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Characterisation of the flower pigments in *Crocus* (Iridaceae) and *Lilium* (Liliaceae) by 1D and 2D NMR techniques and other spectral evidence and their use as chemotaxonomical markers

Ministry of Food, Agriculture and Fisheries
Danish Institute of Agricultural Sciences

Characterisation of the flower pigments in *Crocus* (Iridaceae) and *Lilium* (Liliaceae) by 1D and 2D NMR techniques and other spectral evidence and their use as chemotaxonomical markers

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Contents

i. Preface and acknowledgement

The present thesis is based on 3 years study performed partly at the Royal Veterinary and Agricultural University, Department of Chemistry and partly at the Danish Institute of Agricultural Sciences, Department of Horticulture. The study included a one-year stay at Chemical Instrument Center, Nagoya University, Japan.

The study was sponsored by the Danish Natural Science Research Council (Grant. No. 9502849).

I wish to express my sincere gratitude to my supervisor Lector Jens Kvist Nielsen for his thoroughly guidance and support during my study.

I sincerely wish to thank my supervisor Senior Scientist Kirsten Brandt for her sincere interest in helping me and for her excellent guidance during my study. Especially, I wish to thank her for visiting me in Japan, autumn 1997.

I wish to thank Professor Niels Jacobsen for his thoroughly botanical guidance and for giving me the opportunity to use the collection of *Crocus* on KVL.

I want to thank Adjunct Marian Ørgaard and Senior Scientist Kell Christiansen for helping me collecting both *Lilium* and *Crocus* flowers.

I wish to express my sincere gratitude to Professor Tadao Kondo, University of Nagoya, Japan for his excellent guidance during my one year stay at Nagoya University and for introducing me to the advanced techniques used for structure elucidation of anthocyanins.
I also want to thank the Japanese staff in the 'anthocyanin group'.

I wish to thank my family and my friend Niels Poulsen for their support during this work.

The Danish Natural Science Research Council have given me the opportunity to do this thesis by giving me a scholarship. I am very thankful.

1. Aim and background of the project

Aim of the project.

The aim has been to provide new chemotaxonomical characters to the genera *Crocus* and *Lilium* and evaluate whether they were useful to resolve ambiguities in the existing classification scheme.

Background of the project

The genus *Crocus*-In an ongoing project The Royal Veterinary and Agricultural University, Copenhagen, Department of Ecology, Botanical section has been collecting existing *Crocus* species and cultivars from as many sources as possible to compare and check all the information available, to see if it is in accordance with the classification by Mathew [1].

The genus is classified into two subgenera. Subgenus *Crocus* is further divided into two sections. Section A contains 6 series (a-f) and section B 9 series (g-o). Subgenus *Crociris* has only one species.

In some series the taxonomy is rather complicated as the various classification characters, i.e. distribution pattern, habitat, various morphological traits and cytological data, contribute confusing, non-correlating data to the problem of systematic and phylogenetic grouping. So, additional independent characters will be useful to supplement existing traits.

Within Series h some taxa are lumped and the classification of species is uncertain. Especially, in the *Crocus chrysanthus-biflorus* cultivars it has been difficult to find out which of the cultivars fit well being selections of either of the two species or hybrids between them [2].

It was reasonably to believe that anthocyanins and other flavonoids could supplement the morphological characters of *Crocus*. From the previous work that I have been involved in, a large variation among species in type and amounts of anthocyanins was found. The study was on another genus from Liliales, *Alstroemeria*, and the work suggested that anthocyanins could be used as potential chemotaxonomical markers [3,4].

From a chemical view the cyanic perianth colours of *Crocus* were interesting. The colour often varies considerably within a species and has been well documented by visual judgements. However, the pigments have never been thoroughly analysed and this point attracted our attention.

The genus *Lilium*- The study of the ancestors of two major sections of *Lilium*, the Asiatic and Oriental hybrids needs further independent characters to supplement what is already known from morphological and cytological data. From the spontaneous occurrence of aneuploidity in natural populations it is also of interest to know more about the evolution of the genus *Lilium*.

Chemotaxonomical markers may supplement what is already known from morphological and cytological data in the genus *Lilium*.

