Phenylphenalenones and related phenolic pigments of the Haemodoraceae: Structure, biosynthesis and accumulation patterns in *Xiphidium caeruleum* and *Wachendorfia thyrsiflora*

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Contents

Abbreviations

ANOVA	analysis of variances
BP	band pass (filter)
CLSM	confocal laser scanning microscopy
COSY	correlated spectroscopy
d	doublet
δ	chemical shift relative to TMS
DAD	diode array detector
dd	double-doublet
ddd	triple-douplet
DMSO	dimethyl sulphoxide
EIMS	electron impact mass spectrometry
ESIMS	electrospray ionisation mass spectrometry
EtOAc	ethyl acetate
fw	fresh weight
HMBC	heteronuclear multiple-bond correlation
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
HREIMS	high resolution electron impact mass spectrometry
HRESIMS	high resolution electrospray ionisation mass spectrometry
J	coupling constant
JA	jasmonic acid
LC-MS	liquid chromatography coupled with mass spectrometry
LP	long pass (filter)
m	multiplet
m/z	mass to charge ration
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MSP	microspectral photometry

N	number (sample size)
NA	‚Naturstoff' reagent A
NMR	nuclear magnetic resonance
ppm	parts per million
RGB	red-green-blue (colour mode of CLSM)
R_t	retention time
s	singulet
SE	standard error
t	triplet
TFA	trifluoracetic acid
TMS	trimethylsilane
UV-B	ultraviolet-B
UV-VIS	ultraviolet and visible

1. Introduction

1.1 General

Secondary metabolites in plants include a vast array of structures with a variety of functions. In addition to terpenoids and nitrogen-containing compounds, phenolics represent a third main class of secondary metabolites (Crawley, 1997). Phenolic compounds play a major role in the interaction of plants with their environment (Harborne, 1994). They can act as attractants for insects and fruitivorous animals, as allelopathic signals between plants and as mediators in the interaction of plants with symbiotic or pathogenic organisms. In this context, phenolic compounds have the potential to protect plants against biotic (bacteria, fungi, herbivores) and abiotic (air pollution, heavy metals, UV-B radiation) stresses.

Within the last decades, great attention has been paid to the investigation of phenolic metabolites such as flavonoids, tannins or lignin, metabolites with nearly ubiquitous distribution within the plant kingdom. Phenylphenalenones are phenolic compounds with a limited distribution and about little is known. In this work, details of structural characteristics, biosynthesis and occurrence in two species of the Haemodoraceae plant family are presented.

1.2 Phenylphenalenones

1.2.1 Occurrence

Phenylphenalenones are phenolic pigments. The term phenylphenalenone derives from the carbon skeleton of a phenalene-body, one keto group and an additional phenyl ring (Fig. 1.1).

Secondary metabolites that contain the phenalenone nucleus were first isolated over fifty years ago from higher plants of the Haemodoraceae and fungi of genera of Hyphomycetes and Discomycetes (Cooke and Segal, 1955b; Harman *et al.*, 1955; Cooke and Edwards, 1981). However, the structures of phenalenones from plants and fungi are different and derived from unrelated biosynthetic pathways (Cooke, 1961; Cooke and Edwards, 1981). Phenylphenalenones have been isolated from roots, flowers, fruits or whole plant material of four closely related monocot plant families. Since Haemocorin, the first described phenylphenalenone in a plant, was isolated from *Haemodorum corymbosum* (Haemodoraceae) (Cooke and Segal, 1955a) a great number of phenylphenalenones and related compounds were described for this plant family (Edwards and Weiss, 1974; Cooke and Dagley, 1979; Hölscher and Schneider, 1997). More recently such metabolites were found in Musaceae (Luis *et al.*, 1993), Pontederiaceae (Della Greca *et al.*, 1993) and Strelitziaceae (Hölscher and Schneider, 2000).

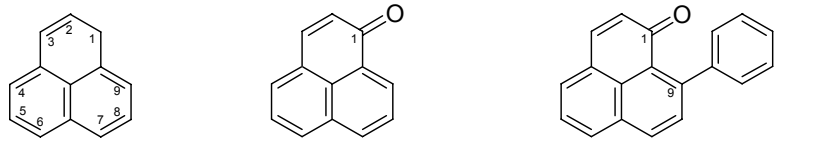


Fig. 1.1: Skeletons of phenalene, phenalenone and phenylphenalenone

1.2.2 Biosynthesis

The shikimic acid pathway delivers the aromatic amino acids phenylalanine and tyrosine, which are precursors for the biosynthesis of phenylphenalenones (Edwards *et al.*,

1972). These amino acids are converted into cinnamic acid by phenylalanine ammonia lyase (PAL) and cumaric acid by tyrosine ammonia lyase. Two units of phenylpropanoic acids, one of cinnamic acid and one of hydroxycinnamates, together with one carbon of acetate combine to form a diarylheptanoid intermediate (Fig. 1.2) (Hölscher and Schneider, 1995a; b; Schmitt *et al.*, 2000). Such diarylheptanoids are widely distributed in the plant families such as Zingiberaceae, Betulaceae or Leguminoseae (Cooke and Edwards, 1981). Finally, a Diels-Alder cycloaddition is proposed for the ring closure to form the C-19 skeleton of a phenylphenalenone (Cooke and Edwards, 1981; Schmitt and Schneider, 1999). A variety of feeding experiments with ^{13}C- and ^{14}C-labelled precursors revealed the origin of the carbon backbone but exact biosynthetic details have yet to be elucidated.

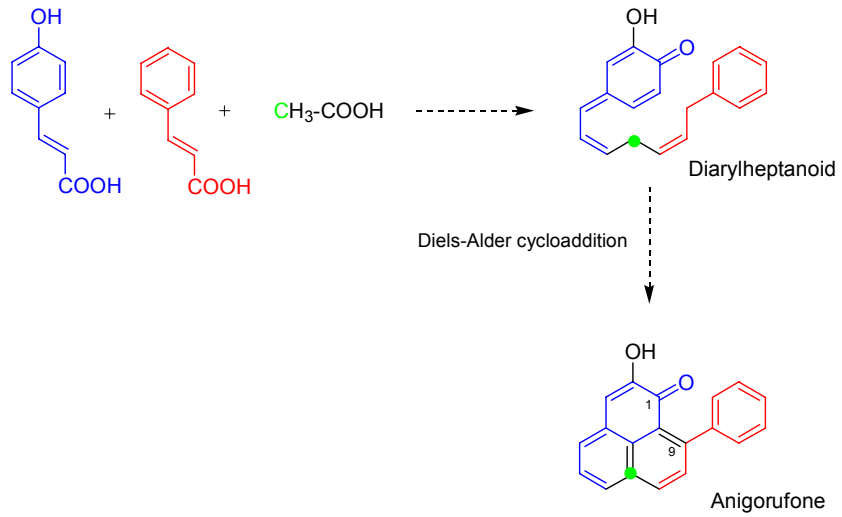


Fig. 1.2: Scheme of the biosynthesis of anigorufone, a 9-phenylphenalenone, starting from phenylpropanoic acids and acetate.

1.2.3 Function

The biological activity of plants of the Haemodoraceae has long been known. Darwin already described the toxic effects to white but not black pigs feeding on *Lachnanthes tinctoria* (Darwin, 1895). Later experiments demonstrated an inducible phototoxicity of *L. tinctoria* extracts to microorganisms (Kornfeld and Edwards, 1972). However, Native Americans used this plant as a narcoticum (Millspaugh, 1887). Parts of other species such as

Australian *Anigozanthos spec*. and South American *Schiekia orinocensis* were eaten and played a role in terms of medical treatment by natives (Millspaugh, 1887; Schultes, 1993).

Haemocorin was the first phenylphenalenone for which antitumor activity and antibacterial properties were described (Schwenk, 1962). In *Musa acuminata* (Musaceae), the biosynthesis of several phenylphenalenones was induced after infection with phytopathogenic fungi or nematodes (Luis *et al.*, 1995; Binks *et al.*, 1997). Since then, phenylphenalenones have been described as metabolites which play a role in inducible (phytoalexines) or constitutive (phytoanticipines) plant defence (Luis *et al.*, 1993; Luis *et al.*, 1997).

1.3 The Haemodoraceae plant family

The Haemodoraceae is a monocot plant family consisting of 14 genera and 103 species, with a close relationship to families such as Philydraceae, Pontederiaceae and possibly Commelinaceae (Simpson, 1998). Its distribution is restricted to tropical and subtropical regions of Australia, South Africa and Latin America with the exception of *Lachnanthes tinctoria*, which occurs partly in temperate regions along the East Coast of Northern America. Most species are found in Australia with seven genera and 84 species.

The Haemodoraceae are divided into two subfamilies, the Conostylidoideae and Haemodoroideae (Simpson, 1998). The Conostylidoideae includes genera such as *Conostylis* (46 species), *Anigozanthos* (11 species) and *Tribonanthes* (5 species), which are exclusively found in South West Australia. In contrast, genera such as *Haemodorum* (20 species), *Wachendorfia* (5 species) or *Lachnanthes* (1 species) belong to the Haemodoroideae subfamily, which is distributed throughout all the mentioned continents.

The Haemodoraceae might have a Gondwanan origin, when the continents now described as Antarctica, Southern America, Africa and Australia formed one common land mass (Simpson, 1990). Since the splitting of Gondwana, Haemodoraceae species of the different continents developed separately from each other reaching the present biogeographic distribution pattern. However, a much older origin of this plant family has been discussed proposing that the present species indicate a largely relictual distribution with a recent radiation in Western Australia (Hopper *et al.*, 1999).

The name Haemodoraceae is due to the red colour of roots from species such as *L. tincoria* or *Wachendorfia thyrsiflora* and is also responsible for the alternative name of the

'blood root' family (Simpson, 1990). The reasons for this colour are phenylphenalenones and related compounds, which represent chemotaxonomic markers of the Haemodoraceae.

1.3.1 *Xiphidium*

The New World genus *Xiphidium* belongs to the Haemodoroideae and consists of two species (Simpson, 1998). Whereas *X. xanthorrhiza* is native to Cuba, *X. caeruleum* (Aubl.) occurs from South Mexico to Brazil. Both are found in moist soils, often along watercourses. Plants of this genus are rhizomatous and stoloniferous herbs reaching a height of up to 0.5 m. They posses lanceolate leaves with minutely cerrulate margins and a sheathing base. Inflorescences are thyrses with lateral helicoid cymes. The 10 mm wide actinomorphic flowers posses white tepals, three free stamina and a superior ovary.

1.3.2 *Wachendorfia*

The four known species of *Wachendorfia* (Burm.) are endemic to the Cape region of South Africa (Helme and Linder, 1992). Whereas three species, *W. paniculata*, *W. brachyandra* and *W. parviflora*, occur in habitats that are at least seasonally dry, *W. thyrsiflora* occupies habitats in perennially wet marshes or along streams. Plants of *Wachendorfia* are rhizomatous herbs up to 2.5 m tall (Simpson, 1998). They posses linear and plicate leaves. The inflorescences are racemes of 3-4 flowered helicoid cymes. The zygomorphic flowers are approximately 2 cm long, possessing yellow to purplish tepals, three free stamina and a superior ovary.

1.4 Scope of this work

In several respects, little is known about phenylphenalenones. The present work is aimed at increasing the knowledge about these phenolics and to provide a base for ongoing investigations.

A main focus of the studies was the isolation and structure elucidation of phenylphenalenone-related compounds from *X. caeruleum*, a species of the Haemodoraceae.

With the exception of one phenylphenalenone, nothing at all had been isolated from this Latin American species. *X. caeruleum* was studied to investigate whether it contains identical or similar compounds of an unusual structural subtype of phenylphenalenones (so called isochromenones), that were found in the North American species *Lachnanthes tinctoria*. It was anticipated that results could give indications if the occurrence of different phenylphenalenones and related compounds in single species reflect biogeographic characteristics of the Haemodoraceae.

The existence of the structural subtype of the isochromenones raises the question about its biosynthetic pathway. All studies on the biosynthesis were made on 'real' phenylphenalenones and hence it was unknown whether the biosynthetic pathway for phenylphenalenones also delivered the isochromenones or if an alternative pathway exists.
Additionally, the distribution patterns of the identified phenylphenalenone-related compounds in *X. caeruleum* were studied. This was focused on the occurrence of these phenolics in plant organs such as roots, stem, leaves and flowers. Special attention was paid to the comparison of the accumulation in wounded and undamaged leaves. Cutting injury experiments were performed to investigate the response of *X. caeruleum* plants to wounding with respect to the level of phenylphenalenones as potential defensive compounds.

To obtain more detailed information about the accumulation of phenylphenalenones within plants, several analytical techniques were applied to study the tissue localisation in root, stem and leave of *X. caeruleum*, notably using strong autofluorescence of phenylphenalenone-related compounds.

1.5 Précis

This work is arranged in chapters of different topics. All compounds are numbered sequentially and continued through the thesis.

Isolation and structure elucidation of compounds from *X. caeruleum* and *W. thyrsiflora* are presented in **Chapter 2**. Studies on the biosynthesis of phenylphenalenone-related compounds are described in **Chapter 3**. The organ-specific distribution pattern of phenylphenalenone-related compounds in *X. caeruleum* is the topic of **Chapter 4**, which includes the cutting injury experiment on leaves. **Chapter 5** deals with the accumulation of the studied compounds in various tissues of *X. caeruleum*. Finally, in **Chapter 6** an overview of the results is given and discussed.

2. Phenylphenalenone-related compounds – chemotaxonomic markers of the Haemodoraceae from *Xiphidium caeruleum* and *Wachendorfia thyrsiflora*

2.1 Introduction

Phenylphenalenones are secondary metabolites of phenylpropanoic origin found in the plant families Haemodoraceae (Cooke and Edwards, 1981), Musaceae (Luis *et al.*, 1993), Pontederiaceae (Della Greca *et al.*, 1993) and Strelitziaceae (Hölscher and Schneider, 2000). These pigments are considered chemotaxonomic markers of the Haemodoraceae genera such as *Anigozanthos* (Cooke and Thomas, 1975; Hölscher and Schneider, 1997), *Dilatris* (Dora *et al.*, 1993), *Haemodorum* (Cooke and Segal, 1955), *Lachnanthes* (Edwards and Weiss, 1970) and *Wachendorfia* (Edwards, 1974). In addition to typical phenylphenalenones, such as 9-

phenylphenalenones with a completely deoxygenated B-ring or tetraoxygenated phenylphenalenones, compounds of the oxabenzochrysenone-type, the phenylbenzoisochromenone-type and the phenylbenzoisochinolinedione-type have also been found (Fig. 2.1). The latter two types only have been isolated from *Lachnanthes tinctoria*, the only well examined American species (Edwards and Weiss, 1972; 1974). Here, several pigments in which O or N replace C-2 were characterised.

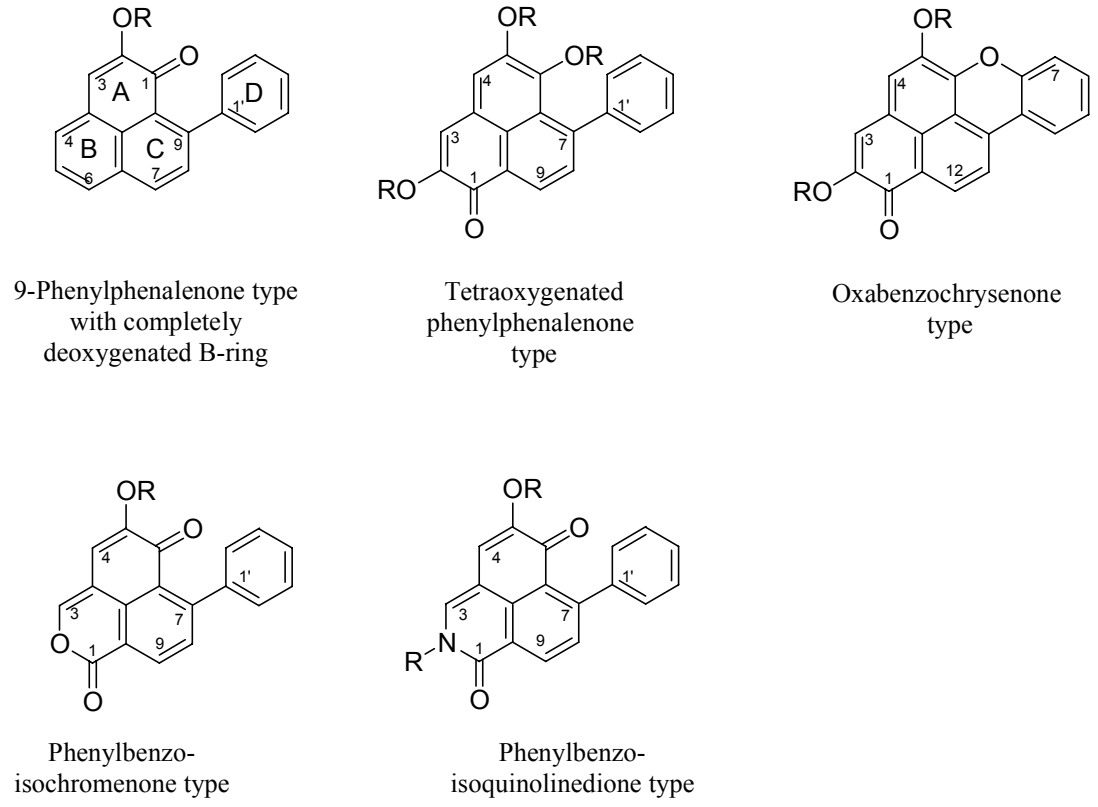
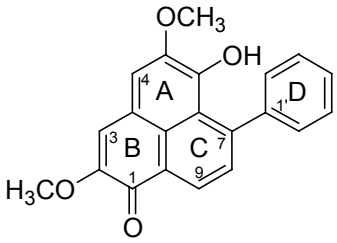


Fig. 2.1: General structure and numbering of subgroups of phenylphenalenone-related compounds.

Xiphidium caeruleum is a neotropical member of the Haemodoraceae native from Mexico to Brazil, from which only one phenylphenalenone, xiphidone (**1**), has been found (Fig. 2.2) (Cremona and Edwards, 1974). To investigate whether phenolic compounds of this species showed structural similarities to those of *L. tinctoria*, further phenylphenalenone-type compounds from plants of *X. caeruleum* were isolated and described based on structure elucidation by NMR and MS methods. Using these compounds as references, sterile root cultures of *Wachendorfia thyrsiflora*, a South African species, were screened for the occurrence of identical pigments. In this context, the characterisation of further

phenylphenalenone related compounds in *X. caeruleum* and *W. thyrsiflora* are of considerable interest with respect to chemotaxonomy and biogeography of the Haemodoraceae.



1: 2,5-Dimethoxy-6-hydroxy-7-phenyl-phenalen-1-one (Xiphidone)

Fig. 2.2: Xiphidone (**1**), the only known phenylphenalenone from *Xiphidium caeruleum*.

2.2 Material and methods

2.2.1 Plant material

Plants of *Xiphidium caeruleum* were obtained from the University of Bochum (Botanical Institute) and maintained at the Botanical Garden of the University of Jena. The plants were transferred to the Max-Planck-Institute for Chemical Ecology Jena and, prior to extraction, maintained in growth chambers for variable time intervals at a minimum temperature of 15°C.

Sterile root cultures of *W. thyrsiflora* were maintained in M3 liquid medium (100 ml in 300 ml Erlenmeyer flasks) at 22°C on a gyratory shaker (100 rpm) under permanent light (600 lux). The root cultures were initiated as described for *A. preissii* (Hölscher and Schneider, 1997).

2.2.2 Isolation and purification

Root (90 g), stem (320 g) and leaf (450 g) material of whole *X. caeruleum* plants were frozen with liquid N_2, ground and exhaustively extracted with MeOH or acetone at room temperature. After evaporation (< 40°C) the extracts were partitioned between *n*-hexane-H_2O, CH_2Cl_2-H_2O and EtOAc-H_2O. The CH_2Cl_2 and EtOAc extracts from roots, stems and leafs were pooled, subjected to MPLC on LiChroprep RP18 (25-40 µm, 230 × 22 mm), and

stepwise eluted using mixtures of MeCN-H_2O 1:4, 1:2, 3:4, 1:1, 4:3, 2:1, 4:1 (v:v, 100-500 ml). Fractions eluting with MeCN-H_2O 2:3 and 1:1 were collected and evaporated to dryness. Further separation was carried out by preparative reversed-phase HPLC on Nucleosil 100-7 C_{18} (7 µm; 250 x 21 mm; flow rate 11.5 ml min^{-1}; UV 254 nm) or LiChrospher 100 RP18 (10 µm; 250 x 10 mm; flow rate 3.5 ml min^{-1}; UV 254 nm). A linear gradient (a) MeCN-H_2O 30:70% → 65:35% in 30 min was used for the CH_2Cl_2 fraction and (b) MeCN-H_2O (0.1% TFA) 5:95% → 50:50% in 40 min for the EtOAc-fraction. Final purification was performed by reversed-phase HPLC on LiChrospher 100 RP-18 (5 µm; 250 x 4 mm) using the same gradients as with the large columns (flow rate 0.8 ml min^{-1}, DAD detection 200-600 nm).

Preparative HPLC was performed on a Merck Hitachi LiChrograph chromatography system (L-6200A gradient pump, L-4250 UV-VIS detector) using a Nucleosil 100-7 C18 (7 µm; 250 × 21 mm) and a LiChrospher 100 RP18 column (10 µm; 250 × 10 mm). Analytical HPLC was carried out on a Agilent 1100 chromatography system (binary pump G1312A, DAD G1315B, autosampler G1313A) using a LiChrospher 100 RP-18 column (5 µm; 250 × 4 mm). Medium pressure liquid chromatography (MPLC) was conducted using a Büchi B-688 pump equipped with a LiChroprep RP18 column (25-40 µm, 230 × 22 mm).

2.2.3 Derivatisation

Acetylation was performed with acetic anhydride in pyridine. Diazomethane in ether was used for methylation. Transesterification of allophanylglucoside **12** to methylallophanate (EIMS *m/z* 118 [M^+] (20), 87 (5), 75 (79), 70 (5), 59 (9), 44 (100); HREIMS *m/z* 118.0378 (calcd for $C_3H_6N_2O_3$, 118.0378) was effected by sodium methoxide. Methylallophanate was prepared for reference purposes from commercially available ethylallophanate (ACROS) using the same procedure.

2.2.4 Mass spectrometry (MS)

Electron impact mass spectra (EIMS) and high-resolution spectra (HREIMS) were recorded on a MasSpec sector field mass spectrometer (Micromass Ltd, Manchester, UK) with direct insertion probe. Electrospray ionisation mass spectra (ESIMS) were recorded on a Micromass Quattro II tandem quadrupole mass spectrometer using the first quadrupole only.

For LC-MS analysis, a Hewlett Packard HP 1100 LC was connected to the Quattro II (flow rate 1 ml min^{-1}). HRESIMS spectra were recorded on the Quattro II or a Micromass LCT mass spectrometer (fitted with a "LockSpray" source) at a resolution of 5000.

2.2.5 Nuclear magnetic resonance spectroscopy (NMR)

A Bruker DRX 500 NMR spectrometer, operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C was used for NMR experiments using standard Bruker pulse sequences. Chemical shifts are given in δ values relative to TMS as internal standard. ^{1}H-NMR, ^{1}H-^{1}H COSY, HMQC and HMBC experiments were recorded in a 2.5 mm inverse detection microprobe head; broadband decoupled ^{13}C spectra were run using a 2.5 mm broadband microprobe head.

2.2.6 UV-VIS absorption spectra

UV-VIS spectra were recorded from an Agilent G1315B diode array detector during analytical HPLC in MeCN-H_2O solvent mixtures between 1:2 and 2:1. All spectra were recorded from 200-700 nm.

2.2.7 Screening of *W. thyrsiflora* root cultures for phenylphenalenones

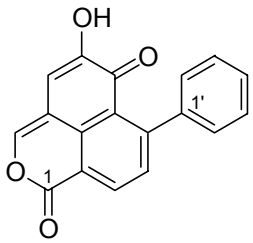
Four root cultures (each approximately 25 g fresh weight) were frozen with liquid N_2, ground and extracted with methanol. After evaporation (< 40°C), the extracts were partitioned between CH_2Cl_2-H_2O and EtOAc-H_2O. Samples of both CH_2Cl_2 and EtOAc fractions were analysed by HPLC. HPLC system and method were the same as for analysis of *X. caeruleum* extracts. Chromatograms of both fractions from the *Wachendorfia*-extract were screened for these compounds previous found in *X. caeruleum*. Compounds isolated from *X. caeruleum* were used as references, and via DAD detection, peaks of correct retention times were checked for identical UV-VIS spectra. Small amounts of these recognised compounds from *W. thyrsiflora* were isolated using preparative HPLC, using the same procedure as for *X. caeruleum*, and their identity was verified by ^{1}H-NMR measurements.

2.3 Results

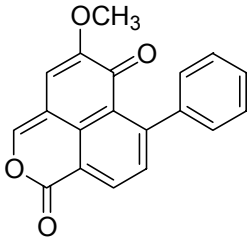
2.3.1 Structure elucidation of isolated compounds

Tetraoxygenated phenylphenalenones and related compounds, almost modified by oxidative conversions in ring B (phenylbenzoisochromenone-type with lactone structure) or formation of an ether bridge between ring A and ring D were found, in the latter case resulting in the formation of oxabenzochrysenone type structures (Fig. 2.3 and 2.5). The ^{1}H NMR spectra all of these compounds are characterized by two singlets of H-3 and H-4, an AB spin system of H-8/H-9 (or H-11/H-12 in the oxabenzochrysenones, respectively). Proton signals of the exocyclic 7-phenyl ring of phenylphenalenones and a well resolved four-spin system of H-7 to H-10 in the oxabenzochrysenones type are also common signals. Structural variations are typically indicated by additional signals of methoxyl groups, sugar units, and changes in chemical shift values caused by additional oxygen substituents at C-3.

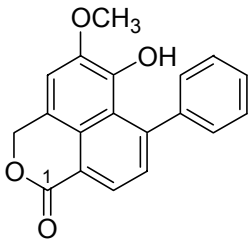
To establish the structures of isolated compounds and to assign ^{1}H and ^{13}C chemical shifts unambiguously, ^{1}H NMR and ^{13}C NMR spectra, ^{1}H-^{1}H COSY, heteronuclear multiple-bond correlation (HMBC), and heteronuclear multiple quantum coherence (HMQC) spectra were measured. The assignment strategy started from the most downfield signal in the ^{1}H NMR spectrum, H-9 (phenylphenalenones) or H-12 (oxabenzochrysenone type). These protons exhibited three-bond HMBC correlations with a carbonyl (C-1), C-7 (or 12d, respectively), and the central carbon atom of the tricyclus, C-9b (12b). The latter carbon atom also showed connectivities through three bonds with H-3 and H-4. The *peri* position of H-3 and H-4 was proved by mutual HMBC cross peaks with the corresponding carbon atoms C-3 and C-4, respectively. Additional HMBC correlations were detected from H-3 with carbonyl C-1 and of H-4 with C-6 (or C-5a in oxabenzochrysenones) through three bonds and C-5 through two bonds. HMBC between H-3 and C-2 was missing when C-2 was substituted. All the isolated compounds share the general NMR data and connectivities described here. Thus, only the type of substituents at positions 2, 3, 5, and 6 of individual compounds remained to be established. Assignments of NMR signals of these variable structural features are discussed below.



