Isolation and Structure Elucidation of Secondary Metabolites from Endophytic Fungi and the Plant *Prismatomeris tetrandra* and Synthesis of (+)-Ochromycinone

Die vom Department Chemie
der Fakultät für Naturwissenschaften
der Universität Paderborn
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
-Dr. rer. nat.-

genehmigte Dissertation

von
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Paderborn 2005

Eingereicht am: 29.06.2005
Mündliche Prüfung: 15.07.2005

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The present study was carried out from August 2001 to March 2005 in the 'Department Organische Chemie der Fakultät Naturwissenschaften der Universität Paderborn' under the supervision of Prof. Dr. Karsten Krohn.

- I am extremely grateful to Prof. Dr. Karsten Krohn, who gave me the opportunity to work with this interesting theme and also enriched it with valuable comments and suggestions. He also very kindly provided the necessary support in all phases of this study.
- I wish to express my gratitude to Prof. Dr. Bernhard Westermann, who gave his kind consent for being the second referee.
- This study involved many different specific tasks, which were performed in cooperation. For this, thanks go to Prof. Dr. H.–J. Aust and all members of his group, especially to PD Dr. B. Schulz for the study related to endophytic fungi; to Prof. Dr. Marsmann, PD Dr. H. Egold, Dr. D. Gehle, and Ms. K. Stolte for recording NMR spectra; to Mr. Jonk and Ms. M. Zukowski for mass spectral measurements; to Dr. U. Flörke for performing the single crystal X-ray analysis; to Ms. C. Neuhaus for help with chiral HPLC; to Dr. T. Kurtán who recorded and helped to prepare the discussion on CD spectra; and to Mr. J. Vetter who provided technical assistance.
- I would like to thank Prof. Dr. van Ree who went through the entire dissertation and helped to minimize the errors.
- I would also like to thank all of my colleagues of the 'Department Organische Chemie' for the many helpful suggestions, especially Dr. K. Steingröver, Dr. B. Elsässer, Dr. M. Riaz, Ms. A. L. -Risse and Mr. M. Al Sahli. I have no word to express my sincere thanks for Mr. Ishtiaq Ahmed and Dr. H. Hussain, who helped me as members of my family.
- I am grateful to Dr. J. Vitz who helped me to prepare the dissertation.
- I wish to express my gratitude to Prof. Dr. C. M. Hasan and Prof. Dr. M. A. Rashid for their many valuable suggestions and constant inspiration.
- I am extremely grateful to all of my family members, to my mother-in-law Prof. Dr. Nilufar Nahar, and also to my two sisters-in-law for their enthusiasm and prayerful concerns. Especially, I am much indebted to my parents for their encouragement and being totally supportive of me throughout my studies. Finally, I wish to express my heartiest gratitude to my wife Farhana Rob Shampa and my two sons M. M. Farhan Saleheen Hossain and M. M. Faizan Hossain for their understanding and endurance.
- Leave with pay from BCSIR is highly acknowledged.

Dedicated to my

Family

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1 Introduction

Disease causing microbes that have become resistant to drug therapy are an increasing public health problem. In spite of the availability of effective drugs and vaccines, the battle against infectious diseases is far from over. Not only do they continue to cause a large number of infections and deaths, particularly in developing countries, but the emergence and spread of antimicrobial resistance is now threatening to undermine our ability to treat infections and save lives.

Respiratory infections, HIV/AIDS, diarrhoeal diseases, tuberculosis, and malaria are the leading killers among the infectious diseases. Resistance to first-line drugs has been observed in all these diseases. In some cases, the level of resistance has forced a change to more expensive second- or third-line agents. When resistance against these drugs also emerges, the world will run out of treatment options.

Antibiotic resistance is inevitable, but there are measures we can take to slow it down. Efforts are under way on several fronts - improving infection control, developing new antibiotics, and using drugs more appropriately.

1.1 Need for new leads from natural products

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and will have a minor environmental impact, respectively. This search is driven by the development of resistance in infectious microorganisms (e.g., *Staphylococcus*, *Mycobacterium*, *Streptococcus*) to existing drugs and by the menacing presence of naturally resistant organisms.[1] The ingress into the human population of new disease-causing agents such as AIDS, Ebola, and SARS requires the discovery and development of new drugs to combat them. Not only do diseases such as AIDS require drugs that target them specifically, but new therapies are needed for treating ancillary infections which are a consequence of a weakened immune system. Furthermore, patients who are immunocompromised (e.g., cancer and organ transplant patients) are at risk of infection by opportunistic pathogens, such as *Aspergillus*, *Cryptococcus*, and *Candida*, that normally are not major problems in the human population.[1] In addition, more drugs are needed to efficiently treat parasitic protozoan and nematodal infections

such as malaria, leishmaniasis, trypanosomiasis, and filariasis. Malaria, by itself, is more effective in claiming lives each year than any other single infectious agent with the exception of AIDS and TB.[2] However, the enteric diseases claim the most lives each year of any other disease complex, and unfortunately, the victims are mostly children.[2]

Finally, because of safety and environmental problems, many synthetic agricultural agents have been and currently are being targeted for removal from the market, which creates a need to find alternative ways to control farm pests and pathogens.[3] Natural products and the organisms that make them continue to offer opportunities for innovation in drug and agrochemical discovery.

1.2 The influence of natural products upon drug discovery

Throughout the ages humans have relied on nature for their basic needs for the production of foodstuffs, shelter, clothing, means of transportation, fertilizers, flavours and fragrances, and, not least, medicines. Nature has been a source of several medicines for treating various types of diseases in humans and animals for many years.[4]

Natural products are naturally derived metabolites and/or byproducts from microorganisms, plants, or animals.[5] These products still play a major role in drug treatment either as the drug, or as a 'forebear' in the synthesis or design of the agent.[6] The world's best known and most universally used medicinal agent is aspirin, which is related to salicin, having its origins in the plant genera *Salix* sp. and *Populus* sp.[1] Examples abound of natural product use, especially in small native populations in a myriad of remote locations on Earth. For instance, certain tribal groups in the Amazon basin, the highland peoples of Papua New Guinea, and the Aborigines of Australia each have identified certain plants to provide relief of symptoms varying from head colds to massive wounds and intestinal ailments.[7]

Even with untold centuries of human experience behind us, and a movement into a modern era of chemistry and automation, it is still evident that natural product-based compounds have had an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them. Furthermore, well over 50% of the new chemical products registered by the FDA as anticancer agents, antimigraine agents, and antihypertensive agents were natural products or derivatives thereof in the time-frame of 1981-2002.[8] Excluding biologics, between 1989 and 1995, 60% of approved drugs and pre-new drug application candidates were of natural origin.[9]

From 1983 to 1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin, as were 78% of all newly approved antibacterial agents.[10] At least 21 natural product and natural product-derived drugs have been launched onto the market in the United States, Europe or Japan since 1998 and there are many natural product-derived compounds in Phase III or registration that may be launched in 2005 and 2006.[11] Many other examples abound that illustrate the value and importance of natural products from plants and microorganisms in modern civilization, and paclitaxel (**1**, Taxol) is the most recent example of an important natural product that has made an enormous impact on medicine (Figure 1).[12,13] It interacts with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and thereby interrupts the cell division.[14] The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well.[1]

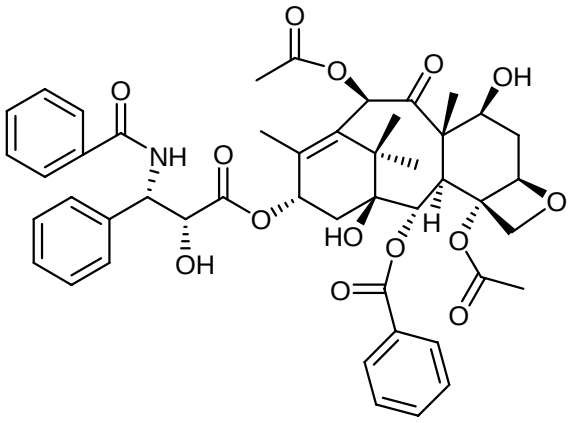

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Figure 1: Paclitaxel (**1**)

In recent years a renewed interest in obtaining biologically active compounds from natural sources has been observed, notwithstanding the impressive progress of new competing methodologies, as for example, combinatorial chemistry and high throughput screening or genetic engineering. Contributing to this world-wide attention towards formulations based on natural products, are their low or absent toxicity, their complete biodegradability, their availability from renewable sources, and, in most cases, their low cost when compared with those of compounds obtained by total chemical synthesis.[15]

1.2.1 Plants: a source of biologically active secondary metabolites

Plants have formed the basis for traditional medicine systems which have been used for thousands of years in countries such as China[16] and India.[17,18] The use of plants in the traditional medicine of many other cultures has been extensively documented.[19] These plant-based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care.[20,21] Plant products also play an important role in the health care systems of the remaining 20% of the population, mainly residing in developed countries. In a study it has been shown that at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs that are in use in one or more countries.[20] Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine.

Examples of traditional medicine providing leads to bioactive natural products abound. Suffice it to point to some recent confirmations of the wealth of this resource. Artemisinine (qinghaosu) (**2**, Figure 2) is the antimalarial sesquiterpene from a Chinese medicinal herb *Artemisia annua* (Wormwood) used in herbal remedies since ancient times.[22,23] Forskolin (**3**, Figure 2) is the antihypertensive agent from *Coleus forskohlii* Briq. (Labiatae), a plant whose use was described in ancient Hindu Ayurvedic texts.[24,25]

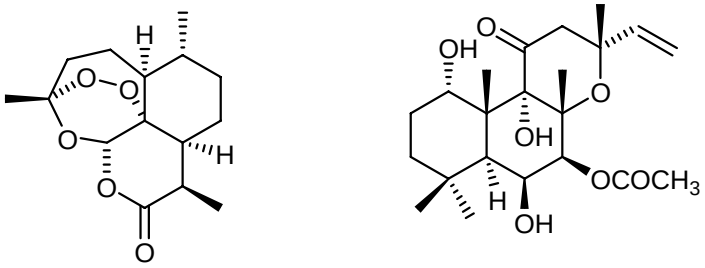
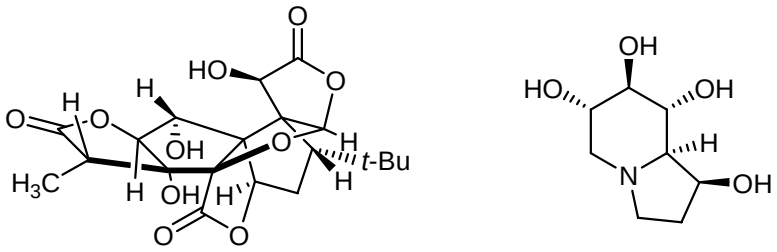


Figure 2: Artemisinin (**2**) and forskolin (**3**)

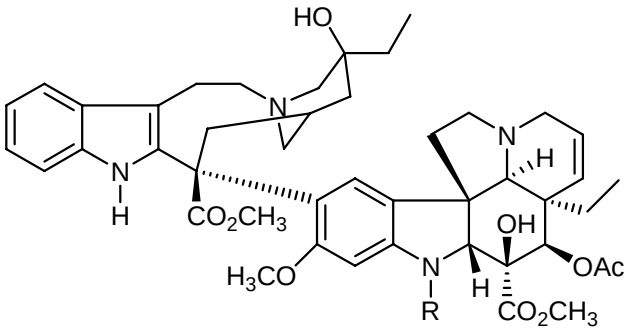
The ginkgo tree, mentioned in Chinese medicinal books from 2800 B.C. and used in antiasthmatic and antitussive preparations, produces the ginkgolides (e.g. **4**, Figure 3), unusual diterpenes containing a tertiary butyl group. Their involvement in the clinical efficacy of ginkgo tree extracts was reported in 1985.[26] Castanospermine (**5**, Figure 3), a tetrahydroxyindolizidine alkaloid isolated from the Australian plant *Castanospermum australe*, inhibits replication of the human immunodeficiency virus (HIV).[27]



4 **5**

Figure 3: A ginkgolide (**4**) and castanospermine (**5**)

A case of serendipity is the discovery of the so-called vinca alkaloids, vincristine (**6**) and vinblastine (**7**), in *Catharanthus roseus*. A random screening programme (conducted at Eli Lilly and Company) of plants with antineoplastic activity found these anticancer agents in the 40th of 200 plants examined. Ethnomedicinal information attributed an anorexigenic effect (i.e. causing anorexia) to an infusion from the plant.[26,28]



6 R = CHO
7 R = CH_3

Figure 4: Vincristine (**6**) and vinblastine (**7**)

Taxol (**1**, Figure 1), the world's first billion-dollar anticancer drug, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia*.[1,12,29]

1.2.2 Fungi: a source of biologically active secondary metabolites

Fungi are a diverse and valuable resource for the discovery of novel beneficial natural products. The chemical potential of fungi is enormous and new approaches need to be devised to efficiently access this genetic and chemical diversity for the development of new medicines. Fungi have been studied for more than 70 years due to the pharmaceutical potential of their secondary metabolites. The search for new drugs from fungi started with the discovery of penicillin, a potent antibiotic active against Gram-positive bacteria, by Fleming from *Penicillium*

notatum in 1928 and reported in the British medical literature in 1929.[30] An excellent reprint has also been issued on 'History of Penicillin Production'[31] and that publication goes into the story in detail from the aspect of large scale production of penicillin G (**8**) and penicillin V (**9**) (Figure 5).

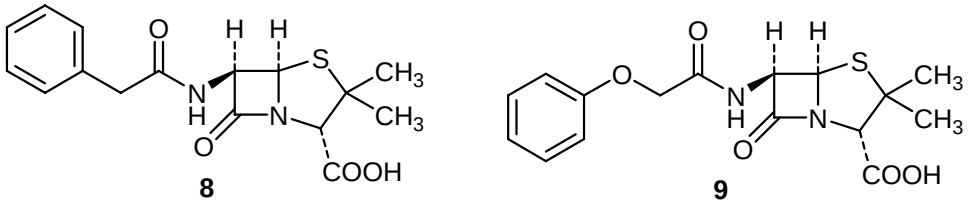
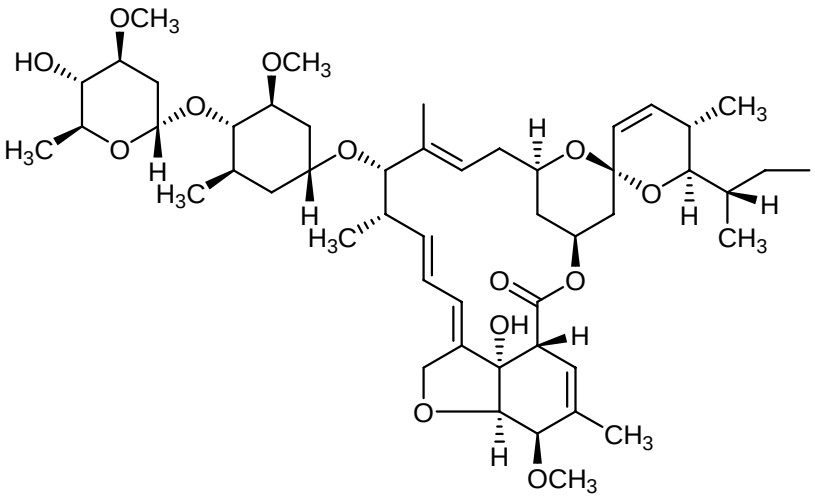


Figure 5: Penicillin G (**8**) and penicillin V (**9**)

A screen designed to find metabolites from microorganisms active against parasitic infections resulted in the detection and isolation of the avermectins, (e.g., avermectin$_{1a}$ **10**, Figure 6). The producer organism *Streptomyces avermitilis* MA-4680 is a soil isolate.[32,33]



10

Figure 6: Avermectin$_{1a}$ (**10**)

Cyclosporin A, a nonpolar cyclic undecapeptide, was isolated from the extract of a soilborne fungus *Tolyplocladium inflatum* (formerly *Trichoderma polysporum*). It was isolated in 1973 and has since become the prototype of a new generation of immunosuppressants that is used in organ transplantation surgery.[34-36] The antifungal agent 'griseofulvin' from *Penicillium griseofulvum*[37] and the cholesterol biosynthesis inhibitor 'lovastatin' from *Aspergillus terreus*[38] are two further examples of important fungal metabolites.

Considering that 6 out of 20 of the most commonly prescribed medications are of fungal origin[39] and only ~ 5% of the fungi have been described,[40,41] fungi offer an enormous potential

for new products. Most fungi studied to date have been isolated from soil and were proven to have a high creative index, i.e. new and interesting secondary metabolites could be isolated. Genera such as *Aspergillus*, *Penicillium*, *Acremonium*, *Fusarium*, all typical soil isolates, are known for their ability to synthesize diverse chemical structures. Dreyfuss, however, described a problem which is often encountered during microbiological screening of fungal isolates for their content of secondary metabolites.[42] Increasingly, known metabolites are rediscovered making screening programmes less efficient. This may be due to the use of the same well-established isolation methods for fungi. Thus, often the same fungal strains are reisolated and this could also result in the rediscovery of known compounds as the same taxa produce the same metabolites with high coincidence.[43]

According to Schulz et al.,[44] in optimizing the search for new bioactive secondary metabolites, it is relevant to consider that: (1) the secondary metabolites a fungus synthesizes may correspond with its respective ecological niche, e.g. the mycotoxins of plant pathogens;[39] and (2) that metabolic interactions may enhance the synthesis of secondary metabolites. Thus, the fungi screened should originate from biotopes from which fungi have not been previously isolated for biochemical purposes and they should have metabolic interactions with their environment. This is an example of intelligent screening and is a strategy for exploiting the untapped potential for secondary metabolites that fungi offer. Endophytic fungi are one source for intelligent screening and fulfil both criteria.

1.2.2.1 Endophytic fungi: a source of biologically active secondary metabolites

Endophytic microorganisms are to be found in virtually every plant on earth. These organisms reside in the living tissues of the host plant and do so in a variety of relationships ranging from symbiotic to pathogenic. Since the discovery of endophytes in Darnel, Germany, in 1904, various investigators have defined endophytes in different ways, usually dependent on the perspective from which the endophytes were being isolated and subsequently examined.[45] Bacon et al. give an inclusive and widely accepted definition of endophytes: “microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects”.[46] While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens.[1]

The most frequently isolated endophytes are the fungi. Dreyfuss and Chapela estimate there may be at least 1 million species of endophytic fungi alone.[47] They grow within their plant hosts without causing apparent disease symptoms[48,49] and growth in this habitat involves continual metabolic interaction between fungus and host and consequently should produce more secondary metabolites.[50] Tan and Zou pointed out that, in comparison to fungal plant pathogens and fungal soil isolates, relatively few secondary metabolites have been isolated from endophytic fungi.[45] Currently, endophytic fungi are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Of the approximately 300 000 higher plant species that exist on the earth, each individual plant, of the millions that exist here, is host to one or more endophytes.[1] It seems obvious that they are a rich and reliable source of genetic diversity and may represent previously undescribed species. Finally, novel microbes (as defined at the morphological and/or molecular levels) often have associated with them novel natural products. This fact alone helps eliminate the problems of dereplication in compound discovery.

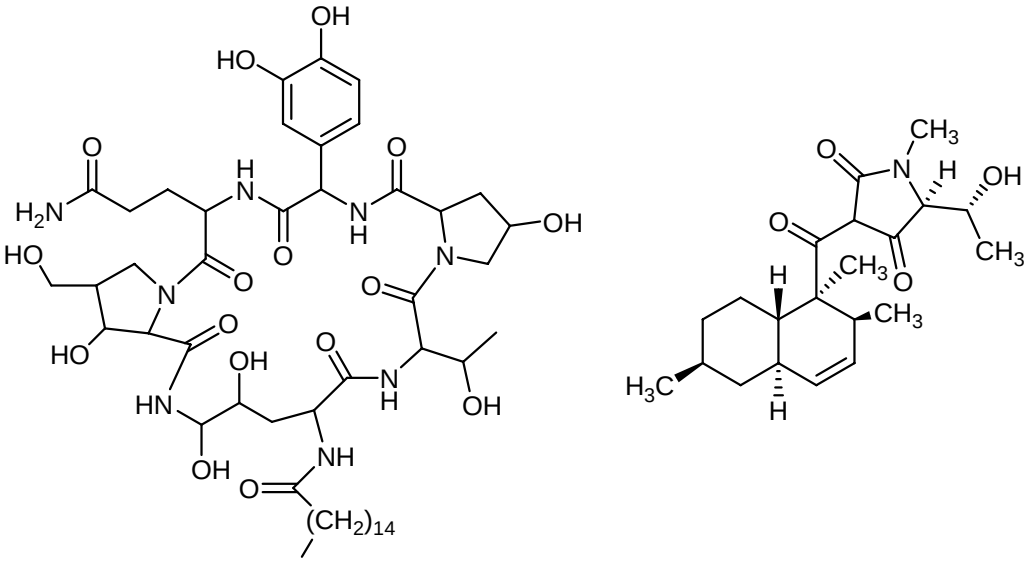
In addition, it is worthy of note that some plants generating bioactive natural products have associated endophytes that produce the same natural products. This is the case with paclitaxel (**1**, Figure 1), a highly functionalized diterpenoid and famed anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.).[29] In 1993, a novel paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized.[51] The concept, that was proposed as a mechanism to explain why *T. andreanae* may be producing paclitaxel, is that some endophytes produce certain phytochemicals, originally characteristic of the host, might be related to a genetic recombination of the endophyte with the host that occurred in evolutionary time.[45,52] Thus, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high value product may be easier and more economical to produce effectively, thereby reducing its market price.

Quite commonly, endophytes do produce secondary metabolites when placed in culture. However, the temperature, the composition of the medium, and the degree of aeration will affect the amount and kinds of compounds that are produced by an endophytic fungus.[1] Sometimes endophytic fungi produce antibiotics. Natural products from endophytic fungi have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to,

phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals.[1]

Tan and Zou recently reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological role.[45] These secondary metabolites of endophytes are synthesized *via* various metabolic pathways,[45] e.g. polyketide, isoprenoid, or amino acid derivation, and belong to diverse structural groups, i.e. steroids, xanthones, phenols, isocoumarins, perylene derivatives, quinines, furandiones, terpenoids, depsipeptides, and cytochalasines.[53-67] Some of them represent novel structural groups, for example the palmarumycins[62,63] and benzopyranone.[68] Endophytes may contribute to their host plant by producing this plethora of substances to provide protection and ultimately survival value to the plant. Ultimately, these compounds, once isolated and characterized, may also have potential for use in modern medicine, agriculture, and industry. Described below are some examples of bioactive products from endophytic fungi and their potential in the pharmaceutical and agrochemical arenas.

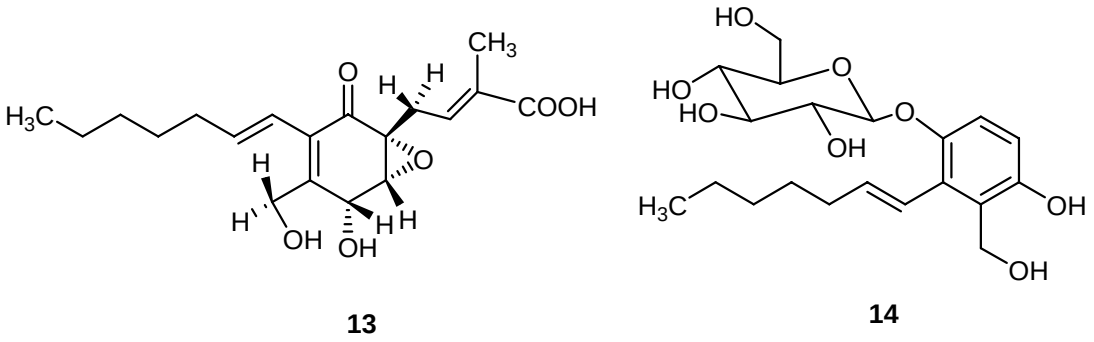
Cryptocandin A (**11**), an antifungal lipopeptide, was isolated and characterized from the endophytic fungus *Cryptosporiopsis quercina*.[69] This compound contains a number of unusual hydroxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxymethylproline (Figure 7). Cryptocandin A is active against some important human fungal pathogens including *Candida albicans* and *Trichophyton* sp. and also against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea*.[1] Cryptocin (**12**), a tetramic acid antifungal compound was also obtained from *C. quercina* (Figure 7).[70] This unusual compound possesses potent activity against *Pyricularia oryzae*, the casual organism of one of the worst plant diseases in the world, as well as a number of other plant pathogenic fungi.[70]



11 **12**

Figure 7: Cryptocandin A (**11**) and cryptocin (**12**)

Ambuic acid (**13**), a highly functionalized cyclohexenone possessing antifungal activity, produced by a number of isolates of the endophytic fungus *Pestalotiopsis microspora* found in rainforests around the world (Figure 8).[71] A strain of *P. microspora*, isolated from the tree *Torreya taxifolia*, produces several compounds having antifungal activity including the glucosylated aromatic compound pestaloside (**14**) (Figure 8).[72]



13 **14**

Figure 8: Ambuic acid (**13**) and pestaloside (**14**)

Pestalotiopsis jesteri is a newly described endophytic fungal species from the Septik river area of Papua New Guinea, and it produces the highly functionalized cyclohexenone epoxides jesterone (**15**, Figure 9) and hydroxyjesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi.[73] Torreyanic acid (**16**), a selectively cytotoxic quinone dimmer and potential anticancer agent, was isolated from a *P. microspora* strain (Figure 9).[74]

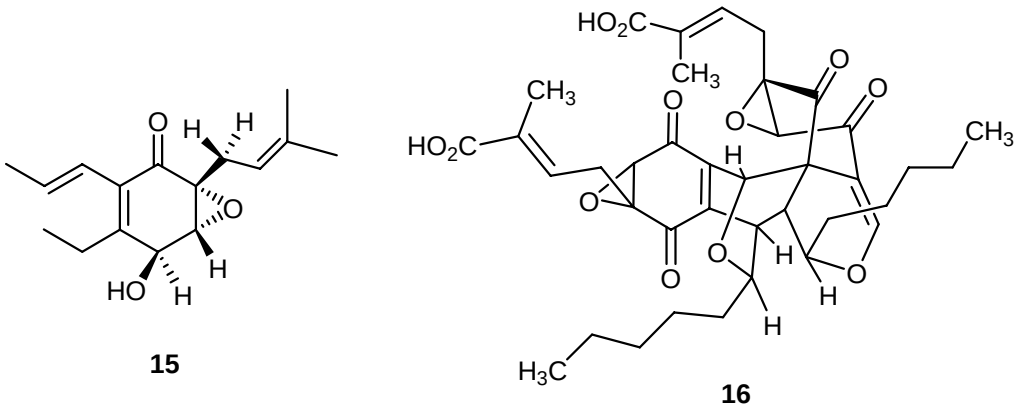
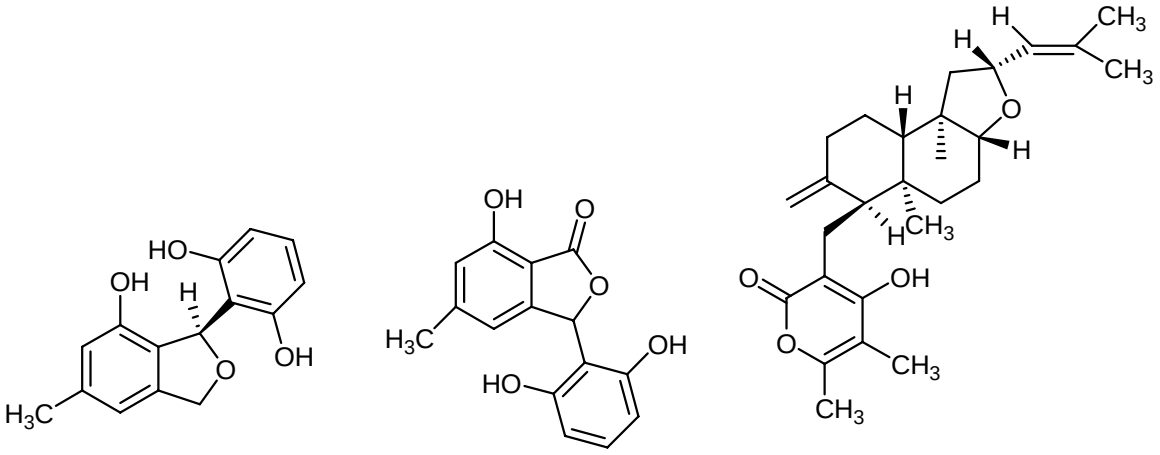


Figure 9: Jesterone (**15**) and torreyanic acid (**16**)

The distribution of the endophytic fungi making paclitaxel (**1**) is worldwide and is not confined to endophytes of yew (*Taxus*) species. The ecological and physiological explanation for the wide distribution of fungi making paclitaxel seems to be related to the fact that paclitaxel is a fungicide, and the most sensitive organisms to it are plant pathogens such as *Pythium* sp. and *Phytophthora* sp.[75] These pythiaceous organisms are among the world's most important plant pathogens and are strong competitors with endophytic fungi for niches within plants.

Pestacin (**17**) and isopestacin (**18**) (Figure 10) have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous tree *Terminalia morobensis*.[76,77] Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. The endophytic fungus *Fusarium subglutinans* produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A (**19**, Figure 10) and B.[78]

The above discussed novel antibiotics, antimycotics, immunosuppressants, and anticancer compounds are only a few examples of what has been found after the isolation and culturing of individual endophytic fungus followed by purification and characterization of some of their natural products. The prospects of finding new drugs that may be effective candidates for treating newly developing diseases in humans, plants, and animals are great. Other applications in industry and agriculture may also be discovered among the novel products produced by endophytes.



17 **18** **19**

Figure 10: Pestacin (**17**), isopestacin (**18**), and subglutinol A (**19**)

1.3 Antibiotics

The word antibiotic has come to refer to a metabolic product of one organism that is detrimental or inhibitory to other microorganisms in very small amounts. Stated differently, an antibiotic is a chemical produced by one microorganism which is inhibitory to other microorganisms.[79] Chemical compounds which have antimicrobial activity, are nontoxic, and are compatible with the host are of great value for the control of microbial infections.

Several thousand antibiotic substances have been isolated and identified since 1940. Not all antibiotics qualify as chemotherapeutic agents; in vitro antimicrobial activity does not guarantee in vivo action. Furthermore, for an antibiotic to be useful for chemotherapy, it must be selective in its toxicity, that is, inhibitory to microorganisms and non-toxic to the host. In practice, this is expressed in terms of therapeutic index – the ratio of the toxic dose to the therapeutic dose. The larger the index, the better is its therapeutic value.[80] Till now, only a small number of antibiotics have proved useful for the treatment of disease. But the few effective substances have radically changed the entire medical practice of treating infectious diseases.

An ideal antibiotic chemotherapeutic agent would have the following qualities:[79,81]

1. It should have the ability to destroy or inhibit specific pathogenic microorganisms. The larger the number of different species affected the better.
2. It should not cause the development of resistant forms of the parasites.
3. It should not produce undesirable side effects in the host, such as allergic reactions, nerve damage, or irritation of the kidneys and gastrointestinal tract.

4. It should not eliminate the normal microbial flora of the host because of the following reasons:
(a) To disturb the normal flora may upset the "balance of nature," permitting normally non-pathogenic microbes or pathogenic forms restrained by the normal flora to establish a new infection. Prolonged use of the broad-spectrum antibiotics, for example, may eliminate the normal bacterial flora but not *Monilia* (fungi) from the intestinal tract. Under these conditions, the *Monilia* may establish an infection. Lactobacilli in the vagina produce acids that protect the vagina from infection by gonococci, the bacteria that cause gonorrhea. Many strains of *Escherichia coli* in the intestines produce colicins, which may protect the intestinal tract from pathogenic intestinal bacteria.
(b) Some of the normal flora are scavengers, using waste material. Many bacteria in the intestine do this.
(c) Some of them play an important role in nutrition of the host. Many intestinal bacteria can synthesize the major B vitamins and vitamins E and K. Vitamins so produced make a significant contribution to the vitamin requirements of the host.
5. It should have a high level of solubility in body fluids.
6. It should be possible to achieve concentrations of the antibiotic in the tissues or blood, which are sufficiently high to inhibit or kill the infectious agent.

One of the major problems associated with the widespread use of the chemotherapeutic agents has been the development of resistance to these drugs by microorganisms. This phenomenon was discovered by Ehrlich with protozoa.[82] Antibiotics once effective for treatment of certain diseases have lost their value for chemotherapy as resistant microbial populations develop. Development of drug resistance is just one example of nature's never-ending processes whereby organisms develop a tolerance for new environmental conditions. The term drug resistance ordinarily refers not to the natural resistance of a species but to acquired genotypic changes, which persist during cultivation in the absence of the drug. The change may be brought about either by mutation, which alters a cell constituent, or by infection by a plasmid, which brings in genes for new enzymes.[83] Penicillin resistance in an organism, for example, may result from the production of penicillinase, an enzyme which inactivates penicillin.[79] On the other hand, some normally susceptible strains of bacteria may acquire resistance to penicillin. Acquired resistance is also due to penicillinase production by the genetically adapted strains of microorganism.[79] Many organisms which do not produce penicillinase are also resistant to penicillin. This suggests that they possess alternative metabolic pathways or enzyme reactions not susceptible to inhibition by penicillin.[79]

Figure 11 represents a typical pattern of drug resistance. In the right hand close-up image (marked by an arrow on the whole plate) some individual mutant cells in the bacterial population were resistant to the antibiotic and have given rise to small colonies in the zone of inhibition.

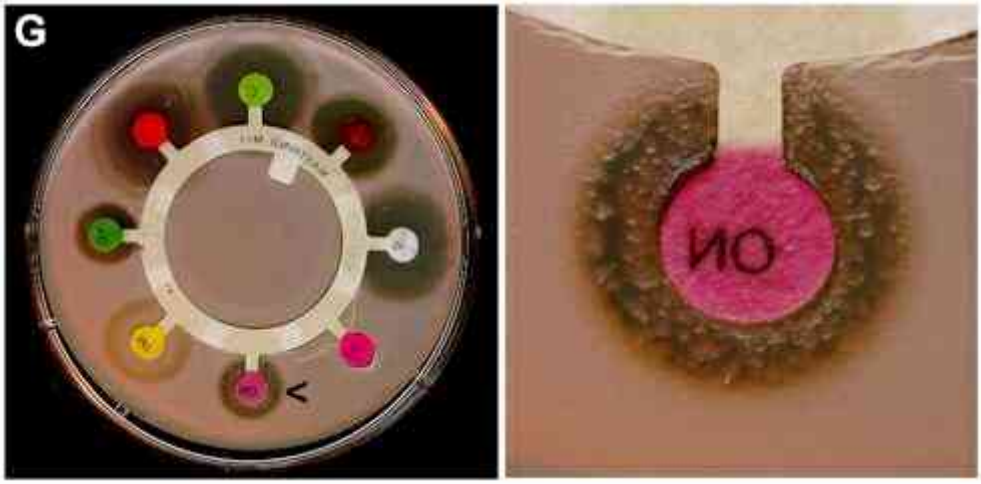


Figure 11: Effects of different antibiotics on growth of a bacterial strain

Antibiotic resistance represents a serious problem for clinicians. The development of resistance can be minimized by[79] (1) avoiding the indiscriminate use of antibiotics where they are of no real clinical value, (2) refraining from the use of antibiotics commonly employed for generalized infections or topical applications, (3) using correct dosages of the right antibiotic to overcome an infection quickly, (4) using combinations of antibiotics of proven effectiveness, and (5) using a different antibiotic when an organism gives evidence of becoming resistant to the one used initially. Furthermore, it points up the continual need for development of new and different drugs to replace those that become ineffective.

1.3.1 Angucycline antibiotics

The angucycline group of antibiotics (reviews[84,85]) are microbial quinone natural products bearing a characteristic four-ring frame of the aglycone moiety, which is assembled in an angular manner. The classification of the angucyclines is related to the tetracyclic benz[*a*]anthracene system (Figure 12) and its derived compounds.

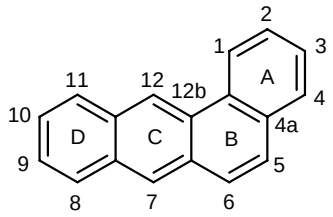


Figure 12: The tetracyclic benz[*a*]anthracene frame

The terms ‘angucycline’ and ‘angucyclinone’ were introduced in 1984.[86] The term angucyclinone includes every natural product consisting of (or derived from) an angular tetracyclic (benz[*a*]anthracene, Figure 12) structural moiety which is biosynthetically derived from a decaketide chain formed via the polyketide biosynthetic pathway.[84,87] The term ‘angucycline’ includes those with hydrolizable sugar moieties, while ‘angucyclinone’ refers to a sugarless compound or a compound with a *C*-glycosidic linked sugar moiety.

Angucyclines have become a rapidly growing group of bioactive natural products and were discovered more or less randomly by diverse screening methods as antibacterials, antitumour, and chemical screens.[84] Angucyclines show a multitude of interesting biological activities such as anticancer, antibacterial, antiviral, enzyme inhibitory, and platelet aggregation inhibitory properties.[84,85] Tetrangomycin (**20**) and tetrangulol (**21**) (Figure 13), the first members of this class of antibiotics, were published by Dann et al. in 1965,[88] and by Kunstmann and Mitscher in 1966,[89] respectively. The structure of ochromycinone (**22**)[90] was described in 1967, followed by aquamycin (**23**)[91,92] (first described without structure in 1968)[91] and rabelomycin (**24**),[93] both in 1970 (Figure 13). Currently, new members of the angucycline group are found by more sophisticated specific screening methods. In the course of screening for anti-*Helicobacter pylori* agents, Taniguchi et al.[94] have isolated a novel antibiotic YM-181741 (**25**) (Figure 13), bearing a hydroxyl group at the C-3 methyl group of the known angucycline antibiotic ochromycinone (**22**),[90] from the culture broth of *Streptomyces* sp. Q57219. The authors also reported that ochromycinone (**22**) and YM-181741 (**25**) showed selective activity against *H. pylori*, the major cause of peptic ulcer,[95-99] with a minimum inhibitory concentration (MIC) value of 0.1 µg/mL and 0.2 µg/mL, respectively.[94]

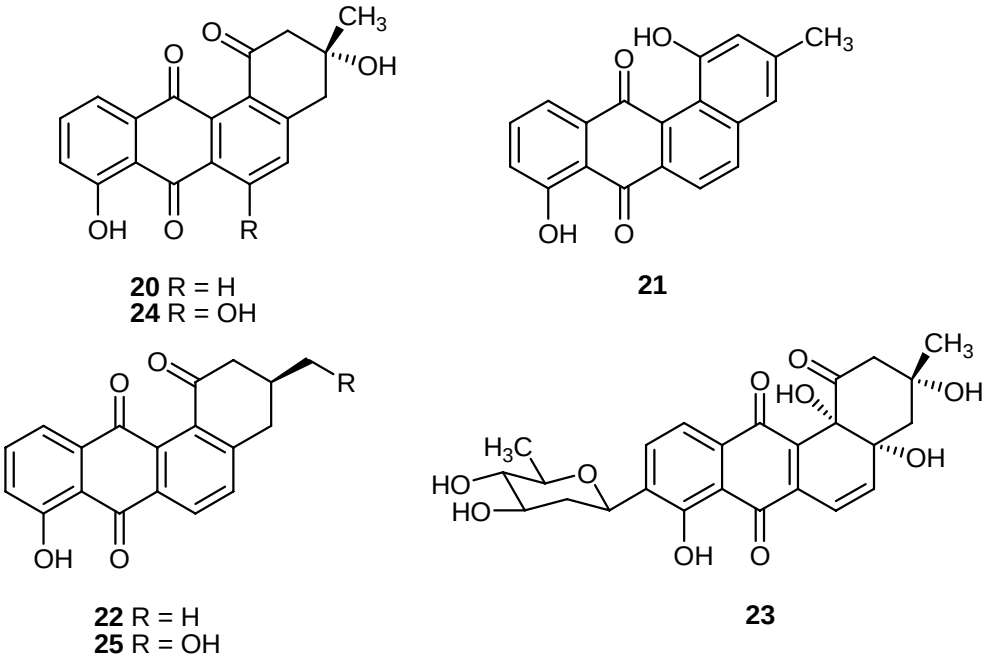


Figure 13: A few representative examples of angucyclinones. Tetrangomycin (**20**), tetrangulol (**21**), ochromycinone (**22**), aquamycin (**23**), rabelomycin (**24**), and YM-181741 (**25**)

1.3.1.1 Classification of the angucycline group of antibiotics

Rohr and Thiericke classified the angucycline group of antibiotics based on the degree of oxygenation and *C*-glycoside formation. An overview of the biosynthetically oriented classification system used for the classification of the angucycline group of antibiotics is summarized in Figure 14.[84] Based on the *C*-glycoside formation the 'classical' angucyclines/angucyclinones were divided into two major types. Both were further subdivided into two types based on the degree of oxygenation.

1.3.1.2 Biosynthesis

Taxonomic classification of the producing organisms of angucyclin(on)es exclusively belong to the group of *Actinomycetes* – Gram-positive, mycelial, sporulating bacteria. Except some exceptions (e.g. sakomycins A – D being produced by the actinomycete *Nocardia* sp.), the majority of these organisms were classified to be *Streptomycetes* of various species.[84]

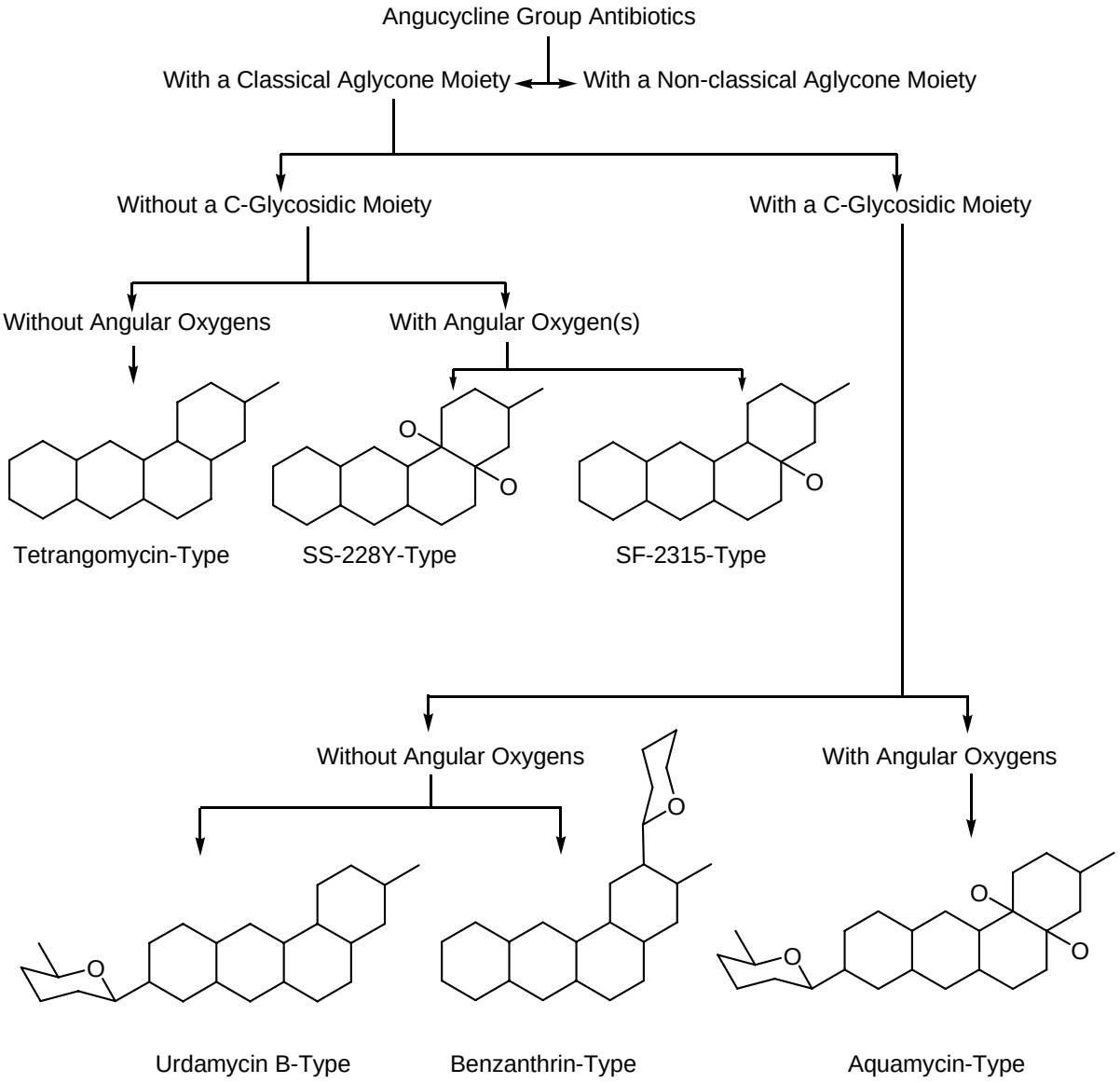
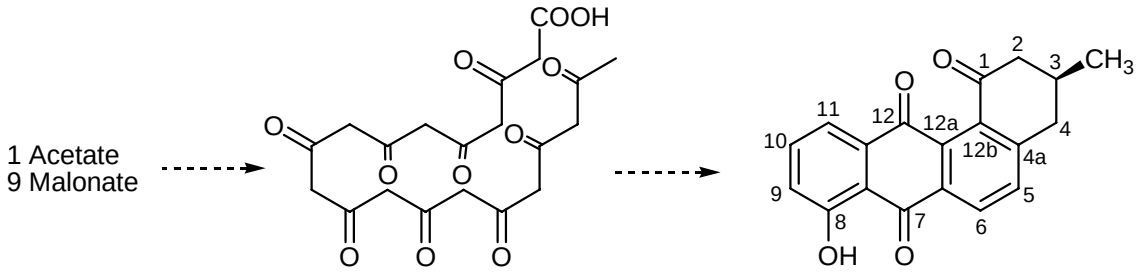


Figure 14: Biogeneric classification of the angucycline group antibiotics[84]

Angucyclines and their most closely related metabolites (anthracyclines and tetracyclines) are biogenetically categorized as aromatic polyketides, i.e., those whose biosynthesis is catalyzed by the polyketide synthases (PKS), namely a type-II PKS.[100-104] This enzyme complex catalyzes the Claisen condensations of the monomeric ketide precursors as well as the folding and ring cyclizations (Aldol reactions) into the final multicyclic form.[101] The biosynthesis of angucyclin(on)es is presumed to occur via a hypothetical decaketide (Scheme 1), which is built up by a starter acetyl-CoA and elongated by nine malonyl-CoA units.[84,85] Exceptions were found for brasiliquinones A-C, which exhibit propionate starter units.[105]



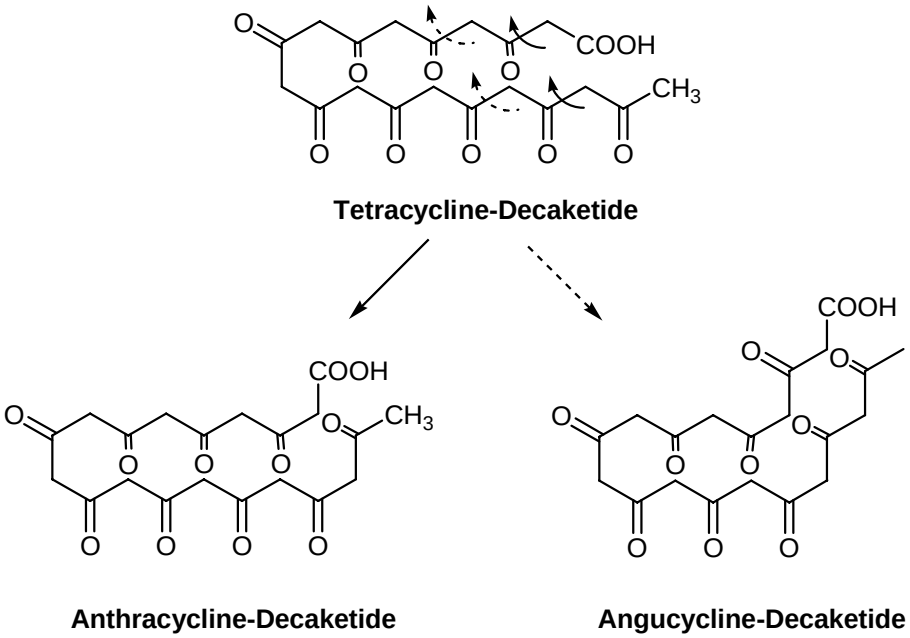
Hypothetical decaketide **22**

Scheme 1: Typical biosynthetic formation of an angucyclinone, e.g. ochromycinone (**22**), from hypothetical decaketide

First biosynthetic studies of angucycline group antibiotics were carried out with the vineomycin family and proved the origin of the entire tetracyclic ring system of vineomycinone A_1 as well as the carbon skeleton of vineomycinone B_2 from a single decaketide chain.[106] In addition, the direction of the acetate derived polyketide chain, which is not obvious due to decarboxylation of the final carboxyl group, was shown to start from 3-CH_3 and to end at C-2.[84] This kind of biosynthetic pattern was also found for several other angucyclin(on)es as dehydrorabelomycin, elmycin D, emycin A, landomycin A, ochromycinone (**22**), and PD 116740.[84,85] Studies on the biogenetic origin of the oxygen atoms of aquamycin (**23**), dehydrorabelomycin, elmycin D, emycin A, and ochromycinone (**22**) showed that all oxygens attached to a former C-1 of an acetate unit deriving from acetate. All other oxygens (attached to a former C-2 of an acetate unit) originate from molecular oxygen.[107-110] In the case of aquamycin (**23**), the oxygens at C-1, C-3, C-4a, C-7, and C-8 derive from acetate and the oxygens at C-12 and C-12b derive from molecular oxygen by monooxygenase.[109] The oxygen at C-12 may be introduced via a monooxygenase during an earlier stage of the biosynthesis. Exceptions were found for landomycin A[102] and PD 116740[87,111] indicating unexpected, prearomatic deoxygenation steps. The oxygens linked at the 6-positions of PD 116740 and landomycin A are assumed to derive from water and molecular oxygen, respectively.

