University of Wollongong
Research Online

University of Wollongong Thesis Collection

University of Wollongong Thesis Collections

2004

Bioactive alkaloids from medicinal plants of Bhutan

Phurpa Wangchuk
University of Wollongong

Recommended Citation

Wangchuk, Phurpa, Bioactive alkaloids from medicinal plants of Bhutan, M.Sc. thesis, Department of Chemistry, University of Wollongong, 2004. http://ro.uow.edu.au/theses/315

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Bioactive Alkaloids from Medicinal Plants of Bhutan

A Thesis Submitted in Fulfillment of the Requirements for the Award of the Degree

Master of Science (Research)

From

The University of Wollongong

By

Phurpa Wangchuk, B.Sc.(Gen.), A.M.C.

Supervisor: Prof. John B. Bremner

Department of Chemistry

University of Wollongong, Australia

June 2004

Declaration

The ideas and the materials of this thesis, submitted in fulfillment of the requirements for the award of the Master of Science by Research (Medicinal Chemistry) from the University of Wollongong, are entirely of my own work and creation unless otherwise referenced or acknowledged.

Phurpa Wangchuk

18 June 2004

Publications

1. Bioactive alkaloids from the Bhutanese medicinal plant *Aconitum orochryseum* Stapf. Three new C_{20} diterpenoid alkaloids (*Journal of Natural Products*, in preparation).
2. Isoquinoline alkaloids, as novel antimalarial leads from the Bhutanese medicinal plant, *Corydalis gerdae* Fedde (*Planta Medica*, in Preparation).

Acknowledgements

I am highly obliged to my government for giving this M.Sc. scholarship. My unfathomable gratitude owes to Prof. John B. Bremner for being my supervisor without whom this work would have been a far distant dream. I am grateful to Dr. Paul Keller for initially coordinating my studies; Dr. John Korth and Larry Hick for training me to use the mass spectrometer and running my samples for GCMS and HRMS; Dr. Wilford Lee and Dr. Sandra Chapman for giving me NMR training and running experiments on the 500 MHz NMR; Dr. David Perkins for helping me with crystal growing and computer modeling of the structures for my alkaloids; Dr. Chris Bourke, Orange Agriculture Institute, Orange, NSW; Dr. Sumalee Kamchonwongpaisan and Ms. Roonglawan Rattanajak, Protein-Ligand Engineering and Antimalarial Screening Laboratories, National Science and Technology Development Agency, Bangkok, for conducting the antimalarial testing for my samples and Amrad, Melbourne for carrying out the antibacterial testing for my samples.

My sincere thanks goes to Mr. Bill Hawkins for being a good office mate and helping a friend in need especially in initializing my first experiment; Mrs. Siritron for providing me her research skills and encouragement; Ms. Waya for helping on the preparation of preparative plates; Mr. Joseph Ambrus for proofreading my thesis; Mr. James for his productive discussion on structure elucidation; Miss Jane and Mr. Johana for their company and to all my fantastic and helpful friends in the Bremner group. I wouldn't have learnt so much had these people not been there for me.

My utmost thanks also goes to the people working in the Institute for Traditional Medicines Services, especially Dungtshos, Mr. Dorji Thinlay and the medicinal plant collection team from the Pharmaceutical and Research Unit of Bhutan for their belief, faith and earnest

support rendered during the time of my studies in the University of Wollongong. Having said this, it won't be justified if Mr. Samten of Pharmaceutical and Research Unit is not brought into the limelight of this success story. He duly did the entire methanol extractions and supplied me the necessary ethno-medical information to which I owe him my heartfelt gratitude.

Lastly, I thank my wife Mrs. Sonam Choden for her evergreen love, care and constant support through out the study and the Bhutanese friends in the University of Wollongong especially Mr. Tshewang Dorji, Mr. Ugyen Dorji, Mr. Khampa Tshering and Mr. Sonam Dukda for their genuine friendship.

List of Abbreviations

AMRAD	Australian Medical Research And Development
bs	Broad singlet
^{13}C-NMR	Carbon Nuclear Magnetic Resonance
CNS	Central Nervous System
COSY	Correlation Spectroscopy
d	Doublet
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DNA	Deoxynucleic Acid
FDA	Fluorescein Diacetate Assay
GCMS	Gas Chromatography Mass Spectrometer
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Correlation
HRCIMS	High Resolution Chemical Ionisation Mass Spectrometry
HREIMS	High Resolution Electron Impact Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
HTS	High Throughput Screening
Hz	Hertz
IC_{50}	Inhibitory Concentration with 50% inhibition
IR	Infrared
LRCIMS	Low Resolution Chemical Ionization Mass Spectrometry
LREIMS	Low Resolution Electron Impact Mass Spectrometry

M	Multiplet
MeOH	Methanol
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
NITM	National Institute of Traditional Medicine
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
ppm	parts per million
PTLC	Preparatory Thin Layer Chromatography
q	Quartet
Q	Quaternary
s	singlet
STD	Sexually Transmitted Disease
TLC	Thin Layer Chromatography
TOCSY	Total Correlation Spectroscopy
US	United States
UV	Ultraviolet
VRE	Vancomycin Resistant Enterococcus
WHO	World Health Organization

