

CONTEMPORARY METHODOLOGICAL APPROACHES IN THE SEARCH FOR NEW LEAD COMPOUNDS FROM HIGHER PLANTS

Emerson F. Queiroz, Andrew Marston and Kurt Hostettmann,

Laboratoire de Pharmacognosie et Phytochimie, Section de Sciences Pharmaceutiques, University of Geneva, 1211 Geneva, Switzerland

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Contents

1. Introduction
 2. Approaches for the discovery of new drugs from higher plants
 3. Selection of plant material
 4. Biological and pharmacological targets
 5. Chemical screening
 - 5.1. Metabolite profiling: a LC-multi-hyphenated strategy
 - 5.2. Problems encountered in metabolite profiling
 - 5.3. Application of LC/UV/MS and LC/NMR in phytochemical analysis
 - 5.3.1. Dereplication of flavanones and isoflavones in crude plant extracts
 - 5.3.2. Stop-flow LC/¹H-NMR with 2D correlation experiments for the structure elucidation of a flavanone of *Monotes englerii*
 - 5.4. Combination of LC/NMR and LC/MS with in-mixture NMR experiments for the investigation of labile constituents
 - 5.4.1. On-line characterisation of unstable cinnamic ester derivatives in *Jamesbrittenia fodina*
 - 5.4.2. Study of epimerisation reactions
 - 5.5. On-line absolute configuration determination
 6. Isolation of active principles and their structure determination
 7. Conclusion
- Acknowledgments
Glossary
Bibliography
Biographical Sketches

Summary

Higher plants represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs. During the last decades, the renewed interest in investigating natural products has led to the introduction of several important drugs, such as the anticancer substances vinblastine, vincristine and taxol, or the antimalarial agent artemisinin. Success in natural products research is conditioned by careful plant selection, based on various criteria such as chemotaxonomic data, information from traditional medicine, field observation or even random collection. One main strategy in the isolation of new lead compounds consists of so-called bioactivity-guided isolation, in which pharmacological or biological assays are used to target the isolation of bioactive compounds. One major drawback of this

strategy is the frequent isolation of known metabolites. Therefore, metabolite profiling using hyphenated techniques such as LC/UV, LC/MS and more recently LC/NMR rapidly provides plenty of structural information, leading to a partial or a complete on-line *de novo* structure determination of the natural products of interest. Complementary to this approach, bioassays performed after LC/microfractionation of the extracts allow an efficient localisation of the bioactive LC-peaks in the chromatograms. The combination of metabolite profiling and LC/bioassays gives the possibility to distinguish between already known bioactive compounds and new molecules directly in crude plant extracts (dereplication). Thus, the tedious isolation of compounds of low interest can be avoided and a targeted isolation of new bioactive products or constituents presenting novel or unusual spectroscopic features can be undertaken. Several examples of rapid localisation of bioactive compounds, based on post-chromatographic bioautographic testing of LC/NMR microfractions and subsequent on-line identification will be illustrated. Application of hyphenated techniques for the efficient characterisation of labile constituents or constituents which are difficult to separate on the preparative scale will also be mentioned. Possibilities and limitations of LC/UV/NMR/MS and LC/bioassay as well as future developments expected in this field will be discussed.

1. Introduction

Higher plants are a source of millions of natural products, with an almost infinite variety of structural variations. These molecules often have specific functions and many of them have biological activities which can be of use to humans. They may provide lead compounds for the development of new drugs or they may be indispensable tools in biomedical research. While many are substrates for life process, there are also toxins, hormones, and molecules with other functions. An assessment of the current interest in natural products can be made by regarding the attempts of the pharmaceutical industry to introduce them into drug discovery programs.

For a long time, plants were the only therapy available to humans. The plant kingdom is still an untapped reservoir of new molecules with potential therapeutic interest and only a relative small percentage of the 350 000 known plant species have been studied from a phytochemical and a pharmacological viewpoint. Research in pharmacognosy has demonstrated that potent bioactive products can be obtained from plants. In the present drug discovery programs, natural products or compounds derived from natural products account for more than 40% of the new registered drugs. A recent statistical investigation into the structural complementarity of natural and synthetic compounds also proved that the potential for new natural products is not exhausted and they still represent an important source for the lead finding process.

