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Exudate flavonoids in Primulaceae: comparative studies
of chemodiversity aspects

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D. lamingtonii (Photo: Eshlaghi)

In loving memory of my father,
who was dearer to me than life.

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Table of contents

Abstract	1
Zusammenfassung.....	2
Co-Authorship Statement	3
Introduction.....	4
Secondary metabolites (SM)	4
Flavonoids.....	4
Exudate flavonoids and glandular trichomes.....	7
Model Systems	8
<i>Primula</i>	8
<i>Dionysia</i>	9
Aims and outline of the thesis	12
References.....	14
Chapter 1: Exudate flavonoids of <i>Primula</i> spp.: Structural and biogenetic chemodiversity	24
Chapter 2: Chemodiversity of exudate flavonoids in <i>Dionysia</i> (Primulaceae): a comparative study	42
Chapter 3: Unusual compounds from exudates of <i>Dionysia diapensifolia</i> and <i>D. gaubae</i> var. <i>megalantha</i> (Primulaceae)	76
Chapter 4: Diversification of exudate flavonoid profiles in further <i>Primula</i> spp.....	91
Chapter 5: Flavonoids in selected <i>Primula</i> spp.: Bridging micromorphology with chemodiversity	103
Chapter 6: Antifungal orphan flavonoids and dihydrochalcones from <i>Primula</i> exudates.....	125
General Conclusion and Perspectives.....	146
Curriculum Vitae	148

Abstract

Exudate flavonoids can be defined as secondary metabolites that are accumulated on aerial surfaces of plants, as a result of excretions associated with the presence of glandular trichomes. Exudate flavonoids are common on alpine plants such as *Primula* L. and *Dionysia* Fenzl., two plant genera dealt with in this thesis. These genera are closely related and their distinction on the generic level is a subject of debate. According to the newest molecular results *Dionysia* should be placed within *Primula* sensu lato. One of the characters of *Primula* and *Dionysia* that catches the eye is the production of mealy or woolly farina. Oily excretions are just as common but not relevant as a character. These excretions, whether mealy, woolly or oily, consist of aberrant flavonoids such as unsubstituted flavone, 2'-OH-flavone, 3',4'-diOH-flavone and 8,2'-diOH-flavone, which vary from the usual flavonoids regarding their substitution pattern and are hence assumed to follow a different and yet unknown biosynthetic pathway. *Dionysia*, however, also exhibits flavonoids in its exudates that arise from the regular biosynthetic pathway. This thesis aims at isolation and identification of exudate flavonoids of these two genera and comparison of their chemosystematics on the behalf of exudate flavonoids. The isolated compounds are further tested for their antifungal properties. Chapters one, four, five and six mainly focus on exudates of *Primula* and discuss their chemodiversity. In chapter five micromorphology is combined with glandular trichome chemistry to study the localization of exudate flavonoids. Chapters two and three on the other hand deal with *Dionysia* and its relationship with *Primula*.

Zusammenfassung

Als Exkretflavonoide kann man jene Sekundärmetabolite bezeichnen, die mittels Drüsenhaare auf der Oberfläche oberirdischer Pflanzenteile akkumuliert werden. Exkretflavonoide kommen häufig bei alpinen Pflanzen vor, wie z.B. *Primula* L. und *Dionysia* Fenzl, den beiden in dieser Arbeit behandelten Pflanzengattungen. Letztere sind eng verwandt und ihre Unterscheidung auf Gattungsebene bleibt umstritten. Nach neuesten molekularen Ergebnissen sollte *Dionysia* innerhalb *Primula* sensu lato platziert werden. Zu den augenfälligsten Eigenschaften von *Primula* und *Dionysia* gehört die Produktion von mehligem oder wolligem Belag. Ölige Exsudate sind ebenso häufig, aber nicht relevant als Merkmal. Diese mehligem, wolligen oder öligen Exsudate bestehen aus aberranten Flavonoiden wie unsubstituiertes Flavon, 2'-OH-Flavon, 3',4'-diOH-Flavon und 8,2'-diOH-Flavon. Diese unterscheiden sich hinsichtlich ihrer Substitutionsmuster von üblichen Flavonoiden, weshalb davon ausgegangen wird, dass ihre Entstehung auf einem anderen und noch unbekannten Biosyntheseweg erfolgt. Die Gattung *Dionysia* weist jedoch auch Flavonoide in ihren Exsudaten auf, die aus dem regulären Biosyntheseweg entstehen. Die vorliegende Dissertation hat das Ziel, Exkretflavonoide dieser beiden Gattungen zu isolieren und identifizieren, und auf dieser Grundlage ihre Chemosystematik zu vergleichen. Die isolierten Verbindungen werden außerdem auf ihre antifungale Wirkung getestet. Kapitel eins, vier, fünf und sechs fokussieren vor allem auf Primelexkrete und diskutieren ihre Chemodiversität. In Kapitel fünf wird Mikromorphologie mit Drüsenchemie verbunden, um die Lokalisation von Exkretflavonoiden zu studieren. Kapitel zwei und drei beschäftigen sich schließlich mit der Gattung *Dionysia* und ihre Verwandtschaftsverhältnisse zu *Primula*.

Co-Authorship Statement

Chapters one to six have been prepared as papers in international journals. Elucidation of chemical structures by 2D NMR and mass spectrometry was always performed by Dr. Lothar Brecker and his team.

Chapter one has been published in Natural Product Communications in 2009, co-authored by Dr. Karin Maria Valant-Vetschera and Dr. Eckhard Wollenweber. My contribution to this manuscript consisted of microscopic work and assistance of manuscript preparation.

Chapter two has been published in Phytochemistry in 2010, co-authored by Dr. Karin Maria Valant-Vetschera and Dr. Eckhard Wollenweber. I was responsible for conducting HPLC analysis and assistance of manuscript preparation.

Chapter three has been published in Natural Product Communications in 2011, co-authored by Dr. Karin Maria Valant-Vetschera, Dr. Eberhard Lorbeer and Dr. Lothar Brecker. As the paper's first author, I was in charge of the lab work, data collection, data analysis, literature review and preparation of the first version of the manuscript.

Chapter four has been published in Natural Product Communications in 2012, co-authored by Dr. Karin Maria Valant-Vetschera. My contribution to this manuscript equals that mentioned for chapter three.

Chapter five has been published in Natural Product Communications in 2012, co-authored by Dr. Karin Maria Valant-Vetschera, Dr. Wolfram Adlassnig and Dr. Lothar Brecker. My contribution to this manuscript equals that mentioned for chapter three.

Chapter six has been submitted as a manuscript, co-authored by Dr. Karin Maria Valant-Vetschera and Dr. Lothar Brecker, to Phytochemistry and is currently under revision. My contribution to this manuscript equals that mentioned for chapter three.

Introduction

Secondary metabolites (SM)

Secondary plant metabolites consist of a large and diverse range of organic compounds, many of which do not appear to participate directly in growth and development or energy metabolism of a plant. More than 100,000 structures, including many nitrogen-containing compounds (such as alkaloids, alkalamides, peptides, amines, cyanogenic glycosides, glucosinolates and non-protein amino acids) and nitrogen-free compounds (such as phenolics, polyacetylenes, polyketides, saponins and terpenes) have been described (Wink, 2008). The real number of SM present in the plant kingdom, however, has been estimated to exceed 200,000. They have apparently evolved in plants as a defense strategy against various herbivores, bacteria, fungi and viruses, as well as against other competing plants (Croteau et al., 2000). SM are therefore of great use to plants and not merely waste products (Hartmann, 2007). Besides being useful as defence compounds, they also serve as chemical characters as shown by Hegnauer in his world-renowned compilation of plant chemotaxonomy (1962-1996). This monumental work focusses on different accumulation tendencies and distribution of secondary compounds within the plant kingdom and gives a very good overview of metabolic activities and their systematic significance in plants. Grayer et al. (1999) compared and found a high proportion of congruence between Hegnauer's chemical data and the molecular information presented by Chase et al. (1993). Chemical profiles can be used successfully to understand the relationships in plants, if used in conjunction with series of other characters (macromolecular, morphological, cytological, etc.) (Essokne et al., 2012).

Flavonoids

Flavonoids comprise a large class of secondary plant metabolites based on a C₆-C₃-C₆ skeleton. For structural formula see Fig. 1. Higher plants, together with bryophytes and pteridophytes, are the natural source of flavonoids. Over nine thousand flavonoids are

known till date (Williams and Grayer, 2004). They are divided into several classes, e.g. anthocyanins, flavones, flavonols, flavanones, dihydroflavonols, chalcones, aurones, flavan and proanthocyanidins, isoflavonoids, bioflavonoids, etc. (Iwashina, 2000). The structural differences between different flavonoid classes are related to the chemistry of the central (C) ring, variations in the number and distribution of phenolic hydroxyl groups across the molecules, and their substitutions (Lee et al., 2005).

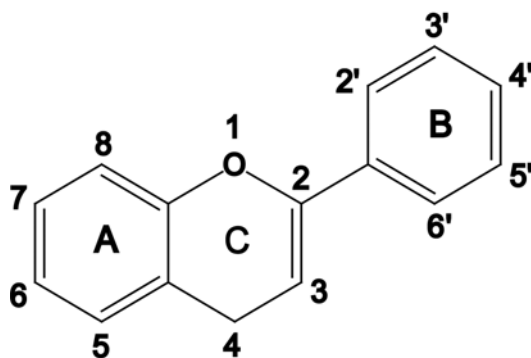


Figure 1: Basic structure of a flavonoid

Flavonoids are derived from phenylalanine and the acetate coenzyme A esters (Fig. 2). Through the enzymatic reaction of phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), 4-coumaroyl-CoA is produced. The latter acts as a substrate to start the flavonoid biosynthesis. Its condensation with three acetate units of malonyl-CoA yields 2',4',6',4'-tetrahydroxychalcone (THC) or naringenin chalcone. This reaction is catalyzed by chalcone synthase (CHS). Naringenin chalcone is the first flavonoid product of this biosynthetic sequence and by the action of chalcone isomerase (CHI) isomerizes to naringenin, a flavanone, which, as the key structure, gives rise to most of the other flavonoid classes (Davies and Schwinn, 2006).

Introduction

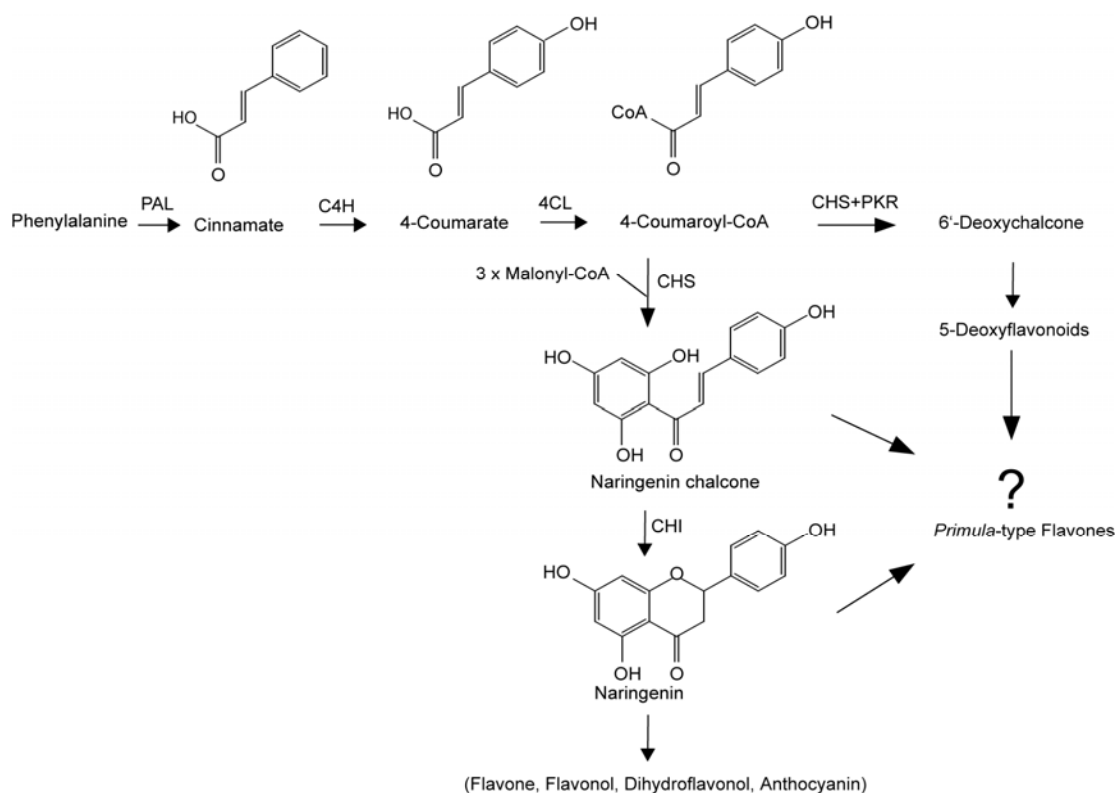


Figure 2: Simplified scheme of flavonoid biosynthesis. Adapted from Davies and Schwinn (2006).

Flavonoids are known to have a diverse range of functions in plants ranging from flower pigmentation, UV-protection, key signalling in plant-microbe interaction (Steinkellner et al., 2007), free radical scavenging, underground signalling in the nitrogen fixing legume-rhizobia symbiosis, defense against microbial pathogens and allelopathy (Star, 1980; Dixon and Steele, 1999; Harborne and Williams, 2000; Winkel-Shirley, 2001; Shaw and Hooker, 2008; Agati et al., 2012). Flavonoids are also known to be beneficial to human health since they possess antioxidant, anti-inflammatory, and anti-carcinogenic activity (Rice-Evans et al., 1996; Korkina, 2007; Werner et al., 2010). They are also known to prevent high blood pressure, harden veins and possess anti-aging and anti-bacterial activities (Yazaki et al., 2009). Much research is therefore being directed toward modifying the types and amounts of flavonoids produced by vegetables (Bovy et al., 2002; Sun et al., 2007; Cartea et al., 2011).

Flavonoids are among the first compounds to be used in taxonomic studies and are still used very frequently, since they exhibit great structural diversity and are widespread in the plant kingdom, chemically stable (well-preserved even in 100-year-old herbarium specimens) and reasonably easy to isolate and identify (Crawford, 1978; Wollenweber, 1989 (a); Essokne et al., 2012).

Exudate flavonoids and glandular trichomes

Exudate flavonoids are flavonoids that are excreted on the surface of aerial plant organs. They are known from several plant genera such as *Aesculus*, *Cheilanthes*, *Cistus*, *Dionysia*, *Eucryphia*, *Grindelia*, *Notholaena*, *Pityrogramma*, *Plectranthus*, *Populus*, *Primula*, *Salvia* and *Uncarina* (Greenaway et al., 1989; Iwashina, 2000; Wollenweber et al., 2000; Chaves et al., 2001; Nikolova et al., 2006; Yamazaki et al., 2007; Krenn et al., 2009; Grayer et al., 2010). Plenty of work has been done on the exudates of ferns (Wollenweber and Schneider, 2000 and references cited therein). Exudate flavonoids have proven useful in taxonomical studies (Wollenweber E. 1989 (b); Valant-Vetschera and Wollenweber, 2001; Grayer et al., 2004), since they exhibit a high degree of chemodiversity due to their structural variation. They are mostly found in the form of aglycones, although flavonoid glycosides have also been occasionally reported in surface exudates (Valant-Vetschera et al., 2003; Wollenweber et al., 2005; Krajsek et al., 2011).

The production of exudate flavonoids is invariably associated with the presence of glandular trichomes. This correlation has been recognized as early as 1945 by Blasdale and has been studied in later works such as Voirin et al., (1993), Bosabalidis et al., (1998), Spring et al., (1999), Fico et al., (2007) and Vitalini et al., (2011). Glandular trichomes are highly specialized epidermal protuberances on the surfaces of aerial plant organs. They can be unicellular or sophisticated multicellular structures exhibiting differentiated basal, stalk and apical secreting cells (Voirin et al., 1993; Werker, 2000; Wagner et al., 2004; Majdi et al., 2011). Glandular trichomes are generally capable of synthesizing, storing and occasionally secreting large quantities and diverse types of specialized metabolites, including various classes of terpenes (Yamaura et al., 1992; Spring et al., 2003; Lange and

Turner, 2012), several types of alkaloids (Zador and Jones, 1986) and flavonoids (Bosabalidis et al., 1998; Göpfert et al., 2006; Budzianowski and Wollenweber, 2007).

Model Systems

As will be demonstrated in the present thesis, *Primula* and *Dionysia* are suitable model genera for studying exudate flavonoids, their distribution, chemosystematics and functional aspects.

Primula

The genus *Primula* (the primroses) is one of the largest genera in Primulaceae Vent., comprising over 450 species divided into 7 subgenera and 38 sections according to Richards (2003). More than half of the species are found in the eastern Himalaya indicating this geographical area as the gene centre of the genus. Only few species are known to occur in South America, in the mountains of Africa (Ethiopia), Java and Sumatra (Richards, 2003). *Primula* species are alpine plants and are highly cultivated for their beautiful and attractive flowers as ornamental pot plants and in gardens (Richards, 2003). They are mostly perennial herbs with leafless stems. Some species have adapted themselves in lowland areas of Europe and North America (Richards, 2003; Jacquemyn et al., 2009; Kelso et al., 2009; Kovtonyuk and Goncharov, 2009). Much molecular research has been done to properly understand their taxonomy (Conti et al., 2000; Källersjö et al., 2000; Mast et al., 2001; Källersjö and Anderberg, 2002; Trift et al., 2002; Zhang et al., 2004; Guggisberg et al., 2006; Mast et al., 2006; Kovtonyuk and Goncharov, 2009). A few phytochemists have focussed on the medicinal value of this genus, which is frequently affiliated to the presence of saponins in rhizome and roots (Morozowska and Wesolowska, 2004; Budzianowski et al. 2005 and refs. cited therein; Kosenkova et al., 2008). Biological characters traditionally used for classifying this genus include heterostyly, chromosome number, pollen type, leaf vernation and presence or absence of farina (Mast et al., 2001; Richards, 2003).

Farina is a mealy excretion on the surface of leaves, stems, calyces or inflorescences. Besides *Primula* it is also known to occur in ferns (Wollenweber and Schneider, 2000 and refs. cited therein) and *Dionysia*. In *Primula*, presence of farina is known since almost a century and was long thought to have consisted only of flavone (Müller, 1915; Brunswik, 1922). Later studies have shown that it also consists of series of unusual substituted flavones such as 2'-OH-flavone, 3',4'-diOH-flavone and 8,2'-diOH-flavone (Wollenweber et al., 1988 (b) and refs. cited therein). Presence of farina is not obligatory for *Primula* species and the difference in the flavonoid composition between farinose and efarinose species is insignificant. However, it should be noted that production of exudate flavonoids, either as farinose or oily excretion, is universal among *Primula* species with glandular trichomes. A variety of cutaneous reactions associated to the compounds produced by the glandular trichomes have been described in several *Primula* species (Hausen et al., 1983; Horper and Marner, 1995; Christensen and Larsen, 2000; Aplin and Lovell, 2001). A number of studies elucidating the exudate flavonoid chemistry of the glandular trichomes in *Primula* species have been carried out (Wollenweber, 1974; Wollenweber and Mann, 1986; Wollenweber et al., 1988 (a), 1988 (b), 1989, 1990; Budzianowski et al., 2005; Budzianowski and Wollenweber, 2007). Biological effects of exudate flavonoids have been demonstrated in *P. denticulata* (Tokalov et al., 2004).

Dionysia

Dionysia is a genus belonging in *Primula* sensu lato. It comprises of about 50 species grouped in three main sections that are further divided into eight subsections (Grey-Wilson, 1989; Trift et al., 2004). *Dionysia* is an Irano-Turanian genus with its centre of diversity in arid mountains of the Iranian highlands. Eleven species have been reported from Afghanistan, three from Turkey, two each from Iraq, Turkmenistan and Tadjikistan, one from Oman and one from Pakistan (Trift et al., 2004). Most of the species are perennial, usually aromatic chasmophytes and grow in the crevices of cliffs and steep rocks as loose tufts or dense cushions (Fig. 3). The systematic position and taxonomic affinities of the genus have been much discussed (Wendelbo, 1961; Trift et al., 2004; Trift and Anderberg, 2006; Liden 2007). At one time, *Dionysia* was merged into *Primula* as a section (Kuntze,

1891). Later authors, however, treated it as a separate genus since they accepted a polyphyletic *Primula* (Liden, 2007 and refs. cited therein). Nevertheless, according to some of the recent molecular studies this genus is deeply nested within the genus *Primula*, its sister group being *Primula* subg. *Sphondylia* (Mast et al., 2001; Trift et al., 2002; Trift et al., 2006). This relationship was earlier refuted by Al Wadi and Richards (1992) on the basis of differentiation in pollen type, farina type, leaf vernation and chromosome number. They further emphasized that the resemblance between *Dionysia* and subg. *Sphondylia* could be a parallel evolution. Yet, due to the similarities and dissimilarities between *Dionysia* and *Primula* the uncertainty whether or not *Dionysia* should be included in *Primula* remains. Like *Primula*, *Dionysia* produces conspicuous excretions. However, in contrast to *Primula*, in which woolly farina is only reported from *P. qinghaiensis* till date, *Dionysia* is well characterized by the presence of woolly farina, with the exception of *D. microphylla*, *D. hedgei* and *D. involucrate*, which produce powdery farina. Furthermore, woody stems are typical for *Dionysia* species but very rare in *Primula* (Grey-Wilson, 1989). *Dionysia* further differs from most *Primula* species in the base chromosome number of 10, a long and narrow corolla tube, a fruit capsule that is few-seeded and splits to the base into 5 valves and a polycolpate pollen type.



Figure 3: a) *P. sikkimensis* b) *P. capitata* (East Sikkim, 4,500 m); c) *D. lamingtonii* d) *D. archibaldii* (Zagros mountains, 2,400 m) in their natural habitats

Aims and outline of the thesis

Comparative studies of organ- and tissue-specific accumulation of secondary metabolites are rarely carried out. Even in the case of plants that are aromatic and sticky and thus would be candidates for production of exudate compounds, no attention is paid to tissue-specific chemistry. Hence, compounds claimed to be present “in” the plant may occur rather “on” the plant. While taking this into consideration, the present thesis analyzes and summarizes the exudate flavonoids of *Primula*, a genus with fascinating exudate chemistry. Exudate flavonoids detected and identified in *Primula* hitherto (Wollenweber and Schnepf, 1970; Wollenweber, 1974; Wollenweber and Mann, 1986; Wollenweber et al., 1988 (a), 1988 (b), 1989, 1990; Iinuma et al., 2006; Budzianowski and Wollenweber, 2007) differ from regular flavonoids, which are produced by the well known biosynthetic pathway. The major aim of this thesis is to verify whether this accumulation trend is regular within the genus *Primula*. If so, is it reasonable to use the study of exudate flavonoids to discuss the chemosystematics of *Primula*.

It is also of interest to see how and where the biosynthesis of these unusual compounds takes place. In order to accomplish this, our regular chemical methods have been augmented by micromorphological analysis of glandular trichomes. Another purpose of this thesis is to study the exudate flavonoids of *Dionysia* and discuss its relationship with *Primula*. Despite its showy farina this genus has not been studied for its composition in detail. For all these works, a vital emphasis is placed on various chemodiversity aspects such as occurrence, organ- and tissue-specificity, and variation among populations by comparing their exudate flavonoid profiles.

This dissertation is arranged in the order of six manuscripts (five published and one under review).

Paper one, **Exudate Flavonoids of *Primula* spp: Structural and Biogenetic Chemodiversity**, focusses on the distribution of exudate flavonoids in several new *Primula*

accessions, including literature data and their correlation with the existing taxonomic views on the infrageneric groupings of *Primula* and related taxa.

Paper two, **Chemodiversity of exudate flavonoids in *Dionysia* (Primulaceae): A comparative study**, deals with 67 accessions of *Dionysia* species, including subspecies and some hybrid taxa, focussing on their chemodiversity with respect to the infraspecific variability and infrageneric distribution.

Paper three, **Unusual compounds from exudates of *Dionysia diapsifolia* and *D. gaubae* var. *megalantha* (Primulaceae)**, reports a finding of (*R*)-(+)-3-acetoxy-3-phenyl-propiophenone as a novel natural product and a sesquiterpene carissone from *D. gaubae* var. *megalantha*.

Paper four, **Diversification of exudate flavonoid profiles in further *Primula* spp.**, gives an overview of exudate flavonoids studied in 20 accessions as a continuation of paper one.

Paper five, **Flavonoids in selected *Primula* spp.: bridging micromorphology with chemodiversity**, reveals exudate flavonoids and their accumulation in glandular trichomes in two *Primula* species using epifluorescence microscopy.

Paper six, **Antifungal orphan flavonoids and dihydrochalcones from *Primula* exudates**, illustrates and discusses the isolation of four antifungal compounds, hitherto unknown from nature.

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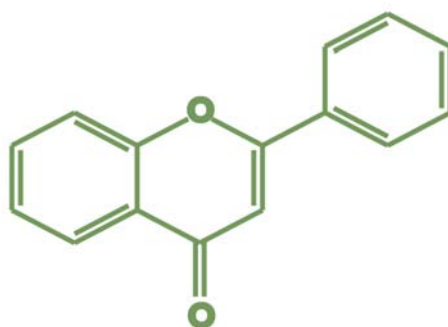
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Chapter 1: Exudate flavonoids of *Primula* spp.: Structural and biogenetic chemodiversity



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ABSTRACT

Several new accessions of the genus *Primula* and of the closely related *Cortusa matthioli* have been studied for their exudate flavonoid profiles. Unsubstituted flavone, 5-hydroxy-, 2'-hydroxyflavone and 5,2'-dihydroxyflavone were found as main components. Several other rather unusual substitution patterns were also found. 8-*O*-Substituted flavones appear to be accumulated more often than 6-*O*-substituted products. Chalcones with corresponding substitution patterns were of scattered occurrence, while flavanones could so far not be detected in exudates of *Primula* species. The distribution of externally accumulated flavonoids, including literature data, is related to current taxonomic views on the infrageneric grouping of *Primula* and related taxa. Organ-specific accumulation, biosynthetic and chemosystematic aspects are briefly addressed.

