity while CDR84 has no activity. The indole derivatives were found to exhibit moderate anti-oxidative properties, based on a combination of DPPH, ABTS and reducing power assays. CDR84 showed the highest antioxidant activity due to both indole and phenolic groups in its structure whilst other β-carboline derivatives do not have the phenolic group. However, none of these derivatives showed the central analgesic activity exhibited by mitragynine.

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Thesis Title

Comment	Page	Correction	Page
Title of the thesis needs to be improved to highlight the chemistry part, which forms a significant part of the work (Title of the thesis does not reflect the chemistry part since this thesis carries quite a lot of chemistry work).	-	Thesis title has been changed to : "Isolation and selective reduction of mitragynine, synthesis and characterization of new indole derivatives and their selected biological activity studies."	-

Table of content

Comment	Page	Correction	Page
Spelling error on overhauser.	Page xxi	Corrected	xx
Wrong name on DPPH-Name should stops at "…hydrazyl".	Page xxi	Corrected	xx
Electrospray is one word.	Page xxi	Corrected	xxi
Include abbreviation for ESI.	Page xxii	Corrected	xxi~~i~~
Correct term for OH is hydroxyl group.	Page xxii	Corrected	xxii
The correct term should be Phosphate Buffered Saline.	Page xxiii	Corrected	xxii
Include abbreviation for hRf (pg61).	Page xxiii	Corrected	xxii

Include abbreviation for SAR (pg 34).	Page xxiii	Corrected	xxii
SI should be Selectivity not selective.	Page xxiii	Corrected	xxii

Ab~~t~~st ract (Both in English and Bahasa Malaysia)

Comment	Page	Correction	Page
The English and Malay versions of the abstract need to be corrected for some wordings.	Abstrak Page xxxii-xxxiii Abstract Page xxxiv-xxxv	Amended	Abstrak Page xxxi-xxxiii Abstract Page xxxiv~~ii~~-xxx~~i~~v
A small improvement might be suitable by mentioning how the absolute configuration of C-1 was determined.	Abstrak Page xxxii-xxxiii Abstract Page xxxiv-xxxv	Amended. The absolute configuration of C-1 was determined using NMR analysis, single crystal data analysis with reference to Cahn-Ingold priority rule.	Abstrak Page xxxi Abstract Page xxxiv~~ii~~
Para 2 of the abstract, 2nd line Rewrite sentence to omit (:) sign.	Page xxxiv	Amended	Page xxxiv~~ii~~

Objective of the study

Comment	Page	Correction	Page
(i) Objective 1-Not convinced with the word "effective", not really development. The isolation method is quite standard. Should be on the "Isolation of mitragynine for further evaluation….. Objectives 1 and 2 suggested to combine. (ii) Objective 2- Needs to be more specific to synthesize all the compounds (4+1). (iii) Objective 3-To identify and characterize (4+1). (iv) Objective 4- Add … Indole derivatives synthesized.	53	Amended. The study objective has been modified accordingly as suggested.	5~~4~~5
In the 1.9 scope of study, only reduction of indole double bonds was mentioned (p. 53). The main objective of chapter 3 is to discover a simple and mild approach to reduce the carbonyl group or the double indole bond of MTG. However, the indole double bond was reduced instead (SRM~~SRM~~). What do you want to suggest, reducing both the carbonyl group and indole double bond or merely indole double bond?	53	Amended. In the revised thesis, with reference to section, 1.9 - Objective of the study, the "reduction of the indole double bond" has been modified to "To develop a simple semisynthetic approach to reduce the isolated mitragynine~~To synthesize and characterize silane reduced mitragynine (CDR80)~~" to generalize the reduction process to tie in with the main objective of chapter 2 -Either to reduce the double bond or carbonyl group.	53,5~~4~~5&5~~6~~8

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Problem Statement

Comment	Page	Correction	Page
The title "Problem statement" should change to ~~highlight the~~ research framework.	51	Amended	52~~3~~

Chapter 1 (Introduction)

Comment	Page	Correction	Page
Need to write the full name of a species if it is at the beginning of a sentence.	1	Amended	1
Should subtitle be changed to 'Toxicological…' instead of 'Pharmacological…' or add both?	2	Amended	2
Para 2, last line Cited ref. should be shifted to earlier sentence.	3	Amended	4~~3~~
Fig. 1.2 Font size of figure is too small Should also include mitragynine with nomenclature label of carbon numbers because in the next few pages you are discussing on the different positional substituents of the indoles & their activity. And also need scientific names in their captions (eg. 6-methoxy-tetrahydro-β-	6	Amended. Font size has been adjusted, mitragynine included with nomenclature labels of carbon numbers. The compounds in figure 1.2 are mitragynine related indole alklaoids and the scientific name of mitragynine	6

carboline derivatives).		has been included.	
Para 1, last line The alkaloids may not be the most potent compounds in that species or when compared to other species?	7	Amended	7
Para 2, 1st and 2nd line Please correct the IUPAC names, no space in substituents name or position number and also no hyphen before "indoles".	11	Amended	11
Delete the hyphen before 'Key…'.	13	Amended	13
Para 1, Last line-Rewrite sentence, Unclear.	16	Amended	16
Para 2, Line 2, Rewrite to omit colon sign :	17	Amended	17
Suggestion for clarity (1.6.1)- Please add a general reduction scheme for the reduction reaction of indole to indoline structure.	18	Amended. A general reduction scheme has been added as shown in Scheme 1.1	19
Add movement of electron i.e. the push arrows. i.e. apply to this Pictet Spengler mechanism scheme catalyzed by H^+.	22	Amended. Movement of electron with push arrow has been added as shown in Figure 1.5	23
Para 2, sentence 1 - rewrite sentence construction to remove brackets.	26	Amended	27
Para 1, Last line Insert ' …. Hotplate method'.	27	Amended	28
Para 1, last line Replace '.. In this thesis' with ' … of this study'.	29	Amended	30

Para 1, last line-correct term, need to add species and replace ‘number’ with ‘concentration’.	30	Amended	312
Give the name of the β-carboline.	38-44	Amended. Their scientific name has been given as shown in Table 1.1.	3940-46

Chapter 2 & Chapter 3

Comment	Page	Correction	Page
Include structure and reaction schemes in chapters 2 and 3.	-	Reaction scheme of mitragynine to silane reduced-mitragynine has been included as shown in scheme 2.1 Structures are as shown in figures 2.3, 2.7 and 2.8.	6972,704, 758, 7881
It is suggested that chapters 2 and 3 be combined.	-	Chapters 2 and 3 have been combined and the title has been changed to ‘Isolation and selective reduction of mitragynine’.	-
It is suggested that MTG extraction process need to be simplified in the form of a flow chart for clearer explanation.	-	Done The MTG extraction process has been explained in text and simplified in the flow chart as shown in Appendix A 10 and Figure 2.1.	5860, 2889

The candidate needs to pay attention to his tendency of repeating things. Conciseness is an important element in good piece of scientific work. The same results/discussion or arguments are repeated several times, need to rectify them. Clarity needed with an improved & rectified figure presentation of the full chemical structure of all synthesized derivatives. There are lots of repetition in the methodology section (Extraction of Mitragynine). For example, the explanation in p. 59 has already been mentioned in p. 55; again the crystallization procedure mentioned on p 56 is repeated on p.60. All the procedures should be mentioned in the methodology chapter while the discussion of the results should be elaborated in a different chapter.	55-56, 59-60	Amended. Chapters 2 and 3 are combined together, revised and the repeated parts have been deleted.	~~59-61~~
Need to define what is 'first column' and 'second column' of the flash chromatography and the mobile phase which is the eluting solvent.	60	The 'first column' and the 'second column' together with the mobile phase eluting solvent have been defined in the foot note of ~~t~~Table 2.1 (Appendix A10, pp.288).	64~~7~~
How did you determine the retention factor hRf? hRf is a ratio and thus should not have unit(cm) value. The number should be less than 100.	61, 63	Homologous retention factor, hRf = [(Distance between starting line and middle of substance spot)/(Distance between starting line and solvent front)]x100 as shown	61, 67~~69-70~~

		in equation 2.1. The unit cm has been deleted in the revised thesis.	
Line 2 How did you get 100 % value as in the table, it is 99 %? Check 99 % versus 100 % of purity for mitragynine.	62	Amended. The purity is 99 %	6770
Table 2.3. What are these columns 2 and 3. Different runs? Different methods?	63	Same method and run, the difference is only in the unit. (Column 2 in g and column 3 in percentage).	66-
In the characterization work using IR (pg. 64, there are two absorptions (1640 and 1704 cm^{-1}) for the carbonyl group off the carboxyl methyl group. Do these two represent the C=O stretching of the carbonyl groups? What is the different between 2870 and 2952.9 cm-1. What do you mean by allo? Rephrase on the spectrum write up about trans double bond geometry	64	There only one absorption peak that represents the C=O stretching group at 1704.5 cm^{-1}. The absorption peak at 1640cm^{-1} represents the C=C stretching. The FTIR spectra shows the symmetry *sp3* hybridised CH stretching band at 2800.0 and 2870.0 cm-1 in addition to the strong asymmetry *sp3* hybridised CH stretching absorption band at 2952.9 cm-1. FTIR alone would not be able to confirm the allo structure and trans double bond geometry. Therefore the sentence of allo has been deleted	767-680 71
Line 10 needs full name of *n*-BS and put abbreviation terms in brackets.	67	Amended as suggested.	557