From a chemical view the perianth colours of *Lilium* were interesting ranging from red, pink to orange, yellow and white flowers.

2. Danish summary

Krokus slægten er delt op i to underslægter. Den ene underslægt, Crocus er igen opdelt i to sektioner, hvor sektion A indeholder serierne (a-f) og sektion B indeholder serierne (g-o). Den anden underslægt Crociris indeholder en enkelt art. Opdelingerne er udarbejdet efter klassiske retningslinier baseret på morfologiske karakterer og kromosomtal, men sammenlagt er karaktererne ofte utilstrækkelige og vanskelige at vurdere i evolutionære sammenhænge.

Ca. 100 krokus sorter eksisterer i dag, som er selekteret ved hybridisering mellem relativt få arter. Der er tvivl om nogle af disse hybriders oprindelse, og hvor vidt der er forskelle mellem arter indenfor Biflori serien (h).

Det er her blevet undersøgt, om kemiske data kan give en bedre forståelse af krokus systematikken ved at anvende blomsterpigmenternes strukturer som kemotyper.

De morfologiske beskrivelser af krokus indeholder beskrivelser af blomsternes farver og aftegninger. Dog findes der kun et begrænset antal ældre værker, alle før 1983, der delvist karakteriserer pigmenterne ud fra deres kemiske strukturer. Blomsterfarven på krokus varierer fra hvide, gule til forskellige blå og lilla nuancer. Anthocyaniner giver anledning til de blå og lilla farver, medens de gule baggrundsstoffer hovedsageligt skyldes karotenoider og muligvis flavonoider.

De svagt gule eller farveløse flavonoider er opbygget af to aromatiske ringe (A og B) sat sammen af en C_3 enhed. Hertil er der bundet sukkerenheder, og til sukkerenhederne kan være bundet syregrupper.

Den farvede anthocyanidin struktur er en 2-phenylbenzopyrylium (eller flavylium) ion, som består af to benzenringe (A og B) koblet sammen via en O-heterocyklisk ring. Ligeledes er der her tale om glykosylering og eventuelt acylering.

Tidligere anvendte ekstraktionsmetoder omfattede hydrolyse, hvorved sukker- og syreenheder fraspaltes fra pigmenternes ringskelet. Man konstaterede herved, at der kun var lille variation i aglykonerne og derfor ikke umiddelbart nogen kemotaksonomiske sammenhæng mellem krokus taxa. Imidlertid er andre ekstraktionsmetoder blevet udviklet, hvor man arbejder med minimal tilsætning af syre, kun lige nok til at holde anthocyaninerne som flavylium ioner. Herved undgår man fraspaltning af sukker- og syreenheder.

HPLC analyser på ekstrakter af kronblade fra 87 arter, 36 kultivarer og 6 hybrider af krokus viste kvalitativt varierede profiler med fra 1-9 forskellige anthocyaniner. Samtlige 18 andre flavonoider, som blev detekteret, fandtes i alle taxa men i varierende mængde.

De detekterede stoffer blev oprenset og identifiseret vha. FAB-MS og 1D, 2D NMR. 6 nye flavonoid strukturer blev bestemt som 3-*O*-α-L-(2-*O*-β-D-glukosyl)rhamnosid-7-*O*-β-D-glukosider af kaempferol, quercetin og myricetin, kaempferol 3-*O*-α-L-(2-*O*-β-D-glukosyl)rhamnosid-7-*O*-(6-*O*-malonyl-β-D-glukosid), kaempferol 3-*O*-α-L-(2-*O*-β-D-glukosyl)rhamnosid-7-*O*-(6-*O*-acetyl-β-Dglukosid) og

kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β–D-glukosyl)rhamnosid. De resterende flavonoider var alle kendte forbindelser: kaempferol 3-*O*-glucosylrhamnosid, 3-*O*-rhamnosylglukosider af kaempferol og isorhamnetin, 3-*O*-sophorosider af kaempferol og quercetin, 3, 4'-*di*-*O*-glukosider af kaempferol, quercetin og isorhamnetin, 3-*O*-glukosider af kaempferol og quercetin, apigenin 7-*O*-glukosid og 2,3-*di*-hydrokaempferol 7-*O*-glukosid.