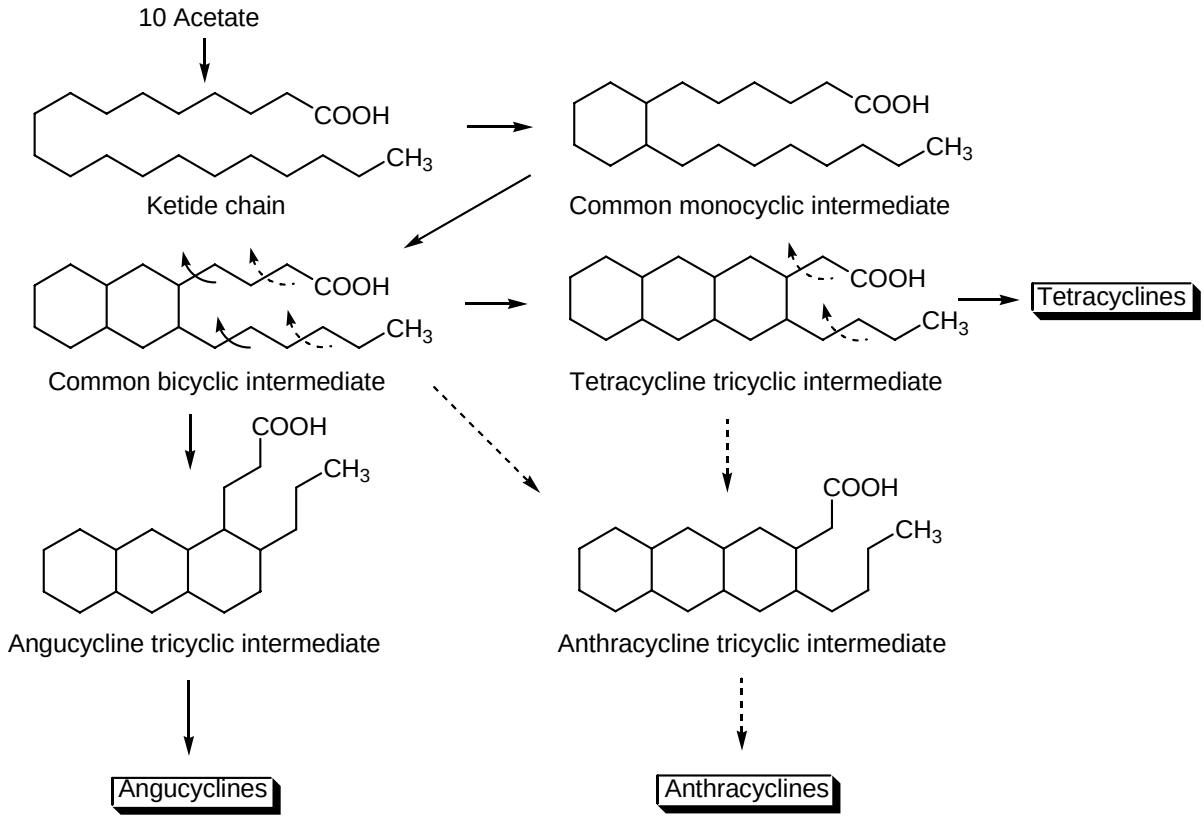
Rohr has presented a folding analysis of polyketides in terms of sequential (*E*) or (*Z*)-enolates.[101] The folding code method, however, is limited to aromatic, multicyclic polyketides, i.e., to those whose biosynthesis is catalyzed by type-II PKS, and which thus derive from an (at least nearly) unreduced polyketide chain. Depending on the differences of the folding codes of decaketides a relationship between the tetracyclines, the anthracyclines, and the angucyclines can be proposed, because there are only two or four differences between any one of the folding

codes, or in other words: only two isomerizations are necessary to change the tetracycline polyketide into the anthracycline and angucycline type, and four to interchange the anthracycline decaketide into the angucycline type (Scheme 2). So, the PKS of this antibiotics group catalyzes the biosynthesis in a similar way.



Scheme 2: Interchange of the decaketides of tetracyclines, anthracyclines, and angucyclines by flipping of all bonds with different folding

Thus, a biosynthesis hypothesis[101] can be proposed, in which tetracyclines, anthracyclines, and angucyclines are biosynthesized in a common pathway up to a bicyclic intermediate, from which the angucyclines branch off (Scheme 3).



Scheme 3: Hypothetical biosynthetic pathway of the tetracyclines, anthracyclines, and angucyclines: successive cyclizations and common mono- and bicyclic intermediates

1.4 Peptic ulcers and *Helicobacter pylori*

The findings that both ochromycinone (**22**) and YM-181741 (**25**) are active against *H. pylori*, the bacterium implicated in peptic ulcers, stimulated our interest to develop a short and efficient total synthesis of enantiomerically pure (+)-ochromycinone. Ulcers are small, open craters or sores that develop in the mucosal lining of the stomach or the duodenum, the first section of the small intestine (Figure 15) and are accompanied by inflammatory responses in the underlying tissues. These are known as stomach and duodenal ulcers and collectively known as peptic ulcers.

The current 'revolution' in ulcer cause and treatment was first noted by Dr. Bary Marshal, who discovered the presence of a small bacterium, under the microscope, attached to the lining of stomach wall samples taken from patients' ulcers.[112] This bacterium is called *Helicobacter pylori*.

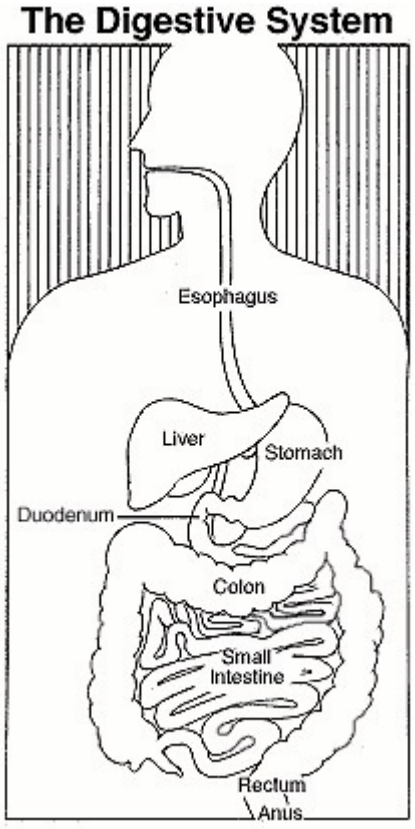


Figure 15: The gastrointestinal tract

H. pylori is a microaerophilic, Gram-negative, spiral-shaped, and flagellated bacterium, which is found only on gastric mucosa.[94] Many epidemiologic studies have shown that this is probably one of the most common bacterial infections throughout the world involving 30% of the population living in developed countries and up to 80 - 90% of the population in developing regions.[113] For example, in Korea, the prevalence of *H. pylori* infection is about 60 - 70% in adults.[114] Many recent studies have shown that peptic ulcer diseases are mainly caused by *H. pylori* infection.[95-99] In Caucasians, about 52% of those with duodenal ulcer and 53% of those with stomach ulcer, respectively, had *H. pylori* infection.[97] In Japan, however, about 97% of duodenal ulcers and 95% of stomach ulcers are positive for *H. pylori* infection.[98,99] *H. pylori* infection is closely correlated not only with peptic ulcers but also with atrophic gastritis, gastric carcinoma, and lymphoma.[115-117]

Eradication of this bacterium heals most ulcers, prevents ulcer relapse, and reduces complications.[94,96,117-119] In 1994 in the United States, the National Institute of Health (NIH) consensus development conference recommended that all patients with documented peptic ulcer who are infected with *H. pylori* should receive antimicrobial therapy to eradicate infection.[120] Some recent studies are consistent with this recommendation of *H. pylori* eradication therapy.

Asaka et al.[119] reported that twelve months after treatment for *H. pylori* eradication, gastric ulcers had recurred in 11.4% of those with successful *H. pylori* eradication and in 64.5% of those with unsuccessful *H. pylori* eradication. Duodenal ulcers had recurred in 6.8% of patients for whom *H. pylori* eradication was successful and in 83.5% of patients in whom eradication failed. Histopathological findings were also improved with regard to inflammation and activity in patients with successful eradication than in those with unsuccessful eradication. In another study,[96] ulcer relapses were absent for up to 9.8 years in the 141 *H. pylori*-eradicated patients with duodenal ulcers and in the 45 *H. pylori*-eradicated patients with gastric ulcers. These findings proved that *H. pylori* eradication significantly reduced ulcer recurrence and also complications associated with the infection and strongly supports the causal role of *H. pylori* in ulcer formation.

Treatment regimens for *H. pylori* eradication, such as the classical triple therapies, which include a proton pump inhibitor and two antimicrobial agents (amoxicillin (**26**), clarithromycin (**27**), or metronidazole (**28**), Figure 16), are currently the most popular first-line treatment.[114,118,121-123] However, these therapies have associated problems including build-up of drug resistance, side effects (e.g. diarrhoea), and poor compliance.

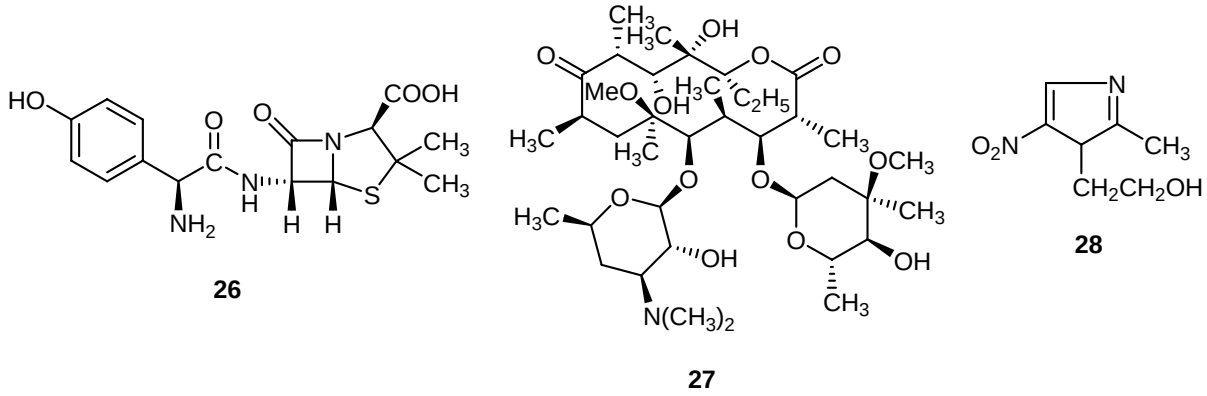


Figure 16: Amoxicillin (**26**), clarithromycin (**27**), and metronidazole (**28**)

Recently, antimicrobial resistance has become a growing problem in *H. pylori* treatment because it is an important cause of eradication failure of *H. pylori* infection.[124] The prevalence of *H. pylori* resistance to metronidazole ranges from about 20% to more than 50% in the United States,[124,125] from 10% to 50% in Europe,[126] and from 26.8% to 49.4% in East Asia.[127,128] The prevalence of *H. pylori* resistance to clarithromycin varies from 7% to 14% in the United States,[124,125] and from 0% to 15% in Europe,[126] and it is around 10% in East Asia.[127,128] In Korea, the resistance rates for amoxicillin, metronidazole, and clarithromycin are reported as 8.5%, 46.8 to 95%, and 3 to 24.3%, respectively.[118] Recently, Eun et al.[114] reported that in

Korea the prevalence of clarithromycin-resistant *H. pylori* strains had increased over the 5-year period and there is an increasing tendency for the emergence of strains with dual resistance to metronidazole and clarithromycin. Smith et al.[123] carried out an antibiotic susceptibility test on the *H. pylori* strains isolated from 56 patients presenting with peptic ulcer and gastritis in Nigeria to find out the reasons for observed treatment failures. The study showed that all the isolates were resistant to amoxicillin (**26**), ampicillin, piperacillin, erythromycin, tetracycline, and metronidazole (**28**), and two of these drugs (amoxicillin and metronidazole) are used for the treatment of *H. pylori* infection in Nigeria.

Therapies with these existing antibiotics are also associated with the high frequency of side-effects. Both amoxicillin (**26**) and clarithromycin (**27**) are broad-spectrum antibiotics and active against a variety of microorganisms and therefore cause diarrhoea as a side-effect by the disturbance of intestinal microbial flora.[94,118,122,129] Besides, allergic skin reaction to amoxicillin,[118,122] increased epigastric pain,[118] nausea,[129] and taste disturbance,[118,129,130] were also reported. A few patients showed increases in alkaline phosphatase levels and decreases in white blood cell counts and uric acid levels.[118]

Although this pathogen can be eradicated by a combination of two or three antibiotics and/or antisecretory agents, many strains resistant to a critical component of the combination therapy have emerged. Some strains with dual resistance are difficult to eradicate. In the future, dual resistant strains might become a major problem in *H. pylori* eradication. Moreover, due to some frequent side-effects associated with the present therapies there is a continuing need for more efficient therapeutic approaches.

1.4.1 Ochromycinone (22) and YM-181741 (25): two selective anti-*H. pylori* agents

It was already mentioned that ochromycinone (**22**) and YM-181741 (**25**), showed selective activity against *H. pylori* with a MIC value of 0.1 µg/mL and 0.2 µg/mL, respectively (Table 1).[94] Both the angucyclinones (**22** and **25**) were inactive against other Gram-positive and Gram-negative bacteria that were tested, in contrast to amoxicillin (**26**) and clarithromycin (**27**), which are active against a variety of microorganisms and therefore cause diarrhoea, the most frequent side-effects associated with the therapy for *H. pylori* infection. These results suggest that both the angucyclinones are selective anti-*H. pylori* agents with a low potential for causing diarrhoea by the disturbance of the intestinal microbial flora.

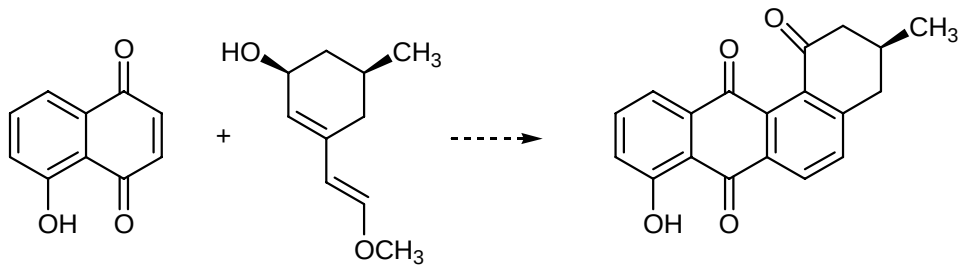
Table 1: Antimicrobial activities of ochromycinone (**22**), YM-181741 (**25**), amoxicillin (**26**), and clarithromycin (**27**)[94]

Test organism	MIC (μg/mL)			
	22	**25**	**26**	**27**
Helicobacter pylori ATCC 43504	0.1	0.2	0.025	0.013
Staphylococcus aureus FDA209P JC-1	>12.5	>12.5	0.39	0.2
Bacillus subtilis ATCC 6633	>12.5	12.5	0.2	0.2
Peptostreptococcus productus CAYA 12-2	>12.5	>12.5	0.05	0.025
Bifidobacterium bifidum CAYA 21-1	>12.5	>12.5	0.39	0.1
Clostridium perfringes CAYA 39-1	>12.5	>12.5	0.1	0.78
Escherichia coli O-1	>12.5	>12.5	1.56	25
Klebsiella pneumoniae ATCC 10031	>12.5	>12.5	50	6.25
Pseudomonas aeruginosa ATCC 10490	>12.5	>12.5	>100	>100
Bacteroides fragilis GAI 5562	12.5	12.5	12.5	0.78

Because of the problems encountered, such as resistant strains, side effects etc. with the present therapies (Section 1.4 and 1.4.1), new antibiotics with selective activity against *H. pylori* are urgently needed. According to Taniguchi et al.[94] both ochromycinone (**22**) and YM-181741 (**25**) showed selective activity against *H. pylori*, whereas ochromycinone (**22**) is twice as active as YM-181741 (**25**) against *H. pylori*. This finding stimulated our interest to develop a short and efficient total synthesis of enantiomerically pure (+)-ochromycinone.

1.5 Known syntheses of ochromycinone (22)

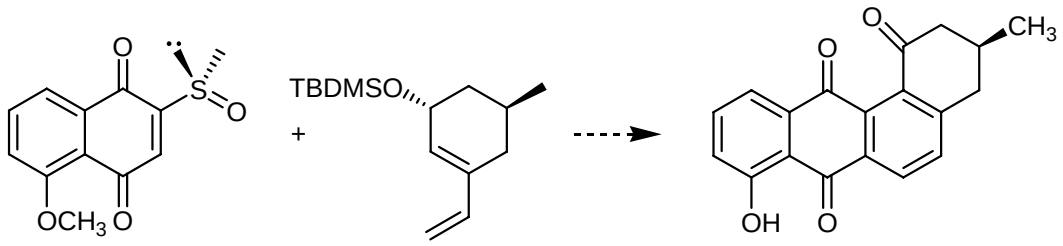
Several syntheses of racemic ochromycinone are known. With the exception of the work of Katsuura and Snieckus,[131,132] who used an aromatic directed metallation strategy, Diels-Alder reaction with juglone derivatives and appropriate dienes was mostly employed to construct the tetrahydrobenz[*a*]anthraquinone skeleton.[87,133,134] Larsen et al.[135] achieved a kinetic resolution with respect to a racemic diene **30** in the Diels-Alder reaction promoted by a chiral Lewis acid prepared from BH_3 in the presence of (*S*)-3-3'-diphenyl-1,1'-binaphthalene-2,2'-diol and juglone (**29**) in their asymmetric synthesis of (+)-ochromycinone (**22**) (Scheme 4).



29 **30** **22**

Scheme 4: Synthesis of (+)-ochromycinone (**22**)[135]

In the work of Carreño et al.,[136] an enantiomerically pure sulfinylquinone dienophile (**31**) was the source of asymmetry to effect a kinetic resolution of a racemic diene (**32**) in their synthesis of enantiomerically enriched (+)-ochromycinone (**22**) (Scheme 5).

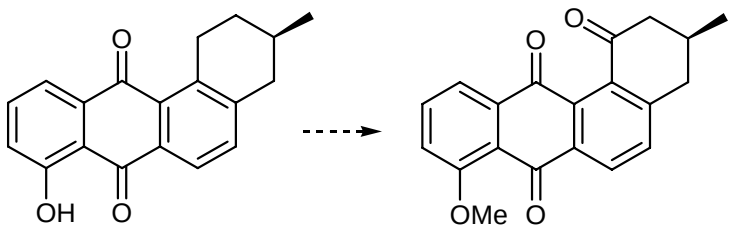


31 **32** **22**

Scheme 5: Synthesis of (+)-ochromycinone (**22**)[136]

Very recently, the related (+)-rubiginone (**34**, *O*-methyl ether of **22**) was prepared via an intramolecular [2+2+2] cycloaddition of a chiral triyne precursor, derived from (+)-citronellal.[137] (+)-Rubiginone (**34**) was also prepared via a BF_3-mediated Diels-Alder reaction of juglone (**29**) and (*R*)-3-methyl-1-vinylcyclohexene, followed by aromatization gave the anthraquinone (**33**), which was converted into **34** after a two-step operation (Scheme 6).[138]

The rubiginones have been shown to potentiate the cytotoxicity of vincristine against vincristine-resistant P388 leukemia and Moser cells.[138]



33 **34**

Scheme 6: Synthesis of (+)-rubiginone (**34**)[138]

1.6 Present study: Aim and Scope

Natural products traditionally have played an important role in drug discovery and were the basis of most early medicines. Over the last few years advances in alternative drug discovery methods such as rational drug design and combinatorial chemistry have placed great pressure upon natural product drug discovery programs and during this period most major pharmaceutical companies have terminated or considerably scaled down their natural product operations. However, despite the promise of these alternative drug discovery methods, there is still a shortage of lead compounds progressing into clinical trials. This is especially the case in therapeutic areas such as oncology, immunosuppression, and metabolic diseases where natural products have played a central role in lead discovery.[11]

Endophytic fungi are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. Although work on the utilization of this vast resource of poorly understood microorganisms has just begun, it has already become obvious that an enormous potential for organism, product, and utilitarian discovery in this field holds exciting promise. This is evidenced by the discovery of a wide range of products and microorganisms that present potential as mentioned in Section 1.2.2.1 and consequently, we were motivated to investigate the secondary metabolism of endophytic fungi with an aim to find the fungal strains able to produce structurally novel and biologically active secondary metabolites.

Many studies have been concerned with ochromycinone from biological and synthetic viewpoints and several reports have described its synthesis. However, there still remains room for further improvement on behalf of a short and economical access to this compound. In

addition, its selective activity against *H. pylori* stimulated our interest to develop a short and efficient total synthesis of enantiomerically pure (+)-ochromycinone.

The present study could be divided into the following two parts:

1. Isolation, characterization, and structure elucidation of secondary metabolites from endophytic fungi and a plant.

2. Enantiospecific synthesis of (+)-ochromycinone.

The study related to the endophytic fungi was performed in cooperation with the research group of Prof. Dr. H.–J. Aust, Institut für Mikrobiologie, TU Braunschweig and BASF AG, Ludwigshafen. Collection and cultivation of the fungal strains as well as the first tests on the crude extracts and pure compounds were performed in the research group of Prof. Dr. Aust under the supervision of PD Dr. Barbara Schulz. The taxonomic identification of the fungal strains was done by Dr. S. Draeger. Bioactivities of some pure compounds were also performed in BASF AG.

2 Results and discussion: metabolites from fungi

2.1 Strain 5681

Strain 5681 was isolated from a leaf of *Salix* species growing in the Harz Mountains in Lower Saxony, Germany. This fungal strain was identified as an endophytic fungus of *Scytalidium* sp.

2.1.1 Isolation of secondary metabolites

Strain 5681 was cultivated at room temperature for 111 days in two different culture media, namely, malt-soya and biomalt semi-solid agar media. The two culture media were then extracted separately, once with petroleum ether and three times with ethyl acetate to obtain the crude extracts. The crude ethyl acetate extracts (E), both from malt-soya and biomalt semi-solid agar culture media, showed inhibitory activities in the agar diffusion assay against *Escherichia coli* (Ec, only biomalt), *Bacillus megaterium* (Bm), *Microbotryum violaceum* (Mv), *Eurotium repens* (Eur), *Mycotypha microspora* (Mm), *Fusarium oxysporum* (Fu) and *Chlorella fusca* (Chl) (Table 2).

Table 2: Agar diffusion assay of crude extracts

Medium	Fraction	Ec	Bm	Mv	Eur	Mm	Fu	*Chl*
Bio	PE	0	0	0	0	0	0	0
	E	0.1-0.3	0.1	0.7	0.4	0.4	0.1	0.5
M/S	PE	0	0	0	0	0	0	0
	E	0	0.2	0.4	0.1	0.2	0.1	0.4

Concentration: 50 µL of the given 40 mg/mL solution per plate; Numbers indicate the radius of zone of inhibition in cm.

Ethyl acetate soluble fractions from both media showed a similar spectrum of compounds on TLC and were therefore combined (4.63 g) and subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane and after that gradients of dichloromethane with up to 10% methanol. A total of 251 fractions were collected. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum or vanillin-sulfuric acid spray reagents. Similar fractions were combined and subjected to preparative TLC on silica gel and crystallization to

isolate the pure compounds **5681-1** (54.3 mg), **5681-2** (9.0 mg), **5681-3** (84.8 mg), **5681-4** (11.1 mg), **5681-5** (12.5 mg), **5681-6** (20.2 mg), **5681-7** (9.1 mg), **5681-8** (3.3 mg), and **5681-9** (73.7 mg). Three compounds (**5681-4, 5681-8**, and **5681-9**) were new natural products. The isolated compounds were numbered according to their polarity on TLC, using dichloromethane/0–8% methanol and toluene/20–40% ethyl acetate as the eluants.

2.1.1.1 Derivatization by acetylation and methylation

To aid structure elucidation, 12.0 and 50.0 mg, respectively, of **5681-9** were acetylated using acetic anhydride/pyridine in dichloromethane, and methylated using an ethereal diazomethane solution, following the standard procedures.[139] Derivatives **5681-5a** (6.0 mg) and **5681-9a** (16.8 mg) were isolated by PTLC as acetylated and methylated derivatives of **5681-9**, respectively. Some very polar fractions obtained by silica gel chromatography (CH_2Cl_2/5-8% MeOH) of the crude extract showed very poor resolution of their compounds on TLC. From the polarity it was assumed that they may contain compounds of polyhydroxyl and/or carboxyl acid derivatives. The entire combined polar fraction was subjected to methylation with diazomethane for two times with the hope that polar phenolic hydroxyl groups and carboxylic acids would be converted to the less polar methyl ethers and esters, which can be purified more easily. In the first methylation, a suspension of 260.0 mg of the crude polar fractions, containing largely brown polymeric material, was treated with 5 ml of ethereal diazomethane solution for a period of three hours at room temperature (20 °C). In the second reaction, 158.0 mg of these combined fractions was similarly methylated for a period of five minutes at about 0 °C. Four pure compounds, **5681-10a** (5.0 mg), **5681-10b** (5.9 mg), **5681-10c** (5.7 mg), and **5681-10d** (6.9 mg) were isolated from these methylated fractions. In the first reaction, **5681-10a** was the minor compound of four and in the second reaction the yield was reversed. A new polar component, **5681-10**, was indirectly identified as a new benzoic acid from these four methylation derivatives. All of the derivatives are reported here for the first time.

2.1.2 Structure elucidation

2.1.2.1 Characterization of 5681-1 as (+)-4,7-dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho-[1,2-*b*]furan-5,6-dicarboxylic anhydride

The least polar constituent **5681-1** (Fig. 17) was isolated from the column fraction by elution with pure CH_2Cl_2. It was obtained as white fine needles with m.p. 251 °C. It appeared as a blue spot on the TLC plate (R_f = 0.43, CH_2Cl_2) under UV light at 254 nm and also exhibited a strong blue fluorescence at 366 nm. It is soluble in CH_2Cl_2 and $CHCl_3$ and insoluble in Et_2O and MeOH. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (75 MHz, $CDCl_3$) of **5681-1** displayed 18 carbon resonances, while the DEPT 135 experiment sorted these signals into 4 methyls, 2 methines and 12 quaternary carbons, *i.e.*, 6 out of the 18 carbons were attached to protons. In the ^1H NMR spectrum (300 MHz, $CDCl_3$) of **5681-1**, three three-proton signals at δ = 1.31, 1.50, and 1.55 could be attributed to three aliphatic methyl groups whereas one three-proton singlet at δ = 2.82 may be attributed to one aromatic or olefinic methyl group. The signal for the methyl protons at δ = 1.50 showed a doublet (J = 6.6 Hz) representing their coupling with the methine proton showing a quartet at δ = 4.72 (J = 6.6 Hz). It can be deduced from the NMR data that the methine hydrogen is geminal to oxygen. The presence of one one-proton singlet at δ = 6.84 is attributable to one aromatic proton. The presence of only one aromatic proton indicated that **5681-1** might carry a pentasubstituted benzene ring. The presence of two sharp one-proton singlets at δ = 11.42 and 11.63 could be attributed to two phenolic chelated hydroxyl groups. The relatively deshielded nature of these hydroxyl groups indicated that each of them might form intramolecular hydrogen bonds with any lone electron pairs of a functional group.

An analysis of the ^{13}C NMR spectrum along with the DEPT 135 experiment sorted the carbons into three groups: 5 sp^2 carbons bound to oxygen (δ = 164.0-166.0); 7 other sp^2 carbons (δ = 93.2-149.7); 6 sp^3 carbons (δ = 14.3-91.8). The resonances at δ = 164.6 and 165.2 could be attributed to two carbonyl carbons of an ester. The presence of two carbonyl carbons of esters was further evident from the two bands in the IR spectrum at 1709 cm^{-1} and 1667 cm^{-1}. It could be assumed that two hydroxyl groups formed two intramolecular hydrogen bonds with these two carbonyls. The resonances at δ = 164.0, 165.7, and 166.0 in conjunction with ^1H NMR data

indicated the presence of three aromatic carbons bearing oxygen. Two of these carbons might be attributed to those carbons bearing the hydroxyl groups and the rest might be that carbon which is geminal to methine's geminal oxygen. The resonances at δ = 93.2, 97.0, 107.9, 117.0, 118.9, 135.1, and 149.7 were attributed to seven aromatic singlets. The resonances at δ = 14.3, 20.5, 23.5, and 25.3 could be attributed to four methyl carbons. Two signals at δ = 91.8 and 43.2 could be attributed to a secondary carbon bonded to oxygen and a quaternary carbon, respectively. The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 329 which, in conjunction with the other spectral data, suggested the molecular formula $C_{18}H_{16}O_6$ for **5681-1**. The optical rotation of **5681-1** was found to be $[\alpha]^{25}_D$ = + 77.2 (*c* 0.46, $CHCl_3$).

The ^1H NMR and ^{13}C NMR data of **5681-1** were found to be identical to those reported for (+)-4,7-dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho-[1,2-*b*]furan-5,6-dicarboxylic anhydride, isolated from the fungi *Aspergillus silvaticus*[140] and *Roesleria pallida*[141] and also to those reported for sclerodin, the levorotatory form of this anhydride, *ent*-**5681-1**, which was found as a constituent of the fungus *Gremmeniella abietina*.[142,143]

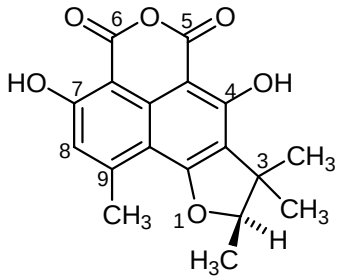


Figure 17: Structure of **5681-1**

The (+)-anhydride **5681-1** was also isolated from *Penicillium herquei*.[144] The relative and also the absolute configuration of the (+)-anhydride were determined by X-ray single crystal analysis.[140]

2.1.2.2 Characterization of 5681-2 as a mixture of epimers of (*R*)-3,5,7-trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-8,9-dihydro-5*H*-phenaleno[1,2-*b*]furan-4,6-dione

Compound **5681-2** (Fig. 18) was isolated from the column fraction by elution with CH_2Cl_2/3% MeOH. It was obtained as a brown semisolid compound. It appeared as a dark spot on the TLC plate (R_f = 0.41, CH_2Cl_2/2% MeOH) under UV light at 254 nm and also exhibited a yellow fluorescence at 366 nm. It is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^1H NMR (300 MHz, $CDCl_3$ and C_6D_6) and ^{13}C NMR (75 MHz, $CDCl_3$ and C_6D_6) spectra of **5681-2**

showed signals representing a minor and a major component in the ratio of approximately 1:1.3. **5681-2** was identified as a mixture of C-5 epimers by comparing the NMR data with those published for these compounds.[142] The ^{13}C NMR spectrum (75 MHz, $CDCl_3$)* (*NMR data of $CDCl_3$ of the major epimer were discussed only) of the major epimer of **5681-2** displayed 22 carbon resonances, while the DEPT 135 experiment sorted these signals into 5 methyls, 1 methylenes, 2 methines, and 14 quaternary carbons.

The structure of **5681-2** was elucidated by direct comparison of its spectral data with those of compound **5681-1**. Although both the ^{1}H NMR and ^{13}C NMR spectra of **5681-2** were in close correspondence to those of **5681-1**, some new signals could be observed, such as the resonances at δ = 3.30 ppm in the ^{1}H NMR and at δ = 51.6 ppm in the ^{13}C NMR spectra. These signals, in conjunction with the DEPT 135 spectrum, proved the presence of a methylene group in **5681-2**. The resonance at δ = 205.7 ppm in the ^{13}C NMR spectrum, and the resonance at δ = 2.24 ppm for a methyl group in the ^{1}H NMR spectrum were characteristic for the presence of an acetyl moiety. Analysis of the ^{13}C NMR spectra of **5681-1** and **5681-2** also revealed that the 2 sp^2 carbons bound to oxygen in the region of δ = 164.0-166.0 were absent in **5681-2**, rather 2 new sp^2 carbons bound to oxygen appeared at δ = 197.0 and 199.0. The downfield shift of two signals for high-field carbonyls, and the presence of signals for additional methylene and acetyl moieties in conjunction with the signal for a new tertiary carbon at δ = 99.0 (C-5) could explain the dione structure of **5681-2** (Fig. 18) instead of the dicarboxylic anhydride of **5681-1** (Fig. 17). This was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 399, in conjunction with the other spectral data, suggesting the molecular formula $C_{22}H_{22}O_7$.

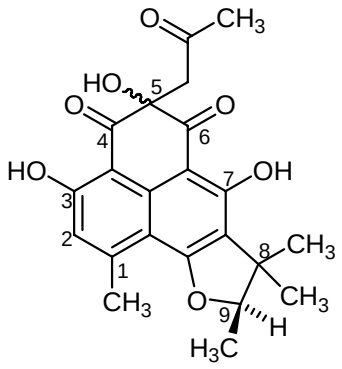
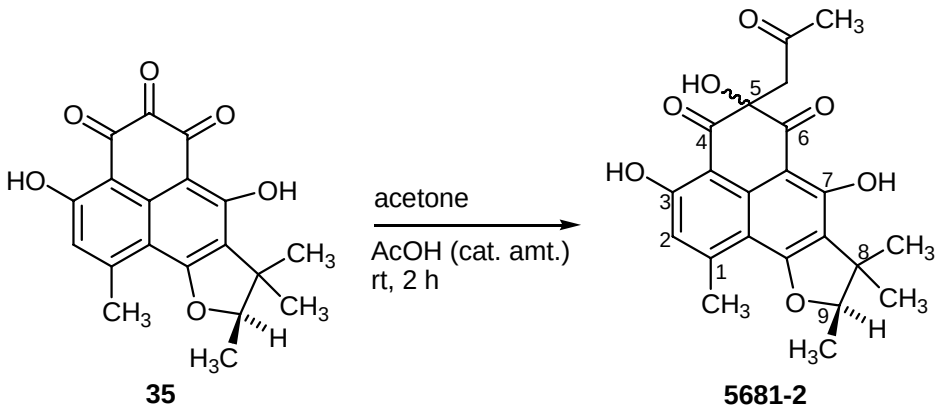


Figure 18: Structure of **5681-2**

It is known from the literature that **5681-2** could be prepared from atrovenetinone (**35**), a metabolite of the fungus *Gremmeniella abietina*.[142] Treatment of **35** with acetone in the

presence of a catalytic amount of acetic acid at room temperature readily gives **5681-2** (Scheme 7).



35 **5681-2**

Scheme 7: Semisynthesis of **5681-2**

2.1.2.3 Characterization of 5681-3 as decarboxycitrinone

Compound **5681-3** (Fig. 19) was isolated from a combined column fraction with the eluant system CH_2Cl_2/0-1% MeOH. It was obtained as white needles with m.p. 223 °C. It appeared as a dark quenching spot on TLC plate (R_f = 0.56, CH_2Cl_2/5% MeOH) under UV light at 254 nm. It is soluble in MeOH, sparingly soluble in $CHCl_3$, and insoluble in CH_2Cl_2 and Et_2O. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{13}C NMR spectrum (50 MHz, $CDCl_3$/2% CD_3OD) of **5681-3** displayed 12 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$/2% CD_3OD) experiment showed signals for one methine and three methyl groups. In the ^1H NMR spectrum (300 MHz, $CDCl_3$/2% CD_3OD) of **5681-3** the presence of one one-proton singlet at δ = 6.34 could be attributed to an aromatic proton. The presence of only one aromatic proton indicated that **5681-3** might carry a pentasubstituted benzene ring.

One one-proton broad singlet at δ = 8.96 and another one-proton sharp singlet at δ = 11.92 could be attributed to two phenolic hydroxyl groups. The relatively deshielded nature of one hydroxyl group (δ = 11.92) indicated that this hydroxyl group might form an intramolecular hydrogen bond with any lone electron pairs of a functional group. In the ^{13}C NMR spectrum the resonance at δ = 167.4 was characteristic for the carbonyl carbon atom of an ester or lactone and it was further evident from a band in the IR spectrum at 1662 cm^{-1}. So it is now obvious that in **5681-3** the carbonyl group formed an intramolecular hydrogen bond with the deshielded hydroxyl group and the hydrogen bond causes the carbonyl group to shift about 40 to 60 cm^{-1} to lower frequency

in the IR spectrum. Again, in the ^{1}H NMR spectrum the presence of three three-proton singlets at δ = 2.20, 2.22, and 2.32 could be attributed to three aromatic and/or olefinic methyl groups.

The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 221 which, in conjunction with the data of other spectra, suggested the molecular formula $C_{12}H_{12}O_4$ for **5681-3**. The ^{1}H NMR and ^{13}C NMR data of **5681-3** were found to be identical to those reported for the isocoumarin 'decarboxycitrinone' isolated from the coprophilous fungus *Cercophora areolata*.[145]

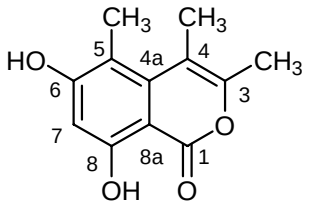


Figure 19: Structure of **5681-3**

This is the first report of **5681-3** from the fungal strain 5681 and the second report of isolation from a natural source.

2.1.2.4 Characterization of 5681-4 as (6,8-dihydroxy-3,5-dimethyl-1-oxo-1*H*-isochromen-4-yl)methyl acetate

Compound **5681-4** (Fig. 20) was isolated from a combined column fraction with the eluant system CH_2Cl_2/0-3% MeOH. It was obtained as white needles with m.p. 230 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.56, CH_2Cl_2/5.2% MeOH) under UV light at 254 nm. It is soluble in MeOH, sparingly soluble in $CHCl_3$, and insoluble in CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (50 MHz, $CDCl_3$/2% CD_3OD) of **5681-4** displayed 14 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$) experiment showed signals for one methine, one methylene, and three methyl groups. The structure of **5681-4** was elucidated by direct comparison of its spectral data with those of compound **5681-3**.[145] Although both the ^{1}H NMR and ^{13}C NMR spectra of **5681-4** were in close correspondence to those of isocoumarin **5681-3**, some new signals could be observed, such as the resonances at δ = 5.10 ppm in the ^{1}H NMR and at δ = 61.3 ppm in the ^{13}C NMR spectra. These signals, in conjunction with the DEPT 135 spectrum, proved the presence of a heteroatom bonded methylene group in **5681-4**, replacing one of the methyl groups in **5681-3**. The resonance at δ = 171.4 ppm in the ^{13}C NMR spectrum, the

carbonyl band at 1740 cm^{-1} in the IR spectrum, and the resonance at δ = 2.07 ppm for a methyl group in the ^1H NMR spectrum were characteristics for the presence of an acetate moiety. This was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 279 which, in conjunction with the data of other spectra, suggested the molecular formula $C_{14}H_{14}O_6$. The relative positions of the ester and methyl groups were determined by a 1D NOE experiment. Irradiation of the methylene protons at δ = 5.10 ppm showed considerable interactions with both the methyl groups at C-3 and C-5. The CH_2OCOCH_3 chain therefore had to be attached at C-4 as shown in structure **5681-4** in Figure 20.

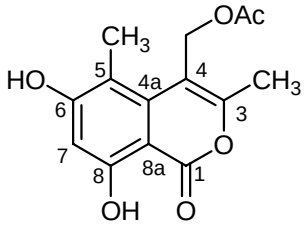


Figure 20: Structure of **5681-4**

Compound **5681-4** is an isocoumarin possessing the same structural skeleton of **5681-3**. A literature survey revealed that **5681-4** is a novel isocoumarin.

2.1.2.5 Characterization of 5681-5 as 4-acetyl-6,8-dihydroxy-5-methyl-1*H*-isochromen-1-one

Compound **5681-5** (Fig. 21) was isolated from a combined column fraction with the eluant system CH_2Cl_2/0-3% MeOH. It was obtained as a white solid mass with m.p. 170 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.52, CH_2Cl_2/5.2% MeOH) under UV light at 254 nm. It is soluble in MeOH and sparingly soluble in $CHCl_3$ and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (50 MHz, $CDCl_3$/2% CD_3OD) of **5681-5** displayed 12 carbon resonances, while the DEPT 135 (50 MHz, $CDCl_3$/2% CD_3OD) experiment showed signals for two methines and two methyl groups, *i.e.*, 4 out of the 12 carbons were attached to protons. The structure of **5681-5** was elucidated as a 4-substituted isocoumarin with no substituent at the 3-position by direct comparison of its ^1H NMR data with that reported for this compound isolated from the fungus *Aspergillus viridinutans*.[146] Both the ^1H NMR (200 MHz, $CDCl_3$/2% CD_3OD) and ^{13}C NMR spectra of **5681-5** were in close correspondence to those of **5681-3** and **5681-4**, but some deviations could be observed due to the different residues at C-3 and C-4, such as the

resonances at δ = 7.44 ppm in the ^{1}H NMR and at δ = 145.4 ppm in the ^{13}C NMR spectra. These signals, in conjunction with the DEPT 135 spectrum, proved the presence of a vinylic proton in **5681-5**. The resonance at δ = 199.2 ppm in the ^{13}C NMR spectrum, and the resonance at δ = 2.53 ppm for a methyl group in the ^{1}H NMR spectrum were characteristics for the presence of an acetyl moiety. On the other hand, the resonances for the C-3 and C-4 subtituents of **5681-3** and **5681-4** were missing in **5681-5** and replaced by the above-mentioned vinylic proton and an acetyl moiety. This was confirmed by the mass spectrum with $[M+H]^{+}$ at *m/z* = 235 which, in conjunction with the other spectral data, suggested the molecular formula $C_{12}H_{10}O_5$.

