Raquel C. Jadulco

Isolation and Structure Elucidation of Bioactive Secondary Metabolites from Marine Sponges and Sponge-derived Fungi

Cuvillier Verlag Göttingen

Isolation and Structure Elucidation of Bioactive Secondary Metabolites from Marine Sponges and Sponge-derived Fungi

(Isolierung und strukturelle Identifizierung von biologisch aktiven Naturstoffen aus marinen Schwämme und aus Schwämmen isolierte Pilze)

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus Bohol, Philippinen

Würzburg 2002

Eingereicht am:

Vorsitzender: Prof. Dr. R. Hedrich
1. Gutachter : Prof. Dr. P. Proksch
2. Gutachter: Prof. Dr. H. Gimmler, vertr. Prof. Dr. W. Kaiser

Tag der Promotionskolloqioms:

Doktorkunde ausgehändigt am:

Erklärung

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation „Isolierung und strukturelle Identifizierung von biologisch aktiven Naturstoffen aus marinen Schwämme und mit Schwämmen assoziierten Pilze“ selbständig angefertigt und keine anderen als die angegebenen Quellen und hilfsmittel benutzt habe. Ich habe diese Dissertation in gleicher oder ähnlicher Form in keinem anderen Prüfungsverfahren vorgelegt. Außerdem erkäre ich, das ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, 08. 07. 2002

Raquel C. Jadulco

Acknowledgment

I would like to acknowledge the following persons without whom this work would not have been made possible:

Prof. Dr. P. Proksch, my “Doktorvater“, adviser, and mentor for providing me the opportunity to be involved in the field of marine natural product research. His unwavering support, constant encouragement, and expert guidance was what inspired me to strive for more achievements.

Prof. Dr. F.-C. Czygan for his support and Prof. Dr. H. Gimmler and Prof. Dr. W. Kaiser for their willingness to evaluate this work.

Former Dean Leticia Barbara B. Gutierrez of the UP College of Pharmacy, Prof. Mildred B. Oliveros, and my department head Prof. Thelma A. Rivera for their support and encouragement during my stay here in Germany.

The DAAD (Deutscher Akademischer Austauschdienst) for the scholarship grant and Dr. Christa Klaus, my DAAD reference person, for her personal guidance and genuine interest in the progress of my research work.

Dr. Karsten Schaumann, Mr. Stefan Steffens (AWI Bremerhaven) and Mr. Jan Hiort (HHU) for the collection, isolation and mass cultivation of Mediterranean Sea-derived fungi; Dr. M. Assman and Dr. Thomas Fendert for the collection of the Indonesian fungi and Prof. Dr. Udo Gräfe (Hans Knoll Institut für Naturstofforschung, Jena) for the mass cultivation of the pure fungal isolates; Mr. Gernot Brauers, Frau Franka Teuscher and Mr. Hefni Effendi for their help in the isolation of pure fungal isolates from the Indonesian- and Philippine-collected fungi.

Dr. R. van Soest (Zoological Museum , University of Amsterdam) for the identification of the sponge materials and Dr. R. A. Samson (Centraalbureau voor Schimmelcultures) for the identification of the fungi species.

Dr. Victor Wray (Gesellschaft für Biotechnologische Forschung, Braunschweig), for the measurement of the NMR spectra and his patient assistance in the interpretation of my data and likewise, Prof. Dr. Peters (Institut für Organische Chemie) for NMR measurements in HHU Düsseldorf.

The late Dr. L. Witte (Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig), Dr. U. Matthiesen, Dr. Albrecht Berg Gräfe (HKI für Naturstofforschung, Jena), and Dr. Peter Tommes (HHU Düsseldorf) for the measurement of the EIMS data and Prof. Dr. Walter Frank (Institut für Anorganische Chemie and Strukturchemie, HHU) for the X-ray crystal analysis.

Dr. U. Hentschel and Ms Anja Friedrich (Institut für Molekulare Infektionsbiologie, Universität Würzburg) for the bacterial cultures used in the antimicrobial assays.

Dr. Klaus Steube of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) for the cytotoxicity tests using human leukemia cell lines.