In order to discover new bioactive compounds from plant sources that could become new leads or new drugs, extracts should be submitted at the same time to a chemical screening and to various biological or pharmacological targets. The chemical screening or metabolite profiling is aimed at distinguishing between already known compounds (dereplication) and new molecules directly in crude plant extracts. Thus, the tedious isolation of known compounds can be avoided and a targeted isolation of constituents presenting novel or unusual spectroscopic features can be undertaken. It has to be noted

that metabolite profiling clearly refers here to the detection and identification of plant metabolites and is different from the metabolic profiling process associated with detection of metabolites issued from a given new lead compound.

Metabolite profiling in crude plant extracts is not an easy task since natural products display a very important structural diversity. For each compound, the orders of the atoms and stereochemical orientations have to be elucidated *de novo* in a complex manner and the compounds cannot simply be sequenced as is the case for genes or proteins. Consequently and unlike genomics and proteomics, a single analytical technique does not exist that is capable of profiling all secondary metabolites in a plant extract.

In order to develop innovative strategies for the metabolite profiling of crude plant extracts, advantage has been taken of the extraordinary development of hyphenated techniques (and particularly LC/MS and LC/NMR) over the last decade for studying their possible application in the on-line identification of natural products. The principle of operation of these latter techniques will not be explained in detail here since different papers in literature have been dedicated to these powerful methodologies.

In this chapter, the emphasis will be put on the strategy developed for efficient dereplication of natural products and for the on-line identification of bioactive constituents based on the combination of LC/UV-DAD, LC/MS, LC/MS/MS and LC/NMR applied to crude plant extract screening. The role of these techniques in the structural investigation of unstable products, or compounds that are difficult to isolate at the preparative level, will also be highlighted by different examples of application.

2. Approaches for the discovery of new drugs from higher plants

- In order to find new plant-based drugs, it is necessary to screen extracts for the presence of novel compounds and to investigate their biological activities. Once novel compounds are suspected, they are generally isolated so that material is available for further biological and toxicological testing (Figure 1). The path that leads from the intact plant to its pure constituents is long.

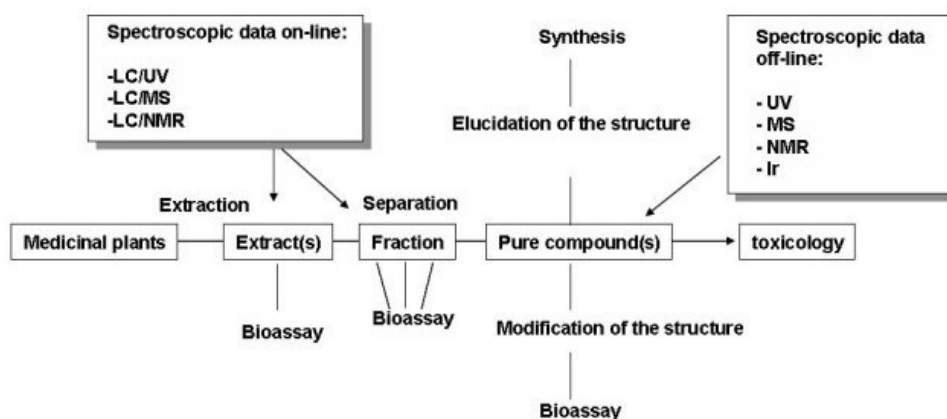


Figure 1. The isolation of pure compounds from plants.

It involves work which might last anything from weeks to years and includes the

following steps:

- Taxonomic identification of the plant with the aid of specialists (botanists).
- Collection and drying of the vegetable material; precautions need to be taken to avoid the formation of artefacts.
- Preparation of extracts using different solvents; analysis of these extracts by different chromatographic methods.
- Fractionation of the extracts by different preparative chromatographic techniques (column chromatography, centrifugal partition chromatography, etc).
- Purity control of the isolated compounds.
- Structure elucidation of the constituents by a combination of diverse spectroscopic techniques (UV/VIS, IR spectrophotometry, carbon and proton nuclear magnetic resonance, mass spectroscopy, X-ray diffraction) and chemical techniques (hydrolyses, formation of derivatives, degradation reaction, etc.).
- Synthesis or semi-synthesis of the natural product.
- Modification of structure with a view to establishing structure-activity relationships.
- Pharmacological and toxicological testing.