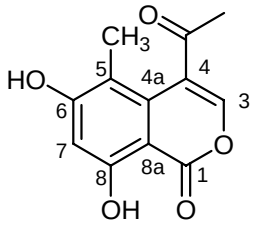


Figure 21: Structure of **5681-5**

2.1.2.6 Characterization of 5681-6 as (3*R*,4*R*)-4-acetyl-6,8-dihydroxy-3-methoxy-5-methyl-3,4-dihydroisochromen-1-one

Compound **5681-6** (Fig. 22) was isolated from a combined column fraction with the eluant system CH_2Cl_2/0-3% MeOH. It was obtained as white needles with m.p. 195 °C. It appeared as a blue spot on the TLC plate (R_f = 0.46, CH_2Cl_2/5.2% MeOH) under UV light at 254 nm and exhibited a blue fluorescence at 366 nm. It is soluble in MeOH and sparingly soluble in $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{13}C NMR spectrum (75 MHz, CD_3OD) of **5681-6** displayed 13 carbon resonances, while the DEPT 135 experiment showed signals for three methines, two methyl and one methoxy groups.

The structure of **5681-6** was elucidated by direct comparison of its spectral data with those of compound **5681-5**. Differences were found in the C-3 and C-4 substituents. Although both the ^{1}H NMR (200 MHz, CD_3OD) and ^{13}C NMR spectra of **5681-6** were in close correspondence to those of **5681-5**, some new signals could be observed, such as the two doublets at δ = 4.04 ($J_{4,3}$ = 1.3 Hz) and δ = 5.60 ($J_{3,4}$ = 1.3 Hz) ppm in the ^{1}H NMR and at δ = 55.2 and δ = 103.7 ppm in the ^{13}C NMR spectra. These signals, in conjunction with the DEPT 135 spectrum, proved the presence of two vicinal methines in **5681-6**. The resonance at δ = 3.57 ppm in the ^{1}H NMR spectrum, and the resonance at δ = 57.6 ppm in the ^{13}C NMR spectrum in conjunction with the

DEPT 135 spectrum proved the presence of an *O*-methyl group. On the other hand, the resonance for the C-3 vinylic proton of **5681-5** was missing in **5681-6**. Other chemical shifts were almost superimposable with those of **5681-5**. This was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 267, suggesting the molecular formula $C_{13}H_{14}O_6$ in agreement with the NMR spectra. The ^{1}H NMR and ^{13}C NMR data of **5681-6** were found identical to those reported for this compound isolated from an endophytic fungus *Mycelia sterila*.[53] The coupling constant of $J_{3,4}$ indicated a *trans*-bisequatorial arrangement of the substituents at C-3 and C-4 on the pyranone ring.

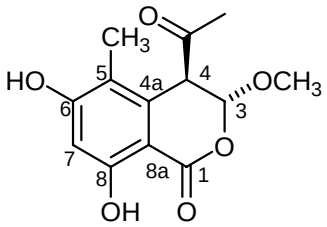


Figure 22: Structure of **5681-6**

2.1.2.7 Characterization of 5681-7 as (*R*)-4-acetyl-6,8-dihydroxy-5-methyl-3,4-dihydroisochromen-1-one

Compound **5681-7** (Fig. 23) was isolated from a combined column fraction with the eluant system CH_2Cl_2/0-3% MeOH. It was obtained as white crystals with m.p. 204 °C. It appeared as a blue spot on the TLC plate (R_f = 0.51, CH_2Cl_2/6% MeOH) under UV light at 254 nm and also exhibited a blue fluorescence at 366 nm. It is soluble in MeOH, sparingly soluble in $CHCl_3$, and insoluble in CH_2Cl_2 and Et_2O. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (50 MHz, CD_3OD) of **5681-7** displayed 12 carbon resonances, while the DEPT 135 experiment showed signals for two methines, one methylene, and two methyls, *i.e.*, 5 out of the 12 carbons were attached to protons. Direct comparison of the spectral data of **5681-7** with those of compound **5681-6** indicated the difference in the C-3 substituent only. In the ^{1}H-NMR spectrum of **5681-7**, the singlet at δ = 3.57 for the methoxyl group, present in the spectrum of **5681-6**, was missing. Instead, the ^{1}H-NMR spectrum of **5681-7** showed two one-proton doublets at δ 4.92 (*J* = 11.0 Hz) and δ 4.17 (*J* = 3.7 Hz) and a one-proton double doublet at δ 4.60 (*J* = 11.0, 3.7 Hz). This data in conjunction with DEPT 135 experiment suggested that the protons of one doublet and the double doublet are due to two non-equivalent geminal protons and the proton for the other doublet corresponds to a non-equivalent methine vicinal to the geminal protons. Other chemical shifts were almost superimposable with those of **5681-6**. This was

confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 237, suggesting the molecular formula $C_{12}H_{12}O_5$ in agreement with the NMR spectra.

The ^1H NMR and ^{13}C NMR data of **5681-7** were found identical to those reported for an isocoumarin isolated from an endophytic fungus *Mycelia sterila*.[53]

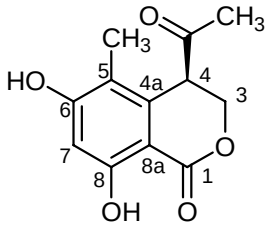
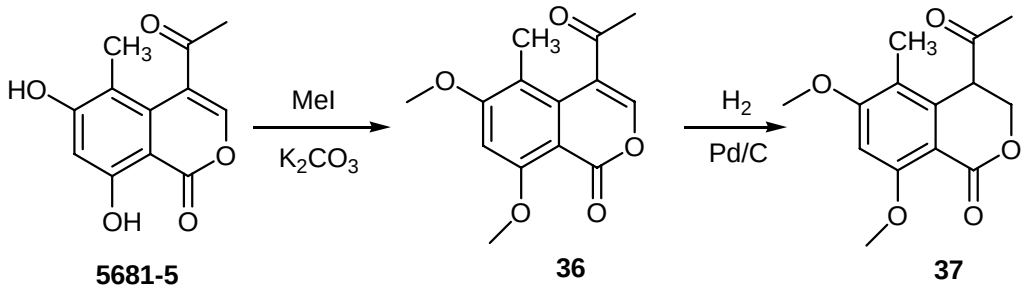


Figure 23: Structure of **5681-7**

Compound **5681-7** was reported as dimethylether **37**, a semisynthetic derivative of **5681-5**, in 1966.[146] Treatment of **5681-5** with methyl iodide in the presence of potassium carbonate readily gives dimethoxycoumarin **36**, which on reduction with hydrogen in presence of palladised charcoal gives **37** (Scheme 8).



Scheme 8: Semisynthesis of 4-acetyl-6,8-dimethoxy-5-methylisocoumarin (**37**)

The 6-methylether of **5681-7** was isolated from the coprophilous fungus *Cercophora areolata*.[145]

2.1.2.8 Characterization of 5681-8 as 6,8-dihydroxy-4-(hydroxymethyl)-3,5-dimethyl-1*H*-isochromen-1-one

Compound **5681-8** (Fig. 24) was isolated from a combined column fraction with the eluant system CH_2Cl_2/3-5% MeOH. It was obtained as brown fine needles with m.p. 245 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.39, CH_2Cl_2/8% MeOH) under UV light at 254 nm. It is soluble in MeOH and insoluble in $CHCl_3$ and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{13}C NMR spectrum (75 MHz, CD_3OD) of **5681-8** displayed 12 carbon resonances,

while the DEPT 135 (75 MHz, CD_3OD) experiment showed signals for one methine, one methylene and two methyl groups, *i.e.*, 4 out of the 12 carbons were attached to protons.

The spectral data of compound **5681-8** closely corresponded to those of both **5681-3** and **5681-4**. The mass spectrum with $[M+H]^+$ at *m/z* = 237 which, in conjunction with the other spectral data, suggested the molecular formula $C_{12}H_{12}O_5$. This showed the presence of an additional oxygen atom for **5681-8** with respect to **5681-3** and the absence of the acetic ester moiety compared to **5681-4**. The absence of the acetic ester moiety was further confirmed by the missing signals for the ester carbonyl carbon atom and the methyl group in the NMR and relevant bands in the IR spectra. The structure of the corresponding alcohol **5681-8**, related to the ester **5681-4**, was further confirmed by the expected upfield shift (δ = 4.52 ppm) of the methylene protons in the ^1H NMR spectrum with respect to the acetate **5681-4** (δ = 5.10 ppm). The location of the hydroxymethyl group was independently determined by a 1D NOE experiment to be located at C-4, as shown by strong interactions of the methylene protons with both the methyl protons at C-3 and C-5. A literature survey revealed **5681-8** as a novel isocoumarin.

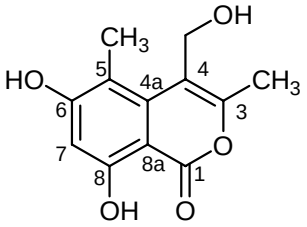
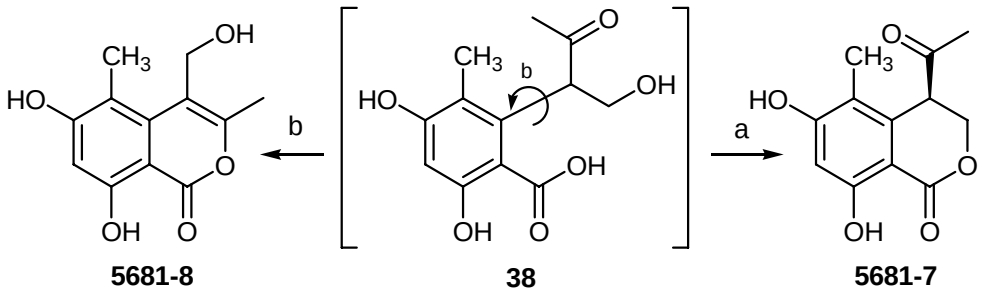


Figure 24: Structure of **5681-8**

The alcohol **5681-8** is closely related to the isocoumarin sescandelin B, isolated by Kimura et al.[147] and recently synthesized by Kim et al.,[148] missing only the methyl group at C-5. On the other hand, there is also a close biosynthetic relationship of the hydroxymethyl isocoumarin **5681-8** with the acetyl compound **5681-7**, also isolated from another endophytic fungus, *Mycelia sterile*.[53] A presumed open chain precursor **38** can either directly be closed to the six-membered lactone (path a) or cyclize after rotation of the side chain via the acetyl enol tautomer to form the hydroxymethyl compound **5681-8** (path b), as shown in Scheme 9.



5681-8 **38** **5681-7**

Scheme 9: Presumed biosynthetic relationship of isocoumarins **5681-7** and **5681-8**

2.1.2.9 Characterization of 5681-9 as 4,6-dihydroxy-2-(1-hydroxy-3-oxobut-1-en-2-yl)-3-methylbenzoic acid

Compound **5681-9** (Fig. 25) was isolated from a combined column fraction with the eluant system CH_2Cl_2/3-5% MeOH. It was obtained as a white solid mass with m.p. 175 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.35, CH_2Cl_2/8% MeOH) under UV light at 254 nm and also exhibited a blue fluorescence at 366 nm. It is soluble in MeOH and insoluble in CH_2Cl_2 and Et_2O. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

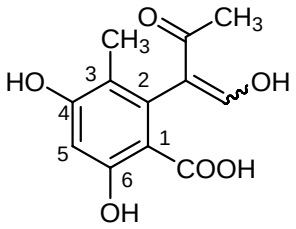
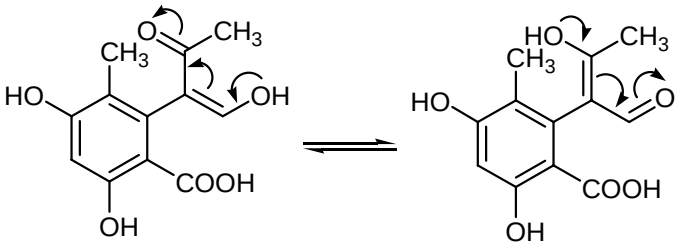


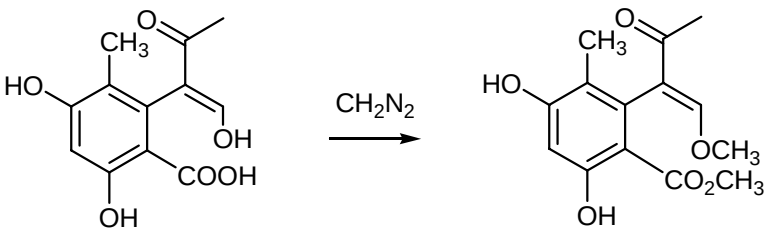
Figure 25: Structure of **5681-9**

The hydroxymethylene ketone **5681-9** is the most polar compound isolated in pure state from the strain 5681. The keto-enol tautomerism (Scheme 10) and thus the occurrence of signals with different integration in the NMR spectra of these tautomers, complicated a straightforward structure elucidation of this polar component. Therefore, the compound was treated with diazomethane for a few minutes (experimental part **7.3.1.10.1**) to trap the enol tautomer (Scheme 11), as described similarly by Aldridge et al. for a related semisynthetic derivative.[146]



Scheme 10: Tautomerism of **5681-9**

Both the ^{1}H NMR and ^{13}C NMR spectra of the major methylation product closely corresponded to those of isocoumarin **5681-5**. However, in the ^{1}H NMR spectrum of **5681-9a**, two three-proton singlets at δ = 3.83 and δ = 3.86 ppm indicated the presence of additional *O*-methyl groups, whereas the position of the signals for the aromatic methyl group at C-4 and the acetyl group remained essentially unchanged.



5681-9 **5681-9a**

Scheme 11: Diazomethane methylation of the hydroxymethylene ketone **5681-9** to the enol ether **5681-9a**.

The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 281 which, in conjunction with the other spectral data, suggested the molecular formula $C_{14}H_{16}O_6$, thus the addition of one methyl and one methoxy group with respect to **5681-5**. The location of the methoxy groups was identified by COLOC experiments. The correlation of one of the methoxy groups with the vinylic proton and the absence of any correlation with the aromatic proton proved the methylation of the conjugated enol system in **5681-9a**. On the other hand, a resonance at δ = 171.6 ppm in the ^{13}C NMR spectrum indicated the presence of an ester group, ca. 6 ppm downfield from the resonance of the corresponding lactone carbonyl in **5681-5** (δ = 165.7 ppm). The resonance of the vinylic carbon (=CHOMe) in the open chain arrangement (δ = 157.3 ppm) also showed a significant downfield shift with respect to that of the cyclized form **5681-5** (δ = 145.4 ppm). All of these data were in agreement with the open chain methyl ester enol ether structure **5681-9a**, originating from β-ketoaldehyde **5681-9**. The displayed $[M+H]^+$ ion at *m/z* = 253 in the mass spectrum of **5681-9** was also in agreement with the proposed structure for this compound. In addition, further evidence for the open chain structure **5681-9** arose from the conversion into the isocoumarin diacetate **5681-5a** upon treatment with acetic anhydride/pyridine (experimental part **7.3.1.10.2**).

A literature survey revealed that **5681-9** is a new compound.

2.1.2.10 Characterization of 5681-10 as 4,6-dihydroxy-3-methyl-2-(2-oxopropanoyl)benzoic acid

The isolation of metabolites from very polar fractions of the silica gel chromatography was hampered by the presence of dark brown polymeric material. The entire polar fractions was subjected to a short methylation with diazomethane (see experimental part **7.3.1.11**) with the hope that polar phenolic hydroxyl groups and carboxylic acids would be converted to the less polar methyl ethers and esters, which can be purified more easily. From this experiment, four

different compounds **10a**–**10d** with very interesting open chain structures were isolated in pure form after extensive preparative TLC of the reaction mixture (Scheme 12). The presence of the diketo acid **5681-10** (Fig. 26) was deduced only indirectly, but the evidence from the structures of the four methylation products **5681-10a** – **5681-10d** unambiguously proved the structure of the precursor **5681-10**.

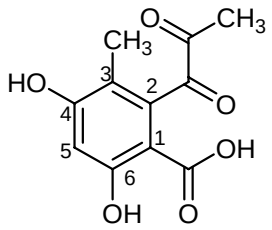
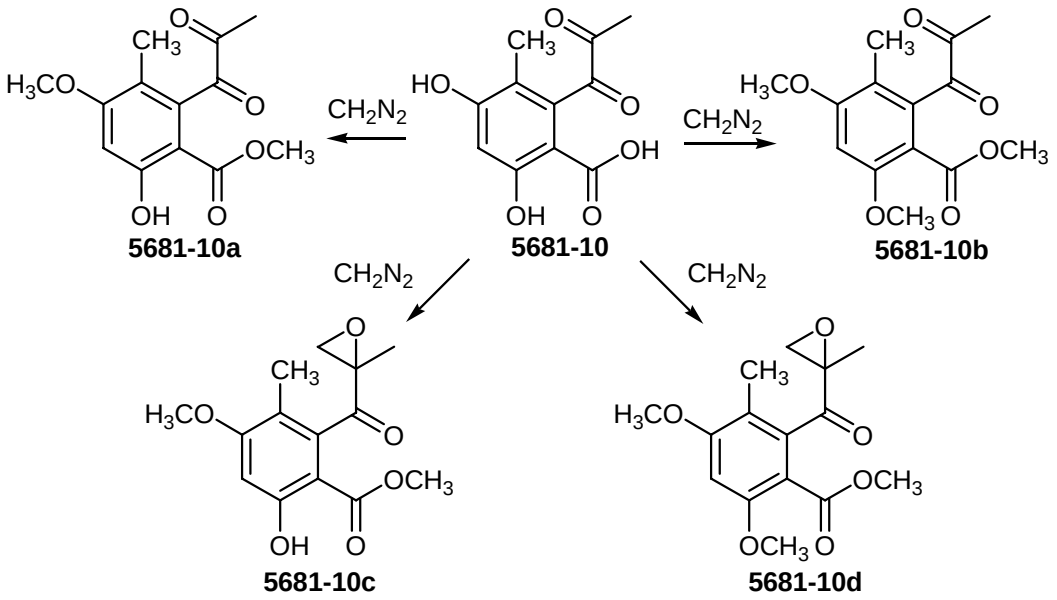


Figure 26: Structure of **5681-10**

The presence of a methyl ester and a free chelated phenolic hydroxy group could be confirmed by comparison of the ^{1}H NMR and ^{13}C NMR spectra of **5681-10a**, the most abundant methylation product, with those of **5681-9a**. The second methyl group was tentatively located at the non-chelated phenolic hydroxy group. No signals for olefinic protons could be detected in **5681-10a**, but instead two resonances at δ = 193.8 and δ = 196.5 ppm indicated the presence of two additional carbonyl groups. Comparison with literature reference[149] of the somewhat unusual chemical shift for these carbonyl groups suggested (in conjunction with the mass spectrum with $[M+H]^+$ at *m/z* = 267 and the molecular formula $C_{13}H_{14}O_6$) the presence of an α-diketo side chain, as shown in the tentatively assumed structure **5681-10a**.

Both the ^{1}H NMR and ^{13}C NMR spectra of **5681-10b** were almost identical to those of **5681-10a** with exception of the signals for an additional methoxy group and a missing chelated phenolic hydroxyl. The methylation of a chelated phenolic hydroxy group was confirmed by the mass difference of 14 units and thus structure **5681-10b** was tentatively assumed for the second methylation product.



Scheme 12: Diazomethane methylation of diketo acid **5681-10** to produce the methylated compounds **5681-10a – 5681-10d**

The chemical nature of the side chain could be determined after analysis of the spectral data of the next two methylation products **5681-10c** and **5681-10d**. They were related to each other as were **5681-10a** and **5681-10b**, also showing the additional methylation of the chelated hydroxyl group in **5681-10d** with respect to **5681-10c**. An additional methylene group and also a quaternary carbon could be detected both in their ^{1}H NMR and ^{13}C NMR spectra, whereas one of the signals for the carbonyl groups present in **5681-10a** and **5681-10b** was missing. The presence of the additional methylene group was also confirmed by the respective mass difference of 14 units. It was thus reasonable to assume the insertion of a methylene carbene to form an oxirane because the chemical shift of the carbon and hydrogen atoms and also the geminal coupling constant of ca. 5.0–5.5 Hz for the methylene protons were in perfect agreement with the presence of oxiranes both in **5681-10c** and **5681-10d**. The upfield shift of the signal for the acetyl methyl groups from δ = 1.94 and 1.97 ppm in **5681-10a** and **5681-10b** to δ = 1.70 and 1.69 ppm in **5681-10c** and **5681-10d** proved the transformation of the external carbonyl group to an oxirane. Evidently, in addition to possible electronic effects, the pentasubstituted benzene ring effectively shields the benzylic carbonyl group. This spectral evidence and also the fact that the methylenation of 1,2-dicarbonyl compounds has ample precedence in the literature,[150] confirms the structures of the methylation products **5681-10a** – **5681-10d** and thus that of the polar natural constituent **5681-10**. A literature survey revealed that **5681-10** is a new compound.

2.1.3 Biological activity of the secondary metabolites

Crude extracts and pure compounds of the strain 5681 were tested for antibacterial, antifungal and antialgal activities by agar diffusion assay method.[151] Sample solutions contained 40 mg/mL for crude extract and 1 or 5 mg/mL for pure compounds. Samples were prepared by taking 50 µL of each solution and pipetting it onto a sterile antibiotic filter disc, which was then placed onto the appropriate agar medium and sprayed with a suspension of the test organism. Growth media, preparation of spraying suspensions, and conditions of incubation were done according to Schulz et al.[151]

The activity of the crude extracts against bacterial, fungal, and algal test organisms was compiled in Table 2 and is discussed in section 2.1.1. The crude ethyl acetate extracts showed moderate activity against the test organisms. Considering the activity of the crude extract three test organisms were selected to study the antibacterial, antifungal, and antialgal activities of the pure compounds by agar diffusion assay method. The results against these organisms are compiled in Table 3.

Three metabolites, **5681-5**, **5681-8** and **5681-9**, were found to be very active against all of the test organisms, exhibiting prominent activities against the Gram-positive bacterium *Bacillus megaterium*, the fungus *Microbotryum violaceum*, and the alga *Chlorella fusca*. Compound **5681-2** showed good antibacterial and antifungal activities. On the other hand, the alga *Chlorella fusca* was found to be resistant to **5681-2**. Compound **5681-3** showed good algicidal activity, whereas the other organisms were found to be resistant to it. Compounds **5681-4** and **5681-6** exhibited only antifungal and antialgal activities, respectively. Compounds **5681-1** and **5681-7** were found to be inactive against all the test organisms. In fact, most of the pure compounds were active against the test organisms as it was assumed from the bioactivity of the crude extract.

Table 3: Biological activity of pure metabolites against microbial test organisms

Metabolites	Concentration (mg/mL)	*Bacillus megaterium*	*Microbotryum violaceum*	*Chlorella fusca*
5681-1	1	0	0	0
	5	0	0	0
5681-2	1	5	5	0
	5	5	6	0
5681-3	1	0	0	5
	5	0	0	5
5681-4	1	0	5	0
	5	0	6	0
5681-5	1	5	7	8
	5	6	10	7
5681-6	1	0	0	6
	5	0	0	7
5681-7	1	0	0	0
	5	0	0	0
5681-8	1	7	5	6
	5*	not done	not done	not done
5681-9	1	5	0	5
	5	5	6	5

Concentration: 50 µL of the given 1 or 5 mg/mL solution per plate; Numbers indicate radius of zone of inhibition in mm; *could not be tested at higher concentration due to the scarcity of sample.

2.2 Strain 6744

Strain 6744, an endophyte, was isolated from the plant *Calestegia sepium* species, growing near the Baltic Sea. Fungal strain 6744 was characterized taxonomically as *Dinemasporium strigosum*.

2.2.1 Isolation of secondary metabolites

Strain 6744 was cultivated at room temperature for 21 days in biomalt semi-solid agar medium. After that, this culture medium was extracted three times with ethyl acetate to obtain the crude extract (16.0 g). The crude extract was subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane, after that gradients of dichloromethane/methanol and finally methanol. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum or vanillin-sulfuric acid spray reagents. Similar fractions were combined and subjected to column chromatography and preparative TLC on silica gel and crystallization to isolate the pure compounds **6744-1**, **6744-2**, **6744-3**, **6744-4**, and **6744-5**. Compounds **6744-4** and **6744-5** were new natural products. The isolated compounds were numbered according to their polarity on TLC.

2.2.1.1 Derivatization by acetylation and benzoylation

Compound **6744-4** was reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-4** (**6744-4a** and **6744-4b**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. With the similar hope, **6744-5** was also reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-5** (**6744-5a** and **6744-5b**). Unfortunately, none of the four bromobenzoates were suitable for X-ray analysis. However, the absolute configuration of **6744-5** was established by 'Exciton Chirality Method' from its dibenzoate derivative **6744-5b**. The determination of the absolute configuration of **6744-4** is underway using the same principle from its dibenzoate derivative **6744-4b**. Besides, the NMR data of all the benzoates contributed to reconfirm their respective parent structures. Some polar fractions of the silica gel chromatography (CH_2Cl_2/1.5-3% MeOH) showed very poor resolution of their compounds on TLC. From the polarity it was assumed that they may contain compounds of polyhydroxyl derivatives. A portion of the polar fractions was therefore subjected to acetylation using acetic anhydride/pyridine in dichloromethane with the hope that polar hydroxyl groups would be converted to the less polar esters, which can be purified more easily. A new component, **6744-6**, was indirectly identified from the acetylated derivative **6744-6a**. All of the derivatives are reported here for the first time.

2.2.2 Structure elucidation

2.2.2.1 Characterization of 6744-1 as palmitic acid

The least polar constituent **6744-1** (Fig. 27) was isolated from the column fraction by elution with petroleum ether/60% CH_2Cl_2. It was obtained as a white powder with m.p. 55 °C. It is not visible under UV light. It is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour (R_f = 0.35, CH_2Cl_2/9% Et_2O).

The ^{13}C NMR spectrum (50 MHz, $CDCl_3$) of **6744-1** displayed 16 carbon resonances, while the DEPT 135 experiment sorted these signals into 1 methyl, 14 methylenes, and 1 quaternary carbon, *i.e.*, 15 out of the 16 carbons were attached to protons. The ^1H NMR spectrum (200 MHz, $CDCl_3$) of **6744-1** showed a triplet at δ = 0.92 (3H, -CH_3 group of aliphatic chain), a multiplet centered at δ = 1.30 (24H, 12 –CH_2 groups of the aliphatic chain), a quintet centered at δ = 1.68 (2H, protons β to the acid group), and a triplet at δ = 2.39, which was assigned to the two protons α to the carboxyl group. The presence of a carboxyl group was further evidenced by the presence of a signal at δ = 180.0 in its ^{13}C NMR spectrum. The signals for methyl and methylene groups were observed at δ = 14.5, 23.1, 25.1, 29.5, 29.6, 29.8 (2 –CH_2 groups), 30.0, 30.1 (5 –CH_2 groups), 32.3, and 34.4, respectively. The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 257 which, in conjunction with the other spectral data, suggested the molecular formula $C_{16}H_{32}O_2$ for **6744-1**.

The ^1H NMR and ^{13}C NMR data of **6744-1** were found to be identical to those reported for palmitic acid.[152,153]

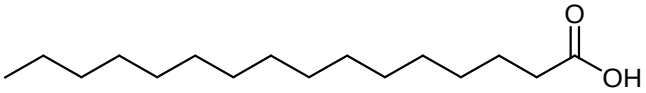


Figure 27: Structure of **6744-1**

2.2.2.2 Characterization of 6744-2 as ergosterol

Compound **6744-2** (Fig. 28) was isolated from the column fraction by elution with petroleum ether/75% CH_2Cl_2. It was obtained as white needles with m.p. 149 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.33, CH_2Cl_2/9% Et_2O) under UV light at 254 nm. It is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (50 MHz, $CDCl_3$) of **6744-2** displayed 28 carbon resonances, while the DEPT 135 experiment sorted these signals into 6 methyls, 7 methylenes, 11 methines, and 4 quaternary carbons, *i.e.*, 24 out of the 28 carbons were attached to protons. The ^{1}H NMR spectrum (200 MHz, $CDCl_3$) of **6744-2** showed two one-proton multiplets at δ = 3.65 and δ = 5.43, typical for the signals of H-3 and H-6 of a steroidal nucleus. The multiplets at δ = 5.23 and δ = 5.36 could be attributed to three olefinic protons at C-7 and in the side chain, as would be expected for ergosterol. The ^{13}C NMR spectral data also suggested that **6744-2** was a sterol with 6 methyl signals at δ = 12.5, 16.7, 18.0, 20.1, 20.4, and 21.5, and an oxygenated methylene signal at δ = 70.9 and 6 olefinic carbon signals at δ = 116.7, 120.0, 132.4, 136.0, 140.2, and 141.8. The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 397 which, in conjunction with the other spectral data, suggested the molecular formula $C_{28}H_{44}O$ for **6744-2**. The fragment ions at *m/z* values of 381 [M^+ - Me], 378 [M^+ - H_2O], 363 [M^+ - (Me + H_2O)], 337 [M^+ - 59], 271 [M^+ - side chain], 253 [M^+ - (side chain + H_2O)], and 211 [M^+ - (side chain + 42 + H_2O)] were in accord with those of ergosterol.[154] Thus, **6744-2** was identified as ergosterol.

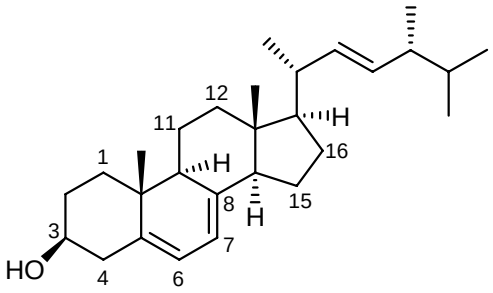


Figure 28: Structure of **6744-2**

2.2.2.3 Characterization of 6744-3 as a mixture of two diastereomers of 4,8-dihydroxy-3-methyl-3,4-dihydroisochromen-1-one (4-hydroxymellein)

Compound **6744-3** (Fig. 29) was isolated as a mixture of two diastereomers from the column fraction by elution with petroleum ether/55% CH_2Cl_2. They appeared as blue spots on the TLC plate (R_f = 0.25 & 0.31, CH_2Cl_2/9% Et_2O) under UV light at 254 nm and also exhibited blue fluorescence at 366 nm. Compound 6744-**3** is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectra of **6744-3** showed signals representing a minor and a major component in the ratio of approximately 1:3.3. **6744-3** was identified as a mixture of two diastereomers of 4-hydroxymellein by comparison with the published NMR data.[155] The ^{13}C NMR spectrum (125 MHz, $CDCl_3$)* (*NMR data of the major diastereomer were discussed only) of the major diastereomer of **6744-3** displayed 10 carbon resonances, while the DEPT 135 experiment sorted these signals into 1 methyl group, 5 methines, and 4 quaternary carbons. In the ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **6744-3** one three-proton doublet at δ = 1.62 (*J* = 6.6 Hz) could be attributed to one aliphatic methyl group. It is shown from the NMR data that two methine protons showing multiplets at δ = 4.73 are geminal to oxygens. The presence of three one-proton signals at δ = 6.95 (d, *J* = 7.3 Hz), δ = 7.06 (d, *J* = 8.5 Hz), and δ = 7.56 (t, *J* = 8.0 Hz) could be attributable to three aromatic protons. The presence of one one-proton sharp singlet at δ = 11.06 could be attributed to one phenolic chelated hydroxyl group. The relatively deshielded nature of this hydroxyl group indicated that it might form intramolecular hydrogen bonds with any lone electron pair of a functional group. In the ^{13}C NMR spectrum (125 MHz, $CDCl_3$) the resonance at δ = 169.1 could be attributed to one carbonyl carbon of an ester or lactone. It could be assumed that the hydroxyl group formed the intramolecular hydrogen bond with this carbonyl. The resonance at δ = 162.2 in conjunction with ^{1}H NMR data indicated the presence of one aromatic carbon bearing oxygen. Two signals at δ = 67.3 and 79.0 could be attributed to two carbons bonded to oxygen.

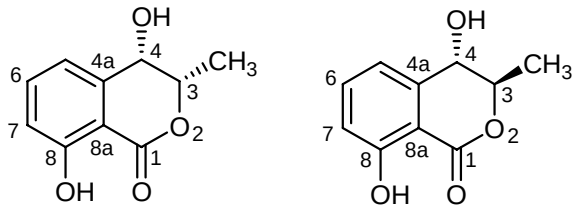


Figure 29: Structures of the diasteromers of **6744-3**

The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 195 which, in conjunction with the other spectral data, suggested the molecular formula $C_{10}H_{10}O_4$ for **6744-3**.

2.2.2.4 Characterization of 6744-4 as 3,11-dihydroxy-2,8-dimethyl-1,7-dioxa-spiro[5,5]undecan-4-one

Compound **6744-4** (Fig. 31) was isolated from the column fraction by elution with CH_2Cl_2/1-2% MeOH). It was obtained as white fine needles with m.p. 149 °C. It is not visible under UV

light. It is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour (R_f = 0.29, CH_2Cl_2/2.9% MeOH). The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6744-4** displayed 11 carbon resonances, while the HMQC experiment indicated that 9 out of the 11 carbons were attached to protons. ^1H NMR (500 MHz, $CDCl_3$) and DEPT 135 (125 MHz, $CDCl_3$) spectra revealed the presence of four oxygenated methines, three methylenes, and two methyl carbons. The ^1H NMR and ^{13}C NMR chemical shifts are shown in Table 4.

The resonance at δ = 205.8 ppm in the ^{13}C NMR spectrum and the carbonyl band at 1722 cm^{-1} in the IR spectrum were characteristic for the presence of a carbonyl carbon. In the ^1H NMR spectrum of **6744-4**, the two one-proton doublets at δ = 2.50 and δ = 3.52 ppm did not show any connectivity with any carbon in the HMQC spectrum. This information, in conjunction with the two bands at 3487 and 3425 cm^{-1} in the IR spectrum, suggested the presence of two hydroxyl groups. The peak, at δ = 101.3 (C-6), appears as a strongly deshielded quaternary carbon, may be tentatively assigned to a ketal function. Analysis of one- and two-dimensional NMR spectra including COSY, HMQC, and HMBC led to the assignment of two partial structures, **a** and **b**, as shown in Figure 30. In partial structure **a**, the C8(CH_3)-C9-C10-C11(OH) portion was assigned by tracing of cross peaks in the COSY spectrum. A tetrahydropyranol ring moiety was disclosed by HMBC correlations (H-8/C-6; CH_3-8/C-6; H-9/C-6; H-10/C-6; OH-11/C-6). In partial structure **b**, the C2(CH_3)-C3(OH)-C4-C5 portion was assigned by tracing of cross peaks in the COSY spectrum. A tetrahydropyranone ring moiety was disclosed by HMBC correlations (H-2/C-4, C-6; CH_3-2/C-4, C-6; H-3/C-4; OH-3/C-4; H-5/C-4, C-6) and the chemical shift of C-4 (δc = 205.8 ppm).

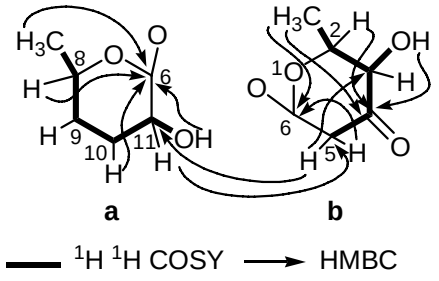


Figure 30: Partial structures (**a** & **b**) of **6744-4**

In both of the partial structures **a** and **b** the C-6 carbon of the ketal function is common, indicating a connection between the partial structures through C-6. Each of the H-5 protons gave a HMBC cross peak with C-11 and also the H-11 gave a HMBC cross peak with C-5, confirming the connection between the partial structures **a** and **b** through the ketal function at C-6, *i.e.*, C-6 is the spiro atom and **6744-4** could be a spiroketal as shown in Figure 31.

Finally, the gross structure of **6744-4** was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 231, suggesting the molecular formula $C_{11}H_{18}O_5$ in agreement with the NMR spectra. Structural rigidity and distortion of the spiro rings in **6744-4** allowed for the observation of W-coupling between H-5 – H-3 (J = 1.1 Hz).

The relative configuration of **6744-4** was determined by a combination of the analysis of the coupling constants and extensive 1D NOE experiments. The large coupling constant $J_{2,3}$ = 9.5 Hz in ring A indicated an antiperiplanar position of these two hydrogen atoms, placing the methyl and hydroxyl groups in *trans* equatorial positions. This was confirmed by a large correlation of the protons of the methyl group at C-2 (δ = 1.47) with the vicinal proton at C-3 at δ = 3.83 in the NOE experiment, proving that these protons are on the same side of the ring (structure **c**, Fig. 31).

The two remaining stereogenic centers in ring B at C-8 and C-11 and the configuration of the acetalic spiro center at C-6, connecting the two rings, were less easily elucidated because the proton coupling system was interrupted by the quaternary spiro center at C-6. Therefore, the only possibility was the analysis of the entire set of the NOE correlations, in particular those between the protons of ring A and ring B.

First, the position of the methyl and hydroxyl group was located at C-8 and C-11, respectively, by a combination of chemical shift, coupling constants, and COSY experiments of ring B NMR resonances (see above). To elucidate the relative configuration of ring B chirality with respect to those of ring A, each stereogenic center at C-6, C-8, and C-11 was then sequentially inverted in the model considerations and analysed for unambiguous agreement with the **entire set** of Overhauser interactions. It must be stated at this point that the NOE data required the existence of two chair conformations of ring B, as shown in Figure 31 (**c** and **d**). The two assumed conformations are very close in energy, assuming either an axial hydroxyl group or an axial methyl group. Interestingtly, these two conformers also occupied to the two energy minima by quantum-mechanical calculations using the Gaussian program.[155a] Here, I like to thank Prof. Dr. van Ree for doing the calculation.

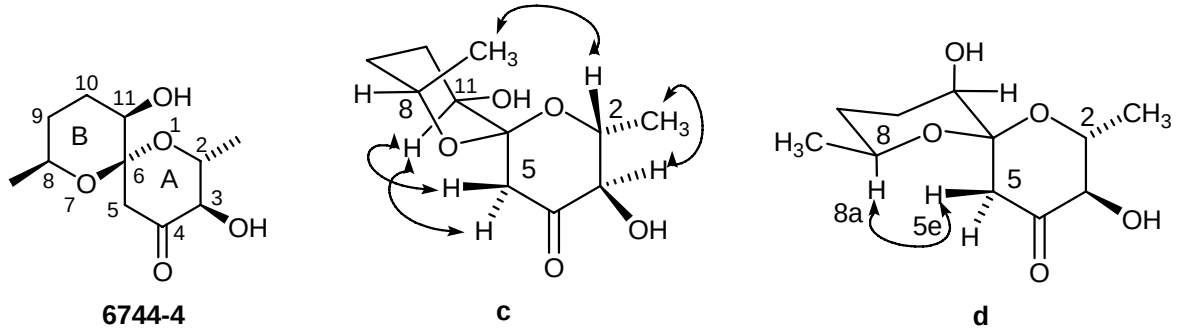


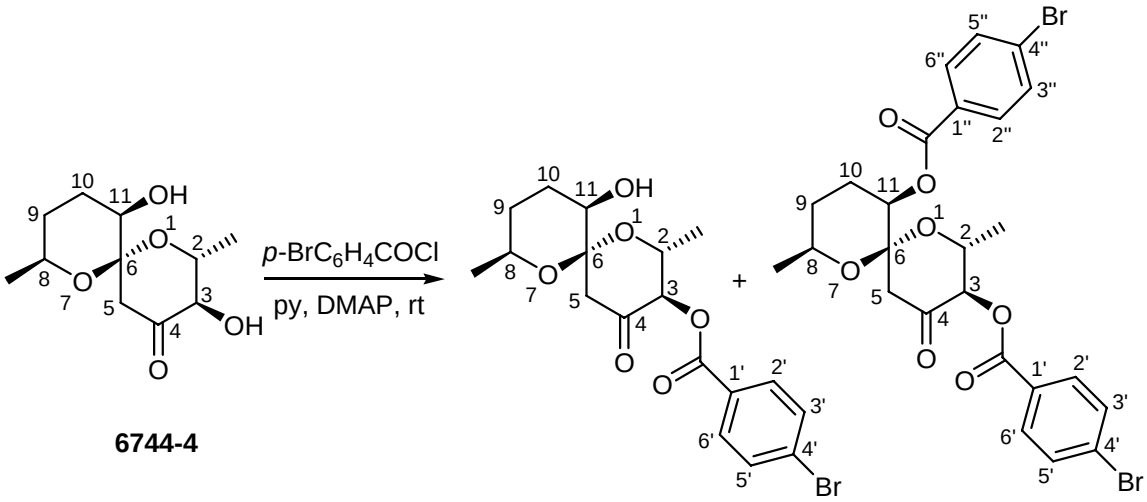
Figure 31: Structure of **6744-4** and relative configuration (**c** and **d**) of **6744-4**

The chirality of the spiroacetalic center was addressed first. The strong NOE correlation between H_a-2 with 8-CH_3 was indicative, requiring an axial position of the methyl group shown in conformation **c**. Model considerations showed, that proximity of H_a-2 with 8-CH_3 was not possible in any possible conformation if the spiroacetalic center was inverted. Similarly, the stereochemistry at C-8 had to be as shown in structure **c**. With an assumed inverted center at C-6, not a single conformation was possible accounting for the strong NOE interaction with long distance (seven bonds!) H_a-2.

On the other hand, a strong correlation of H_a-8 with 5_e-H was also observed (structure **d**). This was not possible with an equatorial H-8 and the alternative chair conformation of ring B with an equatorial 8-CH_3 had to be assumed to account for this strong five bond distant correlation.

Whereas the NOE data left no alternative for the C-6 and C-8 chirality centers, the configuration of the C-11 hydroxyl group was less easily assigned. The only experimental evidence were the **equally strong** NOE interactions of 11-H in ring B with **both** H_a-5 and H_e-5 in ring A (structure **c**). With assumed *cis* configuration of 8-CH_3 and 11-OH and an equatorial hydroxyl group, 11-H is nicely intersecting H_a-5 and H_e-5 with close and equal distance. The alternative chair configuration with axial OH has only proximity with H_a-5 in all conformations, in contrast to the observed correlation with **both** H_a-5 and H_e-5. Thus, based on this data, we propose a structure with *cis* configuration of 8-CH_3 and 11-OH as shown in structure **c**. However, further evidence (not available in the moment from NOE) has to be found to unambiguously confirm the configuration at C-11 of spiro compound **6744-4**.

Compound **6744-4** was reacted with 4-bromobenzoyl chloride (experimental part **7.3.2.5.1**) to afford the 4-bromobenzoates **6744-4a** and **6744-4b** (Scheme 13) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. Unfortunately, none of the 4-bromobenzoates were suitable for X-ray analysis. However, the analyses of the NMR spectra of **6744-4a** and **6744-4b** were in total agreement with the proposed structure of **6744-4** and thus reconfirm the structure of the spiroketal **6744-4**. The elucidation of the relative configuration of the other stereocenters (C-6, C-8, and C-11) and also the absolute configuration of **6744-4** is underway. A literature survey revealed that **6744-4** is a novel compound.



Scheme 13. *p*-Bromobenzoylation of **6744-4**

Table 4: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data for **6744-4**

no.	δ_H (mult., *J* in Hz)	δc	COSY	HMBC
2	4.07 (1H, qq, $J_{2,3}$ = 9.5, $J_{2,2}$ = 6.2)	72.5	C*H*$_3$-2, H3	*C*H$_3$-2, C3, C4, C6
2-CH_3	1.47 (3H, d, $J_{2,2}$ = 6.2)	18.7	H2	C2, C3, C4, C6
3	3.83 (1H, dddd, $J_{3,2}$ = 9.5, $J_{3,OH}$ = 4.1, $J_{3,5}$ = 1.1)	78.0	H2, O*H*-3, H5	C2, *C*H$_3$-2, C4
3-OH	3.52 (1H, d, $J_{OH,3}$ = 4.1)		H3	C2, C3, C4
4		205.8		
5	2.90 (1H, d, $J_{5,5}$ = 13.8)	45.0	H3, H5	C3, C4, C6, C11
	2.83 (1H, dd, $J_{5,5}$ = 13.8, $J_{5,3}$ = 1.1)			
6		101.3		
8	3.90 (1H, sextet, *J* = 6.1)	69.9	C*H*$_3$-8, H9	C6, *C*H$_3$-8, C10
8-CH_3	1.26 (3H, d, $J_{8,8}$ = 6.5)	20.7	H8	C6, C8, C9
9	1.67 (2H, q, *J* = 6.1)	27.6	H8, H10	C6, C8, *C*H$_3$-8, C10, C11,
10	1.99 (1H, m)	24.8	H9, H10, H11	C6, C8, C9, C11
	1.76 (1H, m)			
11	3.55 (1H, sextet, $J_{11,10}$ = 10.3, $J_{11,OH}$ = 6.4, $J_{11,10}$ = 3.4)	69.7	H10, O*H*-11	C5, C9, C10
11-OH	2.50 (1H, d, $J_{OH,11}$ = 6.4)		H11	C6, C10, C11

2.2.2.5 Characterization of 6744-5 as (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one

Compound **6744-5** (Fig. 32) was isolated from the column fraction by elution with CH_2Cl_2/2% MeOH. It was obtained as white fine needles with m.p. 159 °C. It is not visible under UV light. It is soluble in CH_2Cl_2 and $CHCl_3$. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour (R_f = 0.23, CH_2Cl_2/3.8% MeOH).