Iblandt anthocyaniner var 4 nye strukturer: 3,7-*di*-*O*-(6-*O*-malonyl-β–D-glukosider) af petunidin og malvidin, delphinidin 3-*O*-β-D-glukosid-5-*O*-(6-*O*-malonyl–β–D-glukosid) og petunidin 3,7-*di*-*O*-β-D-glukosid. De kendte anthocyaniner var delphinidin 3,7-*di*-*O*-glukosid, 3,5-*di*-*O*-glukosider og 3-*O*-rutinosider af petunidin og delphinidin.

Fra forskellige glykosyleringsmønstre blev der opdelt i 5 anthocyaninkemotyper. Fire kemotyper adskilte sig fra hinanden ved at have forskellige glykosyleringsmønstre, medens den femte type indeholdt anthocyaniner substitueret med malonsyre.

Taxa blev yderligere opdelt i 4 andre grupper efter det kvantitative indhold af flavonoider med forskellige glykosyleringsmønstre.

Variationen af kemiske strukturer i familien gjorde det muligt at bruge kemotyperne som kemotaksonomiske markører, specielt for anthocyanin. Anthocyaninkemotyper og flavonoidgrupper hver for sig understøtter Mathews klassifikation af krokus, og de to forskellige typer af resultater bekræfter gensidigt hinanden.

De 4 nye anthocyanin strukturer og 6 flavonoler med et unikt glykosyleringsmønster findes vidt udbredt i slægten. Dette kan være et kendetegn for krokus familien.

Kemotyperne indenfor en serie var mere ens end på tværs af serierne, og de kemiske data var ensartede indenfor hver underart, hvis man ser bort fra Biflori serien (h). Dog var der 7 arter, hvor der var uoverensstemmelse mellem de kemiske data og klassifikationen. Her vil det være en fordel at supplere med andre karakterer eller *in-situ* hybridisering for at bestemme deres placering i Mathews konstellation.

Kemotypeforskelle mellem *C. biflorus* underarter var lige så store som mellem arter i forskellige andre serier.

Man er interesseret i at kende herkomsten af de mange og meget forskellige Asiatiske og Orientalske lilje hybrider, som er fremkommet ved at krydse forskellige arter. Samtidig har man ud fra enkelte fundne tilfælde af aneuploiditet interesse i at kende til evolutionsmønstret for slægten. Ønsket var at undersøge om anthocyanin data kunne belyse denne problemstilling.

Analyser af blomsterekstrakter fra 8 asiatiske og 3 orientalske hybrider viste, til trods for de mange røde farvenuancer og tegninger, kun to anthocyaniner. Et nyt anthocyanin, cyanidin 3-*O*–β–D-rutinosid-7-*O*-β-D-glukosid, og cyanidin 3-*O*-β-D-rutinosid blev detekteret i flere, ikke tæt relaterede, lilje arter.

Det nye anthocyanin kan være karakteristisk for slægten *Lilium*, men variationen indenfor slægten var for lille til kemotaksonomisk anvendelse.

3. English summary

The rather complicated taxonomy of the genus *Crocus* based on distribution pattern, habitat, various morphological traits and cytological data have been supplemented with additional independent characters to find out whether they were useful to resolve ambiguities in the existing classification scheme.

The anthocyanins causing cyanic colours and the colourless flavonoids were analysed in perianth segments of 87 species, 36 cultivars and 6 hybrids of *Crocus*. Analytical HPLC showed the presence of the same 18 flavonoids in every taxon and a variable pattern of nine anthocyanins. All compounds were isolated and identified by techniques such as FAB-MS and 1D- and 2D-NMR.