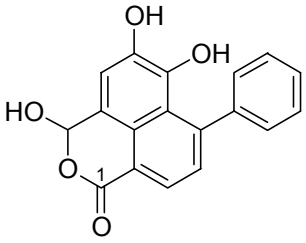
2: 5-Hydroxy-7-phenyl-benzo[*de*]-isochromene-1,6-dione (lachnanthopyrone)



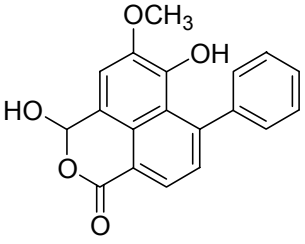
3: 5-Methoxy-7-phenyl-benzo[*de*]-isochromene-1,6-dione



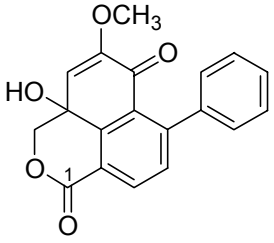
4: 6-Hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one



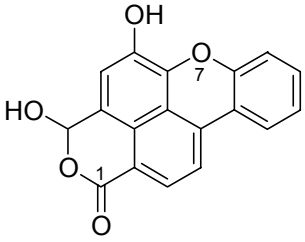
5: 3,5,6-Trihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one



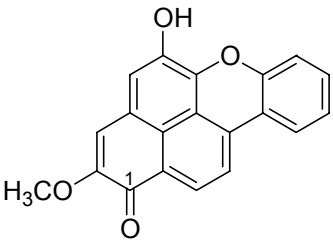
6: 3,6-Dihydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromene-1-one



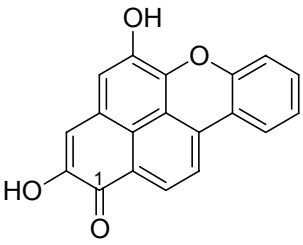
7: 3a-Hydroxy-5-methoxy-7-phenyl-3,3a-dihydro-benzo[*de*]isochromene-1,6-dione



8: 3,5-Dihydroxy-3*H*-2.6-dioxa-benzo[*def*]chrysen-1-one



9: 5-Hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one



10: 2,5-Dihydroxy-6-oxa-benzo[*def*]chrysen-1-one (lachnanthofluorone)

Fig. 2.3: Phenylphenalenone-related aglycones from *Xiphidium caeruleum*.

Aglycones

The common signals described above were found in the NMR spectra of 5-hydroxy-7-phenyl-benzo[*de*]isochromene-1,6-dione (lachnanthopyrone, **2**), which was first isolated from flowers of *Lachnanthes tinctoria* (Edwards and Weiss, 1972). ^{1}H NMR data are shown in Table 2.1 for comparison purposes. ^{13}C NMR data (Table 2.2) are listed here for the first time.

Table 2.1: ^{1}H NMR data (500 MHz, TMS int. standard) of compounds **2 - 7** from *Xiphidium caeruleum*. Compounds **2**, **3**, **4**, **6**, and **7** were measured in acetone-d_6, **5** in methanol-d_4.

	2	**3**	**4**	**5**	**6**	**7**
position	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)
3	8.10 (s)	8.09 (s)	5.79 (s)	6.45 (s)	6.85 (s)	4.72 (d, 11.8) 4.59 (d, 11.8)
4	6.98 (s)	6.97 (s)	7.55 (s)	7.54 (s)	7.71 (s)	6.03 (s)
8	7.68 (d, 8.1)	7.66 (d, 8.1)	7.36 (d, 7.4)	7.36 (d, 7.4)	7.36 (d, 7.3)	7.49 (d, 8.0)
9	8.56 (d, 8.1)	8.54 (d, 8.1)	8.18 (d, 7.4)	8.19 (d, 7.4)	8.23 (d, 7.3)	8.21 (d, 8.0)
2'/6'	7.36 (m)	7.35 (m)	7.37 (m)	7.40 (m)	7.36 (m)	7.26 (dd, 7.7, 2.0)
3'/4'/5'	7.43 (m)	7.41 (m)				7.40 (m)
5-OMe		3.81 (s)	3.98 (s)		4.01 (s)	3.71 (s)

The NMR data of compound **3** (Table 2.1 and Table 2.2) closely resembled those of **2**. The methoxyl group rather than the hydroxyl at position C-5 (δ 152.5) was indicated by the singlet at δ 3.81 and its HMBC correlation with C-5. The structure of compound **3** as 5-methoxy-7-phenyl-benzo[*de*]isochromene-1,6-dione was supported by the $[M-1]^+$ ion (*m/z* 303) in the EIMS, which is typical for the *peri* position of carbonyl with phenyl ring. HREIMS confirmed the suggested structure.

The singlet of H-3 of compound **4** which corresponded to two protons and therefore to a methylene group, appeared downfield at δ 5.79, which is consistent with an oxygen function at C-3. The signal at δ 3.98 indicated a methoxyl at C-5. The hydroxyl group at C-6 was established by means of the acetyl derivative **4a**, which exhibited an acetyl signal at δ 1.39. ^{1}H NMR Data of **4** are given in Table 1. ^{1}H NMR and ^{13}C NMR data of the acetyl derivative **4a** are shown in the Appendix. EIMS (*m/z* 306 $[M^+]$) and HREIMS of **4** confirmed the structure as 6-hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one. This compound has been isolated previously from roots of *L. tinctoria* (Edwards and Weiss, 1972; 1974).

The chemical shift value of the signal of H-3 (δ 6.45) in compound **5**, which integrated to one proton only (Table 2.1), was consistent with a hydroxymethine functionality at C-3 (δ 103.8) (Table 2.2). The acetyl derivative **5a** exhibited three acetyl signals (δ 2.11, 2.29, 1.45) from which three hydroxy groups at the positions C-3, C-5 and C-6, respectively, of compound **5** were deduced (for NMR data see Appendix). Both LCEIMS (*m/z* 308 $[M^+]$) and HREIMS data of **5** were in agreement with the suggested structure of 3,5,6-trihydroxy-7-phenyl-*3H*-benzo[*de*]isochromen-1-one (**5**). This compound has already been proposed as an intermediate in the biosynthesis of a naphthalic anhydride and lachnanthopyrone (**2**) in *L. tinctoria* (Edwards and Weiss, 1974) but hitherto was not found in any plant.

The ^{1}H NMR spectrum of compound **6** differed from that of the trihydroxy compound **5** by an additional methoxyl resonance (δ 4.01, Table 2.1) at C-5 (δ 144.3, Table 2.2), the position of which was established by a corresponding HMBC cross signal. The LCMS data of compound **6** (*m/z* 322 $[M^+]$) and a pseudo-molecular ion *m/z* 320 $[M-2]^+$ in the HREIMS also indicated dehydrogenation to give an isochromene-1,6-dione or naphthalic anhydride as previously discussed for compound **5**. Diazomethane treatment of compound **6** gave 8-formyl-5,6-dimethoxy-4-phenyl-naphthalene-1-carboxylic acid methyl ester (**6a**) (EIMS: *m/z* 350 $[M^+]$) (Fig. 2.4). In addition to the anticipated methylation in *peri* position to the phenyl ring (δ 3.29 / δ 60.7) at C-5 (δ 149.9), the lactone ring was opened to generate a methyl ester

Table 2.2: ^{13}C NMR data (125 MHz, TMS int. standard) of compounds **2, 3** and **5** – **7** from *Xiphidium caeruleum*. Compounds **2**, **3**, **6**, and **7**, were measured in acetone-d_6, compounds **5** in methanol-d_4.

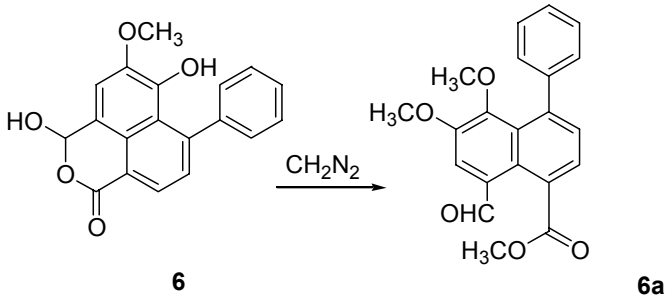
	2	**3**	**5**	**6**	**7**
position	δ_C	δ_C	δ_C	δ_C	δ_C
1	160.6	160.1	166.4	164.1	163.6
3	148.8	145.7	103.8	96.8	74.7
3a	112.8	111.9	119.6	122.0	63.7
4	107.7	104.8	118.7	114.7	110.0
5	150.1	152.5	143.1*	144.3	154.9
6	179.2	177.7	142.9*	143.8	179.2
6a	125.1	125.6	119.4	121.4	125.0
7	152.0	152.0	146.6	145.5	148.9
8	134.1	133.6	129.7	129.7	133.7*
9	134.6	133.8	127.9	127.7	133.8*
9a	120.6	119.2	122.6	120.2	127.7
9b	134.6	133.3	125.0	125.0	145.3
1'	142.1	140.7	144.7	144.5	142.0
2',6'	129.1	128.0	130.0	129.6	129.1
3',5'	128.6	127.9	128.2	127.8	128.7
4'	128.4	127.8	129.3	127.5	128.2
5-OMe		55.7		57.8	55.7

* May be reversed in one column.

and an aldehyde function. The methyl ester gave NMR signals at δ 3.86 / δ 52.4 exhibiting a HMBC correlation with the ester carbonyl carbon (δ 169.7). The low field proton (δ 10.1) and its HMBC cross peak with C-7 (δ 123.1) and C-8b (δ 124.9) proved the presence of an aldehyde function.

Compound **7** is different from **2**-**6** as it bears a tertiary hydroxyl group at C-3a. This was deduced from the chemical shift (δ 63.7) of this ^{13}C signal, which is inconsistent with an aromatic carbon resonance but is indicative of an oxygenated aliphatic carbon atom. As a consequence of the sp^3 hybridization of C-3a, the conjugated system is interrupted. C-6 was found to be a carbonyl carbon (confirmed by the ^{13}C chemical shift of δ 179.2) and C-3 a

methylene group (δ 74.7). The proton signals of H-3a (δ 4.72) and H-3b (δ 4.59) at the prochiral C-3 appeared as well resolved doublets. Attachment of the methoxyl (δ 3.71 / δ 55.7) to C-5 (δ 154.9) was established by means of HMBC correlation. EIMS (*m/z* 322) and HREIMS data were in accord with the structure of **7** as 3a-hydroxy-5-methoxy-7-phenyl-3,3a-dihydro-benzo[*de*]isochromene-1,6-dione.



6 **6a**

Fig. 2.4: Formation of 8-formyl-5,6-dimethoxy-4-phenyl-naphthalene-1-carboxylic acid methyl ester (**6a**) from phenyl-benzoisochromenone **6** by treatment with diazomethane.

The ^{1}H NMR spectrum of compound **8** (Table 2.3) showed signals of an AB spin system of H-11 (δ 7.86) / H-12 (δ 8.07), and singlets of H-3 (δ 6.82) and H-4 (δ 7.50), which bore a striking analogy to the ^{1}H NMR spectrum of the benzo[*de*]isochromen-1-one **5**. However, well-resolved signals of a four-spin system were detected rather than the broad phenyl ring signal of compound **5**, indicating absence of a proton in ortho-position due to substitution or ring closure with the adjacent tricyclic nucleus. HMBC cross signals of H-8 (δ 7.46) and H-10 (δ 8.10) with the low field shifted C-6a (δ 153.3) provided evidence for an electronegative substituent in this position (^{13}C NMR data are shown in Table 2.4). The molecular ion of *m/z* 306, obtained from EIMS, and HREIMS data gave the molecular formula of $C_{18}H_{10}O_5$, which excluded an additional substituent. Thus, a phenol ether bridge must exist between H-5a and H-6a. ^{13}C NMR data and further HMBC and HMQC connectivities proved the structure of **8** to be 3,5-dihydroxy-3*H*-2.6-dioxa-benzo[*def*]chrysen-1-one. This compound is the first 2.6-dioxa-benzochrysenone found as a natural product in plants.

The four-spin system of H-7–H-10 in the ^{1}H NMR spectrum of compound **9** and HMBC connectivities in that part of the molecule similar to those of compound **8** are

Table 2.3: ^{1}H NMR data (500 MHz, TMS int. standard) of compounds **8-10** from *Xiphidium caeruleum*. Compounds **8** and **10** were measured in acetone-d_6 and compound **9** in DMSO-d_6.

position	**8** δ_H (*J*, Hz)	**9** δ_H (*J*, Hz)	**10** δ_H (*J*, Hz)
3	6.82 (s)	7.37 (s)	7.36 (s)
4	7.50 (s)	7.79 (s)	7.84 (s)
7	7.22 (dd, 8.2, 1.2)	7.49 (dd, 7.8, 1.2)	7.52 (dd, 8.1, 1.2)
8	7.46 (ddd, 7.6, 8.2, 1.2)	7.62 (ddd, 7.8, 7.5, 1.2)	7.66 (ddd, 8.1, 7.5, 1.2)
9	7.26 (ddd, 7.8, 7.6, 1.2)	7.39 (ddd, 7.5, 8.1, 1.2)	7.45 (ddd, 7.5, 7.8, 1.2)
10	8.10 (dd, 7.8, 1.2)	8.38 (dd, 8.1, 1.2)	8.45 (dd, 7.8, 1.2)
11	7.86 (d, 7.6)	8.31 (d, 7.8)	8.39 (d, 7.7)
12	8.07 (d, 7.6)	8.51 (d, 7.8)	8.73 (d, 7.7)
2-OMe		3.86 (s)	

Table 2.4: ^{13}C NMR data (125 MHz, TMS int. standard) of compounds **8** - **10**. Compounds **8** and **10** were measured in acetone-d_6 and compound **9** in DMSO-d_6.

position	**8** δ_C	**9** δ_C	**10** δ_C
1	163.4	176.8	178.8
2		152.5	150.9
3	97.0	112.7	113.5
3a	119.1	120.1	n.d.
4	119.2	122.0	123.0
5	140.7	140.1	141.2
5a	137.5	137.2	138.4
6a	153.3	151.5	152.8
7	118.4	117.7	118.6
8	132.6	132.3	132.9
9	125.3	124.6	125.4
10	124.8	124.5	125.1
10a	120.5	118.7	119.9
10b	132.4	132.7	134.7
11	115.4	116.1	116.8
12	129.0	130.0	131.5
12a	118.9	119.7	120.5
12b	123.3	117.0	118.2
12c	122.0	127.0	126.8
2-OMe		55.4	

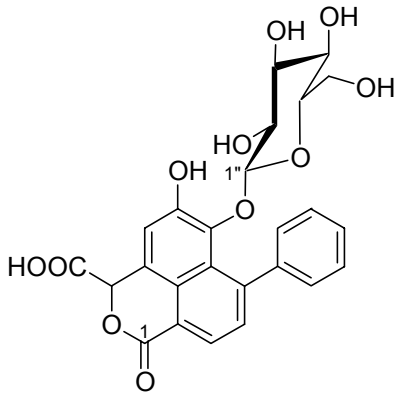
n.d. = not detected

indicative of a structure closely related to **8**. Differences are apparent in positions 2 and 3. Lowfield signals of H-3 (δ 7.37) and C-3 (δ 112.7) are inconsistent with sp^3 hybridization and indicate a protonated sp^2 carbon. This requires a double bond to be accommodated, which is only possible between C-2 and C-3. The C-2 carbon atom was detected in the ^{13}C NMR spectrum at δ 152.5 and assigned by HMBC two-bond correlation with H-3 (δ 7.37) in addition to a HMBC three-bond correlation with protons of a methoxyl group (δ 3.86), which therefore must be attached to C-2. EIMS (*m/z* 316 [M^+]) and HREIMS confirmed the suggested structure of 5-hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one (**9**). Compounds of this type have been described, and designated as naphthoxanthenones, first in *L. tinctoria* (Edwards and Weiss, 1974) and afterwards in other members of the Haemodoracea (Opitz *et al.*, 2002).

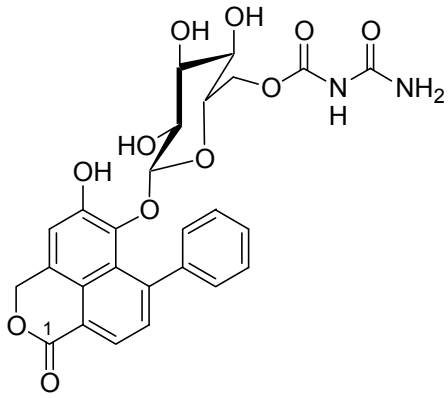
The NMR spectra of compound **10** resembled those of **9** with the only exception of missing methoxyl group resonances. Thus, the structure of **10** (EIMS: *m/z* 302 [M^+]) is 2,5-dihydroxy-6-oxa-benzo[*def*]chrysen-1-one, which was previously described as lachnanthofluorone from roots of *L. tinctoria* (Edwards and Weiss, 1974). The ^1H and ^{13}C NMR spectra reported here unambiguously prove the structure proposed by these authors, although their ^1H NMR data are inconsistent and have been subject to discussion because the synthetic 2,5-dimethoxyl derivative gave different results. The ^1H NMR data published by the latter authors for the dimethoxyl compound are in accord with those described here for compound **10** from *X. caeruleum*.

Glucosides

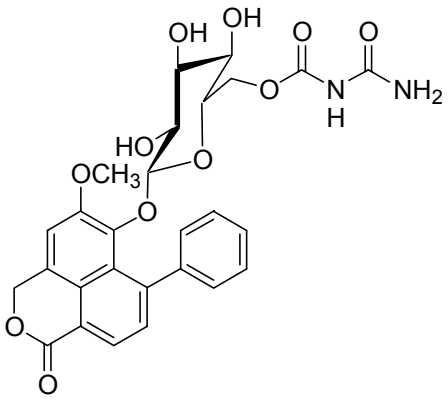
Five glycosidic compounds **11-15** were isolated from the ethyl acetate extract of *X. caeruleum*. The aglycone of compound **11** was identified by means of the NMR and MS data (ESIMS: *m/z* 499 $[M+H]^+$, HREIMS) as 3-carboxy-5,6-dihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one. The carboxyl group at C-3 (δ 82.2) was established by means of a HMBC correlation between H-3 (δ 6.03) and the carboxyl carbon (δ 174.1). The sugar moiety of **11** was identified as β-D-glucose by means of ^1H NMR (Table 5), ^{13}C NMR data (Table 2.6) and a complete set of HMBC and HMQC correlations. The doublet of H-1" at δ 4.75 showed a coupling constant of 7.9 Hz, indicating β-configuration of the anomeric proton. Coupling constants $^3J_{H\text{-}2\text{–}H\text{-}3}$, $^3J_{H\text{-}3\text{–}H\text{-}4,}$ and $^3J_{H\text{-}4\text{–}H\text{-}5}$ of approximately 9 Hz showed that all protons were axial. The HMBC correlation between H-1" and C-6 (δ 139.0) established



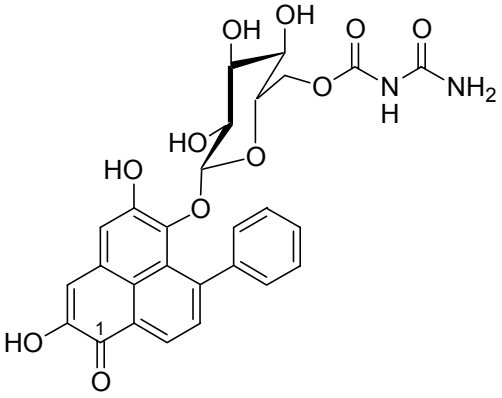
11: 3-Carboxy-5-hydroxy-6-*O*-β-D-glucopyranosyl-7-phenyl-3*H*-benzo[*de*]isochromen-1-one



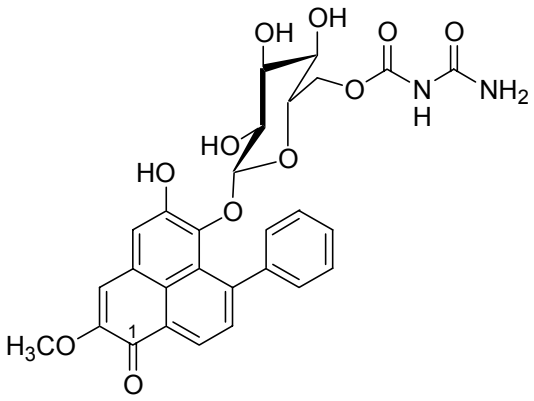
12: 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one



13: 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one



14: 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenyl-phenalen-1-one



15: 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenyl-phenalen-1-one

Fig. 2.5: Phenylphenalenone-related glucosides from *Xiphidium caeruleum*

attachment of the glucose unit to C-6. Mutual interactions between the aromatic ring and the glucose unit in *peri* position were observed. Due to the shielding effect of the phenyl ring, the two doublets of H-2" (δ 2.39) and H-4" (δ 2.97) appeared at unusual high field. Similar high field shift of glucosides (Pauli, 2000) has been observed already for such protons of the antibiotic moenomycin A (Hennig *et al.*, 1998) and benzoxazinone-N-glucosides (Sicker *et al.*, 2001). Notably, H-2" of glucoside **11** was drastically shifted to high field, while the corresponding carbon signals of C-2" (δ 74.7) and C-4" (δ 71.4) appeared within the usual shift range. Electronic effects of the chiral sugar unit, which is in close proximity to the phenyl ring, caused significant signal broadening. Moreover, separate signals of magnetically inequivalent pairs of protons H-2' (δ 7.31)/H-6' (δ 7.35), H-3' (δ 7.48)/H-5' (δ 7.26) and carbon atoms C-2' (δ 127.3)/C-6' (δ 128.1) and C-3' (δ 130.6) /C-5' (δ 131.4) were observed. From these data, the structure of compound **11** was elucidated to be 3-carboxy-5-hydroxy-6-*O*-β-D-glucopyranosyl-7-phenyl-3*H*-benzo[*de*]isochromen-1-one. Attempts to hydrolyze this glucoside by means of ß-glucosidase were not successful. This is in accord with reported resistance to various glycosidases of 1-*O*-glucoside of haemocorin aglycone (Dora *et al.*, 1993), which is very likely to be due to steric hindrance effects of a phenyl *peri* to the bulky sugar unit.

The aglycone of glycoside **12** was identified by means of the NMR data as 5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one. It differed from that of **11** only in the missing carboxyl group at C-3 (δ 70.7), which was replaced by the methylene singlet of H-3 at δ 5.75. Again, separate signals of magnetically inequivalent pairs of phenyl protons and phenyl carbon atoms were observed, together with an HMBC correlation of H-1" (δ 4.60) with C-6 (δ 138.4) proving attachment of the hexose unit to this carbon atom. This sugar moiety was identified as β-D-glucose by ^{1}H-, ^{13}C NMR, and HMBC signals. However, two further low-field ^{13}C signals at δ 172.4 and 170.0, the latter being connected to H-6" (δ 4.16/3.91) of the glucose unit by HMBC, suggested a two-carbon substituent at C-6"-OH. The mass spectrum of **12** (ESIMS: *m/z* 541 $[M+1]^+$, HREIMS) indicated the molecular formula $C_{26}H_{24}N_2O_{11}$. After identification of the aglycone and the glucose moiety, a $C_2H_3N_2O_2$ substituent was suggested, indicating an aminocarbonyl carbamic acid (allophanyl) unit. However, owing to the instability of allophanic acid, a fragment of an intact allophanyl unit was not detectable in the mass spectra. To confirm the allophanyl substituent, compound **12** was transesterified by sodium methoxide and analysed by MS (Fig. 2.6). The molecular ion (EIMS: *m/z* 118 $[M]^+$) and fragmentation pattern of the resulting methyl ester were the same as methyl allophanate,

Table 2.5: ^{1}H NMR data (500 MHz, methanol-d_4, TMS int. standard) of glucosides **11**-**15** from plants of *X. caeruleum*.

	11	**12**	**13**	**14**	**15**
position	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)	δ_H (*J*, Hz)
3	6.03 (s)	5.75 (s)	5.79 (s)	7.04 (s)	7.13 (s)
4	7.48 (s)	7.22 (s)	7.46 (s)	7.46 (s)	7.57 (s)
8	7.37 (d, 7.6)	7.40 (d, 7.4)	7.35 (d, 7.4)	7.54 (d, 7.6)	7.56 (d, 7.5)
9	8.14 (d, 7.6)	8.15 (d, 7.4)	8.12 (d, 7.4)	8.47 (d, 7.6)	8.48 (d, 7.5)
2'	7.31 (br)	7.32 (br)	7.33 (br)	7.33 (br)	7.33 (br)
3'	7.48 (br)	7.60 (br)	7.46 (br)	7.43 (br)	7.46 (br)
4'	7.31 (br)	7.32 (br)	7.33 (br)	7.33 (br)	7.35 (br)
5'	7.26 (br)	7.20 (br)	7.20 (br)	7.22 (br)	7.23 (br)
6'	7.35 (br)	7.37 (br)	7.41 (br)	7.39 (br)	7.40 (br)
1"	4.75 (d, 7.9)	4.60 (d, 7.9)	4.82 (d, 7.7)	4.67 (d, 7.9)	4.66 (d, 7.7)
2"	2.39 (dd, 7.9, 9.3)	2.35 (dd, 7.9, 9.3)	2.07 (dd, 7.7, 9.1)	2.36 (dd, 7.9, 9.0)	2.37 (dd, 7.7, 8.9)
3"	3.18 (dd, 9.3, 9.5)	3.17 (dd, 9.3, 8.4)	3.17 (dd, 9.1, 8.8)	3.19 (dd, 9.0, 9.3)	3.19 (dd, 8.9, 9.0)
4"	2.97 (dd, 9.5, 9.8)	} 3.05 (m)	3.01 (dd, 8.8, 9.7)	3.04 (dd, 9.3, 9.0)	3.00 (dd, 9.0, 9.2)
5"	2.92 (ddd, 9.8, 5.3, 2.2)		4.27 (ddd, 9.7, 5.4, 2.0)	3.09 (ddd, 9.0, 6.5, 2.2)	3.10 (ddd, 9.2, 6.6, 2.0)
6"a	3.47 (dd, 11.8, 5.3)	4.16 (dd, 11.5, 4.8)	4.21 (dd, 11.5, 5.4)	4.18 (dd, 11.8, 6.5)	4.17 (dd, 11.8, 6.6)
6"b	3.57 (dd, 11.8, 2.2)	3.91 (dd, 11.5, 2.0)	3.93 (dd, 11.5, 2.0)	3.96 (dd, 11.8, 2.2)	4.00 (dd, 11.8, 2.0)
2-OMe					3.94 (s)
5-OMe			3.96 (s)		

br = broad signal

Table 2.6: ^{13}C NMR data (125 MHz, methanol-d_4, TMS int. standard) of glucosides **11**-**15** from *Xiphidium caeruleum*.

	11	**12**	**13**	**14**	**15**
position	δ_C	δ_C	δ_C	δ_C	δ_C
1	167.1	166.9	166.7	182.2	181.9
2				151.2	153.6
3	82.2	70.7	70.8	116.3	115.0
3a	126.2	126.4	126.0	128.5	129.0*
4	118.6	116.6	112.9	124.2	124.6
5	148.6	148.9	151.0	149.0	149.0
6	139.0	138.4	139.9	141.0	141.3
6a	121.2	120.0	120.0	127.8*	127.4*
7	145.5	146.1	146.6	148.8	148.7
8	131.7	131.8	131.8	132.3	132.4
9	126.5	127.0	127.6	128.6	128.8
9a	128.4	128.9	129.9	130.0*	129.1*
9b	125.5	125.8	126.0	121.7	121.8
1'	145.1	144.9	145.2	145.3	145.1
2'	127.3 (br)	127.3 (br)	127.3 (br)	127.3 (br)	127.4 (br)
3'	130.6 (br)	130.9 (br)	130.6 (br)	130.7 (br)	130.7 (br)
4'	127.6	127.8	127.6	127.8	127.8
5'	131.4 (br)	131.6 (br)	131.3 (br)	131.8 (br)	131.9 (br)
6'	128.1 (br)	128.2 (br)	127.6 (br)	128.3 (br)	128.2 (br)
1"	103.9	104.0	102.9	104.1	104.1
2"	74.7	74.6	74.2	74.6	74.5
3"	77.6	77.5	77.4	77.6	77.6
4"	71.4	71.1	71.2	71.1	71.3
5"	77.8	75.1	74.6	75.2	75.0
6"	62.7	64.8	65.3	64.9	65.2
1'"		170.0	168.0	169.3	168.2
2'"		172.4	169.7	171.5	169.9
3-COOH	174.1				
2-OMe					56.1
5-OMe			57.5		

* May be reversed in one column. br = broad signal

obtained by transesterification from commercially available ethylallophanate. HREIMS confirmed the structure. Compound **12**, 6-*O*-[(6"-*O*-allophanyl)-β-D-glucopyranosyl]-5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one is the first allophanylglucoside detected as a natural product. Derivatives of allophanic acid hitherto have been found only in two plant

species, *Butea monosperma* (Dhar *et al.*, 1982; Porwal *et al.*, 1988) and *Echinops echinatus* (Sharma *et al.*, 1988).

