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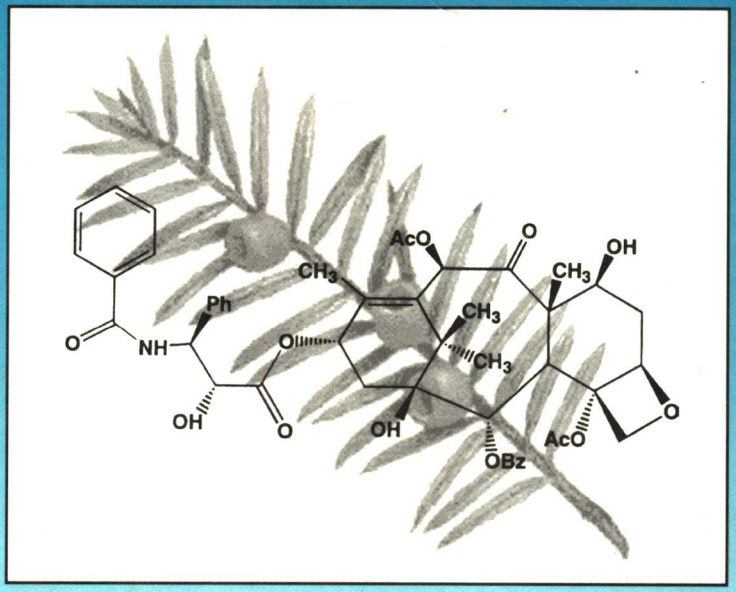
Natural Products Isolation

Edited by

Richard J. P. Cannell



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Humana Press  **Totowa, New Jersey**

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

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ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover design by Patricia F. Cleary.

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Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods in molecular biology™.

Natural products isolation/edited by Richard J. P. Cannell.
p. cm.—(Methods in biotechnology; 4)
Includes index.
ISBN 0-89603-362-7 (alk. paper)
1. Natural products. 2. Extraction (Chemistry). I. Cannell, Richard J. P. II. Series: Methods in biotechnology (Totowa, NJ); 4.
QD415.N355 1998
547.7—dc21 98-16651
CIP

Preface

Biodiversity is a term commonly used to denote the variety of species and the multiplicity of forms of life. But this variety is deeper than is generally imagined. In addition to the processes of primary metabolism that involve essentially the same chemistry across great swathes of life, there are a myriad of secondary metabolites—natural products—usually confined to a particular group of organisms, or to a single species, or even to a single strain growing under certain conditions. In most cases we do not really know what biological role these compounds play, except that they represent a treasure trove of chemistry that can be of both interest and benefit to us. Tens of thousands of natural products have been described, but in a world where we are not even close to documenting all the extant species, there are almost certainly many more thousands of compounds waiting to be discovered.

The purpose of *Natural Products Isolation* is to give some practical guidance in the process of extraction and isolation of natural products. Literature reports tend to focus on natural products once they have been isolated—on their structural elucidation, or their biological or chemical properties. Extraction details are usually minimal and sometimes nonexistent, except for a mention of the general techniques used. Even when particular conditions of a separation are reported, they assume knowledge of the practical methodology required to carry out the experiment, and of the reasoning behind the conditions used. *Natural Products Isolation* aims to provide the foundation of this knowledge. Following an introduction to the isolation process, there are a series of chapters dealing with the major techniques used, followed by chapters on other aspects of isolation, such as those related to particular sample types, taking short cuts, or making the most of the isolation process. The emphasis is not so much on the isolation of a known natural product for which there may already be reported methods, but on the isolation of compounds of unknown identity.

Every natural product isolation is different and so the process is not really suited to a practical manual that gives detailed recipe-style methods. However, the aim has been to give as much practical direction and advice as possible, together with examples, so that the potential extractor can at least make a reasonable attempt at an isolation.

Natural Products Isolation is aimed mainly at scientists with little experience of natural products extraction, such as research students undertaking natural products-based research, or scientists from other disciplines who find they wish to isolate a small molecule from a biological mixture. However, there may also be something of interest for more experienced natural products scientists who wish to explore other methods of extraction, or use the book as a general reference. In particular, it is hoped that the book will be of value to scientists in less scientifically developed countries, where there is little experience of natural products work, but where there is great biodiversity and, hence, great potential for utilizing and sustaining that biodiversity through the discovery of novel, useful natural products.

