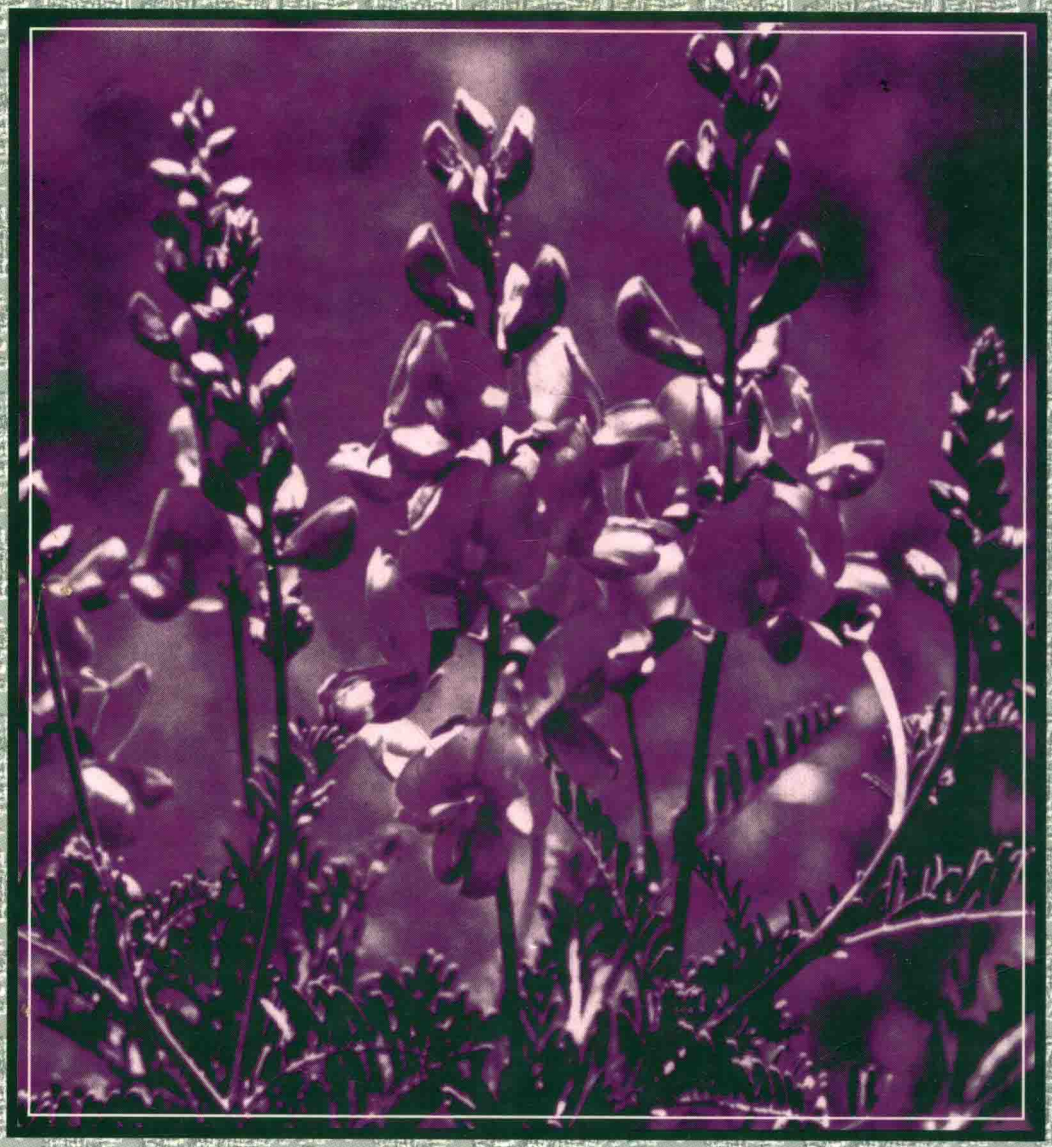
BIOACTIVE NATURAL PRODUCTS

Detection, Isolation, and Structural Determination



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Steven M. Colegate, Ph.D., earned a doctorate in Organic Chemistry (Synthesis, Compound Isolation, Structural Elucidation, and ESR Spectroscopy) at the University of Western Australia in 1978. The bulk of his postdoctoral work to date has been as part of a multidisciplinary team, within the School of Veterinary Studies at Murdoch University, investigating West Australian plants that are toxic to livestock.