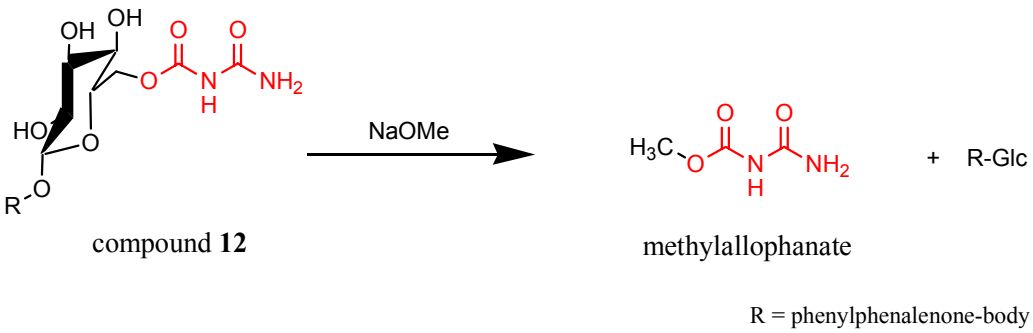


Fig. 2.6: Transesterification of 6-*O*-[(6"-*O*-allophanyl)-β-D-glucopyranosyl]-5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**12**) to methylallophanate.

The spectra of compound **13** were the same as those of **12,** except the additional methoxyl signal at position C-5 (δ 151.0). The aglycone of **13** was readily assigned by means of NMR data (Table 2.5, Table 2.6) as compound **4.** The NMR spectra of the acetyl derivative **13a** (see Appendix), exhibited additional signals of three acetyl groups on the glucose unit, confirming substitution by the allophanyl moiety. Mass spectra (ESIMS: *m/z* 555 $[M+H]^+$, HREIMS) confirmed the structure of **13** as 6-*O*-[(6"-*O*-allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one.

Signals of the allophanylglucosyl moiety were found also in compound **14**. An HMBC correlation of H-1" (δ 4.67) with C-6 (δ 141.0) proved attachment of the glucose to the aglycon at this position. The ^{1}H NMR spectrum (Table 2.5) exhibited aglycon signals, namely two singlets of H-4 (δ 7.46) and H-3 (δ 7.04), the AB spin system of H-8 (δ 7.54)/H-9 (δ 8.47), and signals of a phenyl group. The chemical shift of H-3 and an additional HMBC correlations through two bonds with the carbon signal at δ 151.2 (Table 2.6) were consistent with a hydroxyl group bearing carbon in position 2. The separate signals of nonequivalent protons and carbon atoms indicated a *peri* position with the phenyl ring. The molecular weight of **14** (ESIMS: *m/z* 553 $[M+H]^+$, HREIMS) confirmed these findings, and proved compound **14** to be 6-*O*-[(6"-*O*-allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenyl-phenalen-1-one. The NMR data of compound **15** resembled those of **14** with the exception of

an additional methoxyl signal (δ 3.94/ δ 56.1), which exhibited HMBC correlations with C-1 (δ 181.9) and C-3 (δ 115.0) and, therefore, was assigned to C-2 (δ 153.6). Thus, compound **15** is 6-*O*-[(6"-*O*-allophanyl)-β-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenyl-phenalen-1-one. Both glucosides, but without the allophanyl group, have been described from *Dilatris viscosa*, a South-African species (Dora *et al.*, 1993). The aglycones of **14** and **15** and compound **1** are the only intact phenylphenalenones that were found in *X. caeruleum*.

2.3.2 Methoxylation

A significant number of natural products were isolated from *X. caeruleum* bearing methoxyl substituents. This finding raised the question of artificial methylation during extraction procedure using methanol as a solvent. To check this possibility, control experiments were carried out using acetone rather than methanol as solvent for extraction. HPLC analysis with a MeCN-H_2O gradient indicated the occurrence of all compounds described in here, confirming that the metabolites are indeed natural products. However, two additional compounds, namely 5,6-dihydroxy-3-methoxy- (**16**) and 5-hydroxy-3,6-dimethoxy-7-phenyl-*3H*-benzo[*de*]isochromen-1-one (**17**), representing methoxy analogues of **5,** were found after extraction with methanol [for data of **16a** (acetylated form of **16**) and **17** see Appendix]. In these experiments, the HPLC peaks representing compound **5** were significantly diminished compared to those from the acetone extract. These findings clearly indicate that the methoxyl groups at C-3 of benzoisochromenones are artifacts of the extraction process (see also chapter 3).

2.3.3 UV-VIS spectra

UV-VIS spectra of phenylphenalenone related compounds showed a strong structural correlation, which enabled to identify subtypes by using HPLC coupled with a DAD (Fig. 2.7). The conjugated system, particularly the B-ring, had the most influence on the spectra of the compounds. Additionally a phenol ether bridge, as in the benzochrysenones **8** – **10**, causes absorption maxima in longer wavelength ranges up to 547 nm for **9**. The occurrence of glucose and/or an allophanyl group also influenced the absorption behaviour, as is evident from a comparison of the compounds **4** and **13**. Although both molecules posses the same

aglycone there are significant differences in their UV-VIS spectra. The substitution of a methoxyl group rather than hydroxyl one influenced the absorption spectra only slightly (compare **2** – **3**; **5** – **6**; **12** – **13**; **14** – **15**) except for compounds **9** and **10** where distinct differences occurred. However, there were doubts about the purity of compound of **10**. Metal complexing due to its α-keto-alcohol group at ring B might be possible and therefore the UV-VIS spectrum of **10** has to be considered with reservation.

2.3.4 Detection of phenylphenalenones in *W. thyrsiflora*

Four of the fourteen compounds previously described from *X. caeruleum* have been found in root cultures of *W. thyrsiflora*. In addition to one compound of the isochromenone-type (**3**) two benzochrysenones (**9** and **10**) and two allophanylglucosides (**13** and **15**) were identified. Additionally, one artificially methylated isochromenone was found, which also has been isolated from *X. caeruleum* previously. ^{1}H-NMR spectra of all five compounds matched with these found in *X. caeruleum*.

2.4 Discussion

Fourteen new compounds belonging to the phenylphenalenone-type, the benzochrysenone-type or the isochromenone-type have been isolated from *X. caerulem*. Three of the aglycones were known already from in *L. tinctoria* (Edwards and Weiss, 1972; 1974). However xiphidone (**1**), which was the only known phenylphenalenone of this plant species (Cremona and Edwards, 1974), was not found. Only glucosides with sugars in the sterically hindered position *peri* to the phenyl ring were isolated. Owing to the lack of a hydroxyl in such a position in oxabenzochrysenones, glucoside formation clearly cannot take place with these compounds in *X. caeruleum*. The allophanylglucosides represent a novel type of conjugates and their role in plants has to be investigated.

The occurrence of members of the same subgroup of phenylphenalenone related compounds, such as tetraoxygenated phenylphenalenones, phenylbenzoisochromenones, oxabenzochrysenones and even identical compounds of these types (*e.g.* **2, 4** and **10**) in *X. caeruleum* and *L. tinctoria*, represents a strong argument for the existence of a very close

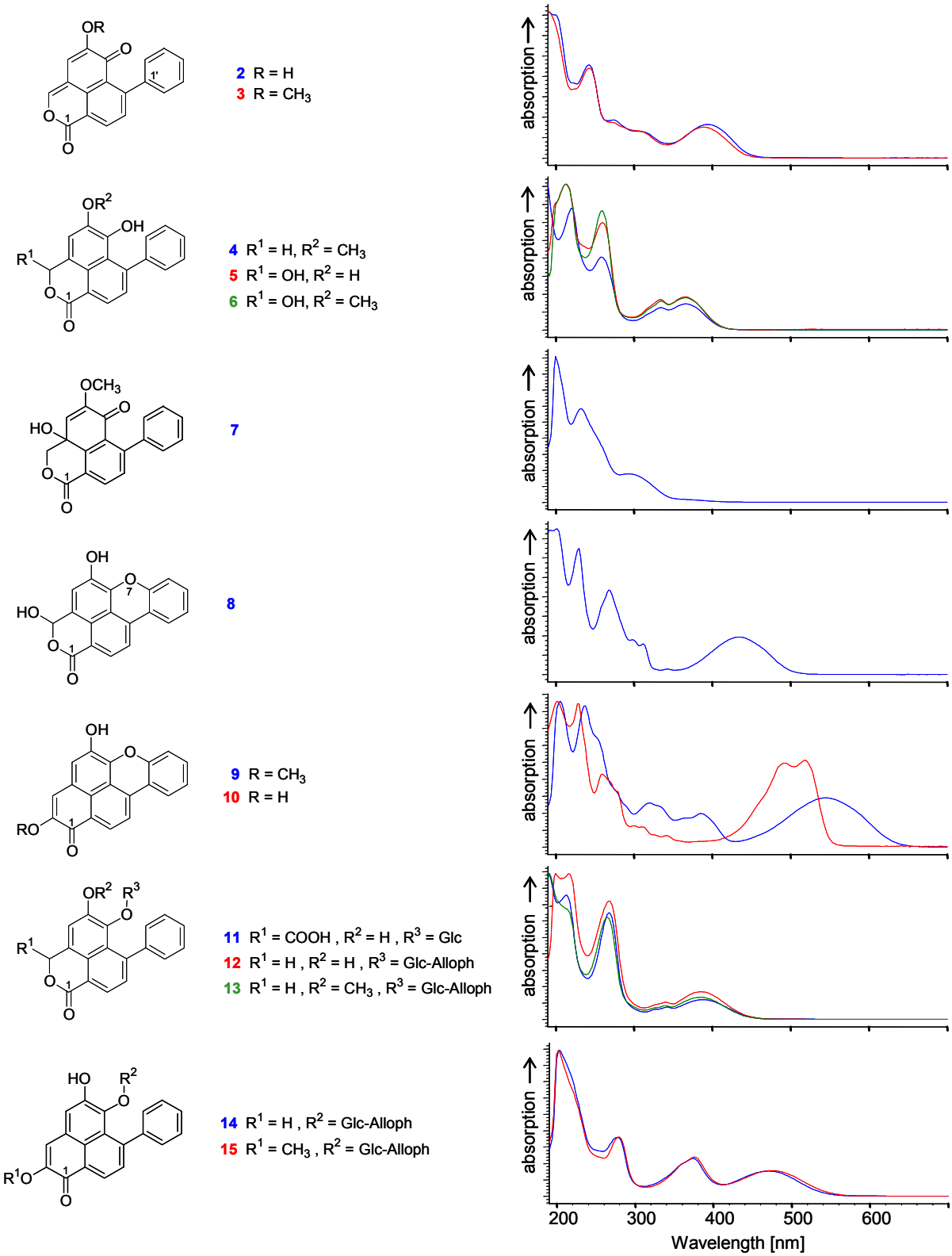


Fig. 2.7: Phenylphenalenone-related natural products from *Xiphidium caeruleum* and their UV-VIS absorption spectra.

relationship between both American species. This has been confirmed recently by the isolation of a phenylbenzoisoquinolinedione from root material of *X. caeruleum*. In addition to *L. tinctoria* (Edwards and Weiss, 1972) this was the second species in which this type of phenolics has been found. The characterisation of further compounds from both *X. caeruleum* and *W. thyrsiflora* has been an important step to construct a distribution pattern of phenylphenalenone-related subtypes of the Haemodoraceae worldwide based on the present level of knowledge (Fig. 2.8). Chemotaxonomic considerations indicated that both tetraoxygenated phenylphenalenones and oxabenzochrysenones might be universal phenolic types of this plant family. By contrast, structural types such as the isochromenones and isochinolinediones are characteristic features of the New World species (although there have been no investigations on genera *Schiekia* and *Pyrrorhiza* from South America). American species seem to contain no 9-phenylphenalenones with a completely deoxygenated B-ring which is a characteristic phenolic type of the Australian genera *Anigozanthos* (Hölscher and Schneider, 1997) and *Conostylis* (Cooke and Edwards, 1981).

The South African species *W. thyrsiflora* produces isochromenones and allophanylglucosides which indicate a close relationship to *L. tinctoria* and *X. caeruleum*, respectively. By contrast, both anigorufone and hydroxyanigorufone (9-phenylphenalenones with a completely deoxygenated B-ring) are known as major compounds of *Anigozanthos* species and were isolated from *W. thyrsiflora* (chapter 3). This in turn indicates a close relationship to some Western Australian Haemodoraceae. Probably Haemodoraceae species of South Africa represent a connecting link between the species of America and Australia. At least *Wachendorfia thyrsiflora* occupies an intermediate position in regard to the occurring phenolic types.

The present level of knowledge about the occurrence of phenylphenalenone-related compounds supports the hypothesis of a Gondwanan origin of the Haemodoraceae based on biogeographic distribution pattern and morphometric studies (Simpson, 1990). Thus, American species would be closer related to South African than to Australian species because of the mutual drift of recent South America and Africa apart from Antarctica and Australia. However molecular phylogenetic analysis revealed a possibly much older origin with a largely relictual distribution (Hopper *et al.*, 1999).

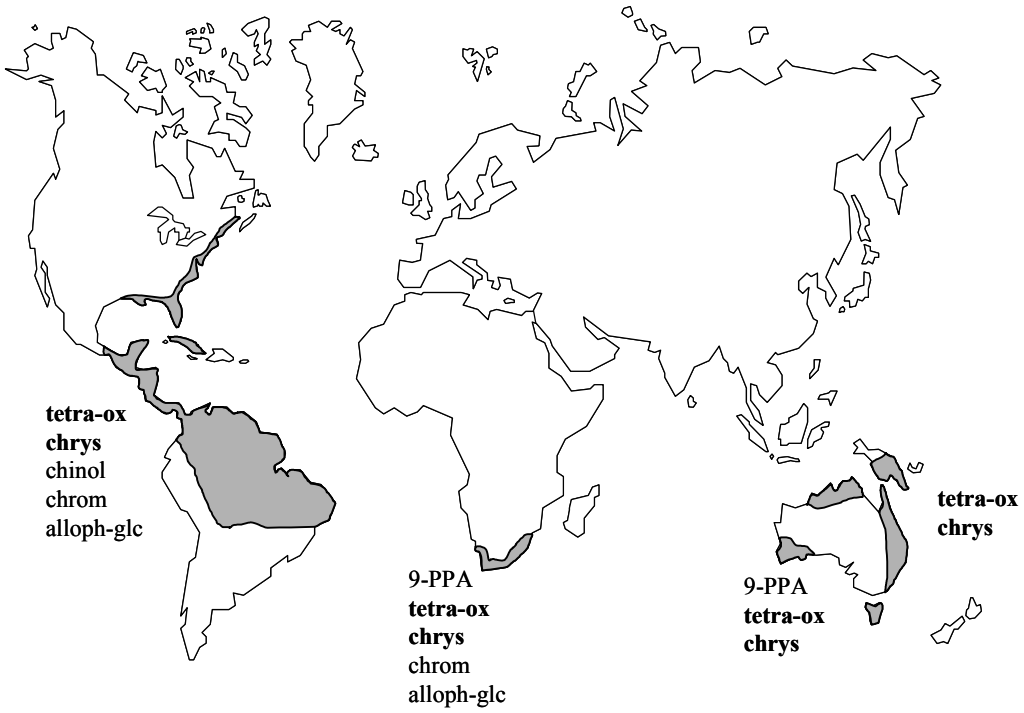


Fig. 2.8: Distribution pattern of phenylphenalenone-related subgroups of the Haemodoraceae worldwide based on current knowledge; grey areas indicate the occurrence of Haemodoraceae species; 9-PPA = 9-phenylphenalenones with a completely deoxygenated B-ring, tetra-ox = tetraoxygenated phenylphenalenones, chrys = chrysenone-type, chinol = isoquinolinedione-type, chrom = isochromenone-type, alloph-glc = allophanyl-glucosides; universally distributed types are indicated in bold.

2.5 Appendix

5-Hydroxy-7-phenyl-benzo[*de*]isochromene-1,6-dione (lachnanthopyrone, **2**). HPLC gradient (a) R_t 23.0 min, isolated amount 1.5 mg; UV (MeCN-H_2O) λ_{max} 243, 395 nm; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* 289 $[M-1]^+$ (100); HREIMS *m/z* 290.0571 (calcd for $C_{18}H_{10}O_4$, 290.0579).

5-Methoxy-7-phenyl-benzo[*de*]isochromene-1,6-dione (**3**). HPLC gradient (a) R_t 22.6 min, isolated amount 1.6 mg; UV (MeCN-H_2O) λ_{max} 243, 390 nm; ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* 303 $[M-1]^+$ (100); HREIMS *m/z* 303.9850 (calcd for $C_{19}H_{12}O_4$, 303.9855).

6-Hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4**). HPLC gradient (a) R_t 24.8 min, isolated amount 1.5 mg; UV (MeCN-H_2O) λ_{max} 214, 268, 388 nm; ^{1}H NMR data, see Table 1; EIMS *m/z* 306 $[M]^+$ (100); HREIMS *m/z* 306.0883 (calcd for $C_{19}H_{14}O_4$, 306.0892).

6-Acetoxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4a**). UV (MeCN-H_2O) λ_{max} 216, 255, 328, 360 nm; ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.21 (1H, d, *J*=7.3, H-9), 7.67 (1H, s, H-4), 7.47 (3H, m, H-3‘,5‘ and H-4‘), 7.44 (1H, d, *J*=7.3, H-8), 7.35 (2H, m, H-2‘,6‘), 5.90 (2H, s, H-3), 3.94 (3H, s, 5-OCH_3), 1.39 (3H, s, 6-OCOCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 168.7 (6-O*C*OCH$_3$), 163.9 (C-1), 150.6 (C-5), 143.3, 143.2 (C-7 and C-1‘), 134.0 (C-6), 131.5 (C-8), 129.6 (C-3a), 128.6 (br, C-2‘,6‘), 128.3 (C-4‘), 128.0 (C-3‘,5‘), 126.7 (C-9), 125.9 (C-6a), 125.4 (C-9b), 121.1 (C-9a), 111.9 (C-4), 70.1 (C-3), 57.2 (5-O*C*H$_3$), 19.4 (6-OCO*C*H$_3$); EIMS *m/z* 348 $[M]^+$ (37), 306 (100), 289 (17); HREIMS *m/z* 348.0993 (calcd for $C_{21}H_{16}O_5$, 348.0998).

3,5,6-Trihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**5**). HPLC gradient (a) R_t 13.3 min, isolated amount 2.0 mg; UV (MeCN-H_2O) λ_{max} 269, 341, 387 nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; LCEIMS *m/z* 308 $[M]^+$; HRESIMS *m/z* 309.0771 (calcd for $[M+H]^+ = C_{18}H_{13}O_5$, 309.0763).

3,5,6-Triacetoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**5a**). UV (MeCN-H_2O) λ_{max} 209, 144, 326 nm; ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.49 (1H, d, *J*=7.7, H-9), 7.87 (1H, s, H-4), 7.80 (1H, s, H-3), 7.64 (1H, d, *J*=7.7, H-8), 7.52 (3H, m, H-3‘,5‘ and H-4‘), 7.39 (2H, m, H-2‘,6‘), 2.29 (3H, s, 5-OCOCH_3), 2.11 (3H, s, 3-OCOCH_3), 1.45 (3H, s, 6-OCOCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 169.3 (3-O*C*OCH$_3$), 168.7 (5-O*C*OCH$_3$), 168.4 (6-O*C*OCH$_3$), 162.0 (C-1), 145.4 (C-7), 142.3 (C-5), 142.3 (C-1‘), 140.2 (C-6), 132.1 (C-8), 130.4 (C-9), 129.7 (br, C-2‘,6‘), 128.8 (C-3a), 128.8 (C-3‘,5‘), 128.6 (C-4‘), 128.0 (C-9b), 125.7 (C-9a), 124.1 (C-4), 119.8 (C-6a), 92.3 (C-3), 20.8 (3-OCO*C*H$_3$), 20.5 (5-OCO*C*H$_3$), 19.3 (6-OCO*C*H$_3$); EIMS *m/z* 434 $[M]^+$ (20), 392 (35), 350 (82), 291 (100); HREIMS *m/z* 434.1008 (calcd for $C_{24}H_{18}O_8$, 434.1002).

3,6-Dihydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromene-1-one (**6**). HPLC gradient (a) R_t 18.0 min, isolated amount 2.5 mg; UV (MeCN-H_2O) λ_{max} 266, 340, 386 nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; LCEIMS *m/z* 322 $[M]^+$ (60), 303 (100), 273 (37), 176 (61); EIMS *m/z* 320 $[M^+]$ (26), 303 (100); HREIMS *m/z* 320.0695 (calcd for $C_{19}H_{12}O_5$, 320.0685).

8-Formyl-5,6-dimethoxy-4-phenyl-naphthalene-1-carboxylic acid methyl ester (**6a**) UV (MeCN-H_2O) λ_{max} 208, 244, 364 nm; ^{1}H NMR (acetone-d_6, 500 MHz) δ 10.12 (1H, s, C*H*O), 8.13 (1H, s, H-7), 7.89 (1H, d, *J*=7.3, H-2), 7.35 (1H, d, *J*=7.3, H-3), 7.32-7.41 (6H, m, H-3, H-2‘–H-6‘), 4.06 (3H, s, 6-OCH_3), 3.86 (3H, s, COOCH_3), 3.29 (3H, s, 5-OCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 190.7 (*C*HO), 169.7 (C-1), 150.1 (C-6), 149.9 (C-5), 145.2 (C-1'), 143.2 (C-4), 131.2 (C-8), 130.3 (C-3), 129.0 (C-2‘,6‘), 129.1 (C-4a), 128.8 (C-2), 127.9 (C-3‘,5‘), 127.1 (C-4‘), 124.9 (C-8a), 123.1 (C-7), 60.7 (5-O*C*H_3), 57.2 (6-O*C*H_3), 52.4 (COO*C*H_3); EIMS *m/z* 350 $[M]^+$ (100), 334 (35), 322 (47), 319 (23) 291 (20), 275 (25), 248 (22); HREIMS *m/z* 350.1139 (calcd for $C_{21}H_{18}O_5$, 350.1154).

3a-Hydroxy-5-methoxy-7-phenyl-3,3a-dihydro-benzo[*de*]isochromene-1,6-dione (**7**). HPLC gradient (a) R_t 13.0 min, isolated amount 1.0 mg; UV (MeCN-H_2O) λ_{max} 200, 232, 296 nm; ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* 322 $[M]^+$ (18), 306 (60), 303 (100), 292 (37), 289 (30), 263 (29); HREIMS *m/z* 322.0885 (calcd for $C_{19}H_{14}O_5$, 322.0841).

3,5-Dihydroxy-3*H*-2.6-dioxa-benzo[*def*]chrysen-1-one (**8**). HPLC gradient (a) R_t 13.9 min, isolated amount 0.8 mg; UV (MeCN-H_2O) λ_{max} 230, 269, 435 nm; ^{1}H NMR data, see Table 3; ^{13}C NMR data, see Table 4; EIMS *m/z* 306 $[M]^+$ (100); HREIMS *m/z* 306.0516 (calcd for $C_{18}H_{10}O_5$, 306.0528).

5-Hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one (**9**). HPLC gradient (a) R_t 20.4 min, isolated amount 0.8 mg; UV (MeCN-H_2O) λ_{max} 237, 320, 386, 547 nm; ^{1}H NMR data, see Table 3; ^{13}C NMR data, see Table 4; EIMS *m/z* 316 $[M]^+$ (100); HREIMS *m/z* 316.0734 (calcd for $C_{20}H_{12}O_4$, 316.0736).

2,5-Dihydroxy-6-oxa-benzo[*def*]chrysen-1-one (**10**). HPLC gradient (a) R_t 24.0 min, isolated amount 0.1 mg; UV (MeCN-H_2O) λ_{max} 238, 320, 396, 553 nm; ^{1}H NMR data, see Table 3; ^{13}C NMR data, see Table 4; EIMS *m/z* 302 $[M]^+$ (100); HREIMS *m/z* 302.0581 (calcd for $C_{19}H_{10}O_4$, 302.0579).

3-Carboxy-5-hydroxy-6-*O*-β-D-glucopyranosyl-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**11**). HPLC gradient (b) R_t 24.9 min, isolated amount 4 mg; UV (MeCN-H_2O) λ_{max} 221, 260, 334, 367 nm; ^{1}H NMR data, see Table 5; ^{13}C NMR data, see Table 6; ESIMS *m/z* 499 $[M+H]^+$ (5), 337 (100); HRESIMS *m/z* 499.1263 (calcd for $[M+H]^+ = C_{25}H_{23}O_{11}$, 499.1240).

6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**12**). HPLC gradient (b) R_t 26.3 min, isolated amount 1.5 mg; UV (MeCN-H_2O) λ_{max} 214, 260, 334, 366 nm; ^{1}H NMR data, see Table 5; ^{13}C NMR data, see

Table 6; ESIMS *m/z* 541 $[M+H]^+$ (18), 293 (100), 231 (9); HRESIMS *m/z* 541.1378 (calcd for $[M+H]^+ = C_{26}H_{25}N_2O_{11}$, 541.1458).

6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**13**). HPLC gradient (b) R_t 26.6 min, isolated amount 2.5 mg; UV (MeCN-H_2O) λ_{max} 199, 260, 336, 370 nm; ^{1}H NMR data, see Table 5; ^{13}C NMR data, see Table 6; ESIMS *m/z* 555 $[M+H]^+$ (4), 307 (100), 231 (5); HRESIMS *m/z* 555.1659 (calcd for $[M+H]^+ = C_{27}H_{27}N_2O_{11}$, 555.1615).

6-*O*-[(2'',3'',4''-Tri-*O*-acetyl-6''-*O*-allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**13a**). UV (MeCN-H_2O) λ_{max} 214, 258, 334, 364 nm; ^{1}H NMR (acetone-d_6, 500 MHz) 8.21 (1H, d, *J*=7.4, H-9), 7.63 (1H, s, H-4), 7.47 (1H, d, *J*=7.4, H-8), 7.30-7.55 (5H, brm, H-2'-H-6'), 5.86 (2H, s, H-3), 5.17 (1H, d, *J*=7.8, H-1''), 5.11 (1H, dd, *J*=9.6, 9.6 H-3''), 4.66 (1H, dd, *J*=9.6, 9.6, H-4''), 4.20 (1H, dd, *J*=12.1, 5.9, H-6''a), 4.03 (3H, s, 5-OCH_3), 3.96 (1H, dd, *J*=7.8, 9.6, H-2''), 3.75 (1H, dd, *J*=12.1, 2.6, H-6''b), 3.59 (1H, ddd, *J*=9.6, 5.9, 2.6, H-5''), 1.97 (3H, COOCH_3), 1.95 (3H, COOCH_3), 1.93 (3H, COOCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 127.0 (C-9), 131.3 (C-8), 112.3 (C-4), 100.5 (C-1''), 72.9 (C-3''), 72.3 (C-2''), 71.5 (C-5''), 69.8 (C-3), 68.9 (C-4''), 63.4 (C-6''), 57.3 (5-O*C*H_3), 20.6 (COO*C*H_3), 20.5 (COO*C*H_3), 20.5 (COO*C*H_3).

6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenyl-phenalen-1-one (**14**). HPLC gradient (b) R_t 26.2 min, isolated amount 0.9 mg; UV (MeCN-H_2O) λ_{max} 211, 279, 378, 477 nm; ^{1}H NMR data, see Table 5; ^{13}C NMR data, see Table 6; ESIMS *m/z* 553 $[M+H]^+$ (4), 305 (100); HRESIMS *m/z* 575.1286 (calcd for $[M+Na]^+ = C_{27}H_{24}N_2O_{11}Na$, 575.1278).

6-*O*-[(6''-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenyl-phenalen-1-one (**15**). HPLC gradient (b) R_t 25.9 min, isolated amount 1.0 mg; UV (MeCN-H_2O) λ_{max} 209, 279, 375, 470 nm; ^{1}H NMR data, see Table 5; ^{13}C NMR data, see Table 6; LCEIMS *m/z* 566 $[M]^+$; HRESIMS *m/z* 567.1607 (calcd for $[M+H]^+ = C_{28}H_{27}N_2O_{11}$, 567.1615).