Keywords: *Primula*, *Cortusa*, flavones, chalcones, unusual substitution patterns.

The genus *Primula* comprises more than 400 species, subdivided into 6 subgenera and 37 sections. More than half of the species occur in the Eastern Sino-Himalaya, which appears to be the primary center of diversification. Several species grow in alpine regions of Europe and North America, and quite a number of species and hybrids are well known ornamentals [1]. A series of papers deals with the molecular systematics of the genus and/or some of its subgeneric groups [2a-2e]. Apart from heterostyly, for which many *Primula* species are well known, another phenomenon catches the eye: the production of farinose glandular secretions, which give the aerial parts a mealy touch. Again, this is not typical of all species: Farinose exudates may be restricted to specific organs, such as calyces, stems, entire inflorescences, and/or leaves. Some taxa or some parts of farinose plants produce less conspicuous oily secretions from glandular structures. The production of farina is not correlated to taxonomic groups, and both farinose and non-farinose species are found in related assemblages [2d]. For a comparative study of the morphology of the glands and the flavonoid chemistry of both oil and farina producing species see [3a]. In continuation of previous research [3b,3c] we wish to report on 17 newly studied species, and on the closely related *Cortusa matthioli* L. The results are compared with published data.

Twenty-seven flavones and 3 chalcones have so far been detected as exudate constituents from aerial parts of 36 *Primula* species. The major exudate constituent is unsubstituted flavone, as has been known for almost 100 years. In contrast to early assumptions, it is accompanied by a series of further flavones [3d]. Typical substitution patterns include inter alia 2'-, 5-, 5,2'-, 5,8- and 5,8,2'-hydroxylations and partly also methoxylation. Early studies revealed the presence of 3',4'-diOH flavone and a possible 5'-OH derivative thereof in several *Primula* spp. and related genera [4a,4b]. It was then assumed that 3',4'-diOH flavone occurs as 4'-glycoside in leaf tissues, and the chemosystematic value was discussed [4b]. 8-*O*-Substitution is found more frequently than 6-*O*-substitution. In some rare cases, methylene- dioxy structures or benzoate occur as substituents. The occurrence of chalcones is very limited, and their substitution patterns

correspond to those of the co-occurring flavones, indicating biogenetic relationships. Earlier, 3,3'-diOH chalcone was reported from *P. macrophylla* D. Don. [4c]. However, the published NMR-data are indicative rather of 2,2'- diOH substitution, and, therefore, 3,3'-hydroxylation can be excluded (F.J.Stevens, pers. comm.). Interestingly, no flavanones have so far been detected in the exudate of *Primula* species.

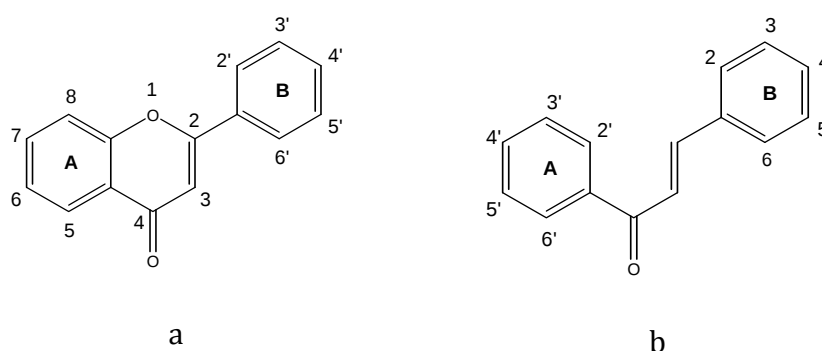


Figure 1: Basic structures of flavone (a) and chalcone (b).

Our current phytochemical results and available published data are combined in Table 1. Some of the *Primula* species studied earlier in EW's lab were now subjected to a more detailed analysis, leading to the detection of several additional compounds. All newly detected flavones, as well as those of newly studied species, are marked in bold letters; taxa of which inflorescences only were analyzed, are marked with an asterisk in Table 1. Two flavones, 5-OH-6,2'-diOMe flavone (trace constituent only in exudates of *P. denticulata* Smith [5a]) and 8,2'-diOH-5'-*O*-benzoate (trace constituent from exudates of *P. palinuri* Petagn. [3c]) were not included in Table 1. So far, the examples studied for organ specific accumulation (leaves/stems/inflorescences) yielded no consistent differences and need to be investigated more deeply, also with respect to glandular hair types. Species arrangement follows phylogenetically based suggestions in large parts [2c,2d]. In cases where no sequence studies were available, sectional alignment follows [1]. Generally, the subgeneric

classification is still controversial, with 6 clades being recognized partly across sectional boundaries. This is especially true for species of the subgenus *Aleuritia*, which come in two clades, separated by species from subgenus *Primula*. The groups which are based upon these recent data [1,2c,2d] are color-coded in Table 1. *Cortusa matthioli* has been included in the present study because of its close affinity with *Primula*, forming a well supported clade, together with species of *Primula* subgen. *Auganthus* [2d]. The exudate flavonoid profiles observed in the subgeneric units will be discussed separately. For basic flavonoid and chalcone structures, see Figure 1.

Subgen. *Sphondylia* (Duby) Rupr.:

This subgenus comprises eight species only [1] and appears to be very close to the genus *Dionysia* [2c]. The present study revealed a relatively simple profile, consisting mainly of flavone, 2'-OH-flavone and its methyl ether, along with 5-OH-flavone (primuletin). *P. verticillata* Forsskal exhibited a more complex profile. 3'-O-Substituted flavones proved to be rather rare in this group. The cultivar *P. kewensis* W.Wats., a cross between *P. floribunda* Wall. and *P. verticillata*, was studied earlier [3d,5b], and corresponds well in its accumulation tendencies.

Subgen. *Aleuritia* (Duby) Wendelbo:

This subgenus is quite large and divided into several sections, being dispersed along the phylogenetic cladogram in 3 different clusters, which is interpreted as an indicator of being polyphyletic. This assumption is further supported on morphological grounds [2c]. The exudate profile of sect. *Sikkimensis* is quite similar to that of the preceding group, except for 5,8,2'-trihydroxy flavone, which was detected in all of the 4 species from this section. The newly analyzed exudate profile of *P. ioessa* Smith, corresponds largely to the known profiles of its sectional mates [3d,5b]. Species from different sections within subgen. *Aleuritia* hardly differ in terms of major substitution tendencies, except for *P. capitata*

Hook., which exhibits a chemically more diverse profile. Some compounds are new for the species studied earlier [3d,5a,5b]. *P. capitata* is also exceptional as it is the only species from this subgenus accumulating a 6-*O*-substituted flavone (5-OH-6-OMe-flavone). Also, the rare compounds 5-OH -8,2'-diOMe flavone and 2'-OH flavone-5-*O*-acetate were detected now, for the first time, in the exudate of this species.

Species from sect. *Oreophlomis* were studied here for the first time. Their exudate profile proved to be quite simple, consisting of flavone, 2'-OH-flavone, 5-OH-flavone and 5,2'-diOH-flavone. Representatives of the following section *Aleuritia* do not appear to be fundamentally different from the other members of subgen. *Aleuritia*. Phylogenetic studies indicate this section to be monophyletic, being one of the most widespread alpine groups within *Primula* in the Eurasiatic Mountains [2b]. *Primula mistassinica* Michaux showed the additional presence of 2'-OH flavone-5-*O*-acetate. *Primula chionantha* Balf.f. & Forrest is the only species studied so far from the larger section *Crystallophlomis*. Its two subspecies cannot be distinguished on the basis of the exudate flavonoid profile. Also, the profile does not allow differentiation from the other members of the subgenus.

Section *Prolifera* is sometimes assumed to be a sister to sect. *Sikkimensis* [2d]. Observed exudate profiles differ by being chemically more diverse than those of species from sect. *Sikkimensis* (Table 1), with main compounds being 5-OH-flavone, 5,2'-diOH-flavone, 5,8-diOH-flavone, and 5,8,2'-triOH-flavone in addition to flavone and 2'-OH-flavone [3a,3d,5b-5e]. *Primula pulverulenta* Duthie and *P. bulleyana* Forrest also accumulate chalcones. 5,8-DiOH flavone (primetin) is reported now for *P. cockburniana* Hemsl., but has not been found in the other species listed here (Table 1).

Subgenus *Primula* L.:

This small subgenus consists of two sections with only a few species, and its members clade together with species from sect. *Prolifera* of subgen. *Aleuritia* [2d].

Flavonoid data exist on the common species *P. elatior* Hill [3b] and *P. veris* L. [6a]. Unusual substitution tendencies are represented by formation of 5-OH-flavone based structures exhibiting unusual 2',6'- substitution in ring B. Furthermore, the typical *Primula* constituent, unsubstituted flavone, could be detected only in trace amounts in *P. elatior* [3b]. There are also further notable differences observed when these species are compared with other taxa of the genus [3b]. This is expressed by a tendency to accumulate a number of 3',4'-disubstituted and 3',4',5'-trisubstituted flavones lacking a 5-OH group in exudates of both species. *P. veris* differs by accumulating flavone and 2'-OH-flavone as major components [6a]. 5-OH flavone and 5,2'-diOH-flavone have not been detected so far in either species. In case this tendency is stable within this subgenus, it might merit systematic significance. It should be mentioned that these taxa bear only oil-secreting glands and hence do not produce farinose excretions like the other species studied here. At present, it cannot be ascertained that the different exudate chemistry is correlated to production of oily secretions in this case.

Subgen. *Auriculastrum* Schott.:

Botanical characters have frequently led to the opinion that subgen. *Auriculastrum* was another basal group in the genus *Primula*, but this assumption could not be confirmed by molecular studies [2d]. In comparison to other studied groups, the exudate composition of the few species analyzed here appears to be chemically less diverse, except for *P. palinuri*, of which the inflorescences exhibit a diversified profile [5b-5d]. Besides chalcones, also complex structures (8,2'-diOH-5'-*O*-benzoate) have been identified from this species recently [3c]. The exudate profile differs largely from that of aerial parts of the closely related *P. auriculata* Lam.

Table 1: Exudate flavonoids from *Primula* spp. and *Cortusa matthioli*.

<i>Primula</i>	Sectional Alignment	2'-β-diOH-chalcone	2'-2-diOH-chalcone	2'-OH-2-OMe-chalcone	flavone	2'-OH-flavone	2'-OMe-flavone	2',5'-diOH-flavone	2',5'-diOMe-flavone	2'-OH-5'-OAc-flavone	8,2'-diOH-flavone	3'-OMe-flavone	3',4'-diOH-flavone	3'-OH-4',5'-diOMe-flavone	3',4'-diOMe-flavone	3',5'-diOH-4'-OMe-flavone	3',4',5'-triOMe-flavone	3'-OH-4',5'-MetDiox-flavone	3'OMe-4',5'-MetDiox-flavone	5-OH-flavone	5,2'-diOH-flavone	5-OH-2'-OMe-flavone	5,2',5'-triOH-flavone	5,8-diOH-flavone	5,8,2'-triOH-flavone	5-OH-8,2'-diOMe-flavone	5-OH-6-OMe-flavone	5,6,2',6'-tetraOMe-flavone	5,6,2',3',6'-pentaOMe-flavone	Flavonoid References	
<i>verticillata</i>	SS				0	0	0	t				0							0	0			0								
<i>boveana</i>	SS				0	0	0												0												
<i>gaubeana</i>	SS				0	0																									
<i>kewensis</i>	SS				0	t						t	t						0					t							3d,5b
<i>florindae</i>	ALS				0	0	0						t							0	0			0	0						3d,5b
<i>loessa</i>	ALS				0	0													0	0			t	0							
<i>waltonii</i>	ALS				0	0							0						0	0			0	0							3d
<i>alpicola</i>	ALS				0	0							t						0	0			0	0							3d
<i>nutans</i>	ALA		t		0	t		t					t						0												5b
<i>capitata</i>	ALC				0	0	0	t	t		t	t	t		t				0	t	t					t	t				3d,5b
<i>denticulata</i>	ALD		0		0	0													0				0								3d,5a,5b
<i>vialii</i>	ALM				0	0							t						0	t			t								3d
<i>auriculata</i>	ALO				0	t													0	t											
<i>luteola</i>	ALO				0	0													0	0											
<i>farinosa</i>	ALAL				0	0							t						0	0			0								3d
<i>frondosa</i>	ALAL				0	0													0	t											3d
<i>halleri</i>	ALAL				0	0	0	0	t										0	0	0		0								3d,5b
<i>mistassinica</i>	ALAL				0	0				t									0	t			0								
<i>vuparensis</i>	ALAL				0	0													0				t								
<i>chionantha subsp. melanops</i>	ALCR				0	0							t						0	t			0								
<i>chionantha subsp. sino-purpurea</i>	ALCR				0	0							t						0	t			0								
<i>elatior</i>	P				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0									0		3b
<i>veris</i>	P				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0								0			6a
<i>cockburniana</i>	ALP				0	0		t			t	t							0	0			0			0					
<i>bulleyana subsp. beesiana</i> *	ALP				0	0	t		0	t		t							0	0											3a,3d,5b,5c
<i>bulleyana</i>	ALP		0		0	0	0	0		0	t		t						0	t											3d,5b,5c,5d
<i>japonica</i>	ALP				0	0	0	0		0			t						t												3d,5b,5d
<i>pulverulenta</i>	ALP		0		0	0		t			t			t					0	0		t									3d,5b,5c,5d,5e
<i>marginata</i>	AURA				0	0													0				0								3d
<i>albenensis</i>	AURA				0	t							t		t																
<i>auricula</i>	AURA				0	0							t		t				0	t			0								3d
<i>palinuri</i> *	AURA	t	0	0	0	0	0	0	0	0	0								0	t					0						3c,5b,5c,5d,5e
<i>rusbyi</i>	AURP				0	0	0	t		t		0							0	0											
<i>forrestii</i>	AUGBU		0		0	0													0	t			0			t					
<i>malacoides</i> *	AUGMO				0	0	t	t						t		t			0	0		t	0	0							3d
<i>polyneura</i>	AUGCO		0	0	0	t	t	t				t		t		t			0												5b
<i>sinensis</i>	AUGAUG				0	0						0							0									0			3d
<i>Cortusa matthioli</i>			0		0		0								t				0		0										
<i>C. matthioli</i> var. <i>turkestanica</i>			t		0														0												

Abbrev.: SS (*Sphondylia* sect. *Sphondylia*), ALS (*Aleuritia* sect. *Sikkimensis*), ALA (*Aleuritia* sect. *Armerina*), ALC (*Aleuritia* sect. *Capitatae*), ALD (*Aleuritia* sect. *Denticulatae*), ALM (*Aleuritia* sect. *Muscaroides*), ALO (*Aleuritia* sect. *Oreophlomis*), ALAL (*Aleuritia* sect. *Aleuritia*), ALCR (*Aleuritia* sect. *Crystallophlomis*), P (*Primula*), ALP (*Aleuritia* sect. *Proliferae*), AURA (*Auriculastrum* sect. *Auricula*), AURP (*Auriculastrum* sect. *Parryi*), AUGBU (*Auganthus* sect. *Bullatae*), AUGMO (*Auganthus* sect. *Monocarpicae*), AUGCO (*Auganthus* sect. *Cortusoides*), AUGAUG (*Auganthus* sect. *Auganthus*). Color coding refers to phylogenetic position estimated from molecular data; bold occurrences indicate new findings. 0= large amounts; t= trace amounts; *=inflorescence.

Subgen. *Auganthus* (Link) Wendelbo:

The species listed here belong to different sections of this subgenus. Major compounds are represented by flavone and 5-OH-flavone in all species studied; 5,2'-diOH flavone is shared by *P. forrestii* Balf.f. and *P. malacoides* Franchet, of which inflorescences were analyzed. The phylogenetic study of [2d] fully supported the inclusion of *Cortusa* in the genus *Primula*, subgen. *Auganthus*, and there appear to be further connections between this subgenus and species of subgen. *Aleuritia*. On the basis of exudate flavonoid composition, *Cortusa matthioli* appears to be quite close to *Primula*, but exhibits a less diversified profile. Both accessions differ, however, in their flavonoid complement, thus indicating variation at the subspecies level.

DISCUSSION

The present results raise a series of questions regarding aspects of chemodiversity. First, the substitution patterns of exudate flavonoids have so far not been observed in exudates of other taxonomic groups [6b]. They thus appear to be unique for *Primula* and its closest allies, despite the fact that only very few species of the large genus *Primula* have been studied so far. Biosynthesis of unsubstituted flavone and related compounds has not yet been elucidated, and an aberrant pathway was suggested [3c]. It may be assumed that a retrochalcone route could be involved which would be suggested by the substitution pattern of ring B (K. Stich, pers. comm.). Substitution patterns of flavones such as in positions 2' and 6' on ring B are of rare occurrence and may be related to the speculated aberrant biosynthetic route. Furthermore, chalcones show similar substitution patterns as the co-occurring flavones. Proof that flavonoid biosynthesis takes place in glandular cells comes from an earlier study by immunofluorescence methods [6c], detecting the presence of chalcone synthase. It should also be noted that corresponding flavanones have so far not been found in exudates of *Primula* species.

Second, organ specificity in terms of both biosynthetic and storage capacity should be considered. Interestingly, the aglycones present in exudates are rarely accumulated as tissue glycosides. Exceptions known are *P. auricula* L., which accumulates 7,2'-diOHflavone as 7-*O*-glucoside in leaf tissue [6d], and earlier reports on the accumulation of presumed 4'-*O*-glycosides of 3',4'-diOH flavone [4b]. Mostly, the "normal" flavones with regular 5,7-substitution are found in glycosylated form [6d]. Studying the biosynthesis in *Primula* glands proves to be quite problematic, since glands are difficult to be isolated physically from leaf surfaces. Since some flavones are auto-fluorescent, they can be detected in intact glands by fluorescence microscopy, as is shown by preliminary studies of *P. vialii* Delavay ex Franch (Figure 2). For morphology of the glands, see also [3a,6c,6d].

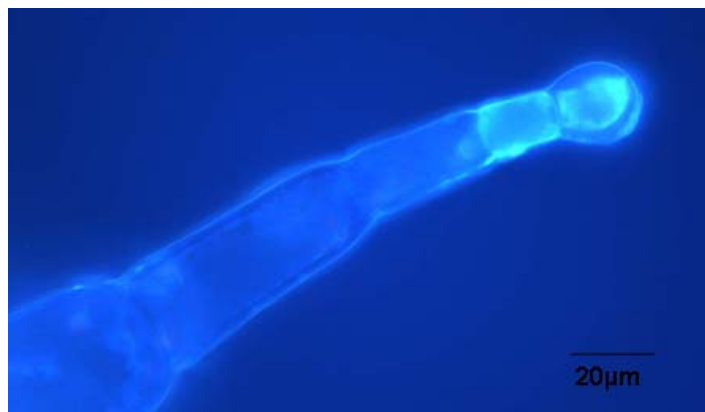


Figure 2: Capitate glandular trichome of *Primula vialii* (leaves).

Third, correlation between morphology of gland types, production of farinose, and exudate flavonoid composition is not clearly seen on the basis of the present results. *Primula palinuri* affords an example of different types of excretion: short glandular hairs on the leaves produce “oily” secretions, while a white meal is found on calyces and stems [1]. The exudate flavonoid profile obtained from the floral region is quite diverse, as stated earlier, containing also chalcones. The latter are not found in exudates from inflorescences of *P. malacoides*, a taxon with mealy stems and flowers, or of *P. bulleyana* Forrest subsp. *beesiana* (Forrest) A.J.Richards, with stems being heavily mealy (Table 1; for botanical characters see [1]). Earlier, the occurrence of 2,2'-diOH chalcone was reported for vegetative parts in several *Primula* spp., but the same compound was not detected in the floral region [5b]. A more detailed analysis to test correlations between chemical and morphological differentiations (glandular structures) will be carried out. Ecological aspects relating to exudate formation merit attention. Many *Primula* spp. occur at higher altitudes, ranging up to 4000 m or higher in Asia. However, they do not grow in dry places, but prefer a certain degree of humidity of soil [1]. Thus, accumulation of anti-oxidative and radical

scavenging flavonoids is most certainly related to increasing UV-radiation at higher altitudes. A study in this respect was recently published [6e].

Finally, the question is raised if substitution profiles may be used as chemical characters for this genus. It is not easy to group the flavonoids according to substitution patterns, particularly as the sequence of progressive oxidation is not clear. It is, for example, difficult to judge if enzymes responsible for B-ring substitutions may use flavone, 5-OH-flavone and 5-OH-8-OMe flavone as substrates. The series of substitution patterns is remarkable: 5,8-substitution, 5,6-substitution on ring A; 3',4',5'-substitutions or 2',5'-substitutions on ring B of flavone (no OH-groups on ring A); or 2',3',6'-substitutions of 5-OH flavone (see also Table 1). Taking these variations of substitution patterns, one can only see clearly that subgen. *Primula* differs from the remaining *Primula* species, and that only species of *Aleuritia* sect. *Sikkimensis* accumulate 5,8,2'-substituted flavone in their exudates. Several species groups appear to be chemically simple in terms of substitution tendencies, such as subgen. *Sphondylia*. By contrast, the studied species of sect. *Proliferae*, which are part of the polyphyletic subgen. *Aleuritia*, exhibit a more diverse profile. Exudate profiles of species from the monophyletic sect. *Aleuritia* of the same subgenus do not differ fundamentally from that of polyphyletic groups, which indicates that the phylogenetic status is not correlated with flavonoid chemistry. Grouping the studied *Primula* species by their exudate flavonoid profiles would lead to a different arrangement in Table 1, both within closely and more distantly related species groups. This observation is paralleled by partly uncorrelated distribution of botanical characters (chromosome numbers, pollen structures, heterostyly and others) in several cases along the DNA-based phylogenetic tree. Genera that are nested within *Primula* clades would be assumed to exhibit similar accumulation tendencies of exudate flavonoids. This is only partly true: *Cortusa* (nested in subgen. *Auganthus*) shows a more simple profile than most *Primula* species. Several *Dionysia* species (grouped with subgen. *Sphondylia*) [2d] also accumulate flavanones with the common substitution pattern, partly in addition to or even instead of the typical *Primula* structures (Wollenweber et al., in prep.). In view of the partly unresolved

phylogenies of *Primula* and related genera, the assessment of the chemosystematic significance of exudate flavonoid profiles is unsatisfactory at present. Further detailed studies are needed to reveal if specific flavonoid structures and maybe also the degree of complexity of profiles can be useful chemical characters. Systematic relationships should become clearer once the biosynthesis of *Primula* flavonoids has been elucidated and more plant material is available for comparative studies.

EXPERIMENTAL

Plant material was received from the Botanical Garden München-Nymphenburg; voucher specimens are deposited in the Herbarium of the Botanische Staatssammlung München (MU). The newly studied species include: *Primula verticillata*, *P. boveana*, *P. gaubeana*, *P. ioessa*, *P. nutans*, *P. auriculata*, *P. luteola*, *P. mistassinica*, *P. yuparensis*, *P. chionantha* subsp. *melanops*, *P. chionantha* subsp. *sino-purpurea*, *P. cockburniana*, *P. albenensis*, *P. rusbyi*, *P. forrestii*, *P. malacoides*, *P. polyneura*, *Cortusa matthioli* and *C. matthioli* var. *turkestanica*.

Aerial parts of the collected plant species were briefly rinsed with acetone in order to dissolve the lipophilic exudate material. The mixture obtained after evaporation was then analysed, as described previously [3b]. Comparative TLC with markers was carried out on polyamide with the solvents: A) Light petroleum₁₀₀₋₁₄₀/toluene/MeCOEt/MeOH 12:6:1:1; B) toluene/light petroleum₁₀₀₋₁₄₀/MeCOEt/MeOH 12:6:2:1; C) toluene/MeCOEt/MeOH 12:5:3; and D) toluene/dioxane/MeOH 8:1:1; and on silica with solvents E) toluene/MeCOEt 9:1; and F) toluene/dioxane/HOAc 18:5:1. Chromatograms were viewed under an UV lamp (366nm) before and after spraying with "Naturstoffreagenz A" (0.2% of diphenyl-boric acid 2-aminoethyl ester in MeOH). Authentic markers for the identification of the flavonoids were available in E.W.'s laboratory. Whenever necessary, UV-spectra and mass spectra were recorded for supplementary structural confirmation. Sections for light microscopy were cut with the help of a razor blade and viewed without staining with a light microscope (Olympus BX41, Epifluorescence) under UV excitation.

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**Chapter 2: Chemodiversity of exudate flavonoids in *Dionysia*
(Primulaceae): a comparative study**



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ABSTRACT

More than 60 accessions of various *Dionysia* spp. were analysed for their exudate flavonoid composition. Many *Dionysia* spp. accumulate the typical *Primula* flavonoids with irregular substitution (unsubstituted flavone, its 2',5'-substituted derivatives and corresponding 5-OH-flavones), but flavones, flavonols and flavanones with regular 5,7-diOH-substitution are also encountered in their exudates. The formation of both types of flavonoids is not mutually exclusive. This paper analyses the chemodiversity of *Dionysia* exudates with respect to infraspecific variability, infrageneric distribution, patterns in hybrid taxa, and comparisons of biogenetic tendencies between *Dionysia* and closest related species of *Primula*. The uniqueness of occurrence of *Primula*-type flavonoids in the family Primulaceae, and their presumed different biosynthetic origin, suggests significance as further character in the *Primula-Dionysia* assemblage. Principal component analysis was applied to test the significance of variation of flavonoid composition across *Dionysia*. Comparative analysis of flavonoid profiles against the current taxonomic views yielded correlations, confined to the level of smaller groups, and only in parts at level of the current infrageneric concept. Flavonoid data are further discussed against the background of morphological and biogeographic differentiation of the genus. Increased diversification of flavonoid profiles may be interpreted as a derived status in *Dionysia*, which agrees with current views on the phylogeny of *Dionysia* as a specialised group within *Primula*. Functional aspects of exudate flavonoid formation are shortly addressed.