Para 2 1st sentence Clarify- '…. Without involving hydrogen'?	68	Amended as suggested	568
Need full name of SRM on title	69	Amended as suggested	5962
Line 1 wrong term- Dichloromethane solution?	69	Amended as suggested	5962
Line 4, Need to add details – 'Flash'	69	Amended as suggested	5962
Line 7, 12 –'.. Mixture of..'	69	Amended as suggested	602
3.2.2.3 Need a much clearer subtitle.	69	Subtitle has been deleted in revised thesis.~~Amended as suggested~~	~~63~~
Instrument name of HPTLC? Need to delete please refer.	70	Full ~~instrument~~ name of HPTLC have been added. Amended	640
3.2.2.6 What is the full terms of DSC?	70	Full term of DSC is differential scanning calorimetry. Amended.	625
3.3.1 Need to add ' respectively.' after mentioning the different reducing agents	71	Amended as suggested	703
Improve the subtitle of the study : Reduction of MTG using *n*-BS	72	Adding in "at different reaction conditions". Amended	703
Para 1, last sentence Explanation unclear-Rewrite in proper chemical mechanism terms	77	Amended	7780

Add movement of electrons i.e. the push arrows. Apply to all mechanism schemes. Either remove that or to maintain it must correct with arrow in the mechanism.	78	Amended	7881
When writing the NMR data, if a compound shows multiplet, the value should be written in range.	6577	Amended. It is triplet, not multipley.corrected	6871
Figures and numbers used for comparison in order to draw the conclusion about the efficiency of isolation of mitragynine are premature.	60-63	Figures and numbers have been amended as suggested. The developed effective method has been changed to a developed simple improved approach.	674, 669-6770
Picric acid is dangerous but it only improves purity by 1 % only?	-	Other organic acid such as acetic acid, acetic anhydride yields no crystals.	-
Why do you not use HPLC instead of GCMS to analyse the compounds qualitatively	-	In HPLC, compounds with the same retention time may not be the same compounds and pure standard is required for comparison. For qualitative identification, in this case GCMS, we can obtain the exact parent mass number and this serves its purpose and therefore is used instead of HPLC	-
Font size of Figure 3.1 not eligible	74	Font size has been corrected accordingly as shown in Figure 2.4	725

Chapter 4

Comment	Page	Correction	Page
Figures Add chiral center to all molecules synthesized	-	Chiral centerre have been added to CDR81, CDR82, CDR83 and CDR84.	1325, 1347, 14851, 14952
Rationale of synthesis Add rationale on why you synthesize the 4 indole derivatives. i.e. how structure difference affects activity. This is lacking in the discussion. This can explain why some derivatives are having good activity and some are not. i.e. do explain the result in connection to the structure (Connect activity with structure-SAR).can be more comprehensive by accommodating sources in area of quantitative-structure activity	-	Amended The rationale of the synthesis is based on designing the molecules with good anti-proliferative activity. Antioxidant and analgesic activity is just value added properties. The discussion on their relationship between structure and activity has been added in the text.	1831-1825
Add a statement about carbonyl activity. Do they have analgesic activity?	85	A statement about the carbonyl activity has been added.	825
Add one page to have all the molecule structure with numbers and with their IUPAC name. Don't use ANI, VAN and etc. Use proper name for the reduced mitragynine product and adopt a numbering system for chemical compounds throughout the thesis	85	Amended SRM, BEN, ANI, ACE and VAN have been changed to CDR80, CRR81, CDR82, CDR83 and CDR84 respectively throughout the thesis. The proper name and numbering of the compounds are shown in Figure 2.7 and 3.1	758, 836

4.2.2 Line 5, Need to add in the actual method name, flash chromatography.	87-90	Amended	859-8892
Line 3 insert 'Mobile phase solvent.	88	Amended	859-8892
Add equation/scheme accordingly for each product synthesized. Where are all your chemical structures for the respective desired products/—Scheme of reaction equations.	87-89	Chemical reactions scheme (Scehme 3.1-3.4) for CDR81, CDR82, CDR83 and CDR 84 have been added to the methodology part of Chapter 3 in revised thesis.	859-8892
Add a reaction scheme for the similar reaction from literature.	94	Chemical reaction scheme for Pictet-Spengler reaction between tryptophan and aldehyde has been added as shown in scheme 3.5	947
Did you check the right chemical structure database '.. Cam Spider.?'	96	Amended	958
Fig. 4.16 Wrong structure for ANI and ``VAN (Both looked like BEN).	134	Amended	1325
Para 2 Line 3-Perhaps useful to refer to chemical structure of Fig 3.4, pg. 78	154	Amended	1527
Para 2 lines 6 & 10. Need to set up equations with designated eq. numbers and define terms.	156	Amended	1547-1558
5.2.2.1.5 line 2 Insert '.. and SRM respectively..'	157	Amended	1558
Line 1-3 discrepancy with values of Table 5.1-All wrong values inserted in statement.	159	The wrong values in the discussion statement have been corrected accordingly as what	157-15860

Formatted Table

		is listed in table 4.1.	
Para Line 4 '..of MTG-related analogues (Fig 5.1).' Actually was it '… MTG and its SRM analogues…'??	160	Amended	15861

Chapter 5

Comment	Page	Correction	Page
Para 2 Either add chemical structure or mention the figures we need to refer to	153-154	The structure-activity relationship of β-carboline derivatives for anti-tumor has been has been revised to refer to Figure 1.7 on page 356 and Figure 2.87 on page 75881.	1514-1525
Explain briefly about apoptosis & necrosis.i.e. add advantage.	163	Apoptosis advantage has been added and amended.	1614
Don't over claim –"Potent lead compounds for the development of future cancer therapeutics".	163	The sentence has been modified accordingly	1614

Chapter 6

Comment	Page	Correction	Page
Revise some of the captions. Captions must be concise, specific and stand alone	164-165, 184-187	Captions have been simplified and revised accordingly.	1625-1636, 1847-18790
Fig 6.1. Draw full proper structures, relevant for pg. 181-182 discussions. Correct the structure of Fig. 6.1. The double bond missing	169	Full proper structures of CDR81, CDR82, CDR83 and CDR 84 have been drawn and shown in Figure 5.1 for the relevant discussion on structure and activity on page 1815-182.6	16770
Line 2(6.2.1) and Line 1 (6.2.3)-Insert '… and VAN respectively.'	170-171	Amended	16871, 1703
Table 6.1 use smaller font size to accommodate column	175	Corrected as shown in Table 5.1	1747
Table 6.4 Column 1-compound names?	179	Corrected as shown in Figure 5.1	17881
Para 2 Wrong chemical substituent designation? Last sentence-'…. The hydroxyl methoxy group at VAN…'??? -did you mean methoxyphenol of VAN?	182	Amended	1814
Para 3 last sentence-Explanation is unclear. Need to rewrite.	182	Amended	1814
Line 5-'.. Both compounds…' So which compounds are these?	188	Amended	18891

Efficiency based on what? Is it SI or $IC_{50.}$	194	Amended. The efficiency is based on IC_{50}	19<u>4</u>~~7~~