The work documented six new flavonoid structures as; 3-*O*-α-L-(2-*O*-β–D-glucosyl)rhamnoside-7-*O*-β-D-glucosides of kaempferol, quercetin and myricetin; kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-O-malonyl-β–D-glucoside); kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-*O*-acetyl-β–D-glucoside) and kaempferol 3-*O*-α (2,3-*di*-*O*-β–D-glucosyl)rhamnoside. They co-occurred with kaempferol 3-*O*-glucosylrhamnoside; 3-*O*-rhamnosylglucosides of isorhamnetin and kaempferol; 3-*O*-sophorosides of kaempferol and quercetin; 3, 4'-*di*-*O*-glucosides of kaempferol, quercetin and isorhamnetin; 3-*O*-glucosides of kaempferol and quercetin; apigenin 7-*O*-glucoside and 2,3 *di*-hydrokaempferol 7-*O*-glucoside.

Four new anthocyanin structures were 3,7-*di*-*O*-(6-*O*-malonyl-β-D-glucosides) of petunidin and malvidin; delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside) and petunidin 3,7-*di*-*O*-β-D-glucoside. Furthermore, delphinidin 3,7-*di*-*O*-glucoside; 3,5-*di*-*O*-glucosides and 3-*O*-rutinosides of petunidin and delphinidin were identified.

The novel flavonols and anthocyanins were widely distributed within the genus and can be used as distinguishing markers for this family.

The diversity of the distribution of the chemical structures within the family made it possible to use them as chemotaxonomical markers, in particular the anthocyanins.

To define the ancestors of two major sections of *Lilium*, the Asiatic and Oriental hybrids, further investigations are needed and chemotaxonomical markers may supplement what is already known from morphological and cytological data.

The lilies have red, pink, orange, yellow or white flowers, often with dark red spots. However, despite of the colour diversity, the HPLC analyse of anthocyanins contents in 8 Asiatic and 3 Oriental hybrids showed only two pigments. The colours were due to a novel anthocyanin, cyanidin 3-*O*-β-D-rutinoside-7-*O*-β-D-glucoside, and cyanidin 3-*O*-β-D-rutinoside. The pigments were found in several distantly related taxa of *Lilium*.

The novel anthocyanin can be characteristic for the genus *Lilium* but the variation within the genus was too small to use this compound as a chemotaxonomical marker.

Keywords: *Crocus*, Iridaceae, *Lilium*, Liliaceae, malonated and acylated flavonoids, malonated anthocyanins, HPLC, NMR, chemotaxonomy.

4. Papers included

1. Nørbæk, R. & Kondo, T. (1999) Flavonol glycosides from flowers of *Crocus speciosus* and *C. antalyensis*. *Phytochemistry* **51**, 1113-1119.

2. Nørbæk, R., Nielsen, J. K. & Kondo, T. (1999) Flavonoids from flowers of two *Crocus* cultivars: 'Eye-catcher' and 'Spring Pearl'. *Phytochemistry* **51**, 1139-1146.

3. Nørbæk, R. and Kondo, T. (1998). Anthocyanins from flowers of *Crocus* (Iridaceae). *Phytochemistry* **47**, 861-864.

4. Nørbæk, R. and Kondo, T. (1999). Further anthocyanins from flowers of *Crocus antalyensis* (Iridaceae). *Phytochemistry* **50**, 325-328.

5. Nørbæk, R. and Kondo, T. (1999). Anthocyanins from flowers of *Lilium*. *Phytochemistry* **50**, 1181-1184.

6. Nørbæk, R., Nielsen, J. K., Brandt, K., Ørgaard, M. & Jacobsen, N. (200X). Flower pigment composition of *Crocus* species and cultivars used for a chemotaxonomic investigation. *Biochemical Systematics and Ecology* (To be submitted).

5. Introduction to the chemistry of flavonols, flavone and anthocyanins

5.1 Flavonol and flavone structures

Flavonoids, yellow or colourless compounds, constitute the largest group of naturally occurring phenolic compounds [5]. Flavonoids are often localised in epidermal cells and more than 4000 different structures have been found [6]. Their structure is based on an aromatic C_{15}-skeleton (fig 5.1).

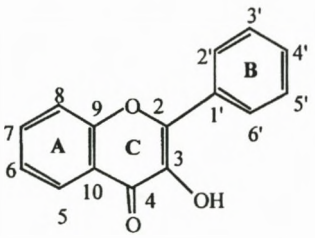


Fig 5.1 The flavonol nucleus. Numbering system of flavonoids.