3. Selection of plant material

When the researcher undertakes a phytochemical investigation of medicinal plants with the aim of isolating and identifying the active substances, the correct choice of vegetable material has to be made. Considering the number of plants which have not yet been studied from both phytochemical and pharmacological points of view, this choice is difficult. The factors to be considered are:

- Chemotaxonomic criteria.
- Information from traditional medicine.
- Field observations.
- Random collection.

Chemotaxonomy, or the science of classification of plants as a function of the structures of their chemical constituents, can introduce useful elements. Constituents are often specific to a given botanical family, to a genus or to a species. If a natural product has interesting therapeutic properties, it may be possible to find analogous substances in species of the same genus or the same family. For example, the gentian family (Gentianaceae), which consists of approximately 1100 different species, is characterised by the presence of bitter compounds in the roots and the leaves which stimulate digestion, and of xanthenes with antidepressant properties. If an investigator is interested in the latter substance class, he can select gentians from different localities in order to increase his chances of discovering new molecules with therapeutic potential. Before beginning a phytochemical and pharmacological study, a literature search is performed on the species in question. Easily accessible data banks facilitate this task. In a short time, it is possible to find all the previous research which has been performed on the plant selected for the study. The distribution of xanthenes is not restricted to gentians. These interesting molecules are also found in the Polygalaceae and

Guttiferaceae (*Hypericum perforatum* L. for example) families. They are absent from most other families.

Selection of plants based on data from traditional medicine can also lead to the discovery of promising new molecules. Plants from tropical and subtropical regions occur in abundance and, further, they have been little studied. They represent an enormous reservoir of new molecules with potential therapeutic activities, waiting to be discovered. Certain representatives of the pharmaceutical industry are aware of this potential and have introduced screening programs for plants from tropical regions. International agreements have been established in order to prevent these natural resources in Third World countries from being excessively exported to industries of the northern hemisphere. Within the framework of these standards, a special place has been reserved for endangered species, to ensure their protection. Meetings between traditional healers and European researchers, in the presence of local representatives (university personnel, botanists, etc.), are arranged to discuss medicinal plants, their identification and use. Preference is of course given to endemic plants which have been investigated. Is the information gathered from these healers reliable? A root decoction which is claimed to be laxative can easily be checked. Plants used for the treatment of sores or wounds are of great interest because wound healing is easy to verify. However, diagnosis by the healers of internal problems is much more difficult to rely on. Moreover, traditional healers often call upon supernatural forces during their treatments. And the placebo effect is often involved. Therefore, a discerning evaluation of this information needs to be made before choosing the plants for study.

During plant collecting expeditions, field observations are obviously very important. A species which grows in a hostile environment (such as tropical forest) in which there is danger of attack from insects, fungi, bacteria or viruses, will attempt to protect itself by synthesizing insecticidal, fungicidal, antibacterial or virucidal constituents. If one observes, for example, that leaves of a plant in such an environment show signs of attack, it could be that the plant contains defensive compounds against insects or microorganisms. Roots often biosynthesize antifungal substances because the soil is rich in pathogenic fungi. These compounds may also have an antifungal effect against human pathogenic fungi. A yellow layer under the bark of a tree can indicate the presence of antifungal polyphenols, as found, for example, in the case of the African species *Brackenridgea zanguebarica* Olivier (Ochnaceae).

Random collection is also indispensable. Given the potential of plants which have not yet been investigated and the effort which needs to be taken to make novel therapeutic agents, action needs to be taken in areas which do not yet have an effective remedy, e.g. AIDS, multiple sclerosis, Parkinson's disease, Alzheimer's disease and certain cancers.

4. Biological and pharmacological targets

Obviously when undertaking an investigation of a plant to identify the active principles, it is impossible to isolate all the constituents. Among the hundreds or thousands of different substances, one or only a few are responsible for the therapeutic action (or the toxic activity, if this is relevant). It is necessary, therefore, to have relatively simple

biological or pharmacological tests available in order to localise the chosen activity in the plant extracts or in the numerous fractions resulting from the different purification steps which lead from the plant to the pure active constituents. These tests have to be very sensitive because the active substances may be present in the plant in very low concentrations. They are also required to be specific for the target involved. The principal targets for biological tests can be divided into 6 groups:

- Lower organisms: microorganisms (bacteria, fungi, viruses).
- Invertebrates: insects, crustaceans, molluscs.
- Isolated subcellular systems: enzymes, receptors.
- Animal or human cell cultures.
- Isolated organs of vertebrates.
- Whole vertebrate animals.