Richard J. P. Cannell

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How to Approach the Isolation of a Natural Product

Richard J. P. Cannell

1. Introduction

It can seem a formidable task, faced with a liter of fermentation broth–a dark, viscous sludge–knowing that in there is one group of molecules that has to be separated from all the rest. Those molecules possibly represent only about 0.0001%, or 1 ppm of the total biomass and are dispersed throughout the organism, possibly intimately bound up with other molecules. Like the proverbial needle in a haystack, you have to remove lot of hay to be left with just the needle, without knowing what the needle looks like or where in the haystack it is.

1.1. What Are Natural Products?

The term "natural product" is perhaps something of a misnomer. Strictly speaking, any biological molecule is a natural product, but the term is usually reserved for secondary metabolites, small molecules (mol wt < 1500 amu approx) produced by an organism but that are not strictly necessary for the survival of the organism, unlike the more prevalent macromolecules such as proteins, nucleic acids, and polysaccharides that make up the basic machinery for the more fundamental processes of life.

Secondary metabolites are a very broad group of metabolites, with no distinct boundaries, and grouped under no single unifying definition. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, or shunt metabolites produced during idiophase, defense mechanisms, regulator molecules, and so on. Perhaps the most cogent theory of secondary metabolism has been put forward by Zähner, who described secondary metabolism as evolutionary "elbow room" *(1)*. If a secondary metabolite has no adverse effect on the producing organism at any of the levels of

From: *Methods in Biotechnology, Vol. 4: Natural Products Isolation*
Edited by: R. J. P. Cannell

differentiation, morphogenesis, transport, regulation, or intermediary metabolism, it may be conserved for a relatively long period during which time it may come to confer a selective advantage. Secondary metabolism therefore provides a kind of testing ground where new metabolites have the opportunity, as it were, to exist without being eliminated, during which time they may find a role that will give an advantage to the producing organism. This is supported by the fact that secondary metabolites are often unique to a particular species or group of organisms and, while many act as antifeedants, sex attractants, or antibiotic agents, many have no apparent biological role. It is likely that all these concepts can play some part in understanding the production of the broad group of compounds that come under the heading of secondary metabolite.

Isolation of natural products differs from that of the more prevalent biological macromolecules because natural products are smaller and chemically more diverse than the relatively homogeneous proteins, nucleic acids and carbohydrates, and isolation methods must take this into account.

1.2. The Aim of the Extraction

The two most fundamental questions that should be asked at the outset of an extraction are:

1. What am I trying to isolate?
   There are a number of possible targets of an isolation:
   a. An unknown compound responsible for a particular biological activity.
   b. A certain compound known to be produced by a particular organism.
   c. A group of compounds within an organism that are all related in some way, such as by a common structural feature.
   d. All of the metabolites produced by one natural product source that are not produced by a different "control" source, e.g., two species of the same genus, or the same organism grown under different conditions.
   e. A chemical "dissection" of an organism, in order to characterize all of its interesting metabolites, usually those secondary metabolites confined to that organism, or group of organisms, and not ubiquitous in all living systems. Such an inventory might be useful for chemical, ecological, or chemotaxonomic reasons, among others.
2. Why am I trying to isolate it?
   The second fundamental question concerns what one is trying ultimately to achieve, for defining the aims can minimize the work required. Reasons for the extraction might be:
   a. To purify sufficient amount of a compound to characterize it partially or fully.
   b. More specifically, to provide sufficient material to allow for confirmation or denial of a proposed structure. As in many cases this does not require mapping out a complete structure from scratch but perhaps simply comparison with a standard of known structure; it may require less material or only par-

tially pure material. There is no point in removing minor impurities if they do not get in the way of ascertaining whether the compound is, or is not, compound X.

c. The generation/production of the maximum amount of a known compound so that it can be used for further work, such as more extensive biological testing. (Alternatively, it may be more efficient to chemically synthesize the compound; any natural product that is of serious interest, i.e., is required in large amounts, will be considered as a target for synthetic chemistry.)