Focusing on the flavonol and flavone nuclei they can be hydroxylated or methoxylated in various positions (Fig. 5.2).

Especially for flavonol glycosides, there is a high degree of similarity between angiosperms in the aglycones they contain. A survey of the leaves of more than 1000 angiosperms showed that kaempferol was found in 48% of the species and quercetin in 56% [7]. The similarity in aglycone structure is outweighted by a great diversity in the number, position and structure of carbohydrates and acyl groups attached to the flavonol aglycones [8]. Flavonols are supposed to be involved in protection against UV-B radiation [9].

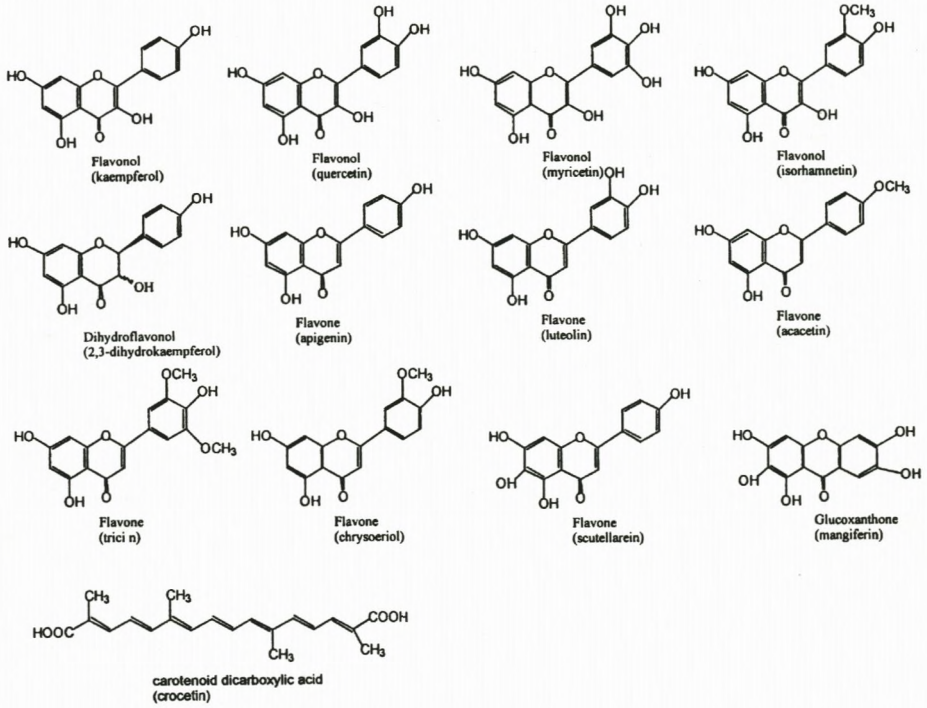


Fig. 5.2 Structures of some common flavonoid nuclei. The glucoxanthone mangiferin and the carotenoiddicarboxylic acid crocetin are also illustrated.

5.2 Anthocyanin structures

The anthocyanins are part of the group of flavonoids. Anthocyanins are responsible for cyanic colours ranging from salmon-pink, red, violet to dark-blue of most flowers, fruits and leaves of angiosperms. They may become visible in roots, tubers, stems, bulbils, etc. and are also found in many gymnosperms, ferns and some bryophytes [10, 11]. Anthocyanins are dissolved in vacuoles or accumulated in vesicles called anthocyanoplasts. Their concentration in pigmented epidermal petal cells are commonly in the order of 10^{-3}–10^{-2} M.

The structure of anthocyanins is based on anthocyanidin, sugars and eventual acyl substituents. The anthocyanidin, commonly referred to as aglycone or flavylium cation, is the aromatic skeleton responsible for the colour of anthocyanins. As with other flavonoids, the aglycone in anthocyanins can be hydroxylated or methoxylated in various positions. The six most common anthocyanidins differ only with respect to the hydroxylation and methoxylation pattern on the B- ring (Fig.5.3).

