New Principles for Targeting Cancer - a Rational Small Molecule Approach

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Center for Microbial Biotechnology
Department of Systems Biology
New principles for targeting cancer
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Preface

This thesis is submitted to the Technical University of Denmark in partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Chemistry. The work was carried out between February 2008 and June 2011, at the Center for Microbial Biotechnology at the Department of Systems Biology and the Department of Chemistry under the supervision of Associate Professor Thomas Ostenfeld Larsen and Associate Professor Mads Hartvig Clausen. January and February 2010 was spent at Deutsches Krebsforschungszentrum (DKFZ), University of Heidelberg, Germany under the supervision of Professor Alwin Krämer, MD. The project was funded by the Danish Research Council (ref. 274-07-0561).

I would like to thank my two supervisors Thomas Ostenfeld Larsen and Mads Hartvig Clausen for three years of incredibly exciting work. You have both been enthusiastic throughout the project and have been an inexhaustible source of ideas for current and future projects, thank you for that. You have also listened when I have told you that I would need another three years to perform all those experiments, for that I am equally thankful. If the rest of my work life proves equally enjoyable, I will be very privileged.

A thank also goes to Alwin Krämer, for letting me work in his research group during my stay in Heidelberg. I learned so incredibly much about biology in general and centrosomal clustering in particular during those two months. I would also like to thank Blanka Leber for not only tutoring me at work but also for opening her and Armin’s home to me. A thank to Simon Anderhub for the hard work he has done in the lab and for swift answers to all the questions I have sent his way. Also thank to Dr. Marc-Steffen Raab, MD for his work with GF15 and GF61 and his contribution to the cooperation in general. I will definitely miss the biannual gatherings, filled with important discussions about the future of the project, but also with sufficient time to go to dinner, have a few beers and talk about life in general.

I have received ample amount of help from a number of people: Lisette and Kir have been a great help for both preparing raw extracts and bringing up fungi from the collection, on top of that they have been great fun to work with and their door has always been figuratively open, even if knocking was mostly answered with a NO! :-). Jesper, Kristian and in particular Hanne have been an indispensable help with running the machine park in 221, thank you for that. I
would also like to thank my office mates in 221, Maria and Jesper for some great years. The office has never been in shortage of professional or personal advice. I would also like to thank the rest of 221 for a great work environment and for the many talks around the coffee machine during the years.

I would like to thank Charlotte and Anne for running those many many NMR experiments and for general hall talks. Also thank to Brian for the help during my PhD and in particular for a number of rotations and IR spectra. I would like to thank Palle and Hélène for the time at Mads’ group. I had the pleasure of sharing a lab with Mathias and although I never knew where my acetone bottle was, or for that matter anything else in the lab, it was a time with lots of organic chemistry talk as well as a fair share of friendly banter. Also a thank to the rest of the people in 201 for making the last three years enjoyable.

Last I would like thank my family for the support during the last three years. That goes for my mom and Niels who took good care of Nanna, Iben and Asger when I worked nights and weekends during the last few months. The same goes for Børge, Hanne, Ellen, Peter and Mette who all visited in the weekends, enabling me to work even more. Thanks to Børge for moral support on the phone when things got rough. Thanks to Andreas and Lea for moving excel data around and for moral support. I am also thankful to Iben and Asger, whom at days of work despair could blow all worries away with a few smiles. Most of all though I am grateful for having such a wonderful wife, she has been an absolutely invaluable help throughout the PhD. Thanks for keeping the house pretty much by yourself for a long time towards the end and thanks for all the professional help that you gave during the three years.
Abstract

Cancer is the leading cause of death in the developed world and research is ongoing both towards curing the disease and understanding the disease on a cellular level. As most cancer cells proliferate very fast, mitosis has often been the target of anti-cancer agents. However, this does not afford cancer specific drugs as healthy cells are also affected, albeit to a lower extent. An ideal anti-cancer drug would target cancer cells specifically, thus obviating side effects.

A difference between healthy and cancerous cells that is currently being explored is the supernumerary centrosomes present in most cancer cells. Centrosomes are the microtubule organizing centers during mitosis and here, one centrosome is situated at each pole of the mitotic spindle. During anaphase, each sister chromosome will be pulled towards the centrosomes in bipolar mitosis. Supernumerary centrosomes enable the cancer cells to undergo multipolar mitosis, introducing chromosomal instability, which would make it unlikely to produce viable progeny. The cancer cells circumvent multipolar mitosis by a mechanism called centrosomal clustering, where any numbers of centrosomes are gathered in two clusters, thus making bipolar mitosis possible. A small molecule that could disrupt centrosomal clustering and force the cells to undergo multipolar mitosis might possess the ability to affect cancer cells specifically.

Prior to the work in this PhD thesis, the fungal metabolite griseofulvin (1) was identified as an anti-cancer compound in a bio-guided screen of fungal raw extracts in a phenotypic based assay. The assay was designed to identify compounds that forced cancer cells to undergo multipolar mitosis and thus apoptosis, using the cancer cell line SCC114 (squamous cell carcinoma cells). In this work two structure activity relationship (SAR) studies have been completed. The first investigated the anti-cancer SAR of griseofulvin analogs in the phenotypic based assay and the other a growth inhibition SAR against the two dermatophytes Trichophyton mentagrophytes and T. rubrum as well as toxicity against the cancer cell line MDA231 (human breast adenocarcinoma).

The studies found a good correlation between the anti-cancer phenotypic IC$_{50}$ values and the cytotoxicity values ($R^2 = 0.70$). General trends showed that alterations of positions 4, 5 and 6 of griseofulvin lower the activity, while elongation of the 2’ position increases the activity. Bulkier substituents increased the activity even further with the 2’ benzyloxy analog (2) being the most potent. The
introduction of an oxime at the 4’ position increase the potency slightly in both assays but did not show any cumulative effect with the 2’ benzyloxy group (3). After extensive synthesis of 2’ analogs, the benzyloxy (2) and benzyloxy oxime (3) analogs were still the best lead compounds.

It is not possible to distinguish between the two aryl methoxy groups in griseofulvin (1) by NMR. To unequivocally prove the position of the two possible phenols and thus the methoxy groups, the two phenols were synthesized and distinguished by single crystal X-ray analysis. In 1H NMR, the 4-methoxy group resonates upfield from the 6-methoxy group. A quantitative yield was obtained for the synthesis of the 4 phenol by treatment of 1 with MgI₂, while the 6 phenol was obtained in 29% yield by treatment of 1 with LiI in pyridine. (+)-Geodin shares the same structural core as griseofulvin (1) and it was isolated from Aspergillus terreus to be tested in the phenotype based assay. The compound showed no induction of multipolar mitoses but was twice as potent in the cytotoxicity assay compared to 1. The unknown absolute structure of (+)-Geodin was assigned by single crystal X-ray analysis.

The compound aurantiamine (4) was isolated from Penicillium cavernicola and found to induce multipolarity in SCC114 cancer cells with an IC₅₀ of 40 ± 4 μM. This was further investigated by fluorescence-activated cell sorting (FACS) analysis showing M phase arrest in SCC114 cells and an increase in the G₀ population indicating apoptosis. Further indications of apoptosis was observed using Western blotting for the p85 fragment of poly (ADP-ribose) polymerase (PARP), a downstream target of activated caspase 3.

In addition to the above mentioned studies, a novel technique for the introduction of radioactive iodide to aryl groups was developed. This was used in scintigraphic in vivo imaging to assess bio-distribution and half-life of a griseofulvin analog. Treating an aryl trimethylsilyl precursor with 2 equiv. of thallium(III)trifluoroacetate in 30% TFA in MeCN for 2 minutes followed by addition of sodium ¹²⁵I or ¹³¹I affords the radio labeled compound in over 95% radioactive yield. This method is applicable for both ortho, meta and para TMS aryl precursors stable in TFA/MeCN (3:7).
Dansk Resumé

Kæft forårsager de fleste dødsfald i den udviklede del af verdenen og kæft-forskning har derfor høj prioritet. Forskningen fokuserer både på at finde midler, der kan helbrede personer ramt af kæft, men også på at forstå hvordan kæft udvikles. De fleste lægemidler i dag er ikke specifikt rettet mod kæftceller men rammer cellernes mitose. Da kæftceller deler sig meget hurtigere end de fleste andre celler i et menneske, bliver kæftcellerne dog hårdere ramt end de raske celler. Ikke desto mindre ønskes et ideelt lægemiddel, der dræber kæftceller og kun kæftceller.

En måde hvorpå at finde denne slags lægemidler er at se på hvilke forskelle, der er på raske celler og kæft celler. En sådan forskel er de u_normalt mange centrosomer, der findes i kæftceller. I raske celler er der to centrosomer under mitosen, hvor de virker som microtubuli organiseringe centre. Under bipolær mitose sørger de for delingen af datterkromosomerne ud i de to datter celler. Kæftcellerne med mange centrosomer har muligheden for at gennemgå en multipolar mitose, hvilket højst sandsynligt ville resultere i ikke levedygtige datter celler. Kæftcellerne undgår dette ved hjælp af centrosomal klyngedannelse, hvor alle centrosomer bliver klumpet sammen i to klynger, der så kan fungere som to poler som i en regulær bipolær mitose. Et molekyle, der kan inhibere denne centrosomale klyngedannelse, kan potentielt virke specifikt mod kæftceller.

Griseofulvin (1, se Figur 2) blev, forud for dette arbejde, identificeret som et anti-kæft stof i en screening af svampe rækstrakter i et phenotype baseret assay. Dette assay er designet til at identificere molekyler, der kan få kæftceller til at gennemgå multipolar mitose. Der er blevet gennemført to struktur-aktivitet sammenhængsundersøgelser i dette arbejde. Det første omhandlede anti-kæft egenskaberne af griseofulvin analoger i det phenotype baserede assay, mens der i det andet blev testet analoger mod to patogene svampe Trichophyton mentagrophytes og T. rubrum samt en cancer cellelinje.

Der var en god korrelation mellem de IC₅₀ værdier, der blev fundet i det phenotype baserede assay, og toksisitets-assay’et mod kæftcelleinjen (R² = 0.70). Ud fra data kunne det konkluderes, at ændringer i positionerne 4, 5 og 6 generelt resulterede i en lavere aktivitet, mens forlængelse af 2’ position øgede aktiviteten. Større grupper øgede aktiviteten yderligere med en 2’ benzyloxy gruppe som den mest aktive. En oxim i 4’ positionen øgede aktiviteten i begge assays, men en
analog med både en 2’ benzylxoy gruppe samt en 4’ oxim viste ikke akkumuleret aktivitet. Efter grundig undersøgelse af 2’ positionens betydning kunne det konkluderes at 2’-benzyloxy analogen samt versionen med en 4’-oxim stadig er de mest potente.

De to methoxy-grupper i griseofulvin kan ikke skelnes ved hjælp af NMR. For at bevise positionen af de to methoxy-grupper blev phenolerne af griseofulvin syntetiseret og positionen af disse blev bestemt ved hjælp af enkeltkrystal røntgenanalyse. På denne måde kunne positionen af methoxy grupperne også bestemmes, og 4-methoxy gruppen er upfield fra 6-methoxy gruppe. 4-phenol analogen blev syntetiseret i kvantitativt udbytte med MgI₂. 6-phenolen blev syntetiseret med LiI i pyridin i 29% udbytte.

(+)-Geodin har samme grundstruktur som 1 og blev derfor isoleret fra Aspergillus terreus for at blive testet. Det viste sig at (+)-geodin ikke inducerede multipolære mitoser i SCC114 kræftceller, dog var (+)-geodin dobbelt så aktiv i toksicitets assayet i forhold til griseofulvin (1). (+)-Geodin’s absolutte struktur var ukendt men blev bestemt ved hjælp af enkeltkrystal røntgenanalyse til at være R ved spirocentret. Aurantiamine (4) blevisolert fra Penicillium cavernicola og inducerede multipolar mitose i SCC114 kræftceller med en IC₅₀ på 40 ± 4 μM. Inddviklingen af 4 på SCC114 celler blev også undersøgt med fluorescens-aktiveret celle-sortering (FACS), og der blev observeret en ophobning af celler i M fase og en øget G₀ population, hvilket kan indikere apoptose. Ved hjælp af Western blotting efter p85 fragmentet af poly (ADP-ribose) polymerase (PARP) blev det yderligere påvist, at cellerne lavede apoptose.

En ny teknik til at mærke aromatiske stoffer med radioaktivt iod blev udviklet. Behandling af en aromatisk trimethylsilyl (TMS) forbindelse med 2 ækvivalenter Tl(OOCCF₃)₃ i MeCN/TFA (7:3) efterfulgt af radioaktivt kalium iodid (¹²⁵I eller ¹³¹I) førte til det radioaktivt mærkede stof i over 95% udbytte. Denne metode er brugbar for både aromatiske ortho-, meta- og para-aryl TMS grupper så længe de er stabile i MeCN/TFA (7:3).
List of Publications


Non peer reviewed publication:

Thesis Outline

The work for this thesis entitled "New principles for targeting cancer - a rational small molecule approach" is based on the cooperation between the Center for Microbial Biotechnology at the Department of Systems Biology and the Department of Chemistry both at the Technical University of Denmark and the German Cancer Research Center at the University of Heidelberg, Germany. The thesis includes work performed at all three locations, in the fields of natural products chemistry, organic chemistry and microbiology.

The thesis consists of nine chapters. Chapter 1 is a short introduction to cancer, centrosomal clustering, and natural products as bioactive compounds. Chapter 2 is a review of the chemistry of griseofulvin, intended for submission to Chemical Reviews upon further work. Chapter 3 is a published paper concerning an anti-cancer structure-activity relationship study of griseofulvin analogs in a phenotypical assay. Chapter 4 is a manuscript concerning the structure-activity relationship study of griseofulvin analogs against two dermatophytes as well as a cancer cell line in two growth inhibition assays, which has been submitted to the Journal of Medicinal Chemistry.

Chapter 5 is a published paper distinguishing between the two aryl methoxy groups of griseofulvin. Chapter 6 presents the work performed on the development of a radiolabeling technique of aryl trimethylsilyl precursors. Chapter 7 is a published paper concerning the isolation of (+)-geodin and the determination of the absolute structure of (+)-geodin. Chapter 8 presents the work concerning the isolation of aurantiamine and the work performed with this natural product at the German Cancer Research Center (DKFZ), University of Heidelberg. Chapter 9 is the final conclusion of the entire work.

A paper with the title "GF-15, a novel inhibitor of centrosomal clustering, suppresses multiple myeloma growth in vitro and in vivo", which has been submitted to Blood is found in Appendix A. Co-author statements for all articles and drafts in the thesis can be found in Appendix B. Supporting information of articles and drafts can be seen in Appendix C-G. To restrain the number of pages in the Appendix most of the supporting information have been modified to only contain the $^1$H and $^{13}$C NMR spectra of synthesized/isolated compounds. Full supporting information for a given article can be obtained at the appropriate journal homepage.
## Contents

1 Introduction .......................................................... 1

1.1 Centrosomal Clustering .................................... 3
1.2 Natural Products with Anti-cancer Properties .......... 8
1.3 Dereplication .................................................. 9
1.4 Secondary Metabolites from Fungi ................... 10
1.5 Work flow .................................................... 11
1.6 Centrosomal Clustering Assay ............................ 11
1.7 Griseofulvin .................................................. 13

2 Chemistry of Griseofulvin ......................................... 19

2.1 Total Syntheses of Griseofulvin ......................... 20
2.2 Total Syntheses of Griseofulvin Analogs ............... 25
2.3 Spectroscopic Studies of Griseofulvin and Analogs .... 28
2.4 Tables of Griseofulvin Analogs .......................... 29

2.4.1 Griseofulvin Analogs with Modifications at the 2’ Position .... 29
2.4.2 Griseofulvin Analogs with Modifications at the 2’ and 3’ position ... 31
2.4.3 Griseofulvin Analogs with Modifications at the 5’ Position .... 33
2.4.4 Griseofulvin Analogs with Modifications at the 4 Position .... 34
2.4.5 Griseofulvin Analogs with Modifications at the 5 Position .... 35
2.4.6 Griseofulvin Analogs with Modifications at the 6 Position .... 35
2.4.7 Griseofulvin Analogs with Modifications at Position 4 and 6 .... 36
2.4.8 Griseofulvin Analogs with Modifications at Position 5 and 7 .... 36
2.4.9 Isogriseofulvin Analogs with Modifications at the 4’ Position ... 37
2.4.10 Isogriseofulvin Analogs with Modifications at the 3’ and 4’ Positions .................................. 39
2.4.11 Isogriseofulvin Analogs with Modifications at the 4, 5 and 6 Positions ................................... 40
2.4.12 Analogs of both Griseofulvin and Isogriseofulvin with Modifications at Ring A and C .............. 41
2.4.13 Griseofulvic Acid with Modifications at the 3’ Position ...................................................... 41
2.4.14 Disubstituted Griseofulvic Acid at the 3’ Position .............................................................. 42
2.4.15 Griseofulvic Acid with Modifications at the 5, 7 and 3’ Positions ........................................ 43
2.4.16 Griseofulvic Acid with Modifications at the 6 position ....................................................... 43

3 Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells 49

4 Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells 57

5 Synthesis and Single Crystal X-ray Analysis of two Griseofulvin metabolites 73

6 A Technique for Selective ipso-Substitution of ortho-, meta- or para-Aryl Trimethylsilyl Groups with Radioactive Iodide in Quantitative Yields 77

7 (+)-Geodin from Aspergillus terreus 83

8 Aurantiamine from Penicillium cavernicola 89
  8.1 Experimental ................................................................. 91
  8.2 2’-Benzyloxy-2’-demethoxy-griseofulvin and inhibition of centrosomal clustering ......................... 93

9 Conclusion 97

A GF-15, a Novel Inhibitor of Centrosomal Clustering, Suppresses Multiple Myeloma Growth in vitro and in vivo 101

B Co-author Statements 133
<table>
<thead>
<tr>
<th>Supporting Information</th>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3</td>
<td>145</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>189</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>235</td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>255</td>
</tr>
<tr>
<td>G</td>
<td>7</td>
<td>267</td>
</tr>
</tbody>
</table>
In developed countries the single highest cause of death for man is cancer and in developing countries it is the second highest cause. Therefore the attention to and funding for cancer research is massive and it has accelerated in the late 20th century with for example the National Cancer Act of 1971 in the United States. The focus in cancer research is both on treating the disease as well as understanding what goes on at the cell biology level.

So what is cancer? In short it is cells that proliferate abnormally and are able to invade other tissue. It involves the transformation from normal to fully malignant cells through a number of mutations (see Figure 1.1). One mutation or combinations of mutations afford the cells with one or more of the malignant phenotypes required for the cells to develop into being fully malignant. Some phenotypes are: insensitivity to antigrowth signals, mitogen-independent growth, evasion of apoptosis, immortalization, sustained angiogenesis and metastasis. It’s well known that smoking increases the risk of lung cancer and another example of an exogenic factor that is life style related is obesity, which increases the risk of renal cancer. However, factors which people have no or little influence on, like ethnicity and socioeconomic status also have a correlation with the risk of developing cancer.

Cancer can be treated by chemotherapy, radiation therapy or surgery and often involves a combination of these treatments. For chemotherapy a popular drug target has been mitosis and although not truly cancer specific, anti-mitotic drugs are expected to affect malignant cells to a greater extent than healthy cells. After all, uncontrolled cell growth and proliferation is a defining phenotype of most malignant cells and thus the effect from disrupting mitosis should be greater on the malignant cells than on healthy, slower proliferating cells. However, some healthy cells also proliferate fast and the side effects of chemotherapy often stem from the drugs effect on these cell types. An example is hair bulb cells that are often affected by cytotoxic anti-cancer drugs resulting in hair loss (alopecia).

Anti-mitotic agents often target tubulin, with two major groups, those that stabilize microtubules (e.g. Taxol, epothilones) and those that destabilize tubulin (e.g. the vinca alkaloids). In both cases microtubule dynamics are greatly reduced, affecting mitosis and resulting in cellular M phase arrest followed by apoptosis.

The ideal anti-cancer drug would target malignant cells and malignant cells only and thus potentially obviate the side effects of chemotherapy. In order to achieve this, differences between malignant cells and healthy cells must be explored and described. A difference in phenotype that is currently being explored is the fact that centrosome amplification is a common feature in human
Figure 1.1: On top normal cells are depicted with one daughter cell showing DNA damage. This cell will undergo apoptosis because of the many tumor suppressor mechanisms present in the cell. On the bottom more cells with DNA damage are shown, which for some reason are not forced to undergo apoptosis, possibly because the damage has affected some of the tumor suppressor genes. This cell population can then acquire more mutations over time and end up with enough cancer phenotypic characteristics to become fully malignant (called the multistep process). Artwork originally created for the National Cancer Institute. Reprinted with permission of the artist, Jeanne Kelly. Copyright 2011.
cancer cells. If not for a phenomenon called centrosomal clustering, cancer cells would undergo multipolar mitoses resulting in aneuploid daughter cells, which are determined to undergo apoptosis. A schematic overview is depicted in Figure 1.2, where a cell with supernumerary centrosomes undergoes bipolar mitosis with the majority of the progeny undergoing apoptosis. There is an odd chance of a daughter cell surviving the multipolar mitosis and regaining chromosomal stability by centrosomal clustering. This cell is now able to undergo bipolar mitosis and to proliferate. A small molecule that inhibits centrosomal clustering would force this initial survivor to continuous multipolar mitosis resulting in unviable progeny at some point. Such a small molecule would potentially offer a specific treatment of cancer.

### 1.1 Centrosomal Clustering

A centrosome (see Figure 1.3) consists of two centrioles surrounded by an electron dense matrix of protein aggregates, referred to as the pericentriolar material (PCM). The centrosomes are microtubule organizing centers and are essential for correct segregation of chromosomes in diploid cells during bipolar mitosis. During mitosis (see Figure 1.4) of healthy cells the two centrosomes are situated at each pole of the mitotic spindle, and in anaphase one copy of each duplicated chromosome will be pulled to each pole. This results in two daughter cells each with one centrosome and one identical set of DNA after cytokinesis. During the next cell cycle the centrosome and DNA will be duplicated exactly once.

Whereas normal cells contain two centrosomes during mitosis it is common that malignant cells contain supernumerary centrosomes. Having more than two centrosomes could facilitate multipolar mitosis, with subsequent aberrant chromosome segregation and the development of aneuploid daughter cells, which most likely will undergo apoptosis. Centrosome amplification can happen through a number of pathways (See Figure 1.5): faulty regulation of centrosome synthesis, abrupted mitosis, fusion of two cells, fragmentation of centrosomes into smaller fractions that are still able to function as microtubule organizing centers, over-expression of certain pericentriolar material proteins producing a-centriolar microtubule organizing centers, de novo synthesis of centrosomes or decoupled DNA and centrosome cycles. Regardless of the path to supernumerary centrosomes, it evokes chromosomal instability upon the cancer cell, and thus a property to acquire more mutations.

There are currently two models for loss and/or gain of chromosomes for cells with supernumerary centrosomes. A regular multipolar mitosis with three or more poles affording aneuploid daughter cells (see Figure 1.6). In this case it is most likely that tripolar mitosis affords aneuploid but viable daughter cells. It is also possible for cells with extra centrosomes to align in a pseudo bipolar fashion (see Figure 1.6). In this case one centrosome could fail to align with the bipolar axis while still being able to nucleate microtubules and capture chromosomes at their kinetochores. During anaphase the misaligned centrosome would pull chromosomes out of the bipolar axis and one daughter cell would receive extra chromosomes while the other would have lost them (see Figure 1.6).
Figure 1.2: (A) The number of centrosomes in a cancer cell is amplified, which can occur through multiple possible pathways (see Figure 1.5). (B) The cell now undergoes multipolar mitosis with most progeny receiving insufficient DNA to survive, leading to apoptosis (C), while it is possible that one cell receives enough DNA to survive and re-enter the cell cycle (D). The cell needs to regain chromosomal stability to be viable and this is achieved by centrosomal clustering, which enables the cancer cell to undergo bipolar mitosis despite its supernumerary centrosomes (E). Reprinted by permission from Macmillan Publishers Ltd: Oncogene\textsuperscript{31}, copyright 2001.
1.1 Centrosomal Clustering

Figure 1.3: A simple presentation of the centrosome. A pair of centrioles perpendicular to each other embedded in a cloud of pericentriolar material (PCM). The centrosomes function as the main microtubule organizing center in the cell. Reprinted by permission from Macmillan Publishers Ltd: Oncogene, copyright 2002.

Figure 1.4: The cell cycle of a healthy cell is depicted. DNA and centrosome replication occur in S phase (not shown), during interphase. In prophase the unordered DNA is folded into sister chromosomes and in metaphase the mitotic spindle is assembled. Here the chromosomes are aligned in between the two centrosomes at each pole. During anaphase each sister chromosome is pulled towards a centrosome followed by telophase, where the contractile ring initiates cytokinesis. After completed cytokinesis two identical daughter cells have been formed, which can now proliferate further. Copyright © 2008 from Molecular Biology of the Cell by Bruce Alberts et al. Reproduced by permission of Garland Science/Taylor & Francis Books, Inc.
Figure 1.5: Four possible pathways to supernumerary centrosomes. (A) A cell that has synthesized extra copies of centrosomes during a single cell cycle will contain extra centrosomes. (B) Aberrant mitosis will leave the cell with two sets of copied chromosomes and two centrosomes that will be duplicated in the next cell cycle leading to multiple centrosomes in a single cell. (C) If splitting of the two centrioles of one centrosome is unsupervised it could result in more than two functional microtubule organizing centers. (D) Increased production of pericentriolar material due to over expression could also result in more than two functional microtubule organizing centers. Reprinted by permission from Macmillan Publishers Ltd: Oncogene, copyright 2002.
Figure 1.6: Multipolar and pseudo-multipolar mitosis with cells harboring supernumerary centrosomes are pictured. (A) Two tripolar metaphase spindles are shown in (a), centrosomes are yellow, microtubules in red and DNA in blue. (b) Three daughter cells from a tripolar mitosis are seen. These three cells should show significant alterations to their DNA content as compared to the mother cell. (B) A cell with supernumerary centrosomes has aligned in a pseudo bipolar fashion, but with a single centrosome out of alignment. During anaphase, this centrosome will pull chromosomes out of the bipolar axis, which results in aneuploid daughter cells as one will have lost and one gained chromosomes. (C) A schematic presentation of the chromosome loss/gain pathway. Reprinted by permission from Macmillan Publishers Ltd: Oncogene, copyright 2002.
In a tripolar mitosis there are still only two copies of DNA and dividing the genes between the three daughter cells would most likely render them all aneuploid leading to apoptosis. It is possible to imagine though, that during the multitude of cell divisions in cancer cells, one daughter cell arising from a multipolar mitosis would receive a sufficient amount of DNA to survive. It is also possible that mutations or genes missing will grant the cell a new cancer phenotype. Continual proliferation of this aneuploid cell is dependent on regaining chromosomal stability, as continual multipolar mitoses would most likely result in apoptosis at some point. An aneuploid daughter cell with supernumerary centrosomes needs to acquire the ability to undergo bipolar mitosis and this can be achieved by centrosomal clustering.

As cancer cells need to cluster their centrosomes to undergo bipolar mitosis and healthy cells do not, a small molecule that inhibits centrosomal clustering would potentially be specific towards cancer cells. A phenotype based assay to search for such small molecules was established by Prof. Dr. Alwin Krämer MD, and Dr. Blanka Rebacz from DKFZ, Germany. See section 1.6 for a short description of the assay.

1.2 Natural Products with Anti-cancer Properties

Medicine derived from nature has most likely been used since before civilization started, with the earliest known records from Egypt around 2900 BC. Today almost 5000 years later, nature is still the major contributor of drugs to mankind. In a period from January 1981 to October 2008, 1024 new anti-cancer drugs were introduced on the market. Of those drugs only 37% were truly synthetic, the rest were in some way inspired by nature. This indicates that looking for novel bioactive compounds in nature is still a valid strategy.

There is a vast pool from which natural products can be derived, compounds have been isolated from plants, insects, bacteria, fungi, and marine organisms (See Figure 1.7 for examples of compounds). In the case of fungi, a raw extract is produced by grinding the fungi and agar in a stomacher with an organic solvent, which upon concentration affords the raw extract. With a raw extract available there are several approaches to the isolation of the natural products, one method is to isolate every novel compounds from a given extract and then possibly test them in various assays afterwards. Another method is the bioguided search for natural products where an assay must be available to test raw extracts. If the target is known, it is possible to do target oriented isolation. A number of raw extracts can then be tested in the assay and hopefully one or more will show activity. With an active raw extract the isolation of the bioactive compound can begin, which is often an iterative process with retesting after each fractionation step. The isolation will most likely be done on a (semi)preparative HPLC system using various normal or reverse-phase chromatography methods. A third option is to use explorative solid phase extraction (E-SPE), where more information about the chemical properties of the target compound is obtained before isolation starts. This is attained by using four orthogonal solid phase columns, utilizing not only polarity but also charge and size of compounds. This method makes it possible to plan an isolation strategy before making a larger raw extract.
1.3 Dereplication

Going through the laborious efforts to make large raw extracts followed by isolation of known compounds is frustrating and can be a potential problem. With isolated bioactive natural products counted in the tenths of thousands, natural product chemists face an ever increasing task in distinguishing between novel and known compounds. Early dereplication (see section 1.3) is vital to natural product chemists and when working with microbial natural products the process can be assisted by e.g. Antibase, which was developed by Prof. Dr. Hartmut Laatsch. It is a program that enables the scientist to compare physical properties, e.g. exact mass or UV spectra of a target natural product, with compounds already available in the database. This search should indicate whether the active compound is a known or novel compound and thus if isolation of the compound should be completed.

A possible problem for bioactive natural products with sufficient potency to go to clinic trials is availability. If isolation from nature only procures minimal amounts, if fermentation is not viable or if total synthesis is too lengthy or inefficient, then availability becomes a bottleneck. This was the case with Taxol, as it was not possible to make this very potent agent available for clinical trials because of its scarce availability. It was not until the isolation of a precursor of Taxol from the needles of Taxus baccata that a semisynthetic route made the compound available in larger quantities.

When a bioactive secondary metabolite has been isolated and characterized, a structure-activity relationship study can be performed pending availability. This is done by altering various positions on the parent compound and subsequently test the analogs in the assay used for the bioguided isolation or in other suitable assays. The modifications can be achieved by total synthesis of the parent compound and analogs, or if enough of the parent compound can be isolated by altering directly on the parent compound. This will yield information about the important positions of the compound and improved potency could result in a new lead compound. This lead compound will then be the basis of a new round of optimization.

1.3 Dereplication

The earlier dereplication can be used to determine if an active fraction is due to a novel or known compound, the more work and time can be saved. Running a raw extract through a HPLC-DAD-HRMS systems will afford a lot of information and data, making the tentative assignment of a given compound in the raw extract plausible. This setup will give a general idea of the polarity of a compound, the UV spectrum affords information about possible functional groups as well as the conjugation of the compound, but most importantly the exact mass of a compound will be of great help in the dereplication of a given raw extract.

When using MS to find the exact mass of a compound, correct interpretation of the MS data is pivotal. The assignment of [M+H]⁺ or [M-H]⁻ can be complicated by minor co-eluting compounds that ionize better than the target compound. The use of adducts (e.g. [M+Na]⁺, [M+H+MeCN]⁺ and/or [M+NH₄⁺]) are of great assistance to correct assignment of the molecular peak. By recognizing the pattern of these adducts it is possible to assign [M+H]⁺ or [M-H]⁻ with
10 Introduction

Figure 1.7: Four anti-cancer agents derived from four different natural sources. Taxol® (1) (plant),
Curacin A (2) (Cyanobacterium), bryostatin 1 (3) (marine symbiot bacteria) and fumagillin (4) (fungi).

There are however some limitations to this approach, compounds that do not ionize in either negative or positive mode and does not have a UV chromophore will not be detected, these could in turn be observed using a light scattering detector.

Depending on the accuracy on the MS equipment the observed mass of a compound could be compatible with several molecular formulas. Even if very high accuracy is obtained there are still different compounds with the same molecular formula. Using all information available: polarity, UC spectra, MS-MS, exact mass it is often possible to discriminate between the hits in antibase acquired from the exact mass.

1.4 Secondary Metabolites from Fungi

Secondary metabolites or natural products can be defined as metabolites not normally involved in primary metabolic processes. The discussion about why secondary metabolites are produced at all, ended with the now accepted notion that natural products evolve under the pressure of nature. Secondary metabolites can be divided into different classes depending on their biosynthetic origin, namely polyketides, terpenes and alkaloids. Natural products can be one of these types solely or any mix of these three. A chromatogram of a raw extract from Aspergillus terreus can be seen in Figure 1.8, with each peak representing a metabolite.

Secondary metabolites fulfills some role in the life of the fungi producing them,
1.5 Work flow

Figure 1.8: HPLC-DAD chromatogram of a raw extract from *Aspergillus terreus*, illustrating peaks corresponding to natural products. The known compounds geodin and dihydro geodin have been assigned.

e.g. by interacting with other microorganisms like bacteria or other fungi. The interaction with bacteria can be extremely helpful for man, as seen when fungi entered the world stage as a source for anti-bacteria agents with the discovery of penicillin. The interaction with other fungi makes it possible that some of these bioactive compounds could have effects on mammalian cells including cancerous cells as both fungi and mammals are eukaryotic; the cell filaments actin and tubulin are for example very conserved in eukaryote cells. An example of a fungal metabolite which shows both anti-fungal and anti-cancer properties is griseofulvin.

Mycotoxins are bioactive compounds that are toxic to humans, these mycotoxins interacts with humans, mostly through food sources or fungi infested living quarters. Some of the more important mycotoxins are the aflatoxins and ochratoxin A. Aflatoxins are produced by fungi contaminating e.g. maize, which can cause liver cancer in humans consuming the maize. Ochratoxin A is found in many consumer products like coffee, wine and meat.