Keywords:

Dionysia; Primulaceae; Exudate constituents: flavones, flavonols, flavanones; Diversification; Taxonomy and phylogeny

1. INTRODUCTION

About fifty species are currently known under the generic name *Dionysia*, which according to recent molecular data, form a monophyletic group deeply nested within groups of the genus *Primula* (Mast et al., 2001; Trift et al., 2004). Even earlier views consider “*Dionysia* represents a blind alley in the scheme of evolution- it is more or less unsuccessful, but so far the best attempt of *Primula* to enter the domaine of the xeromorphs” (Wendelbo, 1961). This quotation refers to the growth habit of most of the *Dionysias*: fruticulose, loose or dense tufts and cushions, reduced leaves – all adaptations to grow under extreme conditions such as in vertical niches or overhanging cliff walls at higher altitudes. However, *Dionysia* spp. are not really xerophytic, but need a certain amount of water supply and shade that is provided in these niches (Wendelbo, 1961). Apart from the extreme growth form, typical characters of *Dionysia* are the base chromosome number of 10, the occurrence of polycolpate pollen, and the five-valved capsule, all uncommon in *Primula* (Trift et al., 2004). The distribution area is Irano-Turanian, with the majority of species found in the Zagros Mountains, partly as endemics. The Iranian Alburz mountain range harbours fewer species, and others occur exclusively in Afghanistan. Only a few taxa extend into Turkey, Turkmenistan, Oman and Pakistan. Recently, the phylogeny of this genus was analysed by sequence data, which were not fully in accordance with morphological differentiations, and there is difficulty finding diagnostic characters that are related to the evolution of this genus (Trift et al., 2004). Analysis of leaf sclereid characters, combined with molecular and biogeographic data (Trift and Anderberg, 2006), gave the basis for a recent revision by Lidén (2007), who maintained *Dionysia* as a genus for convenience.

The production of waxy or farinose coatings on aerial parts including the floral region is a major character of *Dionysia* and *Primula*. In *Dionysia*, farina may have either a woolly or powdery consistency, but some of the species are efarinose, a character sometimes used for infrageneric classification (Trift et al., 2004). In contrast to *Primula*,

Dionysia leaves exhibit an aromatic smell (Melchior, 1943). In *Primula*, farina and oily exudates consist of unsubstituted flavone and some further biogenetically rare types of flavones (Valant-Vetschera et al., 2009 and references cited therein). Earlier, the presence of some of these flavones was reported in hydrolysates of some *Primula* and *Dionysia* species (Harborne 1968, 1971). Whether the anti-tumour activity of the Iranian medicinal plant *Dionysia termeana* Wendelbo is related to the presence of flavonoids, has not been specified (Zahra et al., 2007). Preliminary analyses of exudate flavonoid composition in *Dionysia* indicated chemical similarities to *Primula* exudates concerning major accumulation tendencies. Larger sampling, consisting in parts of the same material that was studied phylogenetically by Trift et al. (2002, 2004) and Trift and Anderberg (2006) became available for comparative studies at the infra- and interspecific level. The results are discussed in relation to morphological features and current taxonomic views, also with regard to the closely related genus *Primula*.

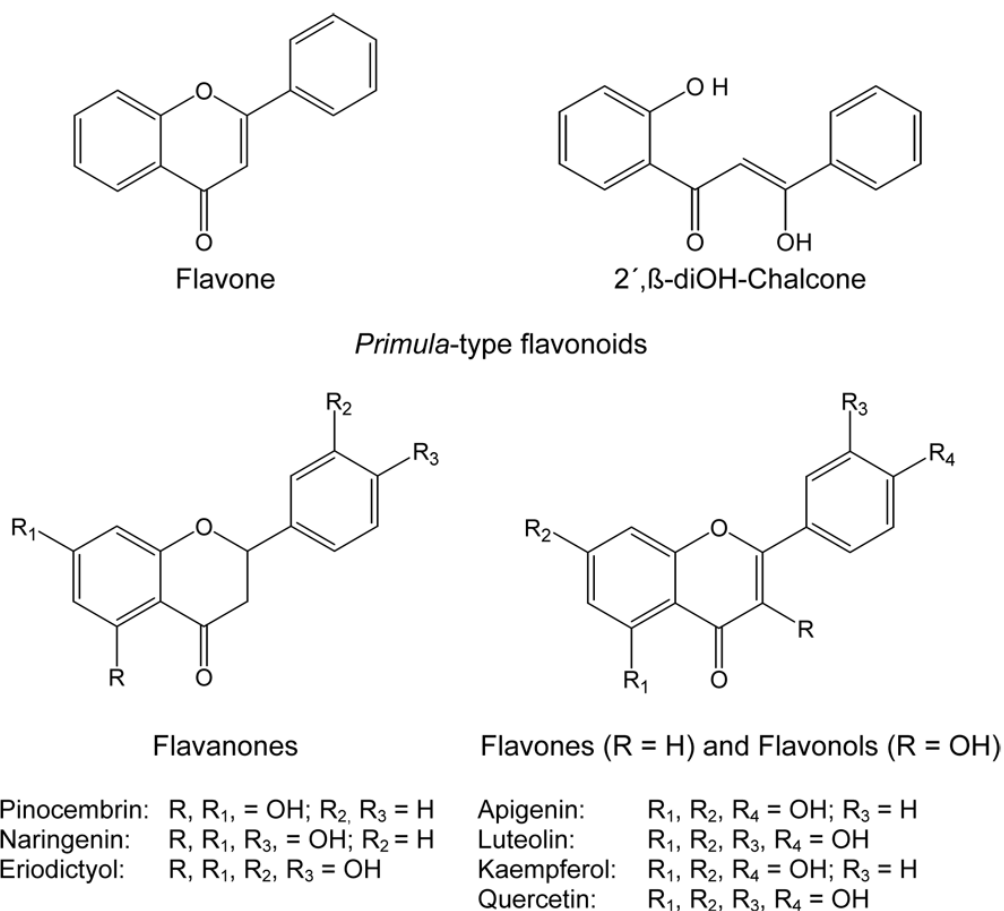
2. RESULTS AND DISCUSSION

2.1. Exudate flavonoid composition

Sixty-seven accessions of *Dionysia* species including subspecies and some hybrid taxa have been analysed for the chemodiversity of exudate flavonoids. The species studied belong to different subgeneric units as described by Lidén (2007). Flavonoid data are summarized in Table 1, in which the species are arranged according to their position based on current phylogenetic data (Trift and Anderberg, 2006). Clades are marked as A, B, C, D, E, F, G and combinations thereof, while section names and their abbreviations follow Lidén (2007). Details are explained in the legend to Table 1. This table also includes data on infraspecific variation in cases where several accessions per taxon were available.

The formation of flavones with the irregular substitution pattern typical of *Primula* exudate flavonoids is also a general feature of *Dionysia* exudates. These flavonoids are marked as “*Primula*-type flavonoids” (in Table 1) and include one chalcone derivative (2', β -diOH-chalcone), further unsubstituted flavone, its 2'- and 5'-*O*-substituted derivatives (5-deoxyflavones), and the structurally related 5-OH-flavones. Occasionally, typical *Primula*-structures such as 3',4'-di-OH-flavone, earlier reported from a few *Dionysia* spp. (Harborne, 1968, 1971), and some of its derivatives are accumulated in trace amounts. While 5-OH-flavone is found in most of the species, 5,8-diOH-flavone is of more restricted distribution, and corresponding 6-*O*-substituted derivatives have not been detected so far. Treatment of these flavones as a separate group (Table 1) is based upon their irregular substitution pattern suggesting a different biosynthetic origin (Valant-Vetschera et al., 2009; for structures see Fig. 1). The formation of flavonoid types with “regular” substitution pattern (i.e. 5,7-OH-substitution in Ring A; 3' and/or 4'-substitution in Ring B) in exudates of *Dionysia* therefore is remarkable. These substitution patterns characterise the occurring flavanones, flavones and flavonols, indicated separately in Table 1. It must be mentioned that both biosynthetic routes are expressed in single species (e.g., *Dionysia diapensifolia*

Boiss.), thus being not mutually exclusive. Structural variation within flavanones and flavone derivatives of apigenin and luteolin is limited. Flavonols based upon quercetin and kaempferol occur rather infrequent, but their structural diversity is greater than that of corresponding flavones. A series of unidentified flavonoids (u1-u6 in Table 1) have been found in addition, but their structures could not be fully determined yet due to lack of material. Nevertheless, they have been included because of their informative value.



Flavonoids not found in *Primula* exudates

Fig. 1: Main structural types of *Dionysia* flavonoids

Table 1: Distribution of exudate flavonoids in sections and clades of *Dionysia*

Source Number	Clade	Dionysia	Sectional Alignment	Primula-type flavonoids														Flavanones			Further Flavones/Flavonols										Unidentified																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																													
1	A	balsamea	non aligned	2',6-diOH-chalcone	Flavone	2'-OH-flavone	2'-OMe-flavone	2',5'-diOH-flavone	2',5'-diOMe-flavone	2'-OH-5'-OAc-flavone	3',4'-diOH-flavone	3'-OMe-4',5'-OCH ₂ -flavone	5-OH-flavone	5,2'-diOH-flavone	5-OH-2'-OMe-flavone	5,2',5'-triOH-flavone	5,8-diOH-flavone	5-OH-8-OMe-flavone	5,8,2'-triOH flavone	5-OH-8,2'-diOMe-flavone	Pinocembrin	Naringenin	Nar-7'-Me	Eriodictyol-7'-Me	Eriod-7',3'-diMe	Apigenin	Ap-7'-Me	Ap-4'-Me	Luteolin-7',3'-diMe	Kaempferol	Kae-3-Me	Kae-7'-Me	Kae-3,7'-diMe	Kae-3,4'-diMe	Kae-7,4'-diMe	Kae-3,7,4'-triMe	Quercetin	Qu-7'-Me	Qu-7',3'-diMe	Qu-7',3',4'-triMe	u1	u2	u3	u4	u5	u6																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
2	A	hissarica	non aligned	O	O	O	O	O	O	t	t	t	O	t	t	O	O	O	t	t	O	O	O	O																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
3	BC	mira	Dionysiopsis	t	O	O	O	O	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t

[illegible]

O, major compounds.

t, minor compounds.

Sources: see Table 3.

Clades according to Trift and Anderberg (2006).

Sectional alignment following Liden (2007): NA, non-aligned; Eastern species: A2-a, sect. *Dionysiastrum* subsect. *Tapetodes*; A2-b, sect. *Dionysiastrum* subsect. *Involucratae*; Western species: B-3, sect. *Dionysiopsis*; B-4, sect. *Zoroasteranthos*; B-5, sect. *Mucida*; B-6, sect. *Dionysia*.

* In earlier papers (Budzianowski and Wollenweber, 2007; Valant-Vetschera et al., 2009) 3'-hydroxy-4',5'-methylenedioxyflavone was erroneously reported instead of 3'-methoxy-4',5'-methylenedioxyflavone from *Primula elatior*.

Distribution of single flavonoids in exudates of *Dionysia* was quantified to be able to visualize the *Dionysia* flavonoid profile composition at large. Detected compounds were coded either as "1" (minor amounts) or "2" (large amounts). Occurrences were summarized and divided by the number of studied taxa, and calculated values were subsequently transformed to a bar chart as shown in Fig. 2. Unsubstituted flavone represents the main accumulation tendency in *Dionysia* exudates, followed by a series of the typical *Primula*-flavonoids. Regular substituted flavanones, flavones and flavonols are minor accumulation tendencies as are the yet unidentified flavonoids (u1-u6).

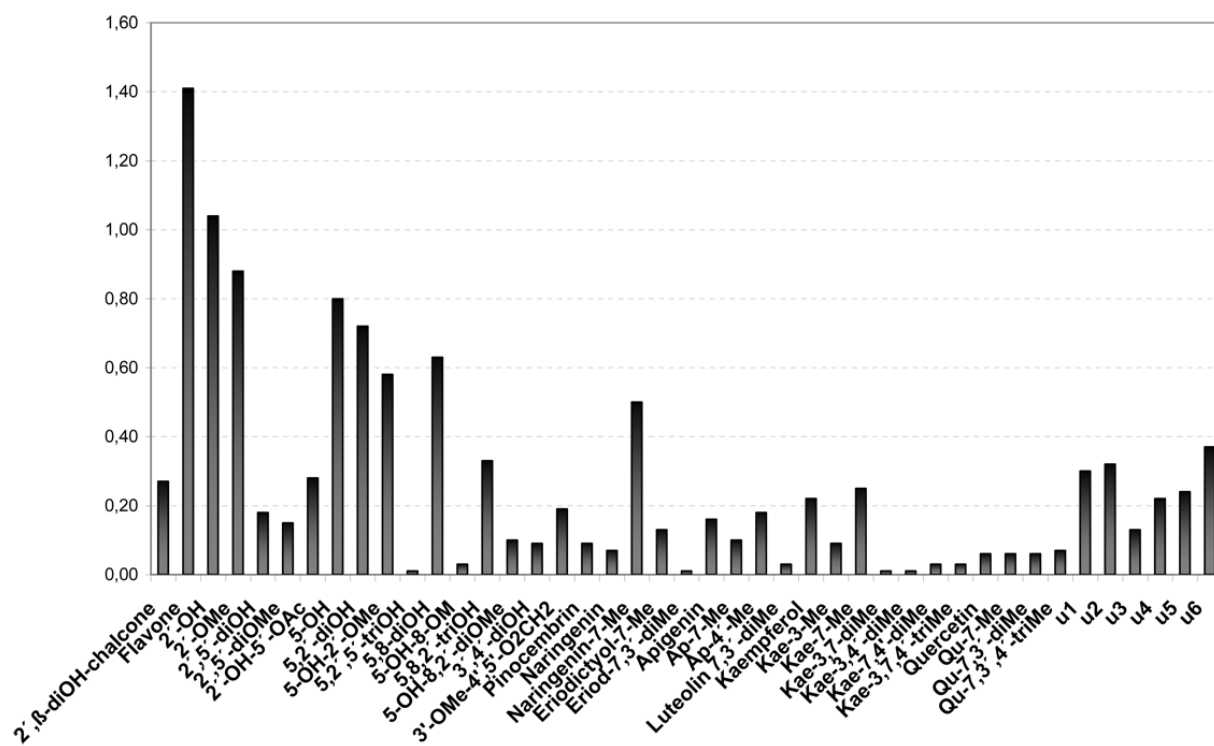


Fig. 2: Frequencies of single flavonoids in exudates of *Dionysia* spp. x-axis: all detected compounds (abbreviations and sequence as in Table 1). y-axis: absolute calculated value (sum of occurrences/number of taxa analysed). Occurrences of single compounds were coded as (2) for large amounts, and as (1) for small amounts. Undetectable amounts were coded as (0).

2.2. Intraspecific variation

The stability of accumulation tendencies at the infraspecific level was studied in different taxonomic groups across the genus (Table 1). Plant source numbers appear in parentheses in the following text; for sources see Table 3. Only two accessions were available for comparison of exudate profiles of *Dionysia esfandiarii* Wendelbo (20, 21) and *Dionysia mozaaffarianii* Lidén (46, 47), which did not exhibit any differences. By contrast, those of *Dionysia hausknechtii* Bornm. et Strauss (51, 52), of *Dionysia freitagii* Wendelbo (7, 8), of *Dionysia lamingtonii* Stapf (55, 56) and of *Dionysia paradoxa* Wendelbo (38, 39)

differed with respect to minor compounds. With exception of *Dionysia lamingtonii*, all material originated from cultivation in Gothenburg Botanic Garden. Accession numbers 30-35 of *Dionysia aretioides* (Lehm.) Boiss. (cultivated material), proved to be quite stable, with only one of the 6 samples deviating lightly. Some variation was noted among 6 samples of *Dionysia tapetodes* Bunge (9-14), but also at a rather low level, and two samples coming from one clone being cultivated in different institutions, showed identical profiles (10,11). Four out of five accessions of *Dionysia archibaldii* Wendelbo (15-18; all cultivated material) proved to be quite uniform, while one collection, which was received as *Dionysia bazoftica* (19; natural habitat), differed by production of 5,8,2'-substituted flavones and of 3 of the unknown compounds. *D. bazoftica*, earlier described by Jamzad (1996) was later considered to be synonym to *D. archibaldii*. Maybe the separation as a distinct taxonomic entity would be rectified, but certainly more material needs to be studied chemically and morphologically.

Larger discrepancies were noted between the two collections of *Dionysia microphylla* Wendelbo (4, 5), concerning the formation of 5-OH-flavones and 3 of the unidentified compounds in one collection. This is the more remarkable since both collections are of the same clone (GWH-1302; Grey-Wilson, 1989), albeit cultivated in different institutions. Similarly, one accession of *Dionysia michauxii* (Duby) Boiss. (62) was found to accumulate the flavanone naringenin-7-Me in addition, while the other accession (63) yielded quercetin-7,3',4'-triMe together with u3, u5, and u6. Both accessions came from cultivation in the Gothenburg Botanic Garden, but material originated from different collections in the wild. The two collections of *Dionysia teucroides* Davis et Wendelbo (27, 28) also yielded quite diversified profiles. They are supposed to be clones, being under cultivation in different institutions.

The reasons for the observed variation even among clones are still obscure. It may only be speculated that culture conditions could play a role. It is known that *Dionysia* species are quite susceptible to fungal infections and also to insecticidal attack (Grey-

Wilson, 1989). Thus, it is conceivable that some material was treated with chemicals which could affect the composition of exudates. It would be interesting to test material from the wild and their offsprings under controlled cultivation conditions, for better assessment of variation. On the other hand, *Primula* species exhibited stable exudate flavonoid profiles even when old herbarium material was analysed (Bhutia, pers. comm.).

2.3. Hybrid taxa

Hybrid taxa are rarely observed to occur in nature due to geographic isolation (Grey-Wilson, 1989). Hybrids produced under cultivation frequently have *D. aretioides* as one parent species. The samples analysed now (Table 2) represent artificial crossings between species of sect. *Dionysiopsis* with those of sect. *Dionysiastrum* (Lidén, 2007). Interpretation of exudate flavonoid profiles is limited since the actual parent species were not available for analysis. Different flavonoid profiles were observed for two accessions of *D. aretioides* x *Dionysia bornmuelleri* (Pax) Clay, but both shared the formation of flavanones with the parent species *D. bornmuelleri*. Also, the hybrid *D. aretioides* x *D. microphylla* exhibited the flavanone naringenin-7-Me found in all accessions *D. aretioides*, but exhibited a reduced profile when compared to the parent species. No fundamental differences were obvious between *D. archibaldii* x *D. microphylla* and *D. microphylla*, and some of the typical compounds from *D. archibaldii* were not detected in this hybrid. It appears that additive flavonoid patterns are not the rule within *Dionysia*, which correlates also to earlier observations in the genus *Achillea* (Valant-Vetschera and Wollenweber, 1988).

Table 2: Exudate flavonoids of artificial hybrids and parent species

Source number	<i>Dionysia</i>	2',β-diOH-chalcone	Flavone	2'-OH-flavone	2'-OMe-flavone	2',5'-diOH-flavone	2'-OH-5'-OAc-flavone	3'-OMe-4',5'-O ₂ CH ₂ -flavone	5-OH-flavone	5,2'-diOH-flavone	5-OH-2'-OMe-flavone	5,8-diOH-flavone	5-OH-8-OMe-flavone	5,8,2'-triOH flavone	5-OH-8,2'-diOMe-flavone	Naringenin-7-Me	Eriodictyol-7-Me	u1	u2	u4	u5	u6
4	<i>microphylla</i>	t	O	t					t	t	t									t	O	O
5	<i>microphylla</i>		O	t		t																
67	<i>archibaldii</i> x <i>microphylla</i>		O	O		t	t		O	O												
15	<i>archibaldii</i>		O	O		t			O	O		O		O								
16	<i>archibaldii</i>		O	O					O	O		O										
17	<i>archibaldii</i>		O	O		t	t		O	O		O										
18	<i>archibaldii</i>		O	O		t	t		O	O		O										
19	<i>archibaldii</i> (sub <i>bazoftica</i>)	t	O	O	O	t		t	O	O	O		t	O	t			O	t			O
29	<i>bornmuelleri</i>		O	O			t									O						
66	<i>bornmuelleri</i> x <i>aretioides</i>		O	O						O				O		O	t					
65	<i>aretioides</i> x <i>bornmuelleri</i>		O	O			t		O			O				O	t		O			
30	<i>aretioides</i>		O	O					O	t		O		O		O	t					
31	<i>aretioides</i>		O			t	t		O	t						O	t					
32	<i>aretioides</i>		O	t		t			O	t		O				O						
33	<i>aretioides</i>		O	t		t			O	t		O				O	t					
34	<i>aretioides</i>		O	t		t			O	t		O				O	t					
35	<i>aretioides</i>		O			t			O	t		O				O	t					
64	<i>aretioides</i> x <i>microphylla</i>		O													O						

2.4. Flavonoid diversification in taxonomic groups of *Dionysia*

Principal Component Analysis (PCo-analysis) was applied in search of distribution patterns and variation of exudate flavonoid distribution within *Dionysia*. Artificial hybrid taxa were excluded, and so was *Dionysia khatamii* Mozaffarian because of coding “zero” in all data. Quantities of accumulation were not taken into account in the binary presence/absence data matrix, as was performed recently (Wollenweber et al., 2008). Variation was sufficiently indicated by values of the first 5 components extracted. Figure 3 results from plotting component 1 against component 2. Cases are represented by species, which were coded with respect to their sectional alignment (Lidén, 2007), corresponding to Table 1. Three main groups are defined: group 1 consists of some species from sect. *Dionysia* (B-6, part 1), with *Dionysia janthina* Bornm. et Wink from sect. *Zoroasteranthos* (B-4) falling into this group. The second group comprises the remaining species from sect. *Dionysia* (B-6, part 2), mixed with several species from sect. *Dionysiopsis* (B-3) and from *Dionysiastrum* subsect. *Involucratae* (A2-b), respectively. Non-aligned taxa (NA) such as *D.*

hissarica fall within taxa of sect. *Dionysiopsis* (B-3), while *D. paradoxa* bridges between species of sect. *Dionysiopsis* (B-3) and sect. *Dionysiastrum* subsect. *Involucratae* (A2-b). While group 1 consists of Western species only, group two is a geographic mix of Eastern and Western taxa. This is also true for group 3, in which species of sect. *Dionysiopsis* subsect. *Tapetodes* (A2-a) and the remaining species of sect. *Dionysiastrum* are grouped. The other non-aligned taxa are now associated with other groups: both accessions of *D. paradoxa* are positioned between *Dionysiopsis* and sect. *Dionysiastrum* subsect. *Involucratae* (A2-b) in group 2, while *Dionysia balsamea* shows up within *Dionysiopsis* (B-3) in group 3. Species of sect. *Dionysia*, which are all efarinose, group together best, which is not true for species from other taxonomic units exhibiting varying types of farina and/or glandular hairs. Flavonoid diversification hardly shows correlation with biogeographic diversification which is basal to the current concept of Lidén (2007). However, the species groups are well supported by their flavonoid accumulation tendencies. Thus, species of group 1 accumulate more of the regular flavones and flavonols and hardly any of the typical *Primula* flavones. Group 2 accumulates both types on a more or less equal level, while species of group 3 tend to accumulate mainly the typical *Primula* flavonoids. Interestingly, the flavanones occur scattered throughout all three groups. The present data suggest that unsubstituted flavone and specific groups of biogenetically defined derivatives represent accumulation tendencies which could be used for interpretation.

The observed diversifications of accumulation tendencies were expected to be possibly associated with characters related to flavonoid production, i.e., production of woolly versus powdery farina, or lack of farina (but presence of glandular hairs). Therefore, farina characters were mapped on the scatterplot (Fig. 3). Whereas species of group 1 (Fig. 3) are all efarinose (sect. *Dionysia*, B-6), combined with *D. janthina* of sect. *Zoroasteranthos* (B-4; reduced presence of glandular hairs; Lidén, 2007), the other two groups are mixed with respect to farina production. Group 2 consists of the remaining species of sect. *Dionysia* (B-6; similarly efarinose), but they are mixed with taxa of woolly and, in one case, of mealy (powdery) farina from sect. *Dionysiopsis*, as well as with two non-aligned taxa *Dionysia hissarica* Lipsky, *D. paradoxa* (NA) both showing woolly farina.