Chapter 7

Comment	Page	Correction	Page
Line 4. So, what is the role of those mineral acids?	198	The mineral Acids serve as catalyst.	<u>197</u>~~200~~
Last sentence, rewrite. Wrong term used?- '.. the derivatives may inherit…'	198	Amended	<u>198 (Line 14-17)</u>~~201~~
IC_{50} value exhibited by of the compounds in the antioxidant assays especially the DPPH assay indicates that they are not active. Check calculation for concentration for IC_{50} in the antioxidant assay.	212 & 213	The concentration for IC_{50} is in µM, not mM and has been modified accordingly.	21<u>3</u>~~4~~-21<u>4</u>~~6~~
Check the unit of the compound concentration.	207 & 210	Amended. It is in µM.	2<u>08, 212</u>~~10~~ & 21<u>3</u>~~3~~
Scale of DPPH activity (Figure 7.2).	211	Scale of DPPH activity has been~~d~~ modified accordingly as shown in Figure 6.2	21<u>2</u>~~4~~
Change the labeling of Figure 7.4.	215	Labeling has been changed accordingly as shown in Figure 6.4	21<u>6</u>~~8~~

Chapter 8

Comment	Page	Correction	Page
Last sentence-how has the dosage range been chosen? Add the rationale of the dose selected, meaning how the dose was calculated.	226	The rationale of the dosage range selection is based on LD_{50} of the mitragynine which is 1098 mg/Kg (Sabetghadam et al., 2013b), and according to the OECD guidelines about 10 times lower is used as ceiling limit of the testing dosage, as revised in thesis.	228~~30~~
Based on Table 8.1, pg. 229- only 3, not 5 different dose were studied as mentioned in line 13 (Pg. 227).	227 & 229	Amended	228~~32~~ & 231~~3~~

Chapter 9

Comment	Page	Correction	Page
For e.g Correlate and postulate whether R is important or not for respective activity. If R is not important for activity, which part of the molecule causes activity.	245-246	Postulation of R (Substituents at C-1) for respective activity has been revised.	a) Anti-Proliferation activity-1830-1825, 2479 b) Analgesic activity-2446-2457, 248450 c) Antioxidant activity-21920-22213, 248450
Line 1-Is this synthesis method has been done beforea novel step?	234	The word 'modified' has been added to the text. The product Synthesized (CDR81, CDR82, CDR83 and CDR 84) is new as published in Goh et al., 2015a and Goh et al., 2015b. These new products were synthesized using modified Pictet-Spengler reactions. [Please refer list of publications No. 6 and No. 10]	234-

General Discussion and Conclusion

Comment	Page	Correction	Page
General Discussion and Conclusion too long and	-	Conclusion separated from general discussion. Done.	-

repetitious, Separate the conclusion.			

References

Comment	Page	Correction	Page
Some book sources need range of page number in their bibliographies. Year of journal not to be repeated.	250, 253, 261, 267, 271, 273, 277 and 278	Amended. The page numbers of the reference book have been added.	2524, 2557, 2635, 26971, 2745, 275, 279, 2807 -and 2812
Some formatting & typing errors in bibliography and citation (ie pg. 196).	196	The errors have been corrected.	196201

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Appendices

Comment	Page	Correction	Page
Correct Appendix A3. Add the molecular structure of mitragynine	280	Corrected	2834
Appendix A5 caption a Path 9b instead of 9a for fraction 4?	281	Amended	2845

Check structure of mitragynine placed on spectra in appendices.	280,283,284	Done.	2834,2867,2878
Indicate & Clarify i.e add solvents.	284	Amended	28790
Update the list of publications	357	The list of publications has been updated accordingly.	3633

CHAPTER 1

INTRODUCTION

1.1 Chemistry of mitragynine (MTG)

Mitragyna speciosa Korth (ketum) is widely recognised in Southeast Asia as a unique medicinal plant because of its opioid-like effects. As the principal indole alkaloid from *M. speciosa*, mitragynine (Figure 1.1) is effective in the treatment of diarrhoea, intestinal infections, muscle pain and to reduce coughing (Suwanlert, 1975; Jansen and Prast, 1988a; Watanabe et al., 1997; Vicknasingam et al., 2010) although it is an illegal and controlled substance (Adkins et al., 2011). It has also been used as a tonic to improve work performance and as an alternative to opium (Burkill and Haniff, 1930; Burkill, 1935; Suwanlert, 1975; Apryani et al., 2010). Mitragynine (MTG) is the main alkaloid derived from *M. speciosa.* It has a molecular formula of $C_{23}H_{30}N_2O_4$ and a molecular weight of 398.50. Mitragynine has a melting point range of 102-106 ºC and boiling point of 230-240 ºC (Jansen and Prast, 1998). It is soluble in chloroform, alcohol and acetic acid. It has UV absorbance with lambda maximum of 254nm due to conjugated indole rings (Jansen and Prast, 1988) and is stable in plasma upon storage for at least 2 days at room temperature and up to 1 month when stored at −20 °C (Parthasarathy et al., 2010).

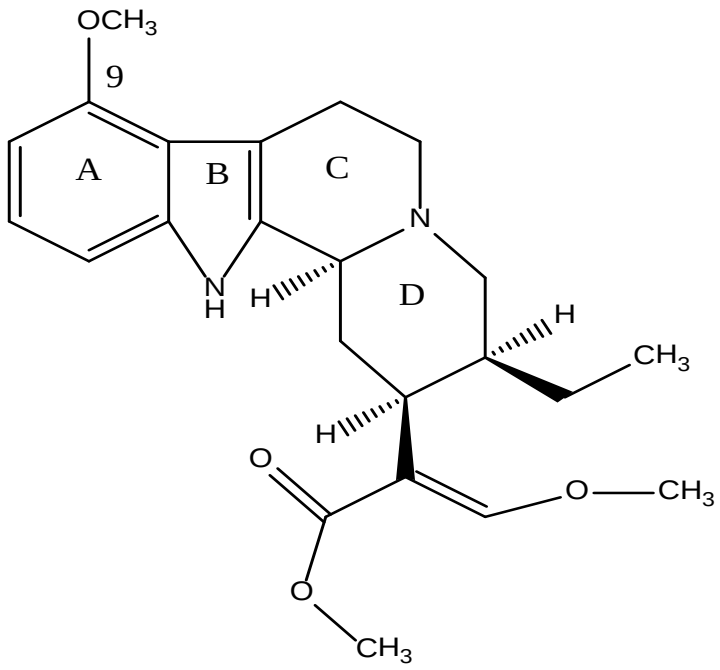


Figure 1.1 Structure of mitragynine (MTG).

1.2 Toxicological and pharmacological effects of MTG

Macko et al. (1972) found no evidence of toxicity when the evaluation measured as tremors or convulsions in dogs at doses as high as 920 mg/kg. Sabetghadam et al. (2013a) demonstrated that MTG is relatively safe at lower sub-chronic doses (1–10mg/kg) but exhibited toxicity at the highest dose of 100mg/kg (sub-chronic: 28 days) in rats. The lethal effects of 200 mg/kg total alkaloid extract of *M. speciosa* and oral dose of 200 mg MTG were reported recently in rats (Janchawee et al., 2007; Azizi et al., 2010). The acute oral LD_{50} toxicity levels in mice for methanolic and alkaloid extracts of *M. speciosa* were reported as 4.90 g/kg and 173.20 mg/kg respectively (Reanmongkol et al., 2007). In addition, Sabetghadam et al. (2013b) found that the oral lethal dosage, $LD_{50,}$ of pure MTG was 1098 mg/kg. MTG has been reported to cause less respiratory depression in animal models as compared to other narcotics (Jansen and Prast, 1988).

Sabetghadam et al. (2013a) discovered significant reduction of relative body weight and food intake in female rats when treated with 100mg/kg dose of MTG. Alterations in biochemical and hematological parameters, together with histopathological changes were observed especially in the high dose (100 mg/kg) treatment group.

Acute oral administration of standardized methanolic extract of *M. speciosa* did not affect spontaneous motor activity, or food and water consumption in rats. The methanolic extract, however, led to a significant increase in alanine transaminase (ALT) and argininosuccinate lyase (ASL) (Harizal et al., 2010; Kapp et al., 2011). Nephrotoxicity was seen only at a 1000 mg/kg dose as evidenced by elevated creatinine levels. A histological examination showed the congestion of sinusoids, hepatocyte haemorrhage, fatty change, centrilobular necrosis and increased number of Kuppfer cells in the liver. However, an acute treatment with the methanolic extract (100, 500 and 1000 mg/kg doses) did not induce damage in the axons and dendrites of hippocampal neurons (Harizal et al., 2010).

Chronic *M. speciosa* users suffered anorexia, weight loss, hyperpigmentation, psychosis, constipation, insomnia, fatigue, poor concentration and hypothyroidism probably due to its suppression effect on thyroid-stimulating hormones (Suwanlert, 1975; Vicknasingam et al., 2010). Besides, *M. speciosa* users have been reported to experience nausea, vomiting, and diarrhoea with occasional reports of nystagmus and tremor (Grewal, 1932). Roche et al. (2008) reported the case of a 32-year-old male who was found having seizure-like movements and mouth-foaming. These movements persisted despite the person being given benzodiazepine treatment and intubation.