The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6744-5** displayed 12 carbon resonances, while the HMQC experiment indicated that 11 out of the 12 carbons were attached to protons. ^{1}H NMR (500 MHz, $CDCl_3$) and DEPT 135 (125 MHz, $CDCl_3$) spectra revealed the presence of eight methines of which two are olefinic and five are oxygenated, one methylene and two methyl carbons. The ^{1}H NMR and ^{13}C NMR chemical shifts are shown in Table 5. The resonance at δ = 173.2 ppm in the ^{13}C NMR spectrum and the carbonyl band at 1732 cm^{-1} in the IR spectrum were characteristics for the presence of a carbonyl carbon of an ester or lactone. In the ^{1}H NMR spectrum of **6744-5**, the one one-proton broad singlet at δ = 2.57 and one one-proton doublet δ = 4.04 ppm did not show any connectivity with any carbon in the HMQC spectrum. This information, in conjunction with the two bands at 3444 and 3390 cm^{-1} in the IR spectrum, suggested the presence of two hydroxyl groups.

Analysis of one- and two-dimensional NMR spectra including COSY, HMQC, and HMBC led to the assignment of the structure '**a**' as shown in Figure 32. In this structure, the C2(C1'-C2'-C3')-C3(OH)-C4(OH)-C4a-C8a-C8-C7(CH_3) portion was assigned by tracing of cross peaks in the COSY spectrum. A tetrahydropyranone ring moiety was disclosed by HMBC correlations (H-4a/C-5; H-8a/C-5 and CH_3-7/C-5) and the chemical shift of C-5 (δc = 173.2 ppm). A tetrahydropyrandiol ring moiety was disclosed by a HMBC correlation of H-8a/C-2.

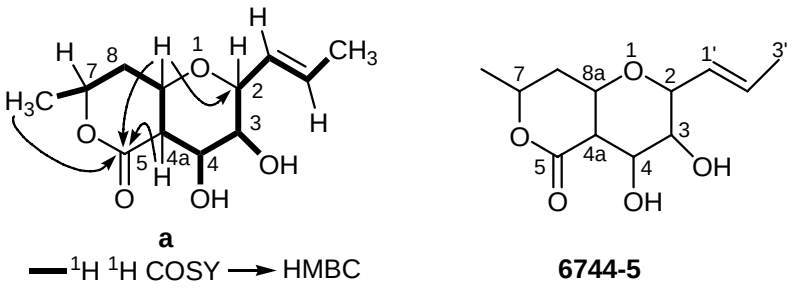
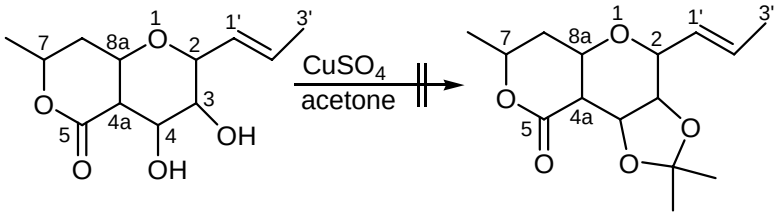


Figure 32: Connectivities of **6744-5** (**a**) and structure of **6744-5**

In both ring moieties, namely tetrahydropyranone and tetrahydropyrandiol, the C-4a and C-8a carbons are common, indicating that the two rings are fused at C-4a and C-8a as shown in Figure 32. Finally, the gross structure of **6744-5** was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 243, suggesting the molecular formula $C_{12}H_{18}O_5$ in agreement with the NMR spectra.

Treatment of **6744-5** with copper sulphate and acetone did not give the expected acetonide (Scheme 14), indicating that **6744-5** is a *trans*-diol.



Scheme 14. Attempted acetonide formation from **6744-5**

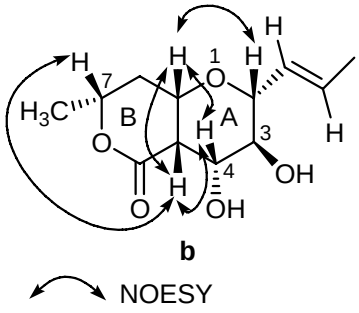


Figure 33: Relative structure of **6744-5** (**b**)

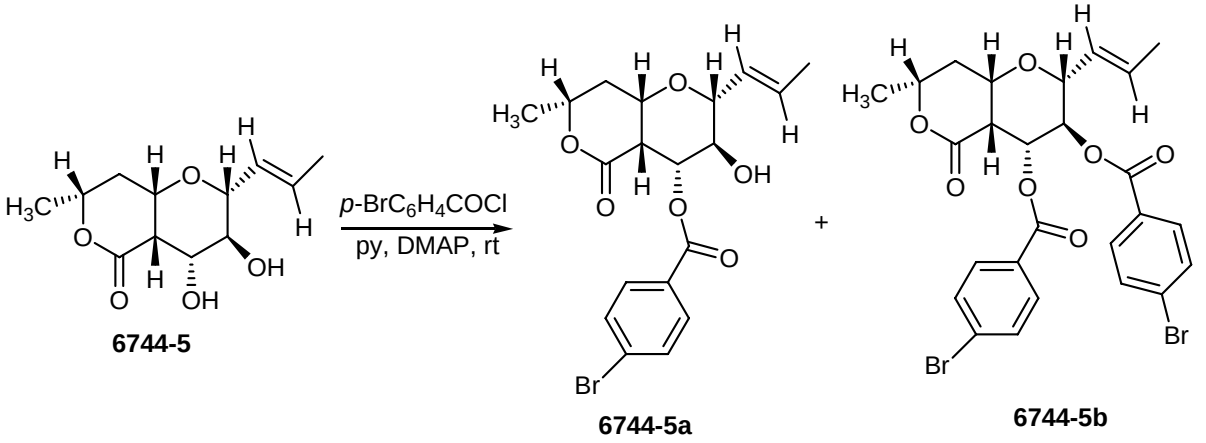
The relative configuration of **6744-5** was determined by tracing of cross peaks in the NOESY spectrum (Figure 33). The cross peaks between H-2/H-8a and vice versa, H-4/H-4a & H-8a and vice versa, and H-4a/H-7 & H-8a and vice versa indicated that the protons H-2, H-4, H-4a, H-7, and H-8a are on the same side of the ring (Structure **b**, Fig. 33). Careful examination of models along with the NOESY interactions and proton-proton coupling constants of **6744-5** allowed the determination of its relative configuration which showed that the two six-membered rings have *syn*-anellation and all four substituents are equatorial. Ring A (Fig. 33) adopted a chair conformation in which 'C_4' and 'O' are above and below the plane, respectively. This is confirmed by the coupling constants of $J_{3,4} = J_{3,2} = 8.5$ Hz, which are possible only when all the three substituents of ring A are equatorial. Finally, the hydrogen bonding between the OH-4 and carbonyl group of the ester moiety confirmed that ring A adopted the chair conformation. On the other hand, ring B (Fig. 33) adopted a boat conformation in order to provide an equatorial arrangement for its methyl group. The

coupling constant of $J_{8,8a}$ = 9.1 Hz is only possible when ring B is in a boat conformation. Besides, the absence of NOE interaction between H-8a and H-7 also indicates that ring B is in a boat conformation. The coupling constant of $J_{7,8}$ = 12.0 Hz indicated an *anti* relationship between the C-7 and one of the C-8 protons, which also indicated the equatorial arrangement of the methyl group and ring B is in a boat conformation. It is further confirmed by a NOE interaction between H-4a and H-7, which is possible, only when ring B could adopt a boat conformation (for discussion of absolute configuration see section 4.3).

Table 5: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data for **6744-5**

no.	δ_H (mult., *J* in Hz)	δc	COSY	HMBC
2	3.57 (1H, t, $J_{2,3} = J_{2,1'} = 8.1$)	81.1	H3, H1’	C3, C4
3	3.66 (1H, t, $J_{3,4} = J_{3,2} = 8.1$)	72.1	H2, O*H*-3, H4	C4
3-OH	2.57 (1H, brs)		H3	
4	3.71 (1H, m)	73.4	H3, O*H*-4, H4a	C3, C5, C8a
4-OH	4.04 (1H, d, $J_{OH,4} = 11.2$)		H4	C4
4a	3.02 (1H, t, $J_{4a,4} = J_{4a,8a} = 3.8$)	44.8	H4, H8a	C3, C4, C5, C7, C8a
5		173.2		
7	4.38 (1H, septet, $J_{7,8}$ = 12.0, $J_{7,7}$ = 6.3, $J_{7,8} = 3.9$)	72.7	C*H*$_3$-7, H8	
7-CH_3	1.44 (3H, d, $J_{7,7} = 6.3$)	20.5	H7	C5, C7, C8, C8a
8	2.48 (1H, dddd, J_{gem} = 15.2, $J_{8,8a}$ = 9.1, $J_{8,7} = 3.9$) 1.81 (1H, dtd, J_{gem} = 15.2, $J_{8,7}$ = 12.0, $J_{8,8a} = 3.0$)	36.9	H7, H8, H8a	C7, C8a
8a	4.16 (1H, sextet, $J_{8a,8}$ = 9.1, $J_{8a,4a}$ = 3.8, $J_{8a,8} = 3.0$)	71.2	H4a, H8	C2, C5, C7
1’	5.52 (1H, qqqq, $J_{1',2'}$ = 15.4, $J_{1',2}$ = 8.1, $J_{1',3'} = 1.4$)	127.7	H2, H2’, C*H*$_3$-3’	C3’
2’	5.89 (1H, qq, $J_{2',1'} = 15.4$, $J_{2',3'} = 6.4$)	131.4	H1’, C*H*$_3$-3’	C2
3’	1.78 (3H, dd, $J_{3',2'} = 6.4$, $J_{3',1'} = 1.4$)	18.0	H1’, H2’	C1’, C2’

Compound **6744-5** was reacted with 4-bromobenzoyl chloride (experimental part **7.3.2.6.1**) to afford the 4-bromobenzoates **6744-5a** and **6744-5b** (Scheme 15) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. Unfortunately, the very fine needles of **6744-5a** and **6744-5b** were not suitable for X-ray analysis. However, the analyses of the NMR spectra of **6744-5a** and **6744-5b** were in total agreement with the proposed structure of **6744-5** and thus reconfirm the structure of **6744-5**.



Scheme 15. *p*-Bromobenzoylation of **6744-5**

A literature survey revealed that **6744-5** is a novel compound.

2.2.2.6 Characterization of 6744-6 as (2*R*,3*S*,4*S*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one

The isolation of metabolites from very polar fractions of the silica gel chromatography was hampered by the presence of dark brown polymeric material. The entire polar fraction was therefore subjected to acetylation using acetic anhydride/pyridine in dichloromethane (experimental part **7.3.2.7**) with the hope that polar hydroxyl groups would be converted to the less polar esters, which can be purified more easily. From this experiment, a nonseparable mixture of two acetylated derivatives (**6744-6a** and **6744-6b**) was isolated (Scheme 16 and 17). The presence of the *cis*-diol **6744-6** (Fig. 34) was deduced indirectly from its acetylated product **6744-6a**.

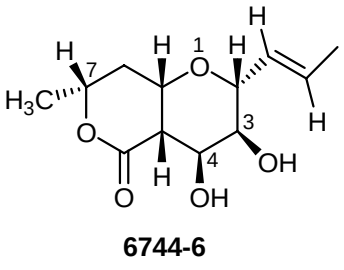


Figure 34: Structure of **6744-6**

The ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectra showed signals representing a minor and a major component of very closely related structures in the ratio of approximately 1:2.4. Both of the derivatives produced NMR spectra very similar to those of **6744-5** (Section **2.2.2.5**), but the presence of two additional acetyl groups was evident from ^{13}C NMR signals* (*NMR data of the minor derivative were discussed only; Table 6) at δ = 169.2 and 169.6 ppm (carbonyl groups), and δ = 20.7 and 20.9 ppm (methyl groups). The latter two corresponded to two additional singlets in ^{1}H NMR at δ = 1.99 and 2.17 ppm (Table 6). HMBC correlations (Fig. 35) were observed between the acetyl carbonyl groups at δ = 169.2 & 169.6 ppm and H-3 & H-4, respectively, indicating acetylation at these positions. This was also evident from the downfield shifts of these two protons in **6744-6a** in comparison with those of **6744-5**. The other NMR data, except the coupling constant of $J_{3,4}$ = 3.3 Hz, were found to be almost superimposable with those of **6744-5**. Finally, the gross structure of **6744-6a** was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 327, suggesting the molecular formula $C_{16}H_{22}O_7$ in agreement with the NMR spectra and the mass difference of 84 units with that of **6744-5**.

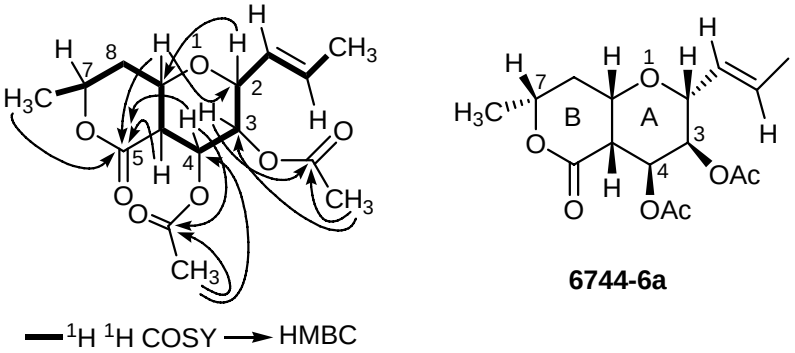
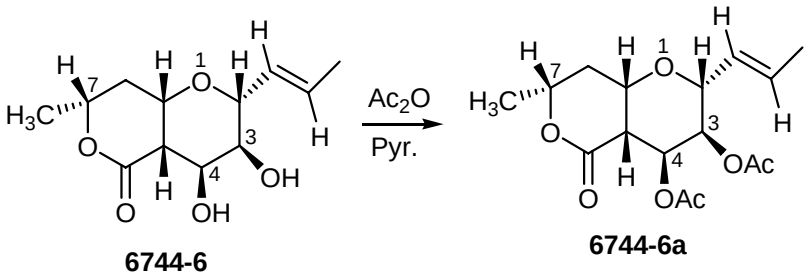


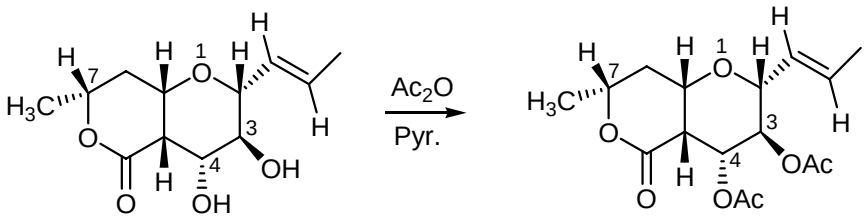
Figure 35: Connectivities of **6744-6a** and structure of **6744-6a**

Compound **6744-6b** has the same structural relationship to **6744-5** as has the **6744-6a** to **6744-5**. In fact, **6744-6b** is closer to **6744-5**, as all its coupling constants, including $J_{3,4}$, are similar to those of **6744-5**. Thus structure **6744-6b** could be attributed as the *trans* diacetylated derivative of **6744-5**, as it was presented in Scheme 17. On the other hand, the coupling constant of $J_{3,4}$ = 3.3 Hz in **6744-6a** indicated that it is a *cis* diacetylated derivative derived from the *cis*-diol **6744-6** as shown in Scheme 16.



6744-6 **6744-6a**

Scheme 16. Acetylation of **6744-6**



6744-5 **6744-6b**

Scheme 17. Acetylation of **6744-5**

The relative and absolute configurations of both **6744-6a** and **6744-6b** were determined by comparison with those of **6744-5**. The almost identical coupling pattern and chemical shifts of signals of **6744-6b** with those of **6744-5** proved the identical relative and absolute configurations of both compounds.

Careful examination of models along with proton-proton coupling constants of **6744-6a** allowed the determination of its relative configuration which showed that the two six-membered rings have *syn*-anellation as it was for **6744-5**. Ring B adopted the boat conformation as it was also for **6744-5**. Ring A (Fig. 35) adopted a chair conformation and thus it allows the equatorial arrangement of its alkenyl and 3-acetyl substituents and axial arrangement for its 4-acetyl substituent. This is confirmed from the coupling constants $J_{3,2}$ = 10.0 and $J_{3,4}$ = 3.3 Hz, which are possible only when the 4-acetyl substituent of ring A is axial and other two substituents are equatorial (for discussion of absolute configuration see section 2.5.4).

A literature survey revealed that **6744-6** is a new compound.

Table 6: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data for **6744-6a**

no.	δ_H (mult., *J* in Hz)	δc	COSY	HMBC
2	4.08 (1H, dd, $J_{2,3}$ = 10.0, $J_{2,1'}$ = 7.7)	76.0	H3, H1'	C3, C4, C8a, C1', C2'
3	5.11 (1H, dd, $J_{3,2}$ = 10.0, $J_{3,4}$ = 3.3)	67.8	H2, H4	C2, 3-O*CO*CH_3, C4, C8a, C1'
3-OCO*CH3*	1.99 (3H, s)	20.7		C3, 3-O*CO*CH_3
3-O*CO*CH_3		169.2		
4	5.83 (1H, t, $J_{4,3}$ = $J_{4,4a}$ = 3.3)	67.0	H3, H4a	C2, C3, C4a, C5, C8a, 4-O*CO*CH_3
4-OCO*CH3*	2.17 (3H, s)	20.9		C4, 4-O*CO*CH_3
4-O*CO*CH_3		169.6		
4a	2.88 (1H, t, $J_{4a,4}$ = $J_{4a,8a}$ = 3.3)	44.6	H4, H8a	C3, C4, C5, C8a
5		169.0		
7	4.32 (1H, m)	72.2	C*H3*-7, H8	*C*H_3-7
7-CH_3	1.41 (3H, d, $J_{7,7}$ = 6.2)	20.5	H7	C5, C7, C8, C8a
8	2.44 (1H, dddd, J_{gem} = 15.3, $J_{8,8a}$ = 9.3, $J_{8,7}$ = 3.6) 1.75 (1H, dtd, J_{gem} = 15.3, $J_{8,7}$ = 12.0, $J_{8,8a}$ = 3.3)	36.7	H7, H8, H8a	C4, C4a, C7, *C*H_3-7, C8a
8a	4.44 (1H, tt, $J_{8a,8}$ = 9.3, $J_{8a,8}$ = $J_{8a,4a}$ = 3.3)	67.9	H4a, H8	C2, C5, C7, C8
1'	5.38 (1H, qqqq, $J_{1',2'}$ = 15.3, $J_{1',2}$ = 7.7, $J_{1',3'}$ = 1.7)	127.4	H2, H2', C*H3*-3'	*C*H_3-3'
2'	5.79 (1H)*	131.5	H1', C*H3*-3'	C2, *C*H_3-3'
3'	1.70 (3H, dd, $J_{3',2'}$ = 6.5, $J_{3',1'}$ = 1.7)	17.9	H1', H2'	C1', C2'

* Overlapped with another signal

2.2.3 Biological activity of the secondary metabolites

Two pure compounds of the strain 6744 were tested for antibacterial, antifungal, and antialgal activities, **6744-4** by the agar diffusion assay method[151] and **6744-5** using the microtiter plate test. The results against these organisms are compiled in Table 7.

Table 7: Biological activity of pure metabolites against microbial test organisms

Metabolites	Concentration (mg/mL)	*Bacillus megaterium*	*Microbotryum violaceum*	*Chlorella fusca*
6744-4	1	0	6	0
	5	6	8	7
6744-5*	0.4	42%	58%	15%

Concentration: 50 µL of the given solution per plate; Numbers for the agar diffusion test indicate radius of zone of inhibition in mm; *those for the microtiter test indicate % inhibition compared to the non-inoculated control

Both the metabolites were found to be active against all the test organisms. At the higher concentration **6744-4** exhibited considerable activities against the Gram-positive bacterium *Bacillus megaterium*, the fungus *Microbotryum violaceum*, and the alga *Chlorella fusca*, whereas at the lower concentration it exhibited good antifungal activity. Compound **6744-5** was active against all the test organisms at very low concentration. In fact, the antifungal and antibacterial activities of **6744-5** are very promising. Both **6744-4** and **6744-5** were new metabolites.

2.3 Strain 6760

Strain 6760 was isolated from a plant, a clover, *Trifolium dubium*, from Wustrow, near the Baltic Sea. This fungal strain did not sporulate and thus the identity could not be determined. The crude extract was found to be very fungicidal (all test organisms) and algicidal, slightly antibacterial, but not herbicidal.

2.3.1 Isolation of secondary metabolites

Strain 6760 was cultivated at room temperature for 28 days in biomalt semi-solid agar medium. After that, this culture medium was extracted three times with ethyl acetate to obtain

the crude extract (9.0 g). The crude extract was subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane, after that gradients of dichloromethane with up to of 20% methanol to afford a total of 41 fractions. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum spray reagents. Similar fractions were combined and subjected to preparative TLC on silica gel and crystallization to isolate the pure compounds **6760-1**, **6760-2**, **6760-3**, and **6760-4**. Compounds **6760-1**, **6760-2**, and **6760-3** were new natural products. The isolated compounds were numbered according to their polarity on TLC.

2.3.2 Structure elucidation

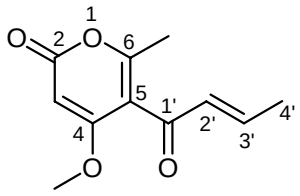
For convenience the discussion on structure elucidation is started from the most polar constituent **6760-4**.

2.3.2.1 Characterization of 6760-4 as 5-((*E*)-but-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (Pyrenocine A)

The most polar constituent **6760-4** (Fig. 36) was isolated from the column fraction by elution with CH_2Cl_2/0-5% MeOH. It was obtained as a white powder with m.p. 100 °C. It appeared as a dark quenching spot on the TLC plate (R_f = 0.30, hexane/50% EtOAc) under UV light at 254 nm. It is soluble in MeOH, $CHCl_3$, and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{1}H NMR and ^{13}C NMR chemical shifts are shown in Table 8.

The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6760-4** displayed 11 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$) experiment showed signals for three methines, one methoxy, and two methyl groups. The resonances at δ = 1.99 and 2.19 ppm in the ^{1}H NMR and at δ = 18.2 and 18.5 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT 135 spectrum, could be attributed for two methyl groups. The resonance at δ = 3.82 ppm in the ^{1}H NMR and at δ = 56.3 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT 135 spectrum, proved the presence of a heteroatom bonded methyl group in **6760-4**. The resonances at δ = 6.33 and 6.81 ppm in the ^{1}H NMR and at δ = 133.1 and 147.2 ppm in the ^{13}C NMR spectra, in conjunction with the DEPT 135 spectrum, could be attributed to two olefinic protons. The cross peak in the COSY spectrum between these two protons indicated that they are vicinal. The coupling constant (J = 15.6 Hz) of these olefinic protons indicated their (*E*)-

configuration. In the ^{13}C NMR spectrum the resonances at δ = 163.0 and 190.5 ppm were characteristic for the carbonyl carbon atom of an ester and ketone, respectively. The mass spectrum displayed the $[M+H]^+$ ion at *m/z* = 209 which, in conjunction with the data of other spectra, suggested the molecular formula $C_{11}H_{12}O_4$ for **6760-4**. Finally, the structure of **6760-4** was confirmed as pyrenocine A by comparison with the published NMR data.[156] Pyrenocine A, a phytotoxin, was also isolated from the fungus *Pyrenochaeta terrestris*.[157,158]



6760-4

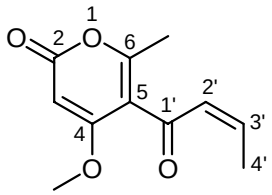
Figure 36: Structure of **6760-4**

2.3.2.2 Characterization of 6760-3 as 5-((*Z*)-but-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one

Compound **6760-3** (Fig. 37) was isolated from the column fraction by elution with CH_2Cl_2/0-5% MeOH. It was obtained as a white semisolid compound. It appeared as a dark quenching spot on the TLC plate (R_f = 0.38, hexane/50% EtOAc) under UV light at 254 nm. It is soluble in MeOH, $CHCl_3$, and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour.

The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6760-3** displayed 11 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$) experiment showed signals for three methines, one methoxy, and two methyl groups. The structure of **6760-3** was elucidated by direct comparison of its spectral data with those of compound **6760-4**. The almost identical NMR coupling pattern and chemical shifts of all signals (Table 8) as well as the same $[M+H]^+$ at *m/z* = 209 proved the identical constitutions of **6760-3** and **6760-4**. The coupling constant between the two olefinic protons at C-2' and C-3' ($J_{2',3'}$ = 11.5 Hz) indicated their (*Z*)-configuration. On this basis, **6760-3** was characterized as 5-((*Z*)-but-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one**.**

Interestingly, the NMR spectra of **6760-3** in $CDCl_3$ indicated that **6760-3** was gradually converted to **6760-4**. The relative ratio of **6760-3**:**6760-4** was 1.6:1 in the NMR spectra. A literature survey revealed that **6760-3** is a novel compound.



6760-3

Figure 37: Structure of **6760-3**

Table 8: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data for **6760-3** and **6760-4**

no.	**6760-3** δ_H (mult., *J* in Hz)	**6760-3** δc	**6760-4** δ_H (mult., *J* in Hz)	**6760-4** δc
2		163.0[a]		163.0
3	5.50 (1H, s)	87.7	5.49 (1H, s)	87.7
4		168.7		168.7
4-OCH_3	3.85 (3H, s)	56.3	3.82 (3H, s)	56.3
5		116.4		114.0
6		162.9[a]		161.4
6-CH_3	2.29 (3H, s)	18.3	2.19 (3H, s)	18.2
1’		190.0		190.5
2’	6.31 (1H, qq, $J_{2',3'}$ = 11.5, $J_{2',4'}$ = 1.5)	129.0	6.33 (1H, qq, $J_{2',3'}$ = 15.6, $J_{2',4'}$ = 1.5)	133.1
3’	6.38 (1H, qq, $J_{3',2'}$ = 11.5, $J_{3',4'}$ = 7.2)	144.9	6.81 (1H, qq, $J_{3',2'}$ = 15.6, $J_{3',4'}$ = 6.9)	147.2
4’	2.20 (3H, dd, $J_{4',3'}$ = 7.2, $J_{4',2'}$ = 1.5)	16.2	1.99 (3H, dd, $J_{4',3'}$ = 6.9, $J_{4',2'}$ = 1.5)	18.5

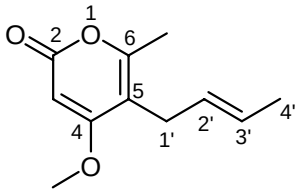
[a] Identical superscripts represent interchangeable assignments.

2.3.2.3 Characterization of 6760-2 as 5-((*E*)-but-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one

Compound **6760-2** (Fig. 38) was isolated from the column fraction by elution with petroleum ether/70-100% CH_2Cl_2. It was obtained as colourless gum. It appeared as a dark quenching spot on the TLC plate (R_f = 0.47, hexane/50% EtOAc) under UV light at 254 nm. It is soluble in MeOH, $CHCl_3$, and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{1}H NMR and ^{13}C NMR chemical shifts are shown in Table 9.

The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6760-2** displayed 11 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$) experiment showed signals for three methines, one methylene, one methoxy, and two methyl groups. The structure of **6760-2** was elucidated by direct comparison of its spectral data with those of compound **6760-4**. Although both the ^{1}H NMR and ^{13}C NMR spectra of **6760-2** were in close correspondence to those of **6760-4**, some differences in the spectra could be observed, such as the resonances at δ = 3.02 ppm in the ^{1}H NMR and at δ = 26.8 ppm in the ^{13}C NMR spectra. These signals, in conjunction with the DEPT 135 spectrum, proved the presence of a methylene group in **6760-2**. On the other hand, the resonance at δ = 190.5 ppm for the carbonyl carbon at C-1' in the ^{13}C NMR spectrum of **6760-4**, was absent in **6760-2**. It could be tentatively assumed that the methylene group of **6760-2** replaced this carbonyl carbon. The cross peaks in the COSY spectrum also confirmed this, showing the connectivities C1'-C2'-C3'-C4' (Table 9). Finally, this was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 195 which, in conjunction with the data of other spectra, suggesting the molecular formula $C_{11}H_{14}O_3$ for **6760-2**.

The (*E*)-configuration for the olefinic protons at C-2' and C-3' of **6760-2** was determined by comparison of its NMR data with those of **6760-1** (Section **2.3.2.4**). A literature survey revealed that **6760-2** is a new compound.



6760-2

Figure 38: Structure of **6760-2**

Table 9: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data for **6760-2**

no.	δ_H (mult., *J* in Hz)	δc	COSY	HMBC
2		164.5		
3	5.46 (1H, s)	87.8		C-2, C-4, C-5, C-1’
4		170.6		
4-OCH_3	3.83 (3H, s)	56.1		C-3, C-4, C-5
5		109.9		
6		158.7		
6-CH_3	2.22 (3H, s)	17.1		C-2, C-3, C-4, C-5, C-6, C-2’
1’	3.02 (2H, dd, $J_{1',2'}$ = 5.2, $J_{1',3'}$ = 1.6)	26.8	H2’, H3’, H4’	C-4, C-5, C-6, C-2’, C-3’
2’	5.40 (1H, m)	127.0[a]	H1’, H3’, H4’	C-5, C-1’, C-4’
3’	5.40 (1H, m)	126.1[a]	H1’, H2’, H4’	C-5, C-1’, C-4’
4’	1.66 (3H, tt, $J_{4',3'}$ = 6.1, $J_{4',2'}$ = 2.6, $J_{4',1'}$ = 1.3)	17.5	H1’, H2’, H3’	C-5, C-1’, C-2’, C-3’

[a] Identical superscripts represent interchangeable assignments.

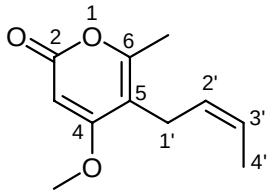
2.3.2.4 Characterization of 6760-1 as 5-((*Z*)-but-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one

Compound **6760-1** (Fig. 39) was isolated from the column fraction by elution with petroleum ether/70-100% CH_2Cl_2. It was obtained as colourless gum. It appeared as a dark quenching spot on TLC plate (R_f = 0.72, hexane/50% EtOAc) under UV light at 254 nm. It is soluble in MeOH, $CHCl_3$, and CH_2Cl_2. Spraying the developed plate with cerium-molybdenum spray reagent, followed by heating, gave a purple colour. The ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of **6760-1** displayed 11 carbon resonances, while the DEPT 135 (75 MHz, $CDCl_3$) experiment showed signals for three methines, one methylene, one methoxy, and two methyl groups.

The structure of **6760-1** was elucidated by direct comparison of its spectral data with those of **6760-2**. The almost identical NMR coupling pattern and chemical shifts of all signals as well as the same $[M+H]^+$ at *m/z* = 195 proved the identical constitutions of **6760-1** and **6760-2**. In fact, **6760-1** has the same structural relationship to **6760-2** as has the **6760-3** to **6760-4**. The coupling constant between the two olefinic protons at C-2’ and C-3’ of **6760-1** and **6760-2**

could not be determined due to the overlapping of their signals and consequently, the (*E*)/(*Z*)-configuration could not be decided. It could be tentatively proposed by comparing their chemical shifts of all signals (especially, the signals of olefinic protons) and polarity that **6760-1** and **6760-2** possess the (*Z*)- and (*E*)-configuration, respectively. On this basis, **6760-1** was characterized as 5-((*Z*)-but-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one**.**

Interestingly, the NMR spectra of **6760-1** in $CDCl_3$ indicated that **6760-1** was gradually converted to **6760-2**, as it was the case also for **6760-3** to **6760-4**. The relative ratio of **6760-1**: **6760-2** was approximately 1:1 in the NMR spectra. A literature survey revealed that **6760-1** is a novel compound.



6760-1

Figure 39: Structure of **6760-1**

2.3.3 Biological activity of the secondary metabolites

The crude extract of the strain 6760 was found to be very fungicidal and algicidal, and slightly antibacterial. Considering the activity of the crude extract three test organisms were selected to study the antibacterial, antifungal, and antialgal activities of the two pure compounds by agar diffusion assay method.[151] The results against these organisms are compiled in Table 10.

Table 10: Biological activity of pure metabolites against microbial test organisms

Metabolite	Concentration (mg/mL)	*Bacillus megaterium*	*Microbotryum violaceum*	*Chlorella fusca*
6760-2	1	0	5	5
	5	5	5	6
6760-4	1	0	6	7
	5	6	7	7

Concentration: 50 µL of the given 1 or 5 mg/mL solution per plate; Numbers indicate radius of zone of inhibition in mm.

Both the metabolites were found to be fungicidal and algicidal, and slightly antibacterial, as expected from the activity of the crude extract. Metabolites **6760-2** and **6760-4** exhibited considerable activities against the fungus *Microbotryum violaceum* and the alga *Chlorella fusca* both at higher and lower concentrations. They also exhibited antibacterial activity at the higher concentration, but at the lower concentration they were found to be inactive against the bacterium *Bacillus megaterium*.

3 Results and discussion: metabolites from the plant *Prismatomeris tetrandra*

Prismatomerin (**PT-1**, Fig. 40) and its glucoside gaertneroside (**PT-2**, Fig. 41) were isolated from a plant *Prismatomeris tetrandra* by Mr. Sujit Kumar Dey, research group of Prof. Dr. M. Mosihuzzaman and Prof. Dr. Nilufar Nahar, Department of Chemistry, University of Dhaka, Bangladesh. Preliminary biotests showed that prismatomerin (**PT-1**) has remarkable anticancer activity. It selectively inhibited renal cancer and also showed activity against non-small cell lung, prostate, melanoma, ovarian, and breast cancer cell lines.[159] It showed potent activity on 60 human tumor cell lines (log_{10} TGI = -5.98; LC_{50} = -4.99). Surprisingly, the specific optical rotations of plumericin (**40**, Fig. 40) (+179 °)[160] and oruwacin (**39**, Fig. 40) (+197 °)[161] are nearly opposite to that of prismatomerin (**PT-1**) (–136 °) with a comparable order of magnitude, though they revealed a good structural similarity and they also have the same relative configurations. This suggests that prismatomerin (**PT-1**) may have the opposite absolute configuration with respect to plumericin (**40**) and oruwacin (**39**). This in itself is highly remarkable. The existence of enantiomeric iridoids suggests the possibility of different biosynthetic pathways. So, the determination of the absolute configuration of this highly active compound is worthwhile. That is why we were motivated to determine the absolute configuration of prismatomerin (**PT-1**).

3.1 *Prismatomeris tetrandra* (Roxb) K. Schum

Prismatomeris tetrandra (Roxb) K. Schum belongs to the family Rubiaceae, a family of about 450 genera and 5500 species[162] distributed mainly in tropical regions, but a small number also grows in the temperate areas. *P. tetrandra* is an evergreen shrub, distributed in Assam, North Bengal, and Andaman Islands of India and it is also available in hilly areas of Bangladesh. *P. tetrandra* was used for the treatment of several ailments in traditional folklore medicine as the juice of leaves in stomach-ache and poultice of leaves in fresh wounds.[163,164] The literature survey revealed that a large number of different alkaloids, anthraquinones, anthraquinols, and iridoids have been isolated from Rubiaceae. Tu *et al.* reported the isolation of eight secondary metabolites from this species.[165]

3.1.1 Isolation of secondary metabolites

Dried and powdered leaves were extracted using a dichloromethane-methanol (1:1) solvent system. The residue obtained from this extract was suspended in water and partitioned with dichloromethane. The residue of the dichloromethane soluble part was suspended in methanol-water (9:1) and partitioned with hexane to separate the fatty and non-polar metabolites. Repeated silica gel chromatography of the residue of the aqueous methanol soluble part afforded the new iridoid prismatomerin (**PT-1**) as white crystals. Chromatographic purification followed by HPLC of one of the more polar fractions of the same residue afforded the glucoside gaertneroside (**PT-2**)[166] as a pale yellow solid.

3.1.1.1 Derivatization by benzoylation

Compound **PT-1** was reacted with 4-bromobenzoyl chloride to afford the 4-bromobenzoate of **PT-1** (**PT-1a**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. Derivative **PT-1a** is reported here for the first time.

3.1.2 Structure elucidation

Dr. Dietmer Gehle, Department of Chemistry, University of Paderborn helped to elucidate the structures isolated from the plant *Prismatomeris tetrandra*.

3.1.2.1 Characterization of PT-1 as prismatomerin

Compound **PT-1** (Fig. 40) was isolated from the aqueous 90% MeOH fraction by repeated silica gel column chromatography. It was obtained as fine needles with m.p. 134-135 °C. It is soluble in CH_2Cl_2, $CHCl_3$, and EtOAc.

The ^{13}C NMR spectrum (75 MHz, $CDCl_3$) of **PT-1** displayed 20 carbon resonances, while the DEPT 135 experiment sorted these signals into 1 methoxy, 12 methines, and 7 quaternary carbons. The key to the assembly of the structure of **PT-1** resided in the connectivities in the ^1H-NMR spectrum starting from the H-1 resonance at δ = 5.67. This resonance was readily identified as the signal for the acetal proton on the basis of the chemical shift of the directly bonded carbon at δ = 102.4. Connectivities within the five spin system containing the H-1 resonance were established from a COSY experiment (H1-H9; H9-H1 and H5; H5-H6, H7 and H9; H6-H5 and H7; H7-H5 and H6, Table 11). The HMBC spectrum also demonstrated some important correlations from H-1 (δ = 5.67) with C-10 (δ = 82.0) and of H-3 (δ = 7.50) with C-1 (δ=102.4). In addition, the correlations between H-10 (δ = 5.25) with C-13 (δ = 144.4) and of H-13 (δ = 7.81) with C-10 (δ = 82.0) demonstrated the connection of the olefin moiety at C-11 of a

tetracyclic ring. Furthermore, the correlations between H-13 (δ = 7.81) with C-2'/6' (δ = 133.3) and of H-2'/6' (δ = 7.71) with C-13 (δ = 144.4) suggested the connection of the phenolic ring with the olefin moiety. The correlation of OC*H*$_3$ (δ = 3.81) with C-14 (δ = 166.8) proved the existence of a methyl ester group and another correlation with C-4 (δ = 109.5) confirmed that this ester moiety is connected at C-4. All of these data suggested the existence of a tetracyclic iridoid skeleton comparable to plumericin (**40**)[167,168] or oruwacin (**39**).[161]

The details of the relative configuration of the new iridoid **PT-1** were then elucidated by comparison of its spectral data with those of oruwacin (**39**)[161] and plumericin (**40**), recently reisolated by our group from *Plumeria rubra*.[160] Although both the ^{1}H-NMR and the ^{13}C-NMR spectra of **PT-1** were in close correspondence with those of **40** and **39**, some deviations could be observed due to the different residues at C-13. In the ^{1}H-NMR spectrum of **PT-1**, the singlet at δ = 3.92 for the aromatic methoxyl group, present in the spectrum of oruwacin (**39**), was missing. Instead, the ^{1}H-NMR spectrum of **PT-1** showed two symmetric pairs of coupled two-proton signals at δ = 7.71 (H-2' and H-6') and at δ = 6.97 (H-3' and H-5') (Table 11), assigned to a 1,4-disubstituted benzene ring present in **PT-1**. The DEPT 135 spectrum of **PT-1** also showed only one resonance for a primary carbon atom at δ = 51.7 for the methyl ester group at C-15. The gross structure of **PT-1**, in comparison to **40** and **39**, was confirmed by the mass spectrum with $[M+H]^+$ at *m/z* = 369, suggesting the molecular formula $C_{20}H_{16}O_7$ in agreement with the NMR spectra, replacing the ferulate group in **39** by a phenolic ring in **PT-1**.

Similarly, the ^{1}H-NMR spectrum of plumericin (**40**) was almost superimposable with the coupling patterns and chemical shifts of the non-phenolic portion of **PT-1**. In the ^{1}H-NMR spectrum of **40**, as expected, the two pairs of coupled two-proton signals of the 1,4-disubstituted benzene ring of **PT-1** were missing and replaced by an additional doublet at δ = 2.09 (*J* = 7 Hz) caused by the methyl group at C-13, resonating at δ = 15.9 in the ^{13}C-NMR spectrum.

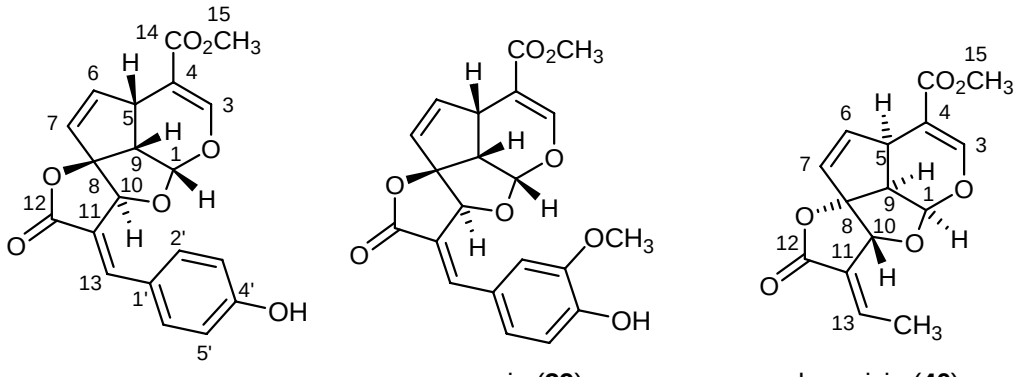
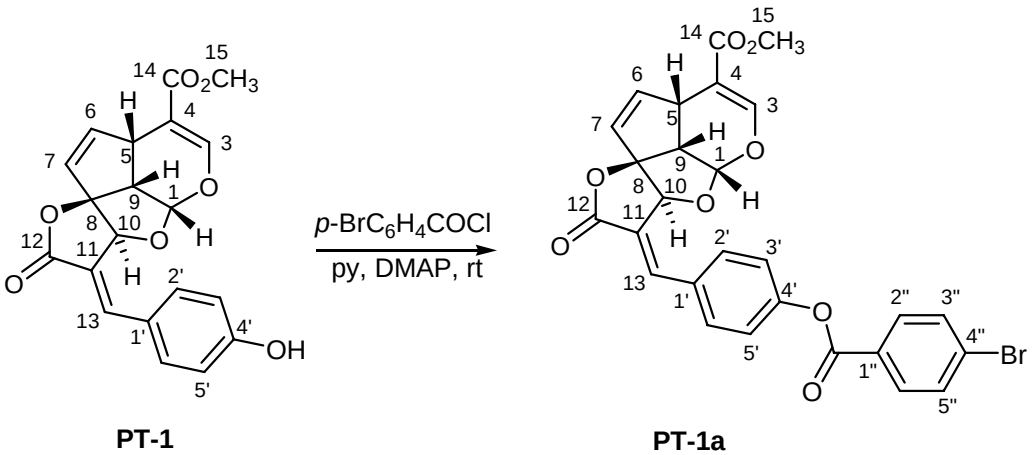


Figure 40: Structures of prismatomerin (**PT-1**), oruwacin (**39**), and plumericin (**40**)

Recently, the relative configuration of plumericin (**40**, Fig. 40) was unambiguously determined by X-ray single crystal analysis.[160] The almost identical NMR coupling pattern and chemical shifts of all signals apart from the phenolic portion of **PT-1** with those of **40** proved the identical relative configuration of both iridoids. This statement was further supported by a 1D NOE experiment of **PT-1**, showing considerable interactions of H-9 with the methine protons H-1 and H-5, indicating that they are on the same side of the ring (Fig. 40). A 1D NOE experiment was also used to determine the *E*-configuration of **PT-1**. Irradiation of the methine proton at δ = 5.25 (H-10) showed considerable interactions with the aromatic protons at δ = 7.71 (2'- and 6'-H) and not with 13-H. This is only possible with an *E*-configuration of the double bond similar as in **40** or **39** with the rotating aromatic ring in **PT-1**.

A literature survey revealed that prismatomerin (**PT-1**) is a new natural product.

Prismatomerin (**PT-1**) was reacted with 4-bromobenzoyl chloride to afford the 4-bromobenzoate **PT-1a** (Scheme 18, experimental part **7.3.4.2.1**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. Unfortunately, the very fine needles of **PT-1a** were not suitable for X-ray analysis. However, the analysis of the NMR spectra of **PT-1a** were in total agreement with the proposed structure and especially all the 1D and 2D NMR data related to H-1 and H-7 contributed to reconfirm the results obtained for **PT-1**.



Scheme 18: *p*-Bromobenzoylation of **PT-1**

Table 11: ^{1}H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data for prismatomerin

no.	δ_H (mult., *J* in Hz)	δc	COSY	HMBC
1	5.67 (1H, d, *J* = 5.8)*	102.4	H9	C8, C9, C10
2				
3	7.5 (1H, s)	152.9		C1, C4, C5, C6, C14
4		109.6		
5	4.08 (1H, tt, *J* = 9.5, 2.0)	38.5	H6, H7, H9	C1, C3, C4, C6, C7, C9
6	6.07 (1H, dd, *J* = 5.4, 2.1)	141.1	H5, H7	C5, C7, C8, C9
7	5.67 (1H)*	126.5	H5, H6	C5, C6, C8, C9
8		104.5		
9	3.56 (1H, dd, *J* = 9.5, 5.8)	54.2	H1, H5	C5, C6, C8
10	5.25 (1H, brs)	82.0		C7, C8, C12, C13
11		120.2		
12		170.2		
13	7.81 (1H, brs)	144.4		C10, C11, C12, C2'/C6'
14		166.8		
15	3.81 (3H, s)	51.7		C14
1'		126.2		
2'/6'	7.71 (2H, d, *J* = 8.6)	133.3	H3'/H5'	C13, C2'/C6', C3'/C5', C4'
3'/5'	6.97 (2H, d, *J* = 8.6)	116.3	H2'/H6'	C1', C3'/C5', C4'
4'	5.73 (1H, brs, 4'-OH)	158.9		

*overlapping each other

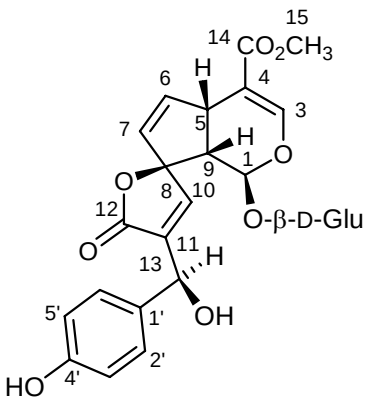
We also tried to assign the absolute configuration of prismatomerin (**PT-1**) based on quantum-mechanical calculation of its CD spectrum and comparison with the experimental CD spectrum. The measured and calculated CD spectra differed from each other significantly so that no statement about the absolute configuration of **PT-1** can be made. Similar attempts with the benzoate **PT-1a** also revealed the same problem.