1.3. Purity

With a clear idea of what one is trying to achieve, one can then question the required level of purity. This in turn might give some indication of the approach to be taken and the purification methods to be employed.

For example, if you are attempting to characterize fully a complex natural product that is present at a low concentration in an extract, you will probably want to produce a compound that is suitable for NMR. The purity needed is dependent on the nature of the compound and of the impurities, but to assign fully a complex structure, material of 95–100% purity is generally required. If the compound is present at high concentration in the starting material and there already exists a standard against which to compare it, structure confirmation can be carried out with less pure material and the purification will probably require fewer steps.

The importance of purity in natural products isolation has been highlighted by Ghisalberti *(2)*, who described two papers that appeared at about the same time, both reporting the isolation from plants of *ent*-kauran-3-oxo-16,17-diol. In one paper, the compound has a melting point of 173–174°C and $[\alpha]_D$–39.2°($CHCl_3$); in the other, no melting point is reported, but the compound has an $[\alpha]_D$–73.1°($CHCl_3$). Either the compounds are different or one is significantly less pure than the other.

If a natural product is required for biological testing, it is important to know at least the degree of purity and, preferably, the nature of the impurities. It is always possible that the impurities are giving rise to all or part of the biological activities in question. If a compound is to be used to generate pharmacological or pharmacokinetic data, it is usually important that the material be very pure (generally >99% pure), particularly if the impurities are analogs of the main compound and may themselves be biologically active.

In some cases, a sample need only be partially purified prior to obtaining sufficient structural information. For example, it may be possible to detect the absence of a certain structural feature in a crude mixture–perhaps by absence of a particular ultraviolet (UV) maximum–and conclude that the mixture does not contain compound A. In other cases, such as X-ray crystallography studies, material will almost certainly be required in an extremely pure state, generally >99.9% pure.

It is worth bearing in mind that the relationship between the degree of purity achieved in a natural product extraction, and the amount of work required to achieve this, is very approximately exponential. It is often relatively easy to start with a crude, complex mixture and eliminate more than half of what is not wanted, but it can be a painstaking chore to remove the minor impurities that will turn a 99.5% pure sample into one that is 99.9% pure. It is also probably true to say that this exponential relationship also often holds for the degree of purity achieved versus the yield of natural product. In the same way that no chemical reaction results in 100% yield, no extraction step results in 100% recovery of the natural product. Compound will be lost at every stage; in many cases it may be that, to achieve very high levels of purity, it is necessary to sacrifice much of the desired material. In order to remove all the impurities it may be necessary to take only the cleanest "cuts" from a separation, thus losing much of the target material in the process (though these side fractions can often be reprocessed).

These factors may, of course, have some bearing on the level of purity deemed satisfactory, and it is useful to ask at each stage of the extraction, whether the natural product is sufficiently pure to answer the questions that are to be asked of it.

At present, there are two main reasons why scientists extract natural products: to find out what they are and/or to carry out further experimental work using the purified compound. In the future, it may be easy to determine structures of compounds in complex mixtures; indeed, it is already possible to do this under some circumstances, but at present, most cases of structural determination of an unknown compound require that it be essentially pure. Similarly, to obtain valid biological or chemical data on a natural product usually requires that it be free from the other experimental variables present in the surrounding biological matrix.

1.4. Fractionation

All separation processes involve the division of a mixture into a number of discrete fractions. These fractions may be obvious, physically discrete divisions, such as the two phases of a liquid–liquid extraction, or they may be the contiguous eluate from a chromatography column that is artificially divided by the extractor into fractions.

The type of fractionation depends on the individual sample and the aims of the separation. Typically, a column is run and the eluate divided into a manageable number of even-sized fractions, followed by analysis of the fractions to determine which contain the desired compounds. (So, the eluate from a silica column with a bed volume of 10 mL becomes, perhaps, 20 × 5-mL fractions.) Obviously, collecting the eluate as a large number of very small fractions means that each fraction is more likely to contain a pure compound, but it requires

more work in analyzing every fraction. This also runs the risk of spreading the target compound over so many fractions that, if originally present in only low concentrations, it may evade detection in any one of the fractions. If the separation process is relatively crude, it is probably more sensible to collect only a few large, relatively crude fractions and quickly home in on those containing the target.