Seizures and coma have been reported in humans when ketum extract was consumed and used whether it being for pain management, in combating opioid withdrawal symptons or as a recreational drug (Chan et al., 2007; Nelson et al., 2010; Vicknasingam et al., 2010). MTG is extensively metabolized in rat and human liver via phase I (hydrolysis and dealkylation) and II (conjugation to glucuronides and sulphates) reactions (Philipp et al., 2009). The MTG elimination half-life in rats was found to be approximately 4–9 h after a single dose (Janchawee et al., 2007). Parthasarathy et al. (2010) found that MTG has a mean half-life of 2.9±2.1 h after intravenous administration. Furthermore, Parthasarathy et al. (2010) has shown MTG has poor calculated absolute oral bioavailability of merely 3.03 % probably due to its poor aqueous solubility. Seizures were observed at very low doses (10 mg/kg) whilst convulsions were evident at a very high dosage (920 mg/kg) (Reanmongkol et al., 2007).

MTG demonstrated cytotoxic effects *in vitro* in human neuronal cells but toxicity could be lessened by using naloxone, an opioid antagonist. However, no genotoxicity was found in the mouse lymphoma gene mutation assay (Saidin et al., 2008; Saidin, 2008). There was also no mutagenic activity using the Ames test in the presence and absence of metabolic activator S9 systems (Ghazali et al., 2011). The aqueous *M. speciosa* extract shows strong antimutagenic properties.

In summary, the various case studies on animals and humans suggest potentially toxic and lethal effects of *M. speciosa* preparations. The reasons are either due to long term consumption and accumulating effects or an acute overdose (Hassan et al., 2013). Therefore, the need for new analogues which possess good pharmacological activity and less toxicity has arisen and this is one of the rationales of the study in this thesis.

1.3 Other indole alkaloids of mitragynine (MTG)

The total alkaloid content in the *M. speciosa* leaves was in the range of 0.5% - 1.5% (Shellard, 1974). Mitragynaline, corynantheidaline, mitragynalinic acid and corynantheidalinic acid were discovered as new types of alkaloidsfrom Malaysian *M. speciosa* (Houghton et al., 1991). Other minor constituents found in mature leaves included mitragynaline, pinoresinol, mitralactonal, mitrasulgynine, 9-methoxymitralactonine and mitralactonine and 3, 4, 5, 6-tetradehydromitragynine (Takayama et al., 1998). Nine corynanthe-type indole alkaloids namely MTG, speciogynine, speciociliatine, paynantheine, 7-hydroxymitragynine, mitragynaline, corynantheidaline, corynantheidine and isocorynoxeine were isolated from the leaves (Takayama et al., 2004) (Figure 1.2). 7-Hydroxyspeciociliatine was another indole alkaloid found in *M. speciosa* fruits (Kitajima et al., 2007). Two major metabolites: mitragynine pseudoindoxyl and 7-hydroxymitragynine, were obtained from microbial transformation (Zarembo et al., 1974) (Figure 1.2).

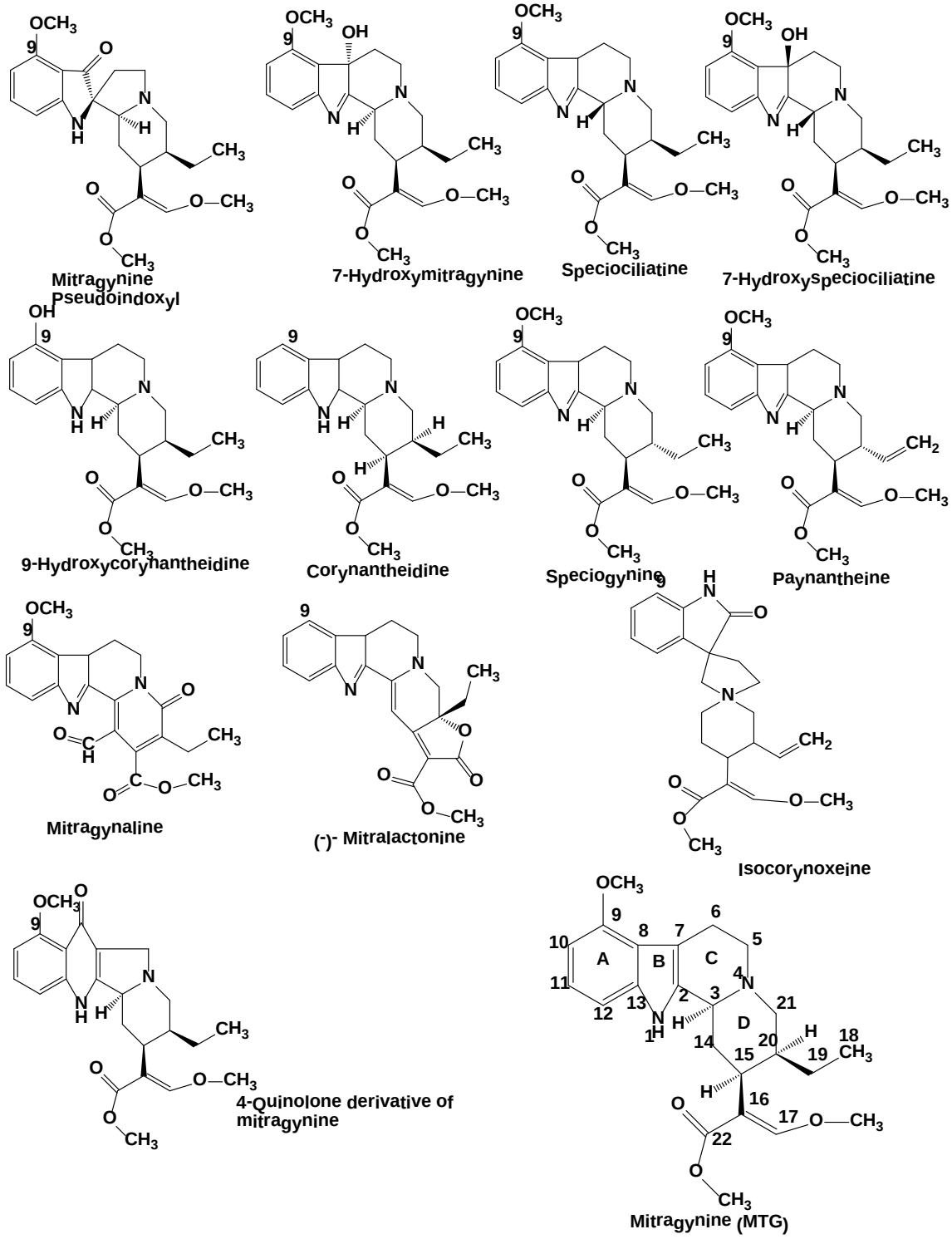


Figure 1.2 Mitragynine [(E)-methyl 2-(3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxyacrylate] related indole alkaloid.

Major indole alkaloids of young leaves of Thai *M. speciosa* are MTG (66%), paynantheine (9%), speciogynine (7%), 7-hydroxmitragynine (2%), and speciociliatine (1%) in which the relative composition varies greatly according to the geographical region and the season (Takayama et al., 2004) (Figure 1.2). Of the above mentioned alkaloids, MTG, paynantheidine, speciogynine, corynantheidine and 7-hydroxmitragynine are known to be pharmacologically active (Takayama et al., 1998; Takayama et al., 2002; Matsumoto et al., 2004; Takayama et al., 2004). MTG possesses analgesic, antitussive, antidiarrheal, adrenergic and antimalarial activities whilst paynatheidine and speciogynine act as smooth muscle relaxants. With 66 % of the total alkaloid mixture, MTG is the main active alkaloid present in *M. speciosa* leaves that triggers opioid agonistic activity though it may not be the most potent psychoactive component when compared to other species (Grewal , 1932; Suwanlert, 1975; Shellard, 1989; Takayama et al., 2002; Takayama et al., 2004).

1.4 Structure and opioid agonist activity of mitragynine-related indole derivatives

Opioid agonistic activity is defined as the inhibition of the twitch contraction, which is reversed by the opioid antagonist naloxone. The opioid agonistic activities of the natural analogues of MTG and semisynthetic MTG derivatives were evaluated in guinea pig ileum contraction induced by electrical stimulation. MTG inhibited the twitch contraction, induced by electrical stimulation in a concentration-dependent manner, which was reversed by the addition of naloxone. However, the potency was one-fourth that of morphine.