1.5 Work flow

Looking for bioactive natural products involves a number of disciplines and processes (see Figure 1.9). The source of the secondary metabolites needs to be handled, the production of a raw extract, testing of the raw extract in a given assay, dereplication to avoid isolating known compounds, isolating a possible novel bioactive compound, structure elucidation and characterizing of the compound. This can then be followed by a structure-activity relationship study of the compound, which could result in a new lead compound.

1.6 Centrosomal Clustering Assay

The assay was designed to identify small molecules that inhibits centrosomal clustering. The cell line initially used was an oral squamous cell carcinoma cell line labeled SCC114, which was cloned to stably express green fluorescent protein (GFP)-α-tubulin. The cell line contained centrosome amplification in 64.5% of the cells but only 3.6% of mitotic cells harbored multipolar spindles. A large por-
Figure 1.9: Starting from the left is the source, in this case fungi, of natural products. Narrowing down from the numerous compounds in a screen with raw extract to a hit, in this case griseofulvin. In the case of griseofulvin tentative identification was achieved by dereplication of the initial raw extract. Had it been a novel compound the process of isolation followed by structure elucidation should have been completed. With the active compound characterized it is possible to do a structure-activity relationship study. This can be done by e.g. total synthesis or in the fortunate case of griseofulvin by modifying directly on the commerically available compound. A SAR study could afford better analogs and a new lead compound.
tion of the cells with supernumerary centrosomes thus underwent bipolar mitosis by centrosomal clustering.

For screening purposes the cells were grown to near confluence and then treated with raw extract at different concentrations for 7 h, fixed, and examined by fluorescence microscopy. Three hundred mitotic cells per well were examined, observing if the cells underwent single, bi or multipolar mitosis, with the readout being the percentage of multipolar mitoses. BJ fibroblasts was used as a noncancerous control cell line to investigate the specificity of a given active extract.

The screening procedure was changed around July 2008 to use the untransformed SCC114 cells. Cells were seeded and incubated for 24 h after which raw extract or griseofulvin analogs were added at various concentrations. After fixation the cells were stained with Eg5 antibodies and evaluated by fluorescence microscopy. The number of cells counted per well was lowered to one hundred, easing the laborious manual counting of cells. Around May 2010 the cell line used for the assay was changed as the SCC114 cells had lost their centrosomal amplification. The SCC114 cells were replaced by a human cell line labeled MDA-MB-231 (breast adenocarcinoma), which at present is the cell line used in the assay.

1.7 Griseofulvin

During the initial screening by Assoc. Prof. Thomas Ostenfeld Larsen, Lisette Knoth-Nielsen (technician) and Blanka Leber (PhD. student at the time), the known natural product griseofulvin was tentatively identified as active in three separate raw extracts (Penicillium berlinense, IBT 18288 and 19440 and P. faroense, IBT 22543). The effect of griseofulvin was further investigated at DKFZ and during my undergraduate work I synthesized three 2’ analogs showing increased activity, which was included in the paper concerning the assay. A large part of the work performed in this work is based on this discovery of griseofulvin in the assay.

Bibliography


[54] Laatsch, H. **2008**.


Chapter 2

Chemistry of Griseofulvin

Griseofulvin (1, see Figure 2.1) was initially isolated from *Penicillium griseofulvum* in 1939 by Oxford et al.\(^1\) and was later isolated from *P. janczewskii* as “Curling Factor” by Brian et al.\(^2,3\) in 1946. The latter described the curling of hyphae induced by 1.\(^2,3\) It was not until 1947 that it was proved that griseofulvin (1) and “Curling Factor” was the same compound.\(^4,5\) The analogous metabolites 7-bromo-7-dechloro-griseofulvin\(^6\) (2, 1954) and dechlorogriseofulvin\(^7\) (3, 1953) were isolated by MacMillan.

The structure of the compound was investigated by means of IR and UV spectroscopy as well as degradation studies.\(^8,9,10,11\) The correct relative structure for griseofulvin (1) was reported in 1952 by Grove et al.\(^12\) followed by a number of papers concerning the chemistry of 1 and synthesis of analogs (1952-1957).\(^13,14,15,16,17\) The absolute structure of 1 was reported in 1959 by MacMillan\(^18\) and has since been supported by a crystal structure.\(^19\) There are two possible epimers of griseofulvin, position 2 and 6’, but through the literature they are both referred to as epigriseofulvin.

The anti-fungal properties of 1 have been known since 1958,\(^20\) but recently potency against cancer\(^21\) and hepatitis C infected cells\(^22\) have renewed the interest in this compound making a review of griseofulvin relevant. In the following sections a number of subjects concerning griseofulvin will be described, including total synthesis, analogs obtained by total synthesis and analog synthesis using 1 as starting material.

Figure 2.1: griseofulvin (1), 7-bromo-7-dechloro-griseofulvin (2) and dechlorogriseofulvin (3).
2.1 Total Syntheses of Griseofulvin

Several different strategies have been utilized in the total synthesis of racemic griseofulvin and the synthesis of the natural product (+)-griseofulvin (1) was published in 1990. The first total synthesis was completed by Day et al. (1960)\textsuperscript{23,24} by oxidative cyclization of a benzophenone precursor (6, see Scheme 1), a strategy based on earlier pondering on the biosynthesis of 1.\textsuperscript{25,26} The two arenes 2-chloro-3,5-dimethoxyphenol\textsuperscript{11} (4) and 2-methoxy-4-((methoxycarbonyl)oxy)-6-methylbenzoic acid\textsuperscript{27} (5), with the latter treated with oxalyl chloride to afford the acid chloride were used as starting materials. The benzophenone (6) was prepared by Fridel-Craft reaction of 4 and the acid chloride of 5 followed by alkaline treatment. The formation of the spiro center was completed by radical formation using potassium ferricyanide affording (±)-dehydrogriseofulvin (7). The final step was hydrogenation of 7 with a preformed catalyst of 3% selenium and 5% rhodium on charcoal affording (±)-griseofulvin (1).

Scheme 1: (a) (COCl)\textsubscript{2}, benzene; (b) AlCl\textsubscript{3}, nitrobenzene; (c) 4M NaOH, ether, nitrobenzene; (d) Na\textsubscript{2}CO\textsubscript{3}, K\textsubscript{3}Fe(CN)\textsubscript{6}, H\textsubscript{2}O; (e) 5% Rh/C containing 3% selenium, H\textsubscript{2}, EtOH

The C ring of griseofulvin (1) was established by a Dieckmann cyclization in the total synthesis by von A. Brossi et al. (1960) (see Scheme 2).\textsuperscript{28,29,30} The synthesis of the substituted 3-coumarone 10 starts with 8, which was treated with methyl 2-bromoacetate to afford 9 setting up the first Dieckman cyclization yielding 10. The 3-coumarone 11 was synthesized by Michael addition to 3-penten-2-one. A second Dieckmann cyclization followed by treatment with diazomethane afforded 2-epi-griseofulvin (12), which was isomerized with sodium methoxide to yield (±)-griseofulvin (1).

Stork and Tomasz (1962)\textsuperscript{31,32} reported the use of a double Michael addition to establish both stereocenters and the 2’ enol ether in a one-pot synthesis (see Scheme 3). The key substrate in this synthesis was an alkoxyethyl vinyl ketone (14) used as a double Michael acceptor. 7-Chloro-4,6-dimethoxy-3-coumaranone (13) was used as precursor and reaction with 14 and potassium t-butoxide in
2.1 Total Syntheses of Griseofulvin

Scheme 2: (a) BrCH₂COOMe, K₂CO₃, DMF; (b) Na, toluene, 100 °C; (c) 3-penten-2-one, Triton B, MeOH; (d) NaOMe, MeOH; (e) CH₂N₂, MeOH; (f) NaOMe, MeOH, 80 °C
diglyme/t-butyl alcohol afforded (±)-griseofulvin (1).

Scheme 3: (a) t-BuOK, 2-methoxyethyl ether, t-BuOH

Utilizing a method described earlier for the synthesis of 6’-desmethyl-griseofulvin and 7-dechloro-7-fluoro-griseofulvin, Taub et al. (1962) used oxidative cyclization to establish the spiro center of (±)-griseofulvin (1) (see Scheme 4). In the early communication the last step was introduction of the chloro substituent, making this a formal total synthesis of 1.
The total synthesis as described in the full paper prepared the benzophenone (6) from two aromatic precursors as by Day et al. The arene (15) destined to become ring C of 1 differed slightly as the phenol was protected by an acetyl group and the acid instead of the acid chloride was used. The coupling with 1 was performed with in situ formation of the mixed anhydride from trifluoracetic anhydride affording both 6 and the ester 16. Transformation of 16 to the desired benzophenone (6) was completed by mild hydrolysis followed by a Fries rearrangement.
For the following ring closure to (±)-dehydrogriseofulvin (7) a number of successful methods were described. Quantitative yields was obtained using potassium
ferricyanide, lead dioxide in ether/acetone or manganese dioxide in ether/acetone. Hydrogenation to 1 was performed in ethyl acetate using 10% palladium on charcoal as catalyst.

Danishefsky and Walker (1979) reported a total synthesis of 1 utilizing a Diels-Alder cycloaddition strategy (see Scheme 5), an improvement of the total synthesis of (+)-2-epigriseofulvin by Danishefsky and Etheredge. The dienophile (19) was synthesized from 17 through double intramolecular nucleophilic attack affording 18, followed by treatment with thiophenol and oxidation with m-chloroperoxybenzoic acid. The dienophile reacted with the diene (20) in toluene at 100-135 °C affording (+)-dehydrogriseofulvin (7), which was hydrogenated to (+)-griseofulvin (1).

A total synthesis of (+)-dechloro-dehydro-griseofulvin (22) was completed by Sargent (1980) (see Scheme 6). Treating the precursor 21 with dry hydrogen chloride and excess titanium tetrachloride in dichloromethane yielded 22 through oxidative cyclization.

An improvement of the method by Stork and Thomas using double Michael addition was published by Yamato et al. (1990) (see Scheme 7). The 3-coumarone (13) was reacted with an altered Michael acceptor (23) yielding 24 and through treatment with activated alumina (±)-2'-demethoxy-2'-methylthio-griseofulvin (25) was formed. Displacement of the thio ether was achieved by oxidizing sulfur with m-chloroperoxybenzoic acid followed by treatment with sodium methoxide affording (±)-griseofulvin (1).

Pirrung et al. (1991) reported the first total synthesis of (+)-griseofulvin (1) (see Scheme 8). Reacting the triether 26, with Mander’s reagent followed by diazo transfer afforded 27, which upon treatment with 5% rhodium pivalate catalyst yielded, through a sigmatropic rearrangement, the 3-coumarone 28. Conversion to 29 followed by Dieckmann cyclisation and diazomethane treatment completed the total synthesis of (+)-griseofulvin (1).
2.1 Total Syntheses of Griseofulvin

Scheme 5: (a) NaH, THF, HMPA; (b) thiophenol, p-toluenesulfonic acid, benzene; (c) m-CPBA, CH₂Cl₂; (d) toluene, 100-135 °C (e) H₂, 10% Pd/C, EtOH

Scheme 6: (a) HCl, TiCl₄, CH₂Cl₂

Scheme 7: (a) t-BuOK, DMF, 0 °C; (b) activated alumina, Et₂O (c) m-CPBA, CH₂Cl₂, 0 °C; (d) MeONa, MeOH, benzene, 0 °C
Scheme 8: (a) LHMDS, NCCO₂Me, THF, -78 °C; (b) MsN₃, Et₃N; (c) Rh₂(piv)₄, benzene; (d) O₃, CH₂Cl₂, MeOH, (CH₃)₂S; (e) Ph₃=C(CH₃)CO₂-t-Bu, THF; (f) TFA; (g) diphenyl phosphorazidate, Et₃N; toluene, then HCl/H₂O; (h) NaOMe, MeOH; (i) CH₂N₂, THF
2.2 Total Syntheses of Griseofulvin Analogs

Ring A and B as well as position 6' of griseofulvin (1) are not easily modified from the natural product and therefore analogs of these types have been obtained by total synthesis. The syntheses have been completed using a number of the methods developed for the total synthesis of griseofulvin. The strategies used are oxidative cyclisation,\textsuperscript{42,43,44,45} Diels-Alder,\textsuperscript{48,46} double Michael addition,\textsuperscript{47} Dieckmann cyclization,\textsuperscript{48} and the improved Michael addition method.\textsuperscript{49} The analogs synthesized by means of total synthesis can be seen in Figure 2.2 and Table 2.1.

Figure 2.2: Racemic analogs synthesized through total synthesis. The structures are shown in Table 2.1.

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Table 2.1: Racemic griseofulvin analogs acquired through total synthesis. See Figure 2.2 for structures.

Scheme 9: (a) R=CN: ethyl cyanoacetate, piperidine; R=COOH: diethyl malonate, piperidine, HOAc, benzene; (b) H$_2$, 10% Pd/C, EtOAc; (c) but-3-en-2-one, 1,2-dimethoxyethane, NaOMe; (d) NaOMe, MeOH; (e) ZnCl$_2$, HCl, ether.
Newman and Angier developed a novel strategy for the preparation of ring B analogs, with a Hoesch condensation as the crucial step (See Scheme 9). In the first step either a cyano or acid group was introduced, the former by condensation of 39 with ethyl cyanoacetate using catalytic amounts of piperidine, which after hydrogenation using 10% palladium on charcoal, afforded the nitrile 40. The latter was synthesized by treatment of 39 with diethyl malonate, piperidine and acetic acid in benzene yielding the acid 41 after hydrogenation. Both 40 and 41 were then reacted further with the Michael acceptor but-3-en-2-one using sodium methoxide as base yielding 42 and 43. A Dieckmann condensation formed the destined ring C (44) and 45 by treatment with sodium methoxide in methanol. The Hoesch condensation was performed by bubling hydrogen chloride through a mixture of 44 or 45 and zinc chloride in ether, affording a position 2 carbon analog of griseofulvin (46). The position 1 carbon and sulphur analogs of griseofulvin can be seen in Figure 2.3 and Table 2.2.

**Figure 2.3:** Ring B analogs synthesized through total synthesis. The compounds are shown in Table 2.2.

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</table>

**Table 2.2:** Ring B analogs prepared by total synthesis. See Figure 2.3 for structures.
2.3 Spectroscopic Studies of Griseofulvin and Analogs

Several articles do not contain synthesis of griseofulvin (1) or analogs thereof, but instead describe spectroscopic studies. Page and Staniforth thoroughly investigated the infrared absorption of a total of 188 griseofulvin analogs.\(^{57,58}\) The IR spectra were acquired in bromoform and very detailed absorption values are listed for all compounds. The authors were able to distinguish between analogs of the griseofulvin or isogriseofulvin form as well as between 1 and the position 2 epimer by IR.

A comprehensive study of the mass spectra of griseofulvin and analogs of griseofulvin have been carried out by Ballantine and Fenwick.\(^{59,60}\) MHz \(^1\)H NMR was used in a study by Arison et al.\(^{60}\) covering griseofulvin and 27 analogs, exploring the at the time, new technique while assigning groups based on differences between the analogs. Another NMR study by Green et al.\(^{61}\) including 43 analogs concluded that the assignment of the 4 and 6 methoxy groups by Arison et al. was faulty. The assignment by Green et al. was confirmed by Rønnest et al.\(^{62}\) by 2D NMR and single crystal X-ray analysis of the 4 and 6 phenols of 1.

The conformation of griseofulvin and the 4'-alcohol analog of 1 in solution (CDCl\(_3\)) was determined by Levine and Hicks\(^{63,64}\) and they proceeded to investigate the \(^{13}\)C-NMR of griseofulvin and four analogs.\(^{65}\) NMR has also been used in the study of the biosynthesis of griseofulvin using deuterium labeled analogs.\(^{66}\) Hansch and Lien\(^{67}\) used griseofulvin analogs in a larger modeling study of the LogP value of anti-fungal agents. These values were later used by Juvale et al.\(^{68}\) in a 2D-QSAR study using the curling of hyphae by the analogs reported by Crosse et al.\(^{69}\) It was concluded by Crosse et al. that there were no correlation between the potency for curling of hyphae and the anti-fungal activity of analogs.
2.4 Tables of Griseofulvin Analogs

Numerous analogs of griseofulvin have been synthesized. In the following tables all occurrences in the literature, in either English or German, of griseofulvin and analogs are shown.

2.4.1 Griseofulvin Analogs with Modifications at the 2’ Position

![Griseofulvin analogs with modifications at the 2’ position](image)

**Figure 2.4:** Griseofulvin analogs with modifications at the 2’ position. See Table 2.3.

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*Continued on next page*
Table 2.3: Griseofulvin analogs with modifications at the 2’ position. See Figure 2.4. A is glucose, B is pyrrolidin-1-yl and C is phthalimido.

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### 2.4 Tables of Griseofulvin Analogs

#### 2.4.2 Griseofulvin Analogs with Modifications at the 2’ and 3’ position

![Figure 2.5](image)

**Figure 2.5**: Griseofulvin analogs with modifications at the 2’ and 3’ position. See Table 2.4.

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*Continued on next page*
Table 2.4: Griseofulvin analogs with modifications at the 2’ and 3’ position. See Figure 2.5.
2.4 Tables of Griseofulvin Analogs

2.4.3 Griseofulvin Analogs with Modifications at the 5’ Position

Figure 2.6: Griseofulvin analogs with modifications at the 5’ position. See Table 2.5.

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Table 2.5: Griseofulvin analogs with modifications at the 5’ position. See Figure 2.6.
2.4.4 Griseofulvin Analogs with Modifications at the 4 Position

![Griseofulvin analogs with modifications at the 4 position. See Table 2.6.](image)

**Figure 2.7:** Griseofulvin analogs with modifications at the 4 position. See Table 2.6.

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**Table 2.6:** Griseofulvin analogs with modifications at the 4 position. See Figure 2.7. A is pyrrolidin-1-yl.
2.4 Tables of Griseofulvin Analogs

2.4.5 Griseofulvin Analogs with Modifications at the 5 Position

Figure 2.8: Griseofulvin analogs with modifications at the 5 position. See Table 2.7.

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Table 2.7: Griseofulvin analogs with modifications at the 5 position. See Figure 2.8.

2.4.6 Griseofulvin Analogs with Modifications at the 6 Position

Figure 2.9: Griseofulvin analogs with modifications at the 6 position. See Table 2.8.

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Table 2.8: Griseofulvin analogs with modifications at the 6 position. See Figure 2.9. A is pyrrolidin-1-yl
2.4.7 Griseofulvin Analogs with Modifications at Position 4 and 6

![Griseofulvin analogs with modifications at positions 4 and 6.](image)

**Figure 2.10:** Griseofulvin analogs with modifications at positions 4 and 6. See Table 2.9.

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</tbody>
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**Table 2.9:** Griseofulvin analogs with modifications at positions 4 and 6. See Figure 2.10.

2.4.8 Griseofulvin Analogs with Modifications at Position 5 and 7

![Griseofulvin analogues with modifications at ring A.](image)

**Figure 2.11:** Griseofulvin analogues with modifications at ring A. See Table 2.10.

<table>
<thead>
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<td>H</td>
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<td>NO₂</td>
<td>81</td>
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</table>

**Table 2.10:** Griseofulvin analogs with modifications at position 5 and 7. See Figure 2.11.
2.4.9 Isogriseofulvin Analogs with Modifications at the 4’ Position

Figure 2.12: Isogriseofulvin analogs with modifications at the 4’ position. See Table 2.11.
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<td>SBn</td>
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Table 2.11: Isogriseofulvin analogs with modifications at the 4’ position. See Figure 2.12. A is phthalimido, B is 2-(2-imidazolyl), C is pyrrolidin-1-yl.
2.4 Tables of Griseofulvin Analogs

2.4.10 Isogriseofulvin Analogs with Modifications at the 3’ and 4’ Positions

Figure 2.13: Isogriseofulvin analogs with modifications at the 3’ and 4’ position. See Table 2.12.

<table>
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<th>Structure</th>
<th>R</th>
<th>R’</th>
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<td>74</td>
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<tr>
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<td>Br</td>
<td>OMe</td>
<td>74</td>
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<td>Cl</td>
<td>OMe</td>
<td>78,61,82,87</td>
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Continued on next page
2.4.11 Isogriseofulvin Analogs with Modifications at the 4, 5 and 6 Positions

Table 2.12: Isogriseofulvin analogs with modifications at the 3’ and 4’ position. See Figure 2.13.

<table>
<thead>
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<th>Reference</th>
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Table 2.13: Isogriseofulvin analogs with modifications at the 4, 5 and 6 positions. See Figure 2.14.

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<td>57</td>
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<td>62</td>
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<td>Cl</td>
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Figure 2.14: Isogriseofulvin analogs with modifications at the 4, 5 and 6 positions. See Table 2.13.
2.4 Tables of Griseofulvin Analogs

2.4.12 Analogs of both Griseofulvin and Isogriseofulvin with Modifications at Ring A and C

Figure 2.15: Analogs of both griseofulvin and isogriseofulvin with modifications at ring A and C. See Table 2.14.

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<td>NC₄H₈</td>
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Table 2.14: Analogs of both griseofulvin and isogriseofulvin with modifications at ring A and C. See Figure 2.15.

2.4.13 Griseofulvic Acid with Modifications at the 3’ Position

Figure 2.16: Analogs of griseofulvic acid with changes at the 3’ position. See Table 2.15.

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Continued on next page
Table 2.15: Analogs of griseofulvic acid with modifications at the 3’ position. See Figure 2.16. A is the sodium salt of the enol form.

2.4.14 Disubstituted Griseofulvic Acid at the 3’ Position

Figure 2.17: Analogs of griseofulvic acid with changes at the 3’ position. See Table 2.16.

Table 2.16: Analogs of griseofulvic acid with modifications at the 3’ position. See Figure 2.17
2.4 Tables of Griseofulvin Analogs

2.4.15 Griseofulvic Acid with Modifications at the 5, 7 and 3’ Positions

![Fig. 2.18](image)

**Figure 2.18:** Analogs of griseofulvic acid with changes at the 5, 7 and 3’ positions. See Table 2.17.

<table>
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<td>Cl</td>
<td>Cl</td>
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**Table 2.17:** Analogs of griseofulvic acid with modifications at the 5, 7 and the 3’ position. See Figure 2.18.

2.4.16 Griseofulvic Acid with Modifications at the 6 position

![Fig. 2.19](image)

**Figure 2.19:** Analogs of griseofulvic acid with modifications at the 6 position. See Table 2.18.

<table>
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*Continued on next page*
Table 2.18: Analogs of griseofulvic acid with modifications at the 6 position. See Figure 2.19. A is pyrrolidin-1-yl

<table>
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<td>88</td>
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</tbody>
</table>

Bibliography


[87] Andres, W. W. US3557151, **1968**.


Chapter 3

Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

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The supporting information from Chapter 3 can be seen in Appendix C
Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells
Synthesis and Structure–Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

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Department of Chemistry, Technical University of Denmark, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark, Center for Microbial Biotechnology, Department of Systems Biology, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark, Clinical Cooperation Unit for Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280 (TP4) 69120 Heidelberg, Germany

Received December 2, 2008

Griseofulvin was identified as an inhibitor of centrosomal clustering in a recently developed assay. Centrosomal clustering is an important cellular event that enables bipolar mitosis for cancer cell lines harboring supernumerary centrosomes. We report herein the synthesis and SAR of 34 griseofulvin analogues as inhibitors of centrosomal clustering. The variations in the griseofulvin structure cover five positions, namely the 4, 5, 2', 3', and 4' positions. Modification of the 4 and 5 positions affords inactive molecules. The enol ether must be at the 2' position, and the 4' position needs to be sp² hybridized. The most active analogues were the 2'-benzoxyl and 2'-4(methylbenzoxyl) analogues as well as the oxime of the former with a 25-fold increase of activity compared to griseofulvin. Comparison of the results obtained in this work with prior reported growth inhibition data for dermatophytic fungi showed both similarities and differences.

Introduction

Griseofulvin (1, Figure 1) is a classic antifungal agent,¹ used clinically for the treatment of dermatomycoses. Since the isolation of griseofulvin in 1939,¹ it has attracted a lot of attention and more than a hundred papers describing analogue synthesis as well as structure–activity relationship (SAR) studies relating to antifungal activity have been published. Since 1950, more than 400 analogues have been disclosed covering most positions and many have displayed significantly increased activity in published antifungal assays. More recently, griseofulvin has been the object of increased interest due to its activity toward a range of mammalian cancer cell lines. Griseofulvin has been shown to be cytotoxic by itself but also to potentiate the activity of another anticancer drug, nocodazole.²,³ The cytotoxicity of three 2' analogues in Chinese hamster V79 cells has previously been reported by Oda.⁴

We have recently developed an assay⁵ to identify small molecules that inhibit centrosomal clustering, a mechanism necessary for successful bipolar mitoses in human cancer cell lines with supernumerary centrosomes.⁶,⁷ Healthy mammalian cells harbor two centrosomes during mitosis that function as mitotic spindle poles to ensure accurate chromosome segregation, whereas the majority of human malignancies contain multiple centrosomes. Supernumerary centrosomes can form multipolar spindles leading to aberrant mitoses with consecutive chromosome missegregation, eventually resulting in apoptosis. In most human cancer cell lines, this spindle multipolarity is overcome through centrosomal clustering, giving rise to two functional spindle poles and thereby allowing for successful mitosis.⁸ As this phenotype is specific to cancer cells, there is an increased focus on identifying small molecules that inhibit centrosomal clustering. The variations in the griseofulvin structure cover five positions, namely the 4, 5, 2', 3', and 4' positions. Modification of the 4 and 5 positions affords inactive molecules. The enol ether must be at the 2' position, and the 4' position needs to be sp² hybridized. The most active analogues were the 2'-benzoxyl and 2'-4(methylbenzoxyl) analogues as well as the oxime of the former with a 25-fold increase of activity compared to griseofulvin. Comparison of the results obtained in this work with prior reported growth inhibition data for dermatophytic fungi showed both similarities and differences.

![Figure 1. Structures of griseofulvin 1, griseofulvic acid 2, and isogriseofulvin 3.](image-url)
Synthesis and SAR of Griseofulvin Analogues

Scheme 1:

Scheme 2:

Scheme 3:

Scheme 4:

Scheme 5:

Compounds 10–15 plus their 4’ enol ether isomers. Dioxane was used as cosolvent for alcohols with higher boiling points, leading to 16 and 17. This method was generally applicable to the synthesis of analogues with straight vinyl ethers in yields of 14–22% (Scheme 2).

Bulkier alcohols like benzyl alcohol and cyclopentanol led to unsatisfactory yields of 2–4%. An attractive alternative was the known\(^1\) route through the 2’ vinyl chloride 18, which relies on a base mediated addition of the alcohol followed by elimination of chloride (Scheme 3). Vinyl chloride 18 is accessible by treatment of 2 with lithium chloride in phosphoryl chloride. We found using dioxane as cosolvent convenient because this allowed us to reduce the amount of phosphoryl chloride to 5 equiv, greatly facilitating the workup procedure and the scale-up to multigram amounts.

The addition of alcohols to 18 performed well in DMF, THF, and dioxane with comparable yields and THF or dioxane was used as solvent for all subsequent experiments. NaH and CsOAc were used as bases, but in the end, diazabicyclo[5.4.0]undecane (DBU) turned out to be the most efficient base and it was used for preparation of analogues 19–25 in yields of 22–93% (Scheme 3).

The 4’ alcohol 26 was derived from 1 using a slight modification of a known method\(^2\) (Scheme 4). Analogue 27 was available by reduction of 1 with hydrogen catalyzed by platinum on charcoal, an improvement of a published procedure.\(^3\) The oximes 28 and 29 were derived from the parent ketones by treatment with hydroxylamine hydrochloride in ethanol and DMSO, a slight modification of the method of Delgado et al.\(^4,5\) and isolated as inseparable 1:1 mixtures of geometrical isomers. In a similar fashion, the hydrazine 30 was synthesized by heating 1 with \(\text{N}_2\text{N}\)-dimethyl hydrazine and acetic acid in toluene. The major product was the \(E\) analogue 30 with the geometry distinguished by NOE in proton NMR. The \(H^7\) proton of the two isomers showed two distinct signals in NMR. The H\(^2\) dipolar coupling between a hydrazine methyl group and H\(^3\) could be observed for the pure analogue 30, but a strong NOE was detected for the isomer.

3’,3’-Dimethyl griseofulvic acid (31) was derived from 2 treated with \(\text{K}_2\text{CO}_3\) and Me\(^\text{I}\)\(^\text{2}\) (Scheme 5). Analogue 32 was synthesized from 2 by reaction with benzyl bromide, NaI, and KOH.\(^6\) Treatment of 1 with \(-\text{iodo}-\text{sucinimide (NIS)}\) and triethylsilyl triflate (TESOTi) afforded 33, and the 3’-iodo analogues 34 and 35 were obtained using the same convenient conditions. This type of compounds have previously been prepared by treating 2 with iodine monochloride and 2 equiv of KOAc,\(^2\) followed by reacting the intermediate with an excess of an appropriate diazo alkane.\(^2\)

Results and Discussion

Compounds 1–35 were evaluated for their ability to inhibit centrosomal clustering in our cellular assay\(^7\) (Table 1). The percentages of cells with multipolar spindles were measured in triplicate at 14 different concentrations (100 \(\mu\text{M}\) to 12 \(\mu\text{M}\)), and IC\(_{50}\) values were calculated with an IC\(_{50}\) of 24 \(\mu\text{M}\) for griseofulvin (1). Analogues with IC\(_{50}\) values higher than 100 \(\mu\text{M}\) are regarded as inactive.
At ring A, the 4 and 5 positions were altered. Elongation at the 4 position resulted in no activity for either the ethyl (1) or benzyl (6) ethers. Introduction of a nitro (7), amine (8), or benzyl amine (9) group at the 5 position negates all activity, indicating that bulker groups at these two positions are not tolerated. Compound 6 has previously been tested for growth inhibition against dermatophytes (Epidermophyton floccosum, Trichophyton interdigitale, T. persicolor, T. mentagrophytes, T. rubrum, and Microsporum canis), and 7 and 8 have been tested against the latter three in addition to T. terrestris and all three were found to be less active than 1.17,25

The 2', 3', and 4' positions of the C ring have been altered in this study. Solvolysis of 2 with a given alcohol affords two isomers with the enol ether positioned at either the 2' or 4' position. We have tested altogether 11 analogues with a 4' enol ether as in isogriseofulvin (3), including the isomers of 10–17 and 20, and all have been inactive (data not shown). This strongly indicates that active analogues should have the enol ether at the 2' position. The activity of the 4' enol ether isomers of 3, 10, 13, and 19 toward a range of dermatophytes (Trichophyton mentagrophytes, T. interdigitale, T. rubrum, T. persicolor, Microsporum canis, and Epidermophyton floccosum) have been published and all showed lower activity than 1.25

Several analogues with modifications at the 2' position have been prepared, and elongation of the methoxy group in 1 (analogues 10–15) increases the activity, with a maximum around 4 carbon atoms.

The activity can be further increased by the introduction of bulker groups. The cyclopropyloxymethoxy (16) and cyclopentoxymethoxy (17) analogues have IC₅₀ values of 1.8 and 1.3 μM, comparable to the phenoxy (19) and benzylthio (21) analogues with 1.5 and 1.3 μM. The most active 2' analogues are the benzoxyl (20) and 4'-methylbenzoxyl (23) compounds, both with an IC₅₀ value of 0.9 μM. While there is little difference between 19 and 20, the phenoxyethane analogue 22 is significantly less potent with an IC₅₀ value of 15 μM. The introduction of even bulker groups such as 4-biphenylmethoxy (24) and adamantylmethoxy (25) affords IC₅₀ values of 2.9 and 3.7 μM, higher than the most active compounds but significantly more potent than 1. Because our assay is whole-cell based, transport over the cell membrane potentially also influences the observed activity. The higher lipophilicity of 24 and 25 could render this transport easier, which would contribute to the observed increase in activity.

Growth inhibition data for the dermatophytes Trichophyton mentagrophytes, T. interdigitale, T. rubrum, T. persicolor, Microsporum canis, and Epidermophyton floccosum has been published for compounds 10, 11, 13, 15, 19, and 20.25 The activities for 10 and 11 were equal or to lower than 1, while 13 was more active against two strains (T. interdigitale, T. persicolor) and similar or less toward the remaining strains. Compound 15 showed increased activity against a single strain (T. interdigitale), and 19 and 20 were inactive.

A different phenotype was seen for 18. Usually, cells are attached to the dishes by focal adhesions. Treatment with 18 led to a detachment of the cell monolayer from the dishes, but, interestingly, the cell–cell contacts were not affected. Because of this phenotype, induction of multipolar spindle formation could not be analyzed.

The 4' alcohol, griseofulvin (26), has no activity and neither has the 2'-methoxy 4'-alcohol analogue (27). The 4' position of 1 was altered to an oxime (28), giving rise to an increase in activity with an IC₅₀ of 17 μM. To check for a possible synergistic effect, the 2' benzoxyl analogue 29 was also tested with an oxime affording 29, but the activity did not exceed that of 20 as the IC₅₀ value remained at 0.9 μM. Introduction of a hydradine group at the 4' position (30) led to a slight decrease in activity with an IC₅₀ of 27 μM. No activity was reported for 26 toward dermatophytes Trichophyton mentagrophytes or T. rubrum, but for 26, the activity toward those two strains and T. terrestris and Microsporum canis was comparable to griseofulvin.17

The results from the 4' analogues indicate that this position is important for the activity, as removal of the ketone renders the compounds (26 and 27) inactive. Taken together with the increase in activity for 28 and 29, it suggests that the 4' position should be sp² hybridized.