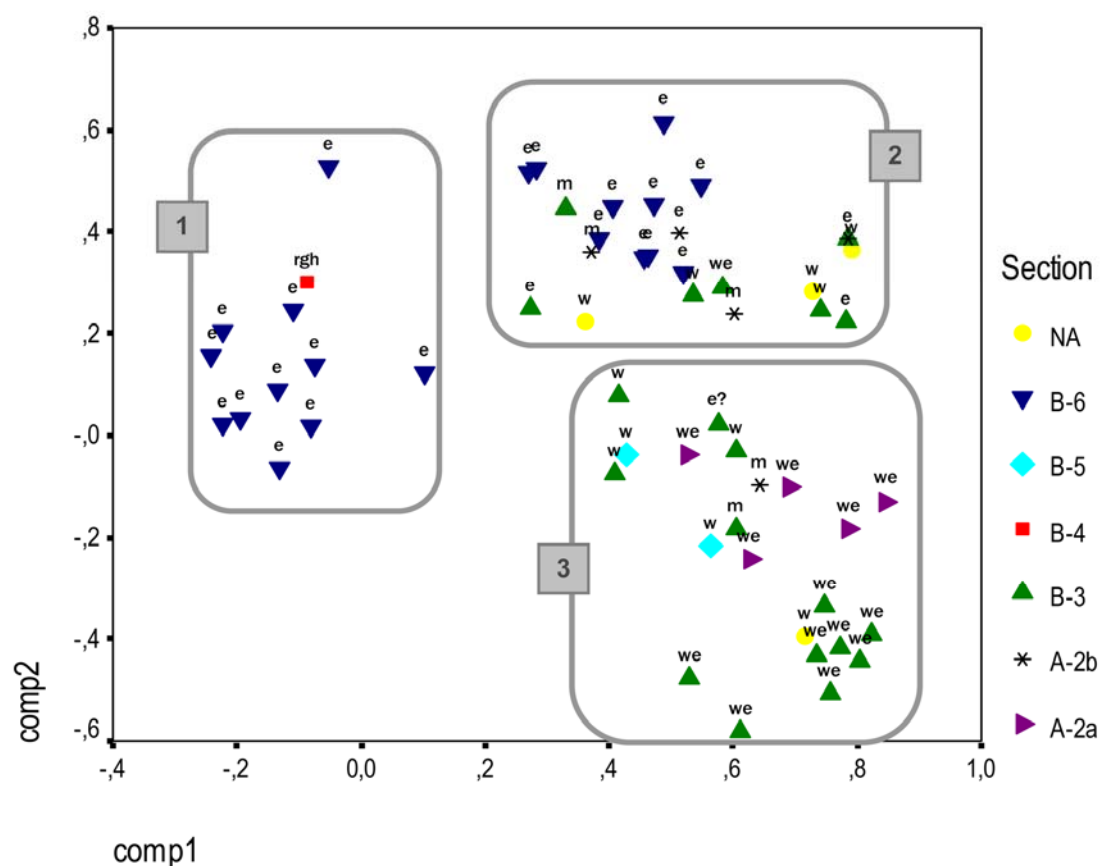


Fig. 3: Principal component analysis of *Dionysia* flavonoids and comparison with farina characters. Sectional alignment following Lidén (2007): NA= non-aligned; Eastern species: A2-a= sect. *Dionysiastrum* subsect. *Tapetodes*; A2-b= sect. *Dionysiastrum* subsect. *Involucratae*; Western species: B-3= sect. *Dionysiopsis*; B-4= sect. *Zoroasteranthos*; B-5= sect. *Mucida*; B-6= sect. *Dionysia*. Farina characters: e= efarinose= lack of farina, but presence of glandular hairs; w= woolly farina; m= mealy (powdery) farina; we = either woolly farina or efarinose; rgh= glandular hairs reduced to specific organ parts in low number. Species of Group 1: *zetterlundii*, *sarvestanica*, *gaubae* var. *megalantha*, *termeana*, *lamingtonii* (all accessions), *odora*, *gaubae*, *iranica*, *cristagalli*, *zagrica*, *janthina*. Species of Group 2: *bryoides*, *diapensifolia*, *sarvestanica* subsp. *spathulata*, *michauxii* (all accessions), *caespitosa* and subsp. *bolivarii*, *mozaffarianii* (all accessions), *haussknechtii* (all accessions), *revoluta* subsp. *revoluta*, *hissarica*, *oreodoxa*, *raptodes*, *esfandiarii* (all accessions), *mira*, *paradoxa* (all accessions), *microphylla* (No.5 in Tab. 1), *involucrata*, *freitagii* (all accessions). Species of Group 3: *viva*, *revoluta* subsp. *canescens*, *lurorum* (incl. sub *aubrietiioides*, *teucroides* (all accessions), *microphylla* (No. 4 in Tab. 1), *tapetodes* (all accessions), *bornmuelleri*, *archibaldii* (all accessions and “*bazoftica*”), *balsamea*, *aretioides* (all accessions).

Group 3 contains species which are described as having both states (woolly farina or efarinose), along with species of the mealy farina type. According to Trift et al. (2004), woolly farina represents an ancestral state, and it is as yet unclear whether “efarinose” represents an advanced character state or not. It appears that the degree of correlation between glandular morphology, production of farina, and flavonoid diversification is equally obscure as was recently found for *Primula* (Valant-Vetschera et al., 2009).

In the following, flavonoid diversification tendencies (Table 1) are discussed against infrageneric (Lidén, 2007) and cladistic concepts (Trift et al., 2004; Trift and Anderberg, 2006) more in detail (Table 1). If not cited otherwise, botanical information mentioned here originates from these publications. Clade A comprises *D. balsamea* Wendelbo & Rech. f. and *D. hissarica*, with strong support as a sister clade to the rest of the genus and considered subsequently as non-aligned. *D. hissarica* (group 2, Fig. 3) differs from *D. balsamea* (group 3, Fig. 3) by the formation of flavonols and of some of the unidentified flavonoids. Thus they are chemically well differentiated and the same applies in parts to their morphological characters (Trift et al., 2004). *Dionysia mira*, another basal taxon being considered to be the closest relative to the genus *Primula* (Wendelbo, 1961), is found as a sister taxon to subclade BCD on the molecular tree (low support). Flavonoid chemistry places this taxon together with *D. paradoxa* and *D. hissarica* in group 2 (Fig. 3). The earlier assumed affinities between *D. balsamea* and *D. paradoxa* (Grey-Wilson, 1989) are neither obvious from chemical nor from molecular analysis. Generally, flavanones and flavonols are quite rare in subclade BCD, and only some of the unknown compounds were found to be occasionally accumulated. The two chemically different accessions of *D. microphylla* are found in group 2 (No. 5, Table 1) and group 3 (No. 4, Table 1), respectively, because of their different flavonoid complement.

In clade BF, *Primula*-type flavones dominate in all of the species, but flavanones and some flavonols were found in a few species, together with some of the unidentified flavonoids. In this clade, *Dionysia oreodoxa* Bornm. and *Dionysia rhapsodes* Bunge form one pair of species, and another group consists of *D. khatamii*, *D. janthina*, and *Dionysia*

curviflora of a separate section named *Zoroasteranthos* (B-4). This section is morphologically well defined, and as far as exudates are concerned, also exceptional. Only one compound of unknown structure (u7 in Table 1), not detected in any of the other *Dionysia* species studied, was found in the exudate of *D. khatamii*, while *D. curviflora* did not yield any exudate at all. Flavonoid profiles had a low degree of similarity in all species from this clade, but infraspecific variation is not known. The closely related *D. aretioides*, the Turkish *D. teucrioides*, and *D. bornmuelleri* exhibit similar flavonoid profiles, and are found in group 3 (Fig. 3).

The following part of Clade B splits up into *Dionysia lurorum* Wendelbo (BE), the sister taxon to the larger group of species of subclade BEG. This clade conforms as a whole to sect. *Dionysia* (B-6 in Fig. 3). *Dionysia lurorum*, being now placed in the new section *Mucida* (B-5; Lidén, 2007), is found in group 3 (Fig. 3) according to its flavonoid accumulation tendencies. Within subclade BEG, a tendency to accumulate flavanones, flavones and flavonols in addition or instead of the *Primula* flavonoids is noted (Table1). Also, unidentified flavonoids are encountered in some of the taxa of this group. These diversifications lead to a split into 2 distinct groups in PCo-analysis as already discussed (see also Fig. 3). Exudates of *Dionysia odora* Fenzl, *Dionysia gaubae* Bornm., *Dionysia zetterlundii* Lidén, *D. lamingtonii*, and *Dionysia sarvestanica* Jamzad et Grey-Wilson, were found to be devoid of the typical *Primula* flavones. However, *D. odora* occupies quite a large distribution area in Iran, and infraspecific variation may occur. The rare flavanone pinocembrin was detected in exudates of *D. odora*, *D. gaubae* var. *megalantha*, and *D. zetterlundii*, respectively. Particularly *D. odora* is seen as close relative to *D. gaubae*. The existing chemical differences between *D. gaubae* and its var. *megalantha* contrast to their small morphological differences which resulted in taxonomic recognition on the level of variety only. Unfortunately, this collection has not yet been included in molecular phylogenetic analysis. The phytochemical differences between *D. sarvestanica* and its subsp. *spathulata* are even greater: *D. sarvestanica* differs by the lack of *Primula* flavones in its exudate and also shows a different composition of flavones and flavonols. While samples of *D. gaubae* originated from the wild, both *D. sarvestanica* collections were grown under

identical conditions in Gothenburg, thus excluding different growth conditions as cause for variation. *D. termeana* is the only species accumulating kaempferol derivatives (3,7-diMe; 7,4'-diMe; 3,7,4'-triMe) in its exudate. In this clade, *D. termeana* is quite close to *D. michauxii*, which is not reflected by the exudate flavonoid composition.

2.5. Exudate flavonoid diversification between *Primula* and *Dionysia*

Accumulation tendencies of *Dionysia* are further compared with those of the designated *Primula* outgroup (species of subgen. *Sphondylia*; Trift et al., 2004; see Fig. 4). 5-Deoxyflavones (DF in Fig. 4) are the major accumulation tendency both in *Dionysia* and in the *Primula* outgroup, followed by the corresponding 5-hydroxyflavones (5F in Fig. 4). *Dionysia* differs by the lack of 5,6-disubstituted 5-hydroxyflavones, and by the accumulation of 2', β -diOH-chalcone, flavanones, regular substituted flavones and flavonols, and the unknown flavonoids from the *Primula* outgroup. Also, quantitative expression of single groups of typical *Primula* flavonoids is different. The degree of exudate diversification in biogenetic terms is larger within *Dionysia* as in the *Primula* outgroup and in other *Primula* spp. studied so far (Valant-Vetschera et al., 2009). However, *Primula* spp. exhibit more complex derivatives of only one biogenetic group of flavones (*Primula*-type flavonoids), and some of these complex derivatives have so far not been detected in the studied species of *Dionysia*.

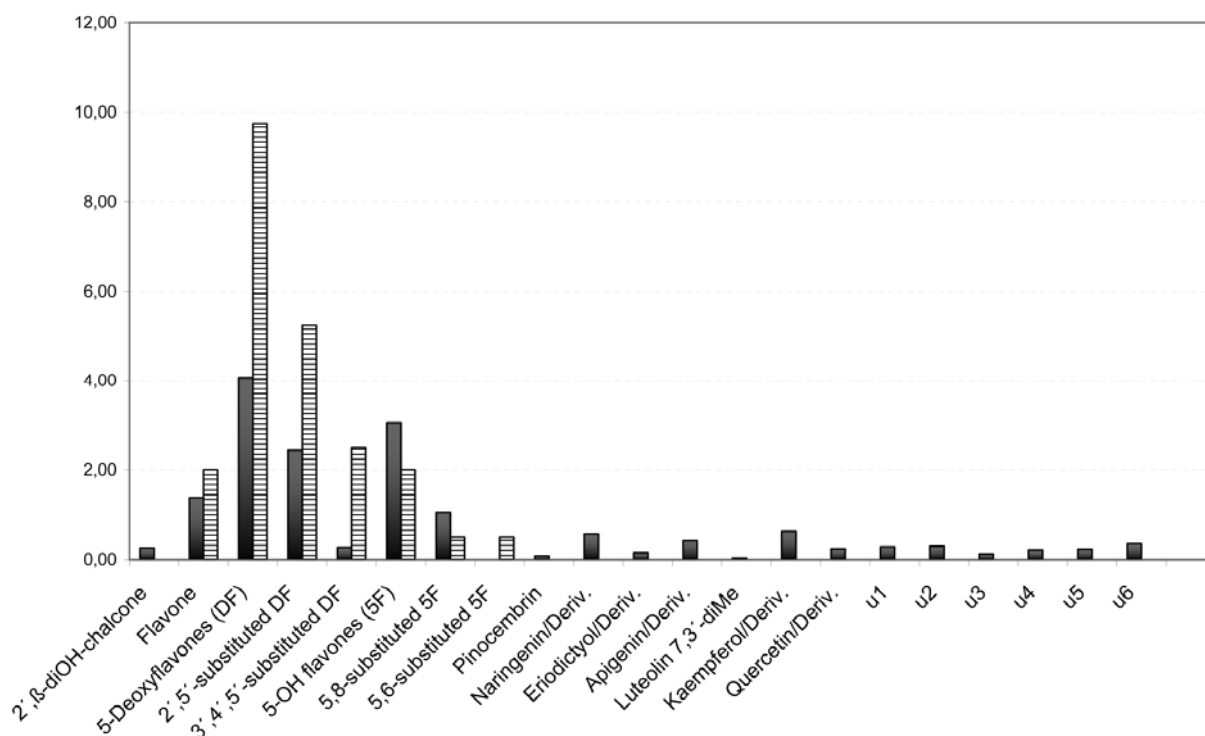


Fig. 4: Comparison of accumulation tendencies between *Primula* (outgroup) and *Dionysia*. x-axis: compounds and derivatives; y-axis: absolute value (calculated: sum of occurrences/number of taxa analysed). black bars: *Dionysia*; hatched bars: *Primula* outgroup; for calculated values see supplementary data.

Based upon current phylogenetic data, *D. tapetodes*, *D. microphylla*, *Dionysia involucrata* Zapr. and *D. janthina* (Trift et al., 2002), or *D. tapetodes* and *D. aretioides* (Mast et al., 2001) are seen as closely allied to *Primula*. Within *Primula*, species of subgen. *Sphondylia* were earlier considered to be the most primitive (Wendelbo, 1961). Their flavonoid profile appears less complex when compared to other members of the genus (Valant-Vetschera et al., 2009). The most “*Primula*-like” profiles were now found in exudates of *D. mira*, *D. microphylla*, *D. freitagii* (p.p.), *D. tapetodes*, *D. archibaldii*, *D. esfandiarrii*, and *D. paradoxa*, of different taxonomic groups within *Dionysia*. If chemical diversity is applied as a character, these species should be regarded as the most primitive ones of the genus. Earlier, Grey-Wilson (1989) had suggested that species of *Primula* sect. *Bullatae* (subgen. *Auganthus*) should be the closest relatives to *Dionysia*. So far, only

Primula forrestii from this group is known for its exudate flavonoid profile (Valant-Vetschera et al., 2009), which would not contradict this assumption.

Correlating exudate flavonoid chemistry with phylogenetic positions derived from DNA-data does not yield satisfactory results for all groups of *Dionysia*. The significance of this observation is not yet clear. It is hard to believe that distinct biosynthetic routes are expressed at random. This assumption is backed by the fact that *Cortusa matthioli*, which is deeply nested within *Primula*, corresponds chemically quite well. Thus, this taxon is more *Primula*-like (Valant-Vetschera et al., 2009) than many of the *Dionysia* species studied. We expect to better understand the evolution of exudate flavonoid pathways once the biosynthesis of the *Primula*-type flavonoids is fully known. In our view, the parallel expression of various accumulation tendencies in *Dionysia* indicates a larger degree of diversification (complexity) and probably a derived state. This is paralleled by the current view of the phylogenetic position of *Dionysia* in the Primulaceae (Trift et al., 2004), and suggests that the genus *Dionysia* represents an evolutionary newer lineage. It certainly will be necessary to study also the genus *Primula* more in detail for a better understanding of relationships in this assemblage. Whether the extreme habitat occupied by *Dionysia* species is causal to the biosynthetic complexity of exudate flavonoid profiles is currently open to speculation. However, anti-oxidative and radical-scavenging activities of these flavonoids may explain their accumulation in plants of arid and/or alpine regions, where increased UV-radiation prevails (Valant-Vetschera and Brem, 2006; Valant-Vetschera et al., 2009). This certainly applies also to our *Dionysia* species, which are so attractive to rock gardeners because of their morphological beauty, and equally to phytochemists because of their interesting biosynthetic capacity.

3. EXPERIMENTAL

3.1. Plant material

Plant material was obtained largely from collections cultivated in green houses at the Gothenburg Botanic Garden, the collection of M. Kammerlander (Würzburg, Germany), Botanischer Garten München, Botanischer Garten der TU Darmstadt, and from collections in the wild from various expeditions carried out by Swedish botanists (see Table 3, material sent by I. Trift (SE) is marked with an asterisk). Respective herbarium specimens are kept in the institutions from which the material was received (M, GB).

3.2. Extraction and identification

Aerial parts of the collected plant species were briefly rinsed with acetone in order to dissolve the lipophilic exudate material. The mixture obtained after evaporation was then analysed, as described previously (Budzianowski and Wollenweber, 2007). Comparative TLC with markers was carried out on polyamide with the solvents: A) petrol₁₀₀₋₁₄₀/toluene/MeCOEt/MeOH 12:6:1:1; B) toluene/ petrol₁₀₀₋₁₄₀/MeCOEt/MeOH 12:6:2:1; C) toluene/ petrol₁₀₀₋₁₄₀/MeCOEt/MeOH 10:25:1:1; D) toluene/MeCOEt/MeOH 12:5:3; E) toluene/dioxane/MeOH 8:1:1; and on silica with solvents F) toluene/MeCOEt 9:1; and G) toluene/dioxane/HOAc 18:5:1. Chromatograms were viewed under UV (366 nm) before and after spraying with “Naturstoffreagenz A” (0.2% of diphenyl-boric acid 2-aminoethyl ester in MeOH). Authentic markers for the identification of the flavonoids were available in E.W.’s laboratory. Their structures have been elucidated previously. Literature references on UV- and MS-data along with retention times from our study are given as supplementary material. Similarly, R_f-values (TLC polyamide DC-11; solvent system C) and colour reactions of compounds u1-u6 may be found in supplementary material.

Table 3: List of analysed material

Number in Tables 1 and 2	<i>Dionysia</i>	Source
1	<i>balsamea</i> Wendelbo & Rech. f.	GWH 580: cultivated; Kammerlander (Würzburg, Germany)
2	<i>hissarica</i> Lipsky*	JJH 918037 Halda 92-0057, Bot. Garden Gothenburg
3	<i>mira</i> Wendelbo	cultivated; Bot. Garden Munich (Germany)
4	<i>microphylla</i> Wendelbo*	Grey-Wilson & Hewer 1302, Bot. Garden Gothenburg
5	<i>microphylla</i> Wendelbo	GWH 1302: cultivated; Kammerlander (Würzburg, Germany)
6	<i>involuta</i> Zapr.*	1992: Bot. Garden Gothenburg
7	<i>freitagii</i> Wendelbo*	Watson P. 84 Grey-Wilson & Hewer 8497: Bot. Garden Gothenburg
8	<i>freitagii</i> Wendelbo*	Watson P. 93 EGW 92/21: Bot. Garden Gothenburg
9	<i>tapetodes</i> Bunge*	T4Z 1086-1 Iran: Khorasan, Kuh-e-Binalud NE Darrud [220-1910]
10	<i>tapetodes</i> Bunge*	Arch. P. 81 Hewer 1164, Bot. Garden Gothenburg
11	<i>tapetodes</i>	H 1164: cultivated; Kammerlander (Würzburg, Germany)
12	<i>tapetodes</i>	cultivated; Bot. Garden Munich (Germany)
13	<i>tapetodes</i>	GWH 780: cultivated; Kammerlander (Würzburg, Germany)
14	<i>tapetodes</i>	MK 9630, seeds derived from cultures Kammerlander
15	<i>archibaldii</i> Wendelbo*	PZ Beuken [2003-865]: Bot. Garden Gothenburg

Chapter 2: Chemodiversity of exudate flavonoids in *Dionysia* spp.

Number in Tables 1 and 2	<i>Dionysia</i>	Source
16	<i>archibaldii</i>	JCA3010: cultivated; Kammerlander (Würzburg, Germany)
17	<i>archibaldii</i>	MK 97-310/2, seeds derived from cultures Kammerlander
18	<i>archibaldii</i>	MK 94-02/1, seeds derived from cultures Kammerlander
19	<i>archibaldii</i> (sub <i>bazoftica</i>)*	87-1 JLMS 02-087 Iran: Bazoft Valley SW-Mayr [2003-193]
20	<i>esfandiarrii</i> Wendelbo*	SLIZE 259-3
21	<i>esfandiarrii</i> Wendelbo*	SLIZE 259-4 [98-1939]
22	<i>viva</i> Lidén & Zetterlund*	T4Z 035 Iran: Fars, Vallyabad between Marwdasht and Amsanjan [2002-1949]
23	<i>revoluta</i> subsp. <i>revoluta</i> Boiss.*	T4Z 030 Iran: Yazd [2002-1943]
24	<i>revoluta</i> subsp. <i>canescens</i> (Boiss.) Wendelbo*	SLIZE 192-3
25	<i>oreodoxa</i> Bornm.*	D. Zschummel 101-06 Iran: Mt Takh Ali [2001-21179]
26	<i>rhaptodes</i> Bunge*	T4Z 1060 Iran: Kerman, Kuh-e-Jupar [2003-1877]
27	<i>teucroides</i> Davis et Wendelbo*	2000 0841 from Bot. Garden Munich (Germany); cultivated Bot. Garden Gothenburg
28	<i>teucroides</i> Davis et Wendelbo	cultivated; Bot. Garden Munich (Germany)
29	<i>bornmuelleri</i> (Pax) Clay.	Rechninger 11456: Bot. Garden Gothenburg
30	<i>aretoides</i> (Lehm.) Boiss.*	SLIZE 035 1998-1857 Lidén [29-9-04]

Chapter 2: Chemodiversity of exudate flavonoids in *Dionysia* spp.

Number in Tables 1 and 2	<i>Dionysia</i>	Source
31	<i>aretioides</i>	cultivated Bot. Garden, TU Darmstadt
32	<i>aretioides</i>	Population a: cultivated; Kammerlander (Würzburg, Germany)
33	<i>aretioides</i>	Population b: cultivated; Kammerlander (Würzburg, Germany)
34	<i>aretioides</i>	Population c: cultivated; Kammerlander (Würzburg, Germany)
35	<i>aretioides</i>	Population d: cultivated; Kammerlander (Würzburg, Germany)
36	<i>khatamii</i> Mozaffarian*	T4Z 13-2 [2002-2200]
37	<i>janthina</i> Bornm. et Wink*	T4Z 007 Iran: Yazd, S of Mehriz, valley to Tang-e-Chenar [2002-1919]
38	<i>paradoxa</i> Wendelbo*	PW 7414: Bot. Garden Gothenburg
39	<i>paradoxa</i> Wendelbo*	PW 744: Bot. Garden Gothenburg
40	<i>lurorum</i> Wendelbo*	T4Z 136-4
41	<i>lurorum</i> (<i>sub aubrietoides</i>) Jamzad et Mozaffarian*	T4Z 136-18 [2002-2059]
42	<i>iranica</i> Jamzad*	T4Z 118 [2002-2036]
43	<i>caespitosa</i> (Duby) Boiss.*	T4Z 155 Iran: Esfahan, above Analajeh, Kuh-e-Dalan [2002-2235]
44	<i>caespitosa</i> subsp. <i>?bolivarii</i> Pau*	T4Z 120 Iran: Esfahan, Karobas Pass, N of tunnel, type loc. [2002-2040]
45	<i>zagrica</i> Grey-Wilson*	SLIZE 176-8
46	<i>mozaffarianii</i> Lidén*	T4Z 100-8 Iran
47	<i>mozaffarianii</i> Lidén*	SLIZE 232-9

Chapter 2: Chemodiversity of exudate flavonoids in *Dionysia* spp.

Number in Tables 1 and 2	<i>Dionysia</i>	Source
48	<i>odora</i> Fenzl*	99-0713 Kammerlander 0713-B, Bot. Garden Gothenburg
49	<i>gaubae</i> Bornm.*	DZ 0038/2
50	<i>gaubae</i> var. <i>megalantha</i> Bornm.*	T4Z 190 Iran: Lorestan [2002-2115]
51	<i>hausknechtii</i> Bornm. et Strauss*	T4Z 175 [2002-2101]
52	<i>hausknechtii</i> Bornm. et Strauss*	SLIZE 322-3 [1998-1973]
53	<i>cristagalli</i> Lidén*	DZ 01-? [2003-859]
54	<i>zetterlundii</i> Lidén*	T4Z 125 Iran: Chaharmahal Va- Bakhtiyari, Karun Valley, Shari Pass [2002-2046]
55	<i>lamingtonii</i> Stapf*	T4Z 138 Iran: Chaharmahal Va- Bakhtiyari, Bazoft Valley [2002- 2061]
56	<i>lamingtonii</i> Stapf*	SLIZE 161-2
57	<i>termeana</i> Wendelbo*	D. Zschummel 00-46-2 [2000-1825]
58	<i>bryoides</i> Boiss*	T4Z 092 Iran: Fraz [2002-2006]
59	<i>diapensifolia</i> Boiss.*	SLIZE 253-2
60	<i>sarvestanica</i> Jamzad et Grey-Wilson*	T4Z 040 Iran: between Kherameh and Sarvestan
61	<i>sarvestanica</i> subsp. <i>spathulata</i> *	T4Z 1044 Iran: Farz, Kuh-e-Sefidar [2003-1861]
62	<i>microchauxii</i> (Duby) Boiss.*	SLIZE 254-11
63	<i>microchauxii</i> (Duby) Boiss.*	SLIZE 254-8 Iran

Number in Tables 1 and 2	<i>Dionysia</i>	Source
<i>Hybrid taxa</i>		
64	<i>aretioides x microphylla</i>	MK-91-25/3, seeds derived from cultures Kammerlander
65	<i>aretioides x bormuelleri</i>	cultivated; Kammerlander (Würzburg, Germany)
66	<i>bormuelleri x aretioides*</i>	1974: Bot. Garden Gothenburg
67	<i>archibaldii x microphylla</i>	MK92-25/1, seeds derived from cultures Kammerlander

* Material received from Ida Trift

3.3. Principal component analysis

Principal components were extracted from a correlation matrix of a binary original data matrix as described in 2.4., with artificial hybrids and *D. khatamii* (yielding zero values) being excluded. The option “Principal components of the factor analysis”, implemented in SPSS (version 10), was applied. Fourteen components were obtained, with the first 5 explaining 60.2% of total variance. Component 1 explained 30.8% of variance, followed by component 2 (10.8%), component 3 (6.9%), component 4 (6.5 %) and component 5 (5.2%). Component 1 and 2 were used for scatterplot illustration (see Fig. 3).