Takayama et al. (2002) found that corynantheidine (Figure 1.2) without a methoxy group at C-9 position (9-demethoxy analogues of MTG) lost agonistic activity in guinea pig ileum preparation when investigating the relationship between opioid agonist activity and the structure of indole alkaloids. This finding has indicated that the methoxy group on C-9 in the MTG indole ring is essential for eliciting analgesic activity (Takayama et al., 2002). Corynantheidine did not show any opioid agonistic activity but had reversed the morphine-inhibited twitch contraction in guinea pig ileum. Takayama et al. (2002) later discovered that corynantheidine has a selective opioid antagonistic property on μ-opioid receptors based on receptor binding assays. Its antagonistic effect was in a concentration-dependent manner. These results suggest that corynantheidine inhibits the effect of morphine through functional antagonism of opioid receptors. Another analogue of MTG, 9-hydroxycorynantheidine (Figure 1.2), demonstrated inhibition on electrically induced twitch contraction in guinea pig ileum, but its percentage of maximum inhibition was less than that of MTG indicating lesser potency (Takayama et al., 2002). Thus, it is suggested that 9-hydroxycorynantheidne is a partial agonist of opioid μ-receptors based on receptor binding assay. Put together, it is an interesting finding showing a shift in activity from full agonist via partial agonist to that of an antagonist on opioid receptors when there is a fine transformation of the functional group on C-9 from OCH_3 (methoxy) to OH (hydroxy) or to H. Hence, it is found that the functional group on C-9 of mitragynine-related compounds determines the relative opioid agonistic activity (Takayama et al., 2002). On the other hand, the replacement of the methoxy functional group by the ethoxy and propoxy groups at C-9 on the indole ring resulted in the reduction in both intrinsic opioid agonistic activity and the potency

as compared with those of MTG (Takayama et al., 2002). These results suggest that the methoxy group at the C-9 position determines its intrinsic compound activities on the opioid receptors and is the most suitable functional group for pharmacophore binding to opioid receptors (Takayama et al., 2002). In summary, although MTG derivatives possess basic skeleton which are completely different from that of morphine, they exhibit potent agonistic properties on opioid receptors in guinea pig ileum. The structural functions of methoxy group at position C-9 of the indole ring of the Corynanthe type indole alkaloid skeleton essentially contribute to the opioid agonistic activity (Takayama et al., 2002). By altering the functional group at the C-9 position, e.g., OCH_3 > OH > H, of MTG, the activities of the compounds dramatically shifted from that of full agonists through partial agonists to that of antagonists on opioid receptors (Takayama et al., 2002).

Speciociliatine (Figure 1.2), which is a minor constituent of *M. speciosa*, is the C-3 stereoisomer of MTG which takes a folded cis-quinolizidine conformation in the C/D-ring junction. The potency of this compound towards opioid receptors was 14-fold weaker than that of MTG in the guinea pig ileum test, indicating that the flat trans-quinolizidine form of MTG was a more efficient conformation for exhibiting opioid agonist activity than the folded cis form of speciociliatine. Besides, the 4-quinolone derivative (Figure 1.2) retained almost the same opioid agonistic activity as that of MTG (Takayama et al., 2002) in the guinea pig ileum test.

The oxidized derivatives of MTG (mitragynine pseudoindoxyl and 7-hydroxymitrgynine) (Figure 1.2), exhibited a higher opioid agonist potency than MTG in the guinea pig ileum contraction test. The 7-hydroxymitragynine which showed full

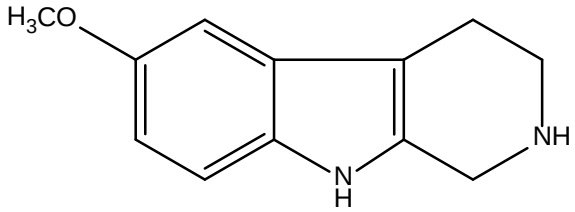
agonistic intrinsic activity on opioid receptors was found to exhibit approximately 13 and 46 times higher potency than those of morphine and MTG respectively. The introduction of a hydroxyl group at the C-7 position led to higher potency as compared with that of MTG. 7-hydroxymitragynine tended to show higher selectivity and affinity for μ receptors than that of MTG. However, the introduction of a methoxy or an ethoxy group at the C-7 position led to a dramatic reduction in both intrinsic activity and potency for opioid receptors. It is concluded that the presence of the hydroxyl functional group at the C-7 position in MTG is essential for its improved potency towards opioid receptors.

1.5 ß-carbolines derivatives

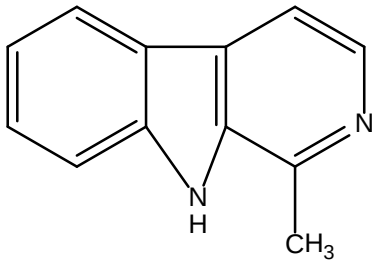
The *de novo* synthesis of MTG suffered the setback of having too many reaction steps (more than 22) and low yields (3-13 %) (Takayama et al., 1995; Ma et al., 2007; Ma et al., 2009; Isabel, 2012). In addition, it requires a laborious purification process in order to obtain pure MTG. Therefore, total synthesis of mitragynine for further structure modification to obtain new analogues is not the objective of this thesis. 6-Methoxy-tetrahydro-β-carboline derivatives (Figure 1.3a) were chosen as targeted synthesized compounds since they resemble intermediate products in the total synthesis of MTG, *trans*-tetrahydro-β-carboline, as shown in Figure 1.3b (Takayama et al., 1995; Ma et al., 2007; Ma et al., 2009, Isabel, 2012). Furthermore, it is interesting to explore whether tetrahydro-β-carboline compounds possess similar analgesic or other useful biological activities as for mitragynine-related compounds. The Pictet-Spengler reaction mediated cyclization which served as an important key step in the total synthesis of MTG, is used

with modification, for the synthesis of 6-methoxy-tetrahydro-β-carboline in this study. The synthesis in this thesis for 6-methoxy-tetrahydro-β-carboline derivatives envisions much simpler reaction schemes with just a one pot reaction step. Therefore, the product yields are expected to be higher compared to that of the reaction involving many steps (Takayama et al., 1995; Ma et al., 2007; Ma et al., 2009, Isabel, 2012).

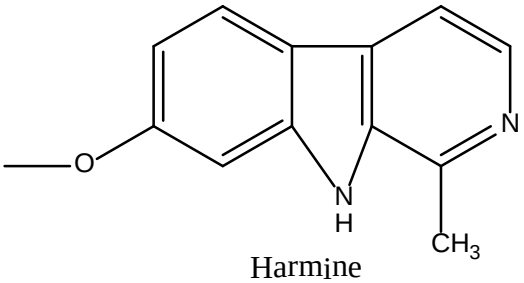
Tetrahydro-ß-carbolines (tetrahydro-pyrido(3,4-b)indole) and ß-carbolines (pyrido(3,4-b)indole) are naturally-occurring indole alkaloids with a β-carboline nucleus (Figure 1.3a and Figure 1.3b). They exhibit a broad range of potent pharmacological and biological activities (Patel et al., 2012). The reported pharmacological effects of this class of compounds comprise of antitumor, antiparasitic, antineoplastic (tubulin binding), anticonvulsive, hypnotic and anxiolytic, antiviral, antimicrobial as well as topoisomerase-II inhibition, cGMP-dependent processes inhibition and antiplasmodial activity (Hamsa et al., 2011,; Frost et al., 2011; Patel et. al., 2011). They may have other endocrinological functions especially 6-methoxytetrahydro-β-carboline derivatives (e.g. pinoline, an example of endogeneous β-carboline), which will be synthesized and studied in this thesis.