Neither griseofulvic acid 2, the 3,3'-dimethyl analogue 31, nor 3'-benzyl griseofulvic acid (32) showed activity in our assay. Introduction of iodine at the 3' position had equivocal effects. 3'-Iodo griseofulvin (33) and 3'-iodo-2'-benzoxyl analogue 35 were inactive. For the 2'-propoxy-3'-iodo analogue 34, the IC₅₀ found was 2.0 μM, only a factor two higher than the most active compounds and lower than the 2'-propoxy analogue 11 itself. In antifungal screens, 33 shows lower activity toward Trichophyton mentagrophytes, T. interdigitale, T. rubrum, T. persicolor, Microsporum canis, and Epidermophyton floccosum.
where 34 has a lower activity against all save T. interdigitale, where the activity is the same as 1.

Conclusion

We report here for the first time a collection of griseofulvin analogues tested for inhibition of centrosomal clustering in cancer cells. Of the 34 analogues tested, 13 were inactive, two had decreased activity, and 18 displayed increased activity as compared to griseofulvin, while one analogue showed a different phenotype characterized by detachment of the cell monolayer from the dish.

All modifications of the 4 and 5 positions resulted in no activity, and thus these positions should not be altered. It appears as if the 4′ position needs to be ε2 hydrazined as reduction of the ketone results in no activity, whereas introduction of either an oxime (28 and 29) or a hydrazine (30) group leads to more active analogues. A total of 11 4′ enol ether analogues have been tested, and all were inactive. The two inactive analogues, 31 and 32 also indicate that modifications must preserve the conjugated system of 1 to be beneficial.

The 2′ position has been explored the most, and elongation at this position increases the activity while introducing bulkier groups do so to an even larger extent. The optimal substituent seems to be an alpha-benzyl group (20) with the possibility of substitution on the aromatic ring. The phenylthio analogues 22 to 15 times less active than 20, and a longer tether is thus not a viable option for increased activity.

The three iodo substituted analogues do not give a clear picture of the effect of substitution at the 3′ position as the activity varies. We speculate that the iodine changes the conformation of the 2′ substituent and that the benzylxoy group is shifted to a less desirable position, whereas the propylxoy group is moved to a more favorable position.

While comparing the SAR data obtained in this work for inhibition of centrosomal clustering with the growth inhibitory effect against seven dermatophytic fungi, some similarities are seen but also notable differences. Modification of the 4 and 5 positions in the A ring renders the analogues inactive with regard to both activities. For the C ring modifications, the 4′ enol ether analogues displayed reduced or no activity in both types of assay. When modifying the 2′ position, the activity toward dermatophytes is retained for the ethyl and propyl analogues. The butyl and hexyl analogues show increased activity toward some fungal strains and lower toward others. The inhibition of centrosomal clustering is increased for all straight chained 2′ analogues, compared to griseofulvin. With bulkier substituents (19 and 20), lower activity is seen toward all dermatophyte strains, while the inhibition of centrosomal clustering is enhanced further.

Reduction of the 4′ ketone makes the analogue inactive both toward dermatophytes and cancer. An oxime on the 4′ position retains activity toward dermatophytes but increases the centrosomal clustering inhibition. Compound 33 with substitution in the 3′ position is less active in our assay as well as against dermatophytes, while 34 has diminished anti-fungal activity but is 12 times more potent than 1 in our assay.

The results described herein demonstrate that the activity of griseofulvin as an inhibitor of centrosomal clustering can be enhanced by structural modifications, which renders this compound class interesting as potential anticancer agents. Further, we have demonstrated that previously published data on the antifungal activity of griseofulvin analogues does not correlate directly to their activity in mammalian cells.

Experimental Section

Starting materials, reagents, and solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Reactions involving air or moisture sensitive reagents were carried out under N2, and flasks were dried by flame heating under reduced pressure. DMF, CH2Cl2, dioxane, and toluene were dried over 3 Å molecular sieves. Evaporation of solvents was done under reduced pressure (in vacuo). Purity of all compounds was found to be greater than 95% by LC-DAD-MS (see below).

NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. 1H NMR spectra were recorded using either a Bruker AC 200 MHz or a Varian Mercury 300 MHz NMR spectrometer. Chemical shifts were given in ppm and coupling constants in Hz. The field is indicated in each case. When CDCl3 was used as solvent, the residual peak was used as internal reference at δ 7.27 for 1H NMR and δ 77.00 for 13C NMR spectra. When DMSO-d6 was used, the values were δ 2.50 for 1H NMR and δ 39.43 for 13C NMR spectra.

IR spectra were recorded using a Perkin-Elmer 1600 series FTIR. All melting points are uncorrected. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (Merck 1.05554.0001). Compounds were visualized by charring after dipping in a solution of 1% KMnO4, 6.7% K2CO3, and 0.08% NaOH in water. UV visualization was done using a model UV-VIS-25 Mineralight lamp.

EIMS were recorded by direct inlet to a GCMS-QP5000 gas chromatograph mass spectrometer from Shimadzu. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (M = H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z, 100—900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetone/methanol (50 ppb TFA) in water gradient starting from 15% to 100% over 25 min with a flow rate of 0.3 mL/min. Microanalyses were obtained from H. Kolbe, Mikroanalytisches Laboratorium, Mülheim/Ruhr, Germany.

For the determination of IC50 values, we tested the compounds in our assay for centrosomal clustering based on Sieri14 cells, an oral squamous cell carcinoma cell line characteristic for this phenotype. Despite the presence of supernumerary centrosomes in 64.5% of Sieri14 cells, only 3.6% of the cells in mitosis harbor multipolar spindles. The rest of the cell population cluster their centrosomes and undergo bipolar mitoses, making this cell line an ideal model system. Sieri14 cells were grown in 96-well plates to near confluence and treated for 24 h with different griseofulvin analogues. The compounds were tested in triplicate at 14 different concentrations (10/5, 25, 6.3, 1.2, 1, 0.4, 0.2, 0.1, 0.05, 0.02, and 0.01 μM). Cells were then fixed and examined by fluorescence microscopy. Then 100 mitotic cells per well were analyzed, with the percentage of mitotic cells with multipolar spindles being the read-out. The relationship between readout and the dose was described by a logistic model (eq 1):

\[
\phi = \frac{1}{1 + \exp(-\alpha - \beta x)}
\]

where ϕ is the percentage of mitotic cells with multipolar spindles and x is the dose scaled according to x = ln(dose + 1). The dose axis was scaled to spread out the low concentration data. The IC50 values and the corresponding 95% confidence intervals were determined from the fitted dose—response curves. The calculations were performed in Matlab 7.0.4.365 (The Mathworks) using the glmfit and glmval functions.

\[
(25,6\text{-R})(7\text{-Chloro-4,6-dimethoxy-5-benzylamino-benzofuran-3-}
\text{-on}-2\text{-spirido-1\text{-}2\text{-methylene-6\text{-methyl-cyclohex-2\text{-one-1\text{-one-the-9\text{-one}}}}})\]
benzaldehyde (0.07 mL, 0.65 mmol) in CHCl₃ (4 mL) was added to a solution of sodium triacetoxoborohydride (104 mg, 0.490 mmol) in CHCl₃ (4 mL) at 0 °C under nitrogen atmosphere. Acetic acid (90%aq. 0.03 mL, 0.65 mmol) was added and the mixture was stirred at 20 °C for 48 h. The mixture was washed with sat. aq NaHCO₃ (15 mL) and water (15 mL). The combined aqueous phases were extracted with CHCl₃ (3 × 30 mL). The combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 30:20:1) to afford the product 9. Yield: 26 mg (17%) (yellow needles); δ (CDCl₃, 50 MHz) δ 7.31 (1H, m), 7.28 (1H, dd, J = 16.5, 6.0 Hz), 6.79 (1H, d, J = 16.5, 3.9 Hz), 3.97 (3H, s), 3.95–3.84 (2H, m), 3.02 (1H, dd, J = 16.5, 13.5 Hz), 2.89–2.75 (2H, m), 2.40 (1H, dd, J = 16.1, 4.1 Hz), 0.89 (3H, d, J = 6.5 Hz) ppm. 13C NMR (75 MHz, CDCl₃) δ 197.0, 192.5, 169.5, 164.5, 157.6, 157.2, 128.8 (2C), 128.3 (12C), 126.4, 105.1, 100.9, 97.1, 89.7, 89.4, 69.9, 57.0, 56.3, 59.9, 36.1, 34.7, 14.1, HRMS (ESI⁺) calcd for [C₁₉H₁₄Cl₂O₂]⁺ 443.1261, found 443.1264.

(25R,6R)-7-Chloro-4-hydroxy-benzofuran-3-one/2-spiro-1'-6'-(methyl-2',4'-pyridyl)-4'-oxime (21). Yield: 218 mg (54%) (white crystals); δ (EtOAc/peptane 5:4) 0.45, mp: 176–178 °C IR (KBr, cm⁻¹) 1709, 1664 1H NMR (300 MHz, CDCl₃) δ 7.38–7.10 (2H, m), 7.06–6.80 (4H, m), 6.89 (2H, s), 6.62 (2H, s), 6.52 (2H, d, J = 6.0 Hz), 3.91 (3H, s), 3.84–3.78 (2H, m), 3.74 (2H, d, J = 6.0 Hz), 3.00 (1H, m), 2.78–2.58 (1H, m), 2.38–2.15 (1H, m), 1.81–1.50 (2H, m), 1.50–1.20 (2H, m), 0.88–0.72 (6H, m), ppm. 13C NMR (75 MHz, CDCl₃) δ 197.0, 192.5, 169.5, 164.5, 157.6, 157.2, 128.8 (2C), 128.3 (12C), 126.4, 105.1, 100.9, 97.1, 89.7, 89.4, 69.9, 57.0, 56.3, 59.9, 36.1, 34.7, 14.1, HRMS (ESI⁺) calcd for [C₁₉H₁₄Cl₂O₂]⁺ 443.1261, found 443.1264.
Synthesis and SAR of Griseofulvin Analogues

(2S,6′R,6′R)-7-Chloro-6,6-dimethyl-7-benzofuran-3-one-2′-spiro-1′-2-carbethoxy-4′-methyl-cyclohex-2′-ene-4′-one-4′-dimethylhydrazyne (3O). To a solution of griseofulvin (1.0 g, 2.83 mmol) in toluene (28.3 mL) was added N,N-dimethylhydroxylamine (0.9 mL, 11.32 mmol) and 90% aq acetic acid (0.5 mL, 8.66 mmol). The mixture was heated to 50 °C for 24 h and cooled to 20 °C. The mixture was diluted with diethyl ether (100 mL), washed with sat. aq NaHCO₃ (50 mL) and brine (150 mL). The combined aqueous phases were extracted with diethyl ether (50 mL), and the combined organic phases were dried (Na₂SO₄) and concentrated. The residue was purified by column chromatography (toluene-CH₂Cl₂:EtOAc 1:1–2) to yield compound 3O. Yield: 208 mg (19%) (orange needles); Rf (EtOAc:heptane 5:1): 0.22; mp: 118 °C.

References


Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells


Chapter 4

Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

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The supporting information for Chapter 4 can be seen in Appendix D
Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells

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Abstract
Griseofulvin (1) and 53 analogs of this compound have been tested against the pathogenic dermatophytes *Trichophyton rubrum* and *T. mentagrophytes* as well as against the breast cancer cell line MDA-MB-231. The modifications to griseofulvin include the 4, 5, 6, 2', 3' and 4' positions. The SAR of the griseofulvin analogs towards the two fungi followed the same trend with the majority being less active than griseofulvin and none had more than twice the potency of the parent compound. A comparison of the anti-fungal and the anti-cancer SAR revealed distinct differences, as the majority of analogs showed increased activity against the cancer cell line MDA-MB-231, highlighted by 2'-benzyloxy-2'-demethoxy-griseofulvin, which showed low activity against both fungi, but was among the most potent compounds against MDA-MB-231 cancer cells. Tubulin has been proposed as the target of griseofulvin in both fungal and mammalian cells, but the differences revealed by this SAR study strongly suggest that the mode-of-action of the compound class towards fungi and mammalian cancer cells is different.

Introduction
Griseofulvin (1, see Figure 1) was one of the first anti-fungal natural products isolated from filamentous fungi and has been known as an anti-fungal agent for decades. The compound was until the approval of Lamisil in 2007, the only drug available for treatment of tinea capitis, a superficial fungal skin infection caused by dermatophytes, which predominantly affects children. Upon administration to man griseofulvin accumulates in the skin (*Stratum corneum*) where it presumably binds to keratin. The mode of action is still not determined but tubulin binding has been proposed. More than four hundred griseofulvin analogs have been reported since its discovery and the activity of these over three hundred of these have been compared with griseofulvin (1) against six dermatophytes in a study by Crosse et al. The curling of hyphae in *Botrytis allii* were also tested, but this phenotype did not correlate with the growth inhibitory effect of the analogs. Griseofulvin analogs with modifications at positions 4, 6, 2' and 3' as well as isogriseofulvin analogs with modifications at the 4, 6, 3' and 4' positions were tested. No 2' analogs excelled in growth inhibition of the dermatophytes, but elongation did increase the curling of hyphae with the optimal analogs the being 2'-propoxy (46) and 2'-butoxy analogs. Most griseofulvin analogs tested showed increased potency against some dermatophytes, but exhibiting lower activity against others. Position 5 has been functionalized with nitro (10) and amine (9) groups rendering the analogs inactive at relevant concentrations against four dermatophytes. An ethoxycarbonyl group has also been introduced in this position and the analog found to be inactive. The 4' position has also been examined with the 4'-alcohol analog (50) being inactive and the 4' oxime (36) being seven fold.

Figure 1. The structure of griseofulvin (1), griseofulvic acid (2) and isogriseofulvin (3). The rings A, B and C as well as the positions modified in this study in 1 are shown.
Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes

Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells

less potent compared to 1, with both analogs tested against Microsporum gypseum.\textsuperscript{11}
Although the initial isolation of 1 was completed in 1939\textsuperscript{1}\nboth anti-cancer\textsuperscript{20}\textsuperscript{23} and anti-viral\textsuperscript{24}\textsuperscript{26} properties of griseofulvin have been discovered recently. Three analogs
tested for the former by Oda et al.\textsuperscript{31} against Chinese hamster
V79 cells showed increased cytotoxicity with 2'-propoxy-
2'demethoxy-griseofulvin being the most potent (\textsuperscript{46}, IC\textsubscript{50}
0.7 \mu M; 1, 8 \mu M) and it was proposed that additional
structural modifications at the 2' position could enhance
activity further. This was supported by ourselves in a whole
cell phenotype-based anti-cancer assay for spindle
multipolarity induction, where increased activity was seen
for analogs with modifications in the 2' position, with the
2'-benzoxyl-2'demethoxy-griseofulvin analog (17) being
the most potent compound tested.\textsuperscript{22} Multiple papers state
that griseofulvin arrests several cancer cell lines in G2/M
phase of the cell cycle.\textsuperscript{11, 23, 25} Several investigators have
proposed tubulin as the main target for griseofulvin although
for mammalian cells this proposition is not undisputed.\textsuperscript{26, 27, 28, 29, 30}
Recently, Panda et al.\textsuperscript{30} proposed two
griseofulvin binding sites on tubulin using molecular
docking studies and similar to Oda\textsuperscript{31} reported that
microtubule dynamics were disrupted by 1. Using the
hepatitis C virus-1b cell culture system Huh7/Rep-Feo, Jin
et al. reported that G2/M phase arrest in infected cells was
induced by griseofulvin (1).\textsuperscript{31} It was speculated that the
effect was due to interaction with microtubule
polymerization.\textsuperscript{23}
Griseofulvin exhibits activity against fungi, mammalian
cancer cells as well as suppressing RNA replication by the
hepatitis C virus, with tubulin having been proposed to be
involved in all three cases. Tubulins are very conserved
within different eukaryotic cell types,\textsuperscript{24} and most of the
variation among different tubulin isoforms is found in the
amino acids near the C-term, which form a ridge on the
surface of microtubules. Therefore, variations among
different isoforms are expected to affect primarily the
association of accessory proteins with the surface of
microtubules, rather than microtubule polymerization \textit{per se}. In
case tubulin is the sole target of griseofulvin in both fungi
and mammalian cells, the activity profile of an array of
analogs against these cell types should be similar. To test
this hypothesis we decided to carry out an SAR study of
griseofulvin analogs. This is to the best of our knowledge
the first study of griseofulvin (1) and analogs thereof, which
compares anti-fungal and anti-cancer SAR from growth
inhibition assays. Griseofulvin (1) and 54 griseofulvin analogs (11 reported
for the first time here) have been tested in an anti-fungal
assay against \textit{T. mentagrophytes} and \textit{T. rubrum}, two
dermatophytes causing \textit{tinea capitis}. All compounds were
also tested in an anti-cancer assay against the human cell
line MDA-MB-231 (breast adenocarcinoma), which was
chosen because this cell line represents a common cancer
type and is known to harbor supernumerary centrosomes
which are regularly clustered into a bipolar mitotic spindle
array in a high percentage of cells.\textsuperscript{35} Analogs with
alterations at the 4, 5, 6, 2', 3' and 4' positions have all been
synthesized from commercially available griseofulvin in one
to five synthetic steps.

\textbf{Results and Discussion}

\textbf{Chemistry.} The 4-phenol (4) was synthesized from 1 by
treatment with freshly prepared MgO, procured by
sonication of Mg and I\textsubscript{2} in Et\textsubscript{2}O/toluene, affording 4 in 99% yield, an improvement on prior methods (Scheme 1).\textsuperscript{36, 37} Alkylation of 4 to synthesize 5 and 6 has previously
been described.\textsuperscript{38, 39} Position 4 analogs 7 and 8 were prepared
from 4 with Ag\textsubscript{2}O and the appropriate alkyl bromide in
dioxane as solvent.\textsuperscript{40} The syntheses of 9 and 10 have already
been described.\textsuperscript{35, 41} For the preparation of 11, 12 and 13 see Rnomest et al.\textsuperscript{42} and
Arkley et al.\textsuperscript{43} Compound 12 was alkylated using Ag\textsubscript{2}O and
Et\textsubscript{2}N followed by repeated solvolysis in MeOH with CSA to
afford 14. The dichloro analogs 16 and 15 were synthesized
using 2,\textsuperscript{44} POCl\textsubscript{3}, LiCl and dioxane, a modification of a
known method (Scheme 2).\textsuperscript{45} The compounds 17-33
were synthesized by 1,4 addition of the corresponding alcohol to
16 using either NaH or DBU as base (Scheme 3).\textsuperscript{46} The isogriseofulvin analogs 3 and 35 were synthesized in the
same manner but from 15. Compound 3 has previously been
prepared by treating 2 with excess diazomethane yielding
both 1 and 3.\textsuperscript{47} Compounds 26 and 38 have been reported in a
patent,\textsuperscript{48} which is also the case for 19, 21 and 22.\textsuperscript{49} The
dimer 34, which was conveniently synthesized together with
22 by a second 1,4-addition of 22 to 16, has been previously
published in a Japanese patent.\textsuperscript{50}
The analogs with an oxime functionality 36, 37 and 38 were
synthesized from the corresponding ketones (1, 17 and 26)
using hydroxylamine hydrochloride in ethanol and
DMSO.\textsuperscript{51, 52} Isoisogriseofulvin (39-44) and griseofulvin analogs
(45-49 and 17) were synthesized simultaneously in pairs by
solvolysis with the respective alcohols and catalytic CSA
and subsequently separated by chromatography (Scheme 4).
Analogs 50-55 were synthesized according to published
procedures.\textsuperscript{53} The fungal secondary metabolite geosmin (50) was
recently isolated from \textit{Aspergillus terreus}.\textsuperscript{54} Please see the
supporting information for H and 13C NMR spectra for
all compounds and experimental for compounds 2, 3, 15,
39-41 and 44.
Scheme 1. (a) MgI$_2$, toluene, Et$_2$O; (b) RBr, Ag$_2$O, dioxane; (c) HOAc, 2 M H$_2$SO$_4$; (d) 0.66 M NaOH; (e) MeOH, CSA; (f) MeOH, CSA ($\uparrow$) or EtBr, Ag$_2$O, dioxane; then MeOH, CSA ($\uparrow$).

Scheme 2. (a) LiCl, POCl$_3$, dioxane.

Scheme 3. (a) ROH, DBU, THF or dioxane. (b) hydroxylamine, NaOAc, EtOH, DMSO.

Scheme 4. (a) ROH, CSA, dioxane (17, 42-44, 48, and 49).

Figure 2. The analogs 50-55 were prepared according to literature procedures.$^{22}$ Geodin (56) was isolated as previously described.$^{36}$

Assays. Griseofulvin (1), geodin (56) and 53 griseofulvin analogs covering variations on six positions (see Figure 1) were tested against two dermatophytes ($T$. rubrum and $T$. mentagrophytes) and against the breast cancer cell line MDA-MB-231 in a cytotoxicity assay. For all the test results the following definitions were used: if no activity was observed at 50 μM a given compound was deemed inactive; if activity was observed but 50% inhibition was not reached at 50 μM a given compound is described as having low activity and no IC$_{50}$ value is calculated (see supporting information for examples). The IC$_{50}$ of griseofulvin (1) was determined to be 0.38 ± 0.048 μM against the $T$. rubrum isolate and 0.058 ± 0.018 μM against the $T$. mentagrophytes isolate. All IC$_{50}$ values and 95% confidence intervals are given in Table 1.

Anti-fungal Structure-Activity Relationship for $T$. rubrum and $T$. mentagrophytes. All compounds in the position 4 series of 4-8 were less active than griseofulvin (1) against the two fungi and 8 was completely inactive. For $T$. rubrum 4 and 6 were inactive, 7 had low activity and 5 was, with an IC$_{50}$ of 2.0 μM, approximately five times less potent than griseofulvin. For $T$. mentagrophytes analog 4...
showed low activity and the rest were less potent than 1: 5 (0.29 μM), 6 (0.25 μM) and 7 (0.17 μM). The activity of 6 has previously been reported against a number of dermatophytes (Epidermophyton floccosum, Microsporum canis, T. interdigitale, T. persicolor, T. mentagrophytes and T. rubrum) and found to be less active than 1 against all of them.11

The nitro (9) and amine (10) position 5 analogs were inactive against both fungi. Compound 10 has also been reported as inactive, but 9 has been reported as weakly active with inhibition for both fungi starting at around 25 μM.11 Other position 5 analogs (ethoxycarbonyl,11 methoxy, methyl or chloro11) have been reported to have lower activity than 1 against dermatophytes.

Whereas the position 6 phenols have been reported to retain the activity of 1 against T. mentagrophytes with an IC50 of 0.06 μM which is in accordance with the literature.11 The 2' series is the most extensively studied due to their anti-cancer phenotype-inducing curling of hyphae, but less active against all dermatophytes except one.11The introduction of an oxime functionality on the parent compounds (17, 26-29, 32 and 33) revealed activity against T. rubrum and T. mentagrophytes with IC50 values of 7.9 μM against T. rubrum and 0.30 μM against T. mentagrophytes. The vinyl sulfide analog 18 (1.3 μM) was as active as 17 against T. rubrum but against T. mentagrophytes the IC50 (0.058 μM) was equal to that of griseofulvin (1). The 3'-dimethyl analog 52 was inactive against both fungi. The series of 3' analogs include three 3'-iodo analogs with 2' modifications, 2'-methoxy (53), 2'-ethoxy (54) and 2'-benzylxoy (55). The 3'-iodo griseofulvin (53) was inactive against T. rubrum and had low activity towards T. mentagrophytes in accordance with Crosse et al.11 The 2'-benzylxoy (55) analog was inactive against T. rubrum and close to twenty times less potent (1.1 μM) than 1 against T. mentagrophytes. Analog 55 was also four times less potent than 17, which has no 3' iodole group. The propoxy (54) analog showed activity against both fungi (T. rubrum 1.6 μM and T. mentagrophytes 0.23 μM) but was less potent than both 1 and the analog 46, which does not contain iodide. Analog 54 has been reported to be the most potent at inducing curling of hyphae, but less active against all dermatophytes. The three 2' pyridine analogs (26-30) had low activity towards T. rubrum and 30 also showed low activity towards T. mentagrophytes. Compounds 29 and 28 had IC50 values of 0.35 μM and 1.3 μM, meaning they were approximately six and 22 times less potent towards T. rubrum compared to 1. The series with ortho-, meta- and para-methoxy groups revealed 33 as inactive against both fungi, 31 with low activity towards T. rubrum and an IC50 of 0.70 μM against T. mentagrophytes. The meta-substituted analog 32 (0.06 μM) was threefold less active against T. rubrum compared to 1 and displayed a twofold decrease in activity against T. mentagrophytes with an IC50 of 0.14 against T. rubrum and T. mentagrophytes. The 2'-phenoxy analog 23 had IC50 values of 6.1 μM against T. rubrum and 1.6 μM against T. mentagrophytes, while the phenylethoxy analog 24 had low activity against T. rubrum and an IC50 of 0.28 μM against T. mentagrophytes. Lower activity for 23 has previously been reported.11 The vinyl sulfide analog 18 (1.3 μM) was as active as 17 against T. rubrum but against T. mentagrophytes the IC50 (0.058 μM) was equal to that of griseofulvin (1). The 3'-dimethyl analog 52 was inactive against both fungi. The series of 3' analogs include three 3'-iodo analogs with 2' modifications, 2'-methoxy (53), 2'-ethoxy (54) and 2'-benzylxoy (55). The 3'-iodo griseofulvin (53) was inactive against T. rubrum and had low activity towards T. mentagrophytes in accordance with Crosse et al.11 The 2'-benzylxoy (55) analog was inactive against T. rubrum and close to twenty times less potent (1.1 μM) than 1 against T. mentagrophytes. Analog 55 was also four times less potent than 17, which has no 3' iodole group. The propoxy (54) analog showed activity against both fungi (T. rubrum 1.6 μM and T. mentagrophytes 0.23 μM) but was less potent than both 1 and the analog 46, which does not contain iodide. Analog 54 has been reported to be the most potent at inducing curling of hyphae, but less active against all dermatophytes.
Table 1. All available IC$_{50}$ values from the screen. If no activity was observed at 50 µM a given compound is deemed inactive. If activity was observed, but 50% inhibition was not reached at 50 µM a given compound is described as having low activity and the IC$_{50}$ value is not calculated.

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Table 1. All available IC$_{50}$ values from the screen. If no activity was observed at 50 µM a given compound is deemed inactive. If activity was observed, but 50% inhibition was not reached at 50 µM a given compound is described as having low activity and the IC$_{50}$ value is not calculated.
Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes
Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells

accordance with the literature, where 3 and 39 have been reported to be less active than griseofulvin against a number of dermatophytes (E. floccosum, M. canis, T. interdigitale, T. mentagrophytes and T. rubrum). 9,10 Griseofulvic acid (2) was inactive against T. rubrum and had low activity against T. mentagrophytes contrary to prior work, which reported 2 to be inactive towards T. mentagrophytes but having visible growth inhibition at 30 μM against T. rubrum. 11 The griseofulvin dimer 34 was inactive against both fungi. The dichloro analog 16 showed low activity towards both fungi while 15 was inactive against both. The reduced analog 54 had low activity against T. rubrum and an IC₅₀ of 3.0 μM against T. mentagrophytes, which is 50 times less potent compared to 1. Geodin (56) was inactive against both fungi.

Of the 55 compounds tested, 27 analogs were inactive or exhibited low activity for both fungi (see Table 1). Nine of the compounds were active against both fungi within the same order of magnitude compared to griseofulvin (17, 20, 21, 23, 26, 32, 36, 49 and 54). There were however some compounds that differed significantly against the two fungi. The compounds 6, 7, 12, 18, 24, 26, 29, 31, 51 and 55 were all inactive or had low activity towards T. rubrum but had IC₅₀ values in the range of 0.25-3.0 μM against T. mentagrophytes. For other analogs (14, 18, 19, 27, 47 and 48) the activity against T. mentagrophytes was similar to that of 1 but against T. rubrum it was significantly lower. This is in accordance with earlier observations by Crosse et al., demonstrating that a given analog was more active against some fungi but less active against others. 15

Anti-cancer Structure-Activity Relationship. Looking at the IC₅₀ values for the 16 analogs (1, 17, 18, 20, 23-25, 27, 36, 37, 45-49 and 54) tested active in both the multipolarity assay 20 and the cytotoxicity based assay there was good correlation between the data with an R² of 0.70 (see supporting information). The IC₅₀ of griseofulvin (1) was determined to be 23 ± 5 μM (20 ± 1 μM in the phenotype-based spindle multipolarity assay 20), while an IC₅₀ of 25 ± 4 μM against HeLa cells was found by Panda et al. 14 In the position 4 series, 4, 5 and 7 were all inactive but 6 and 8 harboring aromatic moieties had activities similar to griseofulvin (1) with IC₅₀ values of 20 (6) and 17 μM (8). This indicates a mode of action that does not involve induction of multipolar mitosis as 6 was inactive in the multipolarity assay. Both the position 5 analogs 9 and 10 were inactive in the cytotoxicity assay as they were in the assay for multipolarity induction. The 6-phenol griseofulvic acid analog 11 was inactive and the 6-phenol griseofulvin analog (13) showed low activity. The 6-ethyl griseofulvin (14) analog was approximately 50% less active than griseofulvin. The two dichloro analogs 15 and 16 were some of the most cytotoxic among the tested analogs with IC₅₀ values of 1.0 and 3.2 μM, respectively.

When elongating the 2’ position from the parent methoxy (1) to ethoxy (45) and propoxy (46) and then further increasing the bulkiness with isopropoxy (47), cyclopentoxy (49) and benzylxoy (17) the activity increased through the series (see Figure 3), plateauing with 17 and 49 at 2.1 and 3.2 μM, respectively. The same trend was seen when these compounds were tested in the phenotype-based multipolarity assay. Moving from the benzylxoy derived analogs to even bulkier groups like the naphthalen-1-ylmethoxy (26, 13 μM), biphenylmethoxy (25, 5.8 μM) and 1-adamantylmethoxy (27, 4.7 μM) analogs, the activity did not increase further. The three compounds were still more potent than griseofulvin though, which was also observed for 25 and 27 in the phenotype-based assay.

Figure 3. The activity increases with the number of carbon atoms at the 2’ position from griseofulvin itself to the 2’ ethoxy (45), propoxy (46), isopropoxy (47) and cyclopentoxy (49) before leveling out with the cyclopentene (49) and benzylxoy (17) analogs. The bulkier analogs 25, 27 and 26 were less active than the benzylxoy analog (17).

A number of variations of 17 have been tested (18-24, 28-30 and 31-33) and although all except 33 (low activity), 28 (32 μM) and (24, 17 μM) 30, 19 μM were more active than 1, only 20 was as active as 17 with an IC₅₀ of 1.8 μM. The difference in activity between the phenox (23, 7.2 μM) and phenylethoxy (24, 17 μM) analogs was less pronounced in the cytotoxicity assay than in the multipolarity assay with two and a half orders of magnitude compared to the approximately seven-fold difference in the phenotype-based assay. The dimer (34) was about twice as active as 1 with an IC₅₀ value of 8.5 μM. The introduction of an oxime functionality at the 4’ position (36) increased potency twofold to 12 μM, an increase in activity that was also seen in the phenotype-based assay. Introducing the oxime to analogs 17 and 26 affording 37 and 38 improved the activity further for both compounds. Apart from 15, 37 is the most active analog in the cytotoxicity assay with an IC₅₀ of 1.4 μM. The stability of the oximes in PBS buffer (pH 7.4) was tested and less than 5% hydrolysis to the parent ketones could be detected after 48 hours (data not shown).
The isogriseofulvin analogs tested in the multipolarity assay (3 and 39-44) were all inactive. In the cytotoxicity assay 35 retained the activity of 1, while the rest were either inactive (3), showed low activity (39, 44) or were less potent (40, 43 μM, 41, 48 μM, 42, 52 μM and 43, 27 μM).

Geodin (56) did not induce multipolar mitoses and was in that respect deemed inactive. Geodin (56) is however twice as cytotoxic as griseofulvin when tested against the MDA-MB-231 cell line, with an IC<sub>50</sub> of 9.9 μM opposed to 18 μM for 1.

The reduced analogs 50 and 51 as well as the 3'-dimethyl analog 52 were inactive in both anti-cancer assays. In the 3'-iodo series, 53 (22 μM) retained activity, the 2' propoxy analog (54, 8.0 μM) had increased activity and 55 exhibited low activity. 53 and 55 were inactive in the phenotype-based assay but 54 was more potent than 1.

Anti-cancer SAR versus anti-fungal SAR. The difference between the anti-fungal and anti-cancer data is illustrated in Figure 4 demonstrating that most of the analogs had increased potency against the cancer cell line but against the two fungal strains the activity was lower than for griseofulvin (1). Looking at the 4 position there were inconsistencies throughout all three cell types. The ethyl analog 5 was active against both fungi but inactive towards the cancer cells, while the bulkier naphyl analog 8 was inactive against both fungi but more potent than griseofulvin against the MDA-MB-231 cells. Analogs 6 and 7 were more potent than 5 against T. mentagrophytes, but both compounds were inactive or showed low activity towards T. rubrum. The most potent analog towards the cancer cells (15) was inactive against both fungi and the isomer (16), which was amongst the most active compounds, only had low activity towards the two fungi. The two bulky analogs 25 and 27 as well as the dimer (34) inactive in the anti-fungal assays but were all more potent than griseofulvin against the cancer cell line. It is also worth noticing that geodin (56) was inactive against both fungi but twice as potent as 1 against the cancer cell line.

Figure 4. The IC<sub>50</sub> values for each cell line have been normalized defining the activity of griseofulvin as having a value of 1. It is seen that of the 53 analogs the majority was less active against the two fungi opposed to the anti-cancer activity where most analogs were more active than griseofulvin.
The introduction of an oxime at the 4’ position (36, 37 and 38) decreased the potency compared to the parent compounds (1, 17 and 26) against both fungi. The opposite effect was seen for the cancer cell line, where the introduction of this moiety increased the activity in all three compounds (36, 37 and 38).