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SUPPLEMENTARY DATA

1) Calculated values to Fig. 2

2) Supplementary to Fig. 4: Table used for calculation

3) Supplementary to Experimental

1) Table ad Fig. 2 part 1

Primula-type flavones																	
	2',8'-diOH-chalcone	Flavone	2'-OH	2'-OMe	2',5'-diOH	2',5'-diOMe	2'-OH-5'-OAc	5-OH	5,2'-diOH	5-OH-2'-OMe	5,2',5'-triOH	5,8-diOH	5-OH-8-OM	5,8,2'-triOH	5-OH-8,2'-diOMe	3',4'-diOH	3'-OMe-4',5'-O ₂ CH ₂
sum of occurrences	18	95	70	59	12	10	19	54	48	39	1	42	2	22	7	6	13
sum/67 (No of taxa)	0,27	1,41	1,04	0,88	0,18	0,15	0,28	0,80	0,72	0,58	0,01	0,63	0,03	0,33	0,10	0,09	0,19

1) Table ad Fig. 2 part 2

Flavanones					Flavones/Flavonols														Unidentified							
Pinocembrin	Naringenin	Naringenin-7-Me	Eriodictyol-7-Me	Eriod-7,3'-diMe	Apigenin	Ap-7-Me	Ap-4'-Me	Luteolin 7,3'-diMe	Kaempferol	Kae-3-Me	Kae-7-Me	Kae-3,7-diMe	Kae-3,4'-diMe	Kae-7,4'-diMe	Kae-3,7,4'-triMe	Quercetin	Qu-7-Me	Qu-7,3'-diMe	Qu-7,3',4'-triMe	u1	u2	u3	u4	u5	u6	
sum of occurrences	6	5	34	9	1	11	7	12	2	15	6	17	1	1	2	2	4	4	4	5	20	21	9	15	16	25
sum/67 (No of taxa)	0,09	0,07	0,50	0,13	0,01	0,16	0,10	0,18	0,03	0,22	0,09	0,25	0,01	0,01	0,03	0,03	0,06	0,06	0,06	0,07	0,30	0,32	0,13	0,22	0,24	0,37

2) Supplementary to Fig. 4: Table used for calculation (excel)

Occurrences of single compounds were coded as (2) for large amounts, and as (1) for small amounts. Undetectable amounts were coded as (0). The value used for graphical illustration is based upon the sum of all occurrences, divided by the number of analysed species of the two genera. Height of bars (in Fig. 4) corresponds to values given below.

	2',6'-diOH-chalcone	Flavone	5-Deoxyflavones (DF)	2',5'-substituted DF	3',4',5'-substituted DF	5-OH flavones (5F)	5,8-substituted 5F	5,6-substituted 5F	Pinocembrin	Naringenin/Deriv.	Eriodictyol/Deriv.	Apigenin/Deriv.	Luteolin 7,3'-diMe	Kaempferol/Deriv.	Quercetin/Deriv.	u1	u2	u3	u4	u5	u6
<i>Dionysia</i>	0,26	1,38	4,07	2,44	0,27	3,05	1,05	0,00	0,08	0,57	0,15	0,43	0,03	0,63	0,24	0,28	0,30	0,13	0,21	0,23	0,36
<i>Primula</i> (outgroup)	0,00	2,00	9,75	5,25	2,50	2,00	0,50	0,50	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

3) Supplementary to Experimental: HPLC and TLC data

Compound	HPLC ^a		TLC			MS-Refs. ^e
	Rt (min)	UV $\lambda_{\max}$ (nm)	SS 1 ^b Rf (×100)	SS 2 ^c Rf (×100)	SS 3 ^d Rf (×100)	
2', β -diOH-chalcone	19.8	250, 342 _{sh} , 364		71		10
Flavone	12.7	252, 296, 308 _{sh}		64		
2'-OH-flavone	11.8	248, 294 _{sh} , 306, 324 _{sh}	27	5	68	1,2
2'-OMe-flavone	13.1	236, 248, 296 _{sh} , 308		56		1
2',5'-diOH-flavone	6.1	246, 296, 360			44	9
2'5'-diOMe-flavone	13.5	244, 294, 350		52		6
2'-OH-5'-OAc-flavone	11.8	248, 296 _{sh} , 306, 324 _{sh}	29		74	9
3',4'-diOH-flavone	7.6	244, 316 _{sh} , 342			49	7
3'-OMe-4'5'-O ₂ CH ₂	13.1	230 _{sh} , 320 _{sh} , 340		42		4
5-OH-flavone	16.4	270, 298, 338		75		
5,2'-diOH-flavone	15.0	268, 342	32		65	2
5-OH-2'-OMe-flavone	16.7	268, 336		73		8
5,2',5'-triOH-flavone	8.8	264, 294 _{sh} , 364			44	10
5,8-diOH-flavone	10.5	280, 364	36	8	72	
5,8,2'-triOH-flavone	8.6	276, 338			42	3
5-OH-8,2'-diOMe-flavone	15.7	276, 332		54		5
5-OH-8-OMe-flavone	15.4	280, 360		58		

^a Measured with Agilent 1100 Series with a UV diode array detector between 200-400 nm on a Hypersil BDS C-18 (RP; 5 μ m; 250 × 4,6 mm) column. The mobile phase consisted of an aqueous buffer (0.015 M O-phosphoric acid, 0.0015 M tetrabutylammonium hydroxide, pH 3) and MeOH using a linear gradient starting with 45 % buffer, decreasing to 10 % buffer at 17 min, and 0 % buffer at 20 min; the subscript _{sh} indicates shoulder

^b Solvent system 1: Toluene - petrol₁₀₀₋₁₄₀ - MeCOEt - MeOH (12:6:2:1.)

^c Solvent system 2: Toluene - petrol₁₀₀₋₁₄₀ - MeCOEt - MeOH (10:25:1:1)

^d Solvent system 3: First ½ with toluene - petrol₁₀₀₋₁₄₀ - MeCOEt - MeOH (10:25:1:1) followed by toluene - MeCOEt - MeOH (12:5:3.)

^e MS References:

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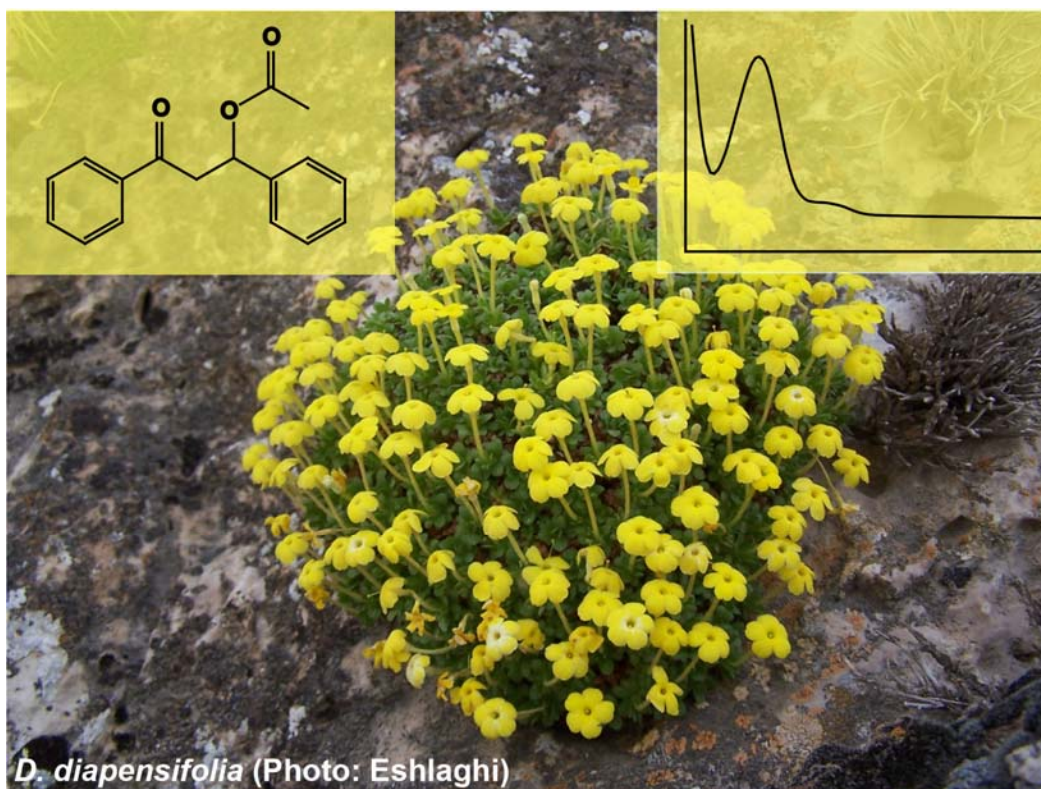
TLC-Data of unknown flavonoids found in exudates of *Dionysia*

Compound	TLC ^a Rf ($\times 100$)	Characteristic colours	
		UV (366 nm)	NA ^b
U1	84	absorbing	no change
U2	79	absorbing	no change
U3	60	bright turquoise fluorescent	no change
U4	26	ochroid	dull pale greenish fluorescent
U5	18	light creamy turquoise fluorescent	no change
U6	10	blue fluorescent	light blue fluorescent

^a Adsorbens: Polyamide 11; Solvent system: Toluene - petrol₁₀₀₋₁₄₀ - MeCOEt - MeOH (10:25:1:1)

^b Detection by UV (366nm) light after spraying with naturstoffreagenz A

Chapter 3: Unusual compounds from exudates of *Dionysia diapensifolia* and *D. gaubae* var. *megalantha* (Primulaceae)



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ABSTRACT

Exudates of *Dionysia diapsifolia* yielded (*R*)-(+)-3-acetoxy-3-phenyl-propiofenone as a new natural product with a basic dihydrochalcone structure, which was elucidated unequivocally by mass spectrometry and NMR spectroscopy. The sesquiterpenoid carissone was found as the major compound in the exudate of *D. gaubae* var. *megalantha*. Sesquiterpenoids have so far not been described as exudate constituents of *Primula* and *Dionysia*. Structural identifications are discussed in detail, and the significance of the occurrence of these unusual compounds in exudates of Primulaceae is shortly addressed.

Keywords: *Dionysia*; exudates, dihydrochalcone, (*R*)-(+)-3-acetoxy-3-phenyl-propiofenone, sesquiterpene, carissone.

The genus *Dionysia* Fenzl comprises about 50 species grouped in three main sections and a number of subsections [1]. *Dionysia* is an Irano-Turanian genus with its centre of diversification in the dry mountains of the Iranian highlands and neighbouring areas [2]. Most of the species are chasmophytes, growing in the crevices of rocks and rock faces, thus contrasting to the species of the closely related genus *Primula* [3-5]. A major characteristic of both genera is the production of conspicuous excretions by glandular trichomes. In *Primula*, excretions may be a powdery farina or simply oily, while in *Dionysia* woolly farina is encountered more frequently than the powdery type. Some species of *Dionysia* are efarinose, but bear glandular hairs and therefore produce exudates. Glandular hairs are the site of production of exudates which consist of flavonoids only in all species studied so far. The genus *Primula* was found to accumulate mainly un-substituted flavone in addition to a series of unusual substituted flavones, which due to their supposed different biosynthetic origin were ascribed recently as “*Primula*-type flavones” [6]. A recent survey indicated that the biosynthetic potential of *Dionysia* is much larger, with additional accumulation of regular substituted flavones, flavonols and flavanones [7]. In continuation of current studies on exudates of *Primula* and *Dionysia*, we wish to report for the first time on new and unusual compounds found in two *Dionysia* species.

New samples of two accessions studied earlier were reanalyzed to identify unknown compounds from their exudates. The current analysis confirmed the presence of several flavonoids, as specified in Table 1. The systematic significance of this flavonoid diversification has been discussed at length by Valant-Vetschera *et al.* [7].

Table 1: Exudate flavonoids of the analyzed species [7].

	<i>D. diapensifolia</i>	<i>D. gaubae</i> var. <i>megalantha</i>
Chalcones	2', β -diOH Chalcone	-
<i>Primula</i> type flavones	Flavone, 2'-OH-flavone, 2'-OMe-flavone, 3'-OMe- 4',5'-O ₂ CH ₂ -flavone, 5- OH-2'-OMe-flavone	-
Regular flavones	Apigenin, Ap-7-Me	Apigenin, Ap- 4'-Me
Flavanones	Naringenin, Nar-7-Me	Pinocembrin
Flavonols	Kaempferol, Kae-3-Me, Kae-7-Me	-

Exudates of *D. diapensifolia* Boiss. yielded an unknown compound (**1**), obvious by comparison of HPLC-spectra and TLC-surveys. Compound **1** has been unambiguously identified by NMR spectroscopy as (*R*)-(+)-3-acetoxy-3-phenyl-propiophenone (Figure 1), which corresponds to a dihydrochalcone structure with an unusual substituent at the β -position. Proton and carbon chemical shifts, as well as ^1H - ^1H coupling constants, are in excellent agreement with those of a prior synthesised racemic mixture of compound **1** [8]. The structure was further proven by the common 2D NMR techniques. These spectra allow the analysis of the dipole-dipole interactions as well as of the scalar $^1J_{\text{H-C}}$ and $^{2/3}J_{\text{H-C}}$ long range heteronuclear couplings, measured by NOESY, HSQC, and HMBC spectra, respectively (Figure 1).

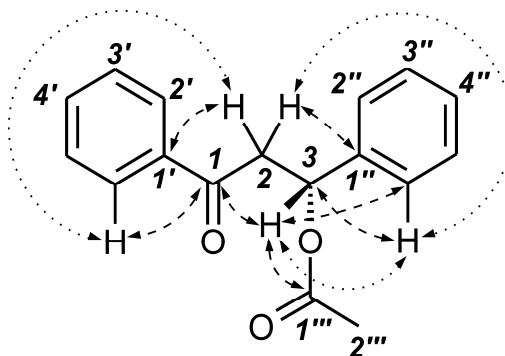
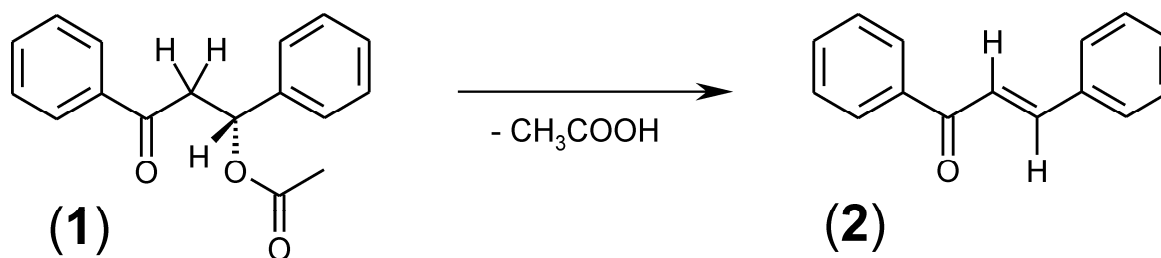


Figure 1: Structure of (*R*)-(+)-3-acetoxy-3-phenyl-propiophenone. Shown are selected NOEs between protons (dotted arrows), as well as indicative $^1J_{H-C}$ and $^2/3J_{H-C}$ -couplings (dashed arrows), which have been used to identify the substance.

Mass spectrometric measurements, however, have been difficult to perform due to the distinct tendency of compound **1** to eliminate acetic acid, even at low temperature (Scheme 1). The resulting benzylidenacetophenone (**2**), structurally corresponding to unsubstituted chalcone, was found in all mass spectrometric measurements, while the molecular ion of **1** was hardly detectable. Even in GC-MS coupling the elimination was detected during the chromatography. However, **1** was also retrieved.



Scheme 1: Thermal induced acetic acid elimination from (*R*)-(+)-3-acetoxy-3-phenyl-propiophenone (**1**) to generate benzylidenacetophenone (**2**).

The enantiomeric purity of compound **1** has been determined by recording ^1H NMR spectra in the presence of a chiral environment, which was induced by addition of substoichiometric amounts of chiral europium tris[3-(heptafluoropropylhydroxymethylene)-(-)-camphorate] ($\text{Eu}(\text{tfc})_3$) [9]. The resulting spectra show small but distinct effects to the shifts of several protons depending on the molar ratio of added $\text{Eu}(\text{tfc})_3$ (Figure 2). However, the consistent changes of all signal group shifts indicate the presence of a very large enantiomeric excess of **1**. The absolute configuration has been determined to be (*R*) by the optical rotation of $[\alpha]_{\text{D}}^{20} = 20.9^\circ$. The optical rotation is slightly smaller than that of earlier reported values, which is caused by small impurities of generated chalcone (**2**) and acetic acid (Figure 2) [10,11].

Although compound **1** has been synthesized for different reasons in the past [8,10,12], it has not yet been known to occur in nature. However, un-substituted dihydrochalcone has been reported for the fungus *Phallus impudicus* [13], together with free acetic acid. As far as substitution patterns are concerned, compound **1** corresponds to 2', β -diOH-chalcone, which is also found in *D. diapsifolia* (Table 1), as shown in our previous study [7]. However, it is not clear how and if these two chalcone structures could be correlated biosynthetically.

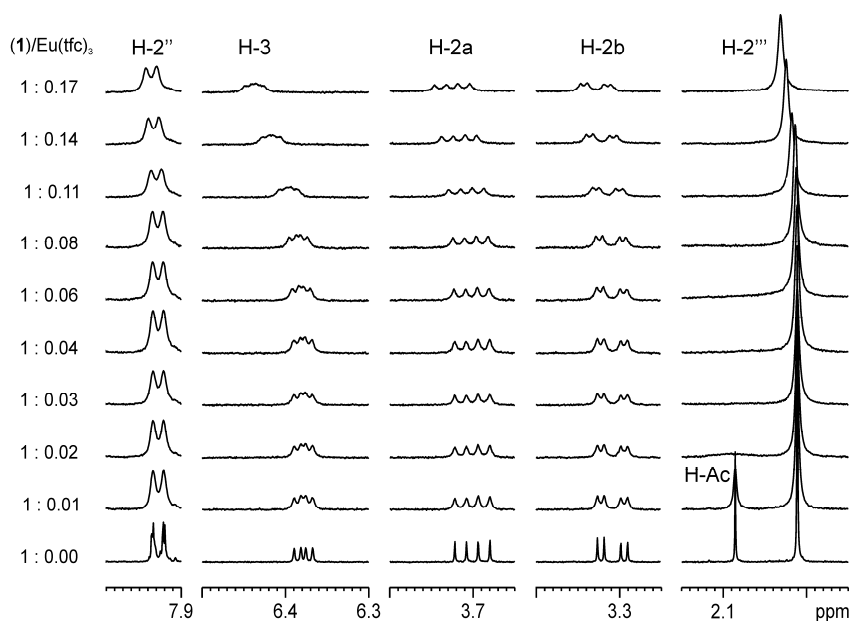


Figure 2: Proton spectra of (*R*)-(+)-3-acetoxy-3-phenyl-propiophenone (**1**) after addition of $\text{Eu}(\text{tfc})_3$. Shown are signals of H-2'', H-3, H-2a, H-2b, and H-2''' as indicative signals. The consistent changes of the different proton shifts indicate one enantiomer of **1** to be present in an excess of >99%. The signal at 2.095 ppm indicates the presence of acetic acid as an impurity in the sample, which has been generated by the elimination procedure shown in Scheme 1.

Compound **1** showed a clear inhibition against *Cladosporium sphaerospermum* in an antifungal bioassay. It further showed a distinct tendency to eliminate acetic acid providing benzylidenacetophenone (**2**), even under moderate conditions. Compound **2** has been reported to possess antibacterial, antiprotozoal and antiparasitic activities [14,15]. It is hence feasible that **1** is produced in the glandular trichomes of *D. diapensifolia* and transported to the surface of the leaves first, but environmental or other abiotic influences could then cause the formation of compound **2**, which may be active directly on the leaf surface.

Preliminary TLC analysis of *D. gaubae* var. *megalantha* Lidén indicated the presence of one unknown compound, probably not of flavonoid nature (Wollenweber, pers. comm.).

Reanalysis of this sample by HPLC revealed, indeed, a new compound, with retention time and UV spectra not correlating to any of the known flavonoids. This compound was isolated by applying the standard separation method (MPLC and prep. TLC), and was identified as carissone (**3**) by comparison of the analytical data with earlier reports [16]. This eudesmol-type sesquiterpene is the first terpenoid structure so far that has been detected in the exudates of Primulaceae studied. This finding is in strong contrast to the average composition of exudates of both *Primula* and *Dionysia*, since they usually accumulate flavonoids as the sole constituents. Therefore, the complete lack of *Primula*-type flavones and the dominating amount of compound **3** in the exudate of this species is a remarkable feature. It should be mentioned that production of larger quantities of terpenoids together with lower amounts of flavonoids in exudates is rather the rule, for example in species of the large families of Lamiaceae and Asteraceae [17,18].

Compound **3** has earlier been isolated from fruits, wood and roots of several plants [19-21], but it has never been reported as an exudate constituent so far. Comparison with *Primula* exudates (Bhutia, in prep.) indicates that this compound is restricted to *Dionysia*. In view of the close relationship between both genera, leading sometimes even to a proposal to merge them [4], this result is of chemotaxonomic significance. However, the already described diversification of biosynthetically different flavonoid types would also support the retention of these two genera as separate taxonomic units.

EXPERIMENTAL

Plant material:

Dried aerial specimens of *D. diapensifolia* and *D. gaubae* var. *megalantha* from the same source as mentioned in our previous study [7] were received from Uppsala University Botanic Garden, Sweden, where also the voucher specimens have been deposited.

Extraction and isolation:

Dried aerial parts were briefly rinsed with acetone in order to dissolve the lipophilic exudate material. The mixture obtained after evaporation was dissolved in MeOH and the compounds were isolated by preparative MPLC and TLC. For MPLC, light petroleum (PE) with increasing amounts of EtOAc (10%, 15%, 20%, 50% and 100%) was used. Compound **1** was eluted with 10% EtOAc in PE. Compound **3** was obtained by prep. TLC (Merck, silica gel 60, toluene: dioxane: HOAc/90:25:5) of the MPLC fraction eluted by 20% EtOAc in PE. Comparative UV-HPLC and TLC with authentic markers were carried out.

HPLC:

Agilent 1100 Series, UV diode array detection at 254 nm, column Hypersil BDS-C18, 250 x 4.6 mm, 5 µm, mobile gradient MeOH 55-100% in aq. buffer (15 mM H₃PO₄, 1.5 mM Bu₄NOH), flow rate 0.5 mL/min.

Antifungal test:

TLC plates (Merck, silica gel 60) were developed with PE: acetone/50:50 and dried for complete removal of solvents. The chromatograms were subsequently sprayed with a spore suspension of *C. sphaerospermum*.

NMR spectroscopy:

Spectra were measured in CDCl₃ using Bruker Topspin 1.3 software on a Bruker DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at either 400.13 MHz (¹H) or 100.61 MHz (¹³C) at 300.0 K +/-0.1 K. For 1D spectra, 64k data points were recorded using a relaxation delay of 1.0 s. Fourier transformation led to spectra with a range of 6000 Hz (¹H) and 24,000 Hz (¹³C). 2D COSY, TOCSY, NOESY, HMQC, and HMBC spectra were measured by 128 experiments with 1024 data points each. Linear forward prediction to 2k and 256 data points in the f₁ and f₂ dimension, respectively, sinusoidal multiplication in both dimensions, and Fourier transformation produced 2D-spectra with a range of 3500 Hz and 17000 Hz for ¹H and ¹³C, respectively. CHCl₃ was used as internal standard for ¹H (δ_H 7.240) and CDCl₃ for ¹³C (δ_C 77.00) measurements.

Mass spectrometry:

Mass spectra were recorded on a 900S Finnigan MAT spectrometer by direct infusion electrospray ionization in positive and negative modes, respectively.

GC-MS coupling:

GC-MS: GC 8000 series gas chromatograph {Fisons Instruments, Milan, Italy: DB-1701 fused silica column (30 m x 0.32 mm, film thickness 0.3 μm, J&W Scientific); carrier gas: helium (80 kPa); injection temperature 230°C; temperature program: 120°C (1 min isotherm), 8°/min up to 250°C (15 min isotherm). MS 800 quadrupole mass spectrometer (Fisons Instruments): ionization energy: 50 eV, ion source temperature: 80°C.

Optical rotation:

This was measured at the sodium D line using a 100 mm path length cell on a Perkin Elmer Automatic Polarimeter 341 (Perkin Elmer Austria).

(R)-(+)-3-Acetoxy-3-phenyl-propiophenone (1)

$[\alpha]_{\text{D}}^{20}$: +20.9 (2.25 g/mL, CH₃OH)

Colorless, clear, and highly viscous liquid.

¹H NMR (CDCl₃) δ : 7.93 (2H, m, H-2''), 7.57 (1H, m, H-4''), 7.45 (2H, m, H-3''), 7.40 (2H, m, H-2'), 7.36 (2H, m, H-3'), 7.31 (1H, m, H-4'), 6.38 (1H, dd, J = 8.4/5.1 Hz, H-3), 3.70 (1H, dd, J = 17.2/8.4 Hz, H-2a), 3.31 (1H, dd, J = 17.2/5.1 Hz, H-2b), 2.01 (3H, s, H-2''').

¹³C NMR (CDCl₃) δ : 196.1, C-1; 169.9, C-1'''; 140.0, C-1''; 136.3, C-1'; 133.3, C-4''; 128.7, C-3'; 128.6, C-4'; 128.2, C-2'; 128.1, C-3''; 126.5, C-2''; 71.9, C-3; 45.1, C-2; 21.1, C-2'''.

ESI MS: pos. mod: m/z (%): 332.1 [M+Na+HCN]⁺ (41%); 291.1 [M+Na]⁺ (100%).

GC-MS: M⁺ m/z = 268 (1.1%), 208 (100), 77 (82), 131 (67), 103 (59), 51 (45).

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Chapter 4: Diversification of exudate flavonoid profiles in further *Primula* spp.



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ABSTRACT

In continuation of previous work, exudate flavonoid profiles of 22 new accessions of the genus *Primula* L. aligned to different subgenera were studied for the first time. Profiling was done by comparative TLC and UV-HPLC against authentic marker compounds. Most of the studied species accumulated the typical *Primula* flavonoids, comprising derivatives of unsubstituted flavone, of 5,8- or 5,6-hydroxyflavones including partly highly oxygenated flavones, together with 2,2'-diOH-chalcone in their exudate. The profile of *P. edelbergii* belonging to subgen. *Sphondylia* differed largely from the majority of *Primula* species studied so far, showing accumulation tendencies similar to those observed earlier for the closely related genus *Dionysia*. The phylogenetic significance of this diversification is shortly addressed.

Keywords: *Primula*; *Dionysia*; Farina; Exudate Flavonoids; Chemodiversity.