5,6-Diacetoxy-3-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**16a**). UV (MeCN-H_2O) λ_{max} 209, 243, 324 nm; ^{1}H NMR (acetone-d_6, 500 MHz) δ 8.44 (1H, d, *J*=7.4, H-9), 7.85 (1H, s, H-4), 7.58 (1H, d, *J*=7.4, H-8), 7.50 (3H, m, H-3',5' and H-4'), 7.37 (2H, m, H-2',6'), 6.62 (1H, s, H-3), 3.72 (3H, s, 3-OCH_3), 2.29 (3H, s, 5-OCOCH_3), 1.43 (3H, s, 6-OCOCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 168.7 (5-O*C*OCH$_3$), 168.4 (6-O*C*OCH$_3$),

163.0 (C-1), 144.9 (C-7), 142.5 (C-1‘), 142.2 (C-5), 139.7 (C-6), 131.8 (C-8), 129.8 (C-9), 129.7 (br, C-2‘,6‘), 128.8 (C-3‘,5‘), 128.4 (C-4‘), 127.8 (C-3a), 127.8 (C-9b), 125.6 (C-9a), 123.7 (C-4), 120.6 (C-6a), 102.2 (C-3), 56.7 (3-O*C*H_3), 20.5 (5-OCO*C*H_3), 19.3 (6-OCO*C*H_3); EIMS *m/z* 406 $[M]^+$ (15), 364 (33), 322 (86), 291 (100); HREIMS *m/z* 406.1057 (calcd for $C_{23}H_{18}O_7$, 406.1052).

5-Hydroxy-3,6-dimethoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**17**). UV (MeCN-H_2O) λ_{max} 266, 340, 386 nm; ^{1}H NMR (methanol-d_4, 500 MHz) δ 8.22 (1H, d, *J*=7.1, H-9), 7.58 (1H, s, H-4), 7.32 (1H, d, *J*=7.1, H-8), 7.34 (5H, m, H-1’,2’,3‘,4’ and H-5‘), 6.47 (1H, s, H-3), 3.67 (3H, s, 3-OCH_3), 3.96 (3H, s, 6-OCH_3); ^{13}C NMR (acetone-d_6, 125 MHz) δ 166.3 (C-1), 147.2 (C-7), 145.2 (5-OH), 145.0 (6-OCH_3), 144.7 (C-1’), 129.8 (C-8), 128.7 (C-9), 128.0 (C-2’ and C-6’), 127.8 (C-3’,4’ and C-5’), 125.6 (C-9b), 122.0 (C-6a), 119.3 (C-9a). 119.0 (C-3a), 115.4 (C-4), 103.9 (C-3), 57.8 (6-O*C*H_3), 56.4 (3-O*C*H_3); EIMS *m/z* 336 $[M]^+$ (50), 305 (100), 289 (30); HREIMS *m/z* 336.0998 (calcd for $C_{20}H_{16}O_5$, 336.0998).

3. Studies on the biosynthesis of phenylphenalenone-related compounds in root cultures of *Wachendorfia thyrsiflora* (Haemodoraceae)

3.1 Introduction

Previously, a variety of feeding experiments were performed using ^{13}C- and ^{14}C-labelled precursors to study the biosynthesis of phenylphenalenones. It has been proved that these phenolics are derived from two C_6-C_3-units and that the central C atom is derived from C-2 of acetate (Edwards *et al.*, 1972; Hölscher and Schneider, 1995a). Additionally, incorporation of stable isotopes from phenylalanine, tyrosine (Edwards *et al.*, 1972) and several phenylpropanoic acids such as cinnamic acid (Hölscher and Schneider, 1995b), coumaric acid (Hölscher and Schneider, 1995a) and caffeic acid (Schmitt *et al.*, 2000) has been observed. After the formation of a diarylheptanoid intermediate, a hypothetical Diels-Alder cycloaddition results in the phenylphenalenone ring system (Fig. 3.1a) (Hölscher and Schneider, 1995b; Schmitt and Schneider, 1999). Although no detailed study has been undertaken, oxygen functions in positions 1 and 6 of the phenylphenalenone type structure shown in Figure 2.1 are supposed to originate from the carboxyl and *p*-hydroxyl groups of the phenylpropanoid precursor, *p*-coumaric acid. In contrast, further oxygenations at the ring

system are considered to occur later in the biosynthesis. The biosynthetic origin of the oxalactone structure in ring B, which is a structural feature of the majority of natural products isolated so far from *Xiphidium caeruleum*, is questioned.

So far, oxa analogues of phenylphenalenones (isochromenone-subtype, phenylnaphthalic anhydrides) have been considered to be oxidation products. The observation of incorporation of ^{13}C from [^{13}C]cinnamic acid into a phenylphenalenone-type anhydride in *Musa acuminata* supports this hypothesis (Kamo *et al.*, 2000). Alternatively, the biosynthesis of oxa analogues may proceed through a Diels-Alder cycloaddition of an ester intermediate (Fig. 3.1b).

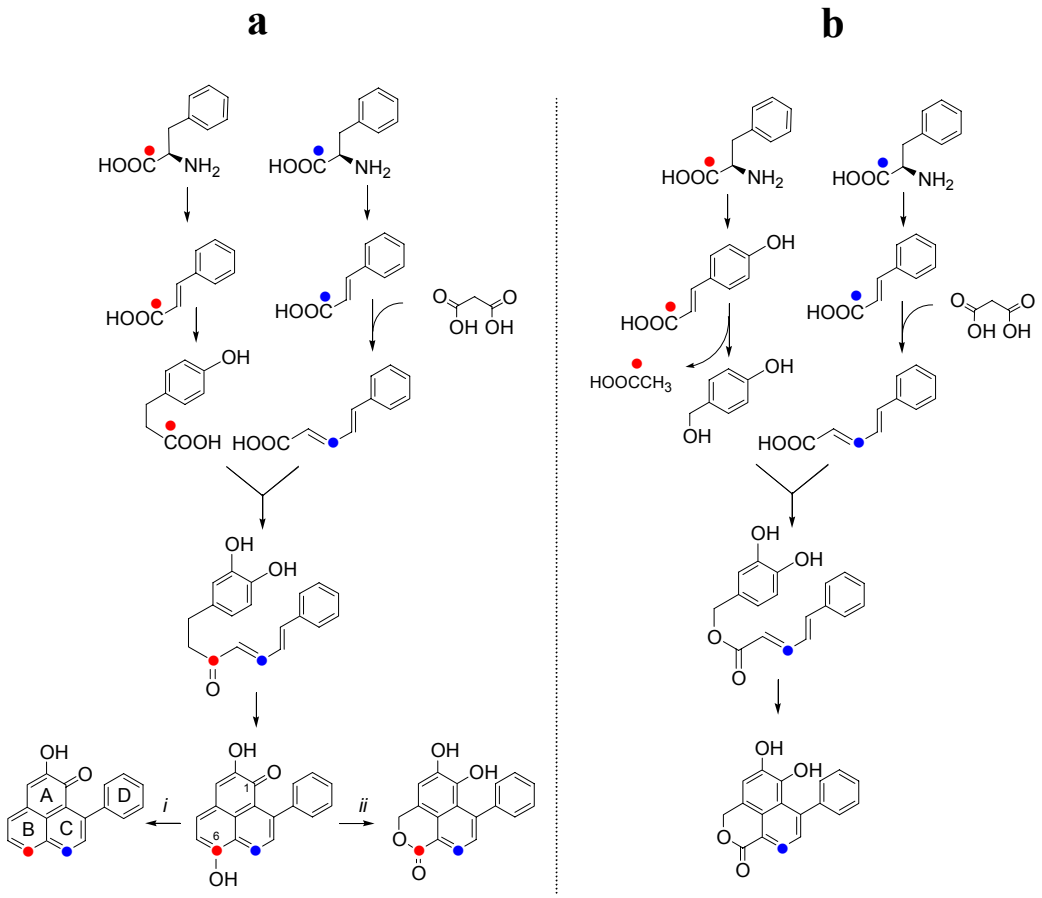


Fig. 3.1: Hypothetical biosynthesis of phenylisochromenones from phenylalanine a) through the phenylphenalenone pathway and subsequent oxidation or b) via a Diels-Alder reaction of an ester intermediate. Coloured dots indicate the position of C-1 of phenylalanine precursors through the pathways. *i* = formation of compounds with deoxygenated B-ring, *ii* = formation of compounds with further oxidation of the B-ring.

Sterile root cultures are a suitable tool for biosynthetic studies. As there was no success with feeding experiments with whole plant material, or to establish sterile root cultures of *X. caeruleum*, biosynthetic studies were performed on available root cultures of *Wachendorfia thyrsiflora*, from which both phenylphenalenones and phenylbenzoisochromenones had been identified (chapter 2). Hence, feeding experiments with sterile root cultures of this species were performed in order to examine incorporation of ^{13}C from [1-^{13}C]phenylalanine into different subtypes of phenylphenalenone-related compounds.

Methionine is a common source for the majority of enzymatic methylations in plants (Ibrahim and Muzac, 2000). To examine the origin of methoxyl substituents of different phenylphenalenones, [*methyl*-^{13}C]methionine was fed to root cultures of *W. thyrsiflora*.

Jasmonic acid (JA) and its methyl ester act as elicitors for plant-defence mechanisms by stimulating the biosynthesis of defending compounds (Farmer and Ryan, 1990) (Gundlach *et al.*, 1992). *W. thyrsiflora* root cultures showed an increase in the amount of several phenylphenalenones after treatment with JA, and hence feeding experiments were performed with the addition of JA to stimulate the biosynthesis of phenylphenalenones and thereby to increase the incorporation level.

3.2 Material and methods

3.2.1 Plant material

Sterile root cultures of *Wachendorfia thyrsiflora* were maintained in M3 (Murashige and Skoog, 1962) liquid medium (100 ml in 300-ml Erlenmeyer flasks) at 22°C on a gyratory shaker (100 rpm) under permanent light (600 lux). Every four weeks, root cultures were divided and transferred into fresh medium. Initiation procedure of the root cultures were the same as for *Anigozanthos preissii* (Hölscher and Schneider, 1997).

3.2.2 Feeding of labelled precursors

Phenylalanine

Before the administration of the labelled compound, the roots were transferred to fresh medium. After two days, [1-^{13}C]phenylalanine (5 mg, Cambridge Isotope Laboratories) was

dissolved in H_2O (1 ml) and fed to a root culture through a membrane filter to a final concentration of 290 µM and incubated for 24 hours (Table 3.1). In three further experiments, in addition to [1-^{13}C]phenylalanine, jasmonic acid (JA) was added simultaneously to a concentration of 50 µM in the medium. The incubation times were 1, 2 and 5 days. Additionally, two root cultures were used as controls without any application of the labelled compound but in one case for an incubation time of 5 days with 50 µM JA.

Methionine

Root cultures were transferred to fresh medium 14 days before the administration of the labelled compound. [*methyl*-^{13}C]Methionine (4 mg, Cambridge Isotope Laboratories) was dissolved in H_2O and fed to a root culture through a membrane filter to a final concentration of 270 µM. Thereafter, jasmonic acid was added in the same manner to obtain a concentration of 80 µM in the medium. Furthermore, two root cultures were used as controls, one with and one without JA treatment (Tab. 3.1).

Table 3.1: Feeding experiments with ^{13}C-labelled precursors and application of jasmonic acid (JA) to root cultures of *Wachendorfia thyrsiflora*.

Expt.	Fresh weight of root cultures [g]	Labelled compound	Days of incubation	Application of JA	Isolated compounds
1	9.1	[1-^{13}C]phenylalanine	1	none	**14**, **18**
2	7.1	[1-^{13}C]phenylalanine	1	50 µM	**14**, **18**
3	11.0	[1-^{13}C]phenylalanine	2	50 µM	**14**, **18**, **19**
4	7.5	[1-^{13}C]phenylalanine	5	50 µM	**13**, **14**, **17**, **18**, **19**
5	9.0	none (control)	5	50 µM	
6	9.5	none (control)	-	none	
7	18.6	[*methyl*-^{13}C]methionine	2	80 µM	**9, 17**, **19**
8	17.5	none (control)	2	80 µM	
9	14.0	none (control)	-	none	

3.2.3 Extraction and isolation of phenylphenalenone-related compounds

After the incubation, roots were frozen in liquid N_2 and extracted with MeOH at room temperature. Samples of each root extract were analysed by analytical HPLC. This was performed by reverse-phase chromatography on a LiChrospher 100 RP-18 column (5 µm; 250 x 5 mm) using a MeCN-H_20 gradient (0.1% TFA) 5:95% → 65:35% in 50 min → 90:10% in 5 min and 90:10% for 5 min (total time 60 min) with a flow rate of 0.8 ml min^{-1} and UV-detection at 254 nm.

After evaporation (< 40°C) the extracts were partitioned between *n*-hexane-H_2O, CH_2Cl_2-H_2O and EtOAc-H_2O. The phenylphenalenone-related compounds were isolated from the CH_2Cl_2- and EtOAc-fractions. The isolation was performed by preparative reversed-phase HPLC on LiChrospher 100 RP18 column (10 µm; 250 x 10 mm; flow rate 3.5 ml min^{-1}; UV 254 nm). A MeCN-H_2O gradient (a) of 30:70% → 65:35% in 30 min → 90:10% in 5 min (total time 35 min) was used for the CH_2Cl_2 fraction. For the EtOAc-fraction, a MeCN-H_2O (0.1% TFA) gradient (b) of 5:95% → 50:50% in 40 min was applied. From root cultures fed with [1-^{13}C]phenylalanine, five compounds, two phenylisochromenones and three phenylphenalenones, were obtained from both the CH_2Cl_2- (gradient a; **17**: R_t 26.0 min, **18**: R_t 31.8 min, **19**: R_t 30.6 min) and EtOAc-fractions (gradient b; **13**: R_t 26.6 min, **14**: R_t 25.9 min) (Fig. 3.2). From the root culture fed with [*methyl*-^{13}C]methionine, three compounds were isolated from the CH_2Cl_2-fraction (**9**: R_t 20.4 min, **17**, **19**).

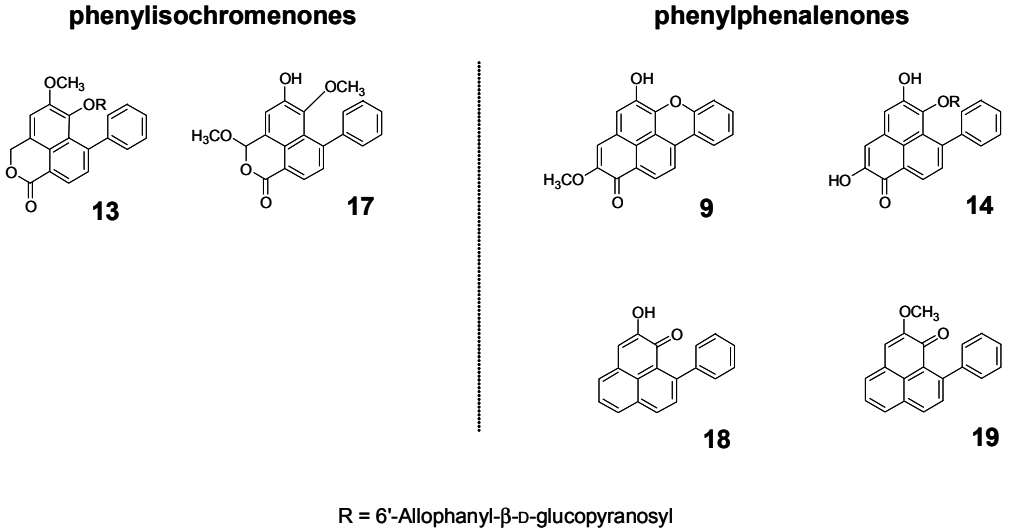


Fig. 3.2: Phenylphenalenone-related compounds from root cultures of *Wachendorfia thyrsiflora* isolated after the application of ^{13}C-labelled precursors.

3.2.4 Spectroscopic methods

NMR spectroscopic analyses were performed on a Bruker 500 NMR spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Chemical shifts are reported relative to tetramethylsilane (TMS). ^{13}C-NMR spectra of crude extracts were run using a 5-mm broadband probe. Spectra of extracts of both treated roots and controls were compared to detect incorporations before the isolation procedure. ^{1}H- and ^{13}C-NMR spectra of isolated compounds were run using a 2.5-mm broadband microprobe. The analytical data of the investigated compounds exactly matched those of authentic references ((Hölscher and Schneider, 1997), see chapter 2). Incorporation levels were estimated on the basis of integral ratios of ^{13}C signals of labelled compounds and non-labelled references. Spectra of both were recorded under identical conditions.

3.3 Results

3.3.1 Incorporation of phenylalanine

Both phenylisochromenones and phenylphenalenones, which were isolated in these experiments, showed approximately equal enhancements of two specific NMR signals (Fig. 3.3).

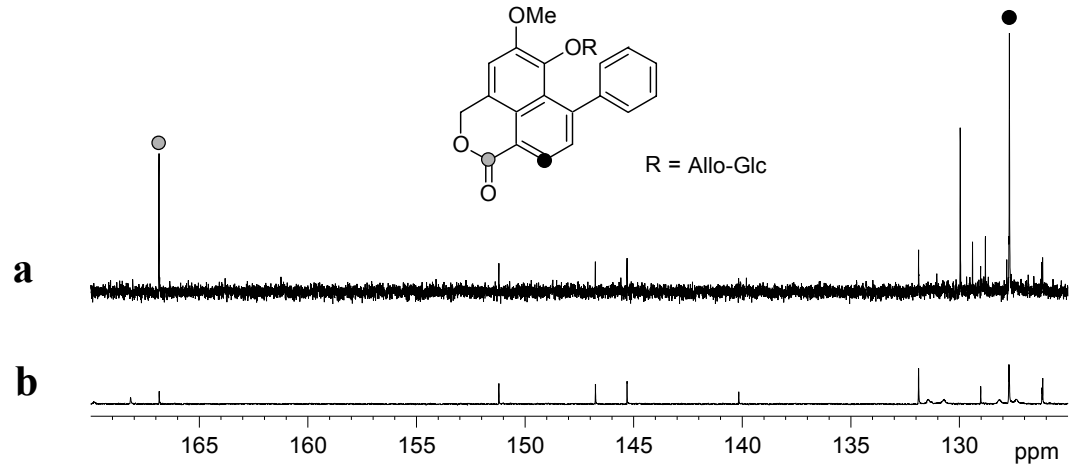


Fig. 3.3: Partial ^{13}C-NMR spectra of the phenylisochromenone **13** isolated from *W. thyrsiflora*. a) after incorporation of [1-^{13}C]phenylalanine; b) unlabelled reference.

This indicated an incorporation of two [1-^{13}C]phenylalanine units of equal proportions into compounds **13**, **14**, **17**, **18** and **19** by root cultures of *W. thyrsiflora* (Fig. 3.4). The highest levels of incorporation after 5 days of incubation were found up to 20 % in anigorufone (**18**) and methoxyanigorufone (**19**).

Compounds **14**, **18** and **19** showed different degrees of labelling after different times (Fig. 3.5). An increase in the incorporation rate into the allophanylglucoside **14** from 11 to 17 % after five days was observed. By contrast, a dramatic decrease was found in anigorufone (**18**) from 49 % (after one day) down to 17 % after five days. Methoxyanigorufone (**19**), which appeared in higher amounts after two days of incubation, showed a decrease in incorporation level from 31 % to 19 % within three days.

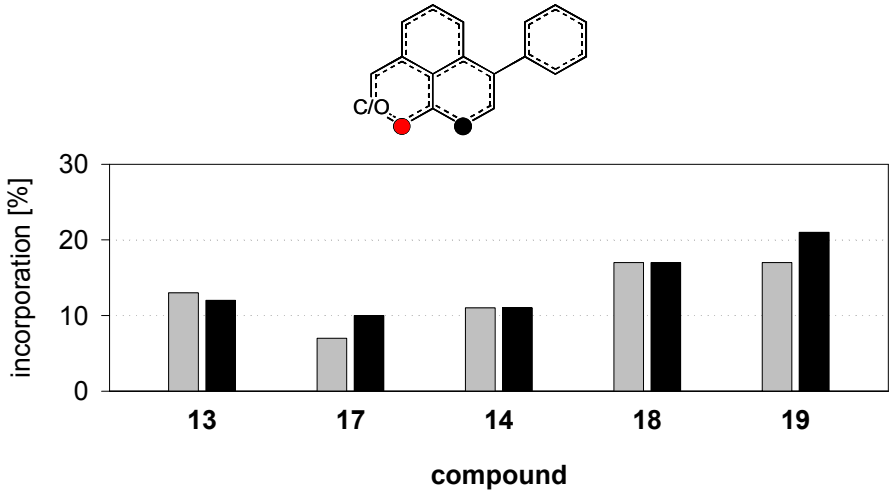


Fig. 3.4: Incorporation levels of [1-^{13}C]phenylalanine into five phenylphenalenone-related compounds after 5 days of incubation. The appropriate grey scale shows incorporation into each half of the molecule.

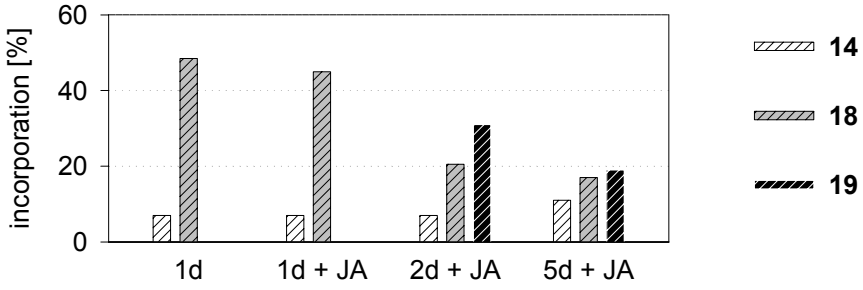


Fig. 3.5: Mean incorporation levels of [1-^{13}C]phenylalanine into the allophanylglucoside **14**, anigorufone (**18**) and methoxyanigorufone (**19**) after incubation times of 1 day (with and without addition of jasmonic acid [JA]), 2 and 5 days (both with JA).

3.3.2 Incorporation of methionine

After the application of [*methyl*-^{13}C]methionine to a root culture (experiment 7), each of the isolated methoxyl compounds **9**, **17** and **19** showed a conspicuous enhancement of one signal which was assigned to a ^{13}C-labelled methoxyl group. This indicated incorporation of the ^{13}C of methionine into methoxyl groups of phenylphenalenones. Incorporation levels varied between 9 % (compound **9**) and 18 % (compound **19**). However, although compound **17** possess two methoxyl substituents, incorporation was observed only into 6-OCH_3 (13 %) but not into 3-OCH_3 (Fig. 3.6).

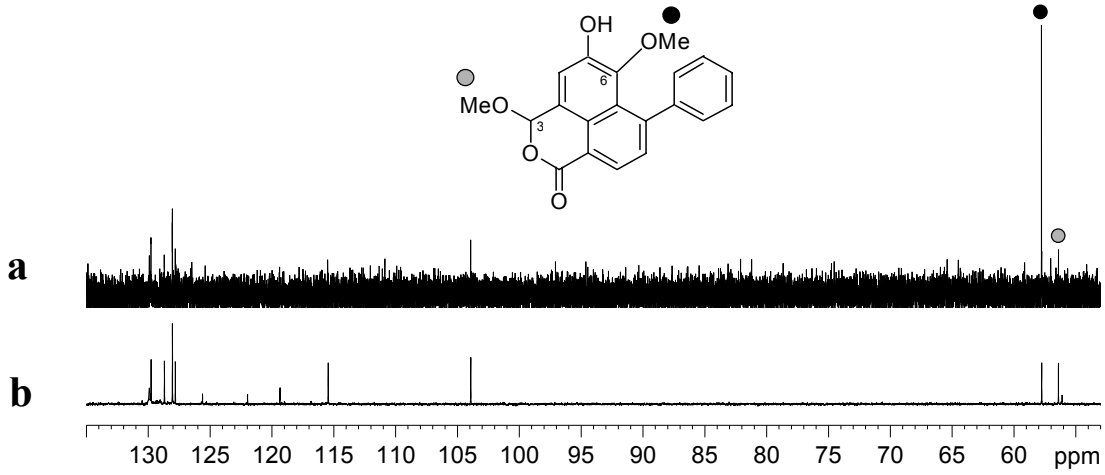


Fig. 3.6: Partial ^{13}C-NMR spectra of the phenylbenzoisochromenone **17** isolated from *W. thyrsiflora*. a) after incorporation of [*methyl*-^{13}C]methionine; b) unlabelled reference.

3.3.3 Impact of jasmonic acid (JA)

For the phenylalanine feeding experiments (Table 3.1, experiments 1-6), the HPLC analysis of the crude extracts showed an influence of JA-treatment on the accumulation of phenylphenalenones **18** and **19** (Fig. 3.7). The amount of anigorufone (**18**) within the root cultures was found to increase significantly after treatment with JA. In addition, there was a strong elicitation of the biosynthesis of methoxyanigorufone (**19**) caused by JA. This compound was not detectable in the control and appeared two days after JA treatment in

sufficient amount for isolation. However, no significant differences in the incorporation of ^{13}C into compounds **14** and **18** were observed between JA treated and untreated root cultures after an incubation period of one day (Fig. 3.5).

The methionine feeding experiments (Table 3.1: experiments 7-9) showed a strong elicitation of the biosynthesis of methoxyanigorufone (**19**) due to the treatment with JA. In contrast to this, no anigorufone (**18**) was detected in these experiments.

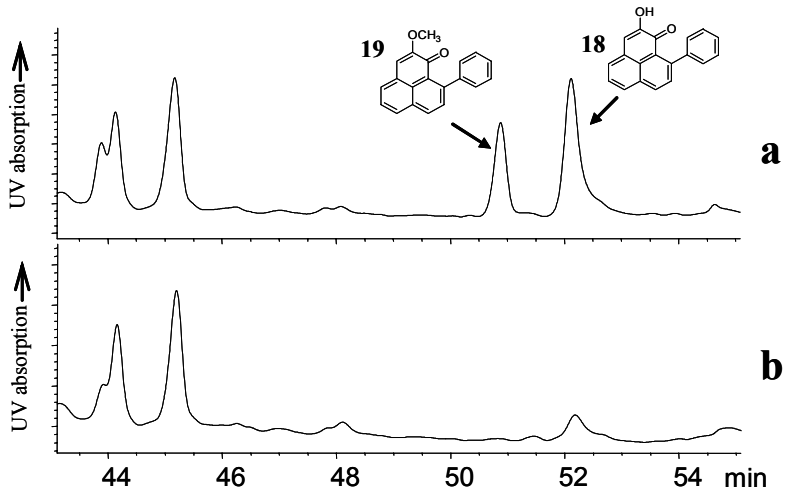


Fig. 3.7: Partial HPLC-chromatogram (UV absorption at 254 nm) of *W. thyrsiflora* root extracts a) after and b) without application of jasmonic acid.

3.4 Discussion

New insights into the biosynthesis of phenylphenalenone-related compounds have been achieved. Whereas feeding experiments with phenylalanine examined the formation of the phenylphenalenone nucleus and its oxidative metabolism, studies on the incorporation of methionine focused on the origin of methoxyl substituents.

The incorporation of ^{13}C from [*methyl*-^{13}C]methionine into these groups proved methionine to be the source of methoxyl substituents on natural phenylphenalenones. In case of compound **17,** which possesses two methoxyl groups, only C-6 was found to be derived from methionine. It has been already suggested that the methoxyl group at C-3 might be an

artefact of methanolic extraction (chapter 2). This lack of incorporation confirms that **17** is not a natural product.

Phenylalanine feeding experiments showed an incorporation of ^{13}C-labelled precursors into all studied compounds. A strong correlation between the time of incubation and the incorporation level was observed for several compounds. The decreasing degree of labelling of anigorufone (**18**) over time can be interpreted as an indication of the flux of the label into downstream metabolites. The highest incorporation level of approximately 50 % after one day confirms, that **18** was rapidly biosynthesised suggesting that anigorufone might be an early product on the phenylphenalenone pathway. By contrast, the allophanylglucoside (**14**) appears to be a later biosynthetic product with much lower incorporation levels, which constantly increased over time.