There were however some similar trends for all three cell types as well. A number of analogs were inactive or had low activity towards both fungi and cancer cells. Amongst these were the position 5 analogs (9, 10), isogriseofulvin (3) and the series of isogriseofulvin analogs (39-44) with 35 as the sole exception. The two 6 phenols (11, 13) were virtually inactive against all cell types, which was the same for 50-52.

Conclusion

The first comparison of anti-fungal and anti-cancer SAR for griseofulvin analogs is presented in this work covering 53 analogs of griseofulvin as well as the natural product geodin (56). All compounds have been tested against T. rubrum, T. mentagrophytes and in a cytotoxicity assay against MDA-MB-231 breast cancer cells.

Even though there were similarities between the SAR of the two fungi, with some compounds showing no activity against both fungi and some active against both, there were also some differences. Analogs 46 and 48 retain the activity of griseofulvin against T. mentagrophytes but were two and four fold less active against T. rubrum than 1, respectively. This is in accordance with reported observations by Crosse et al. that analogs differ in activity against different dermatophytes.11

We show that there is a good correlation between the IC50 values from analogs that were active in both the phenotype-based assay for spindle multipolarity7 and the cytotoxicity assay used in this work with an R2 of 0.70. Comparing the two fungal SAR’s with the anti-cancer cytotoxicity SAR afforded distinct differences. The analogs 8, 15, 27 and 38 were inactive or had low potency against both fungi, but either retained the activity (8) or were at least twofold more active against the cancer cells compared to 1. The 2’-benezoxyl analog (17) was less active against both fungi in this study and against six dermatophytes in the study by Crosse et al.11 This analog is however nine times more potent than 1 against the cancer cell line MDA-MB-231. It is noteworthy that the two dichloro compounds 15 and 16 were inactive against both fungi, but featured as some of the most cytotoxic agents against the cancer cell line.

The differences in activity observed for the two fungi and the MDA-MB-231 cell line could arise from a number of factors, such as transport over the cell membrane, efflux pumps or different modes of action. We find it unlikely that transport or efflux pumps could explain that the majority of analogs were more active against MDA-MB-231 cells and less active against both fungi. Our conclusion therefore is that the mode-of-action of griseofulvin(s) towards fungal and mammalian cells is different, making it unlikely that tubulin itself constitutes the main cellular target in both fungi and mammalian cells. Since it has been shown that griseofulvin leads to mitotic arrest in both fungal and mammalian cells, an alternative explanation is that griseofulvin disrupts microtubule dynamics without directly interacting with tubulin, e.g. through interaction with microtubule-associated proteins (MAPs).

Experimental Section

The fungal micro broth dilution assay was performed in sterile flat bottomed microplates (cat.# 655101) and lida (cat.# 656161) from Greiner Bio-One GmbH (Frickenhausen, Germany). Each microplate accommodated five dilution series starting at 50 μM of a given analog. Each plate was also fitted with four negative and four positive control wells. The fungi T. rubrum (IBT29284) and T. mentagrophytes (IBT2724) are kept in the IBT fungal collection at department of Systems Biology at the Technical University of Denmark. The correct identity of the fungal cultures were verified by microscopy after the last test round in the antifungal assay. For testing against T. rubrum a fourfold dilution series was used, resulting in ten concentrations with the lowest at 0.2 μM. For T. mentagrophytes an eightfold dilution series was used resulting in 10 concentrations with the last one at 0.4 μM.

Inoculum preparation was performed in accordance with the document M38-A2 from the “Committee for Clinical Laboratory Standards”. Inoculum was adjusted to 0.90-1.5 OD at 530 nM on a Genesis 10uv from Thermo Electron Corporation (Waltham, MA, US). The inoculums were diluted fiftyfold with RPMI media (with L-glutamine, without NaClO3, cat.# R6504 in MOPS buffer (cat.# 69947)) both from Sigma-Aldrich (St Louis, MO, US). The pH was adjusted to 7.00 with 1 M NaOH and the volume with MilliQ water from a Milli-Q gradient fitted with a Millipore 0.22 μm filter (cat.# MPGL04001) both from Millipore (Billerica, MA, US).

Each well contained 99μL RPMI media with inoculum, 99μL MilliQ water and 2 μL DMSO. Microplates were incubated for 7 days at 37 °C in a WB binder incubator from VWR (Radnor, PA, US) which point the OD of the walls were measured at 530 μM on a BioTek® Synergy 2 (Bromby, Denmark) using Gen5TM 1.10.8 software. The half maximal inhibitory concentration (IC50) values were obtained with GraphPad Prism5 (ver. 5.04) from GraphPad Software (La Jolla, CA, US) using the “nonlinear regression, sigmoidal dose-response (variable slope)” function. The fitting method used was “automatic outlier elimination,”14 which eliminated less than 1.1% data points.

1H NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer both from Agilent (Santa Clara, CA, US). 13C NMR spectra were recorded using either a Varian Mercury 300 MHz or a Bruker AC 200 MHz from Bruker Optics (Ettlingen, Germany). Chemical shifts were measured in ppm and coupling constants in Hz. When (DCl) was used
as solvent the residual peak was used as internal reference at 6.727 for 1H NMR and 6.770 for 13C NMR spectra. IR spectra were recorded using a Bruker Alpha ATR and measured in cm⁻¹. All melting points are uncorrected. TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merck 1.05554.0001).

High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe (Agilent 5690A). HRMS analysis was performed using a Z-spray electrospray ionisation (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Agilent 1100) system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Agilent 1100).

**General Procedure for the Synthesis of position 4 ethers (7 and 8)**

The appropriate alkyl bromide (1.2 mmol, 3 equiv.) was added to a solution of 4 (0.4 mmol, 1 equiv), Ag₂O (1.2 mmol, 3 equiv), and 1,4-dioxane (5 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), the combined organic phases were washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), the combined organic phases were dried (MgSO₄), and concentrated. The following process was repeated three times: The residue was dissolved in MeOH (10 mL) and CSA (0.1 mmol) was added, the mixture was heated to 65 °C for 18 h. EtOAc (10 mL) was added to the solution and the mixture was washed with sat. aq. NaHCO₃ (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), the combined organic phases were dried (MgSO₄), and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product.

**General Procedure for the synthesis of 1'-2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one (14)**

Ethyl bromide (0.9 mmol, 3 equiv) was added to a solution of 12 (0.3 mmol, 1 equiv), Ag₂O (0.9 mmol, 3 equiv), and 1,4-dioxane (3 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), the combined organic phases were dried (MgSO₄), and concentrated. The following process was repeated three times: The residue was dissolved in MeOH (10 mL) and CSA (0.1 mmol) was added, the mixture was heated to 65 °C for 18 h. EtOAc (10 mL) was added to the solution and the mixture was washed with sat. aq. NaHCO₃ (15 mL). The aqueous phase was extracted with EtOAc (2 × 10 mL), the combined organic phases were dried (MgSO₄), and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product.
T. rubrum and MDA-MB-231 Cancer Cells

Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes

**Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells**
Yield: 62 mg (% white crystals); R-value (EtOAc/heptane, 5:1): 0.43; mp. 92-95 °C; IR (neat, cm\(^{-1}\)): 1705, 1660, 1613, 1589; \( ^1H \) NMR (500 MHz, CDCl\(_3\)): \( \delta \) 7.23 (1H, m), 7.09 (1H, m), 6.86 (1H, m), 6.81 (1H, s), 6.11 (1H, s), 5.64 (1H, s), 4.95 (1H, d, J = 13.2 Hz), 4.88 (1H, d, J = 13.2 Hz), 4.03 (1H, s), 3.96 (1H, s), 3.04 (2H, dd, J = 16.7, 13.4 Hz), 2.85 (2H, dd, J = 13.4, 4.7-6.7 Hz), 2.43 (2H, dd, J = 16.7, 4.7 Hz), 0.99 (6H, d, J = 6.6 Hz); \( ^1C \) NMR (CDCl\(_3\), 50 MHz): \( \delta \) 197.0, 192.3, 169.6, 169.5, 157.6, 156.3, 129.0, 127.3, 122.9, 120.4, 110.0, 105.6, 105.1, 97.1, 90.7, 89.4, 66.2, 56.9, 56.2, 55.2, 39.9, 36.2, 14.2; [\( \eta \]^n = +124\(^\circ\) (c = 1 in CHCl\(_3\)); HRMS (ESI\(^+\)) calcd for [M+H]/[C\(_{24}\)H\(_{37}\)ClO\(_{7}\)]: 419.1211, found 419.1212.

(2,5,8,10,13,16,18,21,24,27,30-tetralone-12,18-dione) 30

Yield: 82 mg (43 %) (white crystals); R-value (EtOAc/heptane, 5:1): 0.43; mp. 92-95 °C; IR (neat, cm\(^{-1}\)): 1705, 1660, 1610, 1588; \( ^1H \) NMR (CDCl\(_3\), 500 MHz): \( \delta \) 7.23 (1H, m), 7.09 (1H, m), 6.86 (1H, m), 6.81 (1H, s), 6.11 (1H, s), 5.64 (1H, s), 4.95 (1H, d, J = 13.2 Hz), 4.88 (1H, d, J = 13.2 Hz), 4.03 (1H, s), 3.96 (1H, s), 3.04 (2H, dd, J = 16.7, 13.5 Hz), 2.87 (1H, dd, J = 13.5, 4.7, 6.6 Hz), 2.43 (1H, dd, J = 16.7, 4.7 Hz), 0.99 (6H, d, J = 6.6 Hz); \( ^1C \) NMR (CDCl\(_3\), 50 MHz): \( \delta \) 197.0, 192.3, 169.6, 169.5, 157.6, 156.3, 129.0, 127.3, 122.9, 120.4, 110.0, 105.6, 105.1, 97.1, 90.7, 89.4, 66.2, 56.9, 56.2, 55.2, 39.9, 36.2, 14.2; [\( \eta \]^n = +124\(^\circ\) (c = 1 in CHCl\(_3\)); HRMS (ESI\(^+\)) calcd for [M+H]/[C\(_{24}\)H\(_{37}\)ClO\(_{7}\)]: 419.1211, found 419.1212.
Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes

Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells

(25.6'R)-7-Chloro-4,6-dimethoxy-benzofuran-3-one-2-spiro-1'-(4'-C-methoxybenzyl)oxy-6'-methyl-cyclohex-3'-en-2'-one 35

Yield: 83 mg (32 %) (white crystals); R-value (EtOAc/heptane, 5:1); m.p. 173-175 °C; IR (neat, cm^-1): 1691, 1653, 1605, 1584; 1H NMR (CDCl3, 300 MHz): δ 7.37-7.27 (1H, m), 7.02-6.84 (3H, m), 6.09 (1H, s), 5.54 (1H, d, J = 1.0 Hz), 5.01-4.85 (2H, m), 4.01 (3H, s), 3.93 (3H, s), 3.84 (3H, s), 3.26 (1H, ddd, J = 17.5, 11.9, 1.0 Hz), 2.96-2.80 (1H, m), 2.54 (1H, dd, J = 17.5, 3.0 Hz), 1.03 (3H, d, J = 6.6 Hz); 13C NMR (CDCl3, 50 MHz): δ 191.7, 188.7, 177.7, 169.7, 164.5, 159.9, 157.7, 136.0, 129.7, 120.0, 114.2, 113.2, 105.1, 108.7, 97.3, 95.1, 98.5, 71.0, 57.0, 56.2, 55.3, 35.2, 32.14.5; [a]21D +179° (c = 0.6 in CHCl3); HRMS (ESI) calel for [M+H]+ [C24H24ClO7] 459.1216, found 459.1216.

General procedure for the synthesis of enol ethers by solvolysis (42 and 43).

CSA (0.1 mmol, 1 equiv.) was added to a solution of griseofulvic acid (10.0 mmol, 1 equiv.) in the appropriate alcohol (6 mL, 0.1 M). The mixture was stirred at 100 °C for 6 hours, and then cooled to 20 °C. EtOAc (20 mL) was added to the solution and the mixture was washed with sat. aq. NaHPO4 (20 mL) and water (20 mL). The combined aqueous phases were extracted with EtOAc (3×20 mL), the combined org. phases were dried (MgSO4) and concentrated. The residue was purified by column chromatography (toulen:CH2Cl2:EtOAc 7:2:1) to afford the desired products. When possible the products were re-crystalized from EtOAc/Heptane.

(25.6'R)-7-Chloro-4,6-dimethoxy-benzofuran-3-one-2-spiro-1'-(4'-cyclopropoxy-methoxy-6'-methyl-cyclohex-3'-en-2'-one) 42

Yield: 520 mg (21 %) (white crystals); R-value (EtOAc/heptane, 5:1); m.p. 160-162 °C; IR (neat, cm^-1): 1703, 1658, 1610, 1592; 1H NMR (CDCl3, 500 MHz): δ 6.06 (1H, s), 5.36 (1H, d, J = 1.2 Hz), 3.99 (3H, s), 3.89 (3H, s), 3.73 (2H, dq, J = 10.4, 7.2 Hz), 3.20 (1H, dd, J = 17.5, 12.2 Hz), 2.88-2.79 (1H, m), 2.49 (1H, dd, J = 17.5, 5.9 Hz), 1.27-1.17 (1H, m), 1.01 (3H, d, J = 6.6 Hz), 0.67-0.60 (2H, m), 0.35-0.29 (2H, m); 13C NMR (CDCl3, 50 MHz): δ 191.7, 188.7, 178.1, 169.6, 164.4, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 74.0, 56.9, 56.2, 35.1, 33.2, 14.4, 9.3, 3.2 (2C); [a]21D +190° (c = 0.4 in CHCl3); HRMS (ESI) calel for [M+H]+ [C24H24ClO7] 459.12.05, found 459.1114.

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Supporting Information Available

Full experimental data for compounds 3, 39-41 and 44 as well as copies of NMR spectra for compounds 3, 7, 8, 15, 19, 21, 22, 26, 28-35 and 38-44. This material is available free of charge via the internet at http://pubs.acs.org.

References

Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes

*Trichophyton mentagrophytes, T. rubrum* and MDA-MB-231 Cancer Cells
Chapter 5

Synthesis and Single Crystal X-ray Analysis of two Griseofulvin metabolites


The supporting information from Chapter 5 can be seen in Appendix E
Synthesis and Single Crystal X-ray Analysis of two Griseofulvin Metabolites
Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

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Abstract

The two phenols, 6-0-desmethyl griseofulvin and 4-0-desmethyl griseofulvin are metabolites of the antifungal drug griseofulvin. Herein, we present an improved synthesis of the 6-phenol derivative, and an unequivocal proof of both structures by single-crystal X-ray analysis.

The natural product griseofulvin (1) (see Fig. 1) was first isolated by Oxford et al. in 1939 and later shown to possess antifungal properties. This antifungal agent is still in clinical use today and is the only orally administered drug approved by the Food and Drug Administration for the treatment of tinea capitis (ringworm of the scalp). Recently, griseofulvin has received renewed attention due to reports of both antiproliferative effects in cancer cells as well as suppression of hepatitis C replication. As a result of its notoriously low water solubility, griseofulvin is furthermore, often used as a benchmark compound in formulation studies and in the development of drug delivery systems.

The metabolism of griseofulvin has been studied both in vitro and in vivo and reported in several publications. In addition to studies in fungi, the in vivo metabolism of griseofulvin has been investigated in rats, mice, rabbits, dogs, and monkeys. Known important metabolites of griseofulvin include 6-0-desmethylgriseofulvin (2) and 4-0-desmethylgriseofulvin (3), but their structures have never been proven unambiguously. In the literature, it is commonly merely stated that the metabolites were compared with authentic samples. Others have used spectroscopic properties and melting points to identify the structures by comparing these data with earlier work. We present herein, the synthesis and crystal structures of both 6-0-desmethylgriseofulvin (2) and 4-0-desmethylgriseofulvin (3), which provide final verification of the structural assignment.

6-0-Desmethylgriseofulvin (2) was first synthesized by Arkley et al. in six steps with an overall yield of 14% (Scheme 1). To confirm the outcome of these transformations, the synthetic route was reproduced and we were actually able to isolate a small amount of 2 at step three (Scheme 1). The lengthy synthesis and poor yield of this route prompted us to search for a more convenient method to access 2. Thus, we were pleased to obtain the desired phenol in 29% yield after the treatment of griseofulvin (1) with LiI in pyridine at 115 °C (Scheme 2). The synthesis of 4-0-desmethylgriseofulvin (3) is described in Scheme 3. The structures of griseofulvin (1), 6-0-desmethylgriseofulvin (2) and 4-0-desmethylgriseofulvin (3) are depicted in Figure 1.
91

Supplementary data

It is not possible to distinguish between the 4 and 6 methoxy groups of 1 by gHMBC as no $^{1}J_{CH}$ correlation is observed. The $^{1}H$ NMR spectrum of 2 (see Supplementary data) does not exhibit a signal for the phenolic proton, due to rapid proton exchange, and thus no heteronuclear correlations can be used to aid in the assignment of the spectrum. For 3, the phenolic proton is observed (see Supplementary data) and the gHMBC contains a single $^{1}J_{HC}$ correlation to C-3a, confirming the position of the phenol. The UV and fluorescence spectra of 2 and 3 were all but identical, and despite small differences in the MS–MS spectra (see Supplementary data), the retention time$^{25}$ is still the most reliable and sensitive analytical method for distinguishing the two phenols.

Acknowledgment

The authors thank the Danish Research Council (ref. 274-07-0561) for the financial support.

Supplementary data

Supplementary data (experimental procedures, characterization, and purity data, HPLC traces, NMR, UV, and MS–MS spectra for compounds 2 and 3, and crystallographic information cif format) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.08.095

References and notes

23. Synthesis of 2 (see Supplementary data). The structures of 2 and 3 were confirmed unequivocally by the procedure originally published by Arkley et al.$^{15}$
24. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CDS 778717 (2) and CDS 779178 (3). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or email: deposit@ccdc.cam.ac.uk).
Chapter 6

A Technique for Selective ipso-Substitution of ortho-, meta- or para-Aryl Trimethylsilyl Groups with Radioactive Iodide in Quantitative Yields

To be communicated.

Supporting information from Chapter 6 can be seen in Appendix F
A Technique for Selective ipso-Substitution of ortho-, meta- or para-Aryl Trimethylsilyl Groups with Radioactive Iodide in Quantitative Yields
A technique for selective ipso-substitution of ortho-, meta- or para-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

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Keywords: Thallium, radiolabeling, regioselectivity, griseofulvin.

A novel technique for the radiolabeling of either ortho-, meta- or para-aryl trimethylsilyl-substituted small molecules was developed. The method takes advantage of the ipso-directing and activating properties of a trimethylsilyl substituent on the aryl. Treatment of a trimethylsilyl precursor with Tl(OOCF$_3$)$_2$ followed by sodium $^{125}$I or $^{131}$I consistently affords radioactive yields over 95% for all three regioisomers.

Introduction

The natural product griseofulvin (1, see Figure 1) was isolated by Oxford et al. in 1939 and besides its long known anti-fungal properties, it has recently been shown to possess anti-cancer as well as anti-viral properties. The 2'-benzyloxy-2'-demethoxy-griseofulvin analog (2), was previously found to be 25 times more active than 1, in a phenotypical whole cell assay against the cancer cell line SCC114 (an oral squamous cell carcinoma cell line). The analog (2) was found to be the most potent amongst the 34 griseofulvin analogs tested.

Figure 1: Griseofulvin (1) and 2'-benzyloxy-2'-demethoxy-griseofulvin (2)

The griseofulvin analog 2 warranted further investigation including $^{125}$I/$^{131}$I scintigraphic in vivo imaging to assess bio-distribution and half-life. McKillop et al. have developed a popular method for the introduction of radioactive iodide by direct electrophilic thallation of aromatic systems with Tl($^{125}$I$^{131}$I)$_2$, followed by addition of potassium $^{125}$I-iodide or $^{131}$I-iodide. The substitution of the aryl thallium(III) bistrifluoroacetate with iodide affords solely the ipso substituted product. However, the initial thallation can yield mixtures of the ortho-, meta- or para-thallium(III) bistrifluoroacetate products, and thus the equivalent iodo products, depending on the substrate and conditions. Using a mixture of radiolabeled compounds is not satisfactory and the separation of such iodo isomers can be laborious. Bell et al. published a study concerning the transformation of aryl trimethylsilanes to aryl thallium(III) bistrifluoroacetates. It was noted that this method proceeds solely with ipso substitution of the TMS group. The reaction is also efficient for deactivated aromatic substrates, while direct thallation of such compounds proceeds slowly. When looking for an efficient route to a radiolabeled analog of 2, we faced with two challenges: the instability of 2 in neat TFA, where hydrolysis to griseofulvic acid took place within minutes, and controlling the regioselectivity of the thallation. We hypothesized that combining the work of McKillop et al. and Bell et al. would afford a simple route from aryl-TMS compounds to aryl iodides, via aryl thallium(III) bistrifluoroacetates, in a regioselective manner, effectively substituting the TMS group for an iodo, while taking advantage of the activating property of the silane to use milder conditions for the thallation. The activities of 10, 11 and 12 in the phenotypic assay for multipolarity are markedly different (data not shown) and we found it prudent to develop a method that would afford a single product, while also enabling us to access either the ortho- (10), meta- (11) or para-iodo (12) isomer. Here we describe the initial results using the thallation/iodation reaction in the preparation of radiolabeled small molecules.

Chemistry

The ortho-, meta- and para- (trimethylsilyl)-benzyl alcohols 3, 4 and 5 were synthesized following a known procedure as was compound 6. The 1,4 conjugate addition of the alcohols to 6 was achieved with diaza(1,3) bicyclo-[5.4.0]undecane (DBU) in dioxane as previously described to yield 7, 8 and 9. The “cold” iodo compounds (10, 11 and 12) were prepared by the same method from the corresponding ortho, meta and para (iodo)-benzyl alcohols (commercially available). The synthesis of the three “cold” iodo compounds were also completed by treating the appropriate TMS analog for 30 min. with 2 equiv. of thallium(III)bistrifluoroacetate in MeCN/TFA (7:3) followed by addition of excess NaI. Experiments were performed with 10, 15 and 20% TFA in MeCN, still using 2 equiv. of Tl(OOCF)$_3$, and excess NaI. At 10% TFA the iodo product was only observed in trace amounts but at 15 and 20% the conversion to the iodo product was 6 and 14%, respectively. It was also
investigated how 0.9 equiv of Tl(OOCCF$_3$)$_3$, NaI, MeCN/TFA (7:3) in MeCN/TFA (7:3) affected the reaction and under these conditions the conversion from 7, 8 and 9 to the iodo products was trace amounts, 7 and 24%, respectively. The “hot” thallation/iodation was performed by treating a substrate with 50 mol% of Tl(OOCCF$_3$)$_3$, followed by addition of sodium $^{125}$I-iodide or $^{131}$I-iodide and after 30 sec. the mixture was purifed by HPLC to afford the radiolabeled compounds.

Scheme 1 (a) DBU, dioxane; (b) Tl(OOCCF$_3$)$_3$, NaI, MeCN/TFA (7:3).

Results and Discussion

The “cold” synthesis of the iodo compounds 10, 11 and 12 from the corresponding TMS precursors by thallation/iodation were completed on a 10 mg scale. The identity of these iodo products were confirmed by comparison (NMR, MS and retention time) with the starting materials and verified iodo products as standards. All experiments were performed with a 2 min. reaction time for the thallation step and 1 min. for the introduction of iodine, which was added in excess.

Treatment of 1 with Tl(OOCCF$_3$)$_3$/NaI in TFA hydrolyses the 2′-enol ether completely and is thus not a viable method for introduction of radioactive iodine for this compound class. Using a mixture of MeCN/TFA (7:3), in which I is stable, with the same conditions resulted in no conversion. However, treating the para-TMS compound (9) with 2 equiv. of Tl(OOCCF$_3$)$_3$/NaI in MeCN/TFA (7:3) afforded 60% conversion to the para iodo compound (12) with starting material as the only other peak. Using the same conditions for 7 and 8 afforded the iodo products in 30% and 50% yields, respectively. The reaction does not proceed without Tl(OOCCF$_3$)$_3$.

As “hot” labeling is performed with sub molar amounts of sodium 125-iodide/131-iodide, these levels of conversions are satisfactory as the starting material and thallation(III) bisthiofluoroacetate intermediates are easily separated from the desired radiolabeled compound. The experiments with “hot” iodide consistently afforded radioactive yields of over 95%.

Conclusion

A novel technique for the introduction of radioactive iodine has been described using thallation/iodation in 30% TFA in MeCN of aryl TMS precursors. This was achieved at both the ortho-, meta- and para-position of the griseofulvin analog, affording the products in over 95% radioactive yields.

Experimental

$^1$H NMR spectra were recorded using a Varian Mercury 300 MHz spectrometer from Agilent (Santa Clara, CA, US). $^{13}$C NMR spectra were recorded using either a Varian Mercury 300 MHz or a Bruker AC 200 MHz from Bruker Optics (Ettlingen, Germany). Chemical shifts were measured in ppm and coupling constants in Hz. The residual peak of the solvent CDCl$_3$ was used as internal reference at $\delta$ 7.27 for $^{1}$H NMR and $\delta$ 77.00 for $^{13}$C NMR spectra. IR spectra were recorded using a Bruker Alpha ATR and measured in cm$^{-1}$. All melting points are uncorrected. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (Merck 1.05554.0001). High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm TFA) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min. Compounds were visualized by charring after dipping in a solution of 1% KmnO$_4$, 6.7% K$_2$CO$_3$ and 0.08% NaOH in water. UV visualization was done using a Model UVGL-25 Mineralight Lamp.

To perform additional purification, all reagents and solvents were purchased from commercial suppliers and were used without further purification. Analysis of the products was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) at 60 °C on a Zorbax Stable Bond C18 1.8 μm, 4.6 × 150 mm column (Agilent) with a gradient of 5-60% B over 120 min (flow rate 250 μL/min; solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetoniitile). Analysis of the radio iodinated compounds were performed by RP-HPLC (Chromolith® Performance RP-18e column, 100 × 4.6 mm) fitted with a Raytest GABI gamma detector (Raytest GmbH, Strasbushardt, Germany) using a gradient of 0-100% B over 5 min (200 μL/min; solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetoniitile UV-absorption = 214 nm, $\gamma$-detection).

General procedure A – Compounds 3, 4 and 5. 2-Bromobenzyl alcohol (200 mg, 1.06 mmol) was mixed with anhydrous Et$_2$O (2 mL) and cooled to 0 °C. BuLi
(1.87 mL, 2.25 mmol) was added drop-wise and the mixture was heated at 0 °C for 30 min. TMSCl (0.68 mL, 5.35 mmol) was added drop-wise and the solution was allowed to reach room temperature. After 20 h the reaction mixture was quenched with sat. aq. NH₄Cl (2 mL), extracted with EtOAc (3×3 mL), and the combined organic phases were dried (MgSO₄) and concentrated. Methanol (20 mL) and a drop of HOAc was added and the mixture stirred for 1 h at room temperature before re-concentration. The residue was purified by column chromatography (EtOAc/heptane 1:12.5) affording the product 3.

2-(Trimethylsilyl)[benzyl] alcohol (3). Yield: 100 mg (52 %) (yellow oil); R-value (EtOAc/heptane, 1:3): 0.32; ¹H NMR (CDCl₃, 300 MHz): δ 7.56 (1H, dd, J = 7.3, 1.4 Hz), 7.49 (1H, dd, J = 7.6, 1.4 Hz), 7.41 (1H, ddd, J = 7.6, 7.3, 1.4 Hz), 7.31 (1H, dt, J = 1.4, 7.3 Hz), 4.77 (2H, s), 2.06 (1H, s), 0.37 (9H, s) (Lit.); ¹³C NMR (CDCl₃, 50 MHz): δ 146.1, 138.0, 134.6, 129.4, 126.9, 65.2, 0.3 (3C) (Lit.).

3-(Trimethylsilyl)[benzyl] alcohol (4). Yield: 74 mg (39 %) (yellow oil); R-value (EtOAc/heptane, 1:3): 0.30; ¹H NMR (CDCl₃, 300 MHz): δ 7.50 (1H, s), 7.48-7.43 (1H, m), 7.36-7.31 (2H, m), 4.65 (2H, s), 2.11 (1H, s), 0.27 (9H, s) (Lit.); ¹³C NMR (CDCl₃, 50 MHz): δ 140.8, 139.9, 132.6, 131.9, 127.9, 127.6, 65.4, -1.2 (3C) (Lit.).

4-(Trimethylsilyl)[benzyl] alcohol (5). Yield: 97 mg (50 %) (yellow oil); R-value (EtOAc/heptane, 1:3): 0.33; ¹H NMR (CDCl₃, 300 MHz): δ 7.54 (2H, d, J = 7.9 Hz), 7.37 (2H, d, J = 7.9 Hz), 4.69 (2H, s), 1.66 (1H, s), 0.27 (9H, s) (Lit.); ¹³C NMR (CDCl₃, 50 MHz): δ 141.3, 139.8, 133.5 (2C), 126.3 (2C), 65.2, -1.3 (3C) (Lit.).

General procedure B – Compounds 7, 8, 9, 10, 11 and 12.

To a solution of 6 (0.65 mmol, 1 equiv) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv) and DBU (1.65 mmol, 2.5 equiv). The mixture was heated to 100 °C and stirred for 18 h. The reaction mixture was cooled to 20 °C, and excess reagent was quenched with sat. aq. NH₄Cl (30 mL). The aqueous phase was extracted with EtOAc (3×30 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (heptane/EtOAc 1:2) affording the product 4. When possible the product was recrystallized from EtOAc/heptane.

(2S,5R,R')-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-[2-(trimethylsilyl)benzyl]oxy-6'-methyl-cyclohex-2'-ene-4'-one (7). Yield: 53 mg (20 %) (yellow oil); R-value (toluene/CH₂Cl₂/EtOAc, 3:3:1): 0.30; IR(neat): 1708, 1663, 1612, 1590; ¹H NMR (CDCl₃, 300 MHz): δ 7.49-7.44 (2H, m), 7.32-7.14 (2H, m), 6.09 (1H, s), 5.61 (1H, s), 4.96 (1H, d, J = 12.1 Hz), 4.88 (1H, d, J = 12.1 Hz), 4.00 (3H, s), 3.95 (1H, s), 3.08 (1H, dd, J = 16.5, 13.4 Hz), 2.89 (1H, ddg, J = 13.4, 4.5, 6.6 Hz), 2.45 (1H, dd, J = 16.5, 4.5 Hz), 0.99 (3H, s), 6.09 (1H, s), 5.61 (1H, s), 2.06 (1H, s), 0.37 (9H, s) (Lit.); ¹³C NMR (CDCl₃, 50 MHz): δ 197.0, 192.2, 169.7, 169.5, 135.4, 137.8, 134.5, 129.4, 127.2, 108.5, 105.2, 97.2, 90.7, 89.5, 71.0, 56.9, 53.6, 40.3, 36.5, 14.2, 0.0 (3C); [α] +162° (c = 1.0 in CHCl₃); HRMS (ESI) calc'd for [M+H]+ [C₂₃H₁₈ClO₂Si]+: 501.1500, found 501.1509.

(2S,5R,R')-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-[2-(2-iodobenzyl)oxy]-6'-methyl-cyclohex-2'-ene-4'-one (8). Yield: 58 mg (32 %) (yellow oil); R-value (toluene/CH₂Cl₂/EtOAc, 3:3:1): 0.31; IR(neat): 1710, 1664, 1613, 1590; ¹H NMR (CDCl₃, 300 MHz): δ 7.43-7.38 (1H, m), 7.30-7.12 (1H, m), 6.10 (1H, s), 5.62 (1H, s), 4.93 (1H, d, J = 12.2 Hz), 4.81 (1H, d, J = 12.2 Hz), 4.01 (3H, s), 3.96 (1H, s), 3.06 (1H, dd, J = 16.4, 13.4 Hz), 2.87 (1H, ddg, J = 13.4, 4.4, 6.6 Hz), 2.44 (1H, dd, J = 16.4, 4.4 Hz), 0.98 (3H, s), 0.20 (9H, s); ¹³C NMR (CDCl₃, 50 MHz): δ 196.9, 192.4, 169.6 (2C), 164.5, 157.7, 149.0, 133.7, 133.0, 131.2, 127.8, 127.0, 105.9, 105.1, 97.2, 90.7, 89.5, 70.8, 56.9, 56.3, 40.0, 36.4, 14.2, -1.3 (3C); [α] +187° (c = 1.0 in CHCl₃); HRMS (ESI) calc'd for [M+H]+ [C₂₃H₁₈ClO₂SI]+: 501.1500, found 501.1494.