In a preceding publication on exudate flavonoids of *Primula* [1], a survey was given on the so far known flavonoids typically found in exudates of *Primula* species. This large genus of about 400 species has its centre of diversification in the eastern Himalaya where almost 75% of the species occur. Numerous species grow in alpine regions in the Northern Hemisphere, and fewer in lowland regions of Europe and North America [2,3,4]. Production of oily or farinose exudates (farina) on aerial surfaces of leaves, stems, calyces and flowers is a conspicuous character for this genus [5,6]. These exudates consist primarily of unsubstituted flavone and of other flavones with unusual substitution patterns, which are probably derived from a still unidentified biogenetic pathway [1]. Similar accumulation tendencies were observed in a comprehensive study on exudates of the closely related genus *Dionysia* [7]. However, a series of *Dionysia* spp. was found to produce also flavonoids derived from the regular biosynthetic pathway, either in addition or instead of the typical *Primula* flavonoids. The significance of this result was discussed recently, particularly in respect to molecular phylogenetic data, which indicated that *Dionysia* should be better incorporated in *Primula* [8]. Biogeographic distribution and molecular data strongly suggest that both *Dionysia* [8] and the Central European *Primula* species [9] bear derived status in the phylogeny of *Primula*. Exudate flavonoid profiles of 22 new accessions from four different subgenera of *Primula* were analyzed now for the first time. Results are summarized in Table 1 and will be discussed in relation to the subgeneric classification of *Primula* and its relationships to the genus *Dionysia*.

Subgen. *Sphondylia* (DUBY) RUPR.:

This subgenus comprises the single section *Sphondylia* only, which consists of eight species and represents a morphologically, geographically and ecologically isolated group [10]. *Primula simensis* Hochst. and *P. edelbergii* O. Schwarz were the two species studied here. While *P. simensis* showed typical *Primula* flavones such as unsubstituted flavone, 2'-OMe-flavone, 2'-OH-5'-OAc-flavone, 3'-OMe-flavone and 5,8-diOH-flavone, *P. edelbergii* exhibited an unusual exudate composition including 2,2'-diOH-chalcone, 5-OH-8-OMe-flavone and 3-acetoxy-3-phenyl-propiophenone, but no trace of unsubstituted flavone,

which is one of the most prominent *Primula* components present in nearly all the species studied so far. In addition, an unknown compound, not yet isolated due to the lack of material, is found to be predominant in this exudate.

Subgen. *Aleuritia* (DUBY) WENDELBO:

This large subgenus is divided into 18 sections, of which species of sections *Minutissimae*, *Petiolares*, *Glabra* and *Amethystina* were studied now. Exudates of *P. primulina* (Sprengel) Hara from sect. *Minutissimae* exhibited flavone, 2'-OH-5'-OAc-flavone and 5,8-diOH-flavone as three major compounds. Two species of sect. *Petiolares*, i.e. *P. bracteosa* Craib (subsect. *Petiolaris*) and *P. calderiana* (subsect. *Griffithii*), accumulated flavone as major compound, followed by 5,8-diOH-flavone and several other flavonoid aglycones (Table 1). Sect. *Glabra* is a small section consisting of only three species. *Primula glabra* Klatt is efarinose like the other two species, and thus its exudate contained only low amounts of flavonoids, with unsubstituted flavone as the only compound present. The exudate profile of *P. dickieana* Watt, belonging to sect. *Amethystina*, showed a more diversified pattern of flavonoid aglycones consisting of flavone, 2'-OMe-flavone, 2'-OH-5'-OAc-flavone, 3'-OMe-flavone and 5-OH-2'-OMe-flavone. Both *P. tibetica* Watt and *P. involucrata* Duby non Sweet of sect. *Armerina* excreted only unsubstituted flavone. By contrast, this compound, together with 5,8,2'-triOH-flavone, were the predominant constituents in exudates of *P. sikkimensis* Hooker f. and *P. firmipes* Balf. f. & Forrest of sect. *Sikkimensis*. The rarely accumulated 2,2'-diOH-chalcone was also detected in the latter species. *Primula glomerata* Pax was the only species of sect. *Capitatae* analyzed now. It showed a similar exudate composition as *P. capitata*, a species belonging to the same section that has been studied earlier [1], with flavone and 5,8-diOH-flavone as major compounds. *Primula rosea* Royle of sect. *Oreophlomis* did not display a wide spectrum of compounds in its exudates. The main components were flavone and 2'-OMe-flavone. Flavone and 5-OH-flavone were the two prominent components in exudates of *P. darialica* Rupr. and *P. specuicola* Rydb. of sect. *Aleuritia*. The major compounds detected in *P. prolifera* Wall. of sect. *Proliferae* were flavone, 5,8-diOH-flavone and 2,2'-diOH-chalcone.

Primula macrophylla D. Don and *P. megalocarpa* Hara of sect. *Crystallophloemis* shared a similar pattern of exudate components, with flavone, 2'-OH-5'-OAc-flavone, 5-OH-flavone and 5,8-diOH-flavone as major compounds. *Primula bellidifolia* King of sect. *Muscaroides* showed a limited excretion capability, exhibiting only flavone and some of its methyl ethers in traces. Although described as being slightly farinose [4], the analyzed sample did not exhibit any farina, which may account for the low amount of obtained exudate.

Subgen. *Auriculastrum* SCHOTT.:

This subgenus comprises 5 sections, one of the largest being sect. *Auricula* with 22 species, growing on the mountains of central and southern Europe. *Primula hirsuta* All. exhibited unsubstituted flavone as the sole compound. Although this species has been studied for both tissue and exudate flavonoids, only the tissue constituents have been structurally identified [11]. These include glycosides of 7,2'-dihydroxyflavone, which is frequently found as aglycone in exudates of *Primula*. The significance of this result is not yet clear, but it certainly shows that biosynthetic enzymes for this compound may also be present in tissue and not only in glandular hairs. *Primula glaucescens* Moretti non Reichb. exhibited unsubstituted flavone accompanied by some other typical *Primula* flavonoids. Profiles of *P. minima*, *P. wulfeniana* and *P. clusiana* (all in subsect. *Arthritica*) revealed none of the typical *Primula* flavonoids, but contained some other unknown compounds. The separation and isolation of these components will be subject of further studies when more material is available.

Subgen. *Auganthus* (LINK) WENDELBO:

This subgenus consists of eight sections. *Primula forbesii* Franchet was the only species of sect. *Monocarpicae* analyzed in this study. Unsubstituted flavone was the major component detected, followed by traces of some of its methyl ethers.

Table 1: Exudate flavonoids from *Primula* spp.

<i>Primula</i>	Sectional alignment	Source	2,2'-diOH-chalcone flavone	2'-OMe-flavone	2',5'-diOH-flavone	2'-OH-5'-OAc-flavone	3'-OMe-flavone	3',4'-diOH-flavone	5-OH-flavone	5,2'-diOH-flavone	5-OH-2'-OMe-flavone	5,8-diOH-flavone	5,8,2'-triOH-flavone	5-OH-8-OMe-flavone	5-OH-8,2'-diOMe-flavone	5,6,2',6'-tetraOMe-flavone	3-acetoxy-3-phenyl-propiofenone
<i>specuicola</i>	ALAL	WU	0			t			t								
<i>darialica</i>	ALAL	ZBG	0			t			0	t							
<i>tibetica</i>	ALAR	KH	t														
<i>involucrata</i>	ALAR	KH	t														
<i>glomerata</i>	ALC	KH	0		t	t			t			0	t				
<i>megalocarpa</i>	ALCR	KH	0		t	0			0	t		0					
<i>macrophylla</i>	ALCR	WU	0			0			0	0		0	t				
<i>glabra</i>	ALGL	KH	t														
<i>bellidifolia</i>	ALM	KH	0			t		t				t					
<i>primulina</i>	ALMI	KH	0			0						0					
<i>rosea</i>	ALO	ZBG	0	0		t			t		t						
<i>prolifera</i>	ALP	KH	0	0		t	t			t							
<i>bracteosa</i>	ALPE	KH	0			t			t			0					
<i>calderiana</i>	ALPE	KH	0		t	t		t		t		0	t				
<i>firmipes</i>	ALS	HBV	0	0	t								t		t	0	
<i>sikkimensis</i>	ALS	HBV	0										0				
<i>dickieana</i>	AUAM	KH	0	0		t	0	t	t		t						
<i>forbesii</i>	AUGMO	ZBG	0		t	t			t	t							
<i>hirsuta</i>	AURA	ZBG	0	t		t						t					
<i>glaucescens</i>	AURA	ZBG	0			t			t			0					
<i>edelbergii</i>	SS	ZBG	0											t			t
<i>simensis</i>	SS	WU	0	t		t	t					t					

Abbrev.: ALAL (*Aleuritia* sect. *Aleuritia*); ALAR (*Aleuritia* sect. *Armerina*); ALC (*Aleuritia* sect. *Capitatae*); ALCR (*Aleuritia* sect. *Crystallophlomis*); ALGL (*Aleuritia* sect. *Glabra*); ALM (*Aleuritia* sect. *Muscaroides*); ALMI (*Aleuritia* sect. *Minutissimae*); ALO (*Aleuritia* sect. *Oreophlomis*); ALP (*Aleuritia* sect. *Proliferae*); ALPE (*Aleuritia* sect. *Petiolares*); ALS (*Aleuritia* sect. *Sikkimensis*); AUAM (*Auriculastrum* sect. *Amethystina*); AUGMO (*Auganthus* sect. *Monocarpicae*); AURA (*Auriculastrum* sect. *Auricula*); SS (*Sphondylia* sect. *Sphondylia*); 0: large amounts; t: trace amounts

HBV: Botanical Garden of the University of Vienna; KH: Herbarium, University of Kyoto; WU: Herbarium, Institute of Botany, University of Vienna; ZBG: Botanical Garden of the University of Zurich

DISCUSSION

In contrast to previous works [1,7] herbarium specimens were analyzed now in addition to material from cultivation. Flavonoids are known to be stable on herbarium sheets that are more than one century old [12], and their stability could also be proved in the present study. Thus, the major exudate compounds were unsubstituted flavone and 5-OH flavone, which were accompanied by their methyl ethers, all of them the typical *Primula* flavonoids with a presumably specific biosynthetic origin. It is highly interesting that, so far, only flavones and structurally related chalcones are found in the exudate profiles, despite the large morphological diversity observed in this genus. However, when species of subgen. *Auriculastrum* are screened, some taxa deviated by lack of *Primula*-type flavonoids. Also, some species such as *P. tibetica*, *P. involucrata* and *P. glabra* had much reduced exudate profiles, consisting sometimes of unsubstituted flavone only. Similar observations have recently been made for some species of *Dionysia* [7]. Eventual correlations with excretion structures and features should be studied in more detail to be able to explain these discrepancies.

Further interesting observations were made in *P. edelbergii* of subgen. *Sphondylia*. Wadi and coworkers have reported earlier that *P. edelbergii* differs palynologically from other *Primula* spp. [10]. This is in line with the results of our study, in which no *Primula* flavonoid but 2,2'-diOH-chalcone could be detected. Furthermore, *P. edelbergii* exhibited 3-acetoxy-3-phenyl-propiofenone and 5-OH-8-OMe-flavone. Presence of these compounds, though in traces, is rather surprising, as they have not been detected in any other *Primula* species analyzed in this and earlier studies [1]. While 5-OH-8-OMe-flavone is well-known from *Dionysia*, and the presumable precursor 5,8-diOH-flavone occurs frequently in *Primula* exudates, 3-acetoxy-3-phenyl-propiofenone isolated for the first time as a natural compound from *D. diapensifolia* [13] is a rare compound of restricted distribution. This particular compound may be a chemical marker in view of the close relationship between

the genus *Dionysia* and *Primula* subgen. *Sphondylia* [8]. *Primula edelbergii*, moreover, is native to Afghanistan, which is one of the major distribution areas of *Dionysia* [14].

Recently 7,3',4'-tri-O-methylquercetin, a flavonol of the regular biosynthetic flavonoid pathway, was surprisingly reported as exudate constituent of *P. spectabilis* Tratt. [15]. This is the first time that a flavonol with a classical 5,7-substitution has been found in exudates of a *Primula* species. However, such compounds do occur in exudates of several *Dionysia* species, so it was only a question of analyzing more samples to find similar structures also in *Primula* exudates. These results challenge the assumption that *Primula* is free from flavonoids arising from normal biosynthetic pathway. Furthermore, preliminary analysis of some other European alpine species also showed some deviation from the exudate flavonoid pattern claimed to be typical of *Primula* (Bhutia, in prep.). This is interesting from the evolutionary point of view as the European alpine *Primula* species are known to be more derived [9]. The parallels with *Dionysia* are intriguing and suggest that in both cases these diversifications may be indicative of the evolution within the *Primula-Dionysia* assemblage. To assess this assumption, further research remains to be done in profiling additional taxa for exudate flavonoids.

EXPERIMENTAL

Plant material:

Fresh samples were collected from plants cultivated in the Botanical Garden of the University of Zurich, Switzerland, as well as from the Botanical Garden of the University of Vienna, Austria, and air dried for analysis. Voucher specimens from the collected plant samples were deposited at the Herbarium WU, University of Vienna, Austria. Herbarium specimens were ordered from Kyoto University Museum, University of Kyoto, Japan, and from Herbarium WU. The source of each specimen is given in Table 1.

Extraction and isolation:

In order to dissolve the lipophilic components excreted on the surfaces, the shade-dried aerial plant materials were rinsed carefully using acetone at room temperature. For rinsing the herbarium specimens, these were removed cautiously from their sheets and subsequently mounted back again in the original position. The acetone filtrate obtained was then dried using a rotary-evaporator to give a crude extract, which was suspended in MeOH and further subjected to TLC and HPLC. Comparative TLC was performed on Merck silica gel 60 using a solvent mixture of Toluene-Dioxane-HOAc/75-25-5. Chromatograms were viewed under UV light, before and after spraying with Naturstoffreagenz A (0.5% of diphenylboric acid 2-aminoethyl ester in MeOH). The identification of the compounds was achieved co-chromatographically with HPLC and TLC by using authentic markers obtained from E. Wollenweber. HPLC analysis was performed with an Agilent 1100 Series equipped with a UV Diode Array Detector using a Hypersil BDS-C18 column (5 μ m, 250 x 4.6 mm, flow rate 0.5 mL/min) with mobile gradient MeOH 55-100% in aq. buffer (15 mM H₃PO₄, 1.5 mM Bu₄NOH). The detection range was 230-360 nm.

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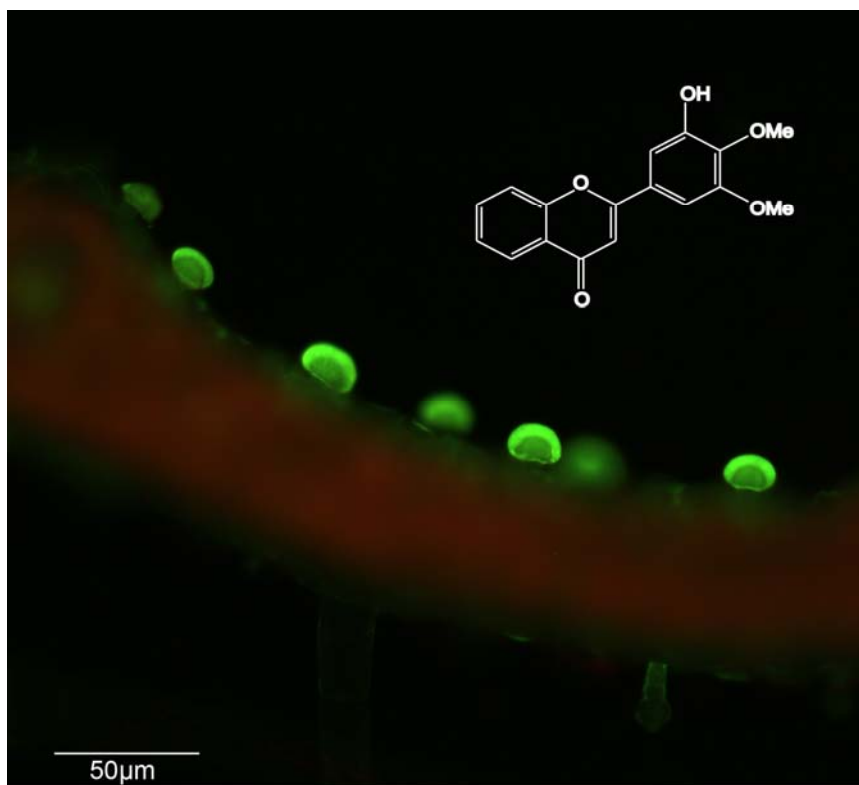
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Chapter 5: Flavonoids in selected *Primula* spp.: Bridging micromorphology with chemodiversity



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ABSTRACT

A combined study was carried out on the micromorphology and chemistry of glandular trichomes with focus on *Primula vialii* and *P. vulgaris*, respectively. Epifluorescence microscopy was applied to study the auto-fluorescent properties of flavonoids and their localization in glandular trichomes. Both species differed in the morphology of the glandular trichomes and in the exudate flavonoid composition. Leaf glands from *P. vialii* and from some species of subgen. *Primula* exhibited uniform glandular fluorescence, but notable differentiation was observed within a single leaf of *P. vulgaris*. Our observations indicate that exudate flavonoids are not transported from the tissue to the glandular hairs. Conversely, only the newly isolated glycoside **1** (kaempferol 3-*O*-(2''-rhamnosyl)-robinobioside) was obtained from leaf tissue of *P. vialii* after removal of the exudate. Its structure was confirmed by NMR and mass spectrometry. This glycoside was not detected in tissue extracts of *P. vulgaris* after similar treatment. The observed chemical diversity is discussed, with focus on possible correlation with glandular structures and tissue differentiation in *Primula*, and also against other studied species. Aspects of biosynthesis in relation to tissue-specific flavonoid diversification are shortly addressed.

Keywords: Autofluorescence, Exudate, Flavonoid, Glandular Trichome, Phytochemistry, *Primula*.

Glandular trichomes are highly specialized epidermal protuberances on the surfaces of leaves, inflorescences and other organs of many plants. Trichome morphology ranges from unicellular to sophisticated multicellular structures that exhibit differentiated basal, stalk and apical secreting cells [1-4]. Glandular trichomes are generally capable of synthesizing, storing and occasionally secreting large quantities and diverse types of specialized metabolites, including various classes of terpenes [5,6], several types of alkaloids [7], and flavonoids [8-10]. Glandular trichomes of *Primula* species are well known to produce lipophilic exudate flavonoids [11], excreted mainly on the surfaces of leaves, stems and inflorescences. The association of exudates and glandular trichomes has been recognized as early as 1945 by Blasdale [12] and has been studied in later works such as Bosabalidis *et al.* [8] and Voirin *et al.* [2]. Microchemical investigations on exudates of *Primula* go back to Brunswik (1922) [13]. Since then, a series of studies elucidating the chemistry of the glandular trichomes in *Primula* species was carried out [9,11,14,15a-f]. However, combined analyses on chemistry and micromorphology or ultrastructure of the glandular trichomes of *Primula* [16-19] are rather rare.

In the course of continued studies on exudate flavonoids in *Primula* [11,20], foliar glandular trichomes of *P. vialii* Delavay ex Franch. revealed a strong auto-fluorescence under an epifluorescence microscope [11]. For the present publication, this phenomenon was studied in more detail with the aim of attaining insight into the localization of the exudate flavonoids in the glandular trichomes of *P. vialii* and *P. vulgaris* Hudson, respectively. To the best of our knowledge, the latter species has not been studied regarding its exudate flavonoids so far. Hence, the main objective of this work was to focus on the micromorphology of the glandular trichomes in these two *Primula* species in combination with analysis of their epicuticular and tissue related flavonoid diversification, and to discuss these results in a broader context.

The novelty of this work is the attempt to visualize the exudate flavonoids of *Primula* spp. using their auto-fluorescence in the absence of chemical processes, i.e., without any pre-treatment, and to correlate the morphological results with the chemical

profile of the exudates of the respective species. Furthermore, the differences between exudate and tissue flavonoid chemistry were checked to confirm further the presence of specific flavonoids solely in glandular trichomes. The species studied differed both in their exudate flavonoid chemistry and in the structure of the glandular hairs containing the flavonoids.

Primula vialii or orchid primrose is endogenous to China, where its population is gradually decreasing due to human activities [21]. This species belongs to sect. *Muscarioides* (subgen. *Aleuritia*) and is very popular in cultivation, being highly attractive as well as robust. *Primula vulgaris* or common primrose is a native plant in western and southern Europe, northwest Africa, and southwest Asia (eastern Turkey to Iran). It belongs to sect. *Primula* (subgen. *Primula*), which is the smallest subgenus comprising currently only seven species. The results from morphological and phytochemical analyses are presented separately; for a combined survey see Table 1.

Table 1: Glandular trichomes in selected *Primula* spp.: Autofluorescence and exudate flavonoids. SG: Sessile glands, CG: Capitate glands, x: present, -: absent. For numbering of flavones see Figure 3.

<i>Primula</i>	SG	CG	UV (360nm)	Blue light (490 nm)	Green light (540nm)	Exudate flavonoids
<i>vialii</i>	x	x	blue	yellowish green	red	flavone, 2'-OMe-flavone, 3',4'-diOH-flavone, 5-OH-flavone, 5,2'-diOH-flavone, 5,8-diOH-flavone
<i>veris</i>	x	x	blue	deep orange	red	flavone, 2'-OH-flavone, 2'-OMe-flavone, 2',5'-diOMe-flavone, 3'-OMe-flavone, 3'-OH-4',5'-diOMe-flavone, 3',4'-diOMe-flavone, 3',4',5'-triOMe-flavone, 3',5'-diOH-4'-OMe-flavone, 3'-OMe-4',5'-MetDiox-flavone
<i>elator</i>	x	x	blue	deep orange	red	2'-OMe-flavone, 2',5'-diOMe-flavone, 3'-OMe-flavone, 3'-OH-4',5'-diOMe-flavone, 3',4'-diOMe-flavone, 3',4',5'-triOMe-flavone, 3',5'-diOH-4'-OMe-flavone, 5,6,2',3',6'-pentaOMe-flavone
<i>vulgaris</i>	x	-	blue	yellowish green/deep orange/red	Red	flavone, 2'-OMe-flavone, 2'-OH-5'-OAc-flavone, 3'-OMe-flavone, 3'-OH-4',5'-diOMe-flavone, 3',4'-diOMe-flavone, 3',4',5'-triOMe-flavone, 5-OH-flavone, 5-OH-2'-OMe-flavone

Morphology and auto-fluorescence of the glands

The leaves of *P. vialii* and *P. vulgaris* showed both non-glandular and glandular trichomes appearing on both sides of the lamina. However, glandular trichomes were concentrated on the abaxial side in both species. The principal morphology of the glandular trichomes studied here follows a scheme widespread in vascular plants [22] and later described for some *Primula* spp. [16]: A base cell carried a stalk consisting of one or a few

cells with a glandular cell at the tip. Leaves of *P. vialii* exhibited two different types of glandular trichomes: a long type with four stalk cells, and a short type with one stalk cell only. The glandular components of the head cells glowed intensely blue upon excitation with UV-light (Figure 1). According to TLC results this auto-fluorescence could be attributed to 2'-OMe-flavone, which, together with flavone and 5-OH-flavone, forms one of the prominent compounds sequestered onto the leaf surfaces of *P. vialii*. The latter compounds lack fluorescent properties, and thus do not contribute to the blue color.



Figure 1: *Primula vialii*: Autofluorescence of a glandular trichome and of 2'-OMe-flavone (detected at 360 nm). Scale bar 20 μ m.

In *P. vulgaris*, the glandular trichomes were morphologically uniform, but they exhibited different fluorescence patterns within one leaf upon excitation with blue light (490 nm). The head cells showed yellowish green, deep orange or red fluorescence, which was correlated with slight differences in the coloration of the cells (Figure 2). Similar differentiations were described for glands from *P. daonensis* from a different taxonomic group of *Primula* [16], but no explanation of this phenomenon was provided.

It is known that glandular trichomes develop from a presecretory to a secretory and finally post-secretory phase, differing in ultrastructure and phytochemistry [10,23]. Exudate flavonoid composition of *Primula* glands could be expected to correlate with respective developmental stages, but this could not be verified in the present study. More insight would come from ultrastructural studies and a more detailed investigation of the glandular constituents *in situ*.

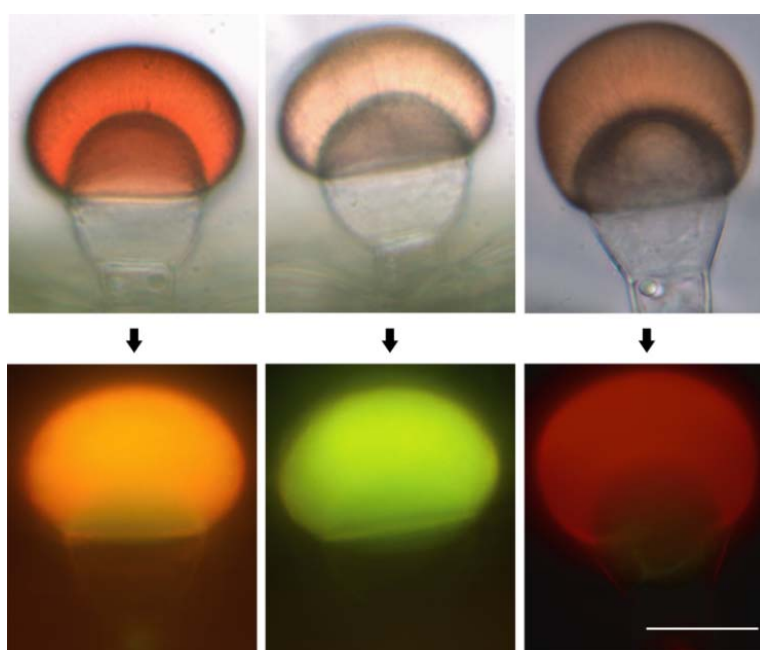


Figure 2: Glandular trichomes of *Primula vulgaris* (upper row: light field; lower row: blue light). Scale bar 20 μm .

Interestingly, *P. elatior* and *P. veris*, both closely related to *P. vulgaris*, exhibited uniform colour schemes upon excitation with various wavelengths (Table 1). So far, no correlation between fluorescence properties and exudate flavonoid chemistry could be established in these species. This is mainly due to the fact that the exudate flavonoid profile of *P. vulgaris*, *P. elatior* and *P. veris* is much more complex, consisting also of compounds that could mask the fluorescence of others. However, in all these species the head cells of the glandular trichomes showed blue autofluorescence which could be correlated to the presence of 2'-OMe-flavone.