If an antifungal or antibacterial activity needs to be investigated, the process is relatively simple. For example, a plant extract or an isolated substance is placed in contact with human pathogenic fungi. It is then straightforward to observe inhibition of spore growth or their death. There is presently a great deal of research underway to develop new antimycotics. This is because of the increased prevalence of systemic mycoses associated with AIDS infections. It is obvious that the fight against viral diseases such as AIDS or herpes is of high priority for numerous research laboratories. Plants are of great potential for their antiviral and antifungal properties. On the other hand, their antibacterial activity, although known for essential oils and for plants such as the bearberry (*Arctostaphylos uva-ursi* (L.) Spreng, Ericaceae), is relatively weak when compared with antibiotics of microbiological origin.

Certain plants have insect repellent or insecticidal properties, while others are active against insect larvae or molluscs (molluscicides). Screening tests for these activities on invertebrates are simple to perform. Insecticidal or larvicidal plants can play an important role in the prevention of tropical parasitic diseases, like malaria or yellow fever, transmitted by mosquitoes. As for molluscicidal plants, they can stop the propagation of schistosomiasis (bilharzia), a parasitic disease with a mollusc (freshwater snail) as intermediate host, which affects many people in Third World countries.

The spectacular progress made during the last few years in cellular biology and molecular pharmacology is of particular importance for biological and pharmacological tests based on mechanisms of action. When the causes of a disease are known, it is possible to act directly on the receptors or enzymes implicated in the etiology of the complication. For example, substances which inhibit cyclo-oxygenase or 5-lipoxygenase, enzymes involved in the process of inflammation, are of great utility in the search for new anti-inflammatory agents. In the war against cancer, inhibitors of the enzymes topoisomerase I and II and protein kinase C, as well as substances which act on the polymerisation of tubulin, are targets for these tests. In the case of benign hyperplasia of the prostate (BPH), which is very frequent in elderly males, inhibitors of enzymes which modify testosterone levels (5 α -reductase, aromatase) are of value. For the treatment of depression, efforts are made to find selective inhibitors of monoamine oxidase, which transforms certain neurotransmitters in the brain. The tests mentioned above are *in vitro* tests, with enzymes of human or animal origin. The substance under

test is placed directly in contact with the target, which is not necessarily the case when taking a medicine orally. The active principle has to be transported in the organism to the target, where it is supposed to exert its effects. Enzymatic tests are generally very specific and very sensitive. They are of special use during the screening of large numbers of samples. The experiments are often relatively easy and require only small amounts of material.

Other *in vitro* tests are made on cell cultures. These are of great importance in cancer research. One of the basic tests is to find cytotoxic molecules or growth inhibitors for tumour cells of human origin. While numerous substances are active *in vitro* on isolated cancer cells, these unfortunately often do not produce useful chemotherapeutic agents. They are toxic to normal cells or do not reach the target tumour.

Sometimes, tests on cell cultures are replaced by investigations on isolated organs of animals. Pharmacological models such as the perfused frog heart have been used for the study of cardiac glycosides. Other tests are carried out on the perfused liver, guinea pig heart, isolated chicken veins etc. The information provided by these tests is often useful but hardly gives any indication of the mode of action of the sample and cannot be extrapolated to the human situation.

Finally, testing on live animals still takes up a large place in the development of new therapeutic agents, even though enormous efforts are being made to find substitutes for this procedure.

5. Chemical screening

When searching for active plant metabolites, biological screening followed by activity-guided fractionation is the standard procedure. Bioassays also serve as a guide during the isolation process. However, the number of available targets is limited. Moreover, bioassays are not always predictive for clinical efficacy. And the bioassay-guided fractionation strategy frequently leads to the isolation of known metabolites. Chemical screening of crude plant extracts therefore constitutes an efficient complementary approach, allowing localisation and targeted isolation of new types of constituents with potential activities. This procedure also enables recognition of known metabolites at the earliest stage of separation, thus avoiding costly and time-consuming isolation of common constituents. The potential of the chemical screening strategy has been considerably increased by the recent development of hyphenated techniques, which are able to provide efficient separation of metabolites and, at the same time, valuable structural information on-line.