High levels of incorporation were estimated not only for anigorufone but for all the studied compounds as well. In several experiments, jasmonic acid (JA) was used to stimulate an incorporation of the applied precursors. It has been shown that JA indeed forces the formation of several compounds, but has no influence on the incorporation levels at least after a short incubation time. In the case of stimulation of the biosynthesis of anigorufone (**18**) caused by JA, intermediates such as phenylpropanoic acids, diarylheptanoids or further phenylphenalenones, which were stored in the roots, might serve as additional sources rather than *de novo* biosynthesis from phenylalanine. These depot compounds should be still unlabelled, resulting in incorporation levels similar to those found for the control (experiment 1).

JA treatment showed a strong elicitation of the biosynthesis of methoxyanigorufone (**19**) in both the methionine and phenylalanine feeding experiments. This compound might derive directly from anigorufone, which implies that a methyltransferase was activated by jasmonic acid. Owing to high incorporation levels of labelled precursors, methoxyanigorufone is a suitable substance for further comparable studies on the biosynthesis of phenylphenalenones using JA as elicitor.

The largely symmetric incorporation of two labelled phenylalanine precursors into all the investigated compounds proved an identical early biosynthetic pathway for both phenylisochromenones and phenylphenalenones, as shown in Figure 3.1a. Hence, diarylheptanoids are formed from two phenylpropanoids and C-2 of acetate resulting in the phenylphenalenone-ring system as shown for a variety of compounds of the phenylphenalenone-subtypes (Edwards *et al.*, 1972; Hölscher and Schneider, 1995a).

There are two routes by which phenylphenalenone-related compounds with a different oxidation status of the B-ring could be formed. (Fig. 3.1). The first intact phenylphenalenone could posses an oxygenated carbon (C-6) of the B-ring, which is derived from the carbonyl group of one phenylpropanoic precursor. Such a compound, lachnanthocarpone, has been isolated from *L. tinctoria* (Edwards and Weiss, 1970). The reduction of this initially formed phenylphenalenone results in the subtype of compounds with a completely deoxygenated B-ring such as in anigorufone (**18**) (Fig. 3.1 *i*). This subtype is represented by structures found in *Anigozanthos* species (Hölscher and Schneider, 1997). On the other hand, compounds belonging to the phenylisochromenone-subtype seem to be oxidation products of the initially formed phenylphenalenones (Fig. 3.1 *ii*). Hence, a carbon of the B-ring can be replaced by oxygen, which is a structural feature of the majority of compounds isolated from *X. caeruleum.* Such substitutions have been already postulated earlier for phenalenones of *L. tinctoria* (Edwards and Weiss, 1974).

This second oxidative route (Fig. 3.1 *ii*) might be universal for the synthesis of all phenylphenalenone-related compounds found in *X. caeruleum* (chapter 2). Figure 3.8 shows in a biosynthetic network how these compounds might be formed. Some compounds are shown, which are still hypothetical (**22**, **25**) or have been isolated from other species such as *L. tinctoria* (**24, 26**) (Edwards and Weiss, 1974), *Haemodorum distichophyllum* (**23**) (Bick and Blackman, 1973), *Wachendorfia thyrsiflora* (**24**) and *W. paniculata* (**24**, **26**) (Edwards, 1974). Intact phenylphenalenones possessing a C-19 skeleton (**23**-**26**) are assumed to function as precursors for the entire set of related structures described here. As phenylbenzoisochromenones (phenylnaphthalides) and other oxalactone compounds represent the most abundant products of *X. caeruleum*, oxidative metabolism of phenalenones appears not to be a spontaneous process, but that the formation of compounds is enzymatically catalysed, at least in the initial step. Oxidation of **25** or **26** adjacent to the carbonyl or hydroxyl function of ring B could be the first step in the hypothetical biosynthetic sequence (Fig. 3.8). This is supported by the occurrence of trioxygenated phenylphenalenones such as lachnanthocarpone (**26**), and tetraoxygenated species such as lachnanthoside aglycone (**24**) from *Lachnanthes tinctoria* (Edwards and Weiss, 1974) and *Wachendorfia paniculata* (Edwards, 1974). Xiphidone (**1**) (Cremona and Edwards, 1974) and the glucosides **14** and **15** might be derived from **23** or, more likely, from its 2,5-dimethoxyl analogue, respectively. Oxidative rearrangement of tautomeric tetraoxygenated phenylphenalenones **23**/ **24** would deliver 3-carboxy-oxalactone type compounds such as **22,** the first phenylisochromenone in the network. Further steps, namely decarboxylation and hydroxylation at C-3 would result in

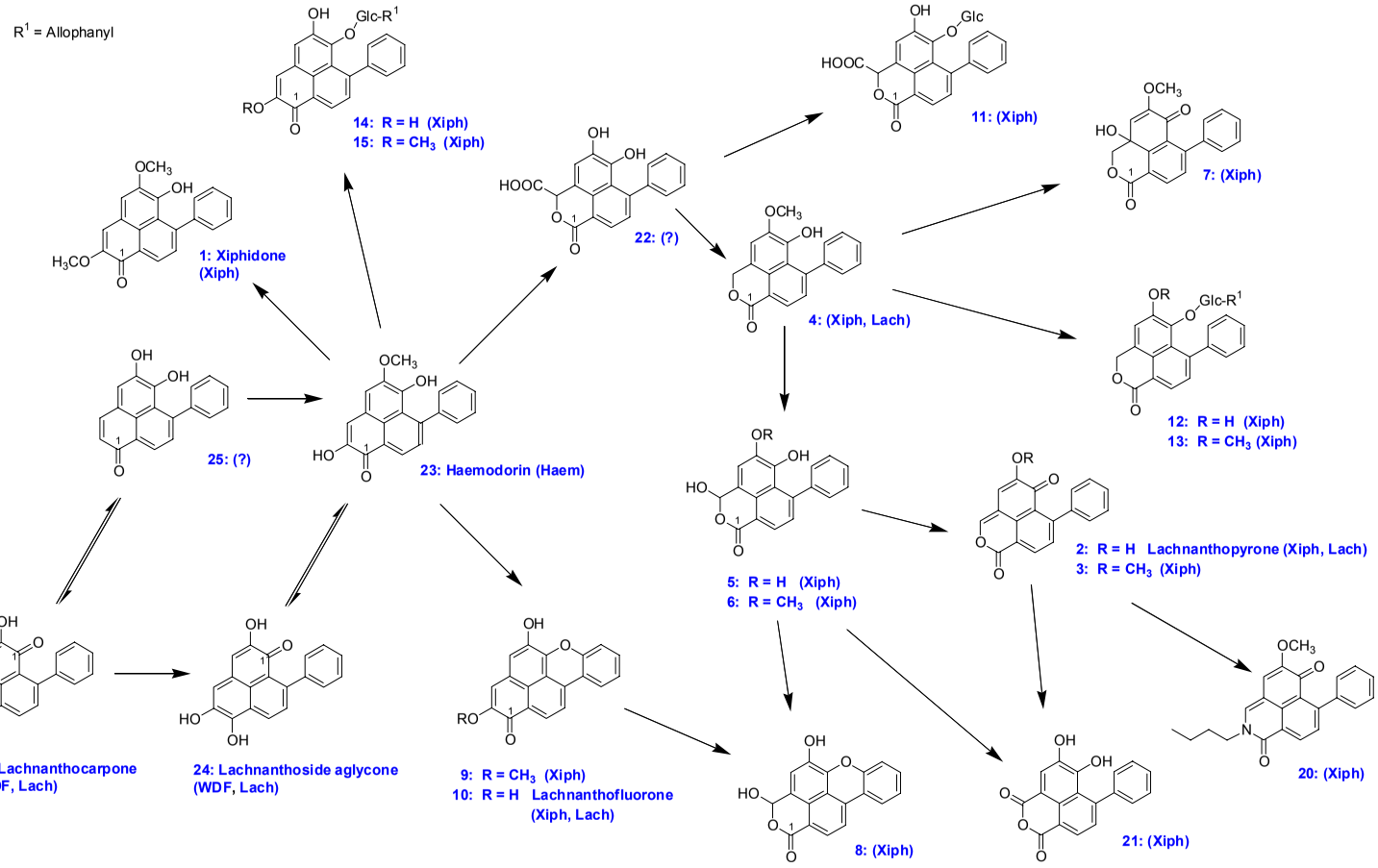


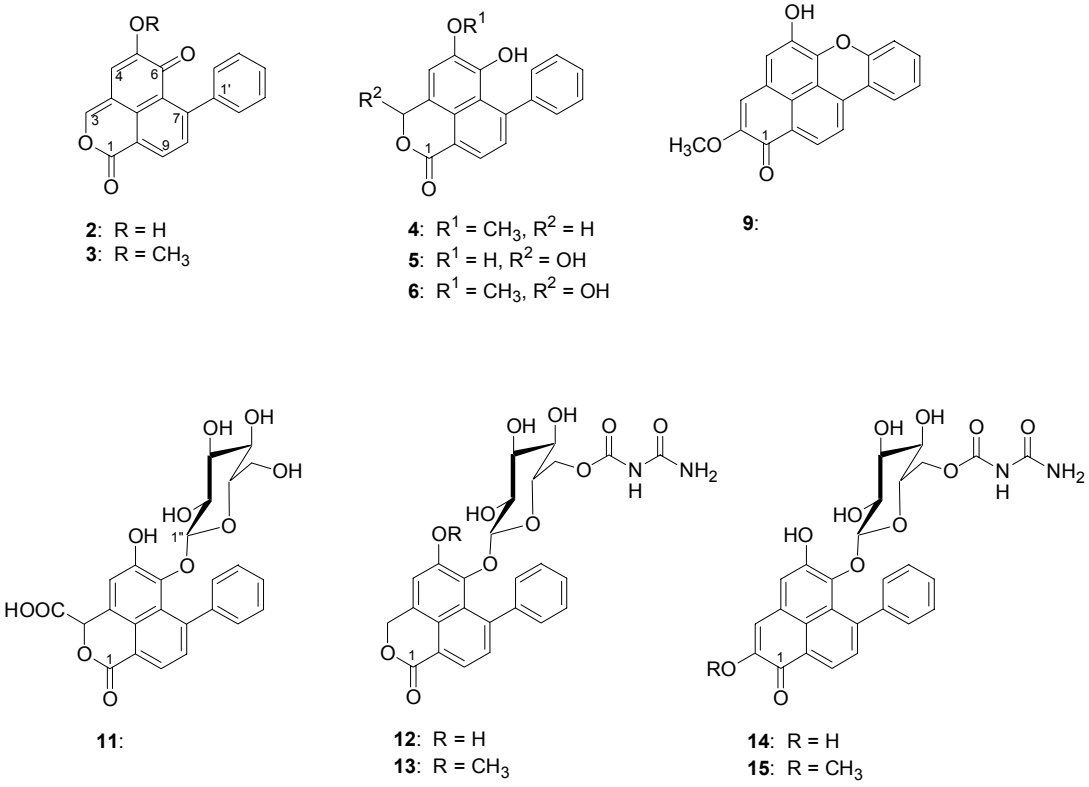
Fig. 3.8: Proposed biosynthetic relationships between phenylphenalenone-related compounds from the Haemodoroideae subfamily of the Haemodoraceae mainly based on compounds found in *Xiphidium caeruleum* (Xiph). Compounds **23**, **24** and **26** were found exclusively in *Lachnanthes tinctoria* (Lach), *Wachendorfia spec*. (WDF) or *Haemodorum distichophyllum* (Haem). Compounds **22,** representing the aglycon of glycoside **11,** and **25** are hypothetical. Parallel pathways involving either methylated or unmethylated compounds may explain the variable methylation pattern.

compound **5**, which is further converted by dehydration and dehydrogenation to lachnanthopyrone (**2**) and the anhydride **21**, respectively. A similar network in part has been previously proposed (Edwards and Weiss, 1974). The majority of further compounds isolated from *X. caeruleum* also fit well into the hypothetical biosynthetic scheme shown in Fig. 3.8. A phenylphenalenone-derived alkaloid, the phenyl-benzoisochinoline-1,6-dione (**20**) is suggested to be formed from compound **3**. Whereas compound **4** might be the precursor for **7** and both glucosides **12** and **13**, glucoside **11** is derived from its aglycone **22**. Starting from compound **23**, a parallel oxidative pathway *via* oxabenzochrysenone type compounds (*e.g.* lachnanthofluorone **10**) to the dioxabenzochrysenone **8** seems to be operative in *X. caeruleum*. Alternatively, **8** may be formed from compound **5** after oxidation of ring B additionally. Since in several cases 5-hydroxy and 5-methoxy compounds coexist, the occurrence of both complete series can be predicted. However, because of the occurrence of several compounds in more than one species, this scheme may be representative for all species belonging to the Haemodoroideae subfamily, which produce metabolites of the phenylbenzoisochromenone-subtype. In this context, further investigations on these species will help to verify and complete the presented scheme.

4. Organ-specific analysis of phenylphenalenone-related compounds in *Xiphidium caeruleum*

4.1 Introduction

A great number of phenylphenalenone-related compounds have been isolated from the monocotyledonous plant families Haemodoraceae (Cooke and Edwards, 1981), Musaceae (Luis *et al.*, 1993), Pontederiaceae (Della Greca *et al.*, 1993) and Strelitziaceae (Hölscher and Schneider, 2000). Roots (Cooke, 1970; Edwards and Weiss, 1974; Dora *et al.*, 1993), shoot material (Bick and Blackman, 1973; Luis *et al.*, 1993), flowers (Bazan and Edwards, 1976), fruits (Edwards and Weiss, 1970; Luis *et al.*, 1993) and sterile root cultures (Hölscher and Schneider, 1997) have been found to be sources of such phenolic compounds. Roots and rhizomes seem to be the richest source, as shown by an intense red pigmentation of these organs in several species due to phenylphenalenones. However, there is little information on how phenylphenalenone-related compounds are distributed within a plant nor there is any quantitative indication of differences between organs. In this work the distribution pattern of up to eleven phenylphenalenone-related compounds (Fig. 4.1) were studied in intact plants of *Xiphidium caeruleum* (Aubl.), a species of the Haemodoraceae. In addition to the comparison



2: R = H
3: R = CH_3

4: R^1 = CH_3, R^2 = H
5: R^1 = H, R^2 = OH
6: R^1 = CH_3, R^2 = OH

9:

11:

12: R = H
13: R = CH_3

14: R = H
15: R = CH_3

2 5-Hydroxy-7-phenyl-benzo[*de*]-isochromene-1,6-dione (lachnanthopyrone)
3 5-Methoxy-7-phenyl-benzo[*de*]-isochromene-1,6-dione
4 6-Hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one
5 3,5,6-Trihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one
6 3,6-Dihydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromene-1-one
9 5-Hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one
11 3-Carboxy-5-hydroxy-6-*O*-β-D-glucopyranosyl-7-phenyl-3*H*-benzo[*de*]isochromen-1-one
12 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one
13 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one
14 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-2,5-dihydroxy-7-phenyl-phenalen-1-one
15 6-*O*-[(6"-*O*-Allophanyl)-β-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenyl-phenalen-1-one

Fig. 4.1: Phenylphenalenone-related compounds from *X. caeruleum*.

of the distribution of compounds among plant organs, particular attention was paid to differences in accumulation among various parts of the leaves.

Secondary metabolites of plants can play a key role in chemical defence against biotic stress factors. Whereas there are compounds, so called phytoanticipines, which effect a continuous protection (constitutive defence), the biosynthesis of a variety of metabolites to act as defending compounds is induced after infection by microorganisms or after physical

damage by herbivores (Gullan, 1994). Phenylphenalenone-related compounds also appear to play a defensive role in plants. As such compounds are induced in *Musa accuminata* after treatment with fungi, they have considered to be phytoalexins (Luis *et al.*, 1993).

Damage to leaves, such as that caused by herbivores, can increase the biosynthesis of defensive metabolites, including terpenoids (Harborne, 1997), alkaloids (Baldwin, 1999) or furanocoumarins (Berenbaum and Zangerl, 1999). In case that phenylphenalenone-related compounds possess defensive properties, one might also expect an increase of accumulation of such compounds after physical damage. Therefore, leaves of *X. caeruleum* plants were injured to study a possible reaction of the plant in regard to the amount of selected phenylphenalenones and related compounds both at the site of damage and distant to it.

4.2 Material and methods

4.2.1 Plant material

Plants of *Xiphidium caeruleum* were obtained from the Botanical Garden (University of Jena). At the Max Planck Institute for Chemical Ecology Jena they were maintained in growth chambers at 28/ 15°C-day/night temperatures, 50/ 85 % relative humidity and a 14-hour light period. For organ-specific distribution studies, plants of ca. 60 cm height were used. Fresh *X. caeruleum* flowers were obtained from the greenhouse of the Botanical Garden Jena. Injury experiments were performed on plants growing in the Palm House of the Botanical Garden Jena.

4.2.2 Sampling

Distribution studies on vegetative organs

Three plants of similar size and age from the growth chamber were studied. Using a razor blade, eight representative samples were taken from each plant (Fig. 4.2). From the shoot, two samples from leaves (of the lamina of two leaves) and two from the middle part of the stem (in distance of 8 cm) were taken. In addition, a mature section of a root (approximately 5 cm from the tip) and the corresponding appropriate root tip of two roots per plant were analysed. In total, six samples per plant site (leaf, stem, mature root and root tip) with a fresh weight between 2 (root) and 80 mg (stem) were taken.

Distribution studies on flowers

Flowers of an inflorescence of a *X. caeruleum* plant from the greenhouse (Botanical Garden Jena) were dissected. Appropriate parts of the flowers were pooled for further analysis resulting in samples of six carpels, 20 stamen and six petals. In addition, samples were taken from the peduncle and a secondary axis of the inflorescence. Fresh sample weights varied between 9 and 42 mg.

Leaf distribution

The second, fourth, sixth and ninth youngest leaves of a plant taken from the growth chamber were analysed. Six cross section samples approximately 6 cm apart were taken from every leaf, three samples from the lamina and three from the sheath (Fig. 4.5, samples I – VI). The first sample from the lamina was taken approximately 6 cm below the leaf tip.

Cutting injury of leaves

Three plants growing in the palm house of the Botanical Garden Jena were wounded for this experiment. The second, fourth and sixth youngest leaves of each plant were injured by removing a 10 cm long part of the tip of the lamina. Immediately after the injury, cross sections were taken at the cut surfaces by using a razor blade (control A at time 0). Further samples were taken in the same manner after 8 and 24 hours accompanied with new wounding. After 48 hours, samples were taken again at the cut surface and additionally at a site near to the base of the leaf sheath. Simultaneously, control samples (control B) were taken from three unwounded plants growing at the same place and resembling the injured plants. From these, cross sections of both the sheath and the lamina were taken.

Each sample was weighed immediately (fresh weight determination) followed by freezing with liquid N_2. Samples were stored at –80 °C until extraction.

4.2.3 Extraction, HPLC analysis and quantification

Each plant sample was placed into a 2.0-ml screwtop microcentrifuge tube containing 0.9 g of Lysing Matrix D (Q-Biogene, Heidelberg, Germany). To each tube, methanol (1 ml) (distribution studies) or acetone (injury experiment) and pyrene (10 µg, Aldrich) in the appropriate solvent as an internal standard were added. The mix was homogenised for 45 s at 6.5 m s^{-1} using a FastPrep® System (FP120; Q-Biogene, Heidelberg, Germany). The homogenate was centrifuged and the supernatant was transferred into a new vial. The cell

debris was extracted once with methanol (1 ml) or acetone, respectively. Both extracts were pooled, dried (Concentrator 5301, Eppendorf, Germany) and frozen at –80 °C. For HPLC analysis, samples were dissolved in DMSO (100 µl). HPLC was performed by reverse-phase chromatography on a LiChrospher 100 RP-18 column (5 µm; 250 x 5 mm) using a MeCN-H_20 gradient (0.1% TFA) 5:95% → 65:35% in 50 min → 90:10% in 5 min and 90:10% for 5 min (total time 60 min) with a flow rate of 0.8 ml min^{-1} and UV-detection at 254 nm.

The retention times [R_t 43.0 min (**2**), 42.6 min (**3**), 44.8 min (**4**), 33.3 min (**5**), 38.0 min (**6**), 40.4 min (**9**), 24.9 min (**11**), 26.3 min (**12**), 26.6 min (**13**), 26.2 min (**14**), 25.8 min (**15**)] and UV-VIS spectra of the analysed compounds were compared with those of authentic reference compounds. HPLC of defined reference concentrations were used to convert absorption integrals into concentrations. Calibration curves of two compounds, showed the linearity of the concentration-absorption ratio up to a concentration of 0.02 µM. During the HPLC analysis of all extracts, the concentrations of the individual compounds did not exceed 0.02 µM.

4.2.4 Statistical analysis

Results of the cutting injury experiment were tested statistically by analysis of variance (ANOVA) and t-test statistic. Samples from identical leaves were analysed using repeated measures (RM) ANOVA. All statistical tests were performed with Sigma Stat 2.03 (SPSS Inc, USA).

4.3 Results

4.3.1 Distribution in vegetative organs

The distribution pattern of analysed phenylphenalenone-related compounds showed considerable differences between the shoot and root system with regard to both the proportion to each other and the amounts (Fig. 4.2). Some compounds were found to be exclusively distributed either above or below the ground, as in case of **2** and **12** (shoot) and **9**, **14** and **15** (root) (Fig. 4.2a). In leaves and stems, compound **11** was the major metabolite and accounted for up to 50 %. However, in the root system other compounds were much more abundant. Similar but less pronounced observations were found for compound **5**.

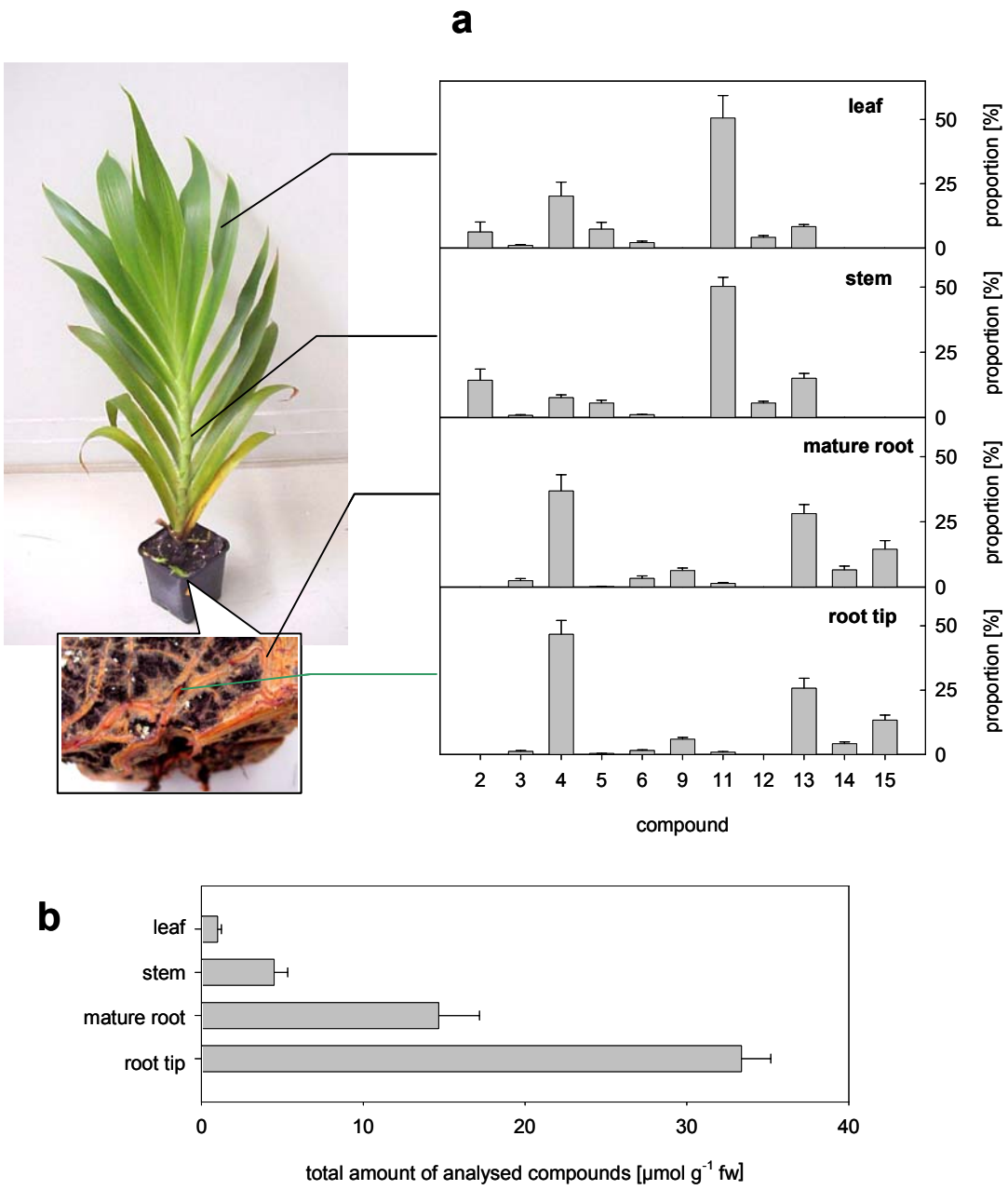


Fig. 4.2: Distribution of phenylphenalenone-related compounds **2** - **6**, **9** and **11** - **15** in vegetative organs of *X. caeruleum*. a) mean (± SE, N = 6) proportion of selected compounds to each other and b) mean (± SE, N = 6) total amounts of analysed compounds within plant organs.

The most apparent difference between the shoot and root system was the level of the metabolites (Fig. 4.2b). An increasing gradient from the leaves via the stem to the root system was found. The highest total amount of the analysed compounds was determined in the root tips with up to a 16 fold higher level than in the leaves. This is twice as much as found in the

mature parts of the roots. The highest amount was calculated for compound **4** to be up to 20 µmol per gram fresh weight in the root tips.

The analysed compounds represented a substantial part of all phenylphenalenone-related compounds in the vegetative organs based on the high proportion (up to 60 %) on UV-absorption signals at 254 nm of the extracts during HPLC analysis.

4.3.2 Distribution pattern of inflorescence

With the exception of **3**, the phenylphenalenone-related compounds which were found in the vegetative shoot organs were also detected in the inflorescence (Fig. 4.3).

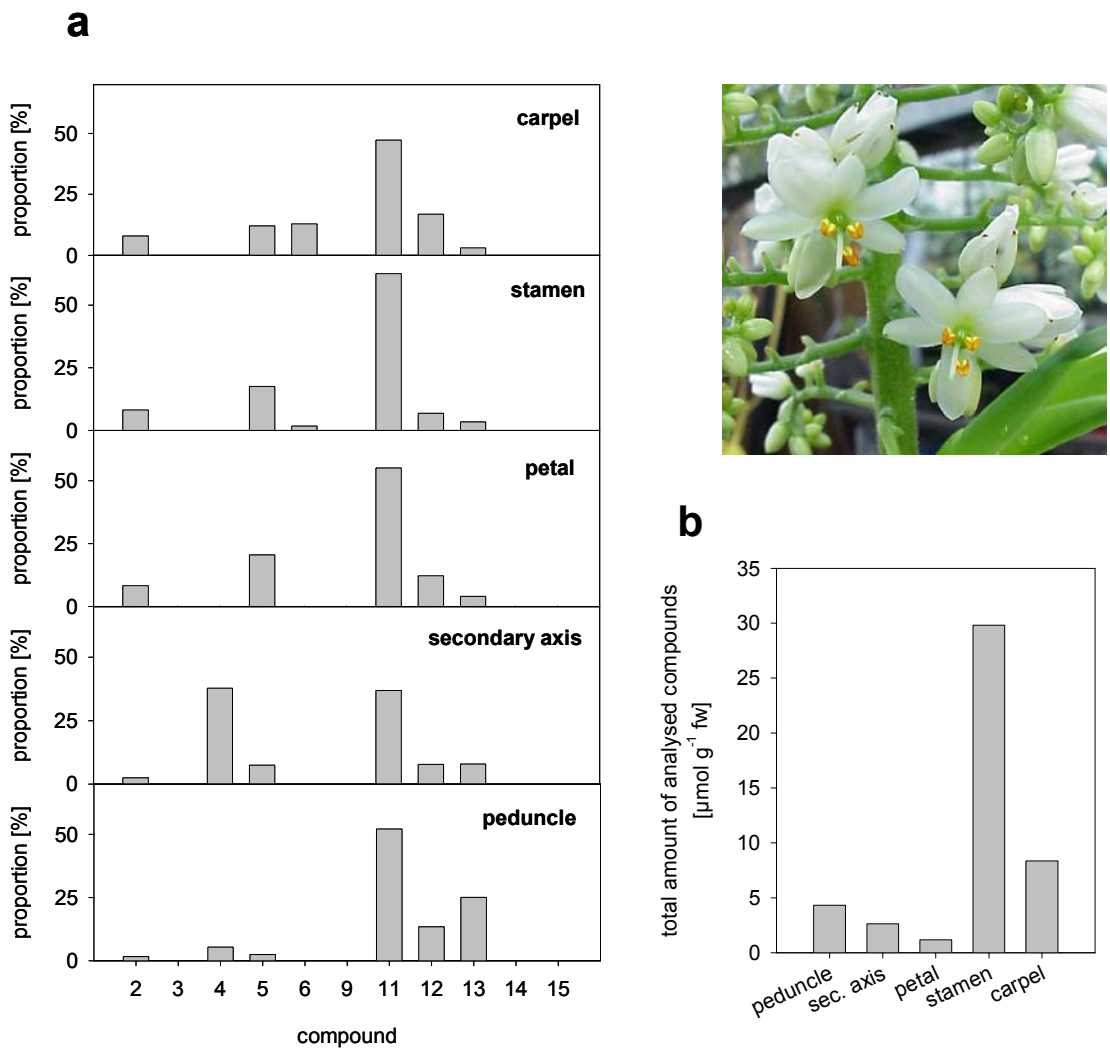


Fig. 4.3: Distribution of phenylphenalenone-related compounds **2** - **6**, **9** and **11** - **15** in reproductive organs of a *X. caeruleum* plant. a) proportion of single compounds to each other and b) total amount of analysed compounds in parts of an inflorescence.