Flavonoid chemistry:

Phytochemical reinvestigation of *P. vialii* exudates yielded 2'-OMe-flavone, but could not confirm the presence of 2'-OH-flavone, as reported earlier from another accession [15a]. All the other compounds detected (Table 1) are in accordance with Wollenweber [15a]. *P. vulgaris* showed a more complex exudate profile compared with that of *P. vialii*. It exhibited, like its closely related species *P. elatior* and *P. veris* [9,14], unsubstituted flavone and series of 3',4' di-substituted and 3',4',5' tri-substituted flavones (Table 1).

As expected, notable differences were observed in both species between the flavonoid composition of leaf exudates and extracts obtained after rinsing off the exudate flavonoids. The major exudate components were present only in traces in the tissue extract, confirming that the exudate was properly removed. In *P. vialii* a prominent polar component was present in the extract, which was completely absent in the exudates. It was isolated as a glycoside and structurally identified as kaempferol 3-*O*-(2'''-rhamnosyl)-robinobioside (**1**) by NMR and mass spectroscopy. The obtained NMR data (chemical shifts, coupling constants, and NOEs) indicated the interglycosidic bonds and anomeric configurations of the sugar units, as shown in Figure 3. On the basis of these data, the structural divergence between **1** and the earlier reported kaempferol 3-*O*-(2''-rhamnosyl)-robinobioside from *Boerhaavia diffusa* L. [24] could be substantiated. This new compound could not be detected in *P. vulgaris* extract after removal of the exudate. HPLC analysis of its tissue extract showed only traces of as yet unidentified flavonol glycosides.

Primula species are not well studied with respect to their flavonoid glycosides, but diversification is quite remarkable, including C-glycosylflavones and flavonol-3-*O*-glycosides in *P. spectabilis* [18] and a series of flavonol-3-*O*-glycosides based upon quercetin, kaempferol and isorhamnetin from a number of other species [16,25]. Glycosides based upon either tamarixetin or myricetin are less frequently reported [16,26]. Several of the flavonol glycosides contain robinobiose as a sugar component, and generally,

a tendency to accumulate di- or triglycosides is observed. The complex sugar moiety of our newly reported kaempferol glycoside (**1**) conforms well to these reports. One 7-*O*-glucopyranoside of 7,2'-diOH flavone was isolated from extracts of *P. macrophylla* [27] and of *P. auricula*, respectively [16].

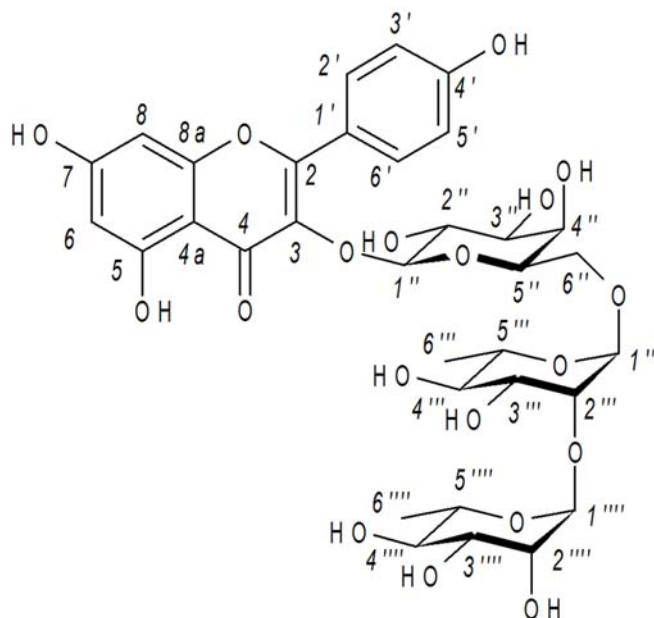


Figure 3: Structure of kaempferol 3-O-(2'''-rhamnosyl)-robinobioside (**1**) with indicated atom numbering of the NMR spectroscopic data.

One reason for the combined analysis of glandular morphology and their chemistry was to find a way to detect the glandular products *in situ*. General exudate flavonoid accumulation trends observed in *Primula* species include unsubstituted flavone and its methyl ethers exhibiting unusual oxygenation patterns, which are most probably derived from a still unidentified pathway [11,20]. Micromorphological analysis has now confirmed that the exudate flavonoids are not transported from the tissues to the glandular trichomes, since auto-fluorescence was limited to the head cells of the glands in both studied species. This is in line with observation on glandular hairs of *Helianthus annuus* [10]. Furthermore, the absence of exudate flavonoids in the tissue extract of *P. vialii* strongly indicates that both biosynthesis and accumulation of exudate components take place locally. Whereas *P. vialii* glands showed no differentiation of excised

glands, those of *P. vulgaris* exhibited different colors, believed to result from different chemical composition.

Comparison of tissue- and glandular flavonoid chemistry revealed interesting differences. So far, none of the aglycones from the glycosides were found as exudate components in *Primula*. This fact strongly favors the assumption of expression of two different biosynthetic routes in tissues and in the glandular hairs. Because of their uncommon substitution patterns, exudate flavonoids of *Primula* are postulated to be biogenetically distant from those of the regular flavonoid pathway [11,28]. Either a completely different pathway is expressed or the classical flavones with regular 5,7-substitution are transformed in the glands due to unknown chemical processes. Hop provides one example for such a transformation of flavonoids, which are prenylated by specialized enzymes present in the glandular trichomes, making them more lipophilic [29].

Interestingly, the 5-deoxycompound 7,2'-diOH flavone is present as glycoside in tissues of some *Primula* spp. [16,27], but not known as an exudate compound. Its biosynthesis, however, may correspond to the known route to 5-deoxyflavones via isoliquiritigenin [30]. It is uncertain whether further modifications of this particular route may lead to the specific flavonoids of *Primula* exudates. It must be mentioned that structural and biogenetic differences between tissue and glandular flavonoids are also observed outside *Primula*. Examples are found *inter alia* in the genera *Uncarina* (Pedaliaceae) [31], *Pulicaria* (Asteraceae) [32] and *Achillea* (Asteraceae) [33a,33b]. Contrary to *Primula*, glandular flavonoids are derived from the regular flavonoid pathway in these cases. Presence of different flavonoid groups and contrasting substitution patterns in tissues and glands indicate specific biosynthesis to take place directly in the glands.

Little information is available on the ultrastructure of *Primula* glands across the genus that may shed some light on glandular flavonoid biosynthesis. Early ultrastructural studies on oil and farina glands revealed the presence of leucoplasts, smooth ER, and microbodies [19]. It is speculative that the leucoplasts are involved in flavonoid biosynthesis, but, in another study, phenolic compounds were proved to be linked to chloroplast membranes in epidermal tissue [34]. Chloroplasts may biosynthesize flavonoids, as is long known from several plant taxa [35]. More detailed and specific ultrastructural studies would be needed to assess the role of chloro- or leucoplasts in glandular flavonoid formation.

Location of biosynthetic enzymes in glandular head cells of *P. kewensis* was earlier studied by immunogold labeling, indicating the presence of phenylalanine ammonia lyase (PAL) in regions of tubular ER and over the cell wall surrounding the head cell, and chalcone synthase (CHS) labeling exclusively with spherical cell compartments [17]. However, this does not explain how the aberrant *Primula* exudate flavonoids are formed, since PAL and CHS are the enzymes with which regular flavonoids are synthesized from the known precursors derived from the acetate and shikimate pathways. Compound **1** would be such a regular flavonoid type, but being a complex glycoside, its occurrence in lipophilic exudates would be unexpected. Simple flavonol glycosides are rarely found as exudate constituents, but have been reported earlier from Solanaceae [36] and recently from *Epilobium hirsutum* [37]. Some species of *Nicotiana* are known to possess two different types of glandular trichomes that produce either hydrophilic or lipophilic components. The hydrophilic compounds are presumed to have been transported to the plant surface via the short type trichomes, whereas the lipophilic compounds are believed to be synthesized in the head cells of the long type trichomes [36]. For *P. vialii*, it is inconceivable that either type of glandular trichomes might transport the glycoside from the leaf tissue to the surface, as in that case, it should have been detected as an exudate component during HPLC profiling.

Studying organ specific accumulation is not only a necessity in chemical systematics, but also informative regarding the functional aspects of production and accumulation of secondary plant metabolites in general and of flavonoids in particular. The strange substitution pattern of exudate flavonoids of *Primula* has not been observed in exudates of other taxonomic groups, hence making it unique to *Primula* and its closest allies.

EXPERIMENTAL

Plant material:

Plant samples were collected from the Botanical Garden of the University of Vienna, Austria. *Primula vulgaris* grew naturally in the meadows of the garden, whereas *P. vialii* was cultivated from seeds obtained from St. Andrews Botanic Garden, Scotland. Voucher specimens were deposited at the Herbarium WU, University of Vienna, Austria.

General:

1D and 2D NMR spectra were measured on a Bruker DRX-600 AVANCE spectrometer at 600.13 MHz (^1H) and 150.90 MHz (^{13}C) at 298.1 K. Mass spectra were recorded on a 900S (Finnigan MAT) by direct infusion electrospray ionization in positive and negative mode, respectively. High resolution mass spectra were performed with the same equipment at a resolution of 10,000.

Micromorphology:

For structural analysis hand sections of fresh leaves were produced using a razor blade. The unstained sections were observed with an epifluorescence microscope (Olympus BX41) using the filter cubes for U-MNU2 (UV excitation), U-MNB2 (blue excitation) and U-MNG2 (green excitation).

Extraction and isolation:

Fresh leaves were rinsed cautiously at room temperature with acetone in order to obtain the lipophilic components excreted on their surfaces. The rinsed

leaves were further chopped and extracted in acetone for 3 days to gain the tissue extract. The acetone filtrates obtained were then dried using a rotary-evaporator to give a crude extract, which was suspended in MeOH and further subjected to TLC and HPLC. Comparative TLC was performed on Merck silica gel 60 using a solvent mixture of toluene-dioxane-acetic acid/75-25-5. Chromatograms were viewed under UV light, before and after spraying with Naturstoffreagenz A (0.5% of diphenylboric acid 2-aminoethyl ester in MeOH). HPLC analysis was performed with an Agilent 1100 Series equipped with a UV Diode Array Detector using a Hypersil BDS-C18 column (5 μ m, 250 x 4.6 mm, flow rate 0.5 mL/min) with mobile gradient MeOH 55-100% in aq. buffer (15 mM H₃PO₄, 1.5 mM Bu₄NOH). The detection range was 230-360 nm. Leaf exudate and tissue extract were compared by means of TLC and HPLC, as mentioned above. The tissue extract of *P. vialii* was further subjected to preparative TLC using toluene-dioxane-acetic acid/90-25-5.

Kaempferol 3-O-(2'''-rhamnosyl)-robinobioside (1)

Yellow-orange amorphous powder.

UV/Vis max: 266 nm, 350 nm.

¹H NMR (CD₃OD): δ = 8.13 (2H, m, H-2' & H-6'), 6.92 (2H, m, H-3' & H-5'), 6.43 (1H, d, J = 1.2, H-8), 6.24 (1H, d, J = 1.2, H-6), 5.03 (1H, d, J = 8.2, H-1''), 4.96 (1H, d, J = 1.1, H-1'''), 4.53 (1H, d, J = 1.1, H-1'''), 3.96 (1H, m, H-2'''), 3.83 (1H, m, H-2''), 3.81 (1H, m, H-4''), 3.75 (1H, m, H-6a''), 3.74 (1H, m, H-3'''), 3.72 (1H, m, H-3'''), 3.69 (1H, m, H-5''), 3.68 (1H, m, H-5'''), 3.62 (1H, m, H-2'''), 3.59 (1H, m, H-3''), 3.58 (1H, m, H-5'''), 3.44 (1H, m, H-4'''), 3.42 (1H, m, H-6b''), 3.37 (1H, m, H-4'''), 1.21 (3H, d, J = 6.2, H-6'''), 1.17 (3H, d, J = 6.2, H-6''').

¹³C NMR (CD₃OD): δ = 179.5, C-4; 167.2, C-2; 162.9, C-5; 161.7, C-4'; 159.4, C-2; 158.6, C-8a; 135.8, C-3; 132.5, C-2'; 122.6, C-1'; 116.1, C-3'; 106.8, C-1''; 105.3, C-4a; 104.4, C-1'''; 102.4, C-1'''; 100.4, C-6; 95.2, C-8; 80.1, C-2''; 75.8, C-5'''; 75.5, C-5'';

74.5, C-4''''; 73.6, C-4'''; 73.4, C-2''; 72.5, C-2''''; 72.5, C-3''''; 72.3, C-3''; 70.7, C-4''; 70.5, C-5''; 70.5, C-3''; 68.0, C-6''; 23.9, C-6'''; 18.1, C-6''''; for numbering see Figure 3.

ESI MS: neg. mod: m/z (%): 739.0 [M-H]⁻ (100); pos. mod: m/z (%): 785.1 [M+2Na-H]⁺ (33), 763.1 [M+Na]⁺ (100).

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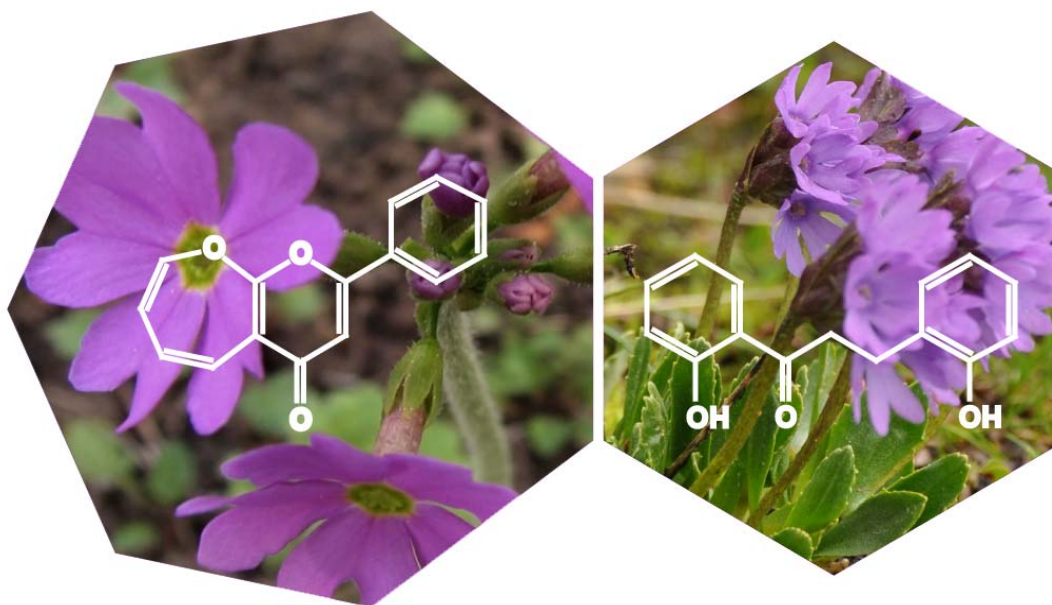
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**Chapter 6: Antifungal orphan flavonoids and dihydrochalcones
from *Primula* exudates**



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(Under revision in *Phytochemistry*)

ABSTRACT

Orphan flavonoids (**1,2**) containing an oxepin structure in ring A were isolated from leaf exudates of *Primula cortusoides*, while *P. glutinosa* exudates yielded two dihydrochalcone derivatives (**3,4**). Chemical structures were elucidated by 2D NMR and mass spectrometry. All four compounds showed antifungal activity against *Cladosporium sphaerospermum*. Possible biosynthesis and nature of compounds **1** and **2** are discussed.

Keywords

Antifungal activity; 1,3-Diphenyl-propan-1-one; Flavonoids; 2-Phenyl-4*H*-oxepino[2,3-*b*]pyran-4-one; *Primula* exudates

1. INTRODUCTION

As part of our ongoing studies on exudate flavonoids on *Primula* spp. (Valant-Vetschera et al., 2009; Bhutia and Valant-Vetschera, 2012), we examined *Primula cortusoides* L., and *P. glutinosa* Wulfen. Both species lack farina, a mealy exudate on aerial surfaces of leaves, stems, calyces and flowers (Richards, 2003). Traditional systematic treatments included presence and absence of farina as one of the few conspicuous characters for the genus *Primula* (Blasdale, 1945). For a comparative study of the glandular structure and flavonoid chemistry of both farina and oil producing species see Wollenweber and Schnepf (1970). Exudates of *Primula* spp. not only consist of unsubstituted flavone, as was believed in the early years (Müller, 1915; Brunswik, 1922), but also of other flavones with unusual substitution patterns such as 2'-OH-flavone, 3',4'-diOH-flavone, 8,2'-diOH-flavone, which are most probably derived from a so far unidentified biosynthetic pathway (Wollenweber and Mann, 1988; Wollenweber et al., 1988; Wollenweber et al., 1990; Valant-Vetschera et al., 2009; Bhutia and Valant-Vetschera, 2012).

Primula cortusoides belongs to Sect. *Cortusoides* of subg. *Auganthus*. It is a deciduous plant with a stout rhizome and is widespread from Eastern Europe to Siberia (Richards, 2003). The exudates produced by the glandular trichomes of *P. cortusoides* were described to cause allergic reactions to human skin (Nestler, 1903). Later work on this species by Wollenweber and Schnepf (1970) revealed the presence of unsubstituted flavone, which is the major component present in almost all of the *Primula* species studied till date and is not known to produce any kind of allergy (Valant-Vetschera et al., 2009; Bhutia and Valant-Vetschera, 2012). *Primula glutinosa* belongs to Sect. *Auricula*, which forms the biggest section of Subg. *Auriculastrum*. It is native to the eastern Alps and has leathery and hairy leaves with sticky secretions (Richards, 2003). To the best of our knowledge, this species has not yet been analyzed for its exudate flavonoids. In the present work isolation of four new compounds (**1-4**) will be discussed in detail.

2. RESULTS

Compounds **1** and **2** were isolated from the leaf exudates of *P. cortusoides* in addition to flavone, 2',5'-diOMe-flavone and traces of 5-OH-flavone, the latter being typical flavonoids of *Primula* at large. To exclude influences of climatic conditions and ontogenetic differences that may affect the composition of exudate flavonoids, accessions of *P. cortusoides* from different localities as well as a herbarium specimen were analyzed. Since compounds **1** and **2** were detected in all the samples (Figure 1), the possibility of these compounds being artifacts could be excluded. Similarly, compounds **3** and **4** were consistently detected in *P. glutinosa*, collected from two different localities, as well as from a herbarium specimen (Figure 1), in addition to 5-OH-flavone and traces of 2',2-diOH-chalcone. Herbarium samples are known to be stable regarding exudates (Wollenweber, 1989; Bhutia and Valant-Vetschera, 2012), for which *P. cortusoides* and *P. glutinosa* afford further examples. The antifungal property of compounds **1-4** was checked, as shown in the experimental part, against the fungus *Cladosporium sphaerospermum* with positive results. All four compounds are of novel character and were not known to occur in nature so far. Structural formulae and UV-spectra of these compounds are illustrated in Figure 2.

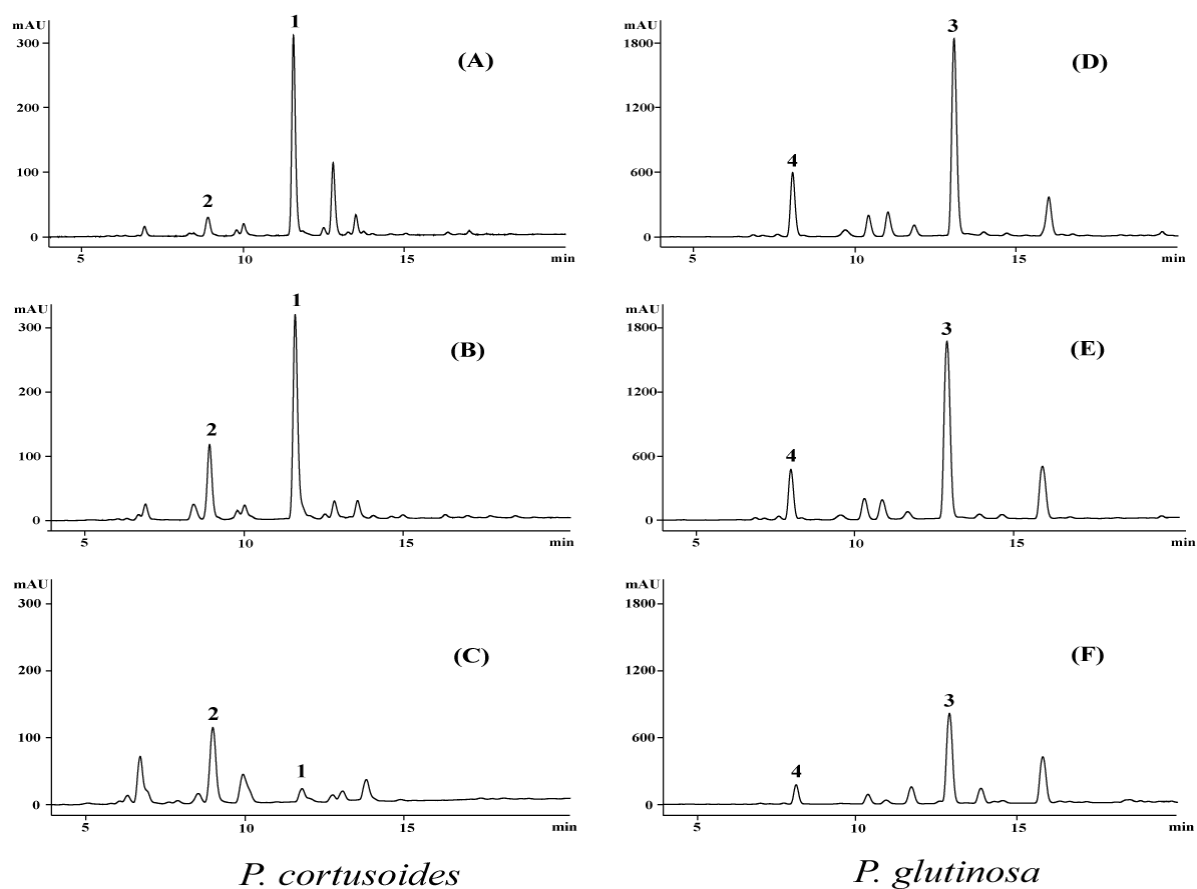


Fig. 1. HPLC chromatograms of *P. cortusoides* [(A): Botanical Garden, University of Vienna; (B): Herbarium specimen (WU545), Altai region, Siberia; (C): Central Siberian Botanical Garden, Novosibirsk)] and *P. glutinosa* [(D): Spittal an der Drau (2050m), Carinthia, Austria; (E): Spittal an der Drau (2290m), Carinthia, Austria; (F): Herbarium specimen (WU003949), Lower Tauern, Styria/Salzburg, Austria].

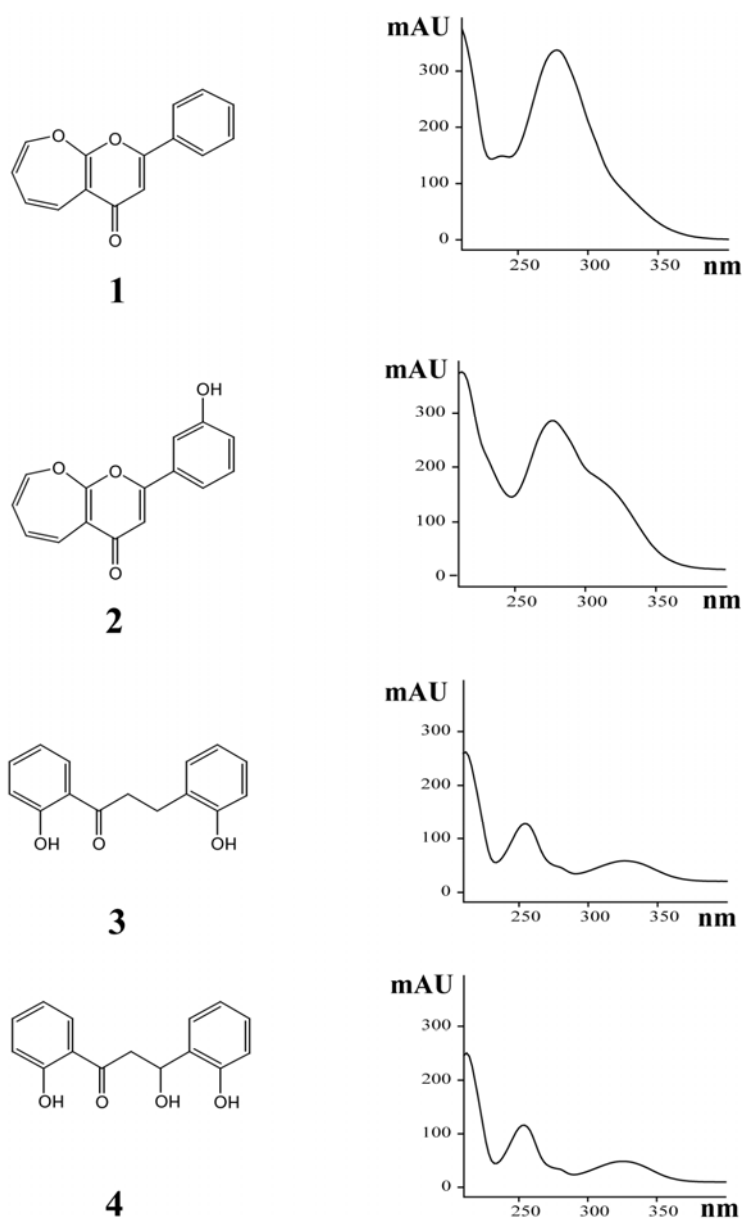


Fig. 2. Structural formulae and UV spectra of compounds **1-4**.