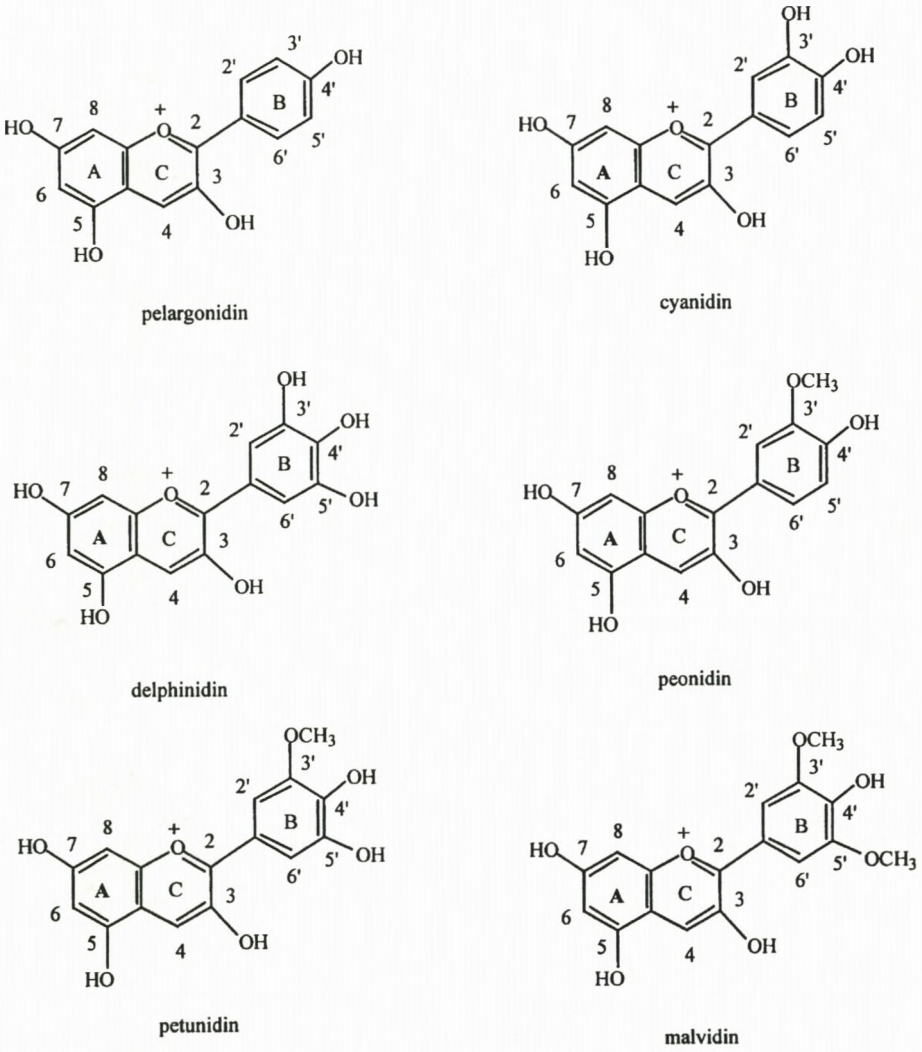
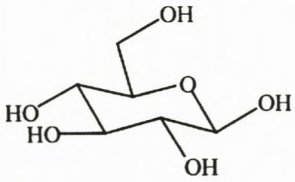
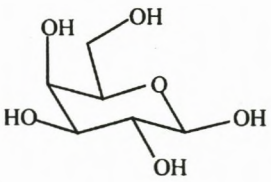


Fig. 5.3 Structures of some of the six most common anthocyanidins arranged by ascending complexity.