3.1.2.2 Characterization of PT-2 as gaertneroside

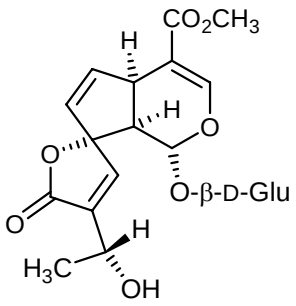
Compound **PT-2** (Fig. 41) was isolated from the aqueous 90% MeOH fraction by repeated silica gel column chromatography followed by HPLC. It was obtained as a pale yellow solid with m.p. 150-151 °C. It is soluble in MeOH.

The ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) spectral data of **PT-2** revealed the presence of an iridoid skeleton with a glucosyl unit and a carbomethoxy group at C-4. The quaternary carbons at δ = 96.7 (C-8), 171.0 (C-12), and 136.5 (C-11), as well as a methine carbon at δ = 148.7 (C-10) were very characteristic for a spiro-lactone ring at C-8, corresponding to a plumieride (**41**, Fig. 41) type iridoid.[168-172] This could also be concluded from the comparison of most other ^{1}H-NMR and ^{13}C-NMR data of **PT-2** and **41**, particularly from the coupling pattern of the iridoid skeleton. Similarly as described for **PT-1** and **40**, the only difference resides in the replacement of the methyl group at C-13 in **41** by a 4-hydroxyphenyl group in **PT-2**. In comparison with **PT-1**, some additional resonances in the ^{1}H-NMR spectrum of **PT-2** at δ = 3.23 (H-2”), 3.29 (H-5”), 3.41 (H-3”), 3.41 (H-4”), 3.72 (H-6”), 3.81 (H-6”), and 4.70 (H-1”) were observed and the chemical shifts of directly bonded carbon of these protons were determined as δ = 73.1 (C-2”), 76.9 (C-5”), 76.4 (C-3”), 69.5 (C-4”), 60.8 (C-6”), and 99.1 (C-1”), respectively. All these data disclosed, along with all the carbon resonances shown for **PT-1**, a glucosyl residue linked at a tricyclic iridoid skeleton. Compound **PT-2** was therefore considered to be a glucoside of the tricyclic counterpart of **PT-1**. A noticeable downfield shift of H-10 and C-10 and upfield shift of H-13 and C-13 were also observed in **PT-2** in comparison with **PT-1**. This could be attributable to the shift of the olefinic double bond of C13-C11 to C11-C10 and to the fact that C-13 is now hydroxylated. In summay, **PT-2** has the same structural relationship to prismatomerin (**PT-1**) as has the glucoside plumieride (**41**) to plumericin (**40**).

The ^{1}H NMR and ^{13}C NMR data of **PT-2** were found to be identical to those reported for the iridoid glucoside ‘gaertneroside’ isolated from *Morinda morindoides*.[166] Compound **PT-2** was found to inhibit the activation of the classical pathway of the complement system (IC_{50} = 58 ± 6 μM).[166] Since the complement system is one of the major effector pathways in the process of inflammation, inhibitors of complement activation, such as gaertneroside (**PT-2**), could be potentially useful in the treatment of inflammatory diseases.



gaertneroside (**PT-2**)



plumieride (**41**)

Figure 41: Structures of gaertneroside (**PT-2**) and plumieride (**41**)

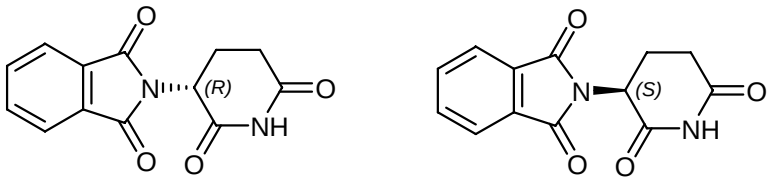
Since prismatomerin (**PT-1**) and gaertneroside (**PT-2**) were isolated from the same plant extract, it is reasonable to assume the same relative and absolute configurations of the relevant identical stereogenic centers. On this basis, the same relative configuration was adopted for both **PT-1** and **PT-2**. Since the absolute configuration of **PT-1** is still unknown, any discussion of the absolute configuration for both iridoids is not possible at this moment.

4 Determination of the Absolute Configuration by the Exciton Chirality Method

4.1 Introduction

The unambiguous knowledge of the stereostructure of biologically active natural products, in particular their absolute configuration, is an important precondition, e.g. for directed structure-activity relationship investigations. While the relative configuration can often quite easily be established spectroscopically (e.g. by NMR) or by X-ray diffraction, the attribution of the absolute stereostructure is sometimes a more difficult task, in particular for novel classes of compounds.

Chirality is a property which often determines the actions and behaviour of molecules in rather unexpected ways. For example, lemons and oranges both contain limonene, the different enantiomers giving rise to subtle differences in the aroma properties of these fruits. Similarly, *R*- and *S*-carvone have different tastes: the former tasting of spearmint and the latter of caraway.[14] The biological effects of chiral substances also depend on their absolute configurations. Two enantiomers can differ significantly in their strength and quality of effect depending considerably on the affinity and selectivity of the active substance for the active site. It is understandable that for the production of these compounds by synthetic means we need to secure them in enantiopure form. We simply cannot face risks similar to the infamous thalidomide (Fig. 42) case of the late 1950s where one enantiomer ((*S*)-enantiomer (**43**, Fig. 42)) of the product turned out to be potently teratogenic.[14]



42
(sedative, hypnotic)

43
(teratogenic)

Figure 42: Structure of (*R*)-thalidomide (**42**) and (*S*)-thalidomide (**43**)

4.2 Exciton chirality method: basic principles

One of the most efficient methods for configurational assignment is the investigation of circular dichroism (CD). With low experimental demand, two enantiomers can be easily distinguished by

their exactly opposite CD spectra. The exciton chirality method, developed from the simple 'dibenzoate chirality method' is a simple and extremely versatile approach for establishing absolute configurations and conformations of organic compounds solely from the measured CD spectrum.[173] The exciton chirality method is based on the nonempirical coupled oscillator theory or group polarizability theory. When two (or more) strongly absorbing chromophores are located nearby in space and constitute a chiral system, their electric transition moments interact spatially so that the energy level of the excited state splits, the result of which is reflected in the UV-VIS and CD spectra. The two chromophores may or may not be identical and, moreover, they do not necessarily have to be in the same molecule to interact.[173]

4.2.1 Exciton coupling between identical chromophores

The coupling of two identical chromophores is illustrated in Figure 43 by *p*-substituted bisbenzoates of a vicinal cyclohaxanediol, for example, a steroidal 2,3-bisbenzoate. Determination of absolute configuration means determination of the absolute sense of chirality between the C2-O and C3-O bonds, which in the case shown is clockwise, defined as a positive chirality; the absolute sense of twist is, of course, the same whether viewed from C3 to C2 or from the opposite direction. Although there may be some free rotation around the two C-O bonds, ester bonds are known to be *s*-trans and the ester carbonyl is *syn* with respect to the methine hydrogens.[173] Hence the direction of the electric transition moment of the benzoate ^1L_a band, which gives rise to the main absorption band, is more or less parallel to the C-O bond in question.

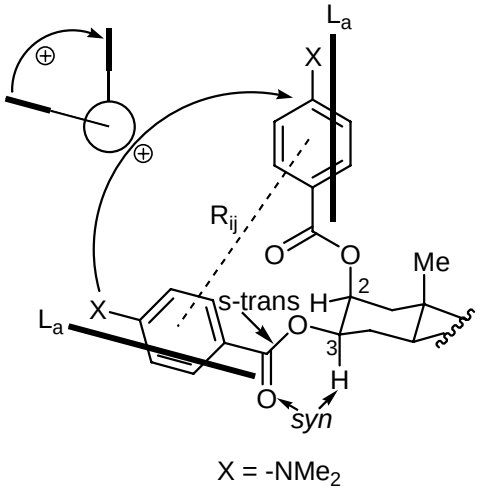


Figure 43: Exciton coupling of two identical chromophores (*i* and *j*) of a steroidal 2,3-bis-*p*-dimethylaminobenzoate with positive chirality and interchromophoric distance R_{ij}

Interaction between the two ^1L_a transition moments results in positive and negative Cotton effects (CEs) in the CD spectrum. If the chirality of the transition moments is clockwise, defined as positive (Fig. 43), the CD becomes a bisignate or 'split' CD and shows a positive first (at longer wavelength) CE and a negative second CE.[173] Such a coupled CD is defined as a positive couplet, and vice versa. The distance between the peak and trough of the split CD curve is called the *amplitude* or *A*, and is positive or negative depending on the sign of the first CE. There is no exception in the signs of bisignate CD curves, positive and negative chiralities always leading to positive and negative couplets, respectively.[173] The following discussion covers some practically pertinent aspects of the exciton chirality method.[173]

4.2.1.1 Some general aspects of the exciton chirality method

This method is very sensitive. The amount of sample required is of the microgram scale. One of the preferred solvents is methylcyclohexane since it does not solvate and distort the spectra; acetonitrile is also a good polar solvent because of its transparency and good solubilizing properties. Methanol should be used with caution because of occurrence of H bonding and occasional ester exchange. Any chromophore with large ε (molar absorptivity) and known direction of μ (transition moment) is suitable for this method. The sign of a split CD is determined by the chirality of the chromophores. The *A* value (amplitude) is maximal at a projection angle of ca. 70°, proportional to ε^2 and inversely proportional to R^2 (R: interchromophoric distance).

4.3 Determination of the absolute configuration of 6744-5

The following discussion on absolute configuration was prepared with the help of Dr. Tibor Kurtán, Department of Organic Chemistry, University of Debrecen, Hungary.

The absolute configuration of **6744-5** (Fig. 44) was determined by the exciton chirality method. Compound **6744-5** was derivatized to the corresponding dibenzoate **6744-5b** (Fig. 44) as described in Scheme 15 (experimental part **7.3.2.6.1**). The absolute configuration was deduced solely from the measured CD spectrum of **6744-5b** (Fig. 45).

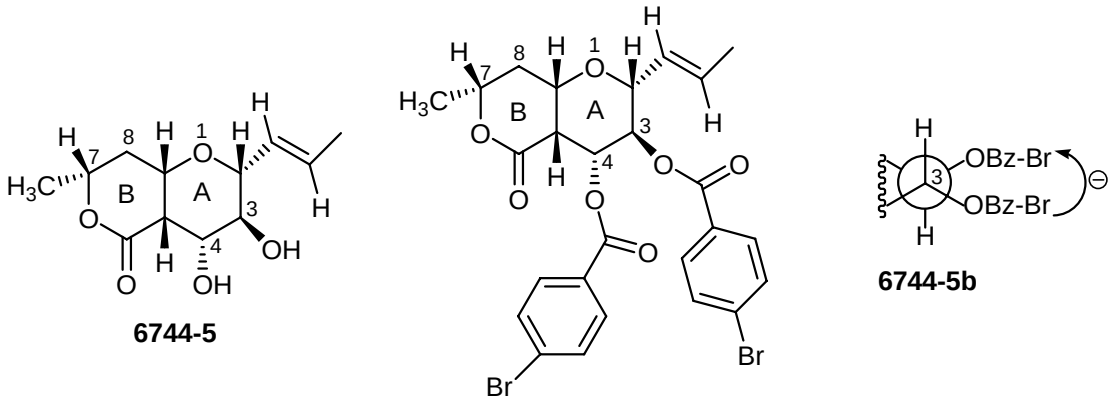


Figure 44: Relative structures of **6744-5** and **6744-5b** and a Newman projection of **6744-5b** showing the relative arrangement of the two ester bonds

The relative configuration of **6744-5** was discussed in details in Section **2.2.2.5**. The same relative configuration is also attributable to its dibenzoate **6744-5b**, as shown in Fig. 44. According to this relative configuration, the two six-membered rings have *syn*-anellation and the four substituents are equatorial. Moreover, ring A has a chair conformation while ring B adopts the boat conformation in order to provide an equatorial arrangement for its methyl group. An exciton coupled interaction takes place between the two vicinal *p*-bromobenzoate chromophores. The direction of the electric transition moment of the benzoate ^1L_a band coincides with the corresponding CH-O ester bond.[173] If the chirality of these transition moments is clockwise (positive chirality), the CD spectrum shows a positive couplet, namely, a positive Cotton effect (CE) at longer wavelength and a negative CE at shorter wavelength, and vice versa.[173]

Since a strong negative CD couplet (amplitude (*A*) = 68) was measured in the ^1L_a region of **6744-5b**, the electric transition moments of the two equatorial *p*-bromobenzoate groups must have negative chirality and hence *R,R* absolute configuration. In Fig. 44, a Newman projection of **6744-5b** shows the relative arrangement of its two ester bonds with their negative chirality. The opposite configuration (*S,S*) would have resulted in a positive couplet. The *p*-bromobenzoate chromophore has a UV maximum at λ = 244 nm (ε = 19500, EtOH)[173] which derives from its ^1L_a band. Accordingly, the CD spectrum of **6744-5b** shows a negative CE above (252 nm (-45.34)) and a positive CE below (235 nm (22.90)) this UV maximum.

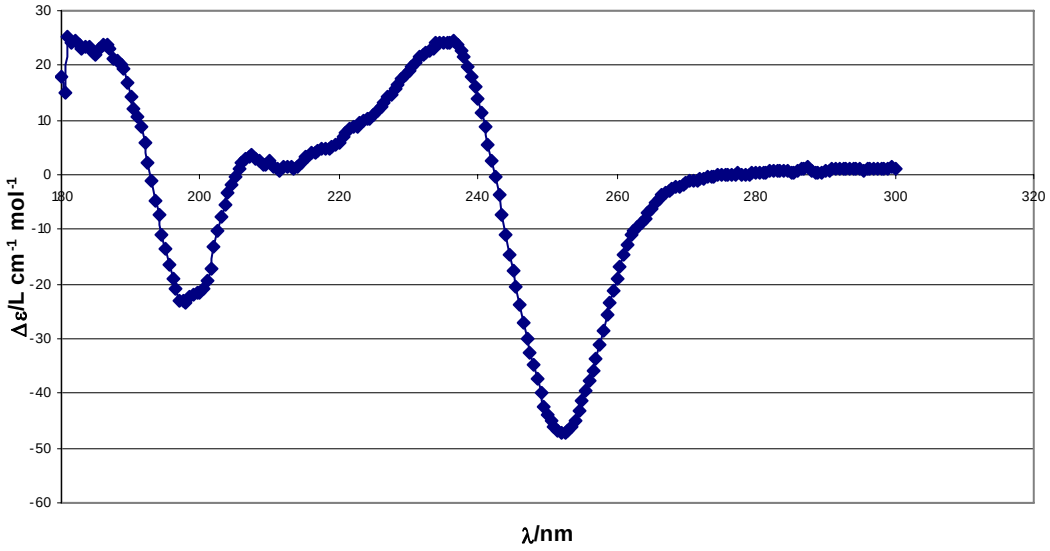


Figure 45: Experimental CD spectrum of **6744-5b**

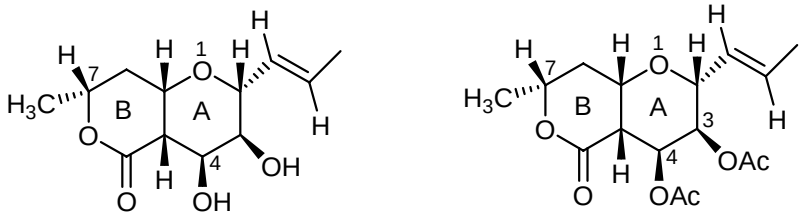
It is also noteworthy that two other CD couplets were also observed in the CD spectrum of **6744-5b**; a positive one around 280 nm and a negative one around 195 nm. The positive CD couplet around 280 nm (283 nm (0.23) and 278 (-0.41)) belongs to the ^1L_b bands of the benzoates. Since the direction of the electric transition moment of the ^1L_b transition is perpendicular to the ^1L_a transition, the chirality of the ^1L_b transitions are opposite to that of the ^1L_a transitions and thus positive CD couplets were measured. The negative couplet around 195 nm (198 nm (-23.25) and 186 (23.88)) belongs to the 1B transition of the benzoates whose electric transition moment has the same orientation as that of the ^1L_a transition which results in a negative couplet.

Any of the observed CD couplets can be used for the configurational assignment, but due to its large amplitude, the one derived from the ^1L_a transition is mainly applied in practice. So, the absolute configuration of **6744-5b** could be attributed as 2*R*,3*R*,4*R*,4a*R*,7*R*,8a*S* and hence the absolute configuration of the parent compound **6744-5** is 2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*.

4.4 Determination of the absolute configuration of 6744-6

The absolute configuration of **6744-6** (Fig. 46) was determined by comparison with that of **6744-5** (Fig. 44). The relative configuration of **6744-6a** (Fig. 46) was discussed in detail in Section **2.2.2.6**. The same relative configuration is also attributable for **6744-6**, as shown in Fig. 46.

According to this relative configuration, the two six-membered rings have *syn*-anellation as it was also for **6744-5**. Ring B adopted the boat conformation as it was also for **6744-5**. Ring A (Fig. 46) adopted a chair conformation and thus it allows the equatorial arrangements of its alkenyl and 3-acetyl substituents and axial arrangement for its 4-acetyl substituent. All but one stereogenic center (C-4) of **6744-6a** remained essentially the same as those of **6744-5**. On this basis, it could be proposed that the absolute configurations for the stereogenic centres of C-2, C-3, C-4a, C-7, and C-8a of **6744-6** are the same as those of **6744-5**, *i.e.*, 2*R*,3*S*,4a*S*,7*R*,8a*S* and for C-4 is opposite, i.e.; 4*S*.



6744-6 **6744-6a**

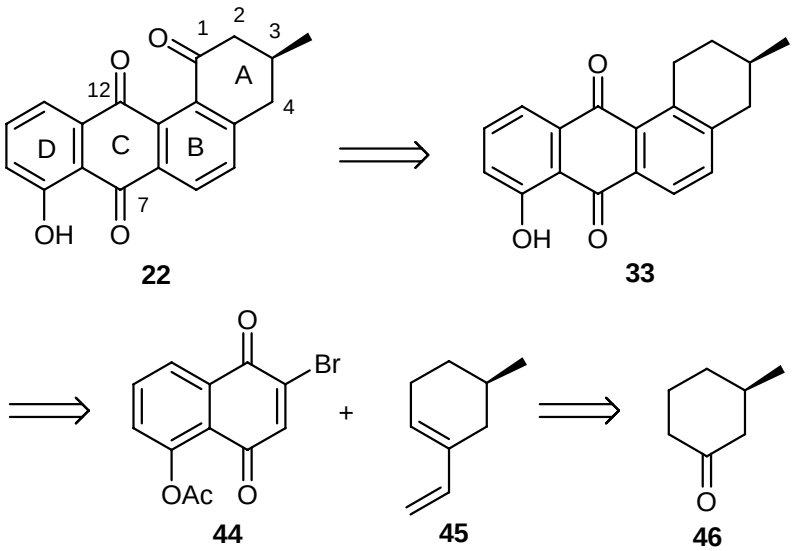
Figure 46: Relative structures of **6744-6** and **6744-6a**

5 Results and discussion: synthetic part

5.1 Synthesis of (+)-ochromycinone

5.1.1 Retrosynthesis

(+)-Ochromycinone was synthesized using the Diels-Alder strategy. Commercially available and surprisingly cheap enantiomerically pure (*R*)-(+)-3-methylcyclohexanone **46** (99% ee) was incorporated as the starting material for the enantiomerically pure diene **45** as shown in the retrosynthetic Scheme 19. 5-Acetoxy-2-bromo-1,4-naphthoquinone (**44**)[174] was used as the dienophile because the bromine atom was known to direct the regiochemistry in the Diels-Alder reaction very efficiently.[175] Thus, the presence of an electron donating substituent such as a methoxy group[134] on the terminal end of the diene can be avoided, reducing the number of synthetic steps. In addition, the presence of the bromine atom in the immediate Diels-Alder adduct facilitates the aromatization of ring B by elimination of hydrogen bromide.[175] The scheme further envisaged the mild photooxidation at C-1[176] in the final step to convert the precursor **33** into (+)-ochromycinone (**22**).



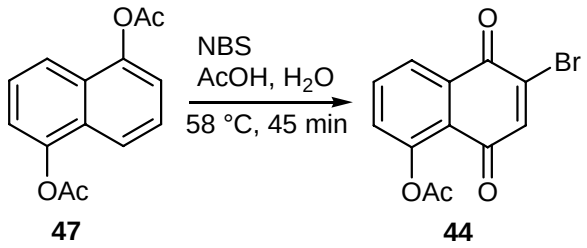
Scheme 19: Retrosynthetic scheme using (*R*)-(+)-3-methylcyclohexanone (**46**) as the source of chirality in the synthesis of (+)-ochromycinone

5.1.2 Results and discussion

The starting materials for the synthesis of (+)-ochromycinone were readily accessible from known compounds.

5.1.2.1 Preparation of 5-acetoxy-2-bromo-1,4-naphthoquinone (**44)**

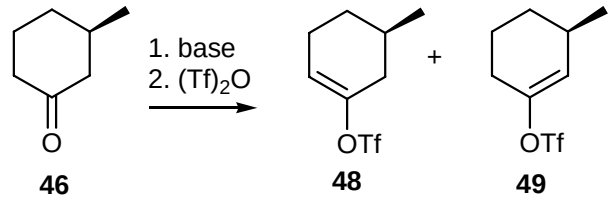
5-Acetoxy-2-bromo-1,4-naphthoquinone (**44**) was prepared by a highly regiospecific one-step procedure following the method of Grunwell and Heinzman.[174] Thus, the reaction between *N*-bromosuccinimide (NBS) dissolved in water and acetic acid, and 1,5-diacetoxynaphthalene (**47**) gave 5-acetoxy-2-bromo-1,4-naphthoquinone (**44**) in 84% yield (Scheme 20).



Scheme 20: Preparation of 5-acetoxy-2-bromo-1,4-naphthoquinone (**44**)[174]

5.1.2.2 Preparation of (*R*)-5-methylcyclohex-1-enyl trifluoromethanesulfonate (**48**) and (*R*)-3-methylcyclohex-1-enyl trifluoromethanesulfonate (**49**)

The synthesis of the required diene **45** started with the deprotonation of enantiomerically pure (*R*)-(+)-3-methylcyclohexanone (**46**) with different bases followed by trapping of the enolates with triflic anhydride to yield the vinyltriflate **48** and its regioisomer **49** (Scheme 21). We expected that the formation of the desired triflate **48** was favoured over the regioisomer **49** due to steric hindrance of the methyl group during kinetically controlled enolate formation.



Scheme 21: Preparation of the vinyltriflate **48** and its regioisomer **49**

Three different bases were tried to investigate the regioselectivity of the enolate formation. The best result was obtained using lithium tetramethylpiperidide (LTMP, **48**:**49** = 5.4:1), followed by lithium diisopropylamide (LDA, **48**:**49** = 2.4:1) and lithium hexamethyldisilazide (LHMDS, **48**:**49** = 2:1) (Table 12).

Antony and Maloney[177] also reported a kinetically controlled reaction with similar results using trityllithium in monoglyme and (±)-3-methylcyclohexanone. Hsung et al.[178] synthesized vinyl triflate **49** (yield 13%) from 3-methylcyclohex-2-enone, but the synthesis of triflate **48** is reported here for the first time.

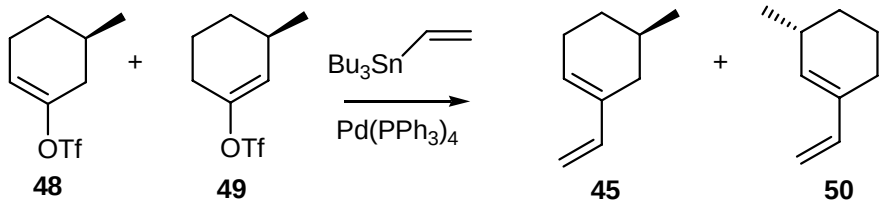
Table 12: Effect of different bases on the regioselectivity of triflate formation

Observation	Base	**48**:**49**	Yield (%)		
			48	**49**	Combined
1	LTMP	5.4:1	60	11	71
2	LDA	2.4:1	31	13	44
3	LHMDS	2:1	---	---	---

The isomer ratio of the enol triflates **48** and **49** was determined by GC. The assignment was possible by analysis of the ^{1}H NMR spectra of the mixtures, showing a broad singlet at δ = 5.78 ppm for the olefinic proton ($CH_2C\mathit{H}$=C) of **48** and a broad singlet at δ = 5.66 ppm for olefinic proton ($CH(CH_3)C\mathit{H}$=C) of **49**.

5.1.2.3 Synthesis of (*R*)-5-methyl-1-vinylcyclohex-1-ene (**45**) and (*R*)-3-methyl-1-vinylcyclohex-1-ene (**50**)

A Stille reaction[179,180] was used to prepare the dienes **45** and **50** by coupling the triflates **48** and **49** (from LDA experiment) and tributylvinylstannane in the presence of a catalytic amount of $Pd(PPh_3)_4$ (2 mol%) in 88% combined yield (Scheme 22), similar to its execution in the deoxybrasiliquinone B synthesis.[181]



48 **49** **45** **50**

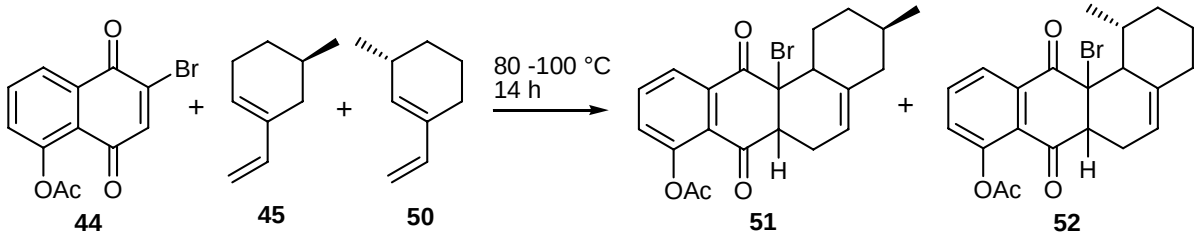
Scheme 22: Synthesis of dienes **45/50** via Stille reaction[179,180] of enoltriflates **48/49** with tributylvinylstannane

The use of α-haloalkanesulfonyl bromides by Block et al.[182] for the racemic vinylcyclohexenes was less convenient for our purpose. The ratio of the dienes **45** and **50** (**45**:**50** = 2.4:1 by NMR) reflected that of the respective triflate starting materials. The respective structures were assigned

by characteristic signals in the ^{1}H NMR spectra for **45** (triplet at δ = 5.78 ppm for CH_2CH=C) or **50** (doublet at δ = 5.64 ppm for $CH(CH_3)CH$=C).

5.1.2.4 Synthesis of (3*R*)-12a-bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-3-methyl-7,12-dioxo-tetraphen-8-yl acetate (**51**) and (1*R*)-12a-bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-1-methyl-7,12-dioxotetraphen-8-yl acetate (**52**)

The Diels-Alder reaction of the dienes **45** and **50** with juglone **44** afforded mixtures (as evidenced from NMR) of two regioisomeric primary Diels-Alder products **51** and **52** in 79% combined yield (Scheme 23). The ratio of the regioisomers improved slightly with respect to the starting dienes **45** and **50** in favour of the desired isomer **51** (**51**:**52** = 2.6:1 by NMR), probably due to steric hindrance of the methyl group in **50** and the bromine atom in **44**.

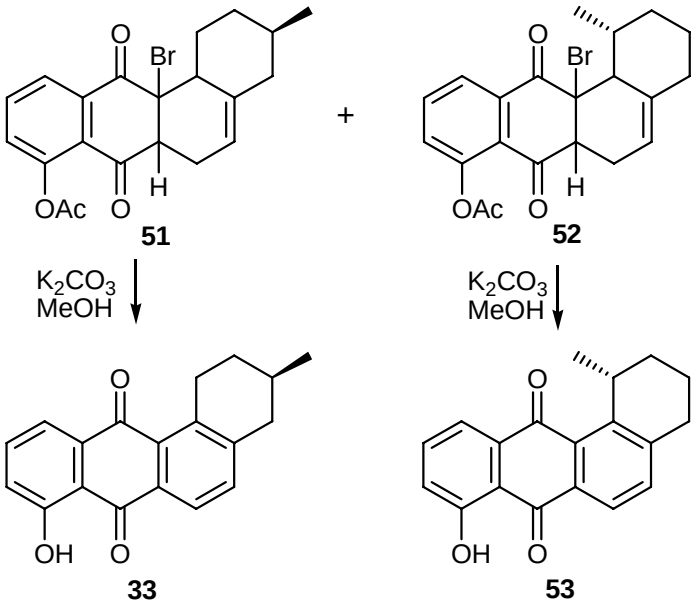


Scheme 23: Synthesis of Diels-Alder products **51** and **52**

5.1.2.5 Synthesis of (*R*)-1,2,3,4-tetrahydro-8-hydroxy-3-methyltetraphene-7,12-dione (**33**) and (*R*)-1,2,3,4-tetrahydro-8-hydroxy-1-methyltetraphene-7,12-dione (**53**)

Dehydrobromination, saponification, and dehydrogenation of **51**/**52** to **33** and **53** were induced in one operation by mild base treatment (K_2CO_3 in methanol) in the presence of air (Scheme 24). Gratifyingly, the major aromatic isomer **33** was isolated in pure form and good yield (48 %) after crystallisation from the mixture of **33** and **53**. The minor isomer **53** was present in the mother liquor and could eventually be obtained in crystalline form after repeated column chromatography and preparative TLC (8%).

Both the aromatic regioisomers **33** and **53** were optically active ($[\alpha]^{25}_D$ = + 114, *c* 0.09, $CHCl_3$ for **33** and $[\alpha]^{25}_D$ = – 173, *c* 0.07, $CHCl_3$ for **53**). The 1D and 2D NMR spectra supported the structures of the respective aromatization products. The structure of the minor product **53** was further confirmed by single crystal X-ray analysis (Figure 47), thus indirectly also proving the structure of the major product **33**.



Scheme 24: Synthesis of the benzo[*a*]anthraquinones **33** and **53**

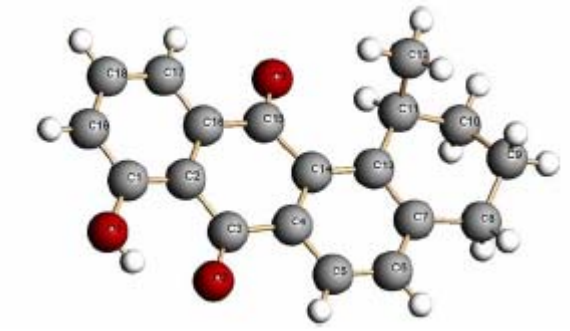


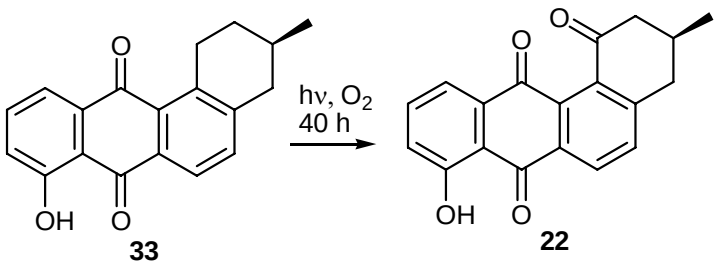
Figure 47: Molecular structure of **53**

5.1.2.6 Photooxidation of (*R*)-1,2,3,4-tetrahydro-8-hydroxy-3-methyltetraphene-7,12-dione (**33**) to (+)-ochromycinone (**22**)

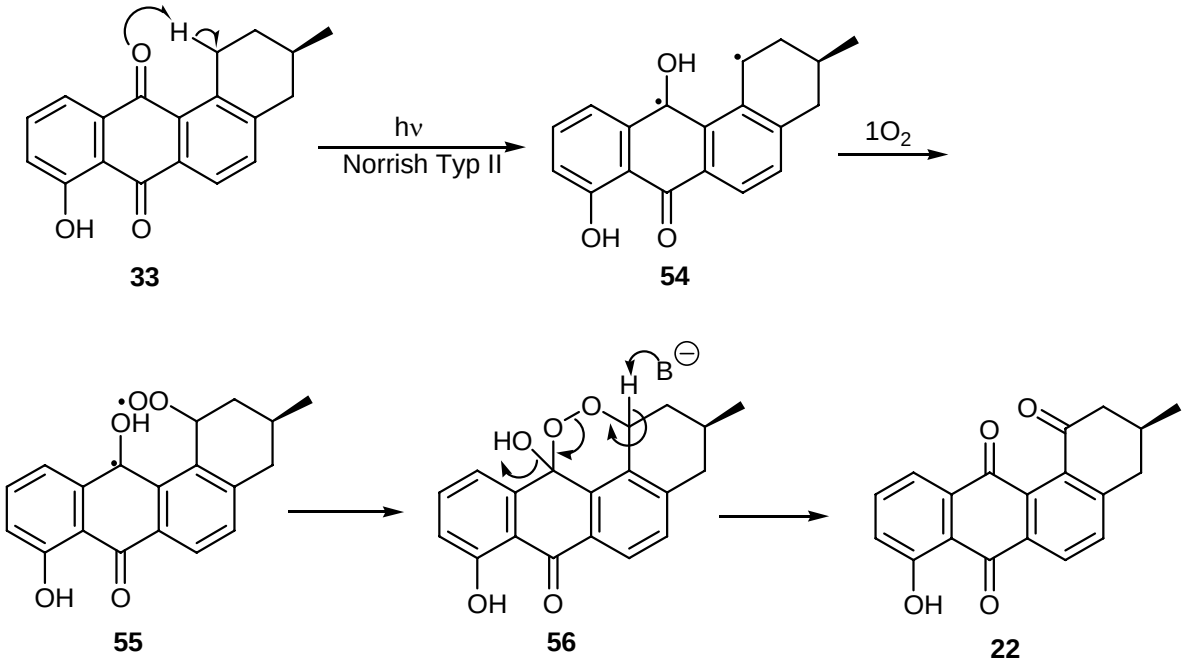
In the final step, the precursor benzo[*a*]anthraquinone **33** was converted to (+)-ochromycinone (**22**) by photooxygenation with diffuse sunlight (Scheme 25) in 70% yield after chromatographic purification followed by crystallization. This mild oxidation reaction was discovered in the laboratory of Prof. Krohn during the synthesis of a daunomycinone/rabelomycinone hybrid[176] (compare[183]) and is now frequently used as the last reaction step in angucycline synthesis.[136,137,184-186] This reaction proved to be of great value for angucycline synthesis. The carbonyl group at C-1 can thus be introduced under mild neutral conditions.

The reaction is initiated by Norrish type II γ-hydrogen abstraction of the excited carbonyl in **33** to yield a diradical **54** as shown in Scheme 26.[85] The H-abstraction requires a very definite steric environment in which the benzylic protons have to be in proximity of the excited carbonyl group. Subsequent addition of the diradical **54** with singlet oxygen is assumed to yield a

peroxodiradical **55** which can cyclize to an unstable hydroxyl-1,2-dioxane **56**. This intermediate is then opened up by proton abstraction to generate the C-1 carbonyl compound (+)-ochromycinone (**22**).



Scheme 25: Photooxidation of **33** to (+)-ochromycinone (**22**)



Scheme 26: Mechanism of photooxidation of **33** to **22**

The relevant anisotropic spectroscopic data of the synthetic material **22** were in agreement with those reported for the natural ochromycinone.[87,108]

5.1.2.7 Determination of the enantiomeric excess (ee) of (+)-ochromycinone (22)

To determine the enantiomeric excess (ee), the specific optical rotation of **22** in chloroform was measured. Surprisingly, a great dependency of the specific optical rotation on the concentration of **22** was found (Table 13, Fig. 48).

Table 13: Dependency of the specific optical rotation on the concentration of (+)-ochromycinone (**22**) in $CHCl_3$

Observation	Concentration (gm/100 mL)	Optical rotation α	Specific optical rotation $[\alpha]^{25}_D$
1	0.012	+00.019	+158.3°
2	0.016	+00.023	+143.8°
3	0.041	+00.045	+109.8°
4	0.069	+00.069	+100.0°
5	0.081	+00.077	+95.1°
6	0.13	+00.114	+87.7°

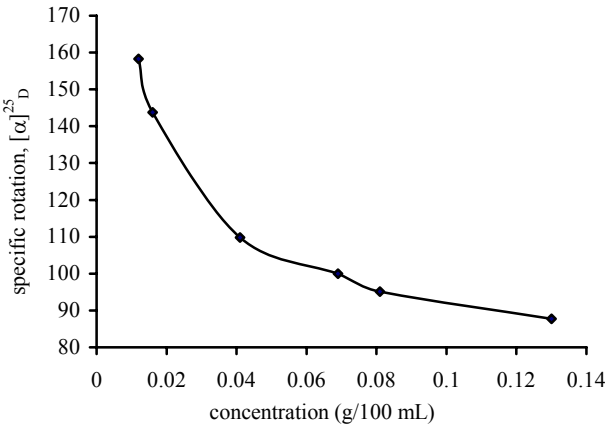


Figure 48: Dependency of the specific optical rotation on the concentration of (+)-ochromycinone (**22**) in $CHCl_3$

To the best of our knowledge, such a pronounced nonlinear effect,[187,188] as shown in Figure 48, has not yet been observed at such low concentrations and may be largely attributed to π-stacking.[189] Interestingly, the effect was not observed in the more polar solvent acetonitrile and is also not seen for the deoxy compound **33**. Evidently, the increase in polarity by the presence of the C-1 carbonyl group contributes to the interaction of the anthracyclinone molecules. In view of these observations, the determination of enanatiomeric excess (ee) in previous enantioselective syntheses by optical rotation[135,136] has to be revisited.

To check the enantioselectivity of **22**, HPLC chromatographic techniques (Ms. Christiane Neuhaus, Department of Chemistry, University of Paderborn helped with chiral HPLC) on an enantioselective stationary phase (Chiracel OD-H-type column) and a partially racemic [+]-ochromycinone (80% ee) as the reference material were used (Fig. 48 and 49). Hexane/20%

isopropanol was used as the mobile phase. Not surprisingly, **22** showed 99% ee (Fig. 49), since the starting material **46** had 99% ee and no racemization-prone step occurred during the synthesis.

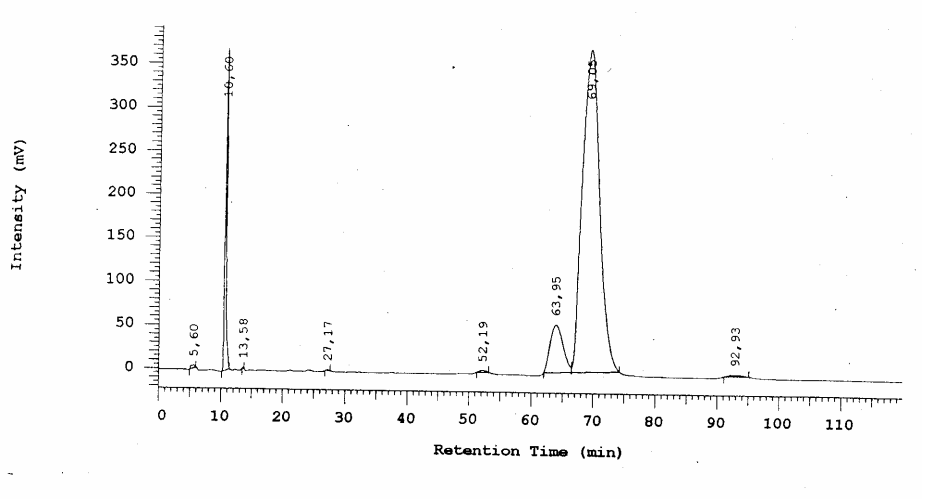


Figure 48: Enantiomeric content of partial racemic (+)-ochromycinone

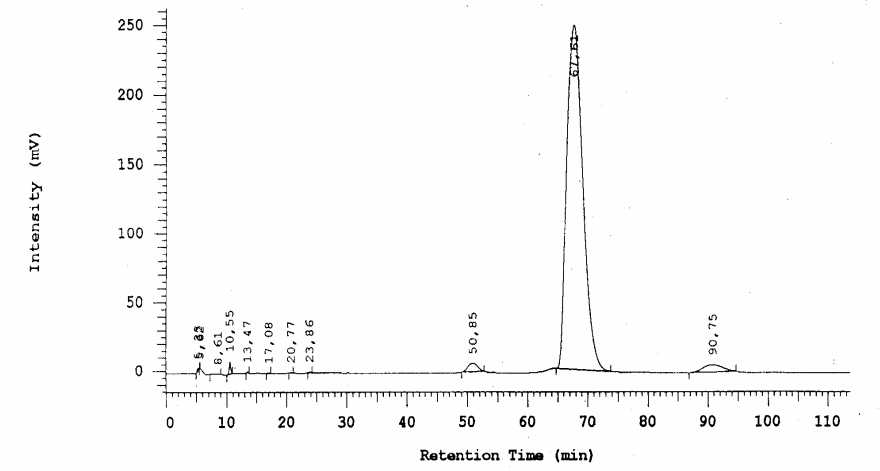


Figure 49: Determination of the enantiomeric excess (ee) of (+)-ochromycinone (**22**)

5.1.2.8 Conclusion

A very short (7 steps from commercially available material) and highly enantiospecific (99% ee) synthesis of (+)-ochromycinone (**22**) in 19% (LTMP as the base) or 12% (LDA as the base) overall yield has been successfully achieved. A large dependency of the specific optical rotation on the concentration of **22** in $CHCl_3$ was observed for the first time for angucyclinones.

6 Summary

The work described in this thesis details the isolation and structure elucidation of secondary metabolites from endophytic fungi and the plant *Prismatomeris tetrandra*, and synthesis of (+)-ochromycinone.

Strain 5681 was identified as an endophytic fungus of *Scytalidium* species. Ten metabolites **5681-1** – **5681-9** were isolated in pure form from the combined crude ethyl acetate extracts by a combination of crystallization, repeated column and preparative TLC chromatography on silica gel. Three compounds (**5681-4**, **5681-8**, and **5681-9**) were new natural products. A polar component was indirectly identified as a new benzoic acid **5681-10** from its four methylation derivatives (**5681-10a** – **5681-10d**). The known metabolites **5681-1** – **5681-3** and **5681-5** – **5681-7** were identified by comparison of their spectral data with those published in the literature. The least polar constituent was identified as (+)-4,7-dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho-[1,2-*b*]furan-5,6-dicarboxylic anhydride (**5681-1**), previously isolated from *Aspergillus silvaticus*[140] and later also from *Roesleria pallida*.[141] The next compound following in polarity, was a related derivative, the dione **5681-2**, found in the levorotatory form in *Gremmeniella abietina*.[142] The dione **5681-2** existed as a mixture of C-5 epimers with the same splitting of some signals in the ^{1}H NMR and ^{13}C NMR spectra. The natural products following in polarity on TLC, were six isocoumarin derivatives. Interestingly, they all had methyl groups at C-5, similar to some natural products previously isolated by Whyte and Gloer,[145] Aldridge et al.,[146] and our group.[53] They were identified as decarboxycitrinone (**5681-3**),[145] 4-acetyl-6,8-dihydroxy-5-methyl-1*H*-isochromen-1-one (**5681-5**),[146] (3*R*,4*R*)-4-acetyl-6,8-dihydroxy-3-methoxy-5-methyl-3,4-dihydroisochromen-1-one (**5681-6**),[53] and (*R*)-4-acetyl-6,8-dihydroxy-5-methyl-3,4-dihydroisochromen-1-one (**5681-7**).[53] The two new isocoumarins were identified as (6,8-dihydroxy-3,5-dimethyl-1-oxo-1*H*-isochromen-4-yl)methyl acetate (**5681-4**) and 6,8-dihydroxy-4-(hydroxymethyl)-3,5-dimethyl-1*H*-isochromen-1-one (**5681-8**). Metabolites **5681-4** and **5681-8** possess an acetoxymethyl and a hydroxymethyl substituent, respectively. The most polar compound, isolated in pure state, was a new highly substituted benzoic acid derivative **5681-9** with unusual hydroxymethylene ketone side chain related in its substitution pattern to isocoumarins. The presence of another new benzoic acid with unusual 1,2-dicarbonyl side chain was indirectly identified as 4,6-dihydroxy-3-methyl-2-(2-oxopropanoyl)benzoic acid from its four methylated derivatives **5681-10a** – **5681-10d** (Sections **2.1.2.10** and **7.3.1.11**). Metabolite **5681-10** is also related in its substitution pattern to isocoumarins.

The crude ethyl acetate extracts showed inhibitory activities in the agar diffusion assay[151] against *Escherichia coli*, *Bacillus megaterium*, *Microbotryum violaceum*, *Eurotium repens*, *Mycothypha microspora*, *Fusarium oxysporum*, and *Chlorella fusca*. Metabolites **5681-5**, **5681-8**, and **5681-9** exhibited prominent activities against the Gram-positive bacterium *Bacillus megaterium*, the fungus *Microbotryum violaceum*, and the alga *Chlorella fusca*. Metabolite **5681-2** showed good antibacterial and antifungal activities. Metabolite **5681-3** showed only good algicidal activity. Metabolites **5681-4** and **5681-6** exhibited only the antifungal and antialgal activities, respectively. Compounds **5681-1** and **5681-7** were found to be inactive against all the test organisms. In fact, most of the pure compounds were active against the test organisms as it was assumed from the bioactivity of the crude extract.