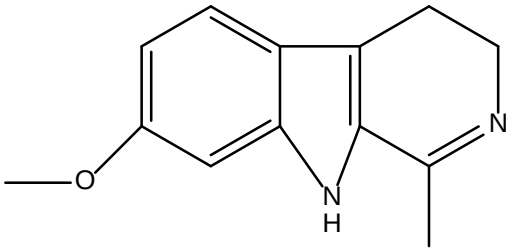
Pinoline (Endogeneous β-carboline)



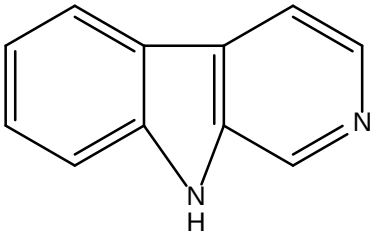
Harman



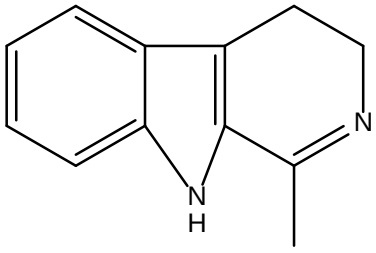
Harmine



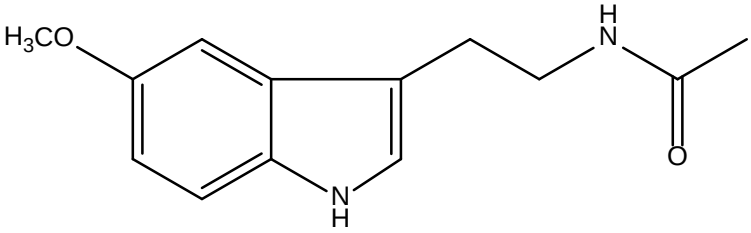
Harmaline



Norharman

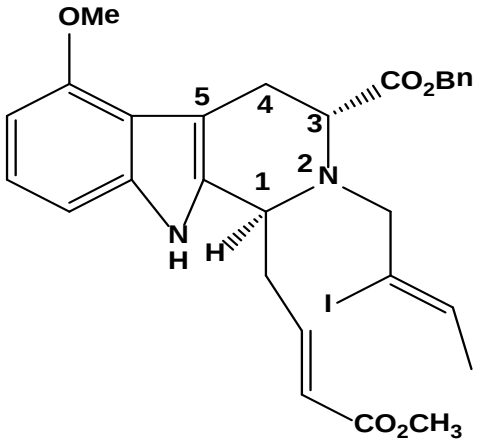


Harmalan

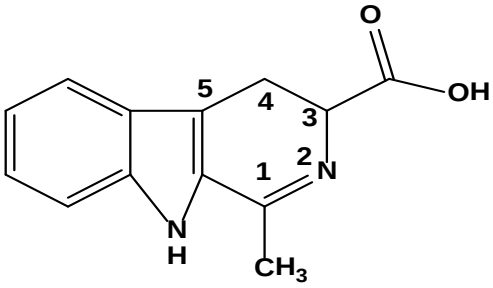


Melatonine (Endogeneous β-carboline)

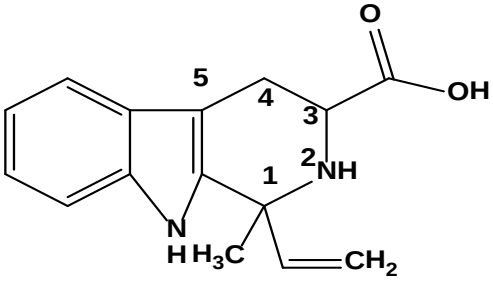
Figure 1.3a Structures and common name of some β-carbolines.



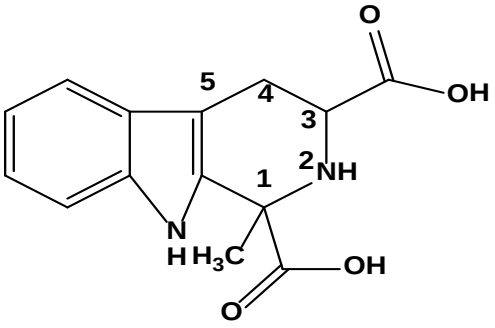
trans-tetrahydro-b-carboline (a,b-unsaturated ester), Key intermediate in mitragynine total synthesis



1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid



1-methyl-2,3,4-tetrahydro-b-carboline-1-vinyl-3-carboxylic acid



1-methyl-2,3,4-tetrahydro-b-carboline-1,3-dicarboxylic acid

Figure 1.3b Structures and IUPAC Scientific name of some β-carbolines.

On the other hand, tetrahydro-ß-carbolines (tetrahydro-pyrido (3, 4-b)-indole) such as harmine and harmaline are central nervous system stimulants and act as selective but reversible inhibitors of MAO-A (RIMAs) (Santos et al., 2011; Santos et al., 2012) that are similar to tyramine-containing foods, as compared to synthetic MAOIs such as phenelzine (Pardo et al., 2012). RIMAs help to replenish the body's supply of monoamines by slowing the breakdown of neurotransmitter and are used as antidepressants. Some examples are moclobemide, tranylcypromine, selegiline and phenelzine. The monoamines include neurotransmitters (serotonin, dopamine) and hormones (melatonin) (Santos et al., 2011; Santos et al., 2012). Harmine and harmaline can be safely consumed together with foods containing tyramine such as wine and aged cheese since they are RIMAs and they do not bind permanently to MAO (Pardo et al., 2012). Therefore, tyramine would be competing with harmaline for binding site on the MAO-A enzyme. Harmala alkaloids (alkaloids from *Perganum harmala* including harmine and harmaline) have been used to treat Parkinson's disease and essential tremor since they are benzodiazepine inverse agonist (Sourkes, 1999).

The BZRs (benzodiazepine receptors) in mammalian CNS (central nervous system) are known to mediate anxiolytic, anticonvulsant and sedative action. ß-carboline alkaloids are non-benzodiazepine antagonists that have been discovered to bind with high affinity to the BZRs and shown to antagonize the principle pharmacological actions of benzodiazepine. For example, 3-(ethoxycarbonyl)-ß-carboline and 3-(methoxycarbonyl)-ß-carboline are benzodiazephine antagonists that block the BZRs and exhibit opposite effects as to those of benzodiazepines in various animal behaviour models (Cao et al., 2007). Although pinoline (an endogeneous β-carboline) does not

have any affinity for the BZRs, significant anxiogenic, anticonvulsive and antidepressant properties of pinoline have also been found in some animal models at pharmacological dosage. These findings have suggested that the neuropharmacological effects of pinoline involve non-BZRs interaction mechanisms (Mcisaac, 1961; Mcissaac et al., 1961; Squires et al., 2004).

ß-carbolines molecules cause various side effects on the CNS including hallucinations, tremors, anxiety, anxiolytic, convulsions, anticonvulsant and sedation (Riba et al., 2001). Oral or intravenous harmine doses ranging from 30–300 mg have been reported to cause agitation, tachycardia, blurred vision and hallucinations. These pharmacological effects are partially due to the interaction of the ß-carboline molecules with various receptor systems in the mammalian CNS, such as 5-HT receptors and BZRs (Giorgio et al.,2004; Cao et al., 2007). Both harmine and harmaline have been proven to act as acetyl cholinesterase inhibitors that stimulate striatal dopamine secretion in rats at high dose, thereby causing hallucinogenic effects in humans (Patel et al., 2012, Geyer and Franz, 2012). However, a lower dose (25-50 mg) indicates CNS stimulation, increases mental activity and produces a pleasant dreamy state for several hours. Whereas a higher doses (200-750 mg) cause the hallucinogenic effects. Harmine also causes some hallucinogenic effects at a dose of 150-200 mg via intravenous administration in humans (Riba et al., 2001; Cao et al., 2007; Santos et al., 2011; Santos et al., 2012). However, harmaline has been found to be hallucinogenic at doses greater than 1mg/kg after intravenous administration. In fact, harmaline is also orally active at a dose of 4mg/kg. Tetrahydroharmine has been reported to induce hallucinogenic effects at 300mg (Cao et al., 2007).

In addition to this, 6-methoxyharmalan produces hallucinogenic effects and induces modest psychoactive action at oral doses of 1.5mg/kg (Cao et al., 2007; Geyer and Franz, 2012). The harmala alkaloids are psychoactive in humans at oral doses of 25-750 mg. Noticeable classic hallucinogens are thought to produce psychoactive effects via interaction with 5-HT2 serotonin receptors in the brain (Cao et al., 2007). However, so far, it has been inconclusive as to whether the ß-carboline alkaloids elicite hallucinogenic actions in a similar way to classical hallucinogen. It seems that the 6-methoxyl moiety contributes to hallucinogenic effects of the compounds and higher conjugation in the tricyclic rings produces higher hallucinogenic effects (Geyer and Franz, 2012).