General procedure C – Compounds 10, 11 and 12. Compound 9 (9.8 mg, 0.02 mmol) was dissolved in 30% TFA in MeCN (340 μL) and triisopropylsilane (2 mL) was added (21 mg, 0.04 mmol, in 210 μL 30% TFA/McCN). After 30 min NaI (11.7 mg, 0.08 mmol) was added and after another 2 min the reaction mixture was quenched with sat. aq. Na₂CO₃ (2 mL). The mixture was extracted with EtOAc (3×30 mL), and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (heptane/EtOAc 2:1) to afford the product 12 (8.7 mg, 35%).
2'-ene-4'-one (11). Yield: 1.1 mg (8 %) (white crystals); Rf-value (EtOAc:heptane, 5:1): 0.45; m.p. 171-174 °C; IR (neat, cm⁻¹): 1703, 1661, 1609, 1586; 1H-NMR (CDCl3): δ 7.59 (1H, d, J = 7.8 Hz), 7.44 (1H, s), 7.13 (1H, d, J = 7.8 Hz), 7.03 (1H, t, J = 7.8 Hz), 6.13 (1H, s), 5.57 (1H, s), 4.85 (1H, d, J = 12.5 Hz), 4.73 (1H, d, J = 12.5 Hz), 4.03 (3H, s), 3.99 (3H, s), 3.08 (1H, dd, J = 16.5, 13.5 Hz), 2.88 (1H, ddg, J = 13.5, 4.5, 6.6 Hz), 2.45 (1H, dd, J = 16.5, 4.5 Hz), 1.01 (1H, d, J = 6.6 Hz); 13C-NMR (CDCl3): δ 196.7, 192.2, 169.4, 169.1, 164.6, 157.7, 157.1, 136.8, 135.2, 130.1, 125.5, 105.7, 105.0, 97.4, 94.2, 90.6, 89.6, 69.2, 56.9, 56.3, 40.0, 36.1, 14.2; Anal. Caled for C23H26O4: C 75.42, H 7.51; Found: C 75.37, H 7.53.

Oral Treatment with Griseofulvin.

2.88 (1H, dd, J = 16.5, 13.5, 4.5, 6.6 Hz), 2.45 (1H, dd, J = 16.5, 4.5 Hz), 1.01 (1H, d, J = 6.6 Hz); 13C-NMR (CDCl3): δ 196.7, 192.2, 169.4, 169.1, 164.6, 157.7, 157.1, 136.8, 135.2, 130.1, 125.5, 105.7, 105.0, 97.4, 94.2, 90.6, 89.6, 69.2, 56.9, 56.3, 40.0, 36.1, 14.2; Anal. Caled for C23H26O4: C 75.42, H 7.51; Found: C 75.37, H 7.53.

We thank the Danish Research Council for financial support.

Supporting Information

1H and 13C NMR spectra for compounds 3-5 and 7-12 as well as UPLC-DAD chromatograms.

References

Chapter 7

(\(+\))-Geodin from \textit{Aspergillus terreus}


A modified version of the supporting information for Chapter 7 can be seen in Appendix G.
(+)-Geodin from *Aspergillus terreus*
Crystal Structure Communications

(+)-Geodin from Aspergillus terreus

Mads H. Rønnest,†a Morton T. Nielsen,b Blanka Leber,b Uffe H. Mortensen,a Mads H. Clausen,a Thomas O. Larsen,a and Pernille Harrisab

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The fungal metabolite (+)-geodin [systematic name: (2R)-methyl 5,7-dichloro-4-hydroxy-6-methoxy-6-methyl-3,4-dioxo-spiro[benzofuran-2,1'-cyclohexa-2,5'-diene]-2'-carboxylate], C17H14Cl4O3, was isolated from Aspergillus terreus. The crystal structure contains two independent molecules in the asymmetric unit. Molecules denoted 1 interact through O—H···O hydrogen bonds creating chains of molecules parallel to the crystallographic c axis, 2 screw axis. Molecules denoted 2 interact through another O—H···O hydrogen bond. The two molecules are similar but molecules 2 have a slightly more planar cyclohexadiene ring than molecules 1. In comparison, cyclohexenone ring C in geodin, (II), consisting of ring systems A, B and C, as shown for (II) in the Scheme below (Grove et al., 1952). Additionally, both compounds (I) and (II) arc dextrorotatory and this general similarity prompted our interest in (I) since (II) has anticancer properties (Ho et al., 2001; Panda et al., 2005). (I) was isolated from A. terreus and tested in our cellular anticancer assay (Rebaza et al., 2007) but did not exhibit any activity (data not shown).

Figure 1

A perspective view of the two independent molecules of geodin. (I), showing the atom-numbering scheme and with displacement ellipsoids drawn at the 50% probability level.
organic compounds

t.r.m.s. deviation of the five atoms of 0.028(3) Å for both molecules. However, atoms O11(O21) and C13(C23) are below the plane and C12(C22) above the plane. In both molecules, there is an intramolecular hydrogen bond from atom O12/O22 to O13/O23 (see Table 2).

The crystal packing down the a axis is shown in Figs. 2 and 3, with the view showing the different packing of molecules 1 (Fig. 3a) and 2 (Fig. 3b) down the c axis. Molecules 1 are hydrogen bonded with a hydrogen bond from atom O12 to atom O15 in a neighbouring molecule (see Table 2, and Figs. 2 and 3a). This creates a chain of molecules along the crystallographic 2 screw axis. A similar hydrogen bond is not found in molecules 2. They are tilted slightly and the distance between atoms O22 and O25(−x + 2, y + 1/2, −z + 2) is 4.521 (4) Å. There are, however, halogen bonds between atoms O23 and Cl21 from neighbouring molecules (see Table 1, and Figs. 2 and 3b) creating chains of molecules along the crystallographic 2 screw axis. Furthermore, molecules of type 2 are oriented so that the AB ring system stacks with the C6 ring from the next molecule in the helix. Molecules 1 and 2 interact via halogen bonds between atoms O27 and Cl12 (see Table 1).

To increase knowledge of the structure–activity relationship of these related compounds (Rønnest et al., 2009) the absolute structure of (I) presented here was determined using anomalous signal from all reflections (Flack, 1983). This showed an R configuration at the spiro centre of both crystallographically independent molecules. In contrast, for (II) the absolute configuration was determined based on alcoholytic reactions (MacMillan, 1959) and later verified by Brown & Sim (1963) by crystal structure determination of a brominated derivative using film data to be S at the spiro centre and R at atom C6.

This structural difference between (I) and (II) could potentially contribute to the observed absence of anticancer activity (Rebacz et al., 2007) for (I).

Based on enzymatic studies of the biosynthesis of geodin, (I) (Fujii et al., 1983), the spirocyclization reaction joining the

---

Figure 2

The molecular packing of (I), showing the hydrogen- and halogen-bond architecture; the view direction is down [100]. Hydrogen and halogen bonds are shown as dashed lines. [Symmetry codes: (i) −x + 1, y + 1/2, −z + 1; (ii) −x + 1, y + 1/2, −z + 1]

Figure 3

Helical chains in (I), viewed in the [001] direction, showing (a) molecules of type 1 and (b) molecules of type 2. [Symmetry codes: (i) −x + 1, y + 1/2, −z + 1; (ii) −x + 1, y + 1/2, −z + 1]
Organic Compounds

Table 1
Selected interatomic distances (Å).

Table 2
Hydrogen-bond geometry (Å, °).

References


Renesse et al. · C2H4Cl3O2


References


References


organic compounds

Chapter 8

Aurantiamine from *Penicillium cavernicola*

Prior to my external stay at DKFZ I worked with the active strain *Penicillium cavernicola* (IBT 3235, CYA) and although the known diketopiperazine aurantiamine was quickly identified as a possible candidate for the activity, the isolation of aurantiamine was initiated.

This was done for a number of reasons, the inhibition of centrosomal clustering had not earlier been associated with aurantiamine, no other active compound was close to being isolated and the reason for the external stay at DKFZ was to learn about the assay and the experiments used for the initial paper by Blanka Rebacz.\(^1\) As those experiments had already been performed with griseofulvin, repeating them again would be unproductive. On those grounds it was decided to isolate aurantiamine and bring it to DKFZ. In the following section experiments have been performed by me under the guidance of Blanka Rebacz except for the centrin staining (in Figure 8.2) and the work on the HeLa, U2OS, MDA-MB-231 and RPE cells which was performed by Simon Anderhub.

*Penicillium cavernicola - aurantiamine*

The alkaloid aurantiamine (1) was first isolated from *Penicillium aurantiogriseum*\(^2\) and HPLC coupled to diode array analysis suggested that it is also produced by *P. cavernicola*.\(^3\) This has been confirmed by the isolation and identification of aurantiamine as a free base (1) in an extract from *P. cavernicola* (IBT 3235, CYA).

Aurantiamine (1) is a diketopiperazine derived from histidine and valine. Diketopiperazines have been isolated from numerous natural sources such as marine derived fungi,\(^4\) sponges\(^5\) and bacteria.\(^6\) They display a broad range of biological activities, including anti-fungal,\(^7\) anti-viral,\(^8\) anti-bacterial\(^9\) and anti-cancer activity.\(^10\)

Aurantiamine is structurally similar to phenylahistin\(^11\) (2, Figure 1), which was initially isolated from *Aspergillus ustus*. Phenylahistin has shown anti-tumor activity with IC\(_{50}\) values in the low micromolar range against several cancer cell lines.\(^11,12,13,14\) Using 2 as a lead compound, an anti-cancer drug candidate (NPI-2358) has been developed and is currently in phase II clinical trials.\(^15,16,17\) The cytotoxic potency of 1 and 2 against P388 murine leukemia cells has been examined and phenylhistin (2) was reported to be forty times more active than aurantiamine (1).\(^18\)

The IC\(_{50}\) of 1, for induction of multipolar mitoses, was determined to be 40 ± 4 μM in SCC114 squamous cell carcinoma cells. Cell cycle analysis was performed
Figure 8.1: The structures of aurantiamine (1) and phenylahistin (2)

Table 8.1: Flow cytometry of SCC114 cells treated with 46 μM aurantiamine

<table>
<thead>
<tr>
<th></th>
<th>Mock 24 h</th>
<th>Mock 48 h</th>
<th>Auran. 24 h</th>
<th>Auran. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-phase</td>
<td>5.3 ± 0.9</td>
<td>4.2 ± 1.2</td>
<td>42.1 ± 8.0</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td>Sub G1</td>
<td>2.0 ± 0.3</td>
<td>3.8 ± 1.3</td>
<td>9.4 ± 1.4</td>
<td>32.2 ± 7.4</td>
</tr>
</tbody>
</table>

to evaluate the effect of aurantiamine (1) on SCC114 cells and BJ fibroblasts. Treated cells were analyzed by flow cytometry following staining with propidium iodide and an antibody to phospho-histone H3 as a mitosis marker (Figure 8.2A, B). Treatment of SCC114 cells with 46 μM aurantiamine for 24 hours led to an increase of cells in M phase of the cell cycle to 42.1 ± 8.0% compared to only 5.3 ± 0.9% of mock-treated cells (see Table 8.1). In contrast, treatment of BJ fibroblasts with the same concentration of 1 for 24 hours led to only 7.2 ± 1.9% of cells in M phase with mock treated fibroblasts having 2.7 ± 1.2% of the population in M phase (see Table 8.2). After 48 hours the percentage of SCC114 cells arrested in mitosis had decreased to 9.4 ± 1.4% compared to 4.2 ± 1.2% for the control, but simultaneously, the percentage of sub-G1 cells had risen to 32.2 ± 7.4%, an effect most likely caused by apoptosis. This phenomenon was further investigated by analyzing cells treated with 1 with an apoptosis marker: whole cell lysates of SCC114 cells treated with 46 μM aurantiamine were evaluated by Western blotting for the presence of the p85 fragment of poly (ADP-ribose) polymerase (PARP), a downstream target of activated caspase 3\textsuperscript{19} (Figure 8.2E). PARP cleavage was already detected after a 24 hour treatment of SCC114 cells with a further increase seen after 48 hours, strongly suggesting that these cells undergo apoptosis. For the mock treated cells no p85 fragment of PARP was seen after either 24 or 48 hours (Figure 8.2D).

The possibility of aurantiamine acting through another mechanism that inhibition of centrosomal clustering was examined by Alwin Krämer and Simon An-

Table 8.2: Flow cytometry of BJ fibroblasts cells treated with 46 μM aurantiamine

<table>
<thead>
<tr>
<th></th>
<th>Mock 24 h</th>
<th>Mock 48 h</th>
<th>Auran. 24 h</th>
<th>Auran. 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-phase</td>
<td>2.7 ± 1.2</td>
<td>0.7 ± 0.6</td>
<td>7.2 ± 1.9</td>
<td>4.1 ± 1.4</td>
</tr>
<tr>
<td>Sub G1</td>
<td>4.5 ± 1.0</td>
<td>4.6 ± 0.9</td>
<td>5.1 ± 1.3</td>
<td>8.6 ± 2.8</td>
</tr>
</tbody>
</table>
derhub. Aurantiamine was tested against HeLa (human cervical cancer), U2OS (human osteosarcoma) and MDA-MB-231 (human breast cancer), showing induction of multipolar mitosis in all three cell lines. However, when tested against another, faster proliferating, non-cancerous control cell line called Retinal Pigment Endothelial (RPE) multipolar mitosis was also induced. This was further investigated by staining MDA-MB-231, SCC114 and RPE cells with centrin antibodies (to label single centrosomes/centrioles) and $\gamma$-tubulin (to label spindlepoles). It was then checked whether each pole in the multipolar cells (after incubation with aurantiamine (1)) also had a centrin signal, which would support the mode of action being centrosomal declustering. This was not the case, instead it was observed that the majority of cells which divide by multipolar mitosis have at least one pole without a centrin signal. So the multipolar mitoses induced by aurantiamine (1) is not a specific inhibition of centrosomal clustering although it is possible that it also includes inhibition of centrosomal clustering.

It was confirmed by isolation and NMR analysis that P. cavernicola produces aurantiamine (1). The IC$_{50}$ value for induction of spindle multipolarity in SCC114 cells for aurantiamine (1) has been determined to be 40 ± 4 $\mu$M. Cell cycle analyses were performed and showed that following exposure to 1, SCC114 cells are arrested in M phase followed by apoptosis, while BJ fibroblasts were affected to a much smaller extent by aurantiamine (1). However RPE cells treated with 1 followed by staining against centrin and $\gamma$-tubulin showed poles with no centrosomes and thus aurantiamine (1) is not a specific inhibitor of centrosomal clustering.

### 8.1 Experimental

Solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. 1D and 2D NMR spectra were recorded using a Varian Unity Inova 500 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz. The residual peak for CDCl$_3$ was used as internal reference at $\sigma$ 7.27 for $^1$H NMR and $\sigma$ 77.00 for $^{13}$C NMR spectra. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray electrospray ionisation (ESI) source, a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software.

SSC114 cells were used for determining IC50 value, cell cycle analysis and Western blotting. Despite the presence of supernumerary centrosomes in 64.5% of SCC114 cells, only 3.6% of the cells in mitosis form multipolar spindles. The remainder of the cell population clusters its centrosomes and undergoes bipolar mitoses, making this cell line an ideal model system.

Flow cytometry to assay cell cycle distribution: trypsinized cells were fixed in 70% aq. ethanol and stained with an antibody to phosphorylated histone H3 (Upstate Biotechnologies, 1:500) for 2 hours at room temperature, followed by a 30 min incubation with secondary antibody Alexa Fluor® 488 (Molecular Probes, 1:1000). Cells were then counterstained with propidium iodide and analyzed on a FACScan flow cytometer (BD Biosciences) using Cellquest software.
**Figure 8.2:** (A) FACS histograms of BJ fibroblasts treated with aurantiamine (1), where a slight increase of cells in mitosis can be seen. (B) SCC114 squamous cell carcinoma cells treated with 1. After 24 hours the population in mitosis has increased significantly, while after 48 hours the sub-G1 population has increased. (C) Mock-treated SCC114 cancer cells and same cells treated with 1. Cells were stained with an anti-centrin mouse monoclonal antibody and an anti-γ-tubulin rabbit polyclonal antibody using goat anti-mouse Cy3 and goat anti-rabbit Alexa 488 as secondary antibodies. Bipolar and multipolar mitosis can be seen, respectively. (D) Dose-response curve for SCC114 cells treated with 1. (E) Western blot (PARP) using whole cell lysates stained with an antibody to full-length and cleaved PARP. Actin provides the loading control.
Western blot (PARP): whole cell protein extracts were prepared by lysis of cells in an appropriate volume of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40 (Genaxxon), 0.5% sodium deoxycholate, 0.1% SDS) supplemented with one Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics) per 50 ml of buffer, followed by mechanical homogenization and collection of the supernatant after centrifugation for 10 min at 20,000 x g. Immunoblotting was performed according to standard protocols. For detection, an antibody to full-length and cleaved PARP (Clone 9532, Cell Signaling) was used following incubation with a HRP-conjugated secondary antibody in BSA.

*P. cavernicola* (IBT 3235) was cultured on 200 plates of Czapek yeast autolysate agar at 25 °C for 7 days and extracted with 1% formic acid ethyl acetate. 1.0 g of the extract (2.6 g total) was fractionated on an IsoleraTM (Biotage) using a gradient from 30 to 100% acetonitrile in water over 30 minutes and a 50 gram SNAP cartridge. The fraction collected from 6.1 to 9.4 minutes was purified on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 4 mL/min H₂O/CH₃CN (Starting at 70/30, increasing to 40/60 over 14 minutes) as the mobile phase to yield 1 (6.2 mg as an yellow oil).

Aurantiamine 1: ¹H NMR (500 MHz, CDCl₃): σ 11.99 (1H, s), 9.01 (1H, br s), 7.55 (1H, s), 6.94 (1H, s), 6.13 (1H, s), 6.04 (1H, dd, J = 17.3, 10.5 Hz), 5.22 (1H, d, J = 10.5 Hz), 5.18 (1H, d, J = 17.3 Hz), 4.06 (1H, t, J = 2.7 Hz), 2.48 (1H, dt, J = 2.7, 7.0 Hz), 1.52 (6 H, s), 1.06 (3H, d, J = 7.0), 0.96 (3H, d, J = 7.0) (in agreement with litt.¹⁸); ¹³C NMR (from gHSQC, gHMBC, 500 MHz, CDCl₃): σ 164.7, 160.6, 144.3, 136.4, 132.1 (2C), 123.6, 113.2, 105.1, 61.0, 37.4, 32.8, 27.8 (2C), 18.5, 15.8 (in agreement with litt.¹⁸); HRMS (ESI+) calcd for [M+H] [C₁₆H₂₃N₄O₂]+ 303.1821, found 303.1814. [α]D²¹ -116 (c 0.10, MeOH) (in agreement with litt.¹⁸).

Aurantiamine was tested in triplicate at 8 different concentrations (80, 60, 40, 20, 10, 5, 2.5, 1 μM). Cells were then fixed and examined by fluorescence microscopy. Two hundred mitotic cells per well were analyzed with the percentage of mitotic cells with multipolar spindles being the read-out. The relationship between readout and the dose was described by a logistic model (see equation 8.1)

\[
p = \frac{1}{1 + \exp(-\alpha - \beta x)}
\]

where p is the percentage of mitotic cells with multipolar spindles and x is the dose scaled according to x=ln(dose+1). The dose axis was scaled to spread out the low concentration data. The IC₅₀ values and the corresponding 95% confidence intervals were estimated based on the fitted dose-response curves. The calculations were performed in Matlab 7.0.4.365 (The Mathworks, Natick, Massachusetts, USA) using the glmfit and glmval functions.

8.2 2’-Benzyloxy-2’-demethoxy-griseofulvin and inhibition of centrosomal clustering

The most potent compound from the initial phenotypical assay, 2’-benzyloxy-2’-demethoxy-griseofulvin (3, see Figure 8.3) was examined further based on the
findings with 1. It was attempted to verify if the multipolarity was indeed inhibition of centrosomal clustering or unspecific induction of multipolarity (For the full manuscript see Appendix A). At lower concentrations 3 showed inhibition of centrosomal clustering, while at higher concentrations unspecific multipolar mitoses were observed. 2'-Benzyloxy-2'-demethoxy-griseofulvin (3) is therefore not 100% specific towards cancer cells, but the difference in activity between cancer cells and healthy cells provide a therapeutic window.

Figure 8.3: 2'-Benzyloxy-2'-demethoxy-griseofulvin 3

Bibliography


Chapter 9

Conclusion

A possible way to specific anti-cancer agents is to explore the differences between healthy cells and cancer cells. One specific difference is the supernumerary centrosomes discovered in cancer cells. This affords the possibility of multipolar mitosis producing unviable progeny. To circumvent this, the cancer cells utilize a mechanism called centrosomal clustering, in order to ensure bipolar mitosis. As healthy cells contain only two centrosomes during mitosis, this mechanism is not used by healthy cells. A small molecule that inhibits centrosomal clustering and forces cancer cells to undergo multipolar mitosis, could therefore be an anti-cancer agent with specificity for cancer. Prior to this project, griseofulvin (1, see Figure 9.1) was identified as a small molecule that induces multipolar mitosis in cancer cells.

The chemistry of griseofulvin (1) was reviewed in Chapter 2, covering the areas of total synthesis, biosynthesis, microbial transformation, spectroscopic studies, and analog synthesis. More than 400 analogs have been synthesized since the discovery of griseofulvin (1) covering most positions of griseofulvin. The review will be of help to other researchers working in the field of griseofulvin, with its overview of more than 120 published papers concerning griseofulvin (1).

In Chapter 3 the structure-activity relationship study of griseofulvin analogs tested in a phenotypical assay was described. A total of 34 (nine novel) griseofulvin analogs were described with a further ten isogriseofulvin analogs tested and found inactive. The analogs covered five positions of griseofulvin (1), namely the 4, 5, 2', 3', and 4' positions. Elongation of the 2' position increased activity and introducing bulkier groups further improved potency with the 2'-benzyloxy (2) analog the most potent with an IC_{50} value of 0.9 μM. Introduction of an oxime to 1 at the 4' position increased activity slightly but did not show a cumulative effect with the 2'-benzyloxy group (3). Alterations at the 4 and 5 positions rendered the analogs inactive and isogriseofulvin analogs were all inactive. This work has contributed to griseofulvin's structure-activity relationship study, an area otherwise poorly examined.

In Chapter 4 55 analogs (11 novel) were tested against two dermatophytes (Trichophyton rubrum and T. mentagrophytes) as well as the cancer cell line MDA231 (human breast adenocarcinoma) in growth inhibition assays. The IC_{50} values from the phenotypical assay was found to correlate well with the values from the cytotoxicity assay (R^2 = 0.70). Tubulin has been proposed as the target for griseofulvin in both fungal cells and mammalian cancer cells. Tubulin is highly
conserved in eucaryotic cells and if tubulin indeed is the target both for fungi and cancer cells, it was anticipated that the structure-activity relationship study would show the same trend, however this was not the case. The vast majority of analogs showed lower potency against the two fungi and higher potency against the cancer cell line. The 2'-benzyloxy-2'-demethoxy-griseofulvin analog (2) was less active against both fungi tested in this work and also less active against six dermatophytes previously tested by Crosse et al (1964). By contrast compound 2 was nine times more potent than griseofulvin (1) in the cytotoxicity assay, highlighting the different SAR and thus indicates different targets in fungi and mammalian cancer cells.

The accepted standards (Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing) for testing compounds against various fungi uses concentrations in mg/L, also for comparing compounds with a variety of molar masses. Visual inspection is used as a mean of evaluating minimum inhibition concentrations. The assay used for testing against fungi in this work used molar concentrations to be able to compare analogs more accurately. The minimum inhibition concentrations were based on spectroscopic measurements, which were processed to IC$_{50}$ values by fitting to a sigmoidal model with the software GraphPad Prism. Using a spectrophotometer as opposed to visual inspection is a more objective way of evaluating raw data. The work presented here is thus a valuable input to the science of antimicrobial susceptibility testing.

In Chapter 5 syntheses of the two phenols of griseofulvin were described. The 4-phenol was obtained in quantitative yields by treatment with MgI$_2$ with a yield of 29% for the 6-phenol by treatment with LiI in pyridine. The position of each phenol was determined by single crystal X-ray analysis and this proved the position of the 4-methoxy group in $^1$H NMR to resonate upfield from the 6-methoxy group. This differentiation of the two methoxy groups will be of help to future synthetic work at ring A of griseofulvin.

The absolute structure of (+)-geodin, isolated from Aspergillus terreus, was determined in Chapter 7 by single crystal X-ray analysis. The spirocenter was found to have the $R$ configuration, by using the Flack parameter. The compound shares the grisan A, B, and C rings of 1 but was tested inactive in the phenotypic assay. However, (+)-geodin was twice as potent when tested in the toxicity
assay against MDA-MB-231 cancer cells in Chapter 4. The determination of the absolute structure will be of interest to research of the biosynthesis of (+)-geodin.

The isolation of aurantiamine (4) from *Penicillium cavernicola* was described in Chapter 8. The compound was tested in a phenotypical assay for multipolarity inducing effects in SCC114 cancer cells (squamous cell carcinoma cells). The compound had an IC<sub>50</sub> of 40 ± 4 μM and by fluorescence-activated cell sorting (FACS) analysis it was observed that aurantiamine (4) induces M phase arrest in the SCC114 cells. It was also observed that the G<sub>0</sub> population increased upon treatment with aurantiamine, which was confirmed using Western blot analysis for the p85 fragment of poly (ADP-ribose) polymerase (PARP), a downstream target of activated caspase 3. The effect on BJ fibroblasts treated with aurantiamine (4) was minor, but RPE cells (Retinal Pigment Endothelial) were affected and multipolar spindles were observed. Aurantiamine (4) was therefore found not to be specific for cancer cells. It was investigated if 2 inhibited centrosomal clustering or like 4 induced unspecific multipolarity in both healthy cells and cancer cells. It was found that 2 at lower concentrations in fact inhibits centrosomal clustering in cancer cells, while at higher concentrations unspecific induction of multipolarity was observed. The analog affected healthy control cells to a lesser extent, providing an overall selectivity index of 10 to 30 fold. Though not truly specific 2 still provides a therapeutic window and at lower concentrations it does inhibit centrosomal clustering.

A novel method for the introduction of radioactive iodide to aromatic rings was described in Chapter 6. This method was used in scintigraphic *in vivo* imaging to assess bio-distribution and half-life of a radioactive griseofulvin analog. The treatment of an aryl trimethylsilyl with thallium(III)trifluoroacetate in 30% TFA in MeCN followed by potassium 125<sup>I</sup>-iodide or 131<sup>I</sup>-iodide affords the radiolabeled compound in over 95% radiochemical yield for all three regioisomers (*ortho*-, *meta*- or *para*-aryl). Since direct thallation/iodation of aryl compounds can yield all three regioisomers, this method offers a more selective approach. The *ortho*-, *meta*- or *para*-ido-aryl of a given compound can be synthesized and tested to assess potency. The most suitable analog can then be chosen and the equivalent TMS compound synthesized. In this way the properties of the radiolabeled analog can be evaluated prior to labeling and only the desired iodo analog is produced.

Regarding future efforts to identify selective inhibitors of centrosomal clustering, the current setup with MDA-MB-231 cells as the cancer cell line screened against, and the RPE cells as controls, holds the foundation of a successful screening strategy. However, the assay is still operated fully manually and thus very time consuming. For the setup to be more successful the assay must be high throughput, making it possible to test a greater number of fungal raw extracts and e.g. synthetic libraries. The fractionation of raw extracts prior to testing should also be attempted. The use of explorative solid phase extraction could be used for initially active compounds to minimize the risk of isolating known anti-cancer compounds, although making this method and the assay compatible would take some work. Using a phenotypical assay, which can identify small molecules
that induces multipolar spindles in cancer cells, is still a rational screening approach, as finding a cancer specific compound is more likely than screening with a simple cytotoxicity assay.
APPENDIX A

GF-15, a Novel Inhibitor of Centrosomal Clustering, Suppresses Multiple Myeloma Growth in vitro and in vivo

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GF-15, a novel inhibitor of centrosomal clustering, suppresses multiple myeloma growth in vitro and in vivo

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Abstract

In contrast to normal cells, malignant plasma cells are highly aneuploid and frequently contain multiple centrosomes. To allow for bipolar mitotic division, supernumerary centrosomes are clustered into two functional spindle poles in many cancer cells. Recently, we have shown that griseofulvin forces tumor cells with supernumerary centrosomes to undergo multipolar mitoses resulting in apoptotic cell death. Here, we describe the characterization of the novel small molecule GF-15, a derivative of griseofulvin, as inhibitor of centrosomal clustering in myeloma cells. In a wide array of myeloma cell lines, the mean inhibitory concentrations (IC50) for proliferation and survival were found to be in the range of 1-5 μM, associated with activation of caspases 8, 9, and 3. GF-15 overcomes the growth advantage conferred by bone marrow stromal and endothelial cell-myeloma co-culture. In addition, treatment with GF-15 was associated with inhibition of VEGF- and IGF1-triggered myeloma cell migration. Importantly, treatment of mouse xenograft models of human myeloma with GF-15 resulted in tumor growth inhibition and significantly prolonged survival. These results demonstrate the in vitro and in vivo anti-tumor efficacy of a prototype small molecule inhibitor of centrosomal clustering and strongly support the further evaluation of this new class of molecules.
Introduction

Although recent advances in the therapeutic management of multiple myeloma (MM) have improved its prognosis, no curative therapy currently exists for this disorder, which is the second most commonly diagnosed hematologic malignancy in the Western world. MM is a plasma cell malignancy characterized almost universally by aneuploidy and chromosomal instability. In hyperdiploid MM, which accounts for approximately 50% of cases, multiple trisomies and monosomies are observed in clonal cells. Even though the mechanisms leading to chromosomal instability in MM are only beginning to be unraveled, accumulating evidence suggests that centrosome amplification might contribute to the acquisition of aneuploidy in MM.

Centrosomes are small cytoplasmic organelles which consist of a pair of centrioles embedded in pericentriolar material and act as microtubule organizing centers. During mitosis, centrosomes function as spindle poles, directing the formation of bipolar spindles, a process essential for accurate chromosome segregation. Centrosomes duplicate precisely once per cell cycle to assure spindle bipolarity, with each daughter cell receiving one centrosome upon cytokinesis. Centrosome amplification is frequent in both solid tumors and hematologic malignancies, and is linked to tumorigenesis and aneuploidy. The extent of centrosomal aberrations correlates with the degree of chromosomal instability and malignant behaviour in tumor cell lines, mouse tumor models, and human tumors.

In mitosis, supernumerary centrosomes can lead to the formation of multipolar spindles, which is a hallmark of many tumor types. Multipolar spindles, however, are antagonistic to cell viability. Most progeny derived from a multipolar mitosis will undergo apoptosis. To circumvent this problem, many cancer cells appear to have mechanisms that suppress spindle multipolarity, the best studied being clustering of supernumerary centrosomes into two spindle poles enabling bipolar division. Bipolar spindle formation via centrosomal clustering is associated with an increased frequency of lagging chromosomes during anaphase, thereby explaining the link between supernumerary centrosomes and chromosomal instability.

The mechanisms of centrosomal clustering in tumor cells are incompletely understood. Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest the involvement of the spindle assembly checkpoint, spindle
tension as controlled by cortical actin cytoskeleton and cell adhesion molecules as well as centrosome and kinetochore components in this process\textsuperscript{19-21}.

Supernumerary centrosomes are almost exclusively found in a wide variety of neoplastic disorders but not in non-transformed cells. Therefore, inhibition of centrosomal clustering with consequential induction of multipolar spindles and subsequent cell death would specifically target tumor cells with no effect on normal cells with regular centrosome content\textsuperscript{9,17}. Using a phenotype-based screening strategy, we have recently identified griseofulvin as chemical inhibitor of centrosomal clustering in human cancer cells\textsuperscript{14}. This compound induced multipolar spindles by inhibition of centrosome coalescence, mitotic arrest, and subsequent cell death in multiple tumor cell lines but not in diploid fibroblasts and keratinocytes with normal centrosome content. Chemical optimization of griseofulvin led to the development of compounds with significantly increased activity and mean inhibitory concentrations (IC\textsubscript{50}) of proliferation and survival in the lower micromolar range when applied to the human squamous cell carcinoma cell line SCC114, which had been used for the initial screening\textsuperscript{22}.

Here, we describe that the griseofulvin derivative GF-15 potently inhibits centrosomal clustering followed by apoptosis induction in MM both \textit{in vitro} and \textit{in vivo}.
Materials and methods

Materials

(2S,6R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-benzylxoy-6'-methylcyclohex-2'-en-4'-one) (2'-benzylxoy-2'-demethoxygriseofulvin; GF-15) was synthesized following the procedures described recently. Other reagents were obtained as follows: VEGF165 and IGF-1 from R&D Systems (Minneapolis, MN); Caspase 8 and PARP antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

All human MM cell lines (RPMI-8226, OPM-2, NCI-H929, OPM-1, KMS-12BM, KMS-12PE, KMS-11, KMS-18, U-266, MM1.S, LR5, Dox40, MM1.R, PAT1) and primary patient MM cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA). Leukemia lines used were HEL, MOLM14, and Ku812 and cultured as described above. Solid tumor cell lines comprised HeLa (cervical carcinoma), HT29, HCT116, SW480 (colorectal carcinoma), PANC1, PACA1 (pancreatic carcinoma), and LN229 (glioblastoma). HS4, KM105, KM104 (bone marrow stromal cells), THLE3 (liver cells), peripheral blood mononuclear cells (PBMC), and primary bone marrow stromal cells (BMSC) served as non-malignant controls. All solid tumor cell lines and non-malignant controls were grown in DMEM medium supplemented with 10% heat-inactivated FBS (Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA).

Isolation of patient tumor cells

After patients provided informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the University of Heidelberg, MM patient cells (96% CD38+ CD45RA-) were obtained as described.

Cell lysis and immunoblotting

Cell lysis and Western blot analysis were performed as described previously.
Evaluation of cell viability
Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma Chemical, St Louis, MO) colorimetric assay, as previously described. Briefly, cells were plated in 96-well microtitre plates at a density of 2-3 x 10^4 cells per well, and each plate was incubated for 24 and/or 48 h, with MTT added to each well for at least 4 h. The absorbance of each well was measured at 570/630 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). Each condition was analyzed in at least three replicates, and the results are presented as the mean ± standard deviation of replicates of a representative experiment that was repeated at least three times.

DNA synthesis and cell proliferation assay
Cell proliferation was assessed by measuring ^3H^-thymidine uptake, as described in prior studies.

Measurement of caspase 3/7 activation
Caspase 3/7 activation was analyzed using the Apoptosis Detection Kit from Promega according to the recommendations of the manufacturer.