Dr. RuAngelie Edrada and Dr. Rainer Ebel for always taking time to give their expert advise and assistance in almost every difficulties encountered in this work, most especially in the interpretation of NMR data.

My colleagues at the Department of Pharmaceutical Chemistry, University of the Philippines (UP) College of Pharmacy: Dr. Alicia P. Catabay, Rhona Limcangco and Rowena Monton for their help in the collection of the sponges and in the isolation of fungi; my colleagues at the Department of Industrial Pharmacy, UP for their help in the freeze drying of the sponge materials and for the use of their laminar flow hood.

My ‘old‘ colleagues from the University of Wuerzburg especially Dr. RuAngelie Edrada, Dr. Bambang Nugroho and Chaidir, as well as Mr. Gernot Brauers and Mr. Jan Hiort from the ‘marine fungi‘ group for guiding me during my first months in the research team; my batch-mate who passed away, Chocho Min, for her friendship and her company; and my ‘new‘ colleagues at the University of Düsseldorf for a pleasant working atmosphere.

To my family and friends who never failed to send their prayers and greetings through letters, phone calls, emails and text messages and showing me with their stories a glimpse of home.

To my Mother

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Zusammenfassung

Niedermolekulare Naturstoffe aus Bakterien, Pilzen, Pflanzen und marinen Organismen weisen eine einzigartige strukturelle Diversität auf, die für die Identifizierung neuer Leitstrukturen für die Entwicklung von Arzneistoffen und Pflanzenschutzmitteln von großer Bedeutung ist. Im Rahmen der Suche nach bioaktiven Verbindungen aus marinen Schwämmen und mit diesen Schwämmen assoziierten Pilzen wurden in dieser Arbeit insgesamt 26 Sekundärstoffe isoliert, wobei es sich bei acht Substanzen um neue Verbindungen handelt. Die Schwämme wurden im indo-pazifischen Gebiet gesammelt, insbesondere aus Indonesien und den Philippinen, so wie aus dem Mittelmeer in der Nähe der Insel Elba in Italien. Für die Entdeckung neuer bioaktiver Substanzen wurde eine Kombination von chemischen und biologischen Methoden angewendet, wodurch Extrakte mit verschiedenen Screening-Methoden auf Bioaktivität getestet worden sind. Zum Einsatz kamen dabei Versuche mit Raupen des polyphagen Nachtfalters *Spodoptera littoralis* (Noctuidae; Lepidoptera) im Hinblick auf potentielle insektizide Wirkungen, antimikrobielle Untersuchungen mit gram-negativen und gram-positiven Bakterien und dem Pilz *Candida albicans*, Zytotoxizitätstests gegenüber menschlichen Krebszellen und Toxizitätstests mit dem Krebs *Artemia salina*. Zusätzlich zur bioaktivitäts-geleiteten Isolierung von Substanzen aus aktiven Extrakten wurden daneben auch DC, UV und MS als Kriterien herangezogen, um die aus chemischer Sicht interessantesten Verbindungen zu isolieren. Damit konnten auch solche Substanzen, die nicht für die Aktivität der Extrakte im Bioscreening verantwortlich waren, weiteren Biotests unterzogen werden.

Im einzelnen wurden die folgenden Verbindungen isoliert, ihre Struktur aufgeklärt, und ihre biologische Aktivität näher charakterisiert:

1. Der antimikrobiell aktive Extrakt aus dem Pilz *Cladosporium herbarum*, der mit dem indonesischen Schwamm *Callyspongia aerizusa* assoziiert ist, ergab sieben Polyketide, die strukturell ähnlich sind, einschließlich der beiden neuen zwölf-gliedrigen Makrolide Pandangolid 3 und Pandangolid 4, sowie ein neues acetyliertes Derivat des bereits bekannten Naturstoffs 5-Hyroxymethyl-2-furancarbonsäure. Beide Furancarbonsäuren zeigten antimikrobielle Aktivität und

dürften deshalb hauptsächlich für die antimikrobielle Aktivität des Extrakts verantwortlich sein.
Daß Cladospolid B, ein bekanntes Phytotoxin, das bereits für die Arten *Cladosporium cladosporoiodes* und *C. tenuissimum* beschrieben wurde, ebenfalls aus *C. herbarum* isoliert wurde, deutet darauf hin, daß Cladospolid B als ein chemotaxonomischer Marker für bestimmte *Cladosporium*-Arten angesehen werden könnte.