Russell J. Molyneux, Ph.D., earned a doctorate in Organic Chemistry (Natural Product Structure Elucidation and Synthesis) at the University of Nottingham in 1963. His program as a Research Chemist at the Western Regional Research Laboratory in Albany, California has focused on the chemistry of toxic range plants, primarily in collaboration with the Poisonous Plant Research Laboratory at Logan, Utah, but also with scientists in many other parts of the world where livestock poisoning is an epidemic problem in livestock production.

THE CONTRIBUTORS

The contributors to this volume have been selected to provide a diverse range of expertise and experience in the general field of bioactive natural products. The aim has been to provide a truly multi-disciplinary approach to the problems of identifying bioactive constituents and subsequently isolating them and establishing their structures. The contributors are all experts within their particular disciplines, with well established reputations. Their contributions have been chosen to illustrate innovative approaches to the problems inherent to research on the diversity of bioactive compounds occurring in living organisms.

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Detection, Isolation, and Structural Determination

CONTENTS

Chapter 1

INTRODUCTION AND OVERVIEW

Steven M. Colegate and Russell J. Molyneux

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I. INTRODUCTION

The investigation of bioactive natural products has, in recent years, assumed a greater sense of urgency in response to the expanding human population and its subsequent demands for food, good health, and increasing areas of land on which to live. The extinction of plant and animal species as mankind encroaches on natural habitats represents lost and irreplaceable resources, the full potential of which is unpredictable. Similarly, the loss of endemic cultures as other cultures become influential in an almost cancerous manner will result in the loss of a fount of empirical ethnobotanical knowledge that has been acquired over the course of thousands of years.

II. THE MULTIDISCIPLINARY APPROACH

Nature recognizes no artificial barriers such as those of the "academic disciplines" and thus it is no surprise to find investigators with quite different academic training studying various aspects of bioactive natural products. It is when such diversely trained investigators come together as a team or, at the very least, collaborate very closely, that the greatest benefit will arise from such studies, since the investigators approach the subject from differing perspectives that will, with a little planning, complement and stimulate each other. This multidisciplinary approach not only facilitates the solution of specific problems, such as those in plant toxicology, but may also enhance the diversity and consequent value of bioactive natural product research.

Successful multidisciplinary collaboration requires that each individual has an understanding and appreciation of the intellectual and technical contributions to the project of the other team members. The sophistication of modern scientific instrumentation is such that the human aspects of using such equipment are frequently overlooked. While almost any research problem can presently be solved by the application of the most powerful equipment on the market, it requires imagination and resourcefulness to achieve results when such techniques are unavailable or too expensive to employ. It is then essential for the collaborators to ask themselves questions concerning the most expeditious approach to be adopted; for example:

1. Can a single bioassay be used, or must more than one be employed to cover the bioactivity of interest?
2. What is the most useful technique for separation of the active compound for both structural determination and possibly large-scale biological testing? Obviously it is inefficient to develop a separation method that yields the milligram quantities necessary for structure elucidation and then have to develop an entirely different method for preparation of gram quantities for *in vivo* and *in vitro* testing.
3. What are the minimal requirements for structural determination? It is an extravagance of time and resources to employ spectroscopic techniques that provide a duplication of evidence, unless the data obtained are equivocal.
4. Can the information regarding separation and structure of the bioactive compound(s) of concern be integrated to give a useful method for detection and analysis? If so, the time required for the developement of such a method may be significantly reduced.

Such questions require that the collaborators have a basic understanding of both the potential and the limitations of the techniques and disciplines that each can bring to bear on a problem. This can only lead to good experimental design that will further the goals of the project. It is the intention of this volume to provide some measure of perception in this regard.

III. WHY ISOLATE BIOLOGICALLY ACTIVE NATURAL PRODUCTS?

The use of herbal and other naturally based medicines has a long history. However, the utilization of whole-plant or other crude preparations for therapeutic or experimental reasons can have several drawbacks, which include

1. Variation in the amount of the active constituent with geographic areas, from one season to another, with different plant parts and morphology, and with climatic and ecologic conditions.
2. Co-occurrence of undesirable compounds causing synergistic, antagonistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity.
3. Losses of bioactivity due to variability in collection, storage, and preparation of the raw material.