Abstract

Natural products had been indispensably used by many cultures and traditions in folklore medicines for thousands of years. These traditional medicines cater to about 85% of the world population for their primary health care needs. Natural products have been intensively explored also for their bioactive pharmacophores by modern pharmaceutical companies. In fact they are the skeletal framework of about 60% of the modern drugs that are available today. Of these, about 80% of antibacterial drugs and 90% of antimalarial drugs are natural derivatives. One of the interesting secondary metabolite groups which provides these potent drug leads are the alkaloids. Owing to their broad range of bioactivities, bioprospecting for these natural products are undertaken in many countries. In this study, on the molecular basis of traditional medicine of Bhutan, particularly those with potential antimicrobial components, four alkaloid positive medicinal plants, i.e. *Aconitum orochryseum* Stapf, *Corydalis gerdae* Fedde, *Rununculus brotherusi* Freyn and an Australian naturalized plant *Tribulus terrestris* Linn, but also used in Bhutan, were selected for phytochemical analysis.

Bhutanese traditional medicines use *Aconitum orochryseum* (whole parts) for the treatment of bilious fever or high fever related to bile disorders including cold and flu, fever of bile (liver), fever caused by malaria, snake bites and for treating blood infections. *Corydalis gerdae* (whole parts) is used for the treatment of malaria and infections. *Ranunculus brotherusi* (aerial parts) is used for treating wounds, pus, infections and as antipyretics. *Tribulus terrestris* (fruit) is used for the treatment of arthritis, kidney diseases and as a diuretic. Though *Tribulus terrestris* and *Ranunculus brotherusi* failed to give any alkaloids, a number of new as well as known alkaloids were isolated from *Aconitum orochryseum* and *Corydalis gerdae*.

Three new hetisane type diterpenoid alkaloids named orochrine, 2-*O*-acetylorochrine, and lingshinaline, together with two known alkaloids atisinium chloride and virescenine and six other unidentified alkaloids, were isolated from *Aconitum orochryseum*. The structures of the new alkaloids were elucidated by ^{1}H-NMR, gCOSY, gNOESY, TOCSY, ^{13}C-NMR, DEPT, gHSQC and gHMBC spectral data analysis. Atisinium chloride, a major alkaloid of the plant, was identified by single crystal X-ray crystallography.

Four known protopine type and protoberberine type isoquinoline alkaloids (protopine, scoulerine, cheilanthifoline, and stylopine) along with one unidentified alkaloid were isolated from *Corydalis gerdae*. The protopine was the major alkaloid of this plant. The alkaloids were identified by MS and ^{1}H-NMR spectral data comparison.

The antibacterial testing of crude methanol extracts, crude alkaloids and the major alkaloids (atisinium chloride, orochrine and protopine) of these two plants were found inactive against the bacterium *Staphylococcus aureus* (MIC = >125 μg/ml) and Vancomycin resistant *Enterococcus faecium* (MIC = >125 μg/ml). But the *in vitro* antimalarial assay of crude methanol extracts, crude alkaloids, atisinium chloride, protopine, and cheilanthifoline gave very significant activity against *Plasmodium falciparum*, TM4 and K1 strains. Crude methanol extract (AO-ME) of *Aconitum orochryseum* was not that active (IC_{50} of >10 μg/ml for both the strains). Increased activity was observed for its crude alkaloid (AO-CEA) with IC_{50} values of 20.40 μg/ml against TM4 and 19.20 μg/ml against K1 strains. The activity was further increased for atisinium chloride with IC_{50} values of 4.02 μM against TM4/8.2 and 3.59 μM against K1CB1 strains.

The antimalarial activity was better for the extracts and alkaloids of *Corydalis gerdae*. Its crude methanol extract (CG-ME) had the IC_{50} values of 1.00 μg/ml against TM4 strain and

2.56 μg/ml against K1 strain. The activity of crude alkaloid of this plant was far better with IC_{50} values of 0.33 μg/ml against TM4 strain and 0.63 μg/ml against K1 strain. Protopine had IC_{50} values of 4.25 μM against TM4/8.2 and 4.29 μM against K1CB1 strains. Cheilanthifoline had the IC_{50} values of 3.76 μM against K1 and 2.78 μM against TM4 strains.

These alkaloids have the potential to become candidates for antimalarial leads. Similar activities may be expected from other alkaloids including new ones isolated from these two plants. Therefore, the combination of these two plant extracts would make the best antimalarial herbal mixture for Bhutanese traditional medicines. The results proved that ethno-directed biorational approach, combined with an alkaloid focus, is an efficient strategy for drug lead discovery. The results also proved for the first time at the molecular level that Bhutanese traditional medicines have clinical efficacy. The medicinal plants of Bhutan thus have potential to provide important new drug leads. Bioprospecting them would benefit local people, pharmaceutical industries and the patients at large.