Interestingly, ß-carboline alkaloids have been demonstrated to play a crucial role in the processes of substance abuse and dependence (Cao et al., 2007). Cappendijk et al. (1993b) has reported that norharman produces prominent inhibitory effects on the naloxone-precipitated withdrawal syndrome in morphine dependent rats. Besides, high plasma levels of harman and norharman have been found in chronic alcoholics and heroin dependent humans. Chronic infusion of harman increases voluntary ethanol intake in rats and rapid discontinuation of these MAO inhibitors can cause a serious withdrawal syndrome. Aricioglu-Kartal et al. (2003) has reported that harman and harmine have some beneficial effects on naloxone-precipitated withdrawal syndrome in rats, and harmine is more effective than harman in reducing the signs of morphine withdrawal syndrome. The use of β-carbolines has an advantage over the conventional treatment approach based on the alleviation of drug withdrawal symptoms (Howard et al., 1997). This group of compounds provides an alternative and effective approach to

interrupting and attenuating the consumption and craving for drugs such as heroin, fentanyl, methadone, codeine, opium, nicotine, amphetamine, caffeine and their combinations which upon ingestion or administration, leads to addiction. These have been shown to be intractable to treatment by other reagents (Howard et al., 1997).

Currently, researches have confirmed the effects of numerous ß-carboline and tetrahydro-ß-carbolines alkaloids of norharman, harman, harmine, harmaline, harmalan on the CNS and their affinity with BZRs and 5-HT receptors (Cappendijk et al., 1993a; Cappendijk et al., 1993b; Yin et al., 2010). They are able to bind to GABA and I2 (Imidazoline) receptors. These compounds might function as mild neuromodulators via inhibition effects on MAO, monoamine uptake and benzodiazepine receptor binding. Simultaneously, they have been increasingly studied in relation to alcoholism, where they might play a role in alcohol addiction either in etiological or in pathological states (Cappendijk et al., 1993a; Yin et al., 2010). Harmaline causes no known physical or psychological dependence. Howard et al. (1997) describes a method for treating various chemical dependencies via the administration of harmaline and other ß-carbolines. Surprisingly, it has been discovered that the β-carboline alkaloids are effective in the treatment of chemical dependency disorders, abuse syndromes or their combination in mammals (Howard et al., 1997).

Collins and his co-workers (1985 and 2009) have reported that *N*-methylated tetrahydro-ß-carbolines and ß-carbolines can be bioactivated to give endogenous neurotoxins. 1-Methyl-1,2,3,4-tetrahydro-*ß*-carboline-3-carboxylic acid is a precursor of mutagenic *N*-nitroso compounds and shows cytogenetic effects, and can cause neuronal cell death. Some ß-carbolines might act as co-mutagenic substances, precursors of *N*-

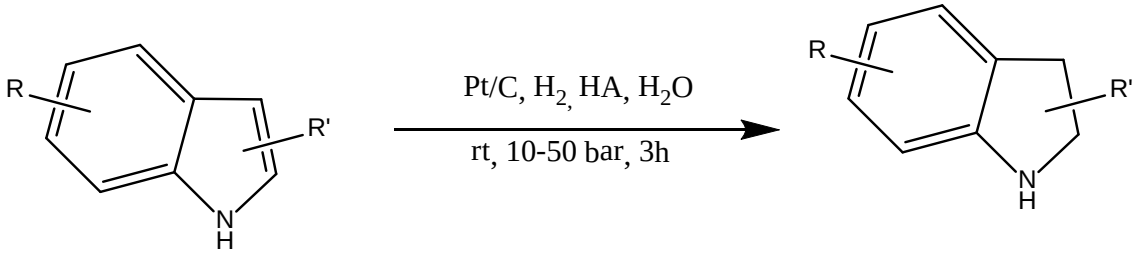
nitroso compounds, and toxic compounds (Jing et al., 2014). Harmaline has both protective and toxic effects on neurons (Lee et al., 2000). A single injection of harmaline at 40 mg/kg in rats has very visible neurotoxic effects. The prototypic β-carboline, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid detected in some food items, is the possible causative substance of the eosinophilia-myalgic syndrome (Pari et al., 2000). Some of the β-carbolines have been shown to be phototoxic to bacteria and insects and this might correlate with their ability to produce reactive oxygen species upon irradiation (Richard et al., 2008). ß-carbolines are DNA intercalating agents and DNA enzymes inhibitors which exhibit toxic and mutagenic effects either in prokaryotic or eukaryotic cells (Pari et al., 2000).

1.6 Synthesis

1.6.1 Reduction of indole to indoline

Lloyd and Gordon (2009) developed an efficient method of chemically reducing indole to indoline by using zinc dust and 85% phosphoric acid without polymerization. It has been reported that an efficient, palladium-catalyzed reduction of *N*-(tert-butoxycarbonyl) indoles gives *N*-(tert-butoxycarbonyl) indolines in good yields, in the presence of polymethylhydrosiloxane (PMHS) as a reducing agent at room temperature (Chandrasekhar et al., 2006). A Brønsted acid catalyzed the process of transfer hydrogenation of indole derivatives with Hantzsch dihydropyridine as the hydrogen source enables an efficient synthesis of various optically active indolines with high enantioselectivities (Rueping and Antonchik, 2010). Sodium borohydride ($NaBH_4$) in neat carboxylic acids sequentially reduces the indole double bond and alkylates nitrogen

atoms to produce *N*-alkylindolines (Gribble, 1998). An environmentally benign procedure for the hydrogenation of unprotected indoles is described by Aditya et al. (2011). The hydrogenation reaction is catalyzed by platinum on activated carbon and accelerated by p-toluenesulfonic acid in water as a solvent. The efficacy of the method is illustrated by the hydrogenation of a variety of substituted indoles to their corresponding indolines which were obtained in excellent yields (Scheme 1.1).



Scheme 1.1 Reduction of indole to indoline using platinum on activated charcoal as catalyst.

Borane-tetrahydrofuran (THF) in trifluoroacetic acid produces acid-stable $H[OC(O)CF_3]_2$.THF and constitutes a convenient, rapid, high yield method for the selective reduction of indoles to indoline in the presence of other functional groups (Bruce and David, 1978). Sodium cyanoborohydride in acetic acid or sodium borohydride in trifluoroacetic acid only reduces the double bond of indole to give indoline (Yatendra and Lennart, 1983). Tetrakis (dimethylamino) ethylene (TDAE), has been exploited for the first time as a mild reagent for the reduction of arenediazonium salts to aryl radical intermediates through a single electron transfer (SET) pathway. Cyclization of the aryl radicals produced in this way has led, in appropriate substrates, to the synthesis of indolines and indoles. There is also a report regarding cascade radical cyclizations of aryl radicals that are derived from arenediazonium salts. The relative ease of removal of the oxidized by-products of TDAE from the reaction mixture makes

the methodology synthetically attractive (Mohan et al., 2009). A method of reducing indole compounds to the corresponding indoline compounds substantially to be free of undesirable side reactions involves contacting an indole compound with a borane complex reagent, dioxyborane, in the presence of trifluoroacetic acid. This method is instantaneously and readily carried out and provides an excellent method for preparing certain indoline compounds from the corresponding indole compounds.

1.6.2 Total synthesis of tetrahydro-β-carbolines via Pictet-Spengler reaction

Modification of the Pictet-Spengler reaction since its invention in 1911 has provided better enantiomeric purity, diastereo-selectivity, simplicity, shorter reaction time and higher yields. The modification process also includes the use of different types of catalysts, reactants, reaction mediums and conditions (Pedro et al., 1990; Mark and Eric, 2004; Tschantz, 2012; Paresh et al., 2013). Bing and Anthony (2002) have designed a modified microwave method using solid wang resin with 1 % TFA in toluene as a catalyst to shorten the reaction time at room temperature significantly from days to minutes with a high yield (> 80 %). Furthermore, tetrahydro-ß-carboline exhibits a broad spectrum of potent biological activities (4). Therefore, a simple and mild Pictet-Spengler procedure has been designed to produce the 6-methoxy-tetrahydro-ß-carboline which contains no carbonyl group.

The Pictet–Spengler reaction, as depicted in Figure 1.4, is a special case of Mannich reaction in which a β-aryl ethylamine, such as tryptamine, undergoes condensation and ring closure with either an aldehyde or ketone (Rodolfo et al., 2013; Agarwal et al., 2013) using acidic catalyst at room or higher temperature (Pirc et al.,

2011; Devesh et al., 2013). Amé Pictet and Theodor Spengler had, in 1911, reported the discovery of this reaction when they isolated 1-methyl-1, 2, 3, 4-tetrahydroisoquinoline from the cyclo condensation of β-phenethylamine with formaldehyde dimethyl acetal in the presence of hydrochloric acid. The reaction was later modified to accept other β-phenethylamines such as *N*-alkyl, *N*-acyl and *N*-sulfonyl derivatives, proceeding via iminium, *N*-alkyliminium, *N*-acyliminium or *N*-sulfonyliminium ion formation, respectively (Larghi et al., 2005; Benjamin et al., 2006).