Strain 6744, an endophyte, was characterized taxonomically as *Dinemasporium strigosum*. From the crude ethyl acetate extract six metabolites **6744-1** – **6744-5** were isolated in pure form by a combination of crystallization, repeated column and preparative TLC chromatography on silica gel. Compounds **6744-4** and **6744-5** were new natural products. A polar component **6744-6** was indirectly identified as a new *cis*-diol from its *cis*-diacetylated derivative **6744-6a**. The known metabolites **6744-1** – **6744-3** were identified by comparison of their spectral data with those published in the literature. The least polar constituent was identified as palmitic acid (**6744-1**).[152,153] The next compound following in polarity was identified as ergosterol.[154] The next compound following in polarity on TLC, was an isocoumarin derivative (**6744-3**). Metabolite **6744-3** was identified as a mixture of two diastereomers (approx. ratio 1:3.3) of 4-hydroxymellein.[155] New metabolites **6744-4** and **6744-5** were identified as 3,11-dihydroxy-2,8-dimethyl-1,7-dioxa-spiro[5,5]undecan-4-one and (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano [4,3-*b*]pyran-5(7*H*)-one, respectively. The presence of a new *cis*-diol **6744-6** was indirectly identified as (2*S*,3*R*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl) pyrano[4,3-*b*]pyran-5(7*H*)-one from its diacetylated derivative **6744-6a** (Sections **2.2.2.6** and **7.3.2.7**). Metabolite **6744-4** was reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-4** (**6744-4a** and **6744-4b**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. With the similar hope, **6744-5** was also reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-5** (**6744-5a** and **6744-5b**). Unfortunately, none of the four bromobenzoates were suitable for X-ray analysis. However, the absolute configuration of **6744-5** was determined by the 'Exciton Chirality Method' from its dibenzoate derivative **6744-5b**.

Metabolites **6744-4** and **6744-5** were tested for antibacterial, antifungal, and antialgal activities by the agar diffusion assay method.[151] At the higher concentration, **6744-4** exhibited considerable activities against the Gram-positive bacterium *Bacillus megaterium*, the fungus *Microbotryum violaceum*, and the alga *Chlorella fusca*, whereas at the lower concentration it exhibited good antifungal activity. Compound **6744-5** was active against all the test organisms at very low concentration. In fact, the antifungal and antibacterial activities of **6744-5** are very promising.

Strain 6760 was isolated from a plant, a clover, *Trifolium dubium*. The identity of this fungal strain could not be determined. From the crude ethyl acetate extract of biomalt semi-solid agar culture medium four metabolites **6760-1** – **6760-4** were isolated in pure form by a combination of crystallization, repeated column and preparative TLC chromatography on silica gel. Compounds **6760-1**, **6760-2**, and **6760-3** were new natural products. The most polar constituent **6760-4** was identified as 5-((*E*)-but-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (pyrenocine A) by comparison of its spectral data with those published in the literature.[156] Pyrenocine A, a phytotoxin, was also isolated from a fungus *Pyrenochaeta terrestris*.[157,158] The three new pyrenocines **6760-1**, **6760-2**, and **6760-3** were identified as 5-((*Z*)-but-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one, 5-((*E*)-but-2-enyl)-4-methoxy-6-methyl-2*H*-paran-2-one, and 5-((*Z*)-but-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one, respectively.

The crude extract was found to be very fungicidal and algicidal, and slightly antibacterial. Metabolites **6760-2** and **6760-4** exhibited considerable activities against the fungus *Microbotryum violaceum* and the alga *Chlorella fusca* both at higher and lower concentrations. They also exhibited antibacterial activity at the higher concentration, but at the lower concentration they were found to be inactive against the bacterium *Bacillus megaterium*.

Prismatomerin (**PT-1**) and its glucoside gaertneroside (**PT-2**) were isolated from a plant *Prismatomeris tetrandra* by Mr. Sujit Kumar Dey, research group of Prof. Dr. M. Mosihuzzaman and Prof. Dr. Nilufar Nahar, Department of Chemistry, University of Dhaka, Bangladesh. Preliminary biotests showed that prismatomerin (**PT-1**) has remarkable anticancer activity. It showed potent activity on 60 human tumor cell lines ($\log_{10}$ TGI = -5.98; LC_{50} = -4.99). An effort was made to assign the absolute configuration of prismatomerin (**PT-1**) based on quantum-mechanical calculation of its CD spectrum and comparison with the experimental CD spectrum. The measured and calculated CD spectra differed from each other significantly, so that no

statement about the absolute configuration of **PT-1** can be made. Prismatomerin (**PT-1**) was reacted with 4-bromobenzoyl chloride to afford the 4-bromobenzoate **PT-1a** (Scheme 18, experimental part **7.3.4.2.1**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated. Unfortunately, the very fine needles of **PT-1a** were not suitable for X-ray analysis. Another effort, based on quantum-mechanical calculation of the benzoate **PT-1a**, revealed the same problem as **PT-1**. Since prismatomerin (**PT-1**) and gaertneroside (**PT-2**) were isolated from the same plant extract, it is reasonable to assume the same relative and absolute configurations of the relevant identical stereogenic centers. Since the absolute configuration of **PT-1** is still unknown, any discussion of the absolute configuration for both iridoids was not possible at this moment.

In this thesis a very short, simple, and highly enantiospecific (seven steps, 99% ee) synthesis of (+)-ochromycinone in 19% (LTMP as the base) or 12% (LDA as the base) overall yield is also reported. In contrast to the two known procedures,[135,190] the commercially available and cheap enantiomerically pure (*R*)-(+)-3-methylcyclohexanone **46** (99% ee) was used as starting material. Key steps are the regioselective Diels-Alder reaction of the juglone derivative **44** with the mixture of dienes **45** and **50** derived from **46**, and the photooxidation of 1-deoxyochromycinone (**33**) to (+)-ochromycinone (**22**). A large dependency of the specific optical rotation on the concentration of **22** in $CHCl_3$ was observed for the first time for angucyclinones. In view of this observation, the determination of ee in previous enantioselective syntheses by optical rotation measurement[135,190] have to be revisited. The intermediate **33** could be converted by a two-step operation to another bioactive angucyclinone (+)-rubiginone (**34**)[138] as shown in Scheme 6, indicating the wide application of our synthetic method.

In short, from the three endophytic fungi 22 secondary metabolites were isolated, of which 10 were new. These secondary metabolites belong to diverse structural groups and most of the tested metabolites were found to be antimicrobial. Some of them exhibited prominent antimicrobial activities, which merit further pharmacological evaluation. This proved the enormous biosynthetic potential of the endophytic fungi, especially in the production of antimicrobial metabolites. A new cytotoxic iridoid **PT-1**, isolated from a plant, is also reported. The absolute configuration of two metabolites (**6744-5** and **6744-6**) was established. Moreover, an efficient enantiospecific synthesis of (+)-ochromycinone (**22**) is reported. The current study could be a good source of reference in the scientific world due to its novel findings, especially the data for a total of 45 compounds, of which 27 are new including natural products and derivatives thereof and intermediates of the synthetic steps.

7 Experimental part

7.1 General methods and Instrumentation

Thin Layer Chromatography (TLC): TLC was carried out using pre-coated silica gel aluminium sheets. Compounds were detected on TLC plates (Silica gel 60 F_{254}; Merck AG, Darmstadt) under UV light at 254 or 366 nm and/or by spraying with cerium-molybdenum (10 g of cerium (IV) sulphate, 25 g of molybdatophosphate, 60 mL of conc. H_2SO_4, and 940 mL of water) and/or vanillin-sulfuric acid[191] (1% vanillin in conc. H_2SO_4) spray reagents.

Column Chromatography: Silica gel 60 (230-400 mesh, 0.040-0.063 mm; Merck AG, Darmstadt) was used as the stationary phase.

Preparative Thin Layer Chromatography (PTLC): Plates (20 × 20 cm) from Macherey-Nagel, Germany (1.0 and 0.5 mm layer thickness) were used for preparative TLC.

Gas Chromatography (GC) analysis: Conditions for the GC analysis: Initial temperature: 50 °C; final temperature: 100 °C; temperature program: 5 °C/min; initial time: 0; end time: 10 min; solvent: CH_2Cl_2; column type: 25 m × 0.25 mm (inner diameter), FS-OV-1-CB- 0.1, CS 32180-72; carrier gas: helium; carrier gas flow rate: 1 mL/min; detector type: FID.

High Performance Liquid Chromatography (HPLC): Conditions for HPLC analysis: Column type: Chiracel OD-H; detector type: UV (operated at 254 nm); pump A type: L-700; solvent A: hexane 80%; solvent B: isopropanol 20%; volume injected: 20 μL; flow rate: 0.7 mL/min.

UV/VIS Spectroscopy: UV/VIS spectra were recorded on a Shimadzu UV-2101 PC double beam spectrophotometer using 1.0 cm quartz cells.

IR Spectra: The spectra were measured as thin films of neat compound on a Nicolet FT-IR 510 p. The absorptions are given in wave numbers (cm^{-1}).

Optical Rotation: Optical rotations were measured using a Perkin-Elmer 241 polarimeter equipped with a standard cuvette (d = 10 cm) and Na lamp (D line, λ = 589 nm).

CD Spectroscopy: CD spectra were recorded on a JASCO J-715/150S spectrometer at 25 °C. Temperature was controlled by Peltier thermostat and CH_3CN was used as the solvent. The spectra were recorded by Dr. Tibor Kurtán, Department of Organic Chemistry, University of Debrecen, Hungary.

Mass Spectra: Mass spectra were recorded on a Fison MD 800 spectrometer. Relative intensity is related to base peak.

NMR spectra: NMR spectra were recorded using the BRUKER ARX 200, AMX 300, and ARX 500 spectrometers. All spectra were recorded at room temperature (RT). The chemical shifts are reported in ppm relative to $SiMe_4$. The ^1H NMR resonance multiplicity is described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Broad resonances are indicated as brs (broad). In ^{13}C NMR, sec. and quat. mean secondary and quaternary C atoms, respectively. Hydrogen connectivity (C, CH, CH_2, CH_3) information was obtained from DEPT-135 experiments. Proton and carbon peak assignments were based on 2D NMR analysis (COSY, HMQC, and HMBC).

X-ray analysis: The single crystal X-ray analysis was performed by Dr. U. Flörke.

Melting Point: Melting points were determined using a Gallenkamp melting point apparatus. One-side open capillaries were used.

Purification of Solvents: Purification of solvents was performed by standard methods.[139,192]

Program for CD Spectra calculation: BDZDO/MCDSPD program package version Z-07A from J. W. Downing, Department of Chemistry and Biochemistry, University of Colorado, Boulder, USA; modified by J. Fleischauer, W. Schlecker, and B. Kramer, or DZDO program version 4.23, ZDO program version of 22.06.2001.

Program to calculate the conformers: Spartan SGI version 5.1.3; Wavefunction Inc., Irvine, USA.

7.2 Microbiological work

Collection and cultivation of the fungal strains as well as the first bioactivity tests of the crude extracts and pure compounds were performed in the research group of Prof. Dr. Aust under the supervision of PD Dr. Barbara Schulz, Institut für Mikrobiologie, TU Braunschweig and BASF AG, Ludwigshafen. Crude extracts and pure compounds were tested for antibacterial, antifungal, and antialgal activities by agar diffusion assay method.[151] The taxonomic identification of the fungal strains was done by Dr. S. Draeger. Bioactivity tests of some pure compounds were also performed by BASF AG.

7.3 Experimental part: Isolation of Natural Products

7.3.1 Metabolites from Strain 5681

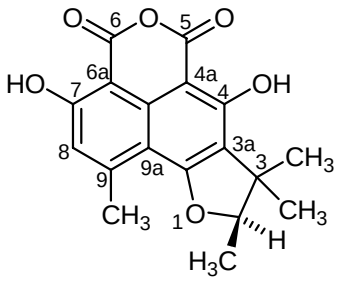
7.3.1.1 Isolation of secondary metabolites

Strain 5681 was cultivated at room temperature for 111 days in two different culture media, namely, malt-soya and biomalt semi-solid agar media. The two culture media were then extracted separately, once with petroleum ether and three times with ethyl acetate to obtain the crude extracts. Ethyl acetate soluble fractions from both media showed a similar spectrum of compounds on TLC and were therefore combined (4.63 g) and subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane, and after that gradients of dichloromethane with up to 10% methanol. A total of 251 fractions were collected. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum or vanillin-sulfuric acid spray reagents. Similar fractions were combined and subjected to preparative TLC on silica gel and crystallization to isolate the pure compounds. After evaporation of solvent, the column fraction eluted with pure CH_2Cl_2 gave slightly impure crystals that were recrystallized from CH_2Cl_2/Et_2O to give **5681-1** (54.3 mg) as white needles. The column fraction of CH_2Cl_2/0-3% MeOH was subjected to preparative TLC on silica gel (toluene/10% EtOAc) to obtain **5681-2** (9.0 mg, brown semisolid), **5681-4** (11.1 mg, white needles), **5681-5** (12.5 mg, white solid mass), **5681-6** (20.2 mg, white needles), and **5681-7** (9.1 mg, white crystals). The slightly impure brown crystals obtained from the column fraction CH_2Cl_2/0-1% MeOH were further purified by washing with different solvents to give **5681-3** (84.8 mg) as white needles. Adopting a similar procedure, crude crystals of the polar fraction CH_2Cl_2/3-5% MeOH were purified to give **5681-9** (73.7 mg, white solid mass). The same polar fraction was subjected to preparative TLC on silica gel to obtain **5681-8** (3.3 mg) as brown fine needles.

To aid structure elucidation, 12.0 and 50.0 mg of **5681-9** respectively, were acetylated using acetic anhydride/pyridine in dichloromethane, and methylated using an ethereal diazomethane solution (Section **7.3.1.10**). Derivatives **5681-5a** (6.0 mg) and **5681-9a** (16.8 mg) were isolated by PTLC as acetylated and methylated derivatives of **5681-9**, respectively. Some very polar fractions of the silica gel chromatography (CH_2Cl_2/5-8% MeOH) showed very poor resolution of their compounds on TLC. From the polarity it was assumed that they may contain compounds of polyhydroxyl and/or carboxyl acid derivatives. The entire polar fraction was subjected to

methylation with diazomethane (Section **7.3.1.11**) for two times with the hope that polar phenolic hydroxyl groups and carboxylic acids would be converted to the less polar methyl ethers and esters, which can be purified more easily. In the first methylation, a suspension of 260.0 mg of the crude polar fractions, containing largely brown polymeric material, was treated with 5 ml of ethereal diazomethane solution for a period of three hours at room temperature (20 °C). In the second reaction, 158.0 mg of these fractions was similarly methylated for a period of five minutes at about 0 °C. Four pure compounds, **5681-10a** (5.0 mg), **5681-10b** (5.9 mg), **5681-10c** (5.7 mg), and **5681-10d** (6.9 mg) were isolated from these methylated fractions. In the first reaction, **5681-10a** was the minor compound of four and in the second reaction the yield was reversed. A new polar component, **5681-10**, was indirectly identified as a new benzoic acid from these four methylation derivatives.

7.3.1.2 (+)-4,7-Dihydroxy-2,3,3,9-tetramethyl-2,3-dihydronaphtho-[1,2-*b*]furan-5,6-dicarboxylic anhydride (**5681-1**)



Molecular formula: $C_{18}H_{16}O_6$.-

Molecular weight: 328.1.-

Melting point: 251 °C (Lit.[140]: 262-263 °C).-

Optical rotation: $[\alpha]^{25}_D$ = + 77.2 (*c* 0.46, $CHCl_3$) (Lit.[140]: + 76 (*c* 0.2, $CHCl_3$)).-

R_f value: 0.43 (CH_2Cl_2).-

IR (KBr): ν = 2919 cm^{-1}, 1709, 1667, 1610, 1460, 1434, 1300.-

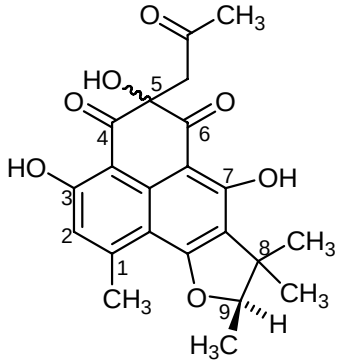
^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.31 (s, 3H, 3-C*H*$_3$), 1.50 (d, $J_{2,2}$ = 6.6 Hz, 3H, 2-C*H*$_3$), 1.55 (s, 3H, 3-C*H*$_3$), 2.82 (s, 3H, 9-C*H*$_3$), 4.72 (q, $J_{2,2}$ = 6.6 Hz, 1H, 2-H), 6.84 (s, 1H, 8-H), 11.42[a] (s, 1H, 4-OH), 11.63[a] (s, 1H, 7-OH).-

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 14.3 (2-*C*H$_3$), 20.5 (3-*C*H$_3$), 23.5 (3-*C*H$_3$), 25.3 (9-*C*H$_3$), 43.2 (C-3), 91.8 (C-2), 93.2 (C-4a), 97.0 (C-6a), 107.9 (C-9a), 117.0 (C-8), 118.9 (C-3a), 135.1 (quat.), 149.7 (C-9), 164.0 (C-4), 164.6 (C-5), 165.2 (C-6), 165.7 (C-7), 166.0 (quat.).-

EIMS (70 eV, <50 °C): *m/z* (%) = 328 [M^+] (12), 313 (48), 285 (10), 269 (13), 63 (6), 43 (11), 32 (22), 28 (100).-

[a] interchangeable assignments.

7.3.1.3 3,5,7-Trihydroxy-1,8,8,9-tetramethyl-5-(2-oxopropyl)-8,9-dihydro-5*H*-phenaleno[1,2-*b*]furan-4,6-dione (**5681-2**)



7.3.1.3.1 Data for major epimer of **5681-2**:

*Molecular formula: $C_{22}H_{22}O_7$.-

*Molecular weight: 398.1.-

*R_f value: 0.41 (CH_2Cl_2/2% MeOH).-

*IR (KBr): ν = 3395 cm^{-1}, 1714, 1605, 1466, 1383, 1336, 1310, 1036.-

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.31 (s, 3H, 8-C*H*$_3$), 1.51 (d, $J_{9,9}$ = 6.7 Hz, 3H, 9-C*H*$_3$), 1.56 (s, 3H, 8-C*H*$_3$), 2.24 (s, 3H, COC*H*$_3$) 2.81 (s, 3H, 1-C*H*$_3$), 3.30 (s, 2H, C*H*$_2$), 3.74 (br s, 5-OH), 4.69 (q, $J_{9,9}$ = 6.7 Hz, 1H, 9-H), 6.79 (s, 1H, 2-H), 12.80[a] (s, 1H, 3-OH), 13.31[a] (s, 1H, 7-OH).-

^{1}H-NMR (300 MHz, C_6D_6): δ = 1.00 (d, $J_{9,9}$ = 6.6 Hz, 3H, 9-C*H*$_3$), 1.10 (s, 3H, 8-C*H*$_3$), 1.29 (s, 3H, 8-C*H*$_3$), 1.53 (s, 3H, COC*H*$_3$), 2.47 (s, 3H, 1-C*H*$_3$), 3.20 (s, 2H, C*H*$_2$), 4.07 (q, $J_{9,9}$ = 6.6 Hz, 1H, 9-H), 6.62 (s, 1H, 2-H), 13.70[b] (s, 1H, 3-OH), 14.25[b] (s, 1H, 7-OH).-

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 14.5 (9-*C*H$_3$), 20.4 (8-*C*H$_3$), 24.0 (8-*C*H$_3$), 25.6 (1-*C*H$_3$), 30.8 (CO*C*H$_3$), 43.1 (C-8), 51.6 (*C*H$_2$), 91.5 (C-9), 99.0 (C-5), 102.5 (quat.), 105.0 (quat.), 109.5 (quat.), 117.8 (C-2), 118.0 (quat.), 137.0 (quat.), 149.0 (C-1), 165.0[c] (C-7), 165.9[c] (C-3), 166.0[c] (quat.), 197.0[d] (C-4), 199.0[d] (C-6), 205.7 (CO).-

^{13}C-NMR (75 MHz, C_6D_6): δ = 14.3 (9-*C*H$_3$), 20.8 (8-*C*H$_3$), 24.2 (8-*C*H$_3$), 25.5 (1-*C*H$_3$), 29.7 (CO*C*H$_3$), 43.5 (C-8), 51.2 (*C*H$_2$), 91.4 (C-9), 103.5 (quat.), 106.3 (quat.), 110.0 (quat.), 118.5 (C-2), 138.5 (quat.), 148.8 (C-1), 164.5[e] (C-7), 165.0[e] (C-3), 166.0[e] (quat.), 198.5[f] (C-4), 200.3[f] (C-6), 205.5 (CO).-

*EIMS (70 eV, <50 °C): *m/z* (%) = 398 [M^+] (94), 383 (14), 355 (93), 313 (98), 312 (99), 297 (100), 257 (69), 149 (29), 69 (41), 43 (55).-

* Data for mixed epimers

[a-f] Identical superscripts represent interchangeable assignments.

7.3.1.3.2 Data for minor epimer of **5681-2**:

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.29 (s, 3H, 8-C*H*$_3$), 1.51 (d, $J_{9,9}$ = 6.7 Hz, 3H, 9-C*H*$_3$), 1.55 (s, 3H, 8-C*H*$_3$), 2.24 (s, 3H, COC*H*$_3$) 2.81 (s, 3H, 1-C*H*$_3$), 3.34 (s, 2H, C*H*$_2$), 3.69 (brs, 5-OH), 4.69 (q, $J_{9,9}$ = 6.7 Hz, 1H, 9-H), 6.79 (s, 1H, 2-H), 12.83[a] (s, 1H, 3-OH), 13.35[a] (s, 1H, 7-OH).-

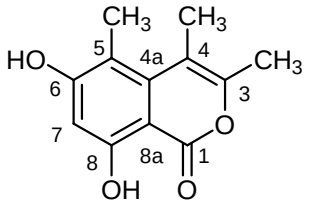
^{1}H-NMR (300 MHz, C_6D_6): δ = 0.98 (d, $J_{9,9}$ = 6.7 Hz, 3H, 9-C*H*$_3$), 1.05 (s, 3H, 8-C*H*$_3$), 1.32 (s, 3H, 8-C*H*$_3$), 1.52 (s, 3H, COC*H*$_3$), 2.47 (s, 3H, 1-C*H*$_3$), 3.22 (s, 2H, C*H*$_2$), 4.09 (q, $J_{9,9}$ = 6.7 Hz, 1H, 9-H), 6.62 (s, 1H, 2-H), 13.71[b] (s, 1H, 3-OH), 14.27[b] (s, 1H, 7-OH).-

^{13}C-NMR (75 MHz, $CDCl_3$): δ = 14.2 (9-*C*H$_3$), 20.4 (8-*C*H$_3$), 24.0 (8-*C*H$_3$), 25.3 (1-*C*H$_3$), 30.9 (CO*C*H$_3$), 43.1 (C-8), 52.0 (*C*H$_2$), 91.4 (C-9), 102.5 (quat.), 105.0 (quat.), 109.5 (quat.), 117.8 (C-2), 118.0 (quat.), 137.0 (quat.), 149.0 (C-1), 165.0[c] (C-7), 165.9[c] (C-3), 166.0[c] (quat.), 197.0[d] (C-4), 199.0[d] (C-6), 205.7 (CO).-

^{13}C-NMR (75 MHz, C_6D_6): δ = 14.6 (9-*C*H$_3$), 20.6 (8-*C*H$_3$), 24.2 (8-*C*H$_3$), 26.0 (1-*C*H$_3$), 30.4 (CO*C*H$_3$), 43.5 (C-8), 50.7 (*C*H$_2$), 91.5 (C-9), 103.5 (quat.), 106.3 (quat.), 110.0 (quat.), 118.5 (C-2), 138.5 (quat.), 148.8 (C-1), 164.5[e] (C-7), 165.0[e] (C-3), 166.0[e] (quat.), 198.5[f] (C-4), 200.3[f] (C-6), 205.4 (CO).-

[a-f] Identical superscripts represent interchangeable assignments.

7.3.1.4 Decarboxycitrinone (**5681-3**)



Molecular formula: $C_{12}H_{12}O_4$.-

Molecular weight: 220.22.-

Melting point: 223 °C (Lit.[145]: 208-210 °C).-

R_f value: 0.56 (CH_2Cl_2/5% MeOH).-

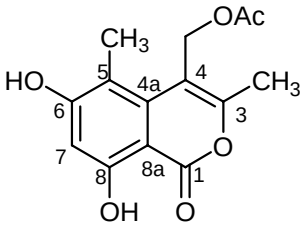
IR (KBr): ν = 1688 cm^{-1}, 1662, 1610, 1471, 1352, 1274.-

^{1}H-NMR (300 MHz, $CDCl_3$/2% CD_3OD): δ = 2.20 (s, 3H, 3-C*H*$_3$), 2.22 (s, 3H, 4-C*H*$_3$), 2.32 (s, 3H, 5-C*H*$_3$), 6.34 (s, 1H, 7-H), 8.96 (brs, 1H, 6-OH), 11.92 (s, 1H, 8-OH).-

^{13}C-NMR (50 MHz, $CDCl_3$/2% CD_3OD): δ = 14.0 (5-*C*H$_3$), 17.9 (3-*C*H$_3$), 18.2 (4-*C*H$_3$), 100.4 (C-8a), 101.5 (C-7), 110.8 (C-4), 111.9 (C-5), 139.8 (C-4a), 149.6 (C-3), 161.9 (C-8), 164.4 (C-6), 167.4 (C-1).-

EIMS (70 eV, <50 °C): *m/z* (%) = 220 [M^+] (42), 205 (19), 191 (6), 178 (15), 177 (24), 149 (17), 91 (10), 77 (14), 69 (14), 51 (11), 43 (28), 32 (22), 28 (100).-

7.3.1.5 (6,8-Dihydroxy-3,5-dimethyl-1-oxo-1*H*-isochromen-4-yl)methyl acetate (**5681-4**)



Molecular formula: $C_{14}H_{14}O_6$.-

Molecular weight: 278.26.-

Melting point: 230 °C.-

R_f value: 0.56 (CH_2Cl_2/5.2% MeOH).-

UV (MeOH): λ_{max} (lg ε) = 242 nm (3.96), 247 (3.96).-

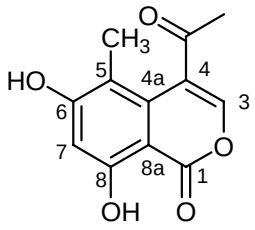
IR (KBr): ν = 1740 cm^{-1}, 1657, 1398, 1269.-

^{1}H NMR (300 MHz, $CDCl_3$/2 % CD_3OD): δ = 2.07 (s, 3H, COC*H*$_3$), 2.26 (s, 3H, 3-C*H*$_3$), 2.29 (s, 3H, 5-C*H*$_3$), 5.10 (s, 2H, C*H*$_2$), 6.38 (s, 1H, 7-H), 8.36 (brs, 1H, 6-OH), 11.65 (s, 1H, 8-OH).-

^{13}C NMR (50 MHz, $CDCl_3$/2% CD_3OD): δ = 12.1 (5-*C*H$_3$), 18.2 (3-*C*H$_3$), 21.4 (CO*C*H$_3$), 61.3 (O*C*H$_2$), 100.4 (C-8a), 102.1 (C-7), 109.9 (C-4), 111.3 (C-5), 137.8 (C-4a), 156.3 (C-3), 162.0 (C-8), 164.2 (C-6), 167.0 (C-1), 171.4 (acetate-CO).-

EIMS (80 eV, 150 °C): *m/z* (%) = 278 [M^+] (78), 236 (6), 219 (58), 218 (100), 217 (37), 193 (33), 175 (36), 148 (15), 43 (16), 28 (4).-

7.3.1.6 4-Acetyl-6,8-dihydroxy-5-methyl-1*H*-isochromen-1-one (**5681-5**)



Molecular formula: $C_{12}H_{10}O_5$.-

Molecular weight: 234.20.-

Melting point: 170 °C (Lit.[146]: 178-179 °C).-

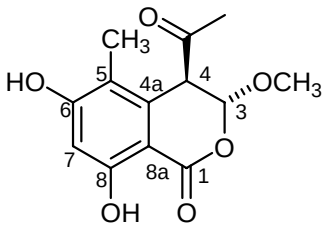
R_f value: 0.52 (CH_2Cl_2/5.2% MeOH).-

^{1}H-NMR (200 MHz, $CDCl_3$/2% CD_3OD): δ = 2.03 (s, 3H, 5-C*H*$_3$), 2.53 (s, 3H, COC*H*$_3$), 6.55 (s, 1H, 7-H), 7.44 (s, 1H, 3-H), 11.22 (brs, 1H, 8-OH).-

^{13}C-NMR (50 MHz, $CDCl_3$/2% CD_3OD): δ = 14.8 (5-*C*H$_3$), 30.9 (CO*C*H$_3$), 100.4 (C-8a), 103.5 (C-7), 112.5 (C-5), 124.4 (C-4), 133.4 (C-4a), 145.4 (C-3), 162.9 (C-8), 164.4 (C-6), 165.7 (C-1), 199.2 (*C*OCH$_3$).-

EIMS (70 eV, 240 °C): *m/z* (%) = 234 [M^+] (100), 219 (45), 217 (77), 206 (29), 191 (74), 189 (39), 175 (39), 174 (34), 163 (46), 135 (42), 77 (28), 69 (35), 43 (53), 28 (37).-

7.3.1.7 (3*R*,4*R*)-4-Acetyl-6,8-dihydroxy-3-methoxy-5-methyl-3,4-dihydroisochromen-1-one (**5681-6**)



Molecular formula: $C_{13}H_{14}O_6$.-

Molecular weight: 266.25.-

Melting point: 195 °C (Lit.[53]: 207 °C).-

R_f value: 0.46 (CH_2Cl_2/5.2% MeOH).-

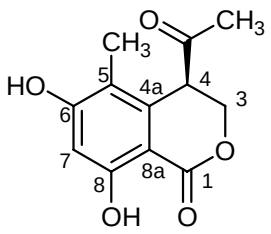
^{1}H-NMR (300 MHz, $CDCl_3$/2% CD_3OD): δ = 2.05 (s, 3H, 5-C*H*$_3$), 2.06 (s, 3H, COC*H*$_3$), 3.55 (s, 3H, OC*H*$_3$), 4.04 (d, $J_{4,3}$ = 1.3 Hz, 1H, 4-H), 5.60 (d, $J_{3,4}$ = 1.3 Hz, 1H, 3-H), 6.39 (s, 1H, 7-H), 8.82 (brs, 1H, 6-OH), 11.16 (brs, 1H, 8-OH).-

^{1}H-NMR (200 MHz, CD_3OD): δ = 2.07 (s, 3H, 5-C*H*$_3$), 2.22 (s, 3H, COC*H*$_3$), 3.57 (s, 3H, OC*H*$_3$), 4.37 (d, $J_{4,3}$ = 1.2 Hz, 1H, 4-H), 5.78 (d, $J_{3,4}$ = 1.2 Hz, 1H, 3-H), 6.37 (s, 1H, 7-H).-

^{13}C-NMR (75 MHz, CD_3OD): δ = 11.5 (5-*C*H$_3$), 29.5 (CO*C*H$_3$), 55.2 (C-4), 57.6 (O*C*H$_3$), 101.0 (C-8a), 102.7 (C-7), 103.7 (C-3), 117.8 (C-5), 137.0 (C-4a), 164.0 (C-8), 165.5 (C-6), 169.0 (C-1), 205.0 (*C*OCH$_3$).-

EIMS (70 eV, <50 °C): *m/z* (%) = 266 [M^+] (39), 224 (20), 207 (14), 206 (100), 193 (34), 192 (52), 178 (13), 174 (12), 164 (42), 163 (26), 135 (17), 77 (11), 69 (14), 43 (64), 28 (58).-

7.3.1.8 (*R*)-4-Acetyl-6,8-dihydroxy-5-methyl-3,4-dihydroisochromen-1-one (**5681-7**)



Molecular formula: $C_{12}H_{12}O_5$.-

Molecular weight: 236.22.-

Melting point: 204 °C (Lit.[53]: 204 °C).-

R_f value: 0.51 (CH_2Cl_2/6% MeOH).-

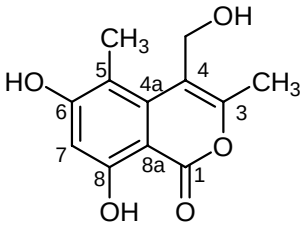
^{1}H-NMR (200 MHz, $CDCl_3$/2% CD_3OD): δ = 2.07 (s, 3H, 5-C*H*$_3$), 2.17 (s, 3H, COC*H*$_3$), 3.83 (d, $J_{4,3b}$ = 3.7 Hz, 1H, 4-H), 4.56 (dd, $J_{3b,3a}$ = 11.5 Hz, $J_{3b,4}$ = 3.7 Hz, 1H, 3b-H), 4.92 (d, $J_{3a,3b}$ = 11.5 Hz, 1H, 3a-H), 6.41 (s, 1H, 7-H), 11.22 (brs, 1H, 8-OH).-

^{1}H-NMR (200 MHz, CD_3OD): δ = 2.07 (s, 3H, 5-C*H*$_3$), 2.27 (s, 3H, COC*H*$_3$), 4.17 (d, $J_{4,3b}$ = 3.7 Hz, 1H, 4-H), 4.60 (dd, $J_{3b,3a}$ = 11.0 Hz, $J_{3b,4}$ = 3.7 Hz, 1H, 3b-H), 4.92 (d, $J_{3a,3b}$ = 11.0 Hz, 1H, 3a-H), 6.38 (s, 1H, 7-H).-

^{13}C-NMR (50 MHz, CD_3OD): δ = 10.0 (5-*C*H$_3$), 27.7 (CO*C*H$_3$), 48.7 (C-4), 68.7 (C-3), 100.4 (C-8a), 101.5 (C-7), 115.4 (C-5), 137.7 (C-4a), 162.9 (C-8), 163.9 (C-6), 170.1 (C-1), 205.7 (*C*OCH$_3$).-

EIMS (70 eV, 240 °C): *m/z* (%) = 236 [M^+] (63), 194 (78), 176 (100), 165 (31), 148 (20), 72 (28), 69 (19), 59 (41), 57 (26), 55 (31), 43 (97), 41 (21), 28 (11), 18 (19).-

7.3.1.9 6,8-Dihydroxy-4-(hydroxymethyl)-3,5-dimethyl-1*H*-isochromen-1-one (**5681-8**)



Molecular formula: $C_{12}H_{12}O_5$.-

Molecular weight: 236.22.-

Melting point: 245 °C.-

R_f value: 0.39 (CH_2Cl_2/8% MeOH).-

UV (MeOH): λ_{max} (lg ε) = 242 nm (3.56), 247 (3.57).-

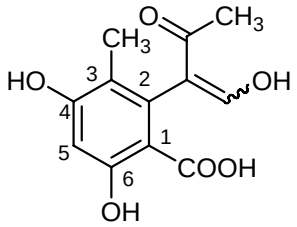
IR (KBr): ν = 2924 cm^{-1}, 2851, 1665, 1612.-

^{1}H NMR (300 MHz, CD_3OD): δ = 2.25 (s, 3H, 3-CH_3), 2.35 (s, 3H, 5-CH_3), 4.52 (s, 2H, CH_2OH), 6.24 (s, 1H, 7-H).-

^{13}C NMR (75 MHz, CD_3OD): δ = 10.9 (3-*C*H_3), 16.3 (5-*C*H_3), 57.3 (*C*H_2OH), 99.4 (C-8a), 100.9 (C-7), 111.7 (C-4), 113.7 (C-5), 137.9 (C-4a), 154.6 (C-3), 162.3 (C-8), 165.2 (C-6), 166.8 (C-1).-

EIMS (70 eV, 240 °C): *m/z* (%) = 236 [M^+] (57), 218 (58), 193 (39), 175 (58), 148 (37), 77 (20), 69 (34), 44 (83), 43 (100), 28 (14).-

7.3.1.10 4,6-Dihydroxy-2-(1-hydroxy-3-oxobut-1-en-2-yl)-3-methylbenzoic acid (**5681-9**)



Molecular formula: $C_{12}H_{12}O_6$.-

Molecular weight: 252.22.-

Melting point: 175 °C.-

R_f value: 0.35 (CH_2Cl_2/8% MeOH).-

UV (MeOH): λ_{max} (lg ε) = 224 nm (3.79), 227 (3.80), 231 (3.78).-

IR (KBr): ν = 2924 cm^{-1}, 1719, 1652, 1616, 1460, 1398, 1274, 1160, 1031.-

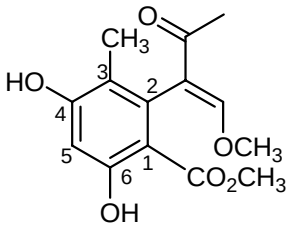
*^{1}H NMR (200 MHz, CD_3OD): δ = 2.07 (s, 3H, 3-CH_3), 2.24 (s, 3H, COCH_3), 6.03 (s, 1H, 5-H), 6.38 (s, 1H, vinyl-H).-

*^{13}C-NMR (50 MHz, CD_3OD): δ = 12.3 (3-*C*H_3), 100.5 (C-1), 101.4 (C-5), 116.3 (*C*=CHOH), 135.5 (C-2), 162.6 (=*C*HOH), 163.8 (C-6), 165.0 (C-4), 204.4 (*C*OCH_3).-

EIMS (80 eV, <50 °C): *m/z* (%) = 252 [M^+] (54), 234 (24), 210 (25), 206 (18), 192 (100), 174 (18), 164 (86), 163 (20), 136 (22), 77 (14), 69 (16), 43 (61), 28 (3).-

*incomplete data

7.3.1.10.1 (*E*)-Methyl 4,6-dihydroxy-2-(1-methoxy-3-oxobut-1-en-2-yl)-3-methylben- zoate (**5681-9a**)



To a solution of **5681-9** (50 mg, 0.2 mmol) in dry ether and MeOH (2 mL, 1:1) was added dropwise an ethereal solution of diazomethane (2 mL, freshly prepared) at 0 °C. The mixture was stirred at 0 °C for 5 min. Acetic acid (0.5 mL) was then added. The mixture was washed thrice with 5% $NaHCO_3$ and then twice with water. The organic layer was extracted with CH_2Cl_2 (10 mL × 3), dried (Na_2SO_4), and concentrated in vacuo to give a semisolid residue, chromatography of which on preparative layer silica gel plates (toluene/24% ethyl acetate) afforded **5681-9a** (16.8 mg, 30%) as white needles.

Molecular formula: $C_{14}H_{16}O_6$.-

Molecular weight: 280.27.-

Melting point: 185 °C.-

R_f value: 0.56 (CH_2Cl_2/8% MeOH).-

UV (MeOH): λ_{max} (lg ε) = 224 nm (3.81), 259 (3.86).-

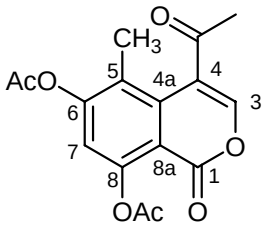
IR (KBr): ν = 2950 cm^{-1}, 2845, 2567, 1728, 1654, 1628, 1612, 1439, 1334, 1250, 1198, 1166, 1150.-

^{1}H NMR (300 MHz, $CDCl_3$/2% CD_3OD): δ = 2.01 (s, 3H, 3-C*H*$_3$), 2.15 (s, 3H, COC*H*$_3$), 3.83 (s, 3H, =COC*H*$_3$), 3.86 (s, 3H, CO_2CH_3), 6.45 (s, 1H, 5-H), 7.41 (s, 1H, vinyl-H), 11.51 (brs, 1H, 6-OH).-

^{13}C NMR (75 MHz, $CDCl_3$/2% CD_3OD): δ = 12.4 (3-*C*H$_3$), 27.4 (CO*C*H$_3$), 52.2 (CO_2CH_3), 62.3 (O*C*H$_3$), 102.9 (C-5), 104.8 (C-1), 118.2 (C-3), 121.7 (*C*=CHOCH$_3$), 136.5 (C-2), 157.3 (=*C*HOCH$_3$), 161.4 (C-6), 162.6 (C-4), 171.6 (CO_2CH_3), 198.7 (*C*OCH$_3$).-

EIMS (70 eV, 240 °C): *m/z* (%) = 280 [M^+] (22), 248 (43), 217 (32), 216 (79), 175 (32), 174 (100), 146 (15), 57 (14), 43 (19), 28 (16).-

7.3.1.10.2 Acetic acid 8-acetoxy-4-acetyl-5-methyl-1-oxo-1*H*-isochromen-6-yl ester (**5681-5a**)



To a solution of **5681-9** (12 mg, 0.048 mmol) in CH_2Cl_2 (1 mL) and pyridine (0.2 mL) were added acetic anhydride (0.1 mL) and DMAP (1 mg), and the mixture was stirred overnight at room temperature. The reaction was worked up by dilution with ice cold 1 N HCl and extraction with CH_2Cl_2 (10 mL × 3). The combined extracts were washed with water, dried (Na_2SO_4), and concentrated in vacuo to give a semisolid residue, the purification of which by preparative TLC (toluene/13% ethyl acetate) afforded **5681-5a** (6 mg, 39.6%) as a white solid.

Molecular formula: $C_{16}H_{14}O_7$.-

Molecular weight: 318.28.-

Melting point: 91 °C.-

R_f value: 0.82 (CH_2Cl_2/8% MeOH).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 239 nm (4.0).-

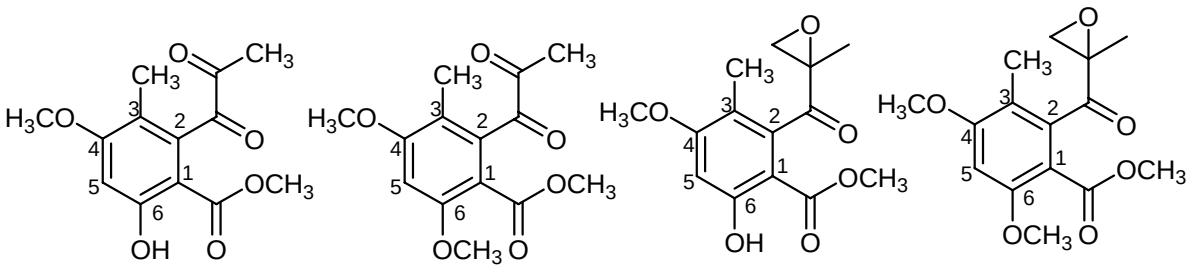
IR (KBr): ν = 3359 cm^{-1}, 2929, 2852, 1771, 1745, 1693, 1626, 1455, 1372, 1316, 1264.-

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.98 (s, 3H, 5-C*H*$_3$), 2.31 (s, 3H, OCOC*H*$_3$), 2.34 (s, 3H, OCOC*H*$_3$), 2.45 (s, 3H, COC*H*$_3$), 7.01 (s, 1H, 7-H), 7.54 (s, 1H, 3-H).-

^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.5 (5-*C*H$_3$), 21.4 (OCO*C*H$_3$), 21.6 (OCO*C*H$_3$), 30.4 (CO*C*H$_3$), 113.3 (C-8a), 119.2 (C-7), 122.7 (C-4), 125.3 (C-5), 136.5 (C-4a), 147.8 (C-3), 151.8 (C-8), 155.6 (C-6), 157.6 (C-1), 168.3 (O*C*OCH$_3$), 169.9 (O*C*OCH$_3$), 197.4 (*C*OCH$_3$).-

EIMS (70 eV, <50 °C): *m/z* (%) = 318 [M^+] (6), 276 (59), 234 (100), 219 (16), 217 (28), 206 (14), 191 (15), 163 (7), 135 (6), 43 (42).-

7.3.1.11 Methyl esters of 4,6-dihydroxy-3-methyl-2-(2-oxopropanoyl)benzoic acid (**5681-10a – 5681-10d**)



5681-10a **5681-10b** **5681-10c** **5681-10d**

To a suspension of 260 mg of a combined column fraction of different polarities (CH_2Cl_2/5-8% MeOH) in dry ether (5 mL), CH_2Cl_2 (0.5 mL), and MeOH (0.5 mL) was added dropwise an ethereal solution of diazomethane (5 mL, freshly prepared) at 20 °C. The mixture was stirred at 20 °C for 3 h. Acetic acid (1 mL) was then added. The mixture was washed thrice with 5% $NaHCO_3$ and then twice with water. The organic layer was extracted with CH_2Cl_2 (10 mL × 3), dried (Na_2SO_4), and concentrated in vacuo to give a semisolid residue. Of the same fraction, a further 158 mg was similarly methylated for a period of 5 min at 0 °C to obtain a semisolid residue. Separate chromatography of both residues on preparative layer silica gel plates (toluene/10% ethyl acetate) afforded **5681-10a** (5 mg, yellow needles), **5681-10b** (5.9 mg, yellow crystals), **5681-10c** (5.7 mg, yellow needles), and **5681-10d** (6.9 mg, yellow solid).