Flow cytometry
Cell cycle analysis by flow cytometry including the quantification of cells in mitosis by phospho-S10-histone H3 staining was done as previously described.

Transwell migration assay
Cell migration was assayed using a modified Boyden chamber assay, as described previously.

Immunofluorescence
Immunofluorescence staining was done as described. The following fluorochrome-conjugated secondary antibodies were used: anti-rabbit Alexa 488 (Molecular Probes) and anti-mouse Cy3 (Jackson ImmunoResearch Laboratories). Immunostained cells were examined using a Zeiss Axiosvert 200 M fluorescence microscope. Images were processed with Photoshop software (Adobe).
**MM xenograft mouse model**

To determine the *in vivo* anti-MM activity of GF-15, beige-nude Xid mice were inoculated s.c. in the right flank with $3 \times 10^6$ OPM2 cells in 100 mL RPMI 1640 medium, together with 100 mL matrigel (Becton Dickinson Biosciences, Bedford, MA). When a tumor was measurable, mice were assigned to a GF-15 treatment group or the control group. GF-15 was dissolved in 100% DMSO and given daily five times a week by i.p. injection for indicated periods. The control group received the carrier alone at the same schedule and route of administration. Tumor burden was measured every alternate day using a calliper (calculated volume = $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$). Animals were sacrificed when their tumor reached 2 cm or when the mice became moribund. Survival was evaluated from the first day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

**Preparation of the radiolabeled GF-15 analogue**

($2S,6'R$)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-$(2'-$(4-(125/I31-iodo)benzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) was prepared by thallation-iodination with $^{125}$I-iodide or $^{131}$I-iodide (Perkin Elmer, Dreieich, Germany) of ($2S,6'R$)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-(4-trimethylsilyl-benzyloxy)-cyclohex-2'-ene-4'-one), which was in turn synthesized from $p$-trimethylsilylbenzyl alcohol$^{26}$ using a known method$^{22}$. The radiolabeled analogue was compared to a sample of ($2S,6'R$)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-$(4$-iodobenzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) prepared from $p$-iodobenzyl alcohol$^{22}$ and found to be identical by HPLC-DAD-MS.

**$^{125}$I scintigraphic *in vivo* imaging**

For imaging studies, 200 µL of a solution of the $^{125}$I-labeled GF-15 analogue (5 MBq/mice) was injected into the tail vein of six week old female NMRI mice. Scintigraphic images were taken using a gamma camera (Biospace, France). The accumulation of the radioactive tracer was monitored by static planar images at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after injection.
Biodistribution studies

$^{131}$I-labeled GF-15 analogue (1 MBq/mice) was injected via the tail vein of six week old female NMRI mice. At the time points specified, the animals were sacrificed, weighed and dissected. Organs or tissues were blotted dry and weighed. The radioactivity was measured in a $\gamma$-counter along with a sample of the injection solution to calculate the percentage of injected dose per gram of tissue (%ID/g).

Stability experiments

The serum stability was determined by incubation of the $^{125}$I-labeled GF-15 analogue in human serum at 37 °C. Aliquots were taken at several points in time, and the degradation was stopped by precipitation of the serum proteins with acetonitrile. After 30 min at 0 °C and a further centrifugation step the clear supernatant was analyzed by reverse-phase HPLC on a Chromolith Performance RP-18e 100 $\times$ 4.6 mm column using water and acetonitrile containing 0.1% trifluoroacetic acid as the eluent.

Statistical analysis

Statistical significance of differences observed in GF-15-treated versus control cell cultures and mice was determined using an unpaired Student’s t test. Overall survival in animal studies was measured using the Kaplan-Meier method. (* $P>0.01$; ** $P>0.001$)
Results

GF-15 leads to multipolar mitosis induction in the upper nanomolar range
Recently, we have shown that especially 2'-modified derivatives of griseofulvin have an enhanced capacity for multipolar mitosis induction as compared to griseofulvin itself\textsuperscript{22}. Initial testing for the ability to inhibit centrosomal clustering was performed in SCC114 cells, an oral squamous cell carcinoma line showing pronounced centrosome amplification\textsuperscript{14,18}. GF-15 (2'-benzyloxy-2'-demethoxygriseofulvin,) is significantly more potent with regard to the induction of spindle multipolarity than griseofulvin (Figure 1A). The EC\textsubscript{50} value of multipolar spindle induction for GF-15 was 900 nM, corresponding to a 27-fold increased activity compared to griseofulvin. Importantly, SCC114 cells that became resistant to GF-15 after long-term culture with increasing doses (0.2 – 1 \textmu M) of the compound over a period of ten weeks, showed significantly less centrosome amplification and formed fewer multipolar spindles upon treatment with therapeutic doses (5 \textmu M) of the drug (Figure 1B, Figure S1).

To test for the contribution of centrosome declustering to total multipolar mitosis induction after treatment with GF-15, the PC-3 prostate carcinoma cell line, which harbors supernumerary centrosomes in 28 ± 4% of the cells, was treated with increasing concentrations of the drug for 24 h (Figure 1C). Intriguingly, at the highest analyzable concentration (1.5 \textmu M), GF-15 induced centrosome declustering – as determined by the detection of two centrioles at each spindle pole (Figure 1D) – in 27 ± 4% of the cells, thereby closely matching the total percentage of cells with supernumerary centrosomes. At the lowest concentration tested (0.375 \textmu M), multipolarity induction was mostly due to inhibition of centrosomal clustering. With increasing doses of GF-15, the contribution of multipolarity induction by other means in cells without supernumerary centrosomes gradually increased. From these results, it may be concluded that mechanisms responsible for holding supernumerary centrosomes together might be similar to the forces that bundle microtubules into a bipolar spindle array in cells with a regular centrosome content. However, different sensitivities of both mechanisms provide a window of opportunity to preferentially target centrosome clustering at certain dose levels.
GF-15 is particularly active against MM cells

Next, we examined the effect of GF-15 on the growth of several different cancer cell lines. GF-15 exhibits potent cytotoxicity in a concentration-dependent manner against a broad spectrum of tumor cell types including colon, cervix, glioblastoma, pancreas, leukemia, and myeloma-derived cell lines (Figure 2A). As compared to solid tumor cell lines, MM cell lines were particularly susceptible to the cytotoxic and anti-proliferative effect of GF-15 with IC$_{50}$ values ranging from 1 - 2.5 µM, including MM cell lines resistant to doxorubicin (Dox40), melphalan (LR5), and dexamethasone (MM1.R) (Figure 2 A, B). Moreover, primary MM cells freshly isolated from the bone marrow of three patients with relapsed myeloma showed marked cytotoxic effects upon treatment with GF-15. This was in stark contrast to their corresponding BMSCs, which displayed virtually no cytotoxicity when exposed to GF-15 (Figure 2C). Similarly, GF-15 did not induce significant cytotoxicity in PBMCs from healthy volunteers even after stimulation with phytohemagglutinin (PHA), providing an overall selectivity index of 10- to 30-fold when compared to IC$_{50}$ values of cancer cell lines (Figure 2D). These data suggest that GF-15 exhibits both potent and selective cytotoxicity against malignant cells.

Induction of spindle multipolarity, mitotic arrest and apoptosis by GF-15

GF-15 induced multipolar mitoses in MM cell lines NCI H929, OPM2, and RPMI 8226 (Figure 3A). After 24 h of treatment with 3 µM GF-15, no significant induction of spindle multipolarity could be detected in primary BMSCs, whereas >80% of mitoses were multipolar in NCI H929, OPM2, and RPMI 8226 cells (Figure 3B). To examine the effect of multipolar mitosis induction on cell cycle progression, starvation-synchronized OPM2 cells were exposed to GF-15, stained with propidium iodide, and subsequently analyzed by flow cytometry. GF-15 induced a pronounced G$_2$/M cell cycle arrest within 12 h of treatment followed by an increase of the sub-G$_1$ population compared to mock-treated cells (Figure 3C, upper panel). Indicating induction of apoptosis, the increase of the sub-G$_1$ population was concentration-dependent (Figure 3C, lower panel), analogous to the effect of griseofulvin in SCC114 cells.$^{14}$ To further verify apoptotic cell death triggered by GF-15, protein profiling in GF-15 treated MM cells showed dose-dependent cleavage of caspase 8, caspase 9, caspase 3, and PARP (Figure 3D). Cleavage fragments of these proteins became detectable at 0.5 µM and strongly increased at 3 µM of GF-15. Importantly, exposure
of primary BMSCs to 3 \( \mu \)M of GF-15 for 24 h did not induce activation of effector caspases 3 and 7 compared to OPM2 cells (Figure 3E).

**Evaluation of combinations of GF-15 with other anti-myeloma agents**

Clinical experience in the therapeutic management of MM patients supports the notion that drug combinations can induce higher response rates when compared with single-agent treatment\(^1\),\(^2\)\(^4\). We therefore evaluated the effects of combinations of GF-15 with other established anti-MM drugs on the viability of MM cells. Specifically, GF-15 was combined with conventional agents (melphalan, dexamethasone) as well as with more recently developed compounds such as bortezomib. While GF-15 together with dexamethasone or bortezomib, respectively, resulted in at least additive effects (data not shown), the combination with melphalan led to a marked abrogation of GF-15 induced cytotoxicity (Figure 3F). This is consistent with an S-phase arrest induced by the DNA-damaging drug melphalan, thereby preventing entry into mitosis of melphalan-exposed cells. This underlines the specificity of GF-15 for cells in G\(_2\)/M-phase and provides important information for its possible use in clinical application.

**GF-15 inhibits myeloma cell growth triggered by bone marrow stromal cells**

In addition to the autocrine/paracrine effects mediated by growth factors and cytokines within the MM bone marrow microenvironment, direct MM-BMSC contact also triggers tumor cell growth. We therefore evaluated the effect of GF-15 on MM cell proliferation induced by the stimulatory effect of BMSCs (Figure 4A, B). Binding of OPM2 or RPMI-8226 cells to primary BMSCs triggered increased MM cell proliferation, which was completely abrogated by GF-15. Importantly, as also shown in Figure 2C, GF-15 did not affect the viability of BMSC lines, as determined by MTT assays.

**GF-15 inhibits cytokine-induced myeloma cell migration**

Migration activity is required for homing of tumor cells to the bone marrow, expansion within the bone marrow microenvironment, and tumor cell egress into the peripheral blood\(^23\),\(^27\). Since centrosomes and microtubules as targets of GF-15 are not only important for mitotic spindle formation but also for interphase cell shape and migration\(^28\), we next investigated whether vascular endothelial growth factor (VEGF)- and insulin-like growth factor-I (IGF-I)-induced migration of MM cells can be
abrogated by pre-treatment with GF-15. Our data show that GF-15 completely abrogates both VEGF/FN- (fibronectin) and IGF-I-triggered MM-cell migration (Figure 4C).

**GF-15 is rapidly eliminated in vivo**

Derived from its parental molecule griseofulvin, GF-15 has been modified at the 2'-position (Figure 1A). We therefore sought to analyze in vivo stability and pharmacokinetics of this new compound. By introducing a p-iodobenzyl group in the 2'-position of the griseofulvin molecule we generated a 125I-labeled GF-15-analogue. HPLC analysis showed only minimal degradation of this molecule in human serum with a half-life of 48 h at 37 °C. The cleavage products resulting from degradation are presumably 4-iodobenzyl alcohol and griseofulvic acid, consistent with the analogue undergoing hydrolysis (data not shown). After i.v.-application of trace amounts of this analogue, rapid renal clearance was observed within the first 6 h after injection (Figure 5). In light of the clearance data and the poor solubility of GF-15 at higher concentrations, we went on to investigate its in vivo efficacy after intraperitoneal (i.p.) application.

**GF-15 exhibits in vivo anti-MM activity in a myeloma xenograft mouse model**

In view of the potent and selective in vitro activity of GF-15 against MM cell lines and freshly isolated primary MM patient cells, we next examined the in vivo effect of GF-15 on human MM growth in immunodeficient mice. A cohort of 30 immunodeficient beige-nude-Xid (BNX) mice was inoculated with 3 x 10^6 OPM2 cells s.c. in the right flank. Treatment with a daily dose of 20 mg/kg (ten mice) or 100 mg/kg (ten mice) i.p. five days per week for two weeks was started when tumors became palpable. Ten mice served as a control cohort and received i.p. injections of the vehicle alone. GF-15 treatment decreased tumor growth in both cohorts of treated mice with a greater effect in the group that received 100 mg/kg i.p. (Figure 6A). Kaplan-Meier and log-rank analysis revealed a significant prolongation of survival for the GF-15 treated groups compared with the vehicle-treated controls (log-rank P<0.001; Figure 6B). The toxicity profile of GF-15 seems to be quite favourable as body weight was not affected by treatment with GF-15 compared with untreated controls (Figure 6C). Importantly, examination of histological tumor sections revealed a dose-dependent,
significant increase of aberrant and multipolar mitoses in the GF-15-treated mice compared with controls (P<0.01 for 20mg/kg, P<0.001 for 100mg/kg; Figure 6D, E).

Discussion

Here, we have shown that treatment of MM cells with GF-15, a derivative of griseofulvin, leads to multipolar spindle formation, centrosomal declustering, mitotic cell cycle arrest, and apoptosis both in tissue culture and in a myeloma xenograft mouse model.

MM, presently an incurable plasma cell malignancy, accounts for approximately 20% of deaths from hematologic malignancies and nearly 2% of deaths from cancer. In virtually all cases, it is preceded by a premalignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which occurs in about 3% of individuals over the age of 50. Virtually all MGUS and MM tumor cells have numeric and/or structural chromosome abnormalities and are characterized by gross chromosomal instability. Using gene expression profiling, we and others have shown that genes relevant for cell cycle progression and chromosomal instability are overexpressed in high-risk MM patients.

Centrosome amplification is common in MM and already present in MGUS. Moreover, its magnitude constitutes an independent prognostic factor in both newly diagnosed and relapsed MM. Mutation or misregulation of a variety of tumor suppressors or oncogenes has been shown to disrupt the normal function and numeral integrity of centrosomes, among them aurora-A, a protein often overexpressed in high-risk MM.

Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest that only an intact spindle assembly checkpoint allows for sufficient time in order for centrosomal clustering to occur and that spindle tension is necessary for clustering of supernumerary centrosomes into a bipolar mitotic spindle array. In this regard, it is interesting to note that gene expression profiling identified overexpression of key regulators that normally maintain faithful segregation of chromosomes, including MAD2, BUBR1, ZWINT, PRC1, survivin, and aurora-B, both as part of a high-risk 15-gene signature in MM and a 70-gene signature of
chromosomal instability associated with poor clinical outcome in multiple cancer types\textsuperscript{32,36}.

Microtubule-interacting drugs are effective in the treatment of both newly diagnosed and relapsed/refractory MM, with clinical activity having been demonstrated for both microtubule-depolymerizing (vincristine) and microtubule-stabilizing agents (paclitaxel, epothilone B)\textsuperscript{37-39}. Also, inhibition of the mitotic kinesin Eg5 effectively kills myeloma cells \textit{in vitro}\textsuperscript{40}. The limited success of paclitaxel in the treatment of MM seems to primarily reflect the fact that it serves as a substrate for the MDR1/Pgp drug efflux pump. The mechanisms by which microtubule-interacting drugs induce cell death are still not completely clear\textsuperscript{41}. Importantly, aside from the formation of stable microtubule bundles associated with G2/M cell cycle arrest and increased microtubule polymer mass at high concentrations, taxanes have been described to affect centrosome organization with subsequent mitotic catastrophe at low nanomolar concentrations\textsuperscript{42}.

Griseofulvin has been used for many years for the treatment of dermatophyte infections\textsuperscript{43}. Mechanistically, it inhibits mitosis in sensitive fungi\textsuperscript{44} and mammalian cells\textsuperscript{45,46} but whether mitotic arrest is a consequence of microtubule depolymerization or some other action on microtubules in both fungi and human cells is still unclear\textsuperscript{47,48}. Although griseofulvin has been reported to bind to mammalian brain tubulin and to inhibit microtubule polymerization \textit{in vitro}, it does so only at concentrations significantly higher than those needed for spindle multipolarity induction in cancer cells with extra centrosomes\textsuperscript{48}. Also, whether griseofulvin binds to tubulin directly or to microtubule associated proteins remains conflicting\textsuperscript{48-51}. Already more than 30 years ago it was reported that griseofulvin treatment induces spindle multipolarity with each mitotic center containing two centrioles in HeLa cells\textsuperscript{45}. While at lower concentrations the drug leads to multipolar spindles with centrosomes at each pole in cells with extra centrosomes, at higher concentrations spindle multipolarity with acentrosomal spindle pole formation is additionally induced. This is consistent with the concept that clustering extra centrosomes in cancer cells might be similar to focusing microtubules into a bipolar spindle array in normal cells. For detailed mechanistic understanding, it will be important to clearly determine the sequence of events: does the drug at low concentrations indeed cause declustering of supernumerary centrosomes with subsequent multipolar spindle formation or does
spindle multipolarity occur first with successive distribution of centrosomes to each pole?

For GF-15, the EC_{50} value for multipolar spindle induction was 900 nM in SCC114 cells, corresponding to a 27-fold increased activity compared to griseofulvin itself. Also, whereas griseofulvin inhibits cell proliferation only weakly with half-maximal inhibition occurring at 25 µM^{48}, GF-15 led to inhibition of MM cell growth \textit{in vitro} at IC_{50} values of 1 to 3 µM.

GF-15 was well tolerated and effective in a murine MM model, as evidenced by significant inhibition of MM tumor growth in mice treated with either 20 or 100 mg/kg GF-15 i.p. daily for seven days a week. Since GF-15 is only poorly soluble in water and its biological half-life is short with little drug left 1 h after administration, it is expected that medicinal chemical optimization will lead to a further improvement in the drugs potency and in this novel therapeutic strategy in general. Indeed, preliminary data suggest that another, slightly modified analogue (GF-61) with improved water solubility exerts anti-myeloma activity in our xenograft mouse model even when administered orally (our unpublished data).

In summary, we have shown that single-agent GF-15 potently inhibits MM cell growth \textit{in vitro} and \textit{in vivo}, overcoming both cell-intrinsic as well as cell-interaction mediated mechanisms of drug resistance. These observations, coupled with GF-15’s lack of major toxicity in a preclinical mouse model and the lack of significant toxicity of griseofulvin in humans, provide the framework for further clinical development of GF-15 in particular and centrosomal cluster inhibitors in general, directed at improving patient outcome in MM.
Acknowledgement
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Conflict-of-interest disclosure
The authors declare no competing financial interests.

Authorship
M.S.R. and I.B. contributed equally to this work. Contribution: M.S.R. designed, performed, and analyzed research and wrote the manuscript; I.B. and M.H.C. designed, performed, and analyzed research; M.H.R., B.L., T.O.L., L.W., S.A., G.K., P.J.H., K.P., J.F., F.N. performed and analyzed research; W.M., U.H., A.D.H., H.G., K.C.A. analyzed data and provided expert advice; A.K. designed the project, analyzed research and wrote the manuscript.
References


Figure 1. GF-15 is a potent and specific inducer of spindle multipolarity. (A) Chemical structures of griseofulvin and GF-15. SCC114 cells stably expressing α-tubulin were treated with increasing concentrations of griseofulvin or GF-15 for 24 h, EC$_{50}$ of spindle multipolarity was assessed by immunofluorescence microscopy. (B) SCC114 cells resistant to GF-15 after long-term culture under increasing concentrations of GF-15 display significantly fewer cells with supernumery centrosomes than wild type SCC114. Centrosomes were counted in interphase by γ-tubulin staining. (C) In PC-3 prostate cancer cells, GF-15 induces centrosomal declustering (declustered) in cells with amplified centrosomes, and spindle multipolarity by other means (aberrant) in cells with regular centrosome content in a concentration-dependent manner. The dashed line depicts the overall percentage of PC-3 cells with centrosome amplification. (D) Spindle phenotypes of PC-3 cells upon
treatment with vehicle only (D', D'') or GF-15 (D''', D'''') according to their centrosome content. Cells were treated with GF-15 or vehicle only for 24 h, spindle poles were counted by γ-tubulin staining, centrioles by centrin staining.

Figure 2. GF-15 selectively inhibits proliferation and survival of tumor cells. (A) IC₅₀ values of cell lines of indicated origins. (B) Dose-related effects of GF-15 on cell survival (upper panel, 48 h) and proliferation (lower panel, 24 h) on indicated MM cell lines. Cell proliferation was assessed by uptake of [³H]-thymidine during the last 8 hrs of 24 h cultures. (C) GF-15 differentially inhibits cell survival of primary cells from MM patients compared to bone marrow stroma cells (BMSCs). (D) Peripheral mononuclear cells (PBMCs) of three healthy donors activated by PHA do not show significant toxicity upon treatment with indicated concentrations of GF-15. Viable cells are expressed as x-fold of respective control. Cell survival: Unless otherwise
indicated, viable cells were measured by MTT cleavage during the last 4 hrs of 48 h cultures. Data shown are the mean +/- SD of experiments performed in triplicates.

Figure 3
A

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B

![Graph showing spindle multiplicity (%) for Control and 3 μM GF-15]

C

![Graphs showing GF-15 (3 μM) and Mock]

24
Figure 3. GF-15 specifically induces spindle multipolarity, cell cycle arrest, and apoptosis in MM cells. (A) MM cell lines display multipolar spindles upon treatment with GF-15. NCI H929 (A'), OPM2 (A''), and RPMI 8226 (A'''') cells were exposed to GF-15 (3 μM; 24 h) and stained for Eg5 (red), γ-tubulin (green), and DAPI (blue). (B) GF-15 selectively induces multipolar mitotic spindles in MM cells (NCI H929, OPM2, RPMI 8226; left to right) compared to three primary BMS Cs. At least 200 mitotic cells were counted for spindle polarity after staining for γ-tubulin, Eg5, and DAPI. (C) Synchronized OPM2 cells arrest in G2/M phase upon treatment with GF-15 followed by increase of sub-G1 population (upper panel). Increase of sub-G1 cells is dose-dependent (lower panel). OPM2 cells were cultured for indicated times to indicated concentrations of GF-15 and stained with propidium iodine after ethanol fixation. (D) GF-15 triggers apoptotic cell death in MM cells. OPM2 cells were exposed to...
indicated concentrations of GF-15 for 24 h, followed either by immunoblot analysis of lysates with indicated antibodies or (E) ELISA-based assessment of activation of effector caspases 3 and 7. Identical treatment of BMSCs showed no significant induction of caspase 3/7 activation compared to OPM2 cells. Results are expressed as x-fold of control. (F) Co-treatment of MM cells with melphalan partially abrogates the growth inhibitory effects of GF-15. OPM2 cells were treated with indicated concentrations of GF-15 with or without 5 μM melphalan for 48 h. MTT cleavage was measured during the last 4 hrs of 48 h cultures.

Data shown are the mean +/- SD of experiments performed in triplicates.

![Figure 4](image)

**Figure 4.** GF-15 abrogates the growth advantage conferred by adhesion to BMSCs, and inhibits cytokine-triggered cell migration. (A-B) Indicated MM cell lines were cultured with or without BMSCs. GF-15 was added at indicated...
concentrations and proliferation was measured by $[^{3}H]$-thymidine uptake during the last 8 hrs of 24 h cultures. (C) GF-15 abrogates VEGF/FN- and IGF-1 – triggered MM-cell migration. Growth factor-deprived GF-15 treated (1, 3, 5 μM) or untreated OPM2 cells were plated on either a fibronectin-coated or non-coated membrane (8 μm pore size) in a Boyden modified chamber and stimulated with the indicated cytokines. Cells in lower chamber were counted with Coulter counter ZBII after 6 hrs of treatment. Data shown are the mean +/- SD of experiments performed in triplicates, representative of three independent experiments.

**Figure 5**

![Whole-body scintigraphic images of beige-nude-Xid mice at indicated times after intravenous injection.](image)

Figure 5. GF-15 is rapidly eliminated after intravenous injection. (A) Whole-body scintigraphic images of beige-nude-Xid mice at indicated times after intravenous
injection of the $^{125}$I-labeled GF-15 analogue. (B) Biodistribution of a $^{131}$I-labeled GF-15 analogue at different times after intravenous administration to beige-nude Xid mice ($n = 12$). Data are expressed as mean %ID/g +/- SD of each time point.

Figure 6

Figure 6. GF-15 induces mitotic aberrations, decreases tumor growth, and prolongs survival in a xenograft mouse model. Beige-nude Xid mice were subcutaneously inoculated in the right flank with $3 \times 10^5$ OPM2 cells. Treatment by intraperitoneal injection (vehicle alone or indicated concentrations) was started when tumors were measurable. Arrows indicates treatment stop. (A) Tumor burden was measured every alternating day using an electronic caliper. Tumor volume is presented as means +/- SE. (B) Survival was evaluated using Kaplan-Meier curves and log-rank analysis. (C) Body weight was evaluated three times per week. (D) In HE-stained tumor sections ($n = 3$ per cohort) at least 200 mitotic cells were counted.
for mitotic aberrations. (E) Dose-dependent effects of GF-15 on mitotic figures. Paraffin-embedded sections of tumor tissue, explanted 24 hrs after last treatment, were HE-stained and analyzed by light microscopy. Representative microscopic images are shown.

Suppl. Fig. 1

Suppl. figure 1. GF-15 does not induce spindle multipolarity in resistant cells
SCC114 cells resistant to EC_{50} concentrations of GF15 after long-term culture under increasing concentrations of GF-15 do not display spindle multipolarity upon treatment with 5µM GF-15 for 24h. Centrosomes were counted in interphase by γ-tubulin staining.
Appendix B

Co-author Statements

In the following are the signed co-author statements from the three accepted publications (Chapters 3, 5 and 7) as well as the two manuscripts in Chapters 4 and 6.
Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: Synthesis and Structure-Activity Relationship of Gilseafulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

Author(s): Mads H. Rannest, Blanka Rebaucz, Lene Mankworth, Anette H. Terp, Thomas D. Larsen, Alwin Krämer, Mads H. Clausen

Journal: Journal of Medicinal Chemistry

PhD-student: Mads H. Rannest

CPR-no.: 050570-3443

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Date: 8 December 2010

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**Mads H. Rennest:** Ideas for as well as writing of the paper. Synthesis and/or purification of compounds 2, 4, 5, 6, 10, 11, 12, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34 and characterization of compounds 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35.

**Blanka Rebacz:** Initial screening of griseofulvin analogues as well as the test in triplicate used for the article. Writing of the paper.

**Lene Markworth:** Synthesis of compounds 2, 7, 8, 9, 13, 14, 15, 18, 19, 21, 23, 28, 29, 30, 32, 33, 34, 35 and characterization of compounds 2, 7, 8, 9, 13, 14, 15, 18, 19, 21, 23, 28, 29, 30, 32, 33, 34, 35.

**Anette H. Terp:** Initial screening of griseofulvin analogues

**Thomas O. Larsen:** Feedback and support on the fungal aspect of the paper.

**Alwin Krämer:** Feedback and support with the assay

**Mads H. Clausen:** Feedback and support on the syntheses present in the paper. Idea for as well as writing of the paper.

All co-authors have been proofreading the manuscript.

Joint author statements shall be delivered to the PhD administration together with the PhD thesis.
Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree.

Title of the article: Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes Trichophyton mentagrophytes, T. rubrum and MDA-MB-231 Cancer Cells

Author(s): Mads H. Rannest, Marc S. Raab, Simon Anderhub, Sven Boesen, Alwin Krämer, Thomas O. Larsen, Mads H. Clausen

Journal: Journal of Medicinal Chemistry

PhD-student: Mads H. Rannest

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Co-author: Mads H. Clausen

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CPR-no.: 050479-3443

Date: 20 June 2011
Description of each author’s contribution to the above-mentioned article:

Mads H. Ronne: Idea for as well as writing of the paper. Synthesis and characterization of all compounds in paper. Initial setup of the anti-fungal assay, finalizing the setup as well as testing all compounds in the anti-fungal assay. All processing of data and IC_{50} calculations.

Marc S. Raab: Idea for as well as feedback and support on the work with the anti-cancer assay

Simon Anderhub: Feedback and support on the work with the anti-cancer assay

Sven Boesen: Initial setup of the anti-fungal assay.

Alwin Krämer: Idea for as well as feedback and support on the work with the anti-cancer assay

Thomas O. Larsen: Idea for as well as feedback and support on the work with the anti-fungal assay.

Mads H. Clausen: Idea for as well as feedback and support on the syntheses present in the paper and the anti-fungal assay.

All co-authors have been proofreading the manuscript.

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Title of the article: Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

Author(s): Mads H. Rønnest, Pernille Harris, Charlotte H. Gotfredsen, Thomas O. Larsen, Mads H. Clausen

Journal: Tetrahedron Letters

PhD-student: Mads H. Rønnest

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Date: 27 October 2010

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Description of each author's contribution to the above-mentioned article:

Mads H. Rønne: Synthesis of all compounds present in the paper, characterization of these, Idea for as well as writing the paper. Proofreading the manuscript.

Pernille Harris: All work concerning X-ray and analysis of data set. Proofreading the manuscript.

Charlotte H. Gøtfredsen: Performed the 2D NMR experiments and gave general feedback and support on the NMR work present in the article. Proofreading the manuscript.

Thomas O. Larsen: Proofreading the manuscript

Mads H. Clausen: Feedback and support on the synthesis present in the paper and idea for as well as writing of the paper. Proofreading the manuscript.

Joint author statements shall be delivered to the PhD administration together with the PhD thesis.
Technical University of Denmark  
Research and Innovation, PhD programme  

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: A technique for selective ipso-substitution of ortho-, meta- or para-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

Author(s): Mads H. Rønnest, Felix Nissen, Thomas Ostenfeld Larsen, Walter Mier, Mads H. Clausen

Journal: To be decided

PhD-student: Mads H. Rønnest  
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Signature: 

1
Description of each author’s contribution to the above-mentioned article:

Mads H. Rønnest: Ideas for as well as writing of the paper. Development of the thallium/iodation mediated radiolabeling procedure. Synthesis and characterization of compounds. “Cold” synthesis of iodo griseofulvin analogs. Proofread the manuscript.

Felix Nissen: Development of the thallium/iodation mediated radiolabeling procedure. “Hot” syntheses of iodo griseofulvin analogs. Proofread the manuscript.

Thomas Ostenfeld Larsen: Proofread the manuscript.

Walter Mier: Development of the thallium/iodation mediated radiolabeling procedure. “Hot” syntheses of iodo griseofulvin analogs. Proofread the manuscript.

Mads H. Clausen: Feedback and support on the syntheses present in the paper. Idea for the project/research and the paper. Proofread the manuscript.
Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2006 about the PhD degree.

Title of the article: (+)-geodin from Aspergillus terreus

Author(s): Mads H. Rennest, Morten T. Nielsen, Elanka Leber, Uffe H. Mortensen, Alwin Krämer, Pamela Harris, Mads H. Clausen, Thomas O. Larsen

Journal: Acta Crystallographica Section C: Crystal Structure Communications

PhD-student: Mads H. Rennest

Signature of the PhD-student: [Signature]

Date: 15 September 2010

Co-author: Morten T. Nielsen

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Description of each author’s contribution to the above-mentioned article:

Mads H. Rønnest: Production of raw extract from *Aspergillus terreus*. Isolation of geodin from raw extract as well as characterization of geodin. Crystallization of crystal for X-ray analysis. The idea for as well as writing of the paper. Proofreading the manuscript.

Morten T. Nielsen: Production of raw extract from *Aspergillus terreus* and writing of the paper. Proofreading the manuscript.

Blanka Leber: Testing of geodin in cancer assay. Proofreading the manuscript.

Uffe H. Mortensen: Feedback and support on the production of raw extract from *Aspergillus terreus*. Proofreading the manuscript.

Alwin Krämer: Feedback and support on testing geodin. Proofreading the manuscript.

Pernille Harris: All work concerning X-ray data and structure analysis. Writing as well as proofreading the manuscript.

Mads H. Clausen: Feedback and support on characterization of geodin as well as idea for the paper. Proofreading the manuscript.

Thomas O. Larsen: Feedback and support on isolation of geodin as well as idea for the paper. Proofreading the manuscript.

Joint author statements shall be delivered to the PhD administration together with the PhD thesis.
Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

Mads H. Rønnest, Blanka Rebacz, Lene Markworth, Anette H. Terp, Alwin Krämer, Thomas O. Larsen, Mads H. Clausen

Supporting Information

S2 Combustion analysis (Table S1).
S3-9 Experimental
S10-S42 NMR spectra of 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35.
Table S1. Combustion analysis.

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<td>C_{13}H_{15}ClO_{6}</td>
<td>C 59.92, H 5.56</td>
<td>59.75, 5.50</td>
</tr>
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<td>C_{13}H_{15}ClO_{6}</td>
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<td>60.71, 5.83</td>
</tr>
<tr>
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<tr>
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<td>60.95, 5.45</td>
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<tr>
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<td>60.95, 5.45</td>
</tr>
<tr>
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<td>C_{19}H_{19}ClO_{6}</td>
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<td>55.60, 4.96</td>
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<tr>
<td>35</td>
<td>C_{20}H_{19}ClO_{6}</td>
<td>C 49.80, H 3.63</td>
<td>49.73, 3.67</td>
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To a solution of griseofulvin (7.0 g, 19.8 mmol) in HOAc (50 mL) was added 2 M HCl (40 mL). The solution was stirred at 20 °C for 24 h. The mixture was diluted with EtOAc (20 mL) and washed with sat. aq. NH₄Cl (20 mL). The aqueous phase was extracted with EtOAc (3×30 mL), the organic layer was dried (MgSO₄), and the residue was purified by column chromatography (C-18, MeOH and water gradient) yielding 4. Yield: 521 mg (18%) (yellow crystals); Rf (MeOH:DCM 1:9, 1% HOAc): 0.45; IR(KBr, cm⁻¹): 1627, 1559; ¹H NMR(500 MHz, CDCl₃): δ 7.68 (1H, s), 6.49 (1H, s), 5.58 (1H, s), 3.89 (3H, s), 3.62 (3H, s), 2.77 (1H, ddq, J = 12.6, 6.5, 4.8 Hz), 2.67 (1H, dd, J = 16.3, 13.4 Hz), 2.33 (1H, dd, J = 16.3, 4.5 Hz), 0.80 (3H, d, J = 6.5 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 195.9, 193.8, 171.1, 170.9, 166.5, 165.8, 106.6, 104.5, 97.3, 89.8 (2C), 56.9, 56.5, 39.4, 35.9, 13.8; HRMS (ESI⁺) calcd for [C₂₈H₂₇ClO₅]⁺ 339.0635, found 339.0633.