An attached proton test (APT) ^{13}C NMR spectrum and a ^1H NMR indicated compound **1** to consist of ten methine groups and five quaternary carbon atoms. Its protonated molecular ion $[\text{M}+\text{H}]^+$, determined by HR-TOF ESI MS in a positive ionization mode, shows a m/z of 239.0696, corresponding to the molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_3$ with a calc. m/z of 239.0702 for the $[\text{M}+\text{H}]^+$. The also measured m/z of

261.0514 corresponds to $[M+Na]^+$ and is in good accordance to the calc. m/z of 261.0522. The 2D NMR spectra indicated the presence of an annulated bicyclic system, based on an unsymmetrical trisubstituted 4*H*-pyran-4-one skeleton. On one of its double bonds a -C₄H₄O- fragment was annulated resulting in an oxepin ring. The representative $^3J_{H-C}$ couplings, in particular from H-3, H-5, and H-8, determined from the HMBC spectrum are shown in Figure 3. All chemical shifts, in particular those of indicative C-4a, C-8, and C-9a, are listed in Tables 1 and 2. These indicative data can only be aligned to the bicyclic structural moiety shown in Figure 3. On the other double bond of the 4*H*-pyran-4-one skeleton an unsubstituted phenyl ring was connected in the β -position to the carbonyl group, comparable to the substitution pattern in flavones.

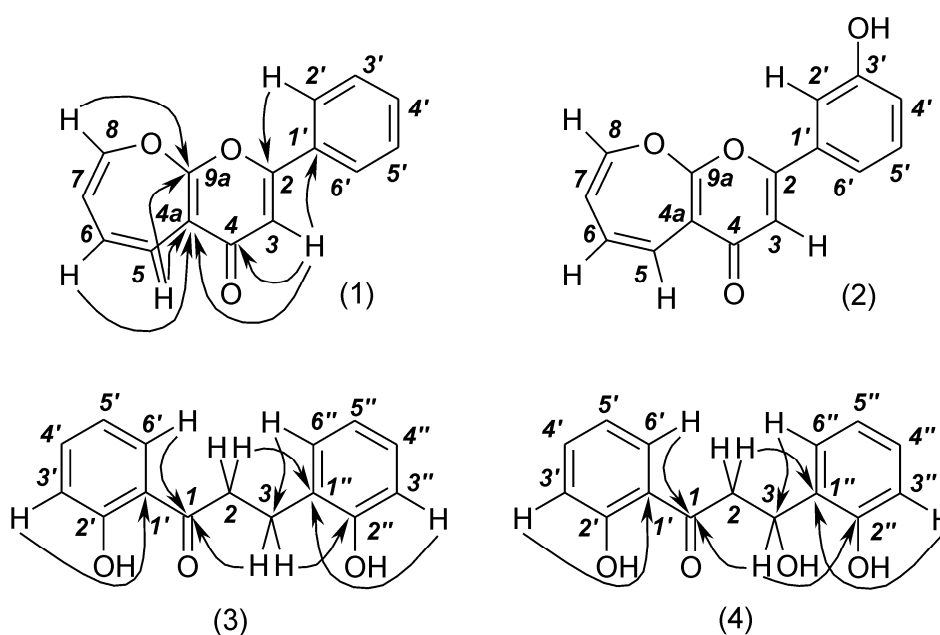


Fig. 3. Structures of compounds **1** to **4**. Atom numbering is indicated according to Tables 1 and 2. Arrows indicate selected representative $^2J_{H-C}$ and $^3J_{H-C}$ long range couplings determined from HMBC measurements for compounds **1**, **3** and **4**.

Table 1

Proton NMR chemical data of compounds **1** to **4**. Chemical shifts [ppm] are amended with integral, multiplicity, and detectable coupling constants [Hz]. Compounds **1**, **3**, and **4** were solved in CDCl₃ and compound **2** was solved in CD₃OD. Indication of the positions is made in accordance to Figure 3.

Pos.	Compound 1	Compound 2	Pos.	Compound 3	Compound 4
2	-	-	1	-	-
3	6.89 (1H, s)	6.77 (1H, s)	2	3.45 (2H, t, 6.6)	3.65 (1H, dd, 18.3, 9.7) 3.44 (1H, dd, 18.3, 2.6)
4	-	-	3	3.05 (2H, t, 6.6)	5.52 (1H, dd, 9.7, 2.6)
4a	-	-	1'	-	-
5	6.83 (1H, d, 10.9)	6.70 (1H, d, 10.8)	2'	-	-
6	6.18 (1H, dd, 10.9, 5.8)	6.21 (1H, dd, 10.8, 5.3)	3'	6.98 (1H, m)	7.02 (1H, m)
7	5.73 (1H, dd, 5.8, 5.7)	5.82 (1H, dd, 5.5, 5.3)	4'	7.47 (1H, m)	7.51 (1H, m)
8	6.11 (1H, d, 5.7)	6.22 (1H, d, 5.5)	5'	6.88 (1H, m)	6.90 (1H, m)
9a	-	-	6'	7.78 (1H, m)	7.69 (1H, m)
1'	-	-	1''	-	-
2'	7.78 (1H, m)	7.21 (1H, m)	2''	-	-
3'	7.49 (1H, m)	-	3''	6.68 (1H, m)	6.93 (1H, m)
4'	7.50 (1H, m)	6.95 (1H, m)	4''	7.11 (1H, m)	7.22 (1H, m)
5'	7.49 (1H, m)	7.31 (1H, m)	5''	6.87 (1H, m)	6.91 (1H, m)
6'	7.78 (1H, m)	7.33 (1H, m)	6''	7.14 (1H, m)	7.03 (1H, m)

Table 2

Carbon NMR chemical data of compounds **1** to **4**. Compounds **1**, **3**, and **4** were solved in CDCl₃ and compound **2** was solved in CD₃OD. Chemical shifts [ppm] and the multiplicities are given. Indication of the positions is made in accordance to Figure 3.

Pos.	Compound 1	Compound 2 *	Pos.	Compound 3	Compound 4
2	161.8, s	nd	1	206.9, s	206.0, s
3	110.1, d	108.8, d	2	39.7, t	44.9, t
4	180.2, s	nd	3	23.6, t	71.6, d
4a	111.2, s	nd	1'	nd**	119.2, d
5	125.5, d	123.9, d	2'	162.5, s	162.6, s
6	126.8, d	126.5, d	3'	118.7, d	118.8, d
7	118.1, d	117.6, d	4'	136.9, d	137.4, d
8	142.2, d	142.0, d	5'	121.0, d	119.5, d
9a	159.6, s	nd	6'	130.1, d	130.1, d
1'	130.5, s	131.8, s	1''	127.6, s	125.5, s
2'	126.2, d	112.1, d	2''	154.5, s	155.7, s
3'	129.6, d	nd	3''	116.8, d	117.7, d
4'	132.1, d	118.7, d	4''	128.1, d	129.5, d
5'	129.6, d	130.0, d	5''	119.1, d	118.9, d
6'	126.2, d	116.5, d	6''	130.6, d	126.8, d

*: Chemical shifts are determined from HSQC. HMBC was not recorded due to low amount of the substance. Chemical shifts of quaternary carbon atoms are hence not determined (nd).

** Not determined due to low s/n ratio

The same uncommon annulated ring system can also be found in compound **2**. It showed very similar chemical proton and carbon shifts for the bicyclic system (Tables 1 and 2). However, according to the HR-TOF ESI MS measurement its molecular ion adduct [M+Na]⁺ showed a *m/z* of 277.0465, which was in good agreement with the calc. *m/z* of 277.0471. This result indicates the molecular formula C₁₅H₁₁O₄. The additional oxygen was located in a hydroxyl group on position 3' of the phenyl ring. Its exact location was determined from the coupling pattern of the aromatic protons in the ¹H NMR spectrum as well as from the chemical proton and carbon shifts.

Compounds **3** and **4** were isolated from the leaf exudates of *P. glutinosa*. The molecular ion $[M+Na]^+$ of compound **3** was determined by HR-TOF ESI MS in a positive ionization mode to have a m/z of 265.0829, which portended the molecular formula of $C_{15}H_{14}O_3$. The according calculated m/z of $[M+Na]^+$ was 265.0835. The APT ^{13}C NMR and the 1H NMR spectra confirmed these finding by indicating two methylen groups, eight methine groups, and five quaternary carbon atoms. 2D NMR spectra revealed the presence of two 1,2-disubstituted benzene rings. Both of them carried one hydroxyl group and were connected to a linear C_3 alkyl linker. One of its carbon atoms directly bound to a phenyl ring was oxidized to a carbonyl group, while the other two were methylene groups. The indicative $^3J_{H-C}$ couplings are presented in Figure 3 and chemical shifts are listed in Tables 1 and 2.

Compound **4** showed the same carbon skeleton and substitution pattern on the phenyl rings as compound **3**. However, apart from the carbonyl group, the linker carried a hydroxyl function on position 3, which was identified by 1H and ^{13}C NMR chemical shifts (Tables 1 and 2) as well as by $^3J_{H-C}$ couplings visualized in Figure 3. HR-TOF ESI MS in a positive ionization mode confirmed these finding by a m/z of 281.0778 for the molecular ion $[M+Na]^+$, which indicated the molecular formula of $C_{15}H_{14}O_4$. The according calculated m/z of $[M+Na]^+$ was 281.0784. The absolute configuration and a possible enantiomeric excess of the chiral center in compound **4** have not been determined.

3. DISCUSSION

Structures of **1** and **2** are completely new in the world of exudate compounds. They differ entirely from flavones and chalcones by bearing an additionally annulated dihydrooxepin ring and, hence, should derive from another biosynthesis (McCormick et al., 1986). The question arises as to why and how these unusual compounds with an oxepin structure are produced. To the best of our knowledge, it is for the first time that such a compound could be isolated from a natural source. Concerning the possible function of both the *Primula*-type flavonoids and the oxepin type compounds, their antifungal properties detected now *in vitro* are well in line with earlier observations in *Dionysia*, a closely related genus (Valant-Vetschera et al., 2010; Bhutia et al., 2011). According to previous works, exudates accumulating on leaf surfaces could probably act as defensive agents against pathogenic fungi (Wollenweber, 1989; Wagner, 1991).

So far, no distinct biosynthetic pathway is known to explain the generation of the orphan type flavonoids **1** and **2** *in planta*. Such a rare annulated oxepin ring has not yet been described in structures that are similar to flavanones or flavones. However, a comparable structural feature has been reported in oxepino[2,3-*b*]benzofuranes, structurally related to dibenzofuranes and in ring enlarged xanthenes (Singh et al., 1994; Singh et al., 1997; Rayne et al., 2005; Krohn et al., 2009). The biogenesis of the latter is assumed to be initiated by an epoxidation of the aromatic moiety (Singh et al., 1997). Similar monooxygenase catalyzed oxidations have been studied in some detail (Boyd et al., 2008). The chemical generation of oxepino[2,3-*b*]benzofuranes, however, starts from 2,2'-biphenylquinones, which derived from irradiation of dibenzo[1,4]dioxins (Rayne et al., 2005). Assuming a similar mechanism in formation of the oxepin ring in the investigated compounds **1** and **2** leads to compounds with 3-hydroxy-1-(2-hydroxyphenyl)-3-phenyl-1-propanone skeletons (dihydrochalcones) as possible starting materials (Figure 4). Very similar structures are found in exudates of *P.*

glutinosa and have recently been described as surface compounds of *D. diapsifolia*, a close relative of *P. cortusoides* (Bhutia et al., 2011). Hence, these partly quite unstable compounds likely also occur on leaf surfaces of other *Primula* species.

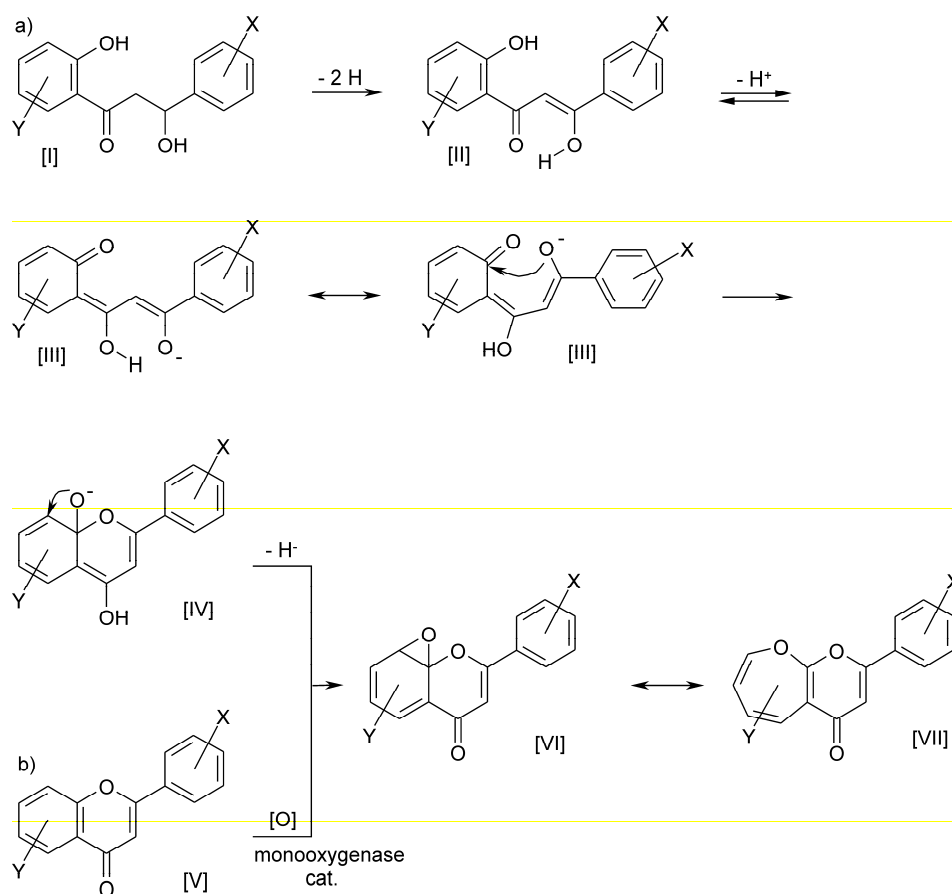


Fig. 4. Possible mechanism of 2-phenyl-4H-oxepino[2,3-b]pyran-4-one generation starting from (a) 3-hydroxy-1-(2-hydroxyphenyl)-3-phenyl-1-propanones **[I]** in accordance to Rayne et al. (2005). After reduction and enolization of the aliphatic hydroxyl group resulting in structure **[II]** a deprotonation of the phenolic proton and tautomerization lead to enolate **[III]**. A subsequent attack to the carbonyl group forms the pyran-4-one ring and a deprotonated hemiketal on a quaternary carbon atom in structure **[IV]**. An oxygen attack to the neighbored double bond, tautomerization and formation of a hydride results in 7-oxabicyclo[4.1.0]hepta-2,4-diene moiety **[VI]**. This system may alternatively be formed *via* (b) by a monooxygenase catalyzed reaction of flavonoid type compound **V** (Boyd et al. 2008). Compound **VI** spontaneously rearranges to the annulated oxepin ring of the final product **VII**. Substituents X and Y on the aromatic moieties represent hydroxyl and

methoxy groups. The reduction and the hydride transfer in natural aqueous conditions in reaction sequence (a) also make an enzyme catalysis of this reaction quite likely.

The twofold oxidation including hydride transfer within the mechanism, however, makes an enzyme catalyzed reaction quite probable. Due to the fact that similar reactions have not been reported in *Primula* species so far, the reaction is quite probably catalyzed by yet unidentified microorganisms located either in the glandular hairs or on the leaf surface. This hypothesis agrees with the fact that the above mentioned oxepin bearing natural products were also generated from fungi (Singh et al., 1994; Singh et al., 1997; Krohn et al., 2009). Taking into account that compounds **1** and **2** were found on leaves of different accessions from *P. cortusoides* (Figure 1), it may be speculated that oxepin generating microorganisms are colonizing leaves of this species, however without causing obvious infections. Although anaerobic microorganisms, endophytic fungi and bacteria, and even viruses are known to transform the structure of flavonoids (Rao and Cooper, 1994; Schneider and Blaut, 2000; Han et al., 2005; Fowler et al., 2011) no compounds resembling **1** and **2** structurally have been reported yet as resulting from such a process. In addition, hydroxylation of compound **2** would be expected to be rather at 4'-position of ring B, when classical flavonoid biosynthesis would have occurred. The structurally resembling 3'-OH flavone is known as synthetic compound only (Nagarathman and Cushman, 1991), and also the respective flavanone as possible precursor has not yet been isolated from natural sources. While 3'-hydroxyl substitution is found only in combination with further substitutions of ring B in *Primula* exudates, a few *Primula* species produce 3'-OMe flavone (Valant-Vetschera et al., 2009). Thus, structural differentiation of exudate flavonoids in analyzed *Primula* species does not provide a clue to biosynthetic relationships, leaving the pathway toward generation of orphan flavonoids speculative.

Even the biosynthesis of unsubstituted flavone as the major compound in *Primula* exudates is still speculative, despite the fact that flavonoid biosynthesis is one of the most well studied pathways (Valant-Vetschera et al., 2009; Yu et al., 2012). Although the key enzyme phenylalanine ammonia-lyase (PAL) has earlier

been detected in glandular hairs of *P. kewensis* (Schöpker et al., 1995), this is not full proof that flavonoid biosynthesis takes place in the glands in the classical way. In *Primula*-type flavonoid biosynthesis, the starter molecule would have to differ from that of the regular pathway leading to 5,7-dihydroxylated flavonoids. However, benzoyl-Co-A is a product of PAL-activity (Ahmed et al., 2002), and it could be a possible candidate. Structurally, such a precursor might explain the lack of hydroxyls in the final flavonoid product. This enzyme is also involved in salicylate biosynthesis (Boatright et al., 2004), and salicylates and other phenolics have been found in the essential oil of *Primula* spp. (e.g. Nan et al., 2002) or of *D. diapsifolia* (Javidnia et al., 2010). Methyl salicylate was used as starting molecule for the chemosynthesis of unsubstituted flavone and its B-ring hydroxylated derivatives via 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones (Nagarathman and Cushman, 1991), a compound resembling **4** from *P. glutinosa*. However, no biosynthetic enzymes are known for a corresponding conversion in nature. Similarly, biosynthetic correlations with the *Primula*-allergen primin (2-methoxy-6-*n*-pentyl-1,4-benzoquinone) are unknown and difficult to assess from comparison of structures. The latter compound was not detected in *P. cortusoides* accessions, and plants were also not found to be contact allergenic (Kovtonyuk, pers. comm.), contrary to an earlier statement (Nestler, 1903).

Biosynthetic enzymes from the type III PKS group catalyzing formation of polyketides (e.g. flavonoids) are primarily known from plants, but they are increasingly reported also from bacteria and fungi (Moore et al., 2002; Yu et al. 2012). It must be mentioned that leaf material of analyzed *Primula* spp. did not show any noticeable sign of infection while examined under a light microscope (Olympus BX41, Epifluorescence). The possible presence of endophytic bacteria or fungi could account, however, for the puzzling flavonoid complement of *Primula* exudates, by involvement in biosynthetic pathways as has been discussed in Wink et al. (2008).

4. EXPERIMENTAL

4.1. General

For NMR spectroscopic measurements each compound was dissolved in methanol-*d*₄ or CDCl₃ (~5.0 mg in 0.7 mL) and transferred into 5 mm high precision NMR sample tubes. Residual CH₃OD or CHCl₃ were used as internal standard in ¹H spectra (δ_{H} 3.34 or 7.24, respectively) and CD₃OD or CDCl₃ in ¹³C spectra (δ_{C} 49.0 or 77.0, respectively). Measurements were performed on a Bruker DRX-600 at 600.13 MHz (¹H) and 150.91 MHz (¹³C) or on a Bruker DRX-400 at 400.13 MHz (¹H) and 100.62 MHz (¹³C), both using the Topspin 1.3 software. 1D spectra were recorded by acquisition of 32k data points and after zero filling to 64k data points and Fourier transformation spectra were performed with a range of 14 ppm (¹H) and 240 ppm (¹³C), respectively. To determine the 2D COSY, TOCSY, NOESY, HMQC, and HMBC spectra 128 experiments with 2048 data points each were recorded and Fourier transformed to 2D-spectra with a range of 10 ppm and up to 220 ppm Hz for ¹H and ¹³C, respectively. Measurement temperature was 298 K +/- 0.05 K.

Mass spectra were measured on a high resolution time-of-flight (HR-TOF) mass spectrometer (maXis, Bruker Daltonics) by direct infusion electrospray ionization (ESI) in positive ionization mode (mass accuracy +/- 5 ppm). TOF MS measurements have been performed within the selected mass range of *m/z* 100-2500. ESI was made by capillary voltage of 4 kV to maintain a (capillary) current between 30-50 nA. Nitrogen temperature was maintained at 180°C using a flow rate of 4.0 L min⁻¹ and the N₂ nebulizer gas pressure at 0.3 bar.

HPLC analysis was performed with an Agilent 1100 Series equipped with a UV Diode Array Detector using a Hypersil BDS-C18 column (250 x 4.6 mm, 5 μ m, flow rate 0.5 mL/min) with mobile gradient MeOH 55-100% in aq. buffer (15 mM H₃PO₄, 1.5 mM Bu₄NOH). The injection volume was 5 μ L and the detection range was 210-300 nm.

4.2. Plant material

Primula cortusoides (WU878) was cultivated in the Botanical Garden of the University of Vienna (HBV) from the seeds obtained from the Bayreuth Botanical Garden, Germany. Additionally, dried leaves of *P. cortusoides* (Sheh2009-NSK) were obtained from plants growing in the Central Siberian Botanical Garden (origin: Altai region). *Primula glutinosa* was collected in Spittal an der Drau, Carinthia, Austria at 2050m (WU0066714) and at 2090m (WU0066705) (Figure 1). Voucher specimens were deposited at the Herbarium WU, University of Vienna, Austria and Herbarium NSK, Central Siberian Botanical Garden, Novosibirsk, Russia.

4.3. Extraction and isolation

Air dried leaves (*P. cortusoides*: 16 g, *P. glutinosa*: 5 g) were briefly rinsed with acetone in order to dissolve the lipophilic exudate material, filtered and evaporated under reduced pressure at 35°C. The obtained residue was dissolved in MeOH and the compounds were isolated by means of preparative MPLC (300×20 mm column, Merck LiChroprep silica gel 60, 25-40 µm) and prep. TLC (Merck silica gel 60, 0.5 mm). In both cases 70 mg of the exudate mixture was subjected to MPLC with increasing amount of EtOAc in light petroleum (PE) as mobile phase. Compound **1** (6 mg) and **2** (1 mg) were eluted as pure substances with 10% EtOAc in PE. Compound **3** and **4** were present as a mixture in the fraction obtained with 10% EtOAc. Therefore a prep. TLC (toluene:dioxane:acetic acid/75:25:5) was performed to afford 10 mg of pure **3** and 7 mg of pure **4**. The identification of the known compounds was achieved co-chromatographically by comparative UV-HPLC and TLC (Merck silica gel 60, 0.2 mm; toluene:dioxane:acetic acid/75:25:5) using authentic markers obtained from E. Wollenweber. TLC chromatograms were viewed under UV light, before and after spraying with Naturstoffreagenz A (0.5% of diphenylboric acid 2-aminoethyl ester in MeOH).

In order to perform the antifungal test TLC plates were developed with PE:acetone /50:50 and dried for complete removal of solvents. The chromatograms were subsequently sprayed with a spore suspension of *C. sphaerospermum*.

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General Conclusion and Perspectives

Several unusual flavonoids could be isolated during the research of this thesis. In general, the flavonoid aglycone profile of *Primula* spp. analyzed is in line with previous studies. Whereas flavonoids, arising from the regular biosynthetic pathway following acetate and shikimate pathways for ring A and B respectively, carry substitutions on the 5th and 7th position, flavonoid aglycones of *Primula* do not concur with this. The substitutions are either lacking completely or are present only on 5th position in combination with an unusual pattern of oxygenation, which appears to be quite unique for the exudates of *Primula* and its closest allies. Thus, the expression “*Primula*-type flavones” has been used for these aberrant flavonoids, in order to distinguish them from the regular flavonoids. Our study on glandular trichomes shows clearly that these unusual flavonoids are not transported from the tissue to the surface, but are indeed produced in the glands, most probably in the head cells. The accumulation of the compounds in the glandular head cells could be shown by using their autofluorescent character. Yet, the biosynthesis of these *Primula*-type flavones remains to be solved. Whereas the formation of 5-deoxyflavonoids is already known (Davies and Schwinn, 2006), it is challenging to determine the enzymes responsible for the biosynthesis of *Primula*-type flavones. Our aim to extract the compounds directly from the glands of the leaves proved to be physically difficult, owing to the minuteness of the glandular heads (~20µm) and the viscous nature of the secretion. Isolation of further exudate compounds, such as the ones in chapter six, can definitely throw some light in this field. One cannot exclude the possibility that endophytic bacteria or fungi are involved in the biosynthetic pathway of *Primula*-type flavonoids. It would therefore be highly interesting to look for the endophytes by different means (in vitro cultivation of *Primula* spp. combined with microscopical methods).

Taken together, one can deduce from the research presented in this thesis that flavonoid aglycones such as the unsubstituted flavone and 2'-OH-flavone could

be seen as chemical markers for the genus *Primula*. *Lysimachia punctata* L., for instance, was moved from Primulaceae to Myrsinaceae by Källersjö et al. (2000) and Mast et al. (2001). Preliminary study on its exudate flavonoid profile shows the presence of some flavonoid aglycones but not a single *Primula*-type flavone (Bhutia in prep.), which corroborates the molecular results.

Exudate flavonoid composition of *Dionysia* and *Primula* differ significantly. While in *Primula* only *Primula* typical flavones are found so far, biosynthetic potential of *Dionysia* is more complex, with additional accumulation of regular substituted flavones, flavonols and flavanones that are derived by the regular and known flavonoid biosynthesis. Hence, in contrast to molecular results according to which *Dionysia* will eventually be included as a subgenus in *Primula*, our results support the retention of *Dionysia* as a separate genus.

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Curriculum Vitae

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EDUCATION

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ACADEMIC WORK EXPERIENCE

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PUBLICATIONS

2012	Bhutia, T.D., Valant-Vetschera, K.M., 2012. Diversification of exudate flavonoid profiles in further <i>Primula</i> spp. Natural Product Communications 7, 587-589.
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