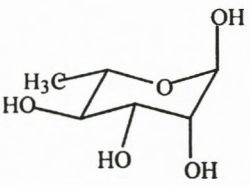
The polyphenolic nucleus is always glycosylated in nature. Anthocyanins are commonly occurring as 3-monosides, 3-biosides and 3-triosides or as 3,5-diglycosides and more as rarely 3,7-diglycosides. Additional substitution of sugars in 5, 7, 3' and 4' positions have been found [12]. The most common sugar residues found in connection to anthocyanins are in order of frequency: Glucose, galactose, rhamnose, xylose and arabinose (Fig. 5.4)



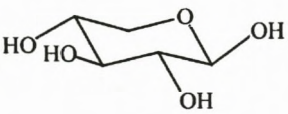
β-D-glucopyranose



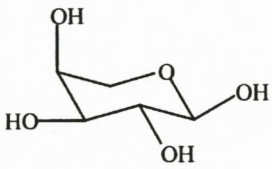
β-D-galactopyranose



α–L-rhamnopyranose



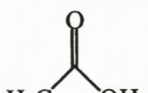
β-D-xylopyranose



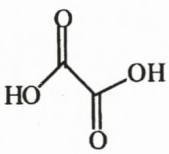
α–L-arabinopyranose

Fig 5.4 Structures of the most common sugar units.

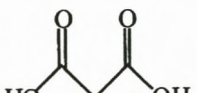
The sugar substituents can be further modified by esterification of one or more of their hydroxyl groups with a variety of aromatic and aliphatic acyl moieties. In recent years, the extraction of anthocyanins under milder conditions has revealed the presence of aliphatic acyl substituents such as acetic, oxalic, malonic and malic acids [12] (Fig 5.5). The most common aromatic acyl groups found in anthocyanins are hydroxycinnamic acids like *p*-coumaric, caffeic, sinapic and ferulic acids (Fig. 5.6) and hydroxybenzoic acids (*p*-hydroxybenzoic, gallic) [13].



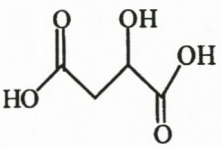
acetic acid



oxalic acid



malonic acid



malic acid

Fig. 5.5 Structures of the most common aliphatic substituents found in anthocyanins.

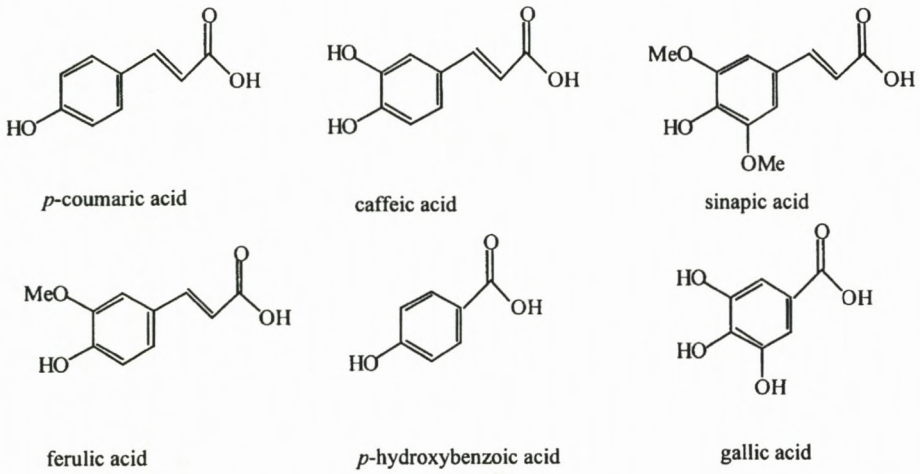


Fig. 5.6 Structures of the most common aromatic acids in anthocyanins occurring as acyl groups.

Acyl groups are most commonly linked to the 6''-position of the sugars, but some anthocyanins with acyl substitution at 2'', 3'' and 4'' sugar hydroxyls have also been reported [14-16].

5.3 Equilibrium forms of anthocyanins

Each anthocyanidin gives rise to several forms with different colours and stability. Based on *in vitro* observations, the anthocyanin exists primarily as a red or purple flavylium cation at pH below 3. As the pH is raised, kinetic and thermodynamic competition occurs between the hydration reaction of the flavylium cation and the proton transfer reactions related to its acidic hydroxyl groups. While the first reaction gives colourless carbinol pseudo-bases, which can undergo ring opening to retro-chalcones, the latter reactions give rise to more violet quinonoidal bases. Further deprotonation of the quinonoidal bases can take place at pH between 6 and 7 with the formation of resonance-stabilised quinonoid anions. (Fig. 5.7) [17, 18, 20].