Thus, the isolation of natural products that have biologic activity toward organisms other than the source has several advantages, including

1. Pure bioactive compounds can be administered in reproducible, accurate doses, with obvious benefits from an experimental or therapeutic point of view.
2. It can lead to the development of analytic assays for particular compounds or for classes of compounds. This is necessary, for example, in the screening of plants for potential toxicity and for quality control of food for human or animal consumption.
3. It permits the structural determination of bioactive compounds that may enable the production of synthetic material, incorporation of structural modifications, and a rationalization of mechanisms of action. This, in turn, will lead to reduced dependency on plants, for example, as sources of bioactive compounds and will enable investigations of structure/activity relationships, facilitating the development of new compounds with similar or more desirable bioactivities.

IV. DETECTION AND ISOLATION

To search for a compound that elicits a particular bioactive response, an appropriate assay is required to screen the source material and to monitor both extracts there from and subsequent purification steps. The assay could, for example, be lethality to a particular species, immunomodulatory activity, anti-inflammatory activity, or production of characteristic lesions in a species. The assay should be as simple, specific, and rapid as possible. An *in vitro* test is more desirable than a bioassay using small laboratory animals, which, in turn, is more desirable than feeding large amounts of valuable and hard-to-obtain extract to larger domestic animals. In addition, *in vivo* tests in mammals are often variable and are highly constrained by ethical considerations of animal welfare. For example, the isolation and purification of swainsonine (**1**) from *Swainsona canescens* was monitored by a simple, rapid enzyme inhibition assay developed as a consequence of a biochemical and pathologic study of the toxicosis.[1] In contrast, stypandrol (**2**), a toxin from *Stypandra imbricata* (blindgrass), was isolated using a time-consuming mouse bioassay in which the occurrence of characteristic lesions was monitored.[2]

Extraction from the plant is a trial-and-error exercise in which different solvents are tried under a variety of conditions such as time and temperature of extraction. The success or failure of the extraction process is monitored by the most appropriate assay. For example, swainsonine

(**1**) can be obtained by extraction of dried, milled *Swainsona canescens* with hot ethyl acetate for 48 h. Stypandrol (**2**), from *Stypandra imbricata*, although unstable at reflux temperatures, can be obtained by room temperature extraction of the dried or fresh plant, whereas stypandrone (**3**), a related naphthoquinone, can only be isolated from fresh plant material.[3]

Once extracted from the plant, the bioactive component then has to be separated from the coextractives. This may involve simple crystallization of the compound from the crude extract, as with the isolation of the glycoside dianellin (**4**) from *Dianella caerulea*.[4] More usually, however, it will involve further solvent partition of the coextractives and extensive chromatography, taking advantage of particular properties of the desired compound, such as acidity, polarity, and molecular size. In some cases the isolation can be assisted by prior derivatization, imparting more easily manageable properties to the desired compound. The isolation of swainsonine, as its triacetate, from *Rhizoctonia leguminicola*[5] is representative of such an approach.

Final purification, to provide compounds of suitable purity for structural analysis, may be accomplished by appropriate techniques such as recrystallization, sublimation, or distillation.

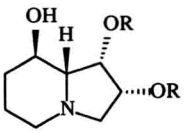
V. STRUCTURE DETERMINATION

The process of structural determination involves accumulating data from numerous sources, each of which gives some structural information, and the assimilation of these data into a chemical structure that rigorously and uniquely fits all the available structural information. There is available today a wide range of spectroscopic instrumentation, such as UV, IR, and visible absorption spectroscopies, nuclear magnetic resonance spectroscopy, and mass spectrometry, forming the backbone of modern structural analysis.

Prior to the availability of such aids to structural determination and in cases where, despite these aids, ambiguity existed, chemical modification or degradation of the unknown compound was necessary. These latter processes involve the treatment of the unknown compound with functional group-specific reagents or degradation of the compound in a predictable manner until a compound of known structure is obtained. Backtracking should then provide a structure for the unknown compound. Apart from being a time-consuming and exacting art, this method can be fraught with difficulty and ambiguity. For example, stypandrol (**2**), the toxin isolated from *Stypandra imbricata* (blindgrass), was apparently first isolated from *Hemerocallis thumbergii* and named hemerocallin, but was assigned the incorrect structure on the basis of ambiguous degradation studies.[6]

The process of spectroscopic structural determination should be closely allied to a familiarity with the scientific literature. If the compound has not already been described, it may be very similar to reported compounds that will assist in the interpretation of the data for the unknown. In this regard, an awareness of the coextractives from the plant may also be of value. For example, the facile structural elucidation of the easily obtained dianellidin (**5**) from *Stypandra imbricata* greatly expedited the structural analysis of the toxin stypandrol, which proved to be a dimer of dianellidin.