General procedure for the synthesis of ring A ethers 5 and 6.

To a solution of 4 (0.7 mmol, 1 equiv.) in DMF (3 mL, 0.2 M) was added NaH (0.7 mmol, 1 equiv.) and tetrabutylammonium iodide (0.1 mmol, 0.1 equiv.). The mixture was stirred at 100 °C and then 20 min. The alkyl bromide (1.1 mmol, 1.5 equiv.) was added. After 24 h the solution was diluted with EtOAc (20 mL) and washed with sat. aq. NH₄Cl (20 mL). The aqueous phase was extracted with EtOAc (3×30 mL), the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (C-18, MeOH and water gradient) (Litt.): 0.69; m.p.: 237-239 °C (Litt.): 253.1108.

(256'R)-(7-Chloro-4-ethoxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) 5.

Yield: 39 mg (15%) (white crystals); Rf (EtOAc:heptane 1:2): 0.55; m.p.: 205-206 °C (Litt.): ¹H NMR(500 MHz, CDCl₃): δ 6.12 (1H, s), 5.53 (1H, s), 4.25-4.15 (2H, m), 4.00 (3H, s), 3.61 (3H, s), 3.04 (1H, d, J = 16.8, 13.5 Hz), 2.84 (1H, ddq, J = 13.5, 4.7, 6.7 Hz), 2.42 (1H, dd, J = 16.8, 4.7 Hz), 1.52 (3H, t, J = 7.0 Hz), 0.96 (3H, d, J = 6.7 Hz) (Litt.): ¹³C NMR(50 MHz, CDCl₃): δ 196.9, 192.2, 170.8, 169.5, 164.4, 157.2, 105.6, 104.7, 97.0, 90.6, 90.3, 65.1, 56.9, 56.6, 40.0, 36.4, 14.3, 14.2; HRMS (ESI⁺) calcd for [C₂₄H₂₆ClO₅]⁺ 367.0948, found 367.0944.

(256'R)-(7-Chloro-4-benzyloxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) 6.

Yield: 40 mg (14%) (white crystals); Rf (EtOAc:heptane 1:2): 0.69; m.p.: 237-239 °C (Litt.): ¹H NMR(500 MHz, CDCl₃): δ 7.49-7.46 (2H, m), 7.42-7.38 (2H, m), 7.36-7.31 (1H, m), 6.17 (1H, s), 5.55 (1H, s), 5.27 (2H, s), 3.94 (3H, s), 3.63 (3H, s), 3.05 (1H, dd, J = 16.6, 13.6 Hz), 2.85 (1H, m), 2.44 (1H, dd, J = 16.6, 4.6 Hz), 0.98 (3H, d, J = 6.7 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 196.9, 192.1, 170.8, 169.4, 164.3, 156.6, 135.4, 128.8 (2C), 128.3, 126.8 (2C), 104.8 (2C), 97.4, 91.5, 90.7, 71.1, 56.9, 56.6, 40.0, 36.5, 14.4; HRMS (ESI⁺) calcd for [C₂₄H₂₆ClO₅]⁺ 429.1105, found 429.1108.

(256'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) 7.
To a solution of griseofulvin (2.0 g, 5.67 mmol) in acetic anhydride (10 mL) was slowly added a solution of nitric acid (2 mL) in acetic anhydride (4 mL) while maintaining the temperature at 0–5 °C. The mixture was stirred at 20 °C for 48 hours and then cooled to 0 °C. A 20% HCl solution was added cautiously to the mixture until a yellow precipitate began to appear, cooling was continued to complete the precipitation of the product and the product was collected by filtration. Additional product was obtained by cautiously adding water to the mother liquor and collected by filtration. The residue was purified by column chromatography (EtOAc:heptane) 1:2 to afford the product 7 which was re-crystallized from EtOAc.

Yield: 744 mg (33%) (yellow needles); Rf (EtOAc:heptane 5:1): 0.76; m.p.: 195-197 °C (Lit.1); IR (KBr, cm⁻¹): 1719, 1669, 1617, 1577 (Lit.1); ¹H NMR (300 MHz, CDCl₃): δ 5.59 (1H, s), δ 4.22 (3H, s), δ 4.14 (3H, s), δ 3.67 (3H, s), δ 2.99-2.95 (1H, m), δ 2.90-2.86 (1H, m), δ 2.49 (1H, dd, J = 14.5, 2.4 Hz), δ 0.98 (3H, d, J = 6.4 Hz) (Lit.1); ¹³C NMR (75 MHz, CDCl₃): δ 196.3, 192.5, 170.0, 169.6, 157.4, 149.5, 105.2, 108.9, 91.2, 91.1, 57.2, 64.5, 63.3, 40.0, 36.6, 14.5; EIMS [C₇H₆CINO₃] 397, found 397; Anal. (C₇H₆CINO₃) C, H.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-5-amino-benzofuran-3-on)-2-spiro-1'-(2'-m ethoxy-6'-m ethyl-cyclohex-2'-ene-4'-one) 8.

Powdered iron (1.458 g, 26.11 mmol) was added over 3 h to a stirred solution of 5-nitro griseofulvin (2.0 g, 5.67 mmol) in 90% aq. acetic acid (18.6 mL). The mixture was refluxed for 1 h and cooled to 20 °C. Powdered iron (1.458 g, 26.11 mmol) was added over 3 h to a stirred solution of 5-nitro griseofulvin (2.0 g, 5.67 mmol) in acetic anhydride (10 mL) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:1) to afford the product 8 which was re-crystallized from EtOAc/heptane. Yield: 369 mg (58%) (yellow crystals); Rf (EtOAc:heptane 5:1): 0.64; m.p.: 169.9, 169.6, 164.4, 157.6, 105.1 (2C), 97.1, 90.8, 89.3, 65.3, 56.9, 56.3, 39.9, 36.3, 14.2, 13.6; HRMS (ESI⁺) calcd for [C₁₅H₁₄O₄ClN] + 367.0949, found 367.0960; Anal. (C₁₅H₁₄O₄ClN) C, H.

General procedure for the synthesis of enol ethers by solvolysis (10-15).

CSA (0.1 mmol, 0.1 equiv.) was added to a solution of griseofulvic acid (0.66 mmol, 1 equiv.) in the appropriate alcohol (6 mL, 0.1 M). The mixture was stirred at 100 °C for 6 hours, and then cooled to 20 °C. EtOAc (20 mL) was added to the solution and the mixture was washed with sat. NaHCO₃ (20 mL) and then water (20 mL). The combined aqueous phases were extracted with EtOAc (3×20 mL), dried (Na₂SO₄) and concentrated.

Yield: 32 mg (14%) (white crystals); Rf (EtOAc:heptane 5:1): 0.59; m.p.: 202-204 °C (Lit.5); IR(KBr, cm⁻¹): 1708, 1661, 1614 (Lit.5); ¹H NMR(500 MHz, CDCl₃): δ 6.12 (1H, s), 5.51 (1H, s), 4.03 (3H, s), 3.98 (3H, s), 3.93-3.74 (2H, m), 3.02 (1H, dd, J = 16.7, 13.4 Hz), 2.91-2.76 (1H, m), 2.42 (1H, dd, J = 16.7, 4.7 Hz), 2.11 (3H, t, J = 7.0 Hz), 0.96 (3H, d, J = 6.7 Hz) (Lit.5); ¹³C NMR(50 MHz, CDCl₃): δ 197.1, 192.6, 169.9, 169.6, 164.4, 157.6, 105.1 (2C), 97.1, 90.8, 89.3, 65.3, 56.9, 56.3, 39.9, 36.3, 14.2, 13.6; HRMS (ESI⁺) calcd for [C₅H₄ClO₂] + 367.0949, found 367.0960; Anal. (C₅H₄ClO₂) C, H.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-ethoxy-6'-methyl-cyclohex-2'-en-4'-one) 10.

Yield: 41 mg (18%) (white crystals); Rf (EtOAc:heptane 5:1): 0.64; m.p.: 151-153 °C (Lit.5); IR(KBr, cm⁻¹): 1706, 1662, 1614 (Lit.5); ¹H NMR(500 MHz, CDCl₃): δ 6.13 (1H, s), 5.51 (1H, s), 4.04 (3H, s), 3.98 (3H, s), 3.79-3.66 (2H, m), 3.05 (1H, dd, J = 16.7, 13.5 Hz), 2.89-2.80 (1H, m), 2.42 (1H, dd, J = 16.7, 4.7 Hz), 1.64-1.55 (2H, m), 0.97 (3H, d, J = 6.7 Hz), 0.82 (3H, t, J = 7.4 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 197.1, 192.6, 170.0 (2C), 164.4, 157.6, 105.1, 104.8, 96.8, 90.8, 89.3, 70.7, 56.9, 56.3, 39.9, 36.2, 21.5, 14.2, 9.9; HRMS (ESI⁺) calcd for [C₁₅H₁₄O₂Cl] + 381.1105, found 381.1105; Anal. (C₁₅H₁₄O₂Cl) C, H.
(25S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-((2'-isopropoxy-6'-methyl-cyclohex-2'-ene-4'-on) 12.

Yield: 50 mg (22%) (off-white crystals); R₁ (EtOAc:heptane 5:1): 0.62; m.p.: 194-197 °C (Lit.); IR (KBr, cm⁻¹): 1714, 1667, 1615 (Lit.); ¹H NMR (500 MHz, CDCl₃): δ 6.12 (1H, s), 5.51 (1H, s), 4.33 (1H, sept, J = 6.1 Hz), 4.03 (3H, s), 3.98 (3H, s), 3.02 (1H, dd, J = 16.7, 13.4 Hz), 2.87-2.77 (1H, m), 2.41 (1H, dd, J = 16.7, 4.7 Hz), 1.23 (3H, d, J = 6.1 Hz), 1.11 (3H, d, J = 6.1 Hz); ¹³C NMR (50 MHz, CDCl₃): δ 197.2, 192.6, 169.6, 169.0, 164.3, 157.5, 155.4 (2C), 97.0, 90.9, 89.2, 72.4, 56.9, 56.5, 39.8, 36.2, 21.2, 20.7, 14.3; HRMS (ESI+) calcd for [C₇H₁₅ClO₃]⁺ 381.1105, found 381.1119; Anal. (C₁₀H₁₂ClO₃) C, H.

(25S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-butoxy-6'-methyl-cyclohex-2'-ene-4'-one) 13.

Yield: 72 mg (14%) (white crystals); R₁ (EtOAc:heptane 5:1): 0.41; m.p.: 152-154 °C (Lit.); IR (KBr, cm⁻¹): 1706, 1662, 1614; ¹H NMR (300 MHz, CDCl₃): δ 6.11 (1H, s), 5.50 (1H, s), 4.03 (3H, s), 3.97 (3H, s), 3.83-3.68 (2H, m), 3.03 (1H, dd, J = 16.5, 13.5 Hz), 2.79 (1H, ddq, J = 13.3, 6.6, 4.7 Hz); ²D 2.41 (1H, dd), δ 1.59-1.49 (2H, m), δ 1.28-1.19 (2H, m), δ 0.95 (3H, d, J = 6.7 Hz), δ 0.74 (3H, t, J = 7.4 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 197.1, 192.9, 170.4, 169.5, 164.7, 157.9, 105.4, 105.2, 97.2, 91.1, 89.6, 69.5, 57.2, 56.6, 40.2, 36.5, 30.2, 19.0, 14.5, 13.8; HRMS (ESI+) calcd for [C₁₀H₁₃ClO₃]⁺ 395.1261, found 395.1263; Anal. (C₁₀H₁₂ClO₃) C, H.

(25S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-pentoxy-cyclohex-2'-ene-4'-one) 14.

Yield: 392 mg (17%) (white crystals); R₁ (EtOAc:heptane 5:1): 0.52; m.p.: 146-148 °C (Lit.); IR (KBr, cm⁻¹): 1701, 1653, 1613; ¹H NMR (300 MHz, CDCl₃): δ 6.11 (1H, s), 5.48 (1H, s), 4.02 (3H, s), 3.96 (3H, s), 3.79-3.66 (2H, m), 3.03 (1H, dd, J = 16.5, 13.5 Hz), 2.82 (1H, ddq, J = 13.5, 6.8, 4.7 Hz), 2.40 (1H, dd, J = 16.5, 4.6 Hz), 1.61-1.51 (2H, m), 1.20-1.15 (4H, m), 0.95 (3H, d, J = 6.7 Hz), 0.77 (3H, t, J = 6.8 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 197.2, 192.8, 170.4, 169.5, 164.7, 157.9, 105.4, 105.1, 97.4, 91.1, 89.5, 69.8, 57.2, 56.6, 40.2, 36.4, 28.0, 27.9, 22.3, 14.5, 14.1; HRMS (ESI+) calcd for [C₁₀H₁₂ClO₃]⁺ 409.1418, found 409.1412; Anal. (C₁₀H₁₂ClO₃) C, H.

Griseofulvic acid (11.3 g, 31.6 mmol) was mixed with LiCl (4.2 g, 0.101 mol) and added to a solution of POCl₃ (15.6 mL, 0.168 mol) in dioxane (40 mL). The mixture was stirred at 100 °C for 30 min, cooled to 0 °C and sat. aq. Na₂CO₃ was added carefully to the solution until slightly basic (pH 7-8). The aqueous phase was extracted with CH₂Cl₂ (4×300 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 35:35:1) affording the desired product 17 and the isomer. The product was re-crystallized from CH₂Cl₂:heptane. Yield: 234 mg (44%) (white crystals); R₁ (EtOAc:heptane 5:1): 0.51; m.p.: 196-198 °C (Lit.); IR (KBr, cm⁻¹): 1698, 1615 (Lit.); ¹H NMR (300 MHz, CDCl₃): δ 6.41 (1H, s), 6.14 (1H, s), 4.03 (3H, s), 3.98 (3H, s), 3.12 (1H, dd, J = 16.7, 13.9 Hz), 2.89 (1H, ddq, J = 13.5, 4.4, 4.7 Hz), 2.45 (1H, dd, J = 16.7, 4.4 Hz), 0.98 (3H, J = 6.7 Hz); ¹³C NMR (50 MHz, CDCl₃): δ 195.0, 191.1, 169.3, 165.3, 158.1, 152.9, 131.6, 105.4,
The mixture was then cooled to 20 °C, diluted with EtOAc (3×30 mL) and the combined organic phases were dried (MgSO₄) and then concentrated. The residue was purified by column chromatography (heptane:EtOAc 3:2) affording the product. When possible the product was re-crystallized from EtOAc/heptane.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzyloxy-6'-methyl-cyclohex-2'-en-4'-one) 19.
Yield: 199 mg (86%); Rₛ (EtOAc:heptane 5:1): 0.50; IR (KBr, cm⁻¹): 1704, 1663; ¹H NMR(300 MHz, CDCl₃): δ 7.36-7.29 (2H, m), 7.22-7.16 (1H, m), 7.00-6.95 (2H, m), 6.13 (1H, s), 5.30 (1H, s), 4.01 (3H, s), 3.99 (3H, s) 3.06 (1H, dd, J = 16.0, 13.5 Hz), 2.99-2.85 (1H, m), 2.42 (1H, dd, J = 16.0, 4.0 Hz), 1.01 (3H, d, J = 6.5 Hz); ¹³C NMR (75 MHz, CDCl₃): 197.0, 192.3, 170.9, 169.6, 164.8, 157.8, 152.6, 130.0 (2C), 126.3, 121.1 (2C), 108.8, 105.2, 97.2, 90.6, 89.5, 57.0, 56.4, 40.3, 36.5, 14.3; HRMS (ESI⁺) calcd for [C₁₂H₁₈ClO₁₂]⁺ 415.0948, found 415.0941.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 20.
Yield: 30 mg (55%) (white crystals); Rₛ (EtOAc:heptane 5:1): 0.49; m.p.: 186-188 °C (Lit.); ¹H NMR(300 MHz, CDCl₃): δ 7.32-7.24 (3H, m), 7.20-7.16 (2H, m), 6.11 (1H, s), 5.60 (1H, s), 4.02 (3H, s), 3.96 (3H, s), 4.92 (1H, d, J = 12.3 Hz), 4.82 (1H, d, J = 12.3 Hz), 3.06 (1H, dd, J = 16.7, 13.4 Hz), 2.87 (1H, dd, J = 13.4, 6.7, 4.8 Hz), 2.44 (1H, dd, J = 16.7, 4.8 Hz), 0.99 (3H, d, J = 6.7 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 196.9, 192.3, 169.5 (2C), 164.5, 157.7, 134.6, 128.5 (2C), 128.1, 126.6 (2C), 105.9, 105.2, 97.2, 90.7, 89.4, 70.6, 56.9, 56.3, 40.0, 36.6, 14.2; HRMS (ESI⁺) calcd for [C₁₂H₁₄ClO₁₂]⁺ 429.1105, found 429.1103.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzylthio-6'-methyl-cyclohex-2'-en-4'-one) 21.
Yield: 349 mg (93%) (white crystals); Rₛ (EtOAc:heptane 5:1): 0.44; m.p.: 208-210 °C (Lit.); IR (KBr, cm⁻¹): 1703, 1665 (Litt.); ¹H NMR (300 MHz, DMSO-d₆): δ 7.33-7.27 (5H, m), 6.51 (1H, s), 6.09 (1H, s), 4.17 (1H, d, J = 12.5 Hz), 4.05 (1H, d, J = 12.5 Hz), 4.03 (3H, s), 3.95 (3H, s), 2.95-2.83 (1H, m), 2.68 (1H, ds, J = 17.3, 13.3 Hz), 2.40 (1H, dd, J = 17.3, 5.3 Hz), 0.77 (3H, d, J = 6.6 Hz); ¹³C NMR (75 MHz, DMSO-d₆): δ 193.4, 191.1, 168.5, 160.2, 158.4, 135.3, 129.8 (2C), 129.4 (2C), 128.4, 122.6, 104.1, 95.8, 92.3, 92.2, 58.3, 57.3, 49.9, 37.4, 35.4, 14.9; HRMS (ESI⁺) calcd for [C₁₂H₁₄ClO₁₁S]⁺ 445.0877, found 445.0872; Anal. (C₉H₈ClOS) C, H.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-ol) 26.
A solution of griseofulvin (212 mg, 0.6 mmol) in MeOH (3 mL) was cooled to –40 °C, CeCl₃ (448 mg, 1.8 mmol) was added and the solution stirred for 10 min. after which NaBH₄ (70 mg, 1.8 mmol) was added. After 3 h excess reagent was quenched by the slow addition of acetone (3 mL) and the mixture was allowed to reach 20 °C, diluted with EtOAc (10 mL) and washed with water (20 mL). The aqueous phase was extracted with EtOAc (5×30 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The compound 26 was re-crystallized from MeOH. Yield: 209 mg (98%) (white crystals); Rₛ (EtOAc:heptane 5:1): 0.25; m.p.: 110-113 °C (Litt.); ¹H NMR(300 MHz, CDCl₃): δ 6.08 (1H, s), 5.11 (1H, d, J = 1.9 Hz), 4.45 (1H, ddd, J = 9.3, 5.8, 1.9 Hz), 4.00 (3H, s), 3.96 (3H, s), 3.44 (3H, s), 2.37 (1H, ddq, J = 13.1, 3.4, 6.8 Hz), 2.10 (1H, dt, J = 9.3, 13.1 Hz), 2.00 (1H, ddd, J = 13.1, 5.8, 3.4, 0.9 Hz), 0.87 (3H, d, J = 6.8 Hz); ¹³C NMR (from gHSQC, gHMBC: 500 MHz, CDCl₃): δ 195.5, 169.6, 164.8, 157.0, 152.6, 105.8, 105.5, 96.9, 92.0, 88.8, 65.9, 56.5, 56.1, 55.0, 36.3, 35.6, 14.2; EIMS [M-H] = 354, found 354.
To a solution of griseofulvin (107 mg, 0.3 mmol) in MeOH (3 mL) was added 5% Pt on charcoal (24 mg). The mixture was stirred at 20 °C for 12 h under a hydrogen atmosphere. EtOAc (20 mL) was added and the mixture was filtered, dried (MgSO₄), and concentrated by column chromatography (EtOAc:heptane 1:1). The product was recrystallized from EtOAc/heptane. Yield: 94 mg (86%) (white crystals); Rf (EtOAc:heptane 4:1): 0.27; m.p.: 188–190 °C (Lit.); IR(KBr, cm⁻¹): 1696; ¹H NMR (500 MHz, CDCl₃): δ 6.06 (1H, s), 3.99 (3H, s), 3.94 (3H, s), 3.83–3.70 (1H, m), 3.62 (1H, dd, J = 12.0, 5.1 Hz), 3.27 (3H, s), 2.29 (1H, m), 2.24 (1H, q, J = 12.0 Hz), 2.09 (2H, m), 1.90–1.78 (1H, m), 0.82 (3H, d, J = 6.1 Hz); ¹³C NMR (50 MHz, CDCl₃): δ 195.8, 169.0, 163.6, 156.9, 107.7, 96.6, 94.8, 88.6, 81.5, 67.4, 58.4, 56.7, 56.0, 37.4, 35.0, 34.9, 14.1; Anal. (C₁₇H₂₂ClO₄): C, H.

To a solution of griseofulvin (514 mg, 1.5 mmol) in THF (10 mL) was added K₂CO₃ (2.54 g, 0.015 mol) for 24 hours. The mixture was cooled to 20 °C and NaI (2.22 g, 0.015 mol) was heated to reflux with copper powder (188 mg, 2.97 mmol) and benzyl bromide (2.54 g, 0.015 mol) for 24 hours. The reaction was quenched with sat. aq. NH₄Cl and MeI (0.2 mL, 3.1 mmol) and the mixture was heated to 80 °C. The solution was cooled to 20 °C after 12 h. The reaction was quenched with sat. aq. NH₄Cl and then extracted with EtOAc (3×30 mL). The combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 2:2:1) to afford the desired product.

Yield: 988 mg (95%) (white needles); Rf (MeOH:CH₂Cl₂:1:10): 0.50; IR (KBr, cm⁻¹): 1706, 1590, 1614 (Lit.); ¹H NMR (300 MHz, CDCl₃): δ 6.62 (0.5H, s), 6.10 (0.5H, s), 6.10 (0.5H, s), 5.59 (0.5H, s), 4.01 (3H, s), 3.96 (1.5H, s), 3.96 (1.5H, s), 3.61 (1.5H, s), 3.55 (1.5H, s), 3.12 (0.5H, dd, J = 16.6, 4.7 Hz), 2.99 (0.5H, dd, J = 15.0, 13.1 Hz), 2.70 (0.5H, dd, J = 16.6, 13.0 Hz), 2.64–2.48 (1H, m), 2.40 (0.5H, dd, J = 15.0, 4.2 Hz), 0.95 (1.5H, d, J = 6.7 Hz), 0.94 (1.5H, d, J = 6.7 Hz) (Lit.); ¹³C NMR (75 MHz, CDCl₃): δ 194.1 (0.5C), 193.9 (0.5C), 169.4 (0.5C), 169.4 (0.5C), 164.3 (0.5C), 164.2 (0.5C), 161.3 (0.5C), 158.6 (0.5C), 157.5 (0.5C), 157.4 (0.5C), 155.0 (0.5C), 151.8 (0.5C), 150.5 (0.5C), 98.9 (0.5C), 97.1, 92.6 (0.5C), 91.4 (0.5C), 91.3 (0.5C), 89.1, 56.9 (0.5C), 56.9 (0.5C), 56.4 (0.5C), 55.9 (0.5C), 36.4 (0.5C), 35.2 (0.5C), 30.8 (0.5C), 25.5 (0.5C), 14.3 (0.5C), 14.2 (0.5C); HRMS (ESI+) calc'd for [C₁₃H₁₇ClNO]⁺ 368.09, found 368.09; Anal. (C₁₃H₁₇ClNO) C, H.

To a solution of griseofulvin (514 mg, 1.5 mmol) in THF (10 mL) was added K₂CO₃ (410 mg, 3.0 mmol) and MeI (0.2 mmol, 1.5 equiv.) in THF (5 mL, 0.03M) and DMSO (2.5 mL, 0.03M) was added hydroxylamine hydrochloride (0.70 mmol, 3.5 equiv.) and sodium acetate (0.86 mmol, 4.3 equiv.). The mixture was stirred at 75 °C for 24 hours, allowed to reach 20 °C and diluted with CH₂Cl₂ (20 mL). The mixture was washed with distilled water (2×15 mL) and then brine (15 mL). The organic phase was dried (MgSO₄) and concentrated. The crude mixture was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 2:2:1) to afford the desired product.

Yield: 75 mg (14%) (yellow oil); Rf (EtOAc:heptane 2:2:1:10): 0.50; IR (KBr, cm⁻¹): 207.2, 201.6, 190.8, 169.2, 164.8, 157.9, 104.5, 97.3, 96.0, 89.6, 60.2, 57.0, 56.3, 40.4, 32.2, 23.6, 23.5, 14.6; EIMS [M⁺] found 368.09, found 368.09; Anal. (C₁₃H₁₅ClNO) C, H.
Yield: 533 mg (8%) (white crystals); Rf (EtOAc:heptane 5:1): 0.33; m.p.: 202-204 °C (Lit.\(^1\)); IR (KBr, cm\(^{-1}\)): 1702, 1616 (Lit.\(^1\)); \(^1\)H NMR (300 MHz, DMSO-d\(_6\)): \(\delta\) 11.6 (1H, s), 7.21-7.16 (2H, m), 7.10-7.05 (3H, m), 6.43 (1H, s), 4.01 (3H, s), 3.88 (3H, s), 3.50 (1H, d, \(J = 14.6\) Hz), 3.41 (1H, d, \(J = 14.6\) Hz), 2.92 (1H, dd, \(J = 16.5, 11.5\) Hz), 2.81-2.70 (1H, m), 2.63 (1H, dd, \(J = 16.5, 5.2\) Hz), 0.84 (3H, d, \(J = 6.4\) Hz); \(^1\)C NMR (75 MHz, DMSO-d\(_6\)): \(\delta\) 190.7, 190.6, 174.4, 168.5, 163.5, 156.9, 140.4, 127.5 (2C), 127.4 (2C), 124.9, 103.8, 95.0, 94.3, 90.4, 90.3, 57.0, 56.0, 34.0, 32.5, 27.3, 13.9; HRMS (ESI+) calcld for [C\(_{23}\)H\(_21\)ClO\(_3\)]\(^{+}\) 429.1105, found 429.1108.

General procedure for the synthesis of 3’-iodo analogues (33-35).

To an ice-cooled solution of griseofulvin (1.4 mmol, 1 equiv.) and N-iodosuccinimide (2.1 mmol, 1.5 equiv.) in anhydrous CH\(_2\)Cl\(_2\) (15 mL, 0.1 M) under an argon atmosphere, was added triethylsilyl trifluoromethanesulphonate (0.35 mmol 0.25 equiv.). The mixture was stirred at 20 °C for 24 h and then diluted with CH\(_2\)Cl\(_2\) (60 mL) and washed with sat. aq. NaHCO\(_3\) (60 mL). The aqueous phase was extracted with CH\(_2\)Cl\(_2\) (50 mL), the combined organic phases were dried (MgSO\(_4\)) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to yield the desired product. When possible the product was re-crystallized from EtOAc/heptane.

\((25,6’R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1’-(3’-iodo-2’-methoxy-6’-methyl-cyclohex-2’-ene-4’-one) 33.

Yield: 126 mg (19%) (white crystals); Rf (EtOAc:heptane 5:1): 0.77; m.p.: 188-190 °C (Lit.\(^2\)); IR (KBr, cm\(^{-1}\)): 1717, 1664 (Lit.\(^2\)); \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 5.56 (1H, s), 4.10 (3H, s), 4.01 (3H, s), 3.63 (3H, s), 2.95 (1H, dd, \(J = 15.6, 13.3\) Hz), 2.90-2.79 (1H, m), 2.46 (1H, dd, \(J = 15.6, 3.6\) Hz), 0.94 (3H, d, \(J = 6.4\) Hz); \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 196.7, 192.5, 170.8, 170.3, 164.6, 157.0, 106.3, 105.4, 97.1, 91.1, 82.1, 63.1, 61.4, 57.1, 40.1, 36.7, 14.6; HRMS (ESI+) calcld for [C\(_{17}\)H\(_{13}\)ClO\(_3\)]\(^{+}\) 478.9758, found 478.9761.

\((25,6’R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1’-(3’-iodo-6’-methyl-2’-propoxy-cyclohex-2’-ene-4’-one) 34.

Yield: 49 mg (19%) (yellow crystals); Rf (EtOAc:heptane 5:1): 0.53; m.p.: 98-100 °C (Lit.\(^2\)); IR (KBr, cm\(^{-1}\)): 1719, 1618 (Lit.\(^2\)); \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 6.13 (1H, s), 4.14-4.11 (2H, m), 4.03 (3H, s), 4.00 (3H, s), 3.80-3.75 (1H, m), 2.94-2.87 (1H, m), 2.92-2.90 (1H, m), 1.75-1.68 (2H, m), 1.05 (3H, d, \(J = 5.8\) Hz), 1.00 (3H, t, \(J = 7.4\) Hz); \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 196.7, 192.1, 188.0, 172.3, 172.3, 165.5, 158.3, 112.2, 103.8, 97.9, 90.1, 67.9, 57.4, 56.8, 39.4, 38.2, 22.3, 12.2, 10.8; HRMS (ESI+) calcld for [C\(_{16}\)H\(_{14}\)ClO\(_3\)]\(^{+}\) 507.0071, found 507.0069.

\((25,6’R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1’-(3’-iodo-2’-benzoxly-6’-methyl-cyclohex-2’-ene-4’-one) 35.

Yield: 56 mg (17%) (white crystals); Rf (EtOAc:heptane 5:1): 0.88; m.p.: 172-175 °C (Lit.\(^2\)); IR (KBr, cm\(^{-1}\)): 1715, 1666 (Lit.\(^2\)); \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.27-7.22 (3H, m), 7.12-7.10 (2H, m), 5.59 (1H, s), 4.90 (1H, d, \(J = 12.3\) Hz), 4.81 (1H, d, \(J = 12.3\) Hz), 4.07 (3H, s), 3.95 (3H, s), 2.95 (1H, dd, \(J = 15.5, 13.2\) Hz), 2.88-2.81 (1H, m), 2.44 (1H, dd, \(J = 15.4, 3.3\) Hz), 0.95 (3H, d, \(J = 6.4\) Hz); \(^1\)C NMR (75 MHz, CDCl\(_3\)): \(\delta\) 196.7, 192.6, 170.9, 168.9, 164.6, 157.0, 134.6, 128.9 (2C), 128.7, 126.9 (2C), 110.4, 106.5, 106.2, 91.2, 82.0, 71.1, 63.1, 61.4, 40.2, 36.5, 14.6; HRMS (ESI+) calcld for [C\(_{23}\)H\(_{19}\)ClO\(_3\)]\(^{+}\) 555.0071, found 555.0071; Anal. (C\(_{23}\)H\(_{19}\)ClO\(_3\)) C, H.