7.3.1.11.1 Methyl 6-hydroxy-4-methoxy-3-methyl-2-(2-oxopropanoyl)benzoate (**5681-10a**)

Molecular formula: $C_{13}H_{14}O_6$.-

Molecular weight: 266.25.-

Melting point: 98 °C.-

R_f value: 0.55 (toluene/20% ethyl acetate).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 231 nm (3.69), 259 (3.66).-

IR (KBr): ν = 2924 cm^{-1}, 2852, 1719, 1703, 1657, 1605, 1584, 1440, 1372, 1347, 1253, 1191, 1160.-

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.94 (s, 3H, 3-C*H*$_3$), 2.55 (s, 3H, COC*H*$_3$), 3.85 (s, 3H, OC*H*$_3$), 3.95 (s, 3H, OC*H*$_3$), 6.53 (s, 1H, 5-H), 10.60 (brs, 1H, 6-OH).-

^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.3 (3-*C*H$_3$), 24.0 (CO*C*H$_3$), 52.9 (O*C*H$_3$), 56.4 (O*C*H$_3$), 100.6 (C-5), 102.5 (C-1), 117.9 (C-3), 139.4 (C-2), 162.4 (C-6), 164.0 (C-4), 168.8 (*C*OOCH$_3$), 193.8 (CO), 196.5 (CO).-

EIMS (70 eV, <50 °C): *m/z* (%) = 266 [M^+] (8), 224 (45), 223 (100), 191 (38), 163 (29), 135 (13), 120 (9), 109 (8), 77 (14), 69 (20), 43 (39).-

7.3.1.11.2 Methyl 4,6-dimethoxy-3-methyl-2-(2-oxopropanoyl)benzoate (**5681-10b**)

Molecular formula: $C_{14}H_{16}O_6$.-

Molecular weight: 280.27.-

Melting point: 134 °C.-

R_f value: 0.41 (toluene/30% ethyl acetate).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 229 nm (3.61).-

IR (KBr): ν = 2924 cm^{-1}, 2847, 1719, 1709, 1647, 1590, 1481, 1440, 1341, 1269, 1207.-

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.97 (s, 3H, 3-CH_3), 2.58 (s, 3H, COCH_3), 3.83 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.53 (s, 1H, 5-H).-

^{13}C NMR (50 MHz, $CDCl_3$): δ = 12.1 (3-*C*H_3), 24.2 (CO*C*H_3), 53.0 (O*C*H_3), 56.2 (O*C*H_3), 56.9 (O*C*H_3), 96.6 (C-5), 109.9 (C-1), 118.0 (C-3), 143.2 (C-2), 160.1 (C-6), 162.9 (C-4), 168.5 (*C*OOCH$_3$), 193.9 (CO), 197.2 (CO).-

EIMS (70 eV, <50 °C): *m/z* (%) = 280 [M^+] (1), 249 (11), 238 (55), 237 (100), 207 (9), 179 (16), 164 (14), 136 (15), 120 (12), 91 (15), 77 (16), 69 (9), 43 (24), 28 (3).-

7.3.1.11.3 Methyl 6-hydroxy-4-methoxy-3-methyl-2-(2-methyloxirane-2-carbonyl) benzoate (**5681-10c**)

Molecular formula: $C_{14}H_{16}O_6$.-

Molecular weight: 280.27.-

Melting point: 134 °C.-

R_f value: 0.48 (toluene/20% ethyl acetate).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 231 nm (3.65).-

IR (KBr): ν = 2919 cm^{-1}, 2857, 1703, 1667, 1605, 1445, 1357, 1253, 1166.-

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.70 (s, 3H, OCCH_3), 1.98 (s, 3H, 3-CH_3), 2.66 (d, J_{gem} = 5.0 Hz, 1H, CH_2), 2.84 (d, J_{gem} = 5.0 Hz, 1H, CH_2), 3.88 (s, 3H, OCH_3), 3.96 (s, 3H, CO_2CH_3), 6.51 (s, 1H, 5-H), 11.13 (brs, 1H, 6-OH).-

^{13}C NMR (75 MHz, $CDCl_3$): δ = 11.9 (3-*C*H_3), 16.4 (OC*C*H_3), 52.1 (*C*H_2), 52.5 (CO_2*C*H_3), 55.6 (O*C*H_3), 60.0 (quat.), 99.4 (C-5), 116.0 (C-1), 122.0 (C-3), 138.0 (C-2), 161.6 (C-6), 162.8 (C-4), 169.0 (*C*O_2CH$_3$), 204.3 (CO).-

EIMS (70 eV, 240 °C): *m/z* (%) = 280 [M^+] (24), 223 (100), 191 (17), 165 (14), 163 (15), 136 (13), 77 (10), 57 (10), 43 (10).-

7.3.1.11.4 Methyl 4,6-dimethoxy-3-methyl-2-(2-methyloxirane-2-carbonyl)benzoate (**5681-10d**)

Molecular formula: $C_{15}H_{18}O_6$.-

Molecular weight: 294.30.-

Melting point: 87 °C.-

R_f value: 0.28 (toluene/30% ethyl acetate).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 231 nm (3.80).-

IR (KBr): ν = 2940 cm^{-1}, 2847, 1734, 1703, 1590, 1476, 1440, 1336, 1269, 1207, 1155, 1021.-

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.69 (s, 3H, OCC*H*$_3$), 2.03 (s, 3-C*H*$_3$), 2.78 (d, J_{gem} = 5.4 Hz, 1H, C*H*$_2$), 2.85 (d, J_{gem} = 5.4 Hz, 1H, C*H*$_2$), 3.89 (s, 3H, OC*H*$_3$), 3.92 (s, 3H, OC*H*$_3$), 3.93 (s, 3H, OC*H*$_3$), 6.50 (s, 1H, 5-H).-

^{13}C NMR (50 MHz, $CDCl_3$): δ = 12.5 (3-*C*H$_3$), 17.2 (OC*C*H$_3$), 52.7 (O*C*H$_3$), 53.5 (*C*H$_2$), 56.1 (O*C*H$_3$), 56.9 (O*C*H$_3$), 60.4 (quat.), 96.4 (C-5), 112.0 (C-1), 117.0 (C-3), 140.6 (C-2), 158.9 (C-6), 161.7 (C-4), 166.9 (CO_2*C*H$_3$), 205.0 (CO).-

EIMS (70 eV, 240 °C): *m/z* (%) = 294 [M^+] (26), 264 (11), 249 (14), 238 (65), 237 (100), 221 (29), 207 (17), 179 (24), 177 (15), 164 (17), 149 (15), 136 (17), 120 (17), 91 (19), 77 (18), 57 (11), 43 (14).-

7.3.2 Metabolites from Strain 6744

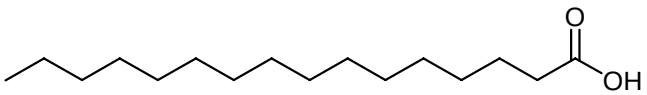
7.3.2.1 Isolation of secondary metabolites

Strain 6744 was cultivated at room temperature for 21 days in biomalt semi-solid agar medium. After that, this culture medium was extracted three times with ethyl acetate to obtain the crude extract (16.0 g). The crude extract was subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane, after that a gradient of dichloromethane/methanol, and finally methanol to afford a total of 15 fractions. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum spray reagents. Methanol was added to the crude solid mass of the column fraction of petroleum ether/60% CH_2Cl_2 and kept several hours at -20 °C. After that the solvent was filtered off to give **6744-1** (35.0 mg) as white powder. The crude mass of the column fraction of petroleum ether/75% CH_2Cl_2 after crystallization from CH_2Cl_2/acetone gave white fine needles of **6744-2** (8.0 mg). The column fraction of petroleum ether/55% CH_2Cl_2 was subjected to preparative TLC on silica gel (CH_2Cl_2/MeOH/AcOH 100:0.6:0.2) to obtain 2.3 mg of **6744-3** as a mixture of two diastereomers. Chromatography of a polar fraction (CH_2Cl_2/1-2% MeOH) on

preparative layer silica gel plate (CH_2Cl_2/acetone/AcOH 100:4:0.2) followed by crystallization from CH_2Cl_2/Et_2O/pentane afforded **6744-4** (14.6 mg) as white fine needles. The crude mass of another polar fraction (CH_2Cl_2/2% MeOH) after crystallization from CH_2Cl_2/Et_2O gave white fine needles of **6744-5** (59.0 mg). Compound **6744-4** was reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-4** (**6744-4a** and **6744-4b**) with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated (Section **7.3.2.5.1**). With the similar hope, **6744-5** was also reacted with 4-bromobenzoyl chloride to afford the mono- and di-4-bromobenzoates of **6744-5** (**6744-5a** and **6744-5b**) (Section **7.3.2.6.1**). The NMR data of all the benzoates contributed to reconfirm their respective parent structures.

Some very polar fractions of the silica gel chromatography (CH_2Cl_2/1.5-3% MeOH) showed very poor resolution of their compounds on TLC. From the polarity it was assumed that they may contain compounds of polyhydroxyl derivatives. A portion of the mixed polar fractions was subjected to acetylation using acetic anhydride/pyridine in dichloromethane with the hope that polar hydroxyl groups would be converted to the less polar esters, which can be purified more easily (Section **7.3.2.7**). A new metabolite, **6744-6**, was indirectly identified from the acetylated derivative **6744-6a**.

7.3.2.2 Palmitic acid (**6744-1**)



Molecular formula: $C_{16}H_{32}O_2$.-

Molecular weight: 256.2.-

Melting point: 55 °C.-

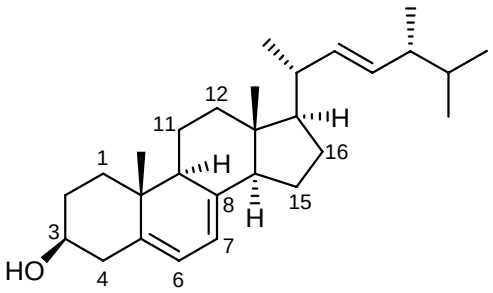
R_f value: 0.35 (CH_2Cl_2/9% Et_2O).-

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 0.92 (t, *J* = 6.3 Hz, 3H, C*H*$_3$), 1.30 (m, 24H, 12 C*H*$_2$ groups), 1.68 (quintet, 2H, C*H*$_2$), 2.39 (t, *J* = 7.5 Hz, 2H, C*H*$_2$).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 14.5 (*C*H$_3$), 23.1 (*C*H$_2$), 25.1 (*C*H$_2$), 29.5 (*C*H$_2$), 29.6 (*C*H$_2$), 29.8 (2 *C*H$_2$ groups), 30.0 (*C*H$_2$), 30.1 (5 *C*H$_2$ groups), 32.3 (*C*H$_2$), 34.4 (*C*H$_2$), 180.0 (-*C*OOH).-

EIMS (70 eV, 170 °C): *m/z* (%) = 256 [M$^+$] (100), 227 (5), 213 (18), 199 (5), 185 (9), 171 (9), 157 (10), 143 (5), 129 (27), 115 (10), 73 (56), 60 (48), 57 (45), 43 (49), 29 (13).-

7.3.2.3 Ergosterol (**6744-2**)



Molecular formula: $C_{28}H_{44}O$.-

Molecular weight: 396.4.-

Melting point: 149 °C.-

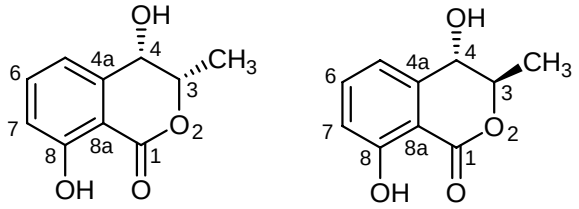
R_f value: 0.33 (CH_2Cl_2/9% Et_2O).-

^{1}H-NMR (200 MHz, $CDCl_3$)*: δ = 3.65 (m, 1H, 3-H), 5.23 (m, 1H, =C*H*), 5.36 (m, 2H, 2 =C*H*), 5.43 (m, 1H, 6-H).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 12.5 (*C*H_3), 16.7 (*C*H_3), 18.0 (*C*H_3), 20.1 (*C*H_3), 20.4 (*C*H_3), 21.5 (*C*H_3), 21.5 (*C*H_2), 23.4 (*C*H_2), 28.7 (*C*H_2), 32.4 (*C*H_2), 33.5 (*C*H), 37.5 (quat.), 38.8 (*C*H_2), 39.5 (*C*H_2), 40.8 (*C*H), 41.2 (*C*H_2), 43.2 (*C*H), 43.2 (quat.), 46.7 (*C*H), 55.0 (*C*H), 56.2 (*C*H), 70.9 (*C*H), 116.7 (*C*H), 120.0 (*C*H), 132.4 (*C*H), 136.0 (*C*H), 140.2 (quat.), 141.8 (quat.).-

EIMS (70 eV, 175 °C): *m/z* (%) = 396 [M^+] (100), 381 (4), 378 (9), 376 (42), 363 (43), 337 (18), 271 (12), 253 (18), 251 (44), 211 (10), 149 (30), 69 (35).-

7.3.2.4 4,8-Dihydroxy-3-methyl-3,4-dihydroisochromen-1-one (4-hydroxymellein) (**6744-3**)



7.3.2.4.1 Data for major diastereomer of **6744-3**:

*Molecular formula: $C_{10}H_{10}O_4$.-

*Molecular weight: 194.1.-

R_f value: 0.31 (CH_2Cl_2/9% Et_2O).-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.62 (d, $J_{3,3}$ = 6.6 Hz, 3H, 3-C*H*$_3$), 4.73 (m, 2H, 3-H, 4-H), 6.95 (d, $J_{5,6}$ = 7.3 Hz, 1H, 5-H) 7.06 (d, $J_{7,6}$ = 8.5 Hz, 1H, 7-H), 7.56 (t, $J_{6,5}$ = $J_{6,7}$ = 8.0 Hz, 1H, 6-H), 11.06 (s, 1H, 8-OH).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 16.0 (3-*C*H_3), 67.3 (C-4), 79.0 (C-3), 106.9 (C-8a), 118.2 (C-7), 118.6 (C-5), 136.8 (C-6), 140.5 (C-4a), 162.2 (C-8), 169.1 (CO).-

*EIMS (70 eV, 175 °C): *m/z* (%) = 194 [M^+] (100), 166 (31), 150 (67), 122 (45), 121 (53), 93 (8), 65 (8).-

* Data for mixed diastereomers

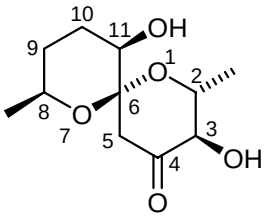
7.3.2.4.2 Data for minor diastereomer of **6744-3**:

R_f value: 0.25 (CH_2Cl_2/9% Et_2O).-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.55 (d, $J_{3,3}$ = 6.0 Hz, 3H, 3-C*H*$_3$), 4.63 (m, 2H, 3-H, 4-H), 6.95 (d, $J_{5,6}$ = 7.3 Hz, 1H, 5-H) 7.03 (d, $J_{7,6}$ = 8.5 Hz, 1H, 7-H), 7.57 (t, $J_{6,5}$ = $J_{6,7}$ = 8.2 Hz, 1H, 6-H), 11.03 (s, 1H, 8-OH).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.9 (3-*C*H_3), 67.5 (C-4), 79.9 (C-3), 106.9 (C-8a), 116.2 (C-7), 117.9 (C-5), 136.9 (C-6), 140.7 (C-4a), 162.2 (C-8), 168.4 (CO).-

7.3.2.5 3,11-Dihydroxy-2,8-dimethyl-1,7-dioxa-spiro[5,5]undecan-4-one (**6744-4**)



Molecular formula: $C_{11}H_{18}O_5$.-

Melting point: 149 °C.-

Molecular weight: 230.1.-

Optical rotation: $[\alpha]^{25}_D$ = - 80.4 (*c* 0.68, $CHCl_3$).-

R_f value: 0.29 (CH_2Cl_2/2.9% MeOH).-

IR (KBr): ν = 3487 cm^{-1}, 3425, 3383, 2976, 2947, 2916, 2866, 1722, 1454, 1402, 1381, 1279, 1254, 1227, 1167, 1134, 1088, 1074, 1063, 1001, 989, 930, 885.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.26 (d, $J_{8,8}$ = 6.5 Hz, 3H, 8-C*H*$_3$), 1.47 (d, $J_{2,2}$ = 6.2 Hz, 3H, 2-C*H*$_3$), 1.67 (q, $J_{9,8}$ = 6.1 Hz, 2H, 9-H), 1.76 (m, 1H, 10-H), 1.99 (m, 1H, 10-H), 2.50 (d, $J_{OH,11}$ = 6.4 Hz, 1H, 11-OH), 2.83 (dd, J_{gem} = 13.8, $J_{5,3}$ = 1.1 Hz, 1H, 5-H), 2.90 (d, J_{gem} = 13.8 Hz, 1H, 5-H), 3.52 (d, $J_{OH,3}$ = 4.1 Hz, 1H, 3-OH), 3.55 (sextet, $J_{11,10}$ = 10.3, $J_{11,OH}$ = 6.4, $J_{11,10}$ = 3.4 Hz, 1H, 11-H), 3.83 (dddd, $J_{3,2}$ = 9.5, $J_{3,OH}$ = 4.1, $J_{3,5}$ = 1.1 Hz, 1H, 3-H), 3.90 (sextet, *J* = 6.1 Hz, 1H, 8-H), 4.07 (qq, $J_{2,3}$ = 9.5, $J_{2,2}$ = 6.2 Hz, 1H, 2-H).-

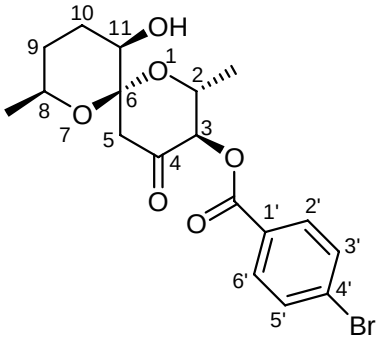
^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.7 (2-*C*H_3), 20.7 (8-*C*H_3), 24.8 (C-10), 27.6 (C-9), 45.0 (C-5), 69.7 (C-11), 69.9 (C-8), 72.5 (C-2), 78.0 (C-3), 101.3 (C-6), 205.8 (C-4).-

EIMS (70 eV, 200 °C): *m/z* (%) = 230 [M^+] (1), 241 (3), 188 (52), 186 (39), 168 (62), 145 (100), 129 (57), 127 (95), 111 (34), 87 (77), 57 (54), 43 (67).-

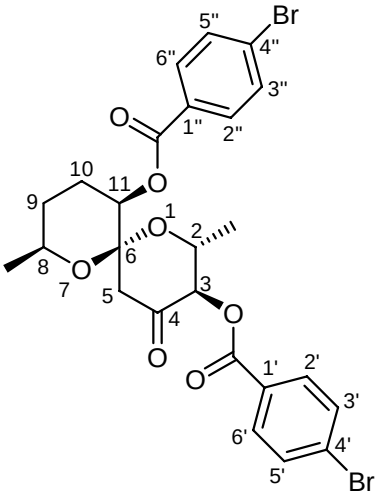
HREIMS: Calcd.: 230.1154

Found: 230.11525

7.3.2.5.1 4-Bromo-benzoic acid 11-hydroxy-2,8-dimethyl-4-oxo-1,7-dioxa-spiro[5,5]-undec-3-yl ester (**6744-4a**) and dibenzoate of 3,11-dihydroxy-2,8-dimethyl-1,7-dioxa-spiro[5,5]undecan-4-one (**6744-4b**)



6744-4a



6744-4b

To a stirred solution of **6744-4** (5.6 mg, 0.024 mmol) in dry pyridine (2.0 mL) was added *p*-bromobenzoyl chloride (16.3 mg) and *N*,*N*-dimethyl-4-aminopyridine (10.0 mg). The reaction mixture was stirred at room temperature for about 1.5 h (TLC monitoring), and was then neutralized by addition of 1 N HCl. The mixture was extracted with CH_2Cl_2, washed with water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The resulting mixture was purified by preparative TLC on silica gel (1 mm, 1 development, CH_2Cl_2) followed by recrystallization (CH_2Cl_2/Et_2O) to afford **6744-4a** (1.9 mg, 20.8%) and **6744-4b** (8.6 mg, 58.3%) as concentrated gums.

7.3.2.5.1.1 4-Bromo-benzoic acid 11-hydroxy-2,8-dimethyl-4-oxo-1,7-dioxa-spiro[5,5]-undec-3-yl ester (**6744-4a**)

Molecular formula: $C_{18}H_{21}BrO_6$.-

Molecular weight: 412.1.-

Optical rotation: $[\alpha]^{25}_D$ = - 68.7 (*c* 0.15, $CHCl_3$).-

R_f value: 0.53 (CH_2Cl_2/2.9% MeOH).-

UV ($CHCl_3$): λ_{max} (lg ε) = 268 nm (3.25).-

IR (KBr): ν = 3504 cm^{-1}, 2926, 2854, 1745, 1728, 1591, 1456, 1275, 1104, 1012, 754.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.29 (d, $J_{8,8}$ = 6.4 Hz, 3H, 8-C*H*$_3$), 1.46 (d, $J_{2,2}$ = 6.2 Hz, 3H, 2-C*H*$_3$), 1.64 (m, 1H, 9-H), 1.71 (m, 1H, 9-H), 1.78 (m, 1H, 10-H), 2.03 (m, 1H, 10-H), 2.55 (d, $J_{OH,11}$ = 4.8 Hz, 1H, 11-OH), 2.86 (dd, J_{gem} = 13.9, $J_{5,3}$ = 0.8 Hz, 1H, 5-H), 2.98 (d, J_{gem} = 13.9 Hz, 1H, 5-H), 3.64 (m, 1H, 11-H), 3.91 (m, 1H, 8-H), 4.59 (qq, $J_{2,3}$ = 10.1, $J_{2,2}$ = 6.2 Hz, 1H, 2-H), 5.17 (dd, $J_{3,2}$ = 10.1, $J_{3,5}$ = 0.8 Hz, 1H, 3-H), 7.63 (d, $J_{3'5',2'6'}$ = 8.6 Hz, 2H, 3'-H, 5'-H), 7.97 (d, $J_{2'6',3'5'}$ = 8.6 Hz, 2H, 2'-H, 6'-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.7 (2-*C*H$_3$), 20.9 (8-*C*H$_3$), 25.1 (C-10), 27.3 (C-9), 45.8 (C-5), 69.2 (C-11), 69.3 (C-2), 70.1 (C-8), 78.8 (C-3), 101.1 (C-6), 128.1[a] (C-4'), 128.7[a] (C-1'), 131.5[b] (C-2', C-6'), 131.8[b] (C-3', C-5'), 164.7 (-*C*OO), 198.3 (C-4).-

EIMS (70 eV, 200 °C): *m/z* (%) = 414 [M^+ + 2] (5), 412 [M^+] (5), 333 (14), 329 (83), 327 (83), 284 (5), 282 (5), 228 (11), 226 (11), 185 (100), 183 (100), 168 (50), 157 (31), 155 (32), 127 (32), 85 (38), 44 (43), 29 (14).-

[a-b] Identical superscripts represent interchangeable assignments.

7.3.2.5.1.2 Dibenzoate of 3,11-dihydroxy-2,8-dimethyl-1,7-dioxa-spiro[5,5]undecan-4-one (**6744-4b**)

Molecular formula: $C_{25}H_{24}Br_2O_7$.-

Molecular weight: 593.9.-

Optical rotation: $[\alpha]^{25}_D$ = - 48.2 (*c* 0.6, $CHCl_3$).-

R_f value: 0.69 (CH_2Cl_2/1.4% MeOH).-

UV ($CHCl_3$): λ_{max} (lg ε) = 272 nm (3.37).-

IR (KBr): ν = 2927 cm^{-1}, 2854, 1747, 1728, 1591, 1485, 1456, 1398, 1273, 1242, 1173, 1117, 1103, 1012, 982, 847, 754.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.38 (d, $J_{8,8}$ = 6.5 Hz, 3H, 8-C*H*$_3$), 1.40 (d, $J_{2,2}$ = 6.2 Hz, 3H, 2-C*H*$_3$), 1.74 (m, 1H, 9-H), 1.87 (m, 1H, 9-H), 1.99 (m, 1H, 10-H), 2.18 (m, 1H, 10-H), 2.82 (dd, J_{gem} = 13.7, $J_{5,3}$ = 0.8 Hz, 1H, 5-H), 2.94 (d, J_{gem} = 13.7 Hz, 1H, 5-H), 4.06 (m, 1H, 8-H), 4.54 (qq, $J_{2,3}$ = 10.1, $J_{2,2}$ = 6.2 Hz, 1H, 2-H), 5.13 (dd, $J_{11,10}$ = 7.5, $J_{11,10}$ = 3.9 Hz, 1H, 11-H), 5.16 (d, $J_{3,2}$ = 10.1 Hz, 1H, 3-H), 7.63[a] (d, $J_{3"5",2"6"}$ = 8.7 Hz, 2H, 3"-H, 5"-H), 7.65[a] (d, $J_{3'5',2'6'}$ = 8.7 Hz, 2H, 3'-H, 5'-H), 7.96[b] (d, $J_{2"6",3"5"}$ = 8.7 Hz, 2H, 2"-H, 6"-H), 7.98[b] (d, $J_{2'6',3'5'}$ = 8.7 Hz, 2H, 2'-H, 6'-H).-

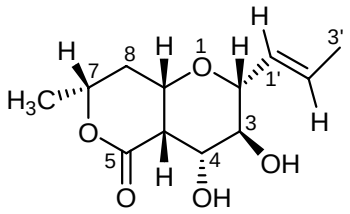
^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.7 (2-*C*H$_3$), 21.0 (8-*C*H$_3$), 22.6 (C-10), 34.1 (C-9), 46.7 (C-5), 69.1 (C-2), 70.0 (C-8), 71.3 (C-11), 78.9 (C-3), 100.0 (C-6), 128.1[c] (C-4"), 128.4[c] (C-1"),

128.7[c] (C-4'), 129.0[c] (C-1'), 131.3[d] (C-2", C-6"), 131.5[d] (C-2', C-6'), 131.8[d] (C-3", C-5"), 131.9[d] (C-3', C-5'), 164.6 (-*C*OO), 165.2 (-*C*OO), 198.0 (C-4).-

EIMS (70 eV): *m/z* (%) = 598 [M^+ + 4] (1), 596 [M^+ + 2] (2), 594 [M^+] (1), 554 (1), 552 (2), 550 (1), 517 (2), 515 (2), 396 (5), 394 (5), 352 (3), 350 (3), 284 (2), 282 (2), 183 (100), 157 (8), 155 (9), 104 (13), 85 (12), 43 (5).-

[a-d] Identical superscripts represent interchangeable assignments.

7.3.2.6 (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-Hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-5**)



Molecular formula: $C_{12}H_{18}O_5$.-

Melting point: 159 °C.-

Molecular weight: 242.1.-

Optical rotation: $[\alpha]^{25}_D$ = - 27 (*c* 0.97, CH_2Cl_2).-

R_f value: 0.23 (CH_2Cl_2/3.8% MeOH).-

IR (KBr): ν = 3444 cm^{-1}, 3390, 2960, 2916, 2854, 1745, 1732, 1385, 1259, 1232, 1205, 1063, 1014, 958, 796.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.44 (d, $J_{7,7}$ = 6.3 Hz, 3H, 7-C*H*$_3$), 1.78 (dd, $J_{3',2'}$ = 6.4, $J_{3',1'}$ = 1.4 Hz, 3H, 3'-C*H*$_3$), 1.81 (dtd, J_{gem} = 15.2, $J_{8,7}$ = 12.0, $J_{8,8a}$ = 3.0 Hz, 1H, 8-H), 2.48 (dddd, J_{gem} = 15.2, $J_{8,8a}$ = 9.1, $J_{8,7}$ = 3.9 Hz, 1H, 8-H), 2.57 (brs, 1H, 3-OH), 3.02 (t, $J_{4a,4}$ = $J_{4a,8a}$ = 3.8 Hz, 1H, 4a-H), 3.57 (t, $J_{2,3}$ = $J_{2,1'}$ = 8.1 Hz, 1H, 2-H), 3.66 (t, $J_{3,4}$ = $J_{3,2}$ = 8.1 Hz, 1H, 3-H), 3.71 (m, 1H, 4-H), 4.04 (d, $J_{OH,4}$ = 11.2 Hz, 1H, 4-OH), 4.16 (sextet, $J_{8a,8}$ = 9.1, $J_{8a,4a}$ = 3.8, $J_{8a,8}$ = 3.0 Hz, 1H, 8a-H), 4.38 (septet, $J_{7,8}$ = 12.0, $J_{7,7}$ = 6.3, $J_{7,8}$ = 3.9 Hz, 1H, 7-H), 5.52 (qqqq, $J_{1',2'}$ = 15.4, $J_{1',2}$ = 8.1, $J_{1',3'}$ = 1.4 Hz, 1H, 1'-H), 5.89 (qq, $J_{2',1'}$ = 15.4, $J_{2',3'}$ = 6.4 Hz, 1H, 2'-H).-

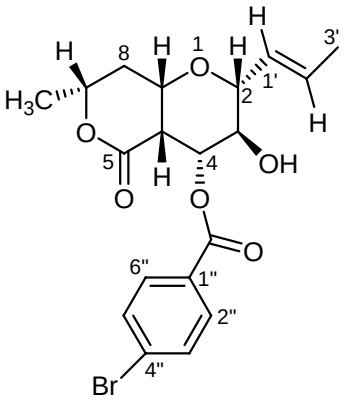
^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.0 (3'-*C*H$_3$), 20.5 (7-*C*H$_3$), 36.9 (C-8), 44.8 (C-4a), 71.2 (C-8a), 72.1 (C-3), 72.7 (C-7), 73.4 (C-4), 81.1 (C-2), 127.7 (C-1'), 131.4 (C-2'), 173.2 (C-5).-

EIMS (70 eV, 175 °C): *m/z* (%) = 242 [M^+] (16), 224 (8), 188 (13), 172 (28), 113 (100), 100 (23), 84 (40), 71 (38), 41 (16).-

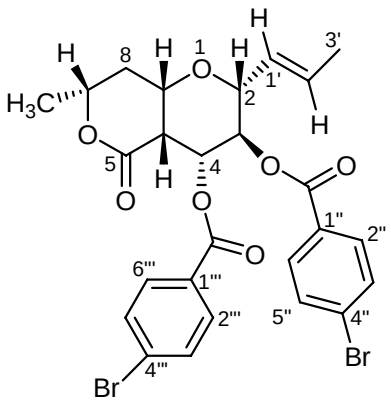
HREIMS: Calcd.: 242.11543

Found: 242.11567

7.3.2.6.1 (2*R*,3*R*,4*R*,4a*R*,7*R*,8a*S*)-Octahydro-3-hydroxy-7-methyl-5-oxo-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-4-yl 4-bromobenzoate (**6744-5a**) and dibenzoate of (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-5b**)



ms-6744-5a



ms-6744-5b

To a stirred solution of **6744-5** (7.5 mg, 0.031 mmol) in dry pyridine (2.0 mL) was added *p*-bromobenzoyl chloride (21.0 mg) and *N*,*N*-dimethyl-4-aminopyridine (10.0 mg). The reaction mixture was stirred at room temperature for about 1.5 h (TLC monitoring), and was then neutralized by addition of 1 N HCl. The mixture was extracted with CH_2Cl_2, washed with water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The resulting mixture was purified by preparative TLC on silica gel (1 mm, 1 development, CH_2Cl_2/0.6% MeOH) followed by recrystallization (CH_2Cl_2/Et_2O) to afford **6744-5a** (6.3 mg, 50%) and **6744-5b** (6.0 mg, 32%) as fine needles.

7.3.2.6.1.1 (2*R*,3*R*,4*R*,4a*R*,7*R*,8a*S*)-Octahydro-3-hydroxy-7-methyl-5-oxo-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-4-yl 4-bromobenzoate (**6744-5a**)

Molecular formula: $C_{19}H_{21}BrO_6$.-

Melting point: 143 °C.-

Molecular weight: 424.1.-

Optical rotation: $[\alpha]^{25}_D$ = - 11.9 (*c* 0.59, $CHCl_3$).-

R_f value: 0.33 (CH_2Cl_2/1.4% MeOH).-

UV ($CHCl_3$): λ_{max} (lg ε) = 268 nm (3.35).-

IR (KBr): ν = 3498 cm^{-1}, 2925, 2854, 1755, 1747, 1714, 1590, 1485, 1398, 1284, 1275, 1196, 1120, 1068, 1012, 962, 762.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.30 (d, $J_{7,7}$ = 6.0 Hz, 3H, 7-C*H*$_3$), 1.69 (dd, $J_{3',2'}$ = 6.5, $J_{3',1'}$ = 1.3 Hz, 3H, 3'-C*H*$_3$), 1.69 (dddd, J_{gem} = 15.3, $J_{8,7}$ = 12.1, $J_{8,8a}$ = 2.7 Hz, 1H, 8-H), 2.03 (brs, 3-OH), 2.39 (dddd, J_{gem} = 15.3, $J_{8,8a}$ = 9.4, $J_{8,7}$ = 3.6 Hz, 1H, 8-H), 3.26 (dd, $J_{4a,4}$ = 4.9, $J_{4a,8a}$= 3.1 Hz, 1H, 4a-H), 3.63 (t, $J_{2,3}$ = $J_{2,1'}$ = 9.2 Hz, 1H, 2-H), 4.15 (t, $J_{3,4}$ = $J_{3,2}$ = 9.2 Hz, 1H, 3-H), 4.22 (sextet, $J_{8a,8}$ = 9.4, $J_{8a,4a}$ = 3.1, $J_{8a,8}$ = 2.7 Hz, 1H, 8a-H), 4.26 (m, 1H, 7-H), 5.09 (dd, $J_{4,3}$ = 9.2, $J_{4,4a}$ = 4.9 Hz, 1H, 4-H), 5.45 (qqqq, $J_{1',2'}$ = 15.4, $J_{1',2}$ = 9.2, $J_{1',3'}$ = 1.3 Hz, 1H, 1'-H), 5.83 (qq, $J_{2',1'}$ = 15.4, $J_{2',3'}$ = 6.5 Hz, 1H, 2'-H), 7.51 (d, $J_{3'',5'',2'',6''}$ = 8.5 Hz, 2H, 3''-H, 5''-H), 7.90 (d, $J_{2'',6'',3'',5''}$ = 8.5 Hz, 2H, 2''-H, 6''-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.0 (3'-*C*H$_3$), 20.3 (7-*C*H$_3$), 37.3 (C-8), 43.9 (C-4a), 68.4 (C-3), 71.7 (C-8a), 72.1 (C-7), 74.3 (C-4), 82.0 (C-2), 127.4 (C-1'), 128.6[a] (C-4''), 128.6[a] (C-1''), 131.6[b] (C-2'', C-6''), 131.8[b] (C-3'', C-5''), 132.4 (C-2'), 166.1 (-*C*OO), 168.5 (C-5).-

EIMS (70 eV, 200 °C): *m/z* (%) = 426 [M^+ + 2] (5), 424 [M^+] (5), 356 (3), 354 (3), 309 (3), 202 (7), 200 (7), 185 (67), 183 (69), 154 (100), 113 (58), 112 (82), 71 (24), 55 (16).-

[a-b] Identical superscripts represent interchangeable assignments.

7.3.2.6.1.2 Dibenzoate of (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-5b**)

Molecular formula: $C_{26}H_{24}Br_2O_7$.-

Melting point: 200 °C.-

Molecular weight: 606.0.-

Optical rotation: $[\alpha]^{25}_D$ = - 95.6 (*c* 0.16, $CHCl_3$).-

R_f value: 0.69 (CH_2Cl_2/1.4% MeOH).-

UV ($CHCl_3$): λ_{max} (lg ε) = 267 nm (3.72).-

IR (KBr): ν = 2924 cm^{-1}, 2854, 1741, 1720, 1589, 1483, 1398, 1286, 1263, 1201, 1174, 1115, 1103, 1070, 1012, 962, 756.-

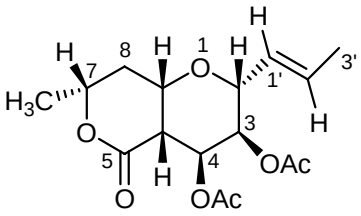
^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.42 (d, $J_{7,7}$ = 6.1 Hz, 3H, 7-C*H*$_3$), 1.62 (dd, $J_{3',2'}$ = 6.6, $J_{3',1'}$ = 1.6 Hz, 3H, 3'-C*H*$_3$), 1.84 (dtd, J_{gem} = 15.4, $J_{8,7}$ = 12.1, $J_{8,8a}$ = 2.8 Hz, 1H, 8-H), 2.53 (dddd, J_{gem} = 15.4, $J_{8,8a}$ = 9.3, $J_{8,7}$ = 3.6 Hz, 1H, 8-H), 3.45 (dd, $J_{4a,4}$ = 5.0, $J_{4a,8a}$ = 3.2 Hz, 1H, 4a-H), 3.95 (t, $J_{2,3}$ = $J_{2,1'}$ = 7.8 Hz, 1H, 2-H), 4.38 (m, 1H, 7-H), 4.38 (sextet, $J_{8a,8}$ = 9.3, $J_{8a,4a}$ = 3.2, $J_{8a,8}$ = 2.8 Hz, 1H, 8a-H), 5.45 (dd, $J_{4,3}$ = 9.6, $J_{4,4a}$ = 5.0 Hz, 1H, 4-H), 5.51 (qqqq, $J_{1',2'}$ = 15.3, $J_{1',2}$ = 7.8, $J_{1',3'}$ = 1.6 Hz, 1H, 1'-H), 5.78 (qq, $J_{2',1'}$ = 15.3, $J_{2',3'}$ = 6.6 Hz, 1H, 2'-H), 5.87 (t, $J_{3,4}$ = $J_{3,2}$ = 9.6 Hz, 1H, 3-H), 7.54 (d, $J_{3'',5'',2'',6''}$ = 8.5 Hz, 2H, 3''-H, 5''-H), 7.54 (d, $J_{3''',5''',2''',6'''}$ = 8.5 Hz, 2H, 3'''-H, 5'''-H), 7.80[a] (d, $J_{2'',6'',3'',5''}$ = 8.5 Hz, 2H, 2''-H, 6''-H), 7.90[a] (d, $J_{2''',6''',3''',5'''}$ = 8.5 Hz, 2H, 2'''-H, 6'''-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.8 (3'-*C*H_3), 20.4 (7-*C*H_3), 37.3 (C-8), 44.1 (C-4a), 69.4 (C-3), 71.9 (C-4), 72.0 (C-7), 72.0 (C-8a), 80.7 (C-2), 126.6 (C-1'), 128.1[b] (C-1"), 128.3[b] (C-4"), 128.5[b] (C-1"'), 128.7[b] (C-4"'), 131.1[c] (C-2", C-6"), 131.5[c] (C-2"', C-6"'), 131.8 (C-3"', C-5"'), 131.8 (C-3", C-5"), 132.4 (C-2'), 164.3 (-*C*OO), 165.8 (-*C*OO), 167.7 (C-5).-

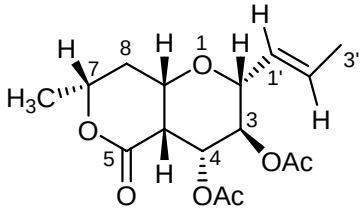
EIMS (70 eV, 200 °C): *m/z* (%) = 610 [M^+ + 4] (2), 608 [M^+ + 2] (3), 606 [M^+] (2), 408 (8), 406 (8), 338 (12), 336 (12), 223 (68), 183 (100), 155 (9), 104 (10), 43 (4).-

[a-c] Identical superscripts represent interchangeable assignments.

7.3.2.7 Diacetates of (2*S*,3*R*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-6a**) and (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-6b**)



6744-6a



6744-6b

To a suspension of 249 mg of a polar fraction (CH_2Cl_2/1.5-3% MeOH) in CH_2Cl_2 (5 mL) and pyridine (2 mL) were added acetic anhydride (0.6 mL) and DMAP (15 mg), and the mixture was stirred at room temperature for 5 h (TLC control). The reaction was worked up by dilution with 2 N HCl and extraction with CH_2Cl_2 (30 mL × 3). The combined extracts were washed with water, dried (Na_2SO_4), and concentrated in vacuo to give a semisolid residue (323 mg). Flash chromatography of the crude extract followed by repeated preparative TLC on silica gel (toluene/20% ethyl acetate) afforded the mixture of **6744-6a** and **6744-6b** (**6744-6b:6744-6a = 2.4:1**; 9.6 mg) as colourless gum.

7.3.2.7.1 Diacetate of (2*S*,3*R*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-6a**)

*Molecular formula: $C_{16}H_{22}O_7$.-

*Molecular weight: 326.2.-

*Optical rotation: $[\alpha]^{25}_D$ = + 3.7 (*c* 0.94, $CHCl_3$).-

*R_f value: 0.28 (CH_2Cl_2/1.4% MeOH).-

*UV ($CHCl_3$): λ_{max} (lg ε) = 270 nm (2.31), 271 (2.30), 274 (2.29).-

*IR (KBr): ν = 2925 cm^{-1}, 1755, 1747, 1732, 1373, 1246, 1230, 1198, 1059, 962.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.41 (d, $J_{7,7}$ = 6.2 Hz, 3H, 7-C*H*$_3$), 1.70 (dd, $J_{3',2'}$ = 6.5, $J_{3',1'}$ = 1.7 Hz, 3H, 3'-C*H*$_3$), 1.75 (dtd, J_{gem} = 15.3, $J_{8,7}$ = 12.0, $J_{8,8a}$ = 3.3 Hz, 1H, 8-H), 1.99 (s, 3H, 3-OCOC*H*$_3$), 2.17 (s, 3H, 4-OCOC*H*$_3$), 2.44 (dddd, J_{gem} = 15.3, $J_{8,8a}$ = 9.3, $J_{8,7}$ = 3.6 Hz, 1H, 8-H), 2.88 (t, $J_{4a,4}$ = $J_{4a,8a}$ = 3.3 Hz, 1H, 4a-H), 4.08 (dd, $J_{2,3}$ = 10.0, $J_{2,1'}$ = 7.7 Hz, 1H, 2-H), 4.32 (m, 1H, 7-H), 4.44 (tt, $J_{8a,8}$ = 9.3, $J_{8a,8}$ = $J_{8a,4a}$ = 3.3 Hz, 1H, 8a-H), 5.11 (dd, $J_{3,2}$ = 10.0, $J_{3,4}$ = 3.3 Hz, 1H, 3-H), 5.38 (qqqq, $J_{1',2'}$ = 15.3, $J_{1',2}$ = 7.7, $J_{1',3'}$ = 1.7 Hz, 1H, 1'-H), 5.79** (1H, 2'-H), 5.83 (t, $J_{4,3}$ = $J_{4,4a}$ = 3.3 Hz, 1H, 4-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.9 (3'-*C*H$_3$), 20.5 (7-*C*H$_3$), 20.7 (3-OCO*C*H$_3$), 20.9 (4-OCO*C*H$_3$), 36.7 (C-8), 44.6 (C-4a), 67.0 (C-4), 67.8 (C-3), 67.9 (C-8a), 72.2 (C-7), 76.0 (C-2), 127.4 (C-1'), 131.5 (C-2'), 169.0 (C-5), 169.2 (3-O*C*OCH$_3$), 169.6 (4-O*C*OCH$_3$).-

*EIMS (70 eV, 200 °C): *m/z* (%) = 326 [M^+] (5), 283 (10), 266 (34), 224 (34), 223 (72), 213 (34), 196 (32), 171 (33), 154 (64), 113 (76), 112 (60), 71 (43), 43 (100).-

*HREIMS: Calcd.: 326.13656

Found: 326.13657

*Data for mixed diacetates

**J value could not be provided due to overlapping with another signal

7.3.2.7.2 (2*R*,3*S*,4*R*,4a*S*,7*R*,8a*S*)-hexahydro-3,4-dihydroxy-7-methyl-2-((*E*)-prop-1-enyl)pyrano[4,3-*b*]pyran-5(7*H*)-one (**6744-6b**)

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.39 (d, $J_{7,7}$ = 6.2 Hz, 3H, 7-C*H*$_3$), 1.69 (dd, $J_{3',2'}$ = 6.4, $J_{3',1'}$ = 1.8 Hz, 3H, 3'-C*H*$_3$), 1.76 (dtd, J_{gem} = 15.2, $J_{8,7}$ = 12.1, $J_{8,8a}$ = 2.9 Hz, 1H, 8-H), 2.01 (s, 3H, 3-OCOC*H*$_3$), 2.11 (s, 3H, 4-OCOC*H*$_3$), 2.45 (dddd, J_{gem} = 15.2, $J_{8,8a}$ = 9.2, $J_{8,7}$ = 3.7 Hz, 1H, 8-H), 3.32 (dd, $J_{4a,4}$ = 5.0, $J_{4a,8a}$ = 2.9 Hz, 1H, 4a-H), 3.72 (t, $J_{2,3}$ = $J_{2,1'}$ = 8.7 Hz, 1H, 2-H), 4.23 (tt, $J_{8a,8}$ = 9.2, $J_{8a,8}$ = $J_{8a,4a}$ = 2.9 Hz, 1H, 8a-H), 4.32 (m, 1H, 7-H), 5.04 (dd, $J_{4,3}$ = 10.0, $J_{4,4a}$ = 5.0 Hz, 1H, 4-H), 5.43 (qqqq, $J_{1',2'}$ = 15.3, $J_{1',2}$ = 7.9, $J_{1',3'}$ = 1.8 Hz, 1H, 1'-H), 5.48 (t, $J_{3,2}$ = $J_{3,4}$ = 9.8 Hz, 1H, 3-H), 5.79 (dqq, $J_{2',1'}$ = 15.3, $J_{2',3'}$ = 6.4, $J_{2',2}$ = 0.7 Hz, 1H, 2'-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.8 (3'-*C*H$_3$), 20.3 (7-*C*H$_3$), 20.8 (3-OCO*C*H$_3$), 21.0 (4-OCO*C*H$_3$), 37.2 (C-8), 43.8 (C-4a), 68.5 (C-3), 71.5 (C-4), 71.8 (C-8a), 71.9 (C-7), 80.8 (C-2), 127.0 (C-1'), 131.9 (C-2'), 167.8 (C-5), 169.1 (3-O*C*OCH$_3$), 171.2 (4-O*C*OCH$_3$).-

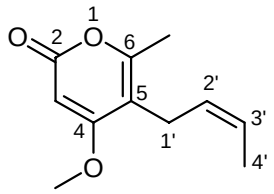
7.3.3 Metabolites from Strain 6760

7.3.3.1 Isolation of secondary metabolites

Strain 6760 was cultivated at room temperature for 28 days in biomalt semi-solid agar medium. After that, this culture medium was extracted three times with ethyl acetate to obtain the crude

extract (9.0 g). The crude extract was subjected to column chromatography for fractionation on silica gel, using gradients of petroleum ether/dichloromethane, then dichloromethane, after that gradients of dichloromethane with up to of 20% methanol to afford a total of 41 fractions. These fractions were screened by TLC on silica gel under UV light and by spraying with cerium-molybdenum spray reagents. The column fraction of petroleum ether/70-100% CH_2Cl_2 was subjected to preparative TLC on silica gel (hexane/20% EtOAc) to obtain **6760-1** (7.0 mg, colourless gum) and **6760-2** (33.6 mg, colourless gum). The column fraction of CH_2Cl_2/0-5% MeOH was subjected to preparative TLC on silica gel (hexane/30% EtOAc) to obtain **6760-3** (3.0 mg, semisolid) and **6760-4** as white solid mass, which after crystallization from CH_2Cl_2/Et_2O gave **6760-4** (52.5 mg) as a white powder.