When analysis of the spectroscopic data for an unknown compound is inconclusive, and if the compound or one of its derivatives is suitably crystalline, a single crystal X-ray diffraction study should be considered. Simplistically, this involves computer-aided analysis of the diffraction pattern obtained when a single crystal is irradiated with X-rays. Correct interpretation of the data will result in a three-dimensional picture of the molecule, including the relative stereochemistry if the molecule is optically active. In some cases the absolute stereochemistry can also be determined. X-ray diffraction studies



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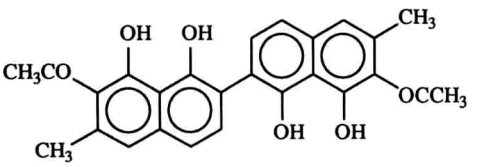

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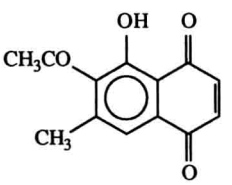

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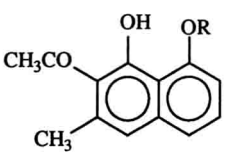

4 R = Rhamnose-Glucose

5 R = H

can give valuable information on the three-dimensional shape of the molecule, bond lengths and angles, and possible intra- and intermolecular interactions. The importance of determining the stereochemical nature of the structure is becoming more self-evident, since enantiomeric (mirror-image) structures may have entirely different biologic properties.[7] It is no longer acceptable to evaluate only the bioactivity of mixtures of such structures.

If structural analysis data are ambiguous and the compound is not amenable to an X-ray diffraction study, then chemical synthesis from precursors of known structure and stereochemistry is usually sufficient to prove or disprove a proposed structure. The chemical literature abounds with examples of how unambiguous synthesis has been vital in finally establishing a structure for an unknown compound.

VI. OVERVIEW

This book comprises chapters written by researchers from a variety of scientific disciplines, each of whom consequently approaches bioactive natural product research from a different perspective. This approach serves to highlight the multidisciplinary nature of this type of research. Despite the fact that most of the chapters in this book deal with the detection, isolation, and structural determination of bioactive compounds from terrestrial plants and fungi, the philosophy and rationale behind the research applies equally to bioactive compounds from aquatic and microbial sources. Indeed, the influence of mankind on aquatic environments parallels that on the terrestrial habitats, with consequently similar concerns for loss of species yet to be investigated or even discovered. Microbial sources of useful bioactive compounds, in conjunction with the capabilities of genetic engineering, are extremely important, especially in large-scale production of such compounds.

The chapters in the first section of this book ("Methods of Detection, Isolation, and Structural Determination") are not intended as definitive texts. Rather, they are reviews with the emphasis being on both the *philosophy and rationale* directing detection and isolation and on the *type and quality* of structural information that can be acquired from the various sources.

The second section in this book ("Specific Case Studies") comprises several chapters that deal with specific bioassays that are used to screen source material, extracts therefrom, or purified compounds, for activity. Where source material is screened, the bioassay is also used to guide the extraction of that material and the subsequent fractionation of the extracts to isolate the active constituents. Some of the chapters in this section concentrate on the bioassay details, while the others emphasize the particular isolation procedure or the structural determination rationale. Several authors in this section review the natural occurrence of compounds with specific activities and describe the rationale guiding the search for such compounds. It must be recognized that this section is not intended to provide a catalog of bioassays, but rather to illustrate the *extreme diversity* of methods and to emphasize the importance of selecting a bioassay *appropriate to the activity* sought.

A. METHODS OF DETECTION, ISOLATION, AND STRUCTURAL DETERMINATION

1. Detection and Isolation

Ghisalberti (Chapter 2) interweaves a great deal of his own extensive experience as a natural products chemist into his chapter describing the detection and isolation of bioactive natural products. This combination therefore provides a critical insight into the diversity and potential complexity of bioactive compounds and into how these compounds are pursued and discovered. Molyneux (Chapter 3) continues this theme by describing the more specific detection and isolation of highly water soluble "cryptic" alkaloids.

2. Structure Determination

Byrne (Chapter 4) describes a strategy for using nuclear magnetic resonance (NMR) spectroscopy in the structural elucidation of compounds. A brief description of NMR fundamentals and techniques is accompanied by extensive referencing to assist those readers who require more detailed information. For simplicity and so as not to detract from the NMR essence of the chapter, Byrne demonstrates this strategy, in the main, through the structural elucidation of one compound. The resulting rational and cumulative acquisition of NMR information gradually, but surely, defines the structure of the compound. This strategy, and