References


(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) 2

$^1$H NMR (500 MHz, DMSO-$d_6$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(25,6'R)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-one)-2-spiro-1'(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 4

$^1$H NMR (500 MHz, DMSO-$d_6$)

$^{13}$C NMR (50 MHz, CDCl$_3$)

S11
(2S,6'R)-(7-Chloro-4-ethoxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 5

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4-benzoxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-((2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 6

^1^H NMR (500 MHz, CDCl₃)

^1^3^C NMR (50 MHz, CDCl₃)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-5-nitro-benzofuran-3-on)-2-spiro-1'-{(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) 7

$^1$H NMR (300 MHz, CDCl$_3$)

$^1$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-5-amino-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one)

\[ \text{H NMR (300 MHz, CDCl}_3 \]}

\[ \text{ppm (f1)} \]

\[ \text{C NMR (75 MHz, CDCl}_3 \]}

\[ \text{ppm (f1)} \]
(2S,6R)-(7-Chloro-4,6-dimethoxy-5-benzylamino-benzofuran-3-on)-2-spiro-1'-{(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one)}

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(25,6'R)(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2''-ethoxy-6''-methyl-cyclohex-2''-en-4''-on) 10

^1H NMR (500 MHz, CDCl3)

^13C NMR (50 MHz, CDCl3)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-[6'-methyl-2'-propoxy-cyclohex-2'-en-4'-on) 11

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-isopropoxy-6'-methyl-cyclohex-2'-en-4'-on) 12

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-butoxy-6'-methyl-cyclohex-2'-ene-4'-one) 13

$^1$H NMR (300 MHz, CDCl$_3$)

$^1$C NMR (75 MHz, CDCl$_3$)
(2S,6'R) (7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-([6'-methyl-2'-pentoxy-cyclohex-2'-ene-4']-one) 14

1H NMR (300 MHz, CDCl₃)

13C NMR (75 MHz, CDCl₃)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'(2'-hexoxy-6'-methyl-cyclohex-2'-ene-4'-one) 15

$^1$H NMR (300 MHz, CDCl$_3$)

$^1$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-2'-cyclopropylmethoxy-6'-methyl-cyclohex-2'-en-4'-one) 16

$^1$H NMR (500 MHz, CDCl$_3$)

$^13$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-cyclopentoxy-6'-methyl-cyclohex-2'-en-4'-one) 17

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-chloro-6'-methyl-cyclohex-2'-ene-4'-one) 18

$^1$H NMR (300 MHz, CDCl$_3$)

$^13$C NMR (50 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'(6'-methyl-2'-phenoxy-cyclohex-2'-ene-4'-one) 19

$^1$H NMR (300 MHz, CDCl$_3$)

$^13$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'(2'-benzyloxy-6'-methyl-cyclohex-2'-en-4'-one) 20

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxybenzofuran-3-one)-2-spiro-1''-(2''-benzylthio-6''-methyl-cyclohex-2''-ene-4''-one) 21

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'(2'- (2-phenylethoxy)-6'-methyl-cyclohex-2'-en-4'-one) 22

$^1$H NMR (300 MHz, CDCl$_3$)

$^1$C NMR (75 MHz, CDCl$_3$)
(25,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-4-methylbenzylxoy-6'-methyl-cyclohex-2'-ene-4'-one) 23

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2'-spiro-1'-(2'-(4-biphenylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) 24

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-2'-(1-adamantylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)

S32
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-[2'-methoxy-6'-methyl-cyclohex-2'-en-4'-ol] 26

$^1$H NMR (300 MHz, CDCl$_3$)

Carbon shifts from gHSQC, gHMBC
(2S,4'S,5'R,7'-Clorotetrahydrofuran-3-ol)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohexan-4'-ol) 27

$^1$H NMR (500 MHz, CDCl$_3$)

$^{13}$C NMR (50 MHz, CDCl$_3$)
(25,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2' methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-oxime) 28

$^1$H NMR (300 MHz, CDCl$_3$)

$^1$C NMR (75 MHz, CDCl$_3$)
(25.6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-({2'-benzyloxy-6'-methylyclohex-2'-ene-4'-one-4'-oxime) 29

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R,E)(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-dimethylhydrazine) 30

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)

S37
\((25,6'R)-(7\text{-Chloro-4,6-dimethoxy-benzofuran-3-on})-2\text{-spiro-1'-(3',3',6'-trimethyl-cyclohex-2',4'-dione})\) 31

\(^1H\) NMR (500 MHz, CDCl\(_3\))

\(^13C\) NMR (75 MHz, CDCl\(_3\))
(2S,6'R)(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'(3'-benzyl-6'-methyl-cyclohex-2',4'-dione) 32

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
(2S,6'R) (7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'- (3'-iodo-2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) \(^{33}\)

\(^1\)H NMR (300 MHz, CDCl\(_3\))

\(^{13}\)C NMR (50 MHz, CDCl\(_3\))
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-6'-methyl-2'-propoxy-cyclohex-2'-ene-4'-one) \(34\)

\(^1\)H NMR (500 MHz, CDCl\(_3\))

\(^{13}\)C NMR (75 MHz, CDCl\(_3\))
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzyloxy-3'-indo-6'-methyl-cyclohex-2'-ene-4'-one) 35

$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
Appendix D

Supporting Information - Chapter 4
Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

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**Supporting Information**

S2-S3 Experimental for compounds 3, 39-41 and 44.
S4-S27 NMR spectra of 3, 7, 8, 15, 19, 21, 22, 26, 28-35 and 38-44.
S28-S41 UPLC-DAD chromatograms of all tested compounds.
S42 Figure S1 and S2.
S43 References.
Griseofulvic acid (2) (3.0 mmol, 1 equiv.) was mixed with LiCl (15 mmol, 5 equiv.) and added to a solution of POCl₃ (15 mmol, 5 equiv.) in dioxane (14 mL). The mixture was stirred at 100 °C for 30 min, cooled to 0 °C and sat. aq. Na₂CO₃ was added carefully to the solution until slightly basic (pH 7-8). The aqueous phase was extracted with CH₂Cl₂ (3×20 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 35:35:1) affording the desired products 15 and 16.

**Yield:** 588 mg (55 %) (white crystals); Rₚ-value (EtOAc/heptane, 5:1): 0.63; m.p. 241-243 °C (Litt.); IR (neat, cm⁻¹): 1699, 1677, 1614, 1596, 1581 (Litt.); ¹H NMR (CDCl₃, 300 MHz): δ 6.28 (1H, d, J = 2.2 Hz), 6.10 (1H, s), 4.01 (3H, s), 3.94 (3H, s), 3.40 (1H, ddd, J = 18.5, 11.2, 2.2 Hz), 2.93 (1 H, ddq, J = 11.2, 5.7, 6.7 Hz), 2.75 (1H, dd, J = 18.5, 5.7 Hz), 1.04 (3H, d, J = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 190.3, 187.0, 169.5, 164.6, 160.0, 157.8, 125.3, 104.7, 97.5, 94.6, 89.6, 57.0, 56.3, 38.6, 36.5, 14.2; [a]²⁰D = +269° (c = 0.5 in CHCl₃); HRMS (ESI⁺) calcd for M⁺H [C₁₆H₁₄Cl₂O₅]+ 357.0297, found 357.0304; Anal. Calcd for C₁₆H₁₄Cl₂O₅: C, 53.80; H, 3.95. Found: C, 53.69; H, 3.88.

**General Procedure for the Synthesis of Enol Ethers by Solvolysis (39-41 and 44).**

Yield: 116 mg (22 %) (white crystals); Rₚ-value (EtOAc/heptane, 5:1): 0.48; m.p. 197-198 °C (Litt.); IR (neat, cm⁻¹): 1701, 1657, 1589; ¹H NMR (CDCl₃, 300 MHz): δ 6.07 (1H, s), 5.44 (1H, d, J = 1.5 Hz), 4.00 (3H, s), 3.76 (3H, s), 3.18 (1H, ddd, J = 17.5, 12.0, 1.5 Hz), 2.83 (1H, m), 2.46 (1H, dd, J = 17.5, 5.7 Hz), 1.01 (3H, d, J = 6.7 Hz) (Litt.); ¹³C NMR (CDCl₃, 50 MHz): δ 191.6, 188.6, 178.8, 169.7, 164.3, 157.6, 145.1, 99.6, 97.3, 95.1, 89.4, 56.9, 56.2 (2C), 35.1, 32.6, 14.4; [a]²⁰D = +142° (c = 0.3 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₁₇H₁₈ClO₆]+ 353.0792, found 353.0790; Anal. Calcd for C₁₇H₁₈ClO₆: C, 57.88; H, 4.86. Found: C, 57.75; H, 4.60.

Yield: 145 mg (64 %) (white crystals); Rₚ-value (EtOAc/heptane, 5:1): 0.67; m.p. 167-168 °C (Litt.); IR (neat, cm⁻¹): 1703, 1657, 1609, 1589 (Litt.); ¹H NMR (CDCl₃, 300 MHz): δ 6.08 (1H, s), 5.41 (1H, d, J = 1.2 Hz), 4.00 (3H, s), 3.98 (2H, q, J = 7.0 Hz), 3.92 (3H, s), 3.18 (1H, ddd, J = 17.6, 12.1, 1.2 Hz), 2.84 (1H, dd, J = 12.1, 5.7, 6.7 Hz), 2.45 (1H, dd, J = 17.6, 5.7 Hz), 1.39 (3H, t, J = 7.0 Hz), 1.02 (3H, d, J = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 191.7, 188.8, 178.0, 169.6, 164.3, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 64.9,
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-propoxy-6'-methyl-cyclohex-3'-en-2'-one) 40

Yield: 146 mg (63%) (white crystals); R_f-value (EtOAc/heptane, 5:1): 0.75; m.p. 176-178 °C (Litt.); IR (neat, cm⁻¹): 1703, 1657, 1609, 1590 (Litt.); ¹H NMR (CDCl₃, 500 MHz): δ 8.08 (1H, s), 5.41 (1H, s), 4.00 (3H, s), 3.92 (3H, s), 3.91-3.82 (2H, m), 3.20 (1H, dd, J = 17.6, 12.1 Hz), 2.83 (1H, ddq, J = 12.1, 6.7, 5.7 Hz), 2.42 (1H, dd, J = 17.6, 5.7 Hz), 1.83-1.74 (2H, m), 1.02 (3H, d, J = 6.7 Hz), 0.99 (3H, t, J = 7.4 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 191.7, 188.8, 178.2, 169.6, 164.4, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 70.7, 56.9, 56.2, 35.1, 33.1, 21.7, 14.4; [α] D²⁰ = +192° (c = 0.5 in CHCl₃); Anal. Calcd for C₁₉H₂₁ClO₆: C, 59.92; H, 5.56. Found: C, 59.84; H, 5.50.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-isopropoxy-6'-methyl-cyclohex-3'-en-2'-one) 41

Yield: 109 mg (47%) (white crystals); R_f-value (EtOAc/heptane, 5:1): 0.71; m.p. 186-187 °C (Litt.); IR (neat, cm⁻¹): 1704, 1657, 1609, 1589 (Litt.); ¹H NMR (CDCl₃, 300 MHz): δ 7.42-7.33 (5H, m), 6.06 (1H, s), 5.53 (1H, s), 4.96 (1H, d, J = 17.2 Hz), 4.94 (1H, d, J = 17.2 Hz), 3.99 (3H, s), 3.86 (3H, s), 3.25 (1H, dd, J = 17.6, 12.0 Hz), 2.80-2.81 (1H, m), 2.53 (1H, dd, J = 17.6, 5.7 Hz), 1.02 (3H, d, J = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 191.6, 188.8, 177.0, 169.7, 164.3, 157.6, 105.2, 100.2, 97.4, 95.1, 89.3, 71.9, 56.9, 56.2, 35.0, 33.6, 21.5, 21.2, 14.4; [α] D²⁰ = +153° (c = 0.4 in CHCl₃); Anal. Calcd for C₁₉H₂₁ClO₆: C, 59.92; H, 5.56. Found: C, 59.84; H, 5.50.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-benzyloxy-6'-methyl-cyclohex-3'-en-2'-one) 44

Yield: 398 mg (31%) (yellow oil); R_f-value (EtOAc/heptane, 5:1): 0.59; IR (neat, cm⁻¹): 1703, 1658, 1609, 1589; ¹H NMR (CDCl₃, 300 MHz): δ 7.42-7.33 (5H, m), 6.06 (1H, s), 5.53 (1H, s), 4.96 (1H, d, J = 17.2 Hz), 4.94 (1H, d, J = 17.2 Hz), 3.99 (3H, s), 3.86 (3H, s), 3.25 (1H, dd, J = 17.6, 12.0 Hz), 2.90-2.81 (1H, m), 2.53 (1H, dd, J = 17.6, 5.7 Hz), 1.02 (3H, d, J = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 191.6, 188.8, 177.7, 169.6, 164.4, 157.6, 134.4, 128.6 (2C), 128.1, 127.8 (2C), 104.9, 100.5, 97.1, 94.9, 89.4, 71.0, 56.9, 56.1, 35.1, 33.1, 14.4; [α] D²⁰ = +307° (c = 0.5 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₂₃H₂₂ClNO₆]⁺ 429.1105, found 429.1107.
$^1$H NMR (300 MHz, CDCl₃) 3

$^{13}$C NMR (50 MHz, CDCl₃) 3
$^1$H NMR (300 MHz, CDCl$_3$) 7

$^{13}$C NMR (50 MHz, CDCl$_3$) 7
$^1$H NMR (300 MHz, CDCl$_3$) 8

$^{13}$C NMR (50 MHz, CDCl$_3$) 8
$^1$H NMR (300 MHz, CDCl$_3$) 14

$^{13}$C NMR (50 MHz, CDCl$_3$) 14
$^1$H NMR (300 MHz, CDCl$_3$) 15

$^{13}$C NMR (75 MHz, CDCl$_3$) 15
$^1$H NMR (300 MHz, CDCl₃) 19

$^{13}$C NMR (50 MHz, CDCl₃) 19
$^1$H NMR (300 MHz, CDCl$_3$) 21

$^{13}$C NMR (50 MHz, CDCl$_3$) 21
\(^1\)H NMR (300 MHz, CDCl\(_3\)) 22

\(^{13}\)C NMR (50 MHz, CDCl\(_3\)) 22
$^1$H NMR (500 MHz, CDCl$_3$) 26

$^{13}$C NMR (75 MHz, CDCl$_3$) 26
$^1$H NMR (300 MHz, CDCl$_3$) 28

$^{13}$C NMR (50 MHz, CDCl$_3$) 28
$^1$H NMR (300 MHz, CDCl$_3$) 29

$^{13}$C NMR (75 MHz, CDCl$_3$) 29
$^1$H NMR (300 MHz, CDCl$_3$) 30

$^{13}$C NMR (50 MHz, CDCl$_3$) 30
$^1$H NMR (300 MHz, CDCl$_3$) 31

$^{13}$C NMR (50 MHz, CDCl$_3$) 31
$^1$H NMR (300 MHz, CDCl$_3$) 32

$^{13}$C NMR (50 MHz, CDCl$_3$) 32
$^1$H NMR (300 MHz, CDCl$_3$) 33

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$^1$H NMR (300 MHz, CDCl$_3$) 34

$^{13}$C NMR (50 MHz, CDCl$_3$) 34
$^1$H NMR (300 MHz, CDCl$_3$) 35

$^{13}$C NMR (50 MHz, CDCl$_3$) 35
$^1$H NMR (500 MHz, CDCl$_3$) 38

$^{13}$C NMR (75 MHz, CDCl$_3$) 38
$^1$H NMR (300 MHz, CDCl$_3$) 39

$^{13}$C NMR (50 MHz, CDCl$_3$) 39
$^1$H NMR (500 MHz, CDCl$_3$) 40

$^{13}$C NMR (50 MHz, CDCl$_3$) 40

S23
$^1$H NMR (500 MHz, CDCl$_3$) 41

$^{13}$C NMR (50 MHz, CDCl$_3$) 41
$^1$H NMR (500 MHz, CDCl$_3$) 42

$^{13}$C NMR (50 MHz, CDCl$_3$) 42
$^1$H NMR (300 MHz, CDCl$_3$) 43

$^{13}$C NMR (50 MHz, CDCl$_3$) 43
$^1$H NMR (300 MHz, CDCl$_3$) 44

$^{13}$C NMR (50 MHz, CDCl$_3$) 44
UPLC-DAD Griseofulvin

UPLC-DAD 2

UPLC-DAD 3

UPLC-DAD 4
UPLC-DAD 5

UPLC-DAD 6

UPLC-DAD 7

UPLC-DAD 8
UPLC-DAD 9

UPLC-DAD 10

UPLC-DAD 11

UPLC-DAD 13
UPLC-DAD 18

UPLC-DAD 19

UPLC-DAD 20

UPLC-DAD 21
UPLC-DAD 26

UPLC-DAD 27

UPLC-DAD 28

UPLC-DAD 29

UPLC-DAD 30
Figure S1 A plot of the IC₅₀ (µM) values for compounds showing activity in both the phenotype based assay and the cytotoxicity assay with correlation at R² = 0.70.

Figure S2. Examples of an active compound (14), a compound with low activity (29) and an inactive compound (2)
References


Supporting Information - Chapter 5
Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

Mads H. Ronnest, Pernille Harris, Charlotte H. Gotfredsen, Thomas O. Larsen, Mads H. Clausen*

Supplementary data

S2-S4 Experimental
S5-S10 HPLC chromatograms and 1D NMR spectra of 2, 3 and S1
S11-S13 gHMBC spectra of 1, 2 and 3 and 2D assignment of 2 and 3
S14 UV spectra of 2 and 3
S15 Fluorescence data of 2 and 3
S16-S18 MS-MS spectra of 2 and 3 at the collision voltages 30, 45 and 60 V
Experimental

$^1$H NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. $^{13}$C NMR spectra were recorded using a Bruker AC 200 MHz. Chemical shifts were measured in ppm and coupling constants in Hz. When CDCl$_3$ was used as solvent the residual peak was used as internal reference at δ 7.27 for $^1$H NMR and δ 77.00 for $^{13}$C NMR spectra. When DMSO-$d_6$ was used the values were δ 2.50 for $^1$H NMR and δ 39.43 for $^{13}$C NMR spectra.

IR spectra were recorded using a Bruker Alpha ATR and measured in cm$^{-1}$. All melting points are uncorrected. TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merck 1.05554.0001).

High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm formic acid) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min.

Compounds were visualized by charring after dipping in a solution of 1% KMnO$_4$, 6.7% K$_2$CO$_3$ and 0.08% NaOH in water. UV visualization was done using a Model UVGL-25 Mineralight Lamp.

Fluorescence spectra was obtained on an Agilent Series 110 FLD.
Method A:
Griseofulvin (10 mg, 0.03 mmol) and LiI (4.7 mg, 0.04 mmol) was dissolved in pyridine (0.5 mL) and heating to 115 °C. After 16 H the reaction was allowed to reach 20 °C and sat. aq. NH$_4$Cl was added. The mixture was extracted 3 times with EtOAc, dried (MgSO$_4$) and concentrated. Purification was performed on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 5 mL/min H$_2$O/CH$_3$CN (isocratic run at 65/35, for 15 minutes) as the mobile phase to yield 2 (2.8 mg, 29 %) as a yellow oil. R$_f$(CH$_2$Cl$_2$:MeOH:HOAc 96:3:1): 0.32; IR(neat): 1707, 1667, 1610; $^1$H NMR (DMSO-$d_6$, 500 MHz) 6.26 (1H, s), 5.59 (1H, s), 3.81 (3H, s), 3.63 (3H, s), 2.78 (1H, ddq, $J = 13.4, 6.5, 4.6$ Hz), 2.68 (1H, dd, $J = 16.4, 13.4$ Hz), 2.33 (1H, dd, $J = 16.4, 4.6$ Hz), 0.80 (3H, d, $J = 6.5$ Hz); $^1$H NMR(CDCl$_3$, 500 MHz): $\delta$ 6.25 (1H, s), 5.57 (1H, s), 3.92 (3H, s), 3.63 (3H, s), 3.04 (1H, dd, $J = 16.8, 13.4$ Hz), 2.83 (1H, ddq, $J = 13.4, 6.5$ Hz), 2.45 (1H, dd, $J = 16.8, 4.7$ Hz), 0.97 (3H, d, $J = 6.5$ Hz); $^{13}$C NMR(DMSO-$d_6$, from gHSQC and gHMBC, 500 MHz): $\delta$ 195.6, 190.6, 170.4, 169.2, 164.5, 156.9, 104.1, 102.9, 94.3, 93.7, 89.8, 56.7, 55.6, 39.2, 35.3, 13.5; $^{13}$C NMR(CDCl$_3$, from gHSQC and gHMBC, 500 MHz): $\delta$ 197.3, 191.8, 170.6, 169.2, 161.4, 157.9, 105.1, 104.6, 96.9, 93.1, 91.0, 56.4, 56.2, 39.7, 36.0, 13.9; $\alpha$ +254° (c = 0.1 in MeOH); HRMS (ESI+) calcd for M+H [C$_{16}$H$_{16}$ClO$_6$]+ 339.0635, found 339.0634.

Method B:
Compound S1 (51 mg, 0.16 mmol) was dissolved in MeOH (2.5 mL), CSA was added (1 mg, 0.01 mmol) and the mixture was refluxed for 20 H. EtOAc was added and the organic phase was washed with sat. aq. NaHCO$_3$. The aqueous phase was extracted three times with EtOAc and the combined organic phases were dried (MgSO$_4$) and concentrated. The residue was purified by column chromatography (CH$_2$Cl$_2$:MeOH:AcOH 97:2:1), yielding 3. A solution of Mgl$_2$ was prepared by adding magnesium turnings (31 mg, 1.3 mmol) and iodine (54 mg, 0.43 mmol) to anhydrous Et$_2$O (2 mL) and toluene (1 mL). This solution was sonicated at 60 °C for 90 min., filtered and added to griseofulvin (1) (100 mg, 0.29 mmol) and heated to 80 °C. The solution was cooled to 20 °C after 20 h, and 0.2 M H$_2$SO$_4$ was added. The mixture was extracted 3 times with EtOAc, dried (MgSO$_4$) and concentrated. The residue was purified by column chromatography (CH$_2$Cl$_2$:MeOH:AcOH 97:2:1), yielding 3, which was recrystallized from EtOAc/heptane to afford yellow crystals (95 mg, 98%). R$_f$(CH$_2$Cl$_2$:MeOH:H$O$Ac 96:3:1): 0.25; m.p: 138-140 °C, IR(neat): 1697, 1620, 1449; $^1$H NMR(DMSO-$d_6$, 500 MHz): $\delta$ 11.53 (1H, s), 6.30 (1H, s), 3.50 (2H, s), 3.37 (3H, s), 2.78 (1H, ddq, $J = 13.4, 6.5$ Hz), 2.68 (1H, dd, $J = 16.3, 4.5$ Hz), 0.81 (3H, d, $J = 6.5$ Hz); $^{13}$C NMR(CDCl$_3$, 300 MHz): $\delta$ 6.18 (1H, s), 5.57 (1H, s), 3.97 (3H, s), 3.65 (3H, s), 2.95 (1H, dd, $J = 15.3, 13.1$ Hz), 2.92-

(25,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 2

\[ \text{(2S,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 3} \]
2.80 (1H, m), 2.48 (1H, dd, J = 15.3, 3.2 Hz), 0.97 (3H, d, J = 6.4 Hz); $^{13}$C NMR (DMSO-$d_6$, 500 MHz): δ 195.5, 191.3, 170.4, 168.1, 163.7, 156.8, 104.5, 103.7, 94.1, 93.6, 89.8, 56.9 (2C), 39.4, 35.6, 13.8; $^{13}$C NMR (CDCl$_3$, 50 MHz): δ 196.8, 195.6, 170.4, 167.3, 165.5, 156.0, 104.8, 103.9, 96.4, 93.7, 91.2, 57.2, 56.7, 40.0, 36.2, 14.2; [α] +310° (c = 0.3 in CCl$_4$); HRMS (ESI$^+$) calcd for M+H [C$_{16}$H$_{16}$ClO$_6$]$^+$ 339.0635, found 339.0633.

(2S,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) S1

Griseofulvic acid$^{1,2}$ (300 mg, 0.88 mol) was dissolved in 0.66 M NaOH (10 mL) and heated to 100 °C for 24 H. The mixture was allowed to reach 20 °C and filtered. The supernatant was acidified with 2 M H$_2$SO$_4$ and the precipitate purified on reverse phase silica (MeOH/H$_2$O). The compound was re-crystallized from MeOH/H$_2$O to afford S1 (123 mg, 43%) as white crystals. R$_f$ (CH$_2$Cl$_2$:MeOH:HOAc 89:10:1): 0.49; m.p: 259-261 °C (decomp.); IR(neat): 3100 (b), 1661, 1632, 1579, 1538; $^1$H NMR (DMSO-$d_6$): δ 11.88 (1H, s), 6.24 (1H, s), 5.30 (1H, s), 3.78 (3H, s), 2.88-2.66 (2H, m), 2.52-2.40 (1H, m), 0.84 (3H, d, J = 6.1 Hz); $^{13}$C NMR (DMSO-$d_6$): δ 190.7, 187.8, 179.3, 170.1, 163.7, 156.9, 103.5, 101.5, 94.3 (2C), 93.6, 55.9, 34.4, 32.9 14.3; [α] +289° (c = 0.3 in MeOH); HRMS (ESI$^+$) calcd for M+H [C$_{15}$H$_{14}$ClO$_6$]$^+$ 325.0479, found 325.0467.

Griseofulvin I

$^1$H NMR (500 MHz, DMSO-$_d_6$)

$^{13}$C NMR (50 MHz, DMSO-$_d_6$)
(25,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'—methoxy-6'-methyl-cyclohex-2'-en-4'-one) 2

$^1$H NMR (500 MHz, DMSO-$d_6$)

Figure 2. $^1$H NMR of 2 in DMSO

LC-DAD chromatogram
(25,6'R)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 3

$^1$H NMR (500 MHz, DMSO-$d_6$)

Figure 3. $^1$H NMR of 3 in DMSO, insert showing spectrum after shaking with D$_2$O.

$^{13}$C NMR (50 MHz, DMSO-$d_6$)
LC-DAD chromatogram
(25,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) S1

$^1$H NMR (300 MHz, DMSO-d$_6$)

$^{13}$C NMR (50 MHz, DMSO-d$_6$)
LC-DAD chromatogram
Figure 4. gHMBC spectrum of griseofulvin, showing that the two methoxy groups (9 and 10) only have one correlation each, which is to the corresponding ipso carbon.
(2S,6'R)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-((2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 2

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<td>2.78 (1H, ddd, $J = 13.4, 6.5$ 4.6 Hz)</td>
<td>35.3</td>
<td>2, 3', 5', 8</td>
<td>5'α, 5'β, 8</td>
</tr>
</tbody>
</table>

Table 1. $^1$H NMR as well as gHMBC and DQF-COSY data for 2 (500 MHz, DMSO-$d_6$)
(25,6'R)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-one)-2-spiro-1’-(2’-methoxy-6’-methyl-cyclohex-2'-en-4’-one) 3

gHMBC (500 MHz, DMSO-d6)

Figure 5. gHMBC of 3. By increasing the intensity, a correlation can be seen from the phenol (4) to the carbon shift 103.7, which corresponds to the 3a position, indicating the position of the phenol.

<table>
<thead>
<tr>
<th>Atom#</th>
<th>H NMR</th>
<th>C NMR</th>
<th>gHMBC</th>
<th>DQF-COSY</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>89.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>191.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>103.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>11.53 (1H, s)</td>
<td>156.8</td>
<td>3a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.26 (1H, s)</td>
<td>94.1</td>
<td>3, 3a, 4, 6, 7, 7a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>163.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>93.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td></td>
<td>168.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.81 (3H, d, J = 6.5 Hz)</td>
<td>13.8</td>
<td>2, 4’, 5’, 6’</td>
<td>6’</td>
</tr>
<tr>
<td>9</td>
<td>3.92 (3H, s)</td>
<td>56.9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3.63 (3H, s)</td>
<td>56.9</td>
<td>2’, 3’</td>
<td></td>
</tr>
<tr>
<td>2’</td>
<td></td>
<td>170.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’</td>
<td>5.60 (1H, s)</td>
<td>104.5</td>
<td>2, 2’, 4’, 5’</td>
<td></td>
</tr>
<tr>
<td>4’</td>
<td></td>
<td>195.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’a</td>
<td>2.34 (1H, dd, J = 16.3, 4.5 Hz)</td>
<td>39.4</td>
<td>2, 3’, 4’, 6’, 8</td>
<td>5’β, 6’</td>
</tr>
<tr>
<td>5’β</td>
<td>2.68 (1H, dd, J = 16.3, 13.4 Hz)</td>
<td>2, 4’, 6’, 8</td>
<td>2, 2’, 3, 5’, 8</td>
<td>5’a, 6’</td>
</tr>
<tr>
<td>6’</td>
<td>2.78 (1H, ddd, J = 13.4, 6.5 4.5 Hz)</td>
<td>35.6</td>
<td>2, 2’, 3, 5’, 8</td>
<td>5’a, 5’β, 8</td>
</tr>
</tbody>
</table>

Table 2. 1H and 13C NMR as well as gHMBC and DQF-COSY data 3 (500 MHz, DMSO-d6)
UV spectrum of 2

UV spectrum of 3
Fluorescence data

<table>
<thead>
<tr>
<th>Excitation</th>
<th>Emission (2)</th>
<th>Emission (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>215 nm</td>
<td>420 nm</td>
<td>424 nm</td>
</tr>
<tr>
<td>237 nm</td>
<td>424 nm</td>
<td>430 nm</td>
</tr>
<tr>
<td>295 nm</td>
<td>427 nm</td>
<td>434 nm</td>
</tr>
</tbody>
</table>
MS-MS spectra of 2 and 3 (collision 30 V)
MS-MS spectra of 2 and 3 (collision 45 V)
MS-MS spectra of 2 and 3 (collision 60 V)
Appendix F

Supporting Information - Chapter 6
A technique for selective ipso-substitution of ortho-, meta- or para-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

Mads H. Rønnest, Felix Nissen, Thomas Ostenfeld Larsen, Walter Mier, Mads H. Clausen

Supporting Information

S2-S10  NMR spectra of 3, 4, 5, 7, 8, 9, 10, 11 and 12.
S11    UPLC-DAD chromatograms of compounds 7, 8, 9, 10, 11 and 12.
$^1$H NMR (300 MHz, CDCl$_3$) 3

$^1$C NMR (75 MHz, CDCl$_3$) 3
$^1$H NMR (300 MHz, CDCl$_3$) 4

$^{13}$C NMR (75 MHz, CDCl$_3$) 4
$^1$H NMR (300 MHz, CDCl$_3$)

$^{13}$C NMR (75 MHz, CDCl$_3$)
$^1$H NMR (300 MHz, CDCl$_3$) 7

$^{13}$C NMR (50 MHz, CDCl$_3$) 7
$^1$H NMR (300 MHz, CDCl$_3$) 8

$^1$C NMR (50 MHz, CDCl$_3$) 8
$^1$H NMR (300 MHz, CDCl$_3$) 9

$^{13}$C NMR (50 MHz, CDCl$_3$) 9
$^1$H NMR (300 MHz, CDCl$_3$) 10

$^{13}$C NMR (50 MHz, CDCl$_3$) 10

$^1$H NMR (300 MHz, CDCl$_3$) 11
$^{13}$C NMR (50 MHz, CDCl$_3$) 11

$^1$H NMR (300 MHz, CDCl$_3$) 12
$^1$C NMR (50 MHz, CDCl$_3$) 12

UPLC-DAD 7
(+)-Geodin from Aspergillus terreus

Mads H. Rønnest, Morten T. Nielsen, Blanka Leber, Uffe H. Mortensen, Alwin Krämer, Mads H. Clausen, Thomas O. Larsen, Pernille Harris*

Supplementary data

S2 Experimental
S3-S4 1D NMR spectra of I and UPLC-DAD chromatogram
S5 References
Experimental

$^1$H NMR spectra were recorded using either a Varian Unity Inova 500 MHz or a Bruker Avance 800 MHz spectrometer. $^{13}$C NMR spectrum were recorded using a Bruker Avance 800 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz. When benzene-$d_6$ was used as solvent the residual peak was used as internal reference at 7.15 for $^1$H NMR and $\delta$ 128.0 for $^{13}$C NMR spectra. For DMSO-$d_6$ the value were $\delta$ 2.50 for $^1$H NMR. The IR spectrum was recorded using a Bruker Alpha ATR and measured in cm$^{-1}$. The melting point is uncorrected. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm formic acid) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min. The following gradient was used:

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>MeCN %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>0.5</td>
<td>25</td>
</tr>
<tr>
<td>6.0</td>
<td>65</td>
</tr>
<tr>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td>8.5</td>
<td>100</td>
</tr>
<tr>
<td>9.0</td>
<td>15</td>
</tr>
<tr>
<td>12.0</td>
<td>15</td>
</tr>
</tbody>
</table>

A. terreus (IBT 28226, culture collection at center for microbial biotechnology (Lyngby, Denmark)) was cultured on 50 plates of yeast extract sucrose agar at 298 °K for 7 days and extracted with ethyl acetate (2L) then concentrated to afford 1.2 g raw extract. The raw extract was dissolved in 10% H$_2$O in MeOH (50 mL) and the aqueous phase was extracted with heptane (50 mL). The water content was increased to 50% by adding 40 mL H$_2$O and shaken with CH$_2$Cl$_2$ (90 mL). The CH$_2$Cl$_2$-phase was concentrated (0.86 g) and further purification was performed on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 5 mL/min H$_2$O/CH$_3$CN (isocratic run at 50/50 for 15 minutes) as the mobile phase to yield 1 (11.6 mg as yellow oil). Geodin (I) was crystallized from EtOAc/heptane to afford yellow crystals.

(R)-Methyl 5,7-dichloro-4-hydroxy-6'-methoxy-6-methyl-3,4'-dioxo-spiro[benzofuran-2,1'-cyclohexa-2',5'-diene]-2'-carboxylate I (geodin)

m.p.: 527-529 °K. (in agreement with litt. (Raistrick & Smith, 1936)). IR(neat): 3396, 1724, 1659, 1610, 1461, 1440; $^1$H NMR (800 MHz, benzene-$d_6$): $\delta$ 7.04 (1H, d, $J = 1.0$ Hz), 5.43 (1H, d, $J = 1.0$ Hz), 2.91 (3H, s), 2.53 (3H, s), 2.00 (3H, s); $^{13}$C NMR (200 MHz, benzene-$d_6$): $\delta$ 194.3, 184.8, 167.8, 166.4, 163.9, 150.4, 147.1, 137.9, 137.7, 115.5, 110.0, 105.1, 85.4, 56.3, 52.5, 18.5; $^1$H NMR (800 MHz, CDCl$_3$): $\delta$ 7.14 (1H, d, $J = 1.5$ Hz), 5.82 (3H, d, $J = 1.5$ Hz), 3.74 (3H, s), 3.70 (3H, s), 2.58 (3H, s) (in agreement with litt. (Sato et al., 2005)); $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 7.05 (1H, d, $J = 1.0$ Hz), 6.05 (1H, d, $J = 1.0$ Hz), 3.72 (3H, s), 3.68 (3H, s), 2.52 (3H, s); HRMS (ESI+) calcd for M + H [C$_{17}$H$_{13}$Cl$_2$O$_7$]$^+$ 399.0038, found 399.0041. [α]$_D^{29}$ +129° (c = 1.16, CHCl$_3$) (in agreement with litt. (Raistrick & Smith, 1936)).
$^1$H NMR (800 MHz, benzene-$d_6$)

$^{13}$C NMR (200 MHz, benzene-$d_6$)
UPLC-DAD chromatogram and UV spectrum

References


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