5.1. Metabolite profiling: a LC-multi-hyphenated strategy

When compared to the classical use of UV, MS and NMR spectroscopy applied to pure natural products, ideally the integration of all these techniques in their hyphenated forms (LC/UV LC/MS and LC/NMR) in a single setup, with centralised acquisition of the spectroscopic data, should permit the complete spectroscopic characterisation of different metabolites in a mixture in a single analysis. Furthermore, other existing hyphenated techniques such as LC/IR or LC/CD may also bring valuable complementary information. In practice, however, many factors may hinder on-line

detection and structure determination of an unknown plant metabolite and often only partial structure information will be obtained. These on-line data however already provide invaluable information for targeting the isolation of new compounds or for the dereplication of known constituents.

In our approach, the LC/NMR analyses are performed independently from the LC/UV/MS runs. LC/UV/MS is used as a first dereplication step for the chemical profiling of crude plant extracts, and compounds are tentatively identified based on molecular weight and fragment information with manual search in natural product libraries as well as on matching with UV in-house spectral libraries. LC/NMR is mainly used in a second step for a more detailed structural investigation of compounds presenting original structural features or displaying interesting activities after LC-bioassays (see Figure 2).

The combination of all these techniques in a single setup is however possible and the creation of a 'total analysis device' has been recently demonstrated in the case of on-line HPLC-UV(DAD)-FT-IR-NMR-MS analyses. The coupling of all these different techniques (especially LC/NMR-MS) is, however, not an easy task to perform since operation conditions that are compatible with all of them have to be found. The possibility of acquiring all data during a unique analysis gives the possibility to efficiently associate the set of on-line spectroscopic data to a given peak and renders the processing more easy to perform.

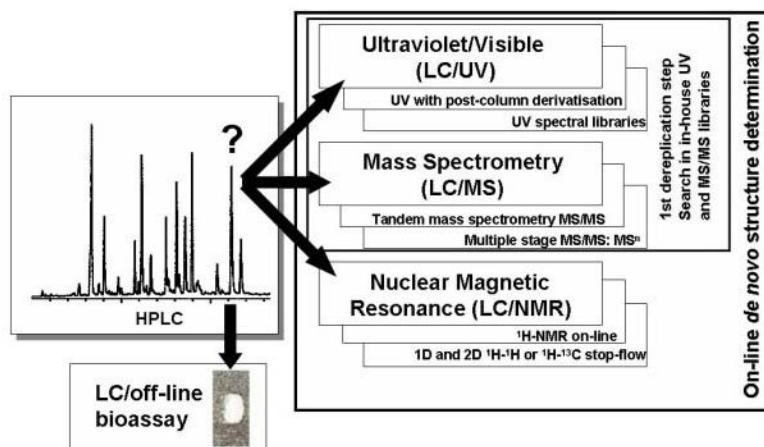


Figure 2. Information that can be obtained from a given LC peak with the different LC hyphenated techniques available. In this approach LC/NMR can be regarded as an efficient complement to LC/UV/MS for *de novo* structure identification of natural products on-line.

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Bibliography

Albert K. (2002) *On-line LC-NMR and Related Techniques*, John Wiley & Sons, Chichester. [This book provides extensive data concerning LC/NMR].

Cogne A.L., Queiroz E.F., Wolfender J.-L., Marston A., Mavi S., Hostettmann K. (2003). On-line identification of unstable catapol derivatives from *Jamesbrittenia fodina* by LC-MS and LC-NMR analysis. *Phytochemical Analysis* **14**, 67-73. [This article provides data concerning the structure elucidation of unstable compounds using the LC/NMR and classical 2D/NMR].

Garo E., Wolfender J.-L., Hostettmann K., Hiller W., Antus S., Mavi S. (1998). Prenylated flavanones from *Monotes engleri*: On-line structure elucidation by LC/UV/NMR. *Helvetica Chimia Acta* **81**, 754-763. [This article provides data concerning the structure elucidation of prenylated flavanones using the LC/NMR].