The distribution pattern showed clear differences between the individual parts of the inflorescence (Fig. 4.3a). Some compounds were localised only in specific organs such as **4** in peduncle and secondary axis of inflorescence and **6** in carpels and stamen. The highest total amount for the analysed compounds was found to be in the stamen with 30 µmol per gram fresh material, a similar dimension as in the root tips (Fig. 4.3b). Glucoside **11** was found to be the most abundant metabolite at 18 µmol per gram fresh weight. The lowest total amount was found in the petals. With the exception of peduncle and secondary axis of the inflorescence, HPLC analysis indicated the occurrence of high levels of further unidentified phenylphenalenone-related compounds (proposed on the basis of UV-VIS-spectra) in all parts of the flowers.

4.3.3 Distribution pattern in leaves

In total, 7 compounds were studied in regard to their overall distribution in leaves (Fig. 4.4). Leaves of different ages showed a noticeable change in the oldest ones (leaves D, Fig. 4.4) with regard to both level and the relative proportion of compounds.

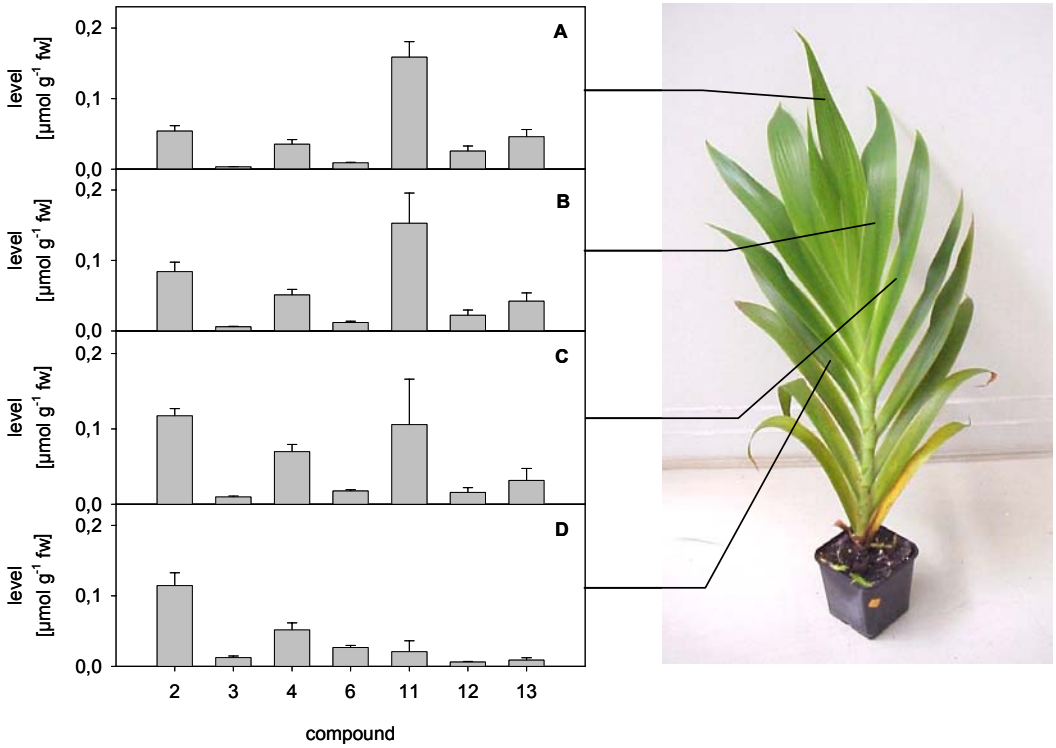


Fig. 4.4: Mean (± SE, N = 6) levels of phenylphenalenone-related compounds **2 - 4**, **6**, and **11 - 13** in four leaves of different age of a *X. caeruleum* plant; A second, B fourth, C sixth and D ninth youngest leaf.

The glucosides (**11**, **12** and **13**) accumulated in much smaller amounts than in younger leaves and hence the total amount of the analysed compounds in old leaves was considerably lower. By contrast levels of aglycones like **2, 3** or **6** increased with leaf age. However, up to the sixth youngest leaf, distribution of the analysed compounds showed a relatively uniform pattern (leaves A – C, Fig. 4.4).

From the base to the top of individual leaves, the distribution pattern of phenylphenalenone-related compounds changed (Fig. 4.5). Decreasing amounts of the glucosides (**11**, **12** and **13**) towards the leaf top were observed. By contrast, the highest amount of the aglycone **4** was found in the upper parts of leaves. Another compound (**2**) showed the lowest amount in the middle part of the leaves. However, the total amount of the analysed compounds was the highest in the samples close to the leaf base (0.56 µmol per gram fresh weight in samples VI) due mainly to the glucosides.

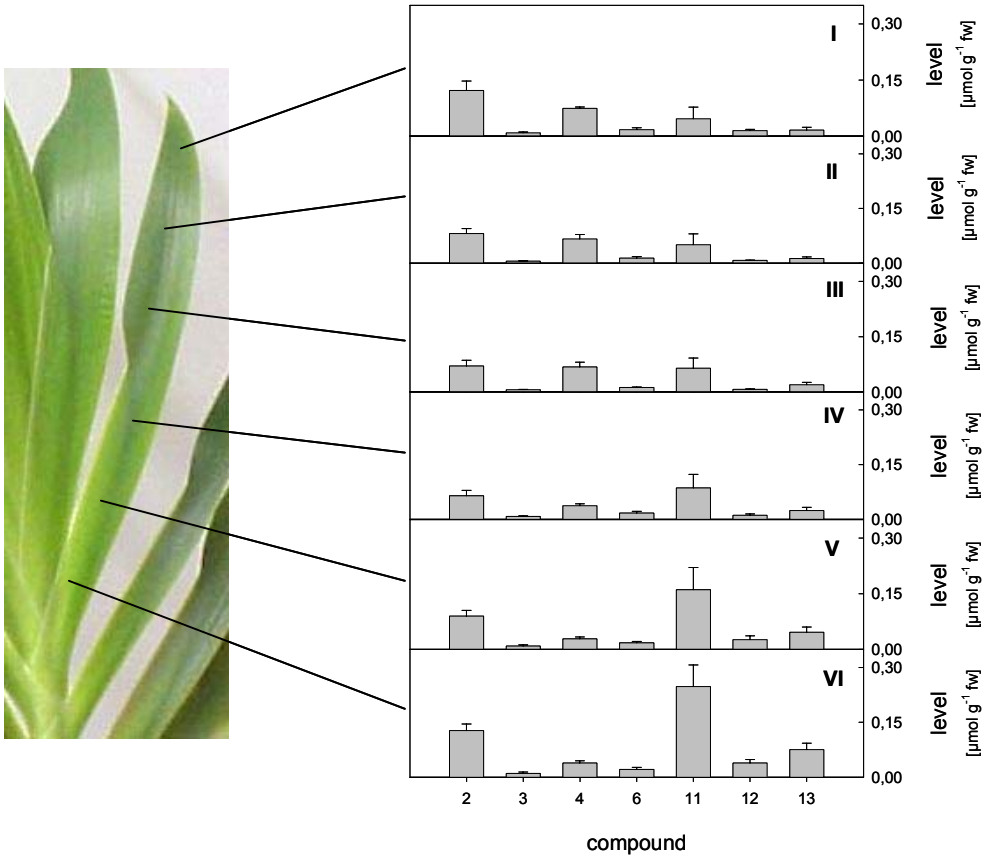


Fig. 4.5: Mean (± SE, N = 4) levels of seven phenylphenalenone-related compounds **2** - **4**, **6**, and **11** - **13** of *X. caeruleum* leaves at six leaf positions beginning near the top (I) and ending near the leaf base (VI).

4.3.4 Analysis after stress treatment

Due to the lack of the glucosides **11**–**13** in the studied plants, the wounding experiment was focused on the amount of the five aglycones **2**-**6** in leaves.

No significant changes in the levels of compound **3**, **4** and **6** were found at the site of injury at any time after treatment (Fig. 4.6). By contrast, the level of **2** and **5** increased over time. Up to 24 hours after the first injury the increase was not significant, but after 48 hours the sites of injury contained significant higher levels of both compounds in comparison to the control A samples (time 0, Fig. 4.6).

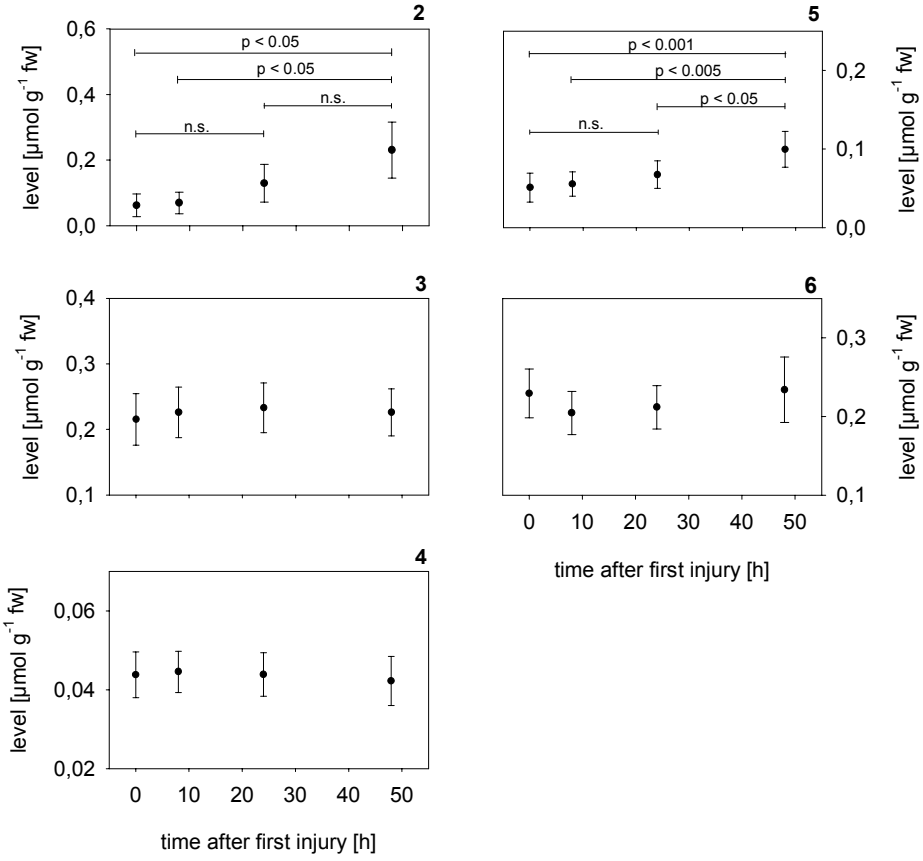


Fig. 4.6: Mean (± SE, N = 9) levels of phenylphenalenone-related compounds **2** - **6** in *X. caeruleum* leaves over time after wounding at time 0. Leaf samples at the site of injury were analysed after 8, 24 and 48 hours accompanied with new wounding. Levels of each compound over time were compared using one way ANOVA with repeated measurements. Significant differences were found for compound **2** and **5** indicated as p-values.

Two way repeated measures ANOVA (one factor repetition) indicated a positive dependency of the amounts of **2** and **5** at both times after injury (described before) and leaf

age (time: **2**: F = 7.883, p < 0.005; **5**: F = 9.033, p < 0.001; leaf age: **2**: F = 7.501, p < 0.05; **5**: F = 38.745, p < 0.001). Comparing the levels in leaves of different age, significantly higher amounts for both compounds were found in the oldest leaves in comparison to the second and fourth youngest ones (one way ANOVA: **2** p < 0.05; **5** p < 0.001). Additionally, the increase in the levels of both compounds within 48 hours of injury was higher in the oldest leaves in comparison to the younger ones, but due to the low sample size, no statistical test was performed for this correlation. However, the increases were independent of plant number (two way repeated measures ANOVA: **2** p = 0.785; **5** p = 0.989).

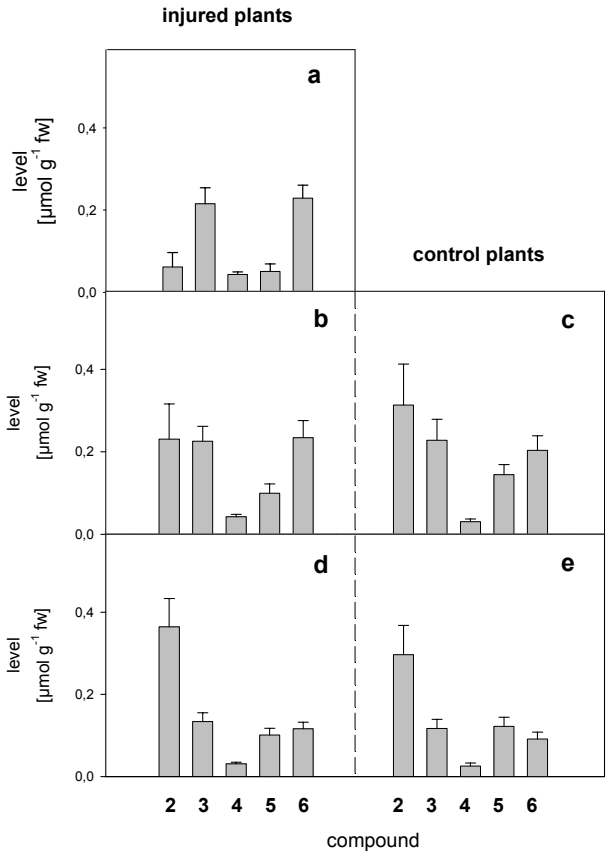


Fig. 4.7: Mean (± SE, N = 9) levels of phenylphenalenone-related compounds **2** - **6** in leaves of injured (a, b, d) and control-B plants (c, e) of *X. caeruleum*. Levels of wounded leaves are shown a) for the site of injury (lamina) at time 0 (control-A), b) for the site of injury after 48 hours and d) for a site near the leaf base (leaf sheath) after 48 hours. Levels in leaves of the control-B plants are shown c) for sites of the lamina and e) the leaf sheath resembling the sample sites of the injured plants. Control-B samples were taken 48 hour after the first injury of the neighbouring plants.

Significant differences in the levels of **2**, **3**, **5** and **6** were found in the injured leaves between samples taken at time zero from the lamina at the site of injury (control A) and those

taken from the sheath near the leaf base 48 h after first injury (Fig. 4.7a, d). Whereas levels of **3** and **6** were lower in the sheath samples (paired t-test: **3** $t = -3.673$, $p < 0.05$; **6** $t = -6.074$, $p < 0.001$), both **2** and **5** showed significantly higher amounts at this part of the leaf in comparison to the lamina (paired t-test: **2** $t = 6.523$, $p < 0.001$; **5** $t = 4.348$, $p < 0.005$). Especially the differences for **2,** with a six fold higher level in the sheath than in the lamina, exceeded the expectation of a "normal" gradient (see 4.3.3.).

Significant differences in the levels of **2** and **5** were found between the control A lamina samples of the injured plants (0 h after injury) and the lamina samples of the control B plants (t-test: **2** $t = 2.385$, $p < 0.05$; **5** $t = 3.069$, $p < 0.05$) (Fig. 4.7a, c). By contrast, there were no significant differences in the levels of any compound between lamina samples of both the injured leaves after 48 h and the control B leaves (t-test: each compound $p > 0.05$) (Fig. 4.7b, c). Additionally, no differences were found in the sheath samples of the injured plants after 48 hours by comparison to the corresponding samples of the control B plants (t-test: for each compound $p > 0.05$) (Fig. 4.7b, c). Hence leaves of wounded plants (48 hours after of injury) and unwounded plants nearby showed comparable pattern of the analysed compounds.

4.4 Discussion

For the first time, the distribution pattern of selected phenylphenalenone-related compounds in a plant, in this case in *Xiphidium caeruleum*, has been investigated. Although there is a lack of information about the complete composition of such phenolics, the substances analysed represent a large proportion of those found in all vegetative plant parts. A different distribution pattern was found in the flowers, where high levels of phenylphenalenone-related compounds of unknown structure were detected and assigned on the basis of similarities of their UV-VIS absorption spectra to those of previously identified compounds (Fig. 2.7, chapter 2).

Comparing the vegetative parts, the highest total amounts of identified compounds were detected in the root system increasing toward the youngest parts (root tips). A similar distribution was found within single leaves with highest total amounts near the growing zone (leaf base). Comparing leaves of different ages, the oldest leaves contained the lowest total amount. Reproductive parts contained high amounts, comparable to the roots, as was shown for the stamina of an inflorescence. Since generative and vegetative organs were studied on plants, which grew under different conditions, direct comparisons must be treated with

caution. However, accumulation of secondary metabolites in young tissue and/or reproductive organs is common for a variety of substances such as alkaloids (Hartmann and Zimmer, 1986; Baldwin, 2001), glucosinolates (Lauda and Mole, 1991; Porter *et al.*, 1991), flavonoids (Harborne, 1991) and terpenoids (Gershenzon and Croteau, 1991). Certainly, the distribution of such compounds depends on the location of their biosynthesis, transport, accumulation and degradation. When looking at individual phenylphenalenone-related substances within the plant, some compounds were distributed exclusively in the root or in the shoot system. Notably, typical phenylphenalenones which contain an intact C-19 carbon skeleton such as **14** and **15**, as well as compound **9** (converted to an oxabenzochrysenone system) were found exclusively in the roots. By contrast, all metabolites detected in above-ground plant parts belong to the phenylbenzoisochromenone subtype, which is formed by oxidative rearrangement from precursors containing the C-19 skeleton of phenylphenalenones (chapter 3). The differential occurrence of phenylphenalenones and phenylbenzoisochromenones could be indicative of biosynthesis in the roots and translocation toward the shoot system. Subsequently, phenylbenzoisochromenones such as **2** and **12** might be formed exclusively in shoots. Furthermore, in contrast to the tendency of highest accumulation in young plant parts, some compounds showed their highest amount in older ones. A possible explanation might be the oxidative degradation (see chapter 3) of originally formed compounds and the accumulation of metabolites in older plant parts.

Water soluble glucosides of phenolic compounds, such as flavonoids, are known as storage forms in vacuoles (Strack, 1997; Harborne and Williams, 2000). The same can be presumed for phenylphenalenone glucosides in *X. caeruleum*, where high proportions of glucosides were found in almost all plant parts. This would require reversibility of the glucosidation process (Schliemann, 1991). However, phenyphenalenone-related glucosides seem to be not hydrolysible under standard chemical and enzymatic conditions (Dora *et al.*, 1993). Additionally, questions arise about the role of the allophanyl group in the glucosides **12** – **15** since nitrogen is the major limiting nutrient for most plants (Wink, 1997). Hence, a simple storage or transport function seems to be rather unlikely. Surprisingly, the glucosides were not found in plants of the Botanical Garden Palm House, which were used for the injury experiment. This demonstrated that the occurrence and accumulation of several phenylphenalenone-related compounds is very sensitive to growing conditions.

It has been well established that wounding by herbivores, as well as other biotic and abiotic factors, causes chemical changes in plants that can increase their resistance to further

damage (Hartley and Jones, 1997). In addition to indirect defence such as the attraction of herbivore enemies caused by release of volatile terpenoids, direct defence responses are known for a variety of secondary metabolites increasing their amounts in the plant after wounding (Sabelis *et al.*, 1999; Baldwin, 2001). In the present work, increasing amounts after wounding have been demonstrated for two compounds belonging to the phenylphenalenone group. The amount of both lachnanthopyrone (compound **2**) and 3,5,6-trihydroxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (compound **5**) were tripled and doubled, respectively, 48 hours after the first damage of *X. caeruleum* leaves at the site of injury. These changes may increase the defensive potential against herbivores or pathogens.

However, in addition to these local reactions to wounding, there is also evidence for systemic responses. The amount of lachnanthopyrone (**2**) in the sheath of an injured leaf (48 hours after injury) was approximately 6 fold higher than in the lamina before the damage (control A). Based on the results of the study on distribution patterns within undamaged leaves, this difference would be unusually high for a "normal" leaf.

Surprisingly, in both the sheath and lamina, the levels of identified phenylphenalenone-related compounds in the control B plants resembled these of the injured ones after 48 hours. A comparison between lamina samples of the control B plants and wounded plants at time 0 (control A) showed differences, although these were not expected because all plants were chosen randomly from the same "habitat" in the greenhouse. However, it must be noted that samples of the control B plants were taken at the end of the experiment 48 hours after the neighbouring plants had been damaged. One might speculate that the control plants were induced by aerial contact with the damaged plants. Volatile signalling compounds released from the damaged plants and perceived by the undamaged ones could be a possible explanation for these results. There is a very limited corpus of information about inter-plant communication by plant-derived volatiles (Agrawal, 2000; Preston *et al.*, 2001). However, it has been demonstrated that undamaged plants respond to cues such as methyl jasmonate or terpenoids which are released by neighbours to accumulate higher levels of resistance against herbivores (Arimura *et al.*, 2000; Karban *et al.*, 2000). Such communication between *X. caeruleum* plants would indicate a high sensitivity to leaf damage. Thus the possible increase of phenylphenalenone-related compounds due to damage of neighbouring plants would support the assumption about the defensive role of compounds like lachnanthopyrone.

Several phenylphenalenone-type phytoalexins and phytoanicipins with antifungal and/or antinematode activity have been already described from *Musa* species (Luis *et al.*, 1993; Binks *et al.*, 1997; Kamo *et al.*, 2001). Elicitors such as kanamycin or jasmonic acid

(JA) stimulate the biosynthesis of several phenylphenalenone-related compounds (Luis *et al.*, 1993) (see chapter 3). Jasmonic acid is known as an essential signalling component responsible for wound-induced increase of secondary metabolites (Baldwin, 2001). In the present study JA might have mediated the wounding reaction in *X. caeruleum*. However, responses to mechanical wounding are not equivalent to herbivore feeding since components of the herbivore oral secretion are known as elicitors of herbivore-specific response (Walling, 2000; Halitschke *et al.*, 2001). Herbivore feeding often causes larger increase of JA leading to higher levels of defensive compounds than wounding alone. In case of the present study, this might mean that plants of *X. caeruleum* would react even more strongly after feeding injury than after mechanical wounding.

5. Histochemical analysis of phenylphenalenone-related compounds in *Xiphidium caeruleum* (Haemodoraceae)

5.1 Introduction

In addition to information about the chemical structure, the biosynthesis and its regulation, knowledge about the tissue distribution of phenolic compounds and their cellular and subcellular localisation within the tissue is an important prerequisite for the understanding of the ecological function of any compound. Phenylphenalenone-related compounds seem to occur in all vegetative and reproductive plant organs of *X. caeruleum* (chapter 4). However, nothing is known about where these phenolics are accumulated within these organs. Phenolic compounds and their respective biosynthetic enzymes can show a high degree of compartmentation within plants (Knogge and Weissenböck, 1986; Haussuhl *et al.*, 1996). A variety of phenylpropanoids and flavonoids accumulate in the central vacuoles of guard cells, epidermal cells and subepidermal cells of leaves (Weissenböck *et al.*, 1986; Schnabl *et al.*,

1989) and shoots (Ozimina *et al.*, 1980). Furthermore some compounds were localised in cell walls, and on surfaces of plant organs (Strack *et al.*, 1988; Cuadra and Harborne, 1996; Fernandez *et al.*, 1999; Karabourniotis *et al.*, 2001). Methods used for such studies include several microscopical techniques (Harris and Hartley, 1976; Charest *et al.*, 1986), enzymatic tissue preparation (Schnitzler *et al.*, 1996) or isolation of organelles and protoplasts (Anhalt and Weissenböck, 1992; Hrazdina, 1992).

Bright field microscopy is the main tool for classical studies, but it is restricted to localising coloured pigments such as anthocyanines. Modern epi-fluorescence microscopy is a more powerful tool since several classes of phenolic compounds such as hydroxycinnamic acids, coumarines, stilbenes and styrylpyrones (Ibrahim and Barron, 1989; Veit *et al.*, 1993; Gorham, 1995) exhibit a strong autofluorescence when irradiated with UV or blue light. To visualize weak or non-fluorescent compounds, immunocytochemical and histochemical methods such as 'Naturstoff' reagent A-staining of flavonoids can be used to induce or enhance fluorescence (Vogt *et al.*, 1994; Hutzler *et al.*, 1998).

Recently, confocal laser scanning microscopy (CLSM) provides the opportunity to localise phenolic compounds in plant tissues in a higher resolution. This technique enables an overlay of serial optical sections to an extended depth of focus and therefore images in the z-axes perpendicular to the system plane (Sheppard, 1993; Fricker *et al.*, 1997; Fernandez *et al.*, 1999).

Phytochemical analysis of *Xiphidium caeruleum* (Aublet), a neotropical member of the Haemodoraceae, resulted in the isolation and identification of a variety of phenylphenalenone related compounds, of which some were capable of intensive autofluorescence. In the present study, this property was used in combination of CLSM, microspectral photometry (MSP) and high pressure liquid chromatography (HPLC) to localise phenylphenalenone-type compounds in *X. caeruleum* plant tissues.

5.2 Materials and methods

5.2.1 Plant material

Plant material of *Xiphidium caeruleum* (Aubl.) was obtained from the University of Bochum (Botanical Institute) and maintained at the Botanical Garden of the University of Jena. Plants were transferred to the Max Planck Institute for Chemical Ecology, Jena and

maintained in growth chambers for variable time intervals at 28/ 15°C day/ night temperature, 50/ 85% relative humidity and a 14 hours day period.

5.2.2 Sample preparation for microscopical analysis

Roots, stem and leaves of *Xiphidium caeruleum* were carefully cut with a razor blade to obtain fresh hand-cut sections. Transverse, longitudinal and whole sections were washed briefly with water and mounted in a droplet of sample buffer (100 mM KP_i pH 6.8, 1% NaCl (w/v)) on a slide using a spacer between slide and coverslip to avoid compression of the sections.

5.2.3 Confocal laser scanning microscopy (CLSM)

Fresh hand-cut sections of *X. caeruleum* plant material were analysed with a Zeiss LSM510 laser scanning microscope (Carl-Zeiss, Jena, Gemany) equipped with a Plan Neofluar 10x/0.3 objective or a C-Apochromat 40x/1.2 water objective. The autofluorescence was visualised in either single optical sections or serial optical sections of roots and leaves. Throughout the work, a single line excitation (UV laser 364 nm) and multiple channel emission technique were used. The thickness of optical sections varied between 1 and 100 µm, and series of 27 – 131 sections were used to obtain extended depth of focus image.