Alternatively, one may monitor "on-line" and fractionate the eluate accordingly. This is generally used at the later stages of separation for separations of less complex mixtures, typically on high-performance liquid chromatography (HPLC) separations monitored by UV, where one can identify and isolate material corresponding to individual peaks.

1.5. Assays

A point that may seem fairly obvious, but worth reiterating, is that, with a complex mixture from which one or a few specific compounds are to be isolated, a means of keeping track of the compound through the extraction process is needed. There are two main ways to follow a compound: (1) physical assay (for example, HPLC, thin-layer chromatography [TLC], liquid chromatography-mass spectometry [LC-MS], and perhaps involving comparison with a standard), or (2) bioactivity assay.

It is not within the scope of this book to discuss at length biological screening and the rapid developments that are being made in this field, but some typical bioactivity screens are listed in **Table 1**.

There are a number of basic points that should be kept in mind when assaying fractions:

1. Samples dissolved or suspended in a solvent different from the original extraction solvent should be filtered or centrifuged to remove any insoluble matter. Assay samples that include a volatile solvent, or different solvents, are usually best dried and redissolved in the original extraction solvent, water, or other solvent in which the compound is known to be soluble. For example, an aliquot of a methanol extract of a broth may be dried, then resuspended and partitioned between water and chloroform. The two phases, or part thereof, can then be redried, redissolved in equal volumes of methanol, and assayed. This may make subsequent assay easier for two reasons:
   a. The test solvent may not be compatible with the assay.
   b. Redissolving the two phases back into the same solvent makes quantitative and qualitative comparisons much easier, particularly if one of the test solvents is very volatile, creating problems with evaporation and differences in concentration.
2. Samples acidified or basified should be readjusted to their original pH to prevent them from interfering with the assay. If volatile acids/bases are present, they may be removed by evaporation.
3. Controls consisting of the solvents and/or buffers, acids, and so on, without sample, should always be carried out to ensure that observed assay results are in

Table 1
Typical Bioactivity Screens

Activity	Common assay form
Antibacterial	Seeded agar diffusion, turbidometric
Antifungal	Seeded agar diffusion, turbidometric
Enzyme inhibitory	UV, colorimetric, radiolabeled, scintillation proximity assay (SPA)
Antitumor	Cell line
Toxicity	Whole organism, e.g., brine shrimp lethality
Antiparasitic	Whole organism, e.g., insect larvae, antihelminth
Receptor binding	Enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), SPA, chemiluminescence, fluorescence
Transcription-based	Chemiluminescence, fluorescence

fact caused by the natural product. The separation may result in fractions that do not have homogeneous "backgrounds" and this may affect the assay. For example, a gradient chromatography system may well result in fractions with increasing organic solvent concentration that might itself affect the assay. In order to allow for the effect of this discrepancy, either a series of control samples should be tested–in this case, fractions from a blank gradient run with no sample–and these results subtracted from the assay results, or, all of the fraction aliquots must be treated in a way that allows them to be presented to the assay in the same form. This might mean drying the samples and redissolving them in the same solvent. Care must be taken to redissolve in a solvent compatible with the assay (e.g., methanol, dimethylsufoxide [DMSO]), and that will solubilize compounds eluting from both the polar and nonpolar ends of a gradient elution. Additionally, for practical reasons, it is often preferable not to take samples to complete dryness as it is sometimes difficult to resolubilize all the components. Samples can be partially dried by evaporation or vacuum centrifugation, such that the more volatile organic solvent is removed leaving only the residual aqueous extract; then volumes can be adjusted to give the same relative concentration. Alternatively, samples may be adsorbed each on its own solid phase extractant (*see* **Subheading 2.3.2.**) and then eluted in a small volume of suitable solvent. This can serve both to concentrate and to further clean the sample by "desalting"–separating the compounds from more polar materials or inorganic components that may have been introduced into the mobile phase to improve chromatography, which may affect the assay.