2. Der antimikrobiell aktive Extrakt aus dem Pilz *Curvularia lunata*, der mit dem indonesischen Schwamm *Niphates olemda* assoziiert ist, ergab drei Substanzen, nämlich das neue antimikrobiell aktive Anthrachinon Lunatin sowie das bereits bekannte Bisanthrachinon Cytoskyrin A, und das bekannte Pflanzenhormon Abscisinsäure. Das gemeinsame Vorkommen der beiden strukturell verwandten Anthranoide könnte ein Indiz dafür sein, daß das Monomer Lunatin eine biogenetische Vorstufe des Bisanthrachinons Cytoskyrin A darstellt.

3. Ein mit dem im Mittelmeer gesammelten Schwamm *Axinella verrucosa* assoziierter Pilz der Gattung *Penicillium* ergab insgesamt sechs Substanzen, im einzelnen das bekannte Antimykotikum Griseofulvin und dessen weniger aktives Dechlor-Derivat, das bekannte Toxin Oxalin, sowie die als zytotoxisch beschriebene Verbindung Communesin B und deren neue Derivate Communesin C und Communesin D. Im Vergleich zu Communesin B erwiesen sich die neuen Communesin-Derivate als weniger aktiv gegenüber dem Krebs *A. salina.*

4. Ein bisher unidentifizierter Pilz aus dem gleichen Schwamm *Axinella verrucosa* lieferte die bekannte Substanz Monocerin, über deren phytotoxische und insektizide Eigenschaften bereits berichtet wurde.

5. Der mit dem philippinischen Schwamm *Hyrtios* aff. *reticulatus* assoziierte Pilz *Aspergillus flavus* ergab das bereits bekannte Toxin α-Cyclopiazonsäure.

6. Der indonesische Schwamm *Agelas nakamurai* lieferte vier bromierte Pyrrol-Alkaloide, nämlich die neue Substanz 4-Brompyrrol-2-carbonsäure sowie die bereits bekannten Verbindungen 4-Brompyrrol-2-carboxamid, Mukanadin B und

Mukanadin C. Alle vier Substanzen außer Mukanadin B zeigten antimikrobielle Aktivität. Bromierte Pyrrol-Alkaloide wurden in vielen Untersuchungen als typische Sekundärstoffe der Schwammgattung *Agelas* beschrieben, die bei der chemischen Verteidigung der Schwämme gegen Fische eine wichtige Rolle spielen.

7. Der indonesiche Schwamm *Jaspis splendens* ergab drei bekannte Substanzen, die für ihre antiproliferative Aktivität bekannt sind, nämlich die Depsipeptide Jaspamid (Jasplakinolid) und dessen Derivate Jaspamid B und Jaspamid C.

I. Introduction

1.1. Significance of the study

1.1.1. The need for lead compounds for drug development

Even today, after more than 100 years of research in pharmaceutical industries, there is still a great need for innovative drugs. Only one third of all diseases can be treated efficiently [Müller *et al*., 2000]. This means that there is a need for new drug entities to enable therapeutic innovations.

The economic importance of natural products is evident in a conference entitled *Profiting from biodiversity by leveraging natural product discovery*, which took place on 28-29 June 1999 in London. It was highlighted that ten of the top 20 selling medicines in 1998 were derived from natural products [Lawrence, 1999]. It was also claimed that drugs derived from natural products acquire a market share of about 80 billion US $ in the world pharmaceutical market [Müller *et al*., 2000]. Furthermore, a study using US-based prescription data from 1993 showed that over 50% of the most-prescribed drugs in the US had a natural product either as the drug, or as a 'forebear' in the synthesis or design of the agent [Grifo *et al*., 1997], thus demonstrating that natural products still play a major role in drug treatment.