For visualisation of multispectral image data, the different channels were associated with a colour value on the display system (RGB colour mode). The three fluorescence channels were assigned to the primary display colours - 'pseudo-colours' - red (longpass filter (LP) >560 nm), green (band pass filter (BP) 505-530 nm) and blue (BP 385-470 nm). All fluorescence channels were measured simultaneously. The visual colour impression from the screen did not necessarily correspond with visual impressions which were obtained with a multiline filter set or an LP filter. Furthermore, the ratios of signals from different channels differed from real time viewing due to filter transmittance, exposure time and detector sensitivity. Image montages were assembled with Powerpoint.

5.2.4 Microspectral photometry (MSP)

UV-absorption spectra from vacuoles of distinct cells were measured with a MSP 800 microspectral photometer from Zeiss using fresh hand-cut sections of roots and leaves. UV-absorption spectra were recorded from 250 to 700 nm, with a 2.5 nm optical bandwidth as a quotient spectrum to compare a region from the vacuole and a region of the same size outside the section. Autofluorescence spectra of vacuoles were recorded from 400 to 700 nm (excited at 365 nm) using a spectrometer "MCS 521 VIS" from Zeiss (Jena, Germany). Spectra were visualised in the program Aspect Plus.

5.2.5 Isolation and characterisation of phenylphenalenones

Isolation and structure elucidation of phenylphenalenone-related compounds have been described in chapter 2. UV spectra of single compounds were obtained using an Agilent G1315B diode array detector during analytical HPLC (Agilent Technologies, Waldbronn, Germany) in MeCN-H_2O solvent mixtures between 1:2 and 2:1. In parallel, the autofluorescence emission spectra were recorded with an Agilent G1321A fluorescence detector (excitation at 364 nm). UV absorption spectra and fluorescence emission spectra were monitored from 200-700 nm and 400-700 nm, respectively.

5.2.6 HPLC analysis of root sections

Ten root tips of approximately 5 mm length were taken from four plants of *X. caeruleum*. Each root tip was cut into eight sections of approximately 0.5 mm with razor blades and were numbered from 1 to 8 (1 = tip). Segments with the same number were pooled to generate eight samples. After weighting, each sample was placed into a 2.0-ml screwtop microcentrifuge tube containing Lysing Matrix D (0.9 g) (Q-Biogene, Heidelberg, Germany). To each tube was added methanol (1 ml) and pyrene (10 µg) in the same solvent as an internal standard. The mixture was homogenised for 45 s at 6.5 m s^{-1} using the FastPrep® System (FP120; Q-Biogene, Heidelberg, Germany). The homogenate was centrifuged and the supernatant was transferred into a new vial. The cell debris was extracted with methanol (1 ml) and both methanolic extracts were pooled and dried (Concentrator 5301, Eppendorf, Germany). The dried samples were dissolved in DMSO (50 µl) for reversed-phase HPLC analysis. Reverse-phase HPLC was performed on a LiChrospher 100 RP-18 column (5 µm; 250 x 5 mm) using a linear MeCN-H_20 gradient (0.1% TFA) 5:95% → 65:35% in 50 min →

90:10% in 55 min → 90:10% in 60 min with a flow rate of 0.8 ml min^{-1} and DAD detection (monitoring wavelength 254 nm). Additionally, a fluorescence detector was used to monitor autofluorescence at 430, 520, 570 and 650 nm with the same excitation wavelength (364 nm) as used for CLSM. Authentic samples of known compounds were used for calibration.

5.2.7 Analysis of leaf extracts

Two extracts of leaf material, one using methanol and one using acetone, were prepared. One hour after extraction, fluorescence spectra (400 - 700 nm) of both extracts were measured using a Jasco-FP-750 fluorescence spectrophotometer (Jasco Labor- und Datentechnik GmbH, Germany) at the excitation wavelength of 364 nm. Both extracts were analysed by HPLC using the same conditions and detection parameters as for root section analysis.

5.2.8 Staining with 'Naturstoff' reagent A

The autofluorescence of a fresh hand-cut section of a leaf was monitored. Afterwards, a droplet of 0.1% (w/v) diphenylboric acid 2-aminoethyl ester ('Naturstoff' reagent A; NA) in sample buffer (prepared immediately before use from a stock solution of 2.5% (w/v) NA in EtOH) was added and sucked under the coverslip. Samples were incubated for 5 min, and excess NA solution under the coverslip was removed by buffer. The fluorescence of the same section of NA-stained phenolic compounds was studied using identical microscopic conditions, and the same leaf area as in the buffer control. Fluorescence intensity of the green channel (505-530 nm) was measured along a line passing through 4-5 epidermal cells (see red line Fig. 5.7). Means of the grey scale values of these cells were calculated before and after NA-staining.

Two samples of a methanolic leaf extract were analysed by HPLC with post column derivatisation using an additional pump system. 0.1 % (w/v) NA was added with a flow rate of 0.2 ml min^{-1} to the eluent for approximately 45 s in the reaction coil before reaching the detector. This procedure was repeated with H_2O as a control. To prove the efficiency of NA-staining, solutions of the flavonoid quercetin (Sigma) were analysed similar without using the column. HPLC equipment and conditions for analysis of leaf samples were the same as described above for root section except that eluents were used without TFA. The use of TFA-free eluents leads to significant peak broadening and shorter retention times R_t of polar

compounds. Fluorescence emission at 520 nm excited at 364 nm as used in CLSM was recorded. The total of fluorescence signals was compared between the samples.

5.3 Results

5.3.1 Localisation of phenolic compounds in roots

The analysis of roots with CLSM showed strong autofluorescence in different wavelength ranges (Fig. 5.1a-f) in all cells. Blue 'pseudo'-colour was caused mainly by propanoic acids bound to cell walls. The vacuoles of the apical meristem, of cortex cells and of root cap cells contained compounds that fluoresced intensely at longer wavelength ranges. Whereas most cells of the cortex showed a yellow emission, a shift to orange and red 'pseudo'-colours was observed towards the outer part of the cortex and the root cap, respectively (Fig. 5.1a-d). The number of orange and red fluorescent cells decreased in the zone of maturation of the root. However, due to their elongation many cells were injured during cross sectioning and lost their contents (Fig. 5.1e). The overlay of serial optical sections of the root surface within the elongation zone exhibited red autofluorescent vacuoles within the root hairs (Fig. 5.1f).

The highest fluorescence intensity was observed in the root tip. This could be due to higher concentrations of autofluorescent compounds in the vacuoles (yellow cells) and/or a higher number of special cells like these of the root cap (red cells) possessing such compounds. In order to correlate the fluorescence intensity with the pattern and amount of known phenylphenalenone type compounds, eight serial sections of 0.5 mm thickness were taken from the tip toward the root hair zone, extracted with MeOH and analysed by HPLC. These HPLC analyses indicated declining amounts of a variety of substances with distance to the root tip. In respect to concentration and fluorescence intensity, four compounds were chosen and identified as 6-hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**4**), 5-hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one (**9**), 6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-*b*enzo[*de*]isochromen-1-one (**13**) and as 6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenylphenalen-1-one (**15**), all belonging to the phenylphenalenone group (Fig. 5.2). HPLC analysis confirmed the microscopic images by showing much higher levels of these compounds in the root tip than in the interior sections (Fig. 5.3). Both glucosides and aglycones showed decreasing amounts

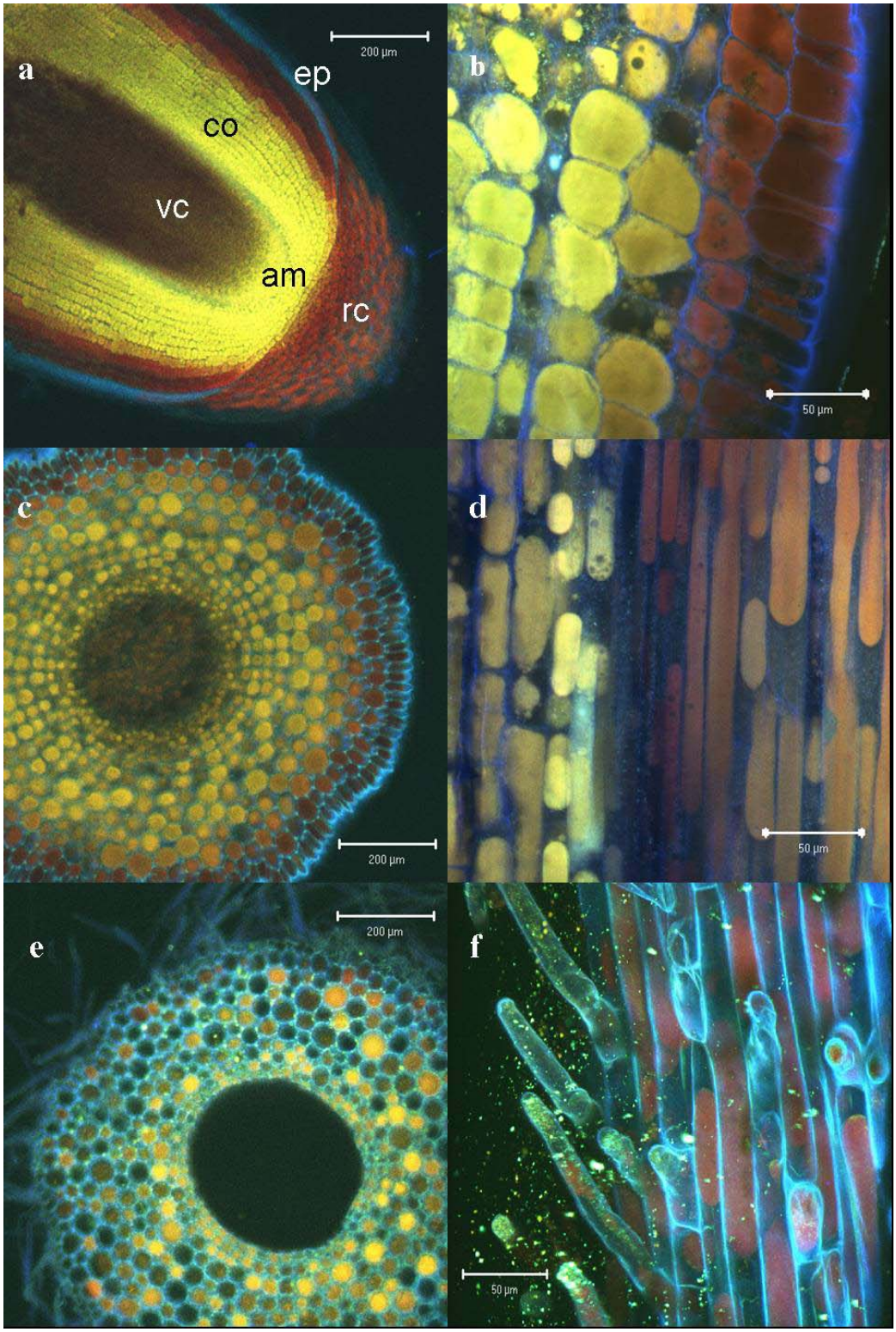


Fig. 5.1: CLSM images of autofluorescence in a root of *X. caeruleum*; RGB-colour mode UV laser-excited (364 nm) autofluorescence of (a) longitudinal section of a root tip: vc – vascular cylinder, am – apical meristem, co – cortex, ep – epidermis, rc – rootcap; (b) longitudinal section within the region of cell division; (c) cross and (d) longitudinal section within the region of cell elongation; (e) cross and (f) surface view within the region of maturation (extended depth of focus).

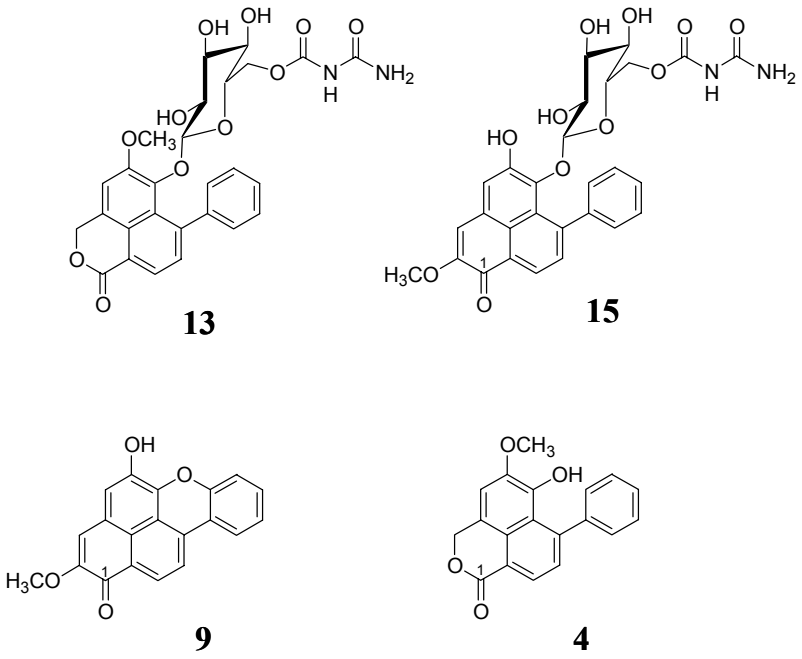


Fig. 5.2: Chemical structures of phenylphenalenone-type compounds from *X. caeruleum*. **13**: 6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one, **15**: 6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-hydroxy-2-methoxy-7-phenylphenalen-1-one, **9**: 5-hydroxy-2-methoxy-6-oxa-benzo[*def*]chrysen-1-one, **4**: 6-hydroxy-5-methoxy-7-phenyl-3*H*-benzo[*de*]iso-chromen-1-one.

toward region 8. The strongest differences in phenylphenalenone levels were observed between section 1 and 2. The differences for the aglycones were less pronounced than for the glucosides that showed a 95 % decline for compound **15**. Compound **4** could be identified as the most abundant phenylphenalenone-related compound reaching levels of 80 µmol g^{-1} fw in the root tip section.

Nevertheless, the HPLC analysis with fluorescence detection showed that glucoside **13** was the major autofluorescencent phenylphenalenone-related compound at both 520 and 570 nm and accounted for up to 85 % of the total fluorescence intensity in the tip section extract. However, fluorescence emission signals were also observed at 630 nm and were at least partly due to the glucoside **15** and aglycone **9**. Although compound **4** was the most abundant phenylphenalenone compound, it showed very weak fluorescence signals.

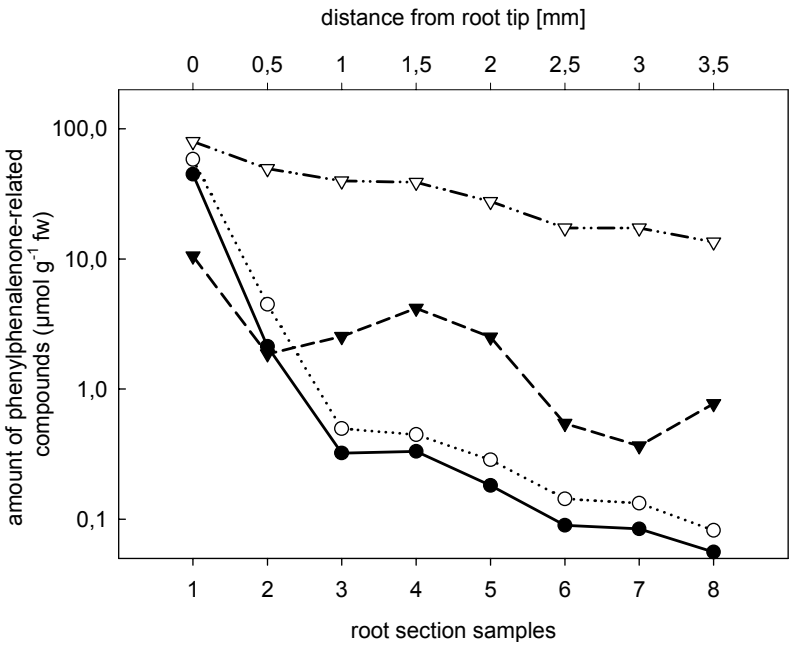


Fig. 5.3: Amounts of four phenylphenalenone-type compounds in serial cross sections of distal root pieces; each sample contains ten sections of the same location (root section sample 1 = tip sections); ○·····○ compound **13**, ●——● compound **15**, ▼----▼ compound **9,** ▽ — · ▽ compound **4**.

The microscopic images and the analysis of the fluorescence characteristics of the different phenolic compounds strongly indicate that the observed pattern of fluorescence distribution in the root of *Xiphidium* is due to a distinct accumulation of different phenylphenalenone-type compounds in the vacuole of specific cells of the root tissue. Hence compound **13** predominately accumulates in the inner cell layers of the root cortex whereas compounds **15** and **9** are more abundant in the epidermal and subepidermal cell layers. However, the bandwidth of the 3 emission channels (RGB) allowed no direct comparison to the fluorescence spectra of the isolated compounds.

To overcome this, the MSP techniques were used to compare the *in situ* fluorescence (excited at the same wavelength as for CLSM and HPLC) and UV-absorption spectra of single yellow, orange and red fluorescent cells and vacuoles in cross sections of roots with that of isolated compounds (Fig. 5.4). The yellow fluorescent cells of the inner root cortex showed two distinct UV absorption maxima between 300 and 400 nm (Fig. 4b). Both maxima were equivalent to the maxima in the spectrum of the isochromenone glucoside **13** ($UV_{MeCN-H2O}$ λ_{max} 260, 336, 370) indicating that **13** is predominately localised in the vacuoles of the

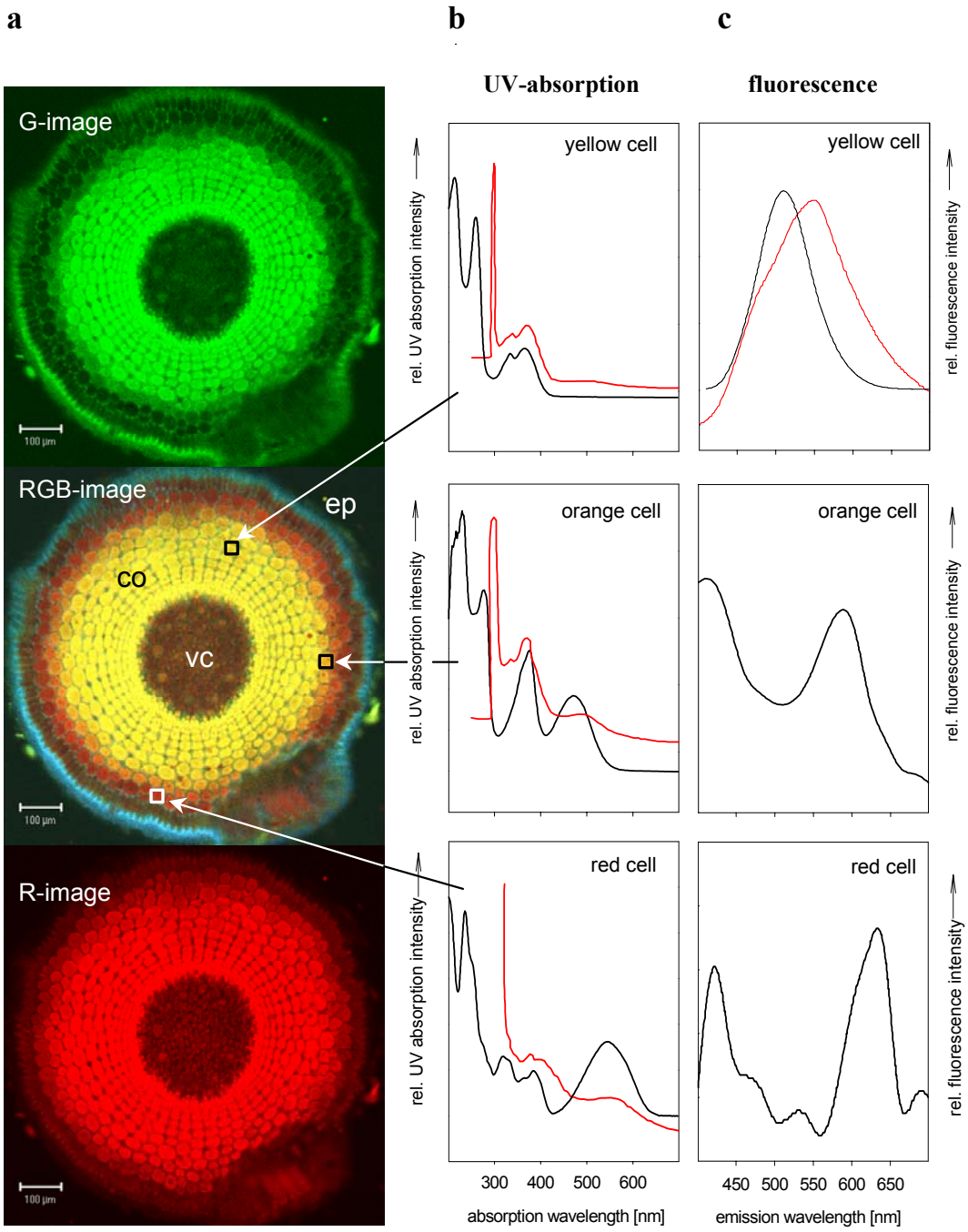


Fig. 5.4: *In situ* measurement of both UV-absorption and fluorescence spectra in cortex cells of a root of *X. caeruleum*. (a) G-, RGB- and R-CLSM colour mode of UV laser-excited (364 nm) fluorescence of a root cross section; vc – vascular cylinder, co – cortex, ep – epidermis; (b) *in situ* UV-absortion and (c) *in situ* emission spectra taken from three cortex cells using MSP (red) compared with spectra (black) of isolated phenylphenalenone-type compound **13** (yellow cell), **15** (orange cell) and **9** (red cell).

inner cortex cells. However, this conclusion could not be confirmed by the *in situ* fluorescence spectra of the yellow cells which showed a maximum which is bathochromically shifted approximately 40 nm compared to the apparent fluorescence maximum of the isolated compounds **13** at 510 nm (Fig. 5.4c).

The maxima between 350 and 600 nm in the *in situ* UV-spectra of orange and red cells can be assigned to glycoside **15** ($UV_{MeCN\text{-}H2O}$ λ_{max} 209, 279, 375, 470) (orange cells) and benzochrysenone **9** ($UV_{MeCN\text{-}H2O}$ λ_{max} 237, 320, 386, 547) (red cells). Orange and red fluorescence intensities were too weak for recording the *in situ* emission spectra of appropriate cells. However, the fluorescence of isolated compounds **15** (maximum at 580 nm) and **9** (maximum at 625 nm) account for the source of the orange and red autofluorescence in cells.

5.3.2 Localisation of phenolic compounds in leaves

CLSM analysis of shoot material showed conspicuous autofluorescence in various tissues (Fig. 5.5a-d). In addition to the blue autofluorescence of cell wall-bound phenylpropanoic acids, cross sections of leaves showed red emissions of the parenchyma cells caused by chlorophyll (Fig. 5.5a). Additionally, green/yellow autofluorescence was visible in vascular bundles and in the epicuticular wax layer. In surface view images of leaves, all epidermal vacuoles contained green/yellow fluorescent compounds (Fig. 5.5b, c), although the highest fluorescence intensities were localised in both the subsidiary and the guard cells of the stomata complex (Fig. 5.5b). In the stem, both epidermis and cortex cells showed green/yellow fluorescent vacuoles (Fig. 5.5d).

A variety of *in situ* UV absorption spectra of epidermal cells, stomata complex, epicuticular layer and vascular bundle cells were recorded by MSP. In all cases, typical absorption spectra of hydroxycinnamic acids such as ferulic or caffeic acid (Fig. 5.6a) were observed. Caffeoyl and feruloyl tartaric acids, which are derivatives of hydroxycinnamic acids, have been isolated from leaf tissue of *Xiphidium caeruleum*, suggesting that these compounds may at least partly be responsible for the characteristic phenylpropanoid absorption.

In situ fluorescence measurements of some epidermal cells showed weak but distinctive spectra including a maximum in the green wavelength range at 525 nm (Fig. 5.6b). Emission maxima of other green/yellow fluorescent tissue varied between 520 and 550 nm. By comparison, spectra of methanolic or acetone leaf extracts consistently showed a distinct

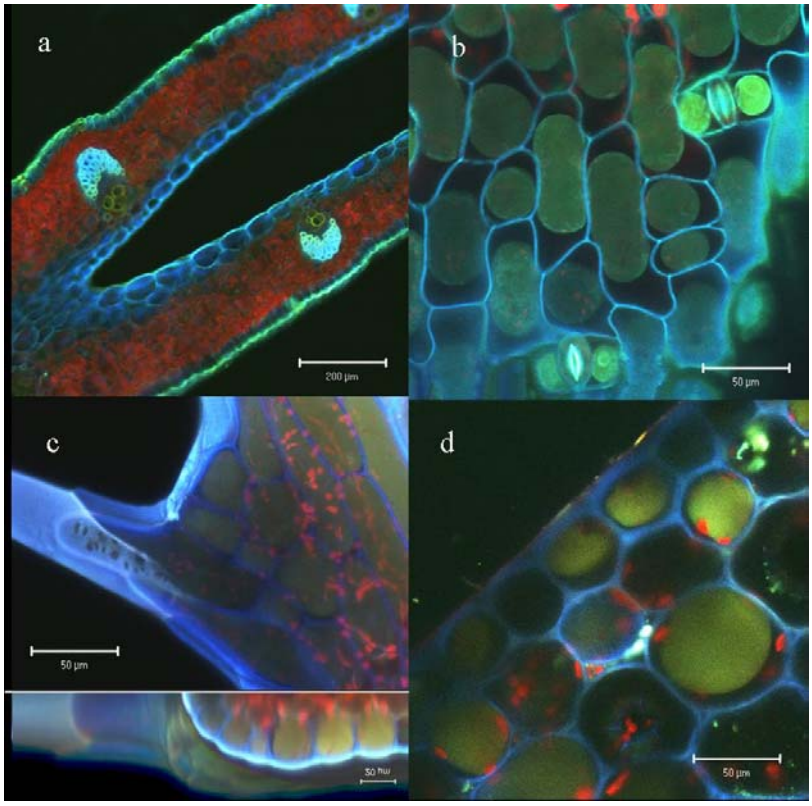


Fig. 5.5: CLSM images of autofluorescence in the shoot of *X. caeruleum*; RGB-colour mode UV laser-excited (364 nm) autofluorescence of a (a) cross section of a leaf sheath, (b) surface view of a leaf section, (c) surface view of a tooth of a cerrulated leaf including a x-z scan of serial optical sections, (d) cross section of a stem.

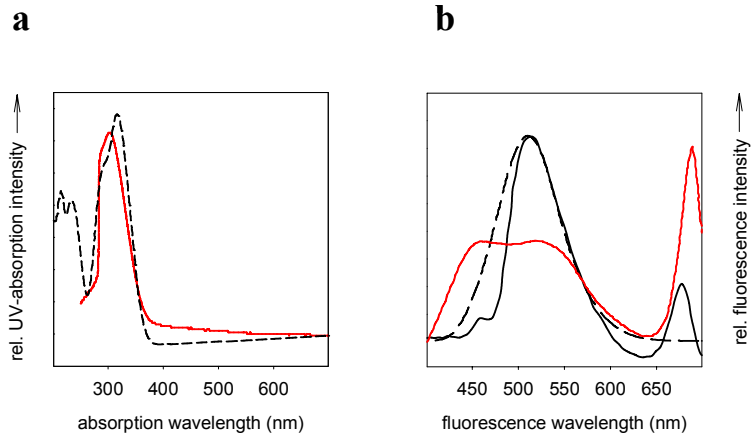


Fig. 5.6: (a) UV-absorption spectra of an epidermal cell using *in situ* measurement by MSP (red) and of ferulic acid recorded with a fluorimeter (black short dashed). (b) autofluorescence spectra of an epidermal cell using *in situ* measurement by MSP (red), of a methanolic leaf extract taken by a fluorimeter (black solid) and of 6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one, the isolated compound **13** (black long dashed).

maximum at 510 nm. HPLC analysis of the same extracts revealed compound **13** as the major phenylphenalenone compound with a fluorescence in the green/yellow wavelength range, which accounted for up to 80 % of the total emission. However, although this measurement revealed that compound **13** was the dominant green/yellow fluorescent substance in the leaf, it was not possible to assign it to certain leaf tissues due to the lack of sharpness of the *in situ* fluorescence spectra.