4. Ideally, the assay should be at least semiquantitative, and/or samples should be assayed at a series of dilutions in order to determine where the majority of the target compound resides. It may well be that the separation process, e.g., the chromatography column, dilutes the activity in a way such that it is not detectable in the assay without concentration, and so the nonappearance of an active fraction may

not mean that the activity is lost but that the assay is insufficiently sensitive for unconcentrated fractions. For this reason, it is always wise to quantify approximately the recovery of compound at each stage.

Such matters may sound obvious and trivial, but preparing fractionation-samples for assay in a suitable way can be a time-consuming and surprisingly troublesome process, often representing a major portion of the work in a bioassay-guided extraction.

1.5.1. Overlay Assay

Sometimes it is possible to combine more closely the separation and the bioassay, as in the case of TLC overlay assays. In this case, the sample may be separated by TLC, the TLC plate dried to remove traces of solvent, and the assay performed *in situ*, on top of the plate. This usually takes the form of the reactants immobilized in a gel poured or sprayed over the plate and the results visualized. The most commonly used form of this assay is an antimicrobial assay in which the plate is covered with agar seeded with microorganism and then incubated, after which microbial growth is seen throughout the agar except over those regions of the chromatogram that contain the antimicrobial components.

As long as the assay can be visualized, either by obvious microbial growth or by the use of a colored reaction product, this principle can be applied to a wide range of assays, including enzyme and receptor-based assays. This principle of immobilizing, or spotting, a small amount of sample onto a TLC plate is one of the quickest and most convenient means of assaying a large number of samples, and this method of overlay assay is widely used for assaying fractions from all types of separation.

1.6. Quantification

During the isolation of a natural product, it is necessary to track the compound and, if possible, obtain some estimate of the recovery at each stage. This can often be done by routine analytical techniques that may involve the use of a standard.

During the isolation of an unknown bioactive compound, the compound is monitored by following the bioactivity at each stage. It is also useful to quantify, at least approximately, this bioactivity at each stage. Approximate quantification is generally carried out by assaying a set of serial dilutions of each fraction at each stage of the separation. To detect the peaks of activity, it is often necessary to assay fractions at a range of dilutions, which serves to indicate the relative amounts of activity/compound present in each fraction. It can then be seen in which fraction(s) the bulk of the active components lie and also allows for some estimation of the total amount of activity recovered, relative to the starting material. Accounting for all the initial activity can be helpful in avoiding potential problems.

For example, one may produce column fractions that obviously contain active compound but which a quick calculation reveals, represent only approx 5% of the activity that went on to the column. There are many possible explanations for such "disappearance" of activity, but essentially, quantification can act as a warning that there is more to look for. Likely explanations may include:

1. There is more than one active component and the major component has not been eluted.
2. Most of the active component has been degraded or modified by the separation process.
3. The starting sample was not prepared so as to be fully compatible with the mobile phase, so that a large proportion of the active component precipitated when loading on to the top of the column.
4. Most of the active component(s) spread across a wide range of fractions in a concentration too low to be detected by the assay.

Quantification also helps to avoid the temptation to assign all the activity of an extract to a particular peak on a chromatogram, when in fact, much of the activity may be represented by a very minor peak or a potent compound present in very low concentrations almost insignificant apart from its bioactivity. These are often more interesting than abundant compounds as they are more bioactive and are less likely to have been previously described.

For similar reasons, it is prudent to retain a reference sample of the mixture at each stage of the process so that it can be assayed alongside the fractions and serve as a record of material recovered at each stage of the process.

2. Where to Start?

How to begin the isolation of a natural product? First, something about the nature of the compound needs to be known so that the approach to take can be determined.

2.1. Determination of the Nature of the Compound

How much needs to be discovered depends on how much is already known and what our aim is. The general features of a molecule that are useful to ascertain at this early stage might include: solubility (hydrophobicity/hydrophilicity), acid/base properties, charge, stability, and size.

1. If the aim is to isolate all of the secondary metabolites of an organism and not to focus on a specific molecule, this information may be less important but still can be useful in getting an idea of the range of compounds being worked with.
2. If the aim is to isolate a known compound(s), much of this information will already be established, or will probably be apparent from the structure. (It may even be that a physical assay exists for the compound and this may provide the basis for an isolation.)
3. If the target is an unknown molecule, it is probable that little is known about the nature of the compound.