The role of natural products in drug discovery is demonstrated by an analysis of the number and sources of anticancer and antiinfective agents, reported mainly in the Annual Reports of Medicinal Chemistry from 1984 to 1995 [Cragg, 1997]. It was observed that over 60% of the approved drugs and pre-NDA (New Drug Applications) candidates (for the period 1989-1995), excluding biologics (vaccines, monocloneals, etc. derived from mammalian sources), developed in these disease areas are of natural origin.

Drugs of natural origin have been classified as original natural products, products derived semisynthetically from natural products, or synthetic products based on natural product models [Cragg, 1997]. Bioactive natural products could thus serve as

lead structures which could then be ‘optimized’ through classical medicinal chemistry techniques and the more recent combinatorial synthesis methods to come up with new agents with improved pharmacokinetics and/or toxicology (Fig. 1.1).

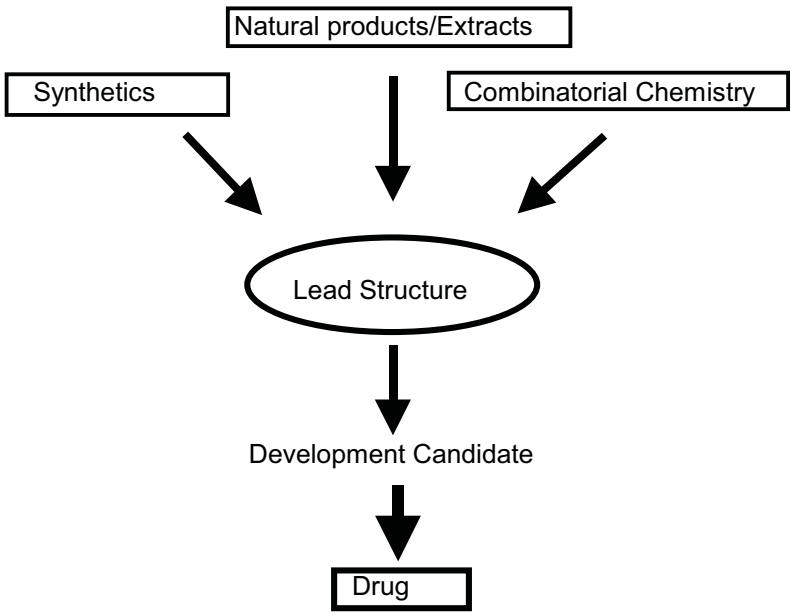

Fig. 1.1: Sources of compounds for drug discovery.

A lead compound is a compound with many of the characteristics of a desired new drug which will be used as a model for chemical modification. It must be potent, but it does not need to possess the potency at the nanomolar or picomolar levels expected of a product candidate. It must be specific for the desired target, but it does not need to possess the exquisite biochemical specificity required for a new drug. Finally, it must be available in sufficient quantities to support the early stages of development, such as biological characterization and toxicity studies, while a total synthesis of the product candidates is being completed.

Low-molecular mass natural products from bacteria, fungi, plants and marine organisms exhibit unique structural diversity, and are of maximum interest for identifying new lead structures. Thomas Henkel (Bayer AG, Wuppertal, Germany) reported that most natural products have a higher molecular weight than their synthetic counterparts, containing more rings and being generally more sterically

complex [Lawrence, 1999]. Furthermore, Henkel found that, on comparison of the compounds in a natural product datase (DNP) with a representative pool of chemical test substances (Synthetics), there was only a 60% homology. Henkel also demonstrated that by comparing the structures of natural products, synthetic drugs, and currently used drugs, there was a much higher incidence of O-containing groups in the natural product compounds. Furthermore, natural product compounds were found to contain more sp^3-hybridized bridgehead atoms. This illustrates the diverse range of compounds that can be gained from natural products that would otherwise be missed using synthetic techniques.