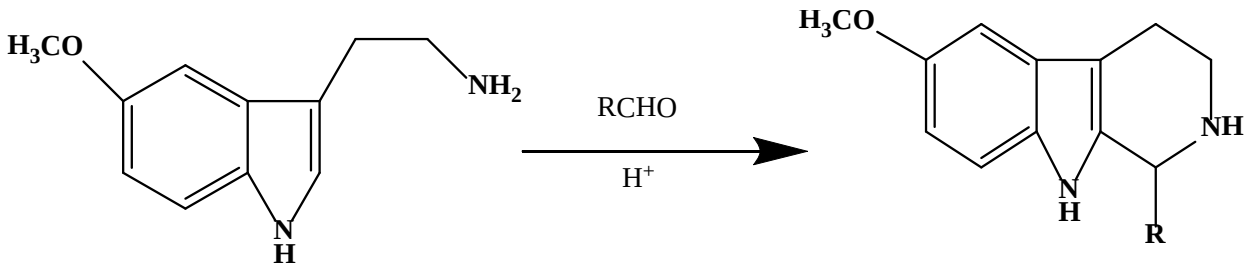


Figure 1.4 Pictet-Spengler Reaction.

Nucleophilic indole or pyrrole rings give products with good yields under mild conditions, whereas less nucleophilic phenylic aromatic rings give poor yields despite the use of higher temperature and stronger acid presence. The original Pictet–Spengler reaction refers to the reaction of β-phenethylamine with dimethyl acetal of formaldehyde using hydrochloric acid as the catalyst to produce tetrahydro isoquinoline (Larghi et al., 2005; Benjamin et al., 2006). Pictet-Spengler reaction tends to show similar trends in reaction rate as the Mannich reaction where the aldehydes give better yields than the ketones. Currently, this reaction has been

successfully applied in solid-phase combinatorial chemistry (Nielsen et al., 2003; Nielsen et al., 2005).

1.6.2.1 Mechanism of Pictet-Spengler reaction

The Pictet-Spengler condensation reaction mechanism to form tetrahydro-β-carboline is characterised by its initial formation of an iminium intermediate ion (4) after the acid catalyses the reaction of a tryptamine derivative and an aldehyde (Figure 1.5) (Larghi et al., 2005). This is followed by 6-*endo* intramolecular cyclization of the imine and electrophilic substitution at the indole double bond (4). After de-protonation (5), the desired product (6) is formed (Larghi et al., 2005). It is the electrophilic imine double bond that drive through the cyclization. The reaction mechanism shown in Figure 1.5 is an example of a 6-endo-trig reaction, which is favoured by Baldwin's ring closure rules.

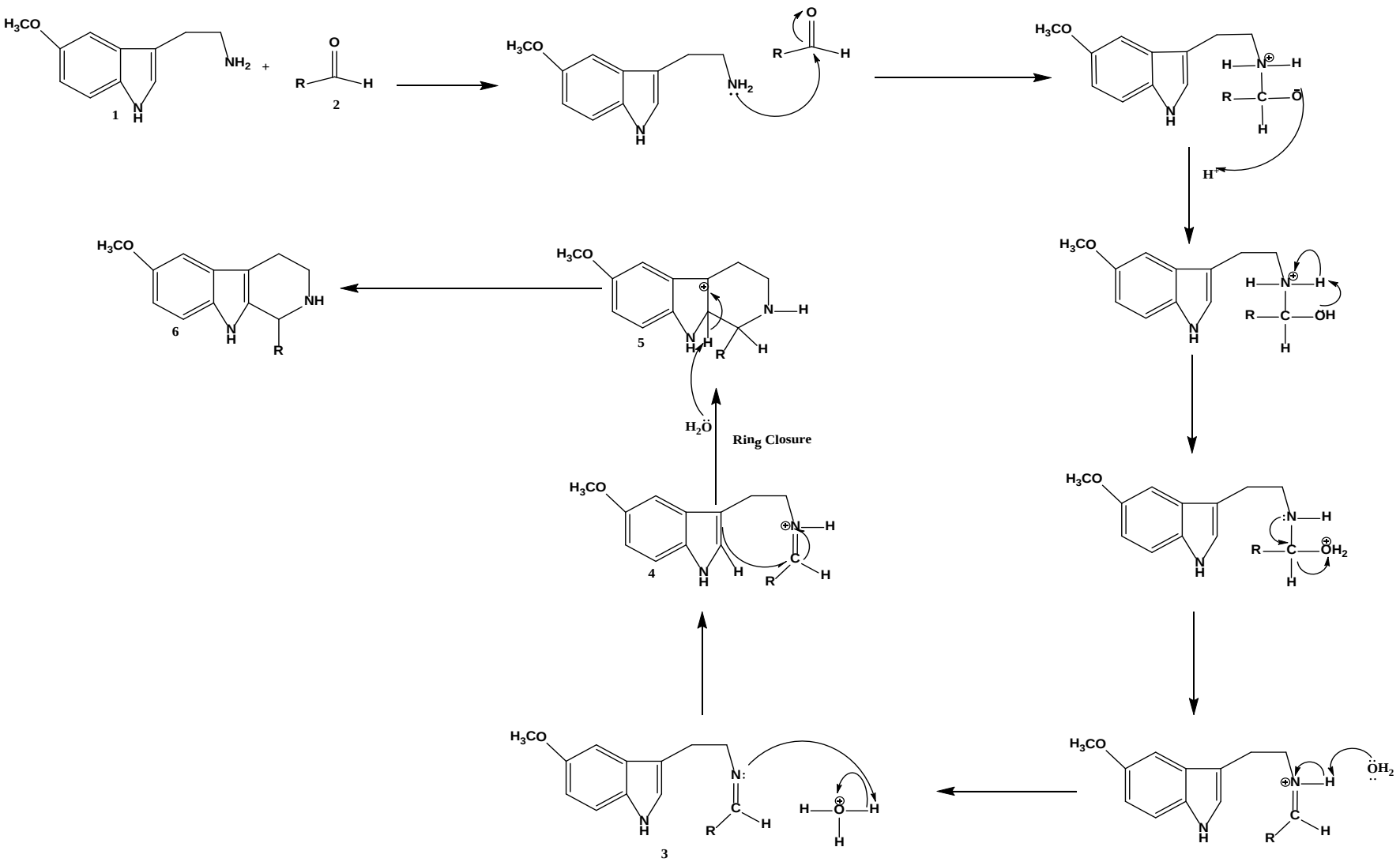


Figure 1.5 Mechanism of Pictet-Spengler reaction.

1.6.2.2 Water as an effective medium of Pictet-Spengler reactions

The Pictet–Spengler reaction strategy in water medium for C–C bond forming would contribute greatly to the development of an environmentally friendly process since it is cheap, readily available, and nontoxic compared to organic solvents. A recent review on C–C bond formation showed a significant improvement of the reaction rate in aqueous conditions which has been attributed to solvent polarity, hydration and hydrogen bonding. Condensation reaction of an amine and an aldehyde to give tetrahydro-β-carbolines in water with an acid catalyst is a mild, convenient and simple procedure and indicates the new variants of modified Pictet-Spengler reactions. Saha et al. (2007) discovered that in 10% TFA–water, aryl aldehydes have either electron-withdrawing or donating groups that undergo the Pictet–Spengler reaction with L-tryptophan or tryptamine to furnish *endo* cyclized products in good yields in comparison to 10% TFA–dichloromethane or *p*-TsOH–toluene.

Unlike the traditional Pictet–Spengler protocol which involves aprotic solvents wherein aldehydes bearing electron-donating substituents have failed to undergo cyclization and furnished imines as the only product. This has been attributed to the decrease in the reactivity of the iminium ion intermediate in the aprotic solvent which in turn prevented the formation of tetrahydro-β-carbolines. Thus, water, probably due to its unique abilities such as hydrogen bonding and high dielectric constant, appears to be a more efficient medium than toluene or dichloromethane in promoting the Pictet-Spengler reaction (Saha et al., 2007). The driving force for *endo* cyclization, contributed by the electrophilicity of the aldiminium ions derived from aryl aldehydes, appears to be enhanced in water. Furthermore, the Pictet–Spengler reaction in water exhibited better