'Naturstoff' reagent A (NA), which enhances fluorescence of flavonoids (Neu, 1957), was used for infiltration of leaf sections. After this treatment, all epidermal vacuoles showed a considerable increase in the intensity of yellow fluorescence (Fig. 5.7a, b). Analysis of the grey scale values of the green emission channel (505-530 nm) showed an approximately 5 fold higher fluorescence within the cells after NA-staining (Fig. 5.7c).

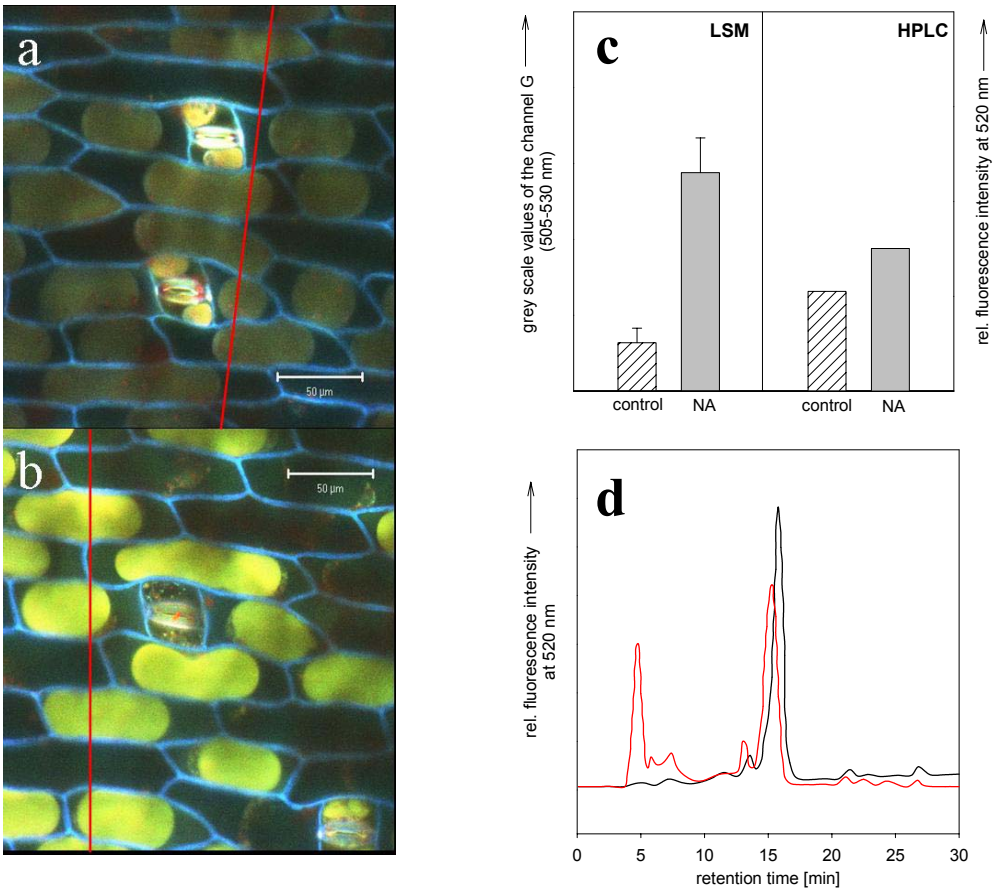


Fig. 5.7: Fluorescence of epidermal leaf cells of *X. caeruleum* before and after staining with 'Naturstoff' reagent A (NA). CLSM images in RGB-colour mode of UV laser-excited (364 nm) fluorescence in epidermal cells before (a) and after (b) NA-staining; (c) comparison of grey scale intensity of the green channel (305-330 nm) before and after NA-staining within epidermal cells along a transect line [red lines in (a) and (b)] of CLSM images (left) and fluorescence intensity of leaf

extracts during HPLC analysis with post column NA-derivatisation; (d) chromatograms of HPLC analysis before (black solid) and after (red solid) treatment with NA.

By comparison, HPLC analysis of leaf extracts confirmed an increase of fluorescence intensity after post column derivatisation with NA. Fluorescence detection at 520 nm showed an increase in the total emission of the whole extract of up to 150 % (Fig. 5.7c). However, the peak area of these compounds (mainly **13**), which are responsible for autofluorescence, were not enhanced after NA treatment (Fig. 5.7d). The increase in fluorescence after addition of NA was due to the staining of substances eluted from the column in the very polar fraction (R_t = 5 min) where no or only weak autofluorescence had been previously detected. These comparisons of microscopic and analytical data give a strong indication of currently unknown flavonoid compounds in the epidermal vacuoles of *Xiphidium caeruleum*.

5.4 Discussion

The present work was aimed at the distribution of phenylphenalenones and related compounds in tissues of *Xiphidium caeruleum*, a species of the Haemodoraceae. Phytochemical studies had revealed already the occurrence of such compounds throughout the whole plant (chapter 4).

A combination of several analytical techniques was applied to study the tissue localisation of these phenolics in root, stem and leaves. The intense autofluorescence of some of these compounds after excitation with UV-light was explored and it was possible to assign yellow to red autofluorescence seen in the CLSM images to specific substances using methods such as MSP and HPLC. More precisely, it was demonstrated that (6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**13**)) plays the key role for the emission in the green to yellow wavelength range in both root and leaf tissue. Although the aglycone (**4**) of glucoside **13** represents the predominately substance in the roots, it was not related to the autofluorescence within the tissue. Fluorescence measurements on both isolated compounds (**13** and **4**) showed an increase of intensity of up to 150 fold due to the attachment of the allophanyl glucose moiety. A largely different fluorescence emission has been described previously for a flavone aglycone and its glycoside (Kartnig and Gobel, 1996). However, the present observation confirms the strong difference

in autofluorescence potential of the phenylphenalenone-related compounds with respect to the occurrence of a variety of these compounds in any plant extract.

The application of MSP was restricted to cells containing sufficient concentrations of green to red fluorescent compounds to overlay the autofluorescence of cell wall bound compounds as in the case of the inner root cortex cells. A limitation of MSP was that spectra represented an integral over a certain distance within the tissue and not a single optical layer of approx. 1 - 2 µm as a CLSM image does. This led to overlaid spectra along the thickness of an illuminated section. However, a comparison of UV-VIS absorption spectra taken *in situ* by MSP with the spectra of isolated compounds was shown to be a suitable method to localise compounds within the cell. By contrast, fluorescence spectra were less specific and differences in the maximum wavelength between *in situ* spectra and those of the isolated compound as in case of **13** are possible. An explanation for such differences might be intermolecular interactions or molecular complexing similar to that described for anthocyanines (Brouillard and Dangles, 1986; Harborne and Williams, 2000).

Phenylphenalenone-related compounds are distributed in the whole plant of *Xiphidium caeruleum*. Within the root system, the highest concentrations were found in vacuoles of the apical meristem and the cortex of the root. Different compounds, but of the same phenolic group, accumulated in the vacuoles of cells of root cap, epidermis and of root hairs. In the shoots phenylphenalenone-type compounds accumulated in the vacuoles of stem cortex and leaf epidermis cells. Soluble phenolic compounds occur predominantly in the form of hydrophilic conjugates in vacuoles of plants (Weissenböck *et al.*, 1984), but they are also known as extraprotoplasmatic, e.g. bound on cell walls (Strack *et al.*, 1988) or on epicuticular wax layer (Karabourniotis *et al.*, 2001; Liakopoulos *et al.*, 2001). In *X. caeruleum*, yellow to red autofluorescence on cell walls of parts of the vascular system in root and shoot, of the endodermis in the stem and of stomata complex were localised. Furthermore, green to yellow emission was observed in the epidermal wax layer of the shoot. It is unclear, however, if phenylphenalenones or other yet unknown phenolic compounds causes this autofluorescence.

Although phytochemical studies (chaper 2) did not succeed in the isolation of flavonoids, their suggested ubiquitous distribution in the plant kingdom cause the question of their occurrence in the leaves of *Xiphidium*. Naturstoffreagent A (NA) is a well-known reagent, which can be used in histochemistry for the detection of flavonoid compounds (Neu and Neuhoff, 1957; Kartnig and Gobel, 1996; Schnitzler *et al.*, 1996) showing no or only a very weak autofluorescence under normal conditions. Although other compounds which posses a catechol structure (necessary for the staining-reaction) can not be excluded for such

reactions, flavonoids are known as the only group of phenols, which give an increase in yellow fluorescence after NA-staining. The NA-positive reaction provided evidence for the occurrence of unknown flavonoids within the vacuoles of the epidermal leaf cells in *X. caeruleum*. Additionally, it was shown that their contribution to the green/yellow autofluorescence of these cells before treatment was insignificant. This strongly supports the hypothesis that phenylphenalenone-related metabolites such as compound **13** indeed cause this autofluorescence in the epidermal leaf cells.

These present results raise the question about the function of phenylphenalenone-related compounds in the plant. Their role as phytoalexines and phytoanticipines are discussed since the production of phenylphenalenones was stimulated or induced after infection by fungi or nematodes in banana plants (Binks *et al.*, 1997; Kamo *et al.*, 1998). High accumulations of phenylphenalenone-related compounds in the region of cell division of *X. caeruleum* roots might indicate an additional protection for apical meristem cells against microorganisms and nematodes similar to root border cells, which play an important role in plant defence (Hawes *et al.*, 2000). However, bioassays had indicated activities of specific phenylphenalenones from banana plants against certain fungi (Luis *et al.*, 1995; Kamo *et al.*, 2001). The possible occurrence of phenylphenalenone-related compounds in the epidermal layer might also indicate a function as UV-B screening pigments as is known for other phenolic compounds such as flavonoids (Olsson *et al.*, 1998; Harborne and Williams, 2000).

6. Final discussion and conclusions

Xiphidium caeruleum was selected as a New World species of Haemodoraceae in order to compare the occurrence of phenylphenalenones and related compounds with those from the North American species *Lachnanthes tinctoria*. After the identification of a variety of phenylbenzoisochromenones in *X. caeruleum*, their structural characteristics raised questions about the biosynthetic relationship to typical phenylphenalenones. After isolation and identification, the distribution of phenylphenalenone-related compounds in *X. caeruleum* was studied.

The isolation and structure elucidation of a variety of phenylphenalenone-related compounds from *X. caeruleum* formed the basis of the research. Fourteen compounds, not including xiphidone as the only example of a phenylphenalenone that had been previously described from this species (Cremona and Edwards, 1974), were found, of which eleven are new natural products. Most notable were four glucosides bearing an allophanyl group, which represented a novel type of conjugates. Previously derivatives of allophanic acid had been isolated from only two species belonging to Asteraceae and Fabaceae plant families (Porwal *et al.*, 1988; Sharma *et al.*, 1988).

With subtypes such as tetraoxygenated phenylphenalenones, phenylbenzoisochromenones, oxabenzochrysenones and several glucosides, phenylphenalenone-related compounds of *X. caeruleum* displayed a great structural diversity. The phenylbenzoisochromenones were found to be the most abundant compounds, which had only been isolated from the North American *Lachnanthes tinctoria* (Edwards and Weiss, 1974). Additionally, three phenylbenzoisochromenones, which were known from *L. tinctoria* (Edwards and Weiss, 1972; 1974) were isolated from *X. caeruleum*, which indicated a close relationship between these species. The hypothesis that the phenylbenzoisochromenone-subtype is a feature exclusively of New World Haemodoraceae was refuted because of the identification of this subtype in the South African species *Wachendorfia thyrsiflora*. As described in chapter 2, this species contains phenylbenzoisochromenones and allophanylglucosides, which only have been found in species of the American continents so far. However, *W. thyrsiflora* also produces 9-phenylphenalenones with a completely deoxygenated B-ring (see chapter 3), which is a characteristic subtype of Australian genera such as *Anigozanthos* (Hölscher and Schneider, 1997). On this basis, the South African *W. thyrsiflora* can be considered to be a link between the Haemodoraceae of Australia and

America with regard to the occurring subtypes of phenylphenalenone-related compounds. The identification of the same phenylphenalenone-related compounds in plants of *X. caeruleum* and root cultures of *W. thyrsiflora* was important to understand the distribution pattern of structural subtypes worldwide. The distribution pattern of the structural subtypes of phenylphenalenone-related compounds supports the hypothesis of a Gondwanan origin of the Haemodoraceae (Simpson, 1990) and therefore reflects biogeographic characteristics of this family.

A variety of previous feeding experiments with ^{13}C and ^{14}C precursors revealed a phenylpropanoic biosynthetic pathway for phenylphenalenone-subtypes (Edwards *et al.*, 1972; Hölscher and Schneider, 1995; Schmitt and Schneider, 1999). However, the occurrence of phenylbenzoisochromenones mainly in the New World species of Haemodoraceae raised the question about a separate biosynthetic pathway for these compounds. After the identification of phenylbenzoisoichromenones in root cultures of *W. thyrsiflora*, feeding experiments with ^{13}C-labelled phenylalanine were performed on this species to answer this question. A comparison of the incorporation of ^{13}C-labelled phenylalanine into compounds of different subtypes proved the identical biosynthetic pathway for phenylphenalenones and phenylbenzoisochromenones, and therefore phenylbenzoisochromenones can be considered as oxidation products of phenylphenalenones. This led to the construction of a biosynthetic network for all phenylphenalenone-related compounds found in *X. caeruleum* (Fig. 3.8, chapter 3), which is similar to degradation patterns of aromatic compounds in plants (Ellis, 1974). Further studies on the isolation of phenylphenalenone-related compounds from different species or synthetic studies will test the validity of this scheme including its applicability to other species of the Haemodoroideae subfamily.

In addition to the origin and oxidative conversion of the tricyclic nucleus, the biosynthetic origin of methoxyl groups of phenylphenalenones was examined. Methionine, the common donor of methyl units (Ibrahim and Muzac, 2000), was shown to be the source of methoxyl substituents of phenylphenalenone-related compounds. Additionally, it was shown that one compound isolated from *X. caereuleum* bears an artificial methoxyl group due to methanolic extraction.

Jasmonic acid (JA) is known to stimulate the biosynthesis of defensive compounds (Farmer and Ryan, 1990). The use of JA in the performed feeding experiments raises some questions. Although JA stimulated strongly the biosynthesis of the phenylphenalenone anigorufone, the incorporation rate of ^{13}C-labelled phenylalanine corresponded to that of the control after one day of incubation. This can be interpreted as an upregulation of the

biosynthesis of this compound using non-labelled intermediates or other phenylphenalenones, which were stored in the plant. However, a longer time of incubation might give a different result since both a short time increase and a delayed increase of anigorufone were observed in root cultures after JA treatment. For a delayed increase, a *de novo* biosynthesis of anigorufone from phenylalanine could operate. In such a case, much higher incorporation rates of labelled phenylalanine into anigorufone caused by JA would occur. Additional kinetic feeding experiments might shed light on the matter.

After the identification of a variety of phenylphenalenone-related compounds from *X. caeruleum*, their distribution pattern in different plant organs was examined. The highest total amounts of the analysed compounds were found to be accumulated in roots, especially in root tips, and in the stamina of the flowers. Analysis of different leaf parts revealed higher total amounts of phenylphenalenone-related compounds in the sheath near the growing zone. The accumulation of the studied phenolics in reproductive organs and young tissues correlated with the observation for other classes of secondary metabolites such as alkaloids or glucosinolates (Rosenthal, 1991).

Some of the analysed phenylphenalenone-related compounds were found exclusively in roots or in shoots. In general, the distribution patterns were different between root and shoot system but more striking differences appeared in flowers, where a variety of unknown phenylphenalenone-related structures were detected. However, since the lack of information about the site of biosynthesis, transport, accumulation, degradation and function of individual compounds, the interpretation of the distribution pattern is problematic. However, these patterns are a prerequisite for further studies.

A possible defensive function was attributed to two phenylbenzoisochromenones as a consequence of a cutting injury experiment on plants of *X. caeruleum*. It was shown for the first time that plants of the Haemodoraceae react to wounding by an increase of phenylphenalenone-related compounds. In addition to the proven local reaction of the plant, strong indication was found for a systemic response. Furthermore, the patterns in the increase of the level of phenylphenalenone-related compounds in neighbouring unwounded plants raised the question about communication between plants of *X. caeruleum*. Such an inter-plant communication could be mediated by plant volatiles (Agrawal, 2000). Some evidences exist for wild tobacco *Nicotiana attenuata* (Karban *et al.*, 2000) and lima bean *Phaseolus lunatus* (Arimura *et al.*, 2000) where undamaged plants response to volatiles, released from wounded neighbors, by higher levels of resistance. The role of volatiles in inter-plant communication in *X. caeruleum* requires further investigations.

Strong autofluorescence of several phenylphenalenone-related compounds enabled microscopical studies on the accumulation of these phenolics in various tissues of *X. caeruleum*. Images of confocal laser scanning microscopy (CLSM) showed distinct autofluorescence mainly in vacuoles of various tissues from green to red wavelength ranges. Using microspectral photometry (MSP) techniques and HPLC-DAD analysis of extracts and isolated compounds, these autofluorescences were assigned to phenylphenalenone-related compounds. A single compound, (6-*O*-[(6”-*O*-allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one (**13**), was identified in yellow fluorescing vacuoles of root cortex cells. This glucoside showed a 150 fold higher fluorescence than its aglucone, which was much highly concentrated in the strong emitting root cells. Hence there is not a necessarily correlation between autofluorescence intensity and total concentration of phenylphenalenone-related compounds within cells or tissues.

MSP techniques were found to be suitable tools for the *in situ* detection of highly concentrated compounds in cells but were of limited value for the detection of low amounts as in case of red fluorescent root cortex cells or green emitting cells of leaf epidermis. However, using ‘Naturstoff’ reagent A, another phenolic group, the flavonoids which show green to yellow fluorescence, was proven to be insignificant for the autofluorescence seen in the CLSM images of leaf epidermis cells.

Phenylphenalenone-related compounds are accumulated in vacuoles of a variety of cells and tissues of the roots such as root cap, apical meristem, cortex, epidermis and root hairs. In the shoots they seem to be concentrated in the vacuoles of stem cortex and leaf epidermis cells. In addition to phenylphenalenone-related compounds within vacuoles, nothing is known about the extraprotoplasmatic occurrence, although other phenolics bind to cell walls or epicuticular wax layers (Strack *et al.*, 1988; Liakopoulos *et al.*, 2001). A similar scenario for phenylphenalenone-related compounds can be envisaged since yellow autofluorescences were seen at the surface of leaves and from cell walls of the vascular tissues.

Organ specific distribution studies revealed the highest accumulation of phenylphenalenone-related compounds in young parts of *X. caeruleum* plants. This was confirmed by the detection of large amounts of fluorescent compounds in the root tip, especially in the cells of the apical meristem. Since phenylphenalenones are described as phytoalexines (Luis *et al.*, 1993) and phytoanticipines (Luis *et al.*, 1997), the accumulation in the root tip might indicate an additional protection of the roots against microorganisms and nematods. The occurrence of phenylphenalenone-related compounds in epidermal cells of leaves raises the question about an additional function of such compounds in protection

against UV-B light, which is already known and studied for other phenolic compounds such as flavonoids (Olsson *et al.*, 1998; Harborne and Williams, 2000). The co-occurrence of phenylphenalenones and other phenolics such as flavonoids and phenylpropanoic acid conjugates within the epidermal cells of *X. caeruleum* could enable comparable studies on the reaction of *X. caeruleum* plants to UV-B light.

The great diversity of biosynthetically routes, as proposed in chapter 3, leads to the occurrence of a vast number of phenylphenalenone-related compounds in one species of the Haemodoraceae. Assuming a defensive function of this phenolic group, the diversity of structures might enable the plant to defend itself more successfully against enemies such as herbivores or pathogens. In terms of plant-herbivore coevolution, plants are forced to produce chemicals as feeding deterrents to which herbivores might respond by generating storage or detoxification mechanisms of these chemicals. The same could apply to plant-pathogen interactions. One consequence of such an arms race could be a diversification of defensive chemicals with various functions and synergistic effects. In this context, it might not surprise that *X. caeruleum* produces such a variety of phenylphenalenone-related compounds, of which only a limited number have been characterised in this work. The structural diversity of phenylphenalenone-related compounds raises questions about the special functions of certain structural types and their contribution to the plant's defence against herbivores, pathogens and UV-B light, as proposed in chapter 5.

Owing to the expense to produce secondary metabolites, a plant has to reach a balance between the costs and benefits of defensive compounds. The need for economy influences the distribution of these metabolites within a plant, leading to strong intraplant heterogeneity. The optimal defence theory predicts that defence metabolites should be located within a plant in proportion to the value of particular tissues for plant fitness and their vulnerability to herbivore or pathogen attack (McKey, 1974; 1979; Wallace and Eigenbrode, 2002). Patterns of accumulation of phenylphenalenone-related compounds, which are presented in this work, are consistent to this theory which predicts highest concentrations of defensive compounds in young growing parts and reproductive organs. In the most vulnerable tissues, damage to expending young tissue of root tips, shoot tips and youngest leaves might disrupt or distort plant's growth, leading to a much higher degree of damage in comparison to consequences of injury in older plant parts. Additionally, young tissue and reproductive organs, which directly contribute to plant fitness, might be especially attractive to herbivores because of its nutrition richness. In this context, a plant has to increase defensive properties in these parts. This

accounts for the organ- and tissue-specific distribution patterns of phenylphenalenones and related compounds, and strongly supports arguments for the defensive role of this phenolic group in plants.

7. Summary

Several aspects of the isolation, biosynthesis and accumulation pattern of phenylphenalenone-related compounds were studied in two species of the Haemodoraceae, *Xiphidium caeruleum* and *Wachendorfia thyrsiflora*. Phytochemical analysis of *X. caeruleum* resulted in the isolation and identification of a variety of phenylphenalenone-related compounds. The structures of four new phenylbenzoisochromenones and two new oxabenzochrysenones were elucidated using MS and NMR spectroscopic techniques. In addition, five new glucosides were identified, among them four allophanyl glucosides, representing a novel type of 6‘-substituted glucosidic natural products. Some of these compounds were also detected in root cultures of *W. thyrsiflora*. Taking phenylphenalenone pattern of further genera of the Haemodoraceae into account, these phenolics and their subtypes, respectively, reflect biogeographic characteristics of this plant family.

Feeding experiments with [1-^{13}C]phenylalanine with root cultures of *W. thyrsiflora* revealed the identical phenylpropanoic biosynthetic pathway for structures of both the phenylphenalenone- and the phenylbenzoisochromenone-subtype. This led to the construction of a biosynthetic network mainly based on all identified compounds found in *X. caeruleum*. Additional feeding experiments proved methionine as the donator for methoxyl substituents on phenylphenalenone-related compounds. Jasmonic acid showed no effect on incorporation rates of labelled precursors into studied compounds although it stimulated the biosynthesis of certain compounds.

The distribution pattern of phenylphenalenone-type compounds was investigated in vegetative and reproductive organs of *Xiphidium caeruleum*. Highest molar amounts of total number of studied compounds were found to be in the root tip and the stamen. In addition to compounds, which were found in all studied organs, certain metabolites were accumulated exclusively in the root or shoot system. Damage to leaves revealed a local reaction of wounded plants via an increase of the biosynthesis of certain phenylphenalenone-related compounds. Additionally, the indication of a systemic reaction and signalling between wounded and non-wounded plants has been discussed concerning the possible role of studied compounds in plant defence.

Exploring the autofluorescence potential of phenylphenalenone-related compounds several analytical techniques such as confocal laser scanning microscopy, microspectral photometry and HPLC analysis were combined to reveal specific distribution of the different

phenyphenalenones in distinct cells of the root and shoot system. 6-*O*-[(6”-*O*-Allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one was identified as the major fluorescent compound, responsible for the strong yellow fluorescence of the inner root cortex cells. Two other phenylphenalenones were attributed to fluorescence in the vacuoles of cells of the root cap and of the root epidermis including the root hairs. The autofluorescence of the vacuoles of leaf epidermal cells was assigned to phenylphenalenone-related compounds because it was demonstrated that flavonoids were not involved in the emission. Results gave new insights into the tissue-specific occurrence of phenylphenalenone-related compounds which are an essential prerequisite towards understanding their function within the plant.

8. Zusammenfassung

In der vorliegenden Arbeit wurden Untersuchungen zu Isolierung, Biosynthese und Speicherung von Phenylphenalenon-artigen Verbindungen an zwei Arten der Haemodoraceae, *Xiphidium caeruleum* und *Wachendorfia thyrsiflora*, durchgeführt. Dabei wurde eine Vielzahl von Phenylphenalenon-artigen Verbindungen aus *X. caeruleum* isoliert und identifiziert. Neben vier neuartigen Phenylbenzoisochromenon- und zwei neuen Oxabenzochrysenon-Verbindungen wurden fünf Glykoside identifiziert, von denen vier eine Allophanyl-Gruppe aufwiesen. Diese stellen eine neuartige Form 6'-substituierter Glykoside dar. Einige der in *X. caeruleum* gefundenen Verbindungen wurden ebenfalls in Wurzelkulturen von *W. thyrsiflora* nachgewiesen. Indem man bekannte Phenylphenalenone weiterer Gattungen in Betracht zog, konnte festgestellt werden, daß das Vorhandensein bestimmter Phenylphenalenon-artiger Verbindungen beziehungsweise deren Strukturtypen in den einzelnen Gattungen die biogeographische Verbreitung der Haemodoraceae widerspiegelt.

Fütterungsexperiments mit markiertem [1-^{13}C]Phenylalanin an Wurzelkulturen von *W. thyrsiflora* zeigten, daß Phenylphenalenone und Phenylbenzoisochromenone über denselben Biosyntheseweg gebildet werden. Darauf aufbauend wurde ein biosynthetisches Schema auf Grundlage aller in *X. caeruleum* gefundenen Verbindungen erstellt. Weitere Fütterungsexperimente wiesen Methionin als Quelle für Methoxyl-Substituenten Phenylphenalenon-artiger Verbindungen nach. Obwohl Jasmonsäure bei allen Fütterungen die Biosynthese bestimmter Verbindungen anregte, wurden damit keine höheren Einbauraten markierter Vorstufen in die Phenylphenalenone erzielt.

Die Verteilungsmuster Phenylphenalenon-artiger Verbindungen in vegetativen und generativen Organen von *X. caeruleum* zeigten die höchsten Gesamtkonzentrationen in Wurzelspitzen und den Staubblättern der Blüten. Während eine Vielzahl der untersuchten Verbindungen in der gesamten Pflanze vorkamen, waren einige ausschließlich in oberirdischen oder unterirdischen Pflanzenteilen zu finden. Nach Verwundung einiger Blätter reagierten Pflanzen von *X. caeruleum* mit einer Akkumulation einzelner Phenylphenalenon-artiger Verbindungen an der Stelle der Verletzung. Neben dieser lokalen Reaktion gab es Hinweise auf eine systemische Antwort der Pflanzen sowie eine mögliche Kommunikation zwischen verletzten und unverletzten Individuen. In diesem Zusammenhang wurde die Rolle Phenylphenalenon-artiger Verbindungen als Pflanzenabwehrstoffe diskutiert.

Unter Ausnutzung eines starken Eigenfluoreszenz-Verhaltens einzelner Phenylphenalenon-artiger Verbindungen wurden verschiedene analytische Techniken, wie z.B. Konfokale Laser Scanning Mikroskopie, Mikrospektral-Photometrie und HPLC, verwendet, um die zu untersuchenden Verbindungen in Zellen und Geweben zu lokalisieren. Dabei wurde 6-*O*-[(6”-*O*-Allophanyl)-ß-D-glucopyranosyl]-5-methoxy-7-phenyl-3*H*-benzo[*de*]isochromen-1-one als die am stärksten gelb-fluoreszierende Verbindung in den inneren Cortex-Zellen der Wurzel identifiziert. Zwei weitere Verbindungen konnten den Zellen der Wurzelhaube, Wurzelepidermis und den Vakuolen der Wurzelhaare zugeordnet werden. Phenylphenalenon-artige Verbindungen wurden als Ursache für Eigenfluoreszenzen in den Vakuolen der Blatterpidermis-Zellen angesehen, nachdem der Nachwies erbracht wurde, daß Flavonoide hierbei keine Rolle spielen. Der Einblick in die Gewebe-spezifische Verteilung stellt eine wichtige Grundlage für zukünftige Untersuchung der Funktionen Phenylphenalenon-artiger Verbindungen dar.

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Ich erkläre, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

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