1.1.2. Strategies for drug development from natural products

Generally, strategies for drug discovery can be separated into three categories: chemically driven, biologically driven and a combination of both [McConnell *et al*., 1994]. In the chemically driven or 'traditional grind-and find' approach, which has been pursued mainly by academic research groups, the object of the search was to find novel compounds from marine sources. Hence, extracts are 'screened' by TLC, ^1H and ^{13}C NMR for unusual and interesting patterns. The next step for this approach is then finding biological properties for purified compounds. The biologically driven strategy is the bioassay-guided approach beginning with crude extracts and has been the preferred method by modern marine natural product researchers.

The biologically driven approach which involves 'screening' crude extracts for biological activity, followed by the crucial work of backtracking the active compounds from the 'hit'-extracts dominate natural products research up to the present. However, a lot of experience is required to exclude both false positive and false negative results. Considerable effort is required to get access to sufficient quantities of raw material for reproduction, isolation, structure elucidation and subsequent verification of biological activity. The complete process proved to be highly time and capacity consuming. Moreover, false positives may result when the activity shown by an extract is attributed to a synergistic effect of more than one constituent in the extract.

It seems advantageous to perform a screening with pure compounds rather than with crude extracts. For individuals, however, the problem arises of getting access to sufficient numbers of natural compounds covering a substantial structural diversity. A new approach utilized by pharmaceutical industries for the discovery of new drugs is the creation of a central natural product pool. With a natural product pool, supplied by the industry and the academic institutions, compounds are getting a more realistic chance to be discovered and highlighted in diverse target directed bioassay systems of therapeutic value. Together with high thoroughput screening (HTS), a greater number of 'hits' of lead compounds have been identified.

The development of new bioassay methods that can selectively detect biologically active molecules at very low levels as well as the advances in chemical instrumentation (e.g. high performance liquid chromatography (HPLC), high performance centrifugal countercurrent chromatography (HPCCC), capillary zone electrophoresis (CZE), high resolution mass spectrometry (HRMS), high field nuclear magnetic resonance (NMR) and X-ray crystallography which now allow the chemist to isolate submilligram quantities of the new compounds, and confidently be able to fully characterize them and identify their structures contributed to the current peak in interest in natural products.

New bioassays which target receptors and enzymes involved in pathogenesis of disease are being developed. These assays reflect new opportunities due to the recent identification of previously unrecognized biomolecular targets for therapy.

1.2. Current status of marine natural product research

Although natural products research was previously focused mainly on plants, growing interests in marine natural products have led to the discovery of an increasing number of potently active agents considered worthy for clinical application. The world's oceans cover more than 70% of the earth's surface represent our greatest resource of new natural products [McConnell *et al*., 1994]. The sea contains well over 200,000 invertebrate and algal species [Ibid]. There exist nearly 150,000 species of algae (sea weed): green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta), and some groups of marine invertebrates in which new chemical structures or

biological activities have been reported: sponges (Porifera), cnidarians or coelenterates [corals, octocorals (including sea fans), hydroids, and sea anemones], nemerteans (worms), bryozoans, ascidians (tunicates including sea squirts), molluscs (sea snails and sea slugs), and echinoderms (brittlestars, sea urchins, starfish, and sea cucumbers) [Ibid].

The earliest findings include the arabinose-nucleosides, known since the 1950's as constituents of the Carribean sponge *Cryptotethya crypta* (Tethydae) which served as lead compounds for the synthesis of analogues, ara-A (Vidarabin, Vidarabin Thilo®) and ara-C (Cytarabin, Alexan®, Udicil®) with improved antiviral and anticancer activity. Since then however, the systematic investigation of marine environments as sources of novel biolgically active agents only began in earnest in the mid-1970s. During the decade from 1977-1987, about 2500 new metabolites were reported from a variety of marine organisms [Newman *et al*., 2000]. Prior to 1995, a total of 6500 marine natural products had been isolated; by January of 1999, this figure had risen to approximately 10,000 [Jaspars, 1999]. These studies have clearly demonstrated that the marine environment is a rich source of bioactive compounds, many of which belong to totally novel chemical classes not found in terrestrial sources.

A recent review provided an updated list of marine natural products which are currently under clinical trials (Table 1) (Fig. 1.2) [Proksch *et al*., 2002].