Cragg, G. M., Newmann, D. J. and Snader, K. M. (1997). Natural products in drug discovery and development. *Journal of Natural Products* **60**, 52-60. [This article provides an overview concerning drug discovery in natural products].

Guilet D., Gunther A., Ioset J.R., Queiroz E.F., Ndjoko K., Foggin C.M., Hostettmann K. (2003). Absolute configuration of a new tetrahydrophenanthrene from *Heliotropium ovalifolium* by LC-NMR of its Mosher esters. *Journal of Natural Products* **66**, 17-20. [This article provides data concerning the determination of absolute configuration by LC/NMR].

Hostettmann, K., Marston, A. (1994). Search for new antifungal compounds from higher plants. *Pure and Applied Chemistry* **66**, 2231-2234. [This article provides data concerning the strategy for isolation of antifungal compounds from higher plants].

Hostettmann, K. (1997). *Tout savoir sur le pouvoir des plantes, sources de médicaments*. Favre SA, Lausanne. [This book provides extensive data concerning plants as a source of drugs].

Hostettmann, K., Marston, A. and Hostettmann, M. (1998). *Preparative Chromatography Techniques: Applications in Natural Product Isolation, 2nd. Ed.*, Springer, Berlin. [This book gives different approaches in natural product isolation].

Markham K.R. (1982). *Techniques of Flavonoid Identification*, Academic Press, London. . [This book provides extensive data on flavonoid chemistry].

Queiroz E.F., Wolfender J.-L., Atindehou K., Traore D., Hostettmann K. (2002). On-line identification of the antifungal constituents of *Erythrina vogelii* by liquid chromatography with tandem mass spectrometry, ultraviolet absorbance detection and nuclear magnetic resonance spectrometry combined with liquid chromatographic micro-fractionation. *Journal of Chromatography A* **974**, 123-134. [This article provides data concerning the structure elucidation of antifungal compounds using the combination of different hyphenated techniques].

Queiroz E.F., Wolfender J.-L., Raelison G.E., Hostettmann K. (2003) Absolute configuration determination of two 6-alkylated -pyrones using LC/NMR. *Phytochemical Analysis* **14**, 34-39. [This article provides data concerning the determination of absolute configuration by LC/NMR].

Biographical Sketches

Emerson F. Queiroz studied pharmacy at University of Paraíba, Brazil. He completed his PhD under the direction of Prof. André Cave at the University of Paris, France. In 1999 he joined the Laboratory of Pharmacognosy and Phytochemistry of University of Lausanne as senior Postdoctoral associate under the direction of Prof. Kurt Hostettmann. His research includes the isolation and the structure elucidation of bioactive natural products from higher plants. He has also been involved in new applications of LC/MS and LC/NMR for the discovery of lead compounds.

Andrew Marston is currently a senior research associate in the Laboratory of Pharmacognosy and Phytochemistry of the University of Geneva, Switzerland. After receiving his PhD in organic chemistry at the University of Liverpool, UK, he was a postdoctoral fellow at the German Cancer Research Institute, Heidelberg. He has been working in the field of medicinal plant research with Kurt Hostettmann since

1983. Dr. Marston is one of the pioneers in the development of preparative separation methods, especially centrifugal partition chromatography (CPC), for the discovery of new bioactive plant metabolites

Kurt Hostettmann studied chemistry at the University of Neuchâtel, Switzerland. After a postdoctoral stay at Colombia University, New York, he joined the Department of Pharmacy of ETH Zürich as senior research associate. In 1981, he was appointed Professor of Pharmacognosy and Phytochemistry at the School of Pharmacy, University of Lausanne. He has been director of this institute ever since, but in September 2004 moved with his whole group to the University of Geneva. He is involved in the phytochemical investigation of plants used in traditional medicine. The aim of his research is to find new lead compounds from Nature which can become drugs. He is also interested in the development of new separation techniques for natural products. He is the author of more than 450 publications, of 60 chapters in books and of 10 books. He has obtained several distinctions, for example Doctor Honoris Causa of the University of Medicine and Pharmacy of Iasi, Romania and the University of Toulouse in France. He is also Honorary Professor at the Institute of Materia Medica of the Chinese Academy of Sciences in Shanghai, at Nanjing University, China and University of Panama.

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