7.3.3.2 5-((*Z*)-But-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (**6760-1**)



Molecular formula: $C_{11}H_{14}O_3$.-

Molecular weight: 194.2.-

R_f value: 0.72 (hexane/50% EtOAc).-

UV ($CHCl_3$): λ_{max} (lg ε) = 272 nm (2.78), 273 (2.78).-

IR (KBr): ν = 2927 cm^{-1}, 2856, 1738, 1732, 1714, 1633, 1462, 1456, 1246, 1238, 1174, 970.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.59 (s, 3H, 6-C*H*$_3$), 1.66 (m, 3H, 4'-C*H*$_3$), 3.03 (m, 2H, C*H*$_2$), 3.72 (s, 3H, 4-OC*H*$_3$), 5.32 (s, 1H, 3-H), 5.36 (m, 2H, 2'-H, 3'-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.9 (4'-*C*H$_3$), 22.2 (6-*C*H$_3$), 27.2 (C-1'), 56.1 (4-O*C*H$_3$), 92.4 (C-3), 103.3 (C-5), 130.0[a] (C-3'), 130.4[a] (C-2'), 158.3 (C-6), 164.0 (C-2), 168.0 (C-4).-

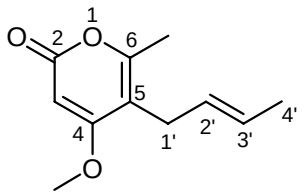
EIMS (70 eV, 200 °C): *m/z* (%) = 194 [M^+] (42), 167 (20), 151 (38), 149 (25), 123 (29), 111 (42), 97 (56), 69 (64), 57 (98), 43 (74), 28 (100).-

HREIMS: Calcd.: 194.0943

Found: 194.19930

[a] Identical superscripts represent interchangeable assignments.

7.3.3.3 5-((*E*)-But-2-enyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (**6760-2**)



Molecular formula: $C_{11}H_{14}O_3$.-

Molecular weight: 194.1.-

R_f value: 0.47 (hexane/50% EtOAc).-

UV ($CHCl_3$): λ_{max} (lg ε) = 288 nm (3.22), 292 (3.23), 293 (3.23).-

IR (KBr): ν = 2941 cm^{-1}, 1722, 1718, 1699, 1643, 1633, 1562, 1456, 1408, 1250, 968, 812.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.66 (tt, , $J_{4',3'}$ = 6.1, $J_{4',2'}$ = 2.6, $J_{4',1'}$ = 1.3 Hz, 3H, 4'-C*H*$_3$), 2.22 (s, 3H, 6-C*H*$_3$), 3.02 (dd, $J_{1',2'}$ = 5.2, $J_{1',3'}$ = 1.6 Hz, 2H, C*H*$_2$), 3.83 (s, 3H, 4-OC*H*$_3$), 5.40 (m, 2H, 2'-H, 3'-H), 5.46 (s, 1H, 3-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 17.1 (6-*C*H$_3$), 17.5 (4'-*C*H$_3$), 26.8 (C-1'), 56.1 (4-O*C*H$_3$), 87.8 (C-3), 109.9 (C-5), 126.1[a] (C-3'), 127.0[a] (C-2'), 158.7 (C-6), 164.5 (C-2), 170.6 (C-4).-

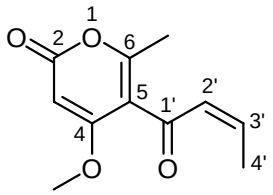
EIMS (70 eV, 200 °C): *m/z* (%) = 194 [M^+] (92), 166 (29), 151 (88), 137 (31), 123 (43), 112 (18), 91 (44), 69 (36), 55 (27), 43 (100), 28 (30).-

HREIMS: Calcd.: 194.0943

Found: 194.09427

[a] Identical superscripts represent interchangeable assignments.

7.3.3.4 5-((*Z*)-But-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (**6760-3**)



Molecular formula: $C_{11}H_{12}O_4$.-

Molecular weight: 208.1.-

R_f value: 0.38 (hexane/50% EtOAc).-

UV ($CHCl_3$): λ_{max} (lg ε) = 290 nm (3.17), 291 (3.17), 292 (3.16).-

IR (KBr): ν = 2929 cm^{-1}, 2856, 1738, 1732, 1714, 1682, 1633, 1564, 1456, 1400, 1255.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 2.20 (dd, $J_{4',3'}$ = 7.2, $J_{4',2'}$ = 1.5 Hz, 3H, 4'-CH_3), 2.29 (s, 3H, 6-CH_3), 3.85 (s, 3H, 4-OCH_3), 5.50 (s, 1H), 6.31 (qq, $J_{2',3'}$ = 11.5, $J_{2',4'}$ = 1.5 Hz, 1H, 2'-H), 6.38 (qq, $J_{3',2'}$ = 11.5, $J_{3',4'}$ = 7.2 Hz, 1H, 3'-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 16.2 (4'-CH$_3$), 18.3 (6-CH$_3$), 56.3 (4-OCH$_3$), 87.7 (C-3), 116.4 (C-5), 129.0 (C-2'), 144.9 (C-3'), 162.9[a] (C-6), 163.0[a] (C-2), 168.7 (C-4), 190.0 (C-1').-

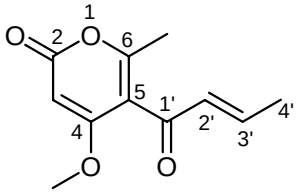
EIMS (70 eV, 200 °C): *m/z* (%) = 208 [M^+] (20), 193 (100), 179 (21), 165 (18), 125 (34), 83 (16), 69 (82), 55 (25), 43 (69), 41 (40).-

HREIMS: Calcd.: 208.07358

Found: 208.07341

[a] Identical superscripts represent interchangeable assignments.

7.3.3.5 5-((*E*)-But-2-enoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (**6760-4**)



Molecular formula: $C_{11}H_{12}O_4$.-

Melting point: 100 °C (Lit.[158]: 110.9-111.6).-

Molecular weight: 208.1.-

R_f value: 0.30 (hexane/50% EtOAc).-

IR (KBr): ν = 2929 cm^{-1}, 2856, 1738, 1732, 1714, 1660, 1633, 1564, 1400, 1296, 1248.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.99 (dd, $J_{4',3'}$ = 6.9, $J_{4',2'}$ = 1.5 Hz, 3H, 4'-CH_3), 2.19 (s, 3H, 6-CH_3), 3.82 (s, 3H, 4-OCH_3), 5.49 (s, 1H), 6.33 (qq, $J_{2',3'}$ = 15.6, $J_{2',4'}$ = 1.5 Hz, 1H, 2'-H), 6.81 (qq, $J_{3',2'}$ = 15.6, $J_{3',4'}$ = 6.9 Hz, 1H, 3'-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 18.2 (6-CH$_3$), 18.5 (4'-CH$_3$), 56.3 (4-OCH$_3$), 87.7 (C-3), 114.0 (C-5), 133.1 (C-2'), 147.2 (C-3'), 161.4 (C-6), 163.0 (C-2), 168.7 (C-4), 190.5 (C-1').-

EIMS (70 eV, 200 °C): *m/z* (%) = 208 [M^+] (65), 194 (55), 193 (88), 165 (59), 161 (33), 125 (64), 109 (16), 77 (14), 69 (100), 43 (78), 41 (70), 28 (15).-

7.3.4 Metabolites from *Prismatomeris tetrandra*

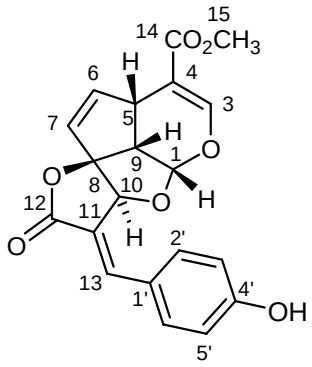
7.3.4.1 Isolation of secondary metabolites

Mr. Sujit Kumar Dey, University of Dhaka, Bangladesh isolated two metabolites, namely prismatomerin (**PT-1**) and gaertneroside (**PT-2**), from *Prismatomeris tetrandra*. Recording of

NMR spectra, discussion on structure elucidation, and derivatization of **PT-1** to the 4-bromobenzoate **PT-1a** were performed by us.

Plant materials of *Prismatomeris tetrandra* were collected from Chokoria, Cox's Bazar, Bangladesh during 02-04 April, 2000 and were identified by the late Professor M. Salar Khan of Bangladesh National Herbarium (BNH). A voucher specimen for this collection is maintained at BNH under the accession number DACB-29513. The air-dried powdered leaves (3.0 kg) were extracted using a 1:1 CH_2Cl_2:MeOH solvent system and the residue obtained from this extract was suspended in water and partitioned with CH_2Cl_2. The residue of the CH_2Cl_2 soluble part was again suspended in aqueous 90% MeOH and partitioned with hexane to separate the fatty and non-polar metabolites. Repeated silica gel chromatography of the residue of the aqueous methanolic soluble part afforded the new iridoid prismatomerin (**PT-1**, 70 mg) as fine needles from 20% ethyl acetate in hexane. Repeated column chromatography followed by HPLC of one of the relatively polar column fractions of the same residue afforded the glucoside gaertneroside (**PT-2**, 15 mg) as a pale yellow solid. Prismatomerin (**PT-1**) was reacted with 4-bromobenzoyl chloride to afford the 4-bromobenzoate **PT-1a** with the hope to establish the absolute configuration by X-ray single crystal analysis with a heavy atom incorporated (Section **7.3.4.2.1**). The analyses of the NMR spectra of **PT-1a** were in total agreement with the proposed structure and especially all the 1D and 2D NMR data related to H-1 and H-7 contributed to reconfirm the results obtained for **PT-1**.

7.3.4.2 Prismatomerin (**PT-1**)



Molecular formula: $C_{20}H_{16}O_7$.-

Melting point: 134-135 °C.-

Molecular weight: 368.1.-

Optical rotation: $[\alpha]^{25}_D$ = - 136 (*c* 0.01, EtOH).-

R_f value: 0.45 (hexane/16.7% EtOAc).-

UV ($CHCl_3$): λ_{max} (lg ε) = 316 nm (4.07), 317 (4.07), 318 (4.07).-

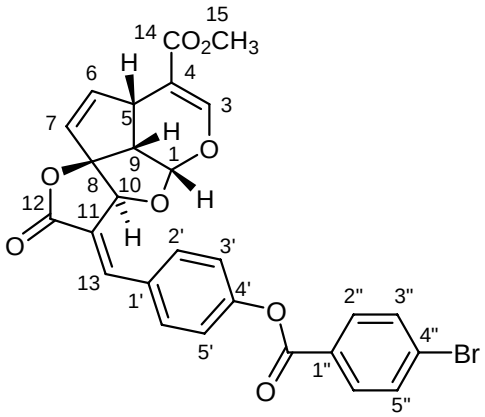
IR (KBr): ν = 3512 cm^{-1}, 3123, 2962, 1732, 1696, 1644.-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 3.56 (dd, *J* = 9.5, 5.8 Hz, 1H, 9-H), 3.81 (s, 3H, 15-OC*H*$_3$), 4.08 (tt, *J* = 9.5, 2.0 Hz, 1H, 5-H), 5.25 (brs, 1H, 10-H), 5.67 (1H, 7-H), 5.67 (d, *J* = 5.8 Hz, 1H, 1-H), 5.73 (brs, 1H, 4'-OH), 6.07 (dd, *J* = 5.4, 2.1 Hz, 1H, 6-H), 6.97 (d, *J* = 8.6 Hz, 2H, 3'-H, 5'-H), 7.50 (s, 1H, 3-H), 7.71 (d, *J* = 8.6 Hz, 2H, 2'-H, 6'-H), 7.81 (brs, 1H, 13-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 38.5 (C-5), 51.7 (15-O*C*H$_3$), 54.2 (C-9), 82.0 (C-10), 102.4 (C-1), 104.5 (C-8), 109.6 (C-4), 116.3 (C-3', C-5'), 120.2 (C-11), 126.2 (C-1'), 126.5 (C-7), 133.3 (C-2', C-6'), 141.1 (C-6), 144.4 (C-13), 152.9 (C-3), 158.9 (C-4'), 166.8 (C-14), 170.2 (C-12).-

ESIMS: *m/z* = 368 [M^+], 351, 337, 319, 309, 291, 275, 249, 199, 175, 163, 139, 133.-

7.3.4.2.1 4-Bromobenzoate of prismatomerin (**PT-1a**)



To a stirred solution of **PT-1** (7.9 mg, 0.021 mmol) in dry pyridine (2.0 mL) was added *p*-bromobenzoyl chloride (8.9 mg) and *N*,*N*-dimethyl-4-aminopyridine (20.0 mg). The reaction mixture was stirred at room temperature for about 0.5 h (TLC monitoring), and was then neutralized by addition of 1 N HCl. The mixture was extracted with CH_2Cl_2, washed with water, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The resulting mixture was purified by preparative TLC on silica gel (CH_2Cl_2/1% MeOH) followed by recrystallization (CH_2Cl_2/Et_2O) to afford **PT-1a** (9.0 mg, 76%) as fine needles.

Molecular formula: $C_{27}H_{19}BrO_8$.-

Melting point: 175 °C.-

Molecular weight: 550.0.-

Optical rotation: $[\alpha]^{25}_D$ = - 56.9 (*c* 0.42, $CHCl_3$).-

R_f value: 0.53 (CH_2Cl_2/1% MeOH).-

UV ($CHCl_3$): λ_{max} (lg ε) = 296 nm (4.47), 299 (4.47), 302 (4.49).-

IR (KBr): ν = 2953 cm^{-1}, 2924, 2854, 1755, 1738, 1705, 1651, 1645, 1589, 1508, 1439, 1398, 1298, 1263, 1211, 1169, 1084, 1022, 1011, 964, 872.-

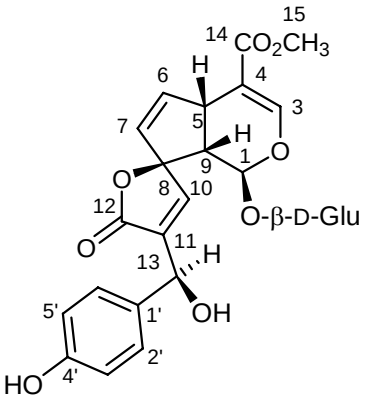
^{1}H-NMR (500 MHz, $CDCl_3$): δ = 3.59 (dd, *J* = 9.5, 5.8 Hz, 1H, 9-H), 3.81 (s, 3H, 15-OC*H*$_3$), 4.08 (tt, *J* = 9.5, 2.2 Hz, 1H, 5-H), 5.23 (s, 1H, 10-H), 5.66 (dd, *J* = 5.5, 2.2 Hz,1H, 7-H), 5.68 (d, *J* = 5.8 Hz, 1H, 1-H), 6.08 (dd, *J* = 5.5, 2.2 Hz, 1H, 6-H), 7.38 (d, *J* = 8.8 Hz, 2H, 3'-H, 5'-H), 7.49 (s, 1H, 3-H), 7.70[a] (d, *J* = 8.5 Hz, 2H, 2"-H, 6"-H), 7.87 (d, *J* = 8.8 Hz, 2H, 2'-H, 6'-H), 7.88 (s, 1H, 13-H), 8.09[a] (d, *J* = 8.5 Hz, 2H, 3"-H, 5"-H).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 38.5 (C-5), 51.7 (15-O*C*H$_3$), 54.2 (C-9), 81.5 (C-10), 102.4 (C-1), 104.7 (C-8), 109.5 (C-4), 122.6 (C-3', C-5'), 123.6 (C-11), 126.3 (C-7), 128.0 (C-1'), 129.3 (C-1"), 131.2 (C-4"), 131.7[b] (C-3", C-5"), 132.1[b] (C-2", C-6"), 132.3 (C-2', C-6'), 141.4 (C-6), 143.4 (C-13), 152.8 (C-3), 153.1 (C-4'), 164.1 (-*C*OO), 166.6 (C-14), 169.4 (C-12).-

EIMS (70 eV, 200 °C): *m/z* (%) = 552 [M^+ + 2] (8), 550 [M^+] (8), 368 (11), 279 (8), 202 (16), 200 (16), 185 (96), 183 (100), 122 (59), 121 (73), 57 (41), 45 (38).-

[a-b] Identical superscripts represent interchangeable assignments.

7.3.4.3 Gaertneroside (**PT-2**)



Molecular formula: $C_{26}H_{28}O_{13}$.-

Melting point: 150-151 °C.-

Molecular weight: 548.0.-

Optical rotation: $[\alpha]^{25}_{D}$ = + 43.1 (*c* 0.75, MeOH) (Lit.[166]: + 24.8 (*c* 0.75, MeOH)).-

R_f value: 0.40 (CH_2Cl_2/4.8% MeOH).-

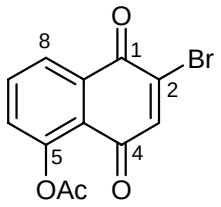
IR (KBr): ν = 3510 cm^{-1}, 3120, 2960, 2872, 1735, 1695.-

^{1}H-NMR (500 MHz, CD_3OD): δ = 2.93 (dd, *J* = 7.6, 4.9 Hz, 1H, 9-H), 3.23 (m, 1H, 2"-H), 3.29 (m, 1H, 5"-H), 3.41 (m, 1H, 3"-H), 3.41 (m, 1H, 4"-H), 3.72 (dd, *J* = 12.2, 4.9 Hz, 1H, 6"-H), 3.78 (s, 3H, 15-OC*H*$_3$), 3.81 (dd, *J* = 12.2, 2.2 Hz, 1H, 6"-H), 3.94 (dddd, *J* = 7.6, 4.0, 2.3 Hz, 1H, 5-H), 4.70 (d, *J* = 7.9 Hz, 1H, 1"-H), 5.18 (d, *J* = 4.9 Hz, 1H, 1-H), 5.38 (s, 1H, 13-H), 5.59

(dd, *J* = 5.6, 2.3 Hz, 1H, 7-H), 6.49 (dd, *J* = 5.6, 2.3 Hz, 1H, 6-H), 6.81 (d, *J* = 8.6 Hz, 2H, 3'-H, 5'-H), 7.32 (d, *J* = 8.6 Hz, 2H, 2'-H, 6'-H), 7.48 (s, 1H, 10-H), 7.55 (d, *J* = 1.6 Hz, 1H, 3-H).-
^{13}C-NMR (125 MHz, CD_3OD): δ = 38.9 (C-5), 49.4 (C-9), 50.6 (15-O*C*H$_3$), 60.8 (C-6"), 68.5 (C-13), 69.5 (C-4"), 73.1 (C-2"), 76.4 (C-3"), 76.9 (C-5"), 93.0 (C-1), 96.7 (C-8), 99.1 (C-1"), 109.5 (C-4), 114.9 (C-3', C-5'), 128.2 (C-2', C-6'), 128.5 (C-7), 131.9 (C-1'), 136.5 (C-11), 140.2 (C-6), 148.7 (C-10), 151.1 (C-3), 157.1 (C-4'), 167.1 (C-14), 171.0 (C-12).-
FAB MS: *m/z* = 549 [M^+ + H], 369, 351, 307, 289, 154, 136, 128, 107.-

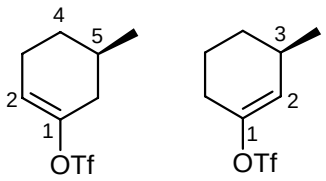
7.4 Experimental part: Synthesis of (+)-Ochromycinone (22)

7.4.1 2-Bromo-1,4-dihydro-1,4-dioxonaphthalen-5-yl-acetate (**44**)



A solution of NBS (14.6 g, 82 mmol) in a mixture of acetic acid (160 mL) and water (320 mL) was heated to 58 °C, and 5 g (20.5 mmol) of **47** as a warm solution in 160 mL acetic acid was added dropwise over a 15-min time period maintaining the reaction temperature. The reaction mixture was stirred for an additional 45 min (TLC control) and subsequently quenched with 320 mL ice-cold water. The resulting solution was extracted with CH_2Cl_2 (3×350 mL) and the combined extracts were washed with ice-cold water, saturated $NaHCO_3$, and saturated NaCl solution. The solution was dried over Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure until crystallization began, which was completed by addition of diethyl ether to afford **44** (5.1 g, 84%) as yellow needles.
Melting point: 155 °C (Lit.[174]: 154.5-156 °C).-
^{1}H-NMR (200 MHz, $CDCl_3$): δ = 2.48 (s, 3H, COC*H*$_3$), 7.43 (s, 1H, 3-H), 7.46 (dd, $J_{6,7}$ = 8.1, $J_{6,8}$ = 1.2 Hz, 1H, 6-H), 7.81 (t, $J_{7,6}$ = $J_{7,8}$ = 7.9 Hz, 1H, 7-H), 8.19 (dd, $J_{8,7}$ = 7.8, $J_{8,6}$ = 1.2 Hz, 1H, 8-H).-

7.4.2 (*R*)-5-Methylcyclohex-1-enyl trifluoromethanesulfonate (**48**) and (*R*)-3-methylcyclohex-1-enyl trifluoromethanesulfonate (**49**)



48 **49**

A solution of 2,2,6,6-tetramethylpiperidine (TMP) (0.84 mL, 0.7 g, 4.9 mmol) in THF (15 mL) was cooled to –10 °C, and *n*-butyllithium in toluene (2.2 mL of a 2.5 M solution) was added slowly. The mixture was stirred for 30 min at –10 °C and subsequently cooled to –78 °C. A solution of the ketone **46** (Aldridge, 0.55 mL, 0.5 g, 4.5 mmol) in THF (10 mL) was added by means of a syringe pump within 1 h with intensive stirring. The mixture was stirred for 2 h at –78 °C, and trifluoromethanesulfonic acid anhydride (0.83 mL, 1.4 g, 4.9 mmol) was added. The mixture was allowed to warm to 20 °C and stirring was continued for an additional 15 h. The reaction mixture was quenched by addition of HCl (0.1 M, 30 mL), neutralized by addition of $NaHCO_3$ (5%, 1.0 mL), extracted with pentane (3×20 mL), dried (Na_2SO_4), filtered, and concentrated at reduced pressure. Flash chromatography of the residue on silica gel (pentane/5% Et_2O) gave a mixture of triflates **48** and **49** as colourless oil. Similar reactions were performed with LDA and LHMDS; the isomer ratios and the yields were determined by GC. (LTMP: **48:49** = 5.4:1, 71% combined isolated yield; LDA: **48:49** = 2.4:1, 44% combined isolated yield; LHMDS: **48:49** = 2:1, by GC).

7.4.2.1 Data for (*R*)-5-methylcyclohex-1-enyl trifluoromethanesulfonate (**48**):

*****IR (KBr): ν = 2961 cm^{-1}, 2934, 2874, 2853, 1694, 1460, 1422, 1368, 1352, 1248, 1221, 1145, 1031.-

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.07 (d, *J* = 6.4 Hz, 3H, 5-C*H*$_3$), 1.17-1.34 (m, 1H, 4-H), 1.65-1.83 (m, 2H, 4-H, 5-H), 1.83-2.16 (m, 1H, 6-H), 2.17-2.58 (m, 3H, 6-H, 3-C*H*$_2$), 5.78 (brs, 1H, 2-H).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 21.3 (*C*H$_3$), 23.8 (sec.), 29.5 (sec.), 29.7 (C-5), 35.9 (sec.), 118.4 (C-2), 122.1 (*C*F$_3$), 149.2 (C-1).-

*EIMS (200 °C): *m/z* (%) = 244 [M^+] (35), 110 (33), 95 (26), 94 (27), 83 (22), 79 (84), 69 (74), 55 (100), 41 (71), 39 (20), 28 (25).-

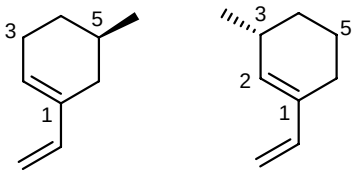
*data for mixed triflates **48** and **49**

7.4.2.2 Data for (*R*)-3-methylcyclohex-1-enyl trifluoromethanesulfonate (**49**):

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.08 (d, *J* = 7.0 Hz, 3H, 3-C*H*$_3$), 1.17-1.34 (m, 1H, 4-H), 1.65-1.83 (m, 2H, 3-H, 4-H), 1.83-2.02 (m, 1H, 5-H), 2.17-2.58 (m, 3H, 5-H, 6-C*H*$_2$), 5.66 (brs, 1H, 2-H).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 21.3 (*C*H$_3$), 21.7 (sec.), 27.9 (sec.), 30.0 (sec.), 30.3 (C-3), 115.8 (*C*F$_3$), 124.4 (*C*-2), 149.5 (C-1).-

7.4.3 (*R*)-5-Methyl-1-vinylcyclohex-1-ene (**45**) and (*R*)-3-methyl-1-vinylcyclohex-1-ene (**50**)



45 **50**

A solution of the mixture of the triflates **48** and **49** (ratio ca. 2.4:1, 2.86 g, 11.7 mmol, from the LDA-experiment) and vinyltributylstannane (3.8 mL, 4.1 g, 12.89 mmol) was added to a slurry of LiCl (5.2 g, 123 mmol) and $Pd(PPh_3)_4$ (0.3 g, 2.0 mol%) in dry THF (110 mL). The mixture was heated under reflux for 15 h, cooled to room temperature, and diluted with pentane. The resulting solution was washed sequentially with a 10% aqueous ammonium hydroxide (3×50 mL) solution and water (3×50 mL). This solution was dried (Na_2SO_4), filtered, and concentrated at reduced pressure. Flash chromatography of the residue on silica gel (pentane) gave a mixture of dienes **45** and **50** as colourless oil (1.26 g, 88% combined yield, **45:50** = 2.4:1 by NMR).

7.4.3.1 (*R*)-5-Methyl-1-vinylcyclohex-1-ene (**45**):

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.07 (d, *J* = 6.0 Hz, 3H, C*H*$_3$), 1.26-1.35 (m, 1H), 1.62-1.94 (m, 3H), 2.05-2.43 (m, 3H), 4.94 (d, $J_{1',2'a}$ = 10.7 Hz, 1H, 2'a-H), 5.12 (d, $J_{1',2'b}$ = 17.5 Hz, 1H, 2'b-H), 5.78 (t, 1H, 2-H), 6.41 (dd, $J_{1',2'b}$ = 17.5, $J_{1',2'a}$ = 10.7 Hz, 1H, 1'-H).-

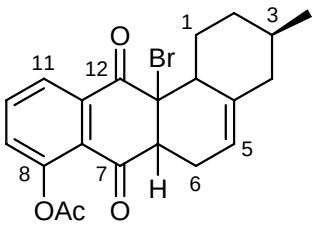
^{13}C-NMR (50 MHz, $CDCl_3$): δ = 22.4 (*C*H$_3$), 26.3 (sec.), 28.8 (C-5), 31.2 (sec.), 32.8 (sec.), 110.0 (C-2'), 129.8 (C-2), 136.2 (C-1), 140.4 (C-1').-

7.4.3.2 (*R*)-3-Methyl-1-vinylcyclohex-1-ene (**50**):

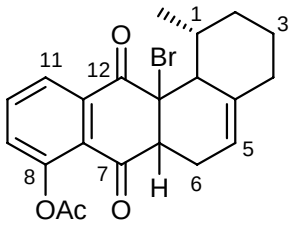
^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.05 (d, *J* = 7.0 Hz, 3H, C*H*$_3$), 1.26-1.35 (m, 1H), 1.62-1.94 (m, 3H), 2.05-2.43 (m, 3H), 4.96 (d, $J_{1',2'a}$ = 10.7 Hz, 1H, 2'a-H), 5.12 (d, $J_{1',2'b}$ = 17.5 Hz, 1H, 2'b-H), 5.64 (d, *J* = 3.9 Hz, 1H, 2-H), 6.38 (dd, $J_{1',2'b}$ = 17.5, $J_{1',2'a}$ = 10.7 Hz, 1H, 1'-H).-

^{13}C-NMR (50 MHz, $CDCl_3$) : δ = 21.7 (sec.), 21.9 (*C*H$_3$.), 24.2 (sec.), 31.3 (C-3), 31.7 (sec.), 110.5 (C-2'), 135.5 (C-1), 136.4 (C-2), 140.5 (C-1').-

7.4.4 (3*R*)-12a-Bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-3-methyl-7,12-dioxotetraphen-8-yl acetate (**51**) and (1*R*)-12a-bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-1-methyl-7,12-dioxotetraphen-8-yl acetate (**52**)



51



52

A solution of **44** (2.4 g, 8.1 mmol) and the mixture of the dienes **45** and **50** (ratio ca. 2.4:1, 0.9 g, 7.4 mmol) in dry toluene (70 mL) was heated under argon for 12 h at 80 °C followed by 2 h at 100 °C (TLC monitoring). The solvent was evaporated at reduced pressure and the resulting material was purified by flash chromatography (CH_2Cl_2) to afford a mixture of cycloadducts **51** and **52** (2.42 g, 79% combined yield, ratio **51**:**52** = 2.6:1 by NMR).

7.4.4.1 Data for (3*R*)-12a-bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-3-methyl-7,12-dioxotetraphen-8-yl acetate (**51**):

*UV (CH_2Cl_2): λ_{max} (lg ε) = 272 nm (3.55), 311 (3.39).-

*****IR (KBr): ν = 2956 cm^{-1}, 2923, 2869, 2847, 1776, 1705, 1667, 1596, 1450, 1368, 1324, 1265, 1237, 1189, 1102, 1015, 911, 868.-

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 0.93 (d, *J* = 6.6 Hz, 3H, 3-C*H*$_3$), 1.42-1.61 (m, 2H), 1.65-1.81 (m, 2H), 2.05 (m, 1H), 2.33-2.38 (m, 2H), 2.41 (s, 3H, COC*H*$_3$), 2.45-2.71 (m, 2H), 2.77 (m, 1H, 12b-H), 3.60 (dd, *J* = 6.2, 2.3 Hz, 1H, 6a-H), 5.46 (t, *J* = 1.9 Hz, 1H, 5-H), 7.40 (dd, $J_{9,10}$ = 7.9, $J_{9,11}$ = 1.1 Hz, 1H, 9-H), 7.77 (t, $J_{9,10}$ = $J_{10,11}$ = 7.9 Hz, 1H, 10-H), 8.09 (dd, $J_{10,11}$ = 7.9, $J_{9,11}$ = 1.1 Hz, 1H, 11-H).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 17.9 (*C*H$_3$), 21.4 (CO*C*H$_3$), 29.0 (C-3), 32.4 (sec.), 35.5 (sec.), 35.7 (sec.), 41.9 (sec.), 50.1 (C-12b), 57.7 (C-6a), 69.8 (quat.), 115.1 (quat.), 117.7 (C-5), 126.6 (C-11), 130.2 (C-9), 135.0 (quat.), 135.3 (C-10), 135.8 (quat.), 149.3 (C-8), 169.7 (*C*OCH$_3$), 191.3 (C-7 or C-12), 193.2 (C-7 or C-12).-

*EIMS (200 °C): *m/z* (%) = 337 [M$^+$ - HBr] (45), 296 (23), 295 (100), 294 (62), 293 (21), 292 (34), 278 (13), 277 (48), 263 (22), 149 (16), 121 (23), 98 (42), 85 (16), 84 (17), 82 (32), 81 (12), 80 (33), 79 (12), 60 (31), 45 (38), 43 (70), 28 (7).-

*Anal. Calcd. For $C_{21}H_{21}BrO_4$: C, 60.44; H, 5.07. Found: C, 60.27; H, 4.79.-

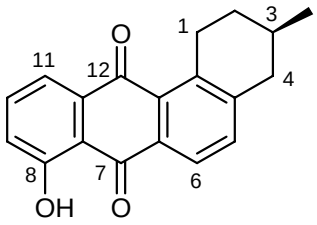
*data for mixed cycloadducts **51** and **52**

7.4.4.2 Data for (1*R*)-12a-bromo-1,2,3,4,6,6a,7,12,12a,12b-decahydro-1-methyl-7,12-dioxotetraphen-8-yl acetate (**52**):

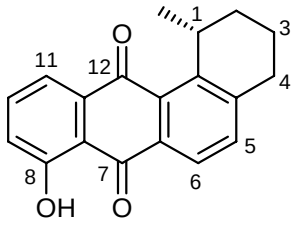
^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.15 (d, *J* = 6.6 Hz, 3H, 1-C*H*$_3$), 1.42-1.61 (m, 2H), 1.65-1.81 (m, 2H), 2.05 (m, 1H), 2.33-2.38 (m, 2H), 2.42 (s, 3H, COC*H*$_3$), 2.45-2.71 (m, 2H), 2.77 (m, 1H, 12b-H), 3.63 (dd, *J* = 6.1, 2.3 Hz, 1H, 6a-H), 5.46 (t, *J* = 1.9 Hz, 1H, 5-H), 7.38 (dd, $J_{9,10}$ = 7.9, $J_{9,11}$ = 1.2 Hz, 1H, 9-H), 7.77 (t, $J_{9,10}$ = $J_{10,11}$ = 7.9 Hz, 1H, 10-H), 8.03 (dd, $J_{10,11}$ = 7.9, $J_{9,11}$ = 1.2 Hz, 1H, 11-H).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 21.4 (CO*C*H$_3$), 24.0 (*C*H$_3$), 24.7 (sec.), 25.8 (sec.), 28.0 (sec.), 35.0 (C-1), 35.3 (sec.), 49.9 (C-12b), 56.2 (C-6a), 69.7 (quat.), 115.1 (quat.), 117.1 (C-5), 126.5 (C-11), 130.0 (C-9), 135.3 (C-10.), 136.2 (quat.), 139.5 (quat.), 149.6 (C-8), 169.7 (*C*OCH$_3$), 190.4 (C-7 or C-12), 194.3 (C-7 or C-12).-

7.4.5 (*R*)-1,2,3,4-Tetrahydro-8-hydroxy-3-methyltetraphene-7,12-dione (**33**) and (*R*)-1,2,3,4-tetrahydro-8-hydroxy-1-methyltetraphene-7,12-dione (**53**)



33



53

A solution of the mixture of **51** and **52** (ratio ca. 2.6:1, 1.42 g, 3.4 mmol) in methanol (10 mL) was treated with 1.6 g of K_2CO_3. The suspension was stirred for 16 h (TLC monitoring) at room temperature in the dark. The alkaline reaction mixture was acidified by addition of HCl (2 N, 5 mL), the suspension was filtered, and the solvent removed under reduced pressure. The crude material was redissolved in CH_2Cl_2, washed with H_2O, dried (Na_2SO_4), filtered, and the solvent was evaporated at reduced pressure to afford a mixture of **33** and **53**. The major isomer **33** (m.p. 162 °C) was isolated by flash chromatography of the crude mixture followed by crystallization of the nonpolar fraction to yield 0.48 g (48%) of **33**. The mother liquor was again purified by flash chromatography on silica gel followed by preparative TLC and crystallization to yield 0.08 g (8%) of **53** (combined isolated yield of **33** and **53** 56%).

7.4.5.1 Data for (*R*)-1,2,3,4-tetrahydro-8-hydroxy-3-methyltetraphene-7,12-dione (**33**):

R_f value: 0.52 (pentane/50% CH_2Cl_2).-

Optical rotation: $[\alpha]^{25}_D$ = + 114 (*c* 0.09, $CHCl_3$).-

UV ($CHCl_3$): λ_{max} (lg ε) = 268 nm (4.24), 285 (4.11), 402 (3.89).-

IR (KBr): ν = 2950 cm^{-1}, 2923, 2363, 2341, 1667, 1634, 1580, 1553, 1482, 1450, 1417, 1373, 1324, 1276, 1243, 1156.-

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.12 (d, *J* = 6.5 Hz, 3H, C*H*$_3$), 1.19-1.50 (m, 1H, 2-H), 1.75-2.13 (m, 2H, 2-H, 3-H), 2.43-2.63 (m, 1H, 4-H), 2.85-3.05 (m, 1H, 4-H), 3.10-3.34 (m, 1H, 1-H), 3.44-3.65 (m, 1H, 1-H), 7.23 (dd, $J_{9,10}$ = 7.9, $J_{9,11}$ = 1.5 Hz, 1H, 9-H), 7.43 (d, $J_{5,6}$ = 8.0 Hz, 1H, 5-H), 7.63 (t, $J_{9,10}$ = $J_{10,11}$ = 7.7 Hz, 1H, 10-H), 7.71 (dd, $J_{10,11}$ = 7.7, $J_{9,11}$ = 1.5 Hz, 1H, 11-H), 8.09 (d, $J_{5,6}$ = 8.0 Hz, 1H, 6-H), 12.53 (s, 1H, 8-OH).-

^{13}C-NMR (50 MHz, $CDCl_3$): δ = 22.0 (*C*H$_3$), 28.2 (C-3), 29.6 (sec.), 31.7 (sec.), 40.2 (sec.), 115.9 (quat.), 119.6 (C-11), 123.4 (C-9), 125.1 (C-6), 131.6 (quat.), 133.1 (quat.), 135.0 (C-5), 135.5 (quat.), 136.9 (C-10), 141.9 (quat.), 146.8 (quat.), 162.1 (C-8), 185.2 (C-7 or C-12), 189.1 (C-7 or C-12).-

EIMS (200 °C): *m/z* (%) = 292 [M^+] (100), 278 (33), 277 (71), 264 (15), 263 (69), 152 (7), 151 (27), 150 (58), 84 (12), 57 (17), 49 (10), 44 (9), 43 (18), 41 (10), 28 (9).-

Anal. Calcd. For $C_{19}H_{16}O_3$: C, 78.06; H, 5.52. Found: C, 77.24; H, 4.91.-

7.4.5.2 Data for (*R*)-1,2,3,4-tetrahydro-8-hydroxy-1-methyltetraphene-7,12-dione (**53**):

R_f value: 0.56 (pentane/50% CH_2Cl_2).-

Optical rotation: $[\alpha]^{25}_D$ = - 173 (*c* 0.07, $CHCl_3$).-

UV (CH_2Cl_2): λ_{max} (lg ε) = 269 nm (4.22), 285 (4.01), 399 (3.76).-

IR (KBr): ν = 2922 cm^{-1}, 2351, 2194, 1664, 1634, 1617, 1582, 1460, 1367, 1268, 1157, 1082, 1041.-

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 1.31 (d, *J* = 6.9 Hz, 3H, C*H*$_3$), 1.80-2.06 (m, 4H), 2.99 (m, 2H), 4.49 (m, 1H, 1-H), 7.28 (dd, $J_{9,10}$ = 8.3, $J_{9,11}$ = 1.2 Hz, 1H, 9-H), 7.51 (d, $J_{5,6}$ = 8.0 Hz, 1H, 5-H), 7.68 (t, $J_{9,10}$ = $J_{10,11}$ = 7.9 Hz, 1H, 10-H), 7.78 (dd, $J_{10,11}$ = 7.6, $J_{9,11}$ = 1.2 Hz, 1H, 11-H), 8.19 (d, $J_{5,6}$ = 8.0 Hz, 1H, 6-H), 12.56 (s, 1H, 8-OH).-

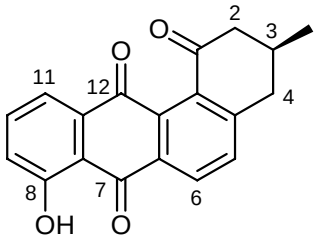
^{13}C-NMR (50 MHz, $CDCl_3$): δ = 17.3 (sec.), 22.6 (*C*H$_3$), 29.8 (C-1), 30.1 (sec.), 31.4 (sec.), 115.9 (quat.), 119.9 (C-11), 123.3 (C-9), 125.3 (C-6), 131.1 (quat.), 133.6 (quat.), 135.6 (C-5), 135.9 (quat.), 136.9 (C-10), 146.2 (quat.), 147.7 (quat.), 162.1 (C-8), 185.2 (C-7 or C-12), 189.3 (C-7 or C-12).-

EIMS (200 °C): *m/z* (%) = 292 [M^+] (100), 277 (42), 264 (19), 263 (71), 98 (20).-

7.4.5.3 Crystal Structure Determination of **53:**[193] $C_{19}H_{16}O_3$, Mr = 292.3, monoclinic, space group P $2_1/c$, a = 8.0593(10), b = 11.7374(14), c = 14.5045(17) Å, β = 90.849(3)°, V = 1371.9(3) Å³, Z = 4, D_x = 1.415 g/cm³, F(000) = 616, T = 120(2) K. Bruker-AXS SMART APEX, graphite monochromator, λ(MoKα) = 0.71073 Å, μ = 0.095 mm^{-1}, yellowish crystal, size 0.20 x 0.12 x 0.08 mm³, 11541 intensities collected $4.4 < 2\theta < 56.8°$, $-10 < h < 10$, $-15 < k < 15$, $-19 < l < 19$. Structure solved by direct methods, full-matrix least-squares refinement based on F^2 and 201 parameters, all but H atoms refined anisotropically, H atoms refined with riding model on idealized positions with U = 1.5 U_{iso}(O, C-methyl) and 1.2 U_{iso}(C). Refinement converged at R1(F) = 0.058, wR2(F^2, all data) = 0.113, S = 0.72, max(δ/σ) < 0.001, min/max height in final ΔF map –0.19/0.21 e/Å³. Figure 1 shows the molecular structure. Programme used: SHELXTL.[193]

Full crystallographic data (excluding structure factors) for **53** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-223554. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44)1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

7.4.6 (*S*)-3,4-Dihydro-8-hydroxy-3-methyltetraphene-1,7,12(2*H*)-trione;
(+)-ochromycinone (**22**)



A solution of the dione **33** (22 mg, 0.075 mmol) in CH_2Cl_2 (15 ml) was exposed to diffuse sunlight for about 40 h in 5 NMR tubes (TLC and NMR monitoring). The combined solutions were concentrated at reduced pressure. The crude reaction mixture was then subjected to flash chromatography followed by recrystallization to afford **22** (16.2 mg, 70%) as yellow needles, m.p. 160 °C. All spectral data of synthetic **22** were identical to those of the natural ochromycinone.[87,108] For measurement of optical rotations, see Table 13 and Figure 48.

Melting point: 160 °C (Lit.[132]: 168-169 °C).-

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.14 (d, $J_{3,3}$ = 6.5 Hz, 1H, 3-C*H*$_3$), 2.40 (m, 1H, 3-H), 2.50 (dd, J_{gem} = 16.0, $J_{2,3}$ = 11.0 Hz, 1H, 2-H), 2.61 (dd, J_{gem} = 16.6, $J_{4,3}$ = 11.0 Hz, 1H, 4-H), 2.95 (m, 2H,

2-H, 4-H), 7.20 (m, 1H, 11-H), 7.47 (d, $J_{5,6}$ = 8.0 Hz, 1H, 5-H), 7.60 (m, 2H, 9-H, 10-H), 8.22 (d, $J_{6,5}$ = 8.0 Hz, 1H, 6-H), 12.21 (s, 1 H, 8-OH).-

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 21.5 (3-*C*H_3), 30.8 (C-3.), 38.4 (C-4.), 47.5 (C-2), 115.4 (C-7a), 119.6 (C-9), 123.6 (C-11), 129.0 (C-6), 133.0 (C-5), 133.5 (C-12a.), 135.1 (C-12b), 136.0 (C-6a), 136.6 (C-11a), 137.0 (C-10), 150.4 (C-4a), 162.1 (C-8), 183.0 (C-7 or C-12), 187.6 (C-7 or C-12), 199.1 (C-1).-

8 Abbreviations

AcOH	acetic acid
Bio	biomalt agar
BuLi	butyllithium
cat.	catalyst, catalytic
CD	circular dichroism
CI	chemical ionization
CoA	coenzyme A
COLOC	correlation spectroscopy via long-range coupling
COSY	correlation spectroscopy
DMAP	*N*,*N*-dimethyl-4-aminopyridine
EI	electron impact ionization
EtOAc	ethyl acetate
EtOH	ethanol
Et_2O	diethylether
GC	gas chromatography
h.	hour(s)
HMBC	heteronuclear multibond correlation
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography
LDA	lithium diisopropylamide
LHMDS	lithium hexamethyldisilazide
LTMP	lithium tetramethylpiperidide
Me	methyl
MeOH	methanol
min.	minute
m.p.	melting point
MS	mass spectrometry
M/S	malt extract soya meal agar
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance spectroscopy
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser spectroscopy

PE	petrolether
PTLC	preparative thin layer chromatography
py	pyridine
rt	room temperature
Tf	trifluoromethanesulfonate
THF	tetrahydro furan
TLC	thin layer chromatography
TMP	2,2,6,6-tetramethylpiperidine
TMS	trimethylsilyl

9 References

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Publications from the present study:

1. Krohn K, Sohrab MH, Aust H-J, Draeger S, Schulz B: Biologically active metabolites from fungi, 19: New isocoumarins and highly substituted benzoic acids from the endophytic fungus, Scytalidium sp.. *Natural Product Research.* 18(3): 277-285, 2004.

2. Krohn K, Sohrab MH, Flörke U: Total synthesis of Angucyclines. Part 18: A short and efficient synthesis of (+)-ochromycinone. *Tetrahedron: Asymmetry*. 15(4): 713-718, 2004.

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