Table of Contents

Page

Declaration i
Publications ii
Acknowledgement iii
List of Abbreviation v
Abstract vii
Table of Contents x

Chapter 1 1
Natural Products-Based Drug Discovery and Recent Developments
1.1 General Introduction 1
1.2 Development of Antibacterial Resistance 1
1.2.1 Mechanism of Bacterial Resistance 3
1.3 Development of Antimalarial Resistance 3
1.3.1 Mechanism of Parasite Resistance 6
1.4 Strategy for Control of Bacterial and Malarial Infections 6
1.5 Development of Drugs from Bioactive Natural Products 7
1.5.1 Application of Alkaloids in Medicine 8
1.6 Natural Products in Traditional Medicines 11
1.7 Natural Products and Bhutanese Traditional Medicine 14
1.8 Research Aims and Objectives 17

Chapter 2 18
Selection and Collection of Bhutanese Medicinal Plants
2.1 General Introduction 18
2.2 Medicinal Plants of Bhutan 20
2.2.1 Sustainability of Medicinal Plants 21
2.3 Selection of Medicinal Plants 22
2.3.1 Criteria for Selecting the Medicinal Plants 22
2.4 Collection of Medicinal Plants 30

Chapter 3 **32**

Alkaloids from *Aconitum orochryseum* Stapf

3.1 Botanical Description and Ethno-medical Use 32

3.2 Diterpenoid Alkaloids: Biosynthesis and Classification 33

3.2.1 Hetisane-type Diterpenoid Alkaloids 36

3.3 Pharmacological Investigation of the Diterpenoid Alkaloids from *Aconitum* species 37

3.4 Isolation, Structure Elucidation and Identification of Diterpenoid Alkaloids from *Aconitum orochryseum* Stapf 38

3.4.1 Orochrine 39

3.4.2 2-*O*-Acetylorochrine 45

3.4.3 Lingshinaline 50

3.4.4 Virescenine 56

3.4.5 Atisinium chloride 60

3.4.6 AO-358 62

3.4.7 AO-340 63

3.4.8 AO-330 63

3.4.9 AO-372 63

3.4.10 AO-414 63

3.4.11 AO-466 64

Chapter 4 **65**

Alkaloids from *Corydalis gerdae* Fedde

4.1 Botanical Description and Ethno-medical Use 65

4.2 Isoquinoline Alkaloids: Biosynthesis and Classifications 65

4.3 Phytochemical and Pharmacological Investigation of Isoquinoline Alkaloids from the genus *Corydalis* 70

4.4 Isolation and Identification of Alkaloids from *Corydalis gerdae* Fedde 71

4.4.1 Protopine 71

4.4.2 Scoulerine 74

4.4.3 Cheilanthifoline 76

4.4.4 Stylopine 78
4.4.5 CG-337 79
4.4.6 9-Octadecenamide 79

Chapter 5 **81**
Alkaloids from *Tribulus terrestris* L
5.1 Botanical Description and Ethno-medical Use 81
5.2 Chemical and Pharmacological Studies of *Tribulus* species 82
5.3 β-Carboline Alkaloids and Classification 84
5.4 Attempted Isolation of β-Carboline Alkaloids from *Tribulus terrestris* Linn 84

Chapter 6 **86**
Investigation of *Ranuculus brotherusi* Freyn for Alkaloids
6.1 General Introduction 86
6.2 Phytochemical and Pharmacological Studies of the genus *Ranunculus* 86
6.3 Investigation of *Ranunculus brotherusi* Freyn for Alkaloids 87

Chapter 7 **89**
Microbiological Bioassays
7.1 General Introduction 89
7.2 Assays for Detecting Antibacterial Activity 90
7.2.1 Results of the Antibacterial Testing 91
7.3 Assays for Detecting Antimalarial Activity 91
7.3.1 Results of Antimalarial Testing 92

Chapter 8 **94**
Conclusions and Future Directions
8.1 Conclusions 94
8.2 Future Directions 96

Chapter 9 **98**

Experimental Section

9.1 Equipment and Chemical Specifications 98

9.1.1 Equipment 98

9.1.2 Chemicals and Solvents 99

9.2 Detection Methods and Spray Reagent 99

9.3 Collection of Plant Material 99

9.4 General Procedure for Extraction and Isolation of Alkaloids 100

9.5 Purification and Structure Elucidation of Alkaloids from *Aconitum orochryseum* Stapf 102

9.6 Purification and Structure Elucidation of Alkaloids from *Corydalis gerdae* Fedde 112

9.7 Attempted Extraction and Isolation of Alkaloids from *Tribulus terrestris* L 115

9.7.1 Attempt-I. 115

9.7.2 Attempt-II 116

9.8 Attempted Isolation of Alkaloid from *Ranunculus brotherusi* Freyn 117

9.9 Antibacterial Assay 117

9.9.1 Sample Preparation 117

9.9.2 Antibacterial Testing Methodology 117

9.9.3 Assay Procedure for 96-well Microtitre Plates 118

9.9.4 Results of Antibacterial Testing 118

9.10 Antimalarial Assay 119

9.10.1 Sample Preparation 119

9.10.2 Antimalarial Assay Method 119

9.10.3 Results of the Antimalarial Assay 120

References **121**

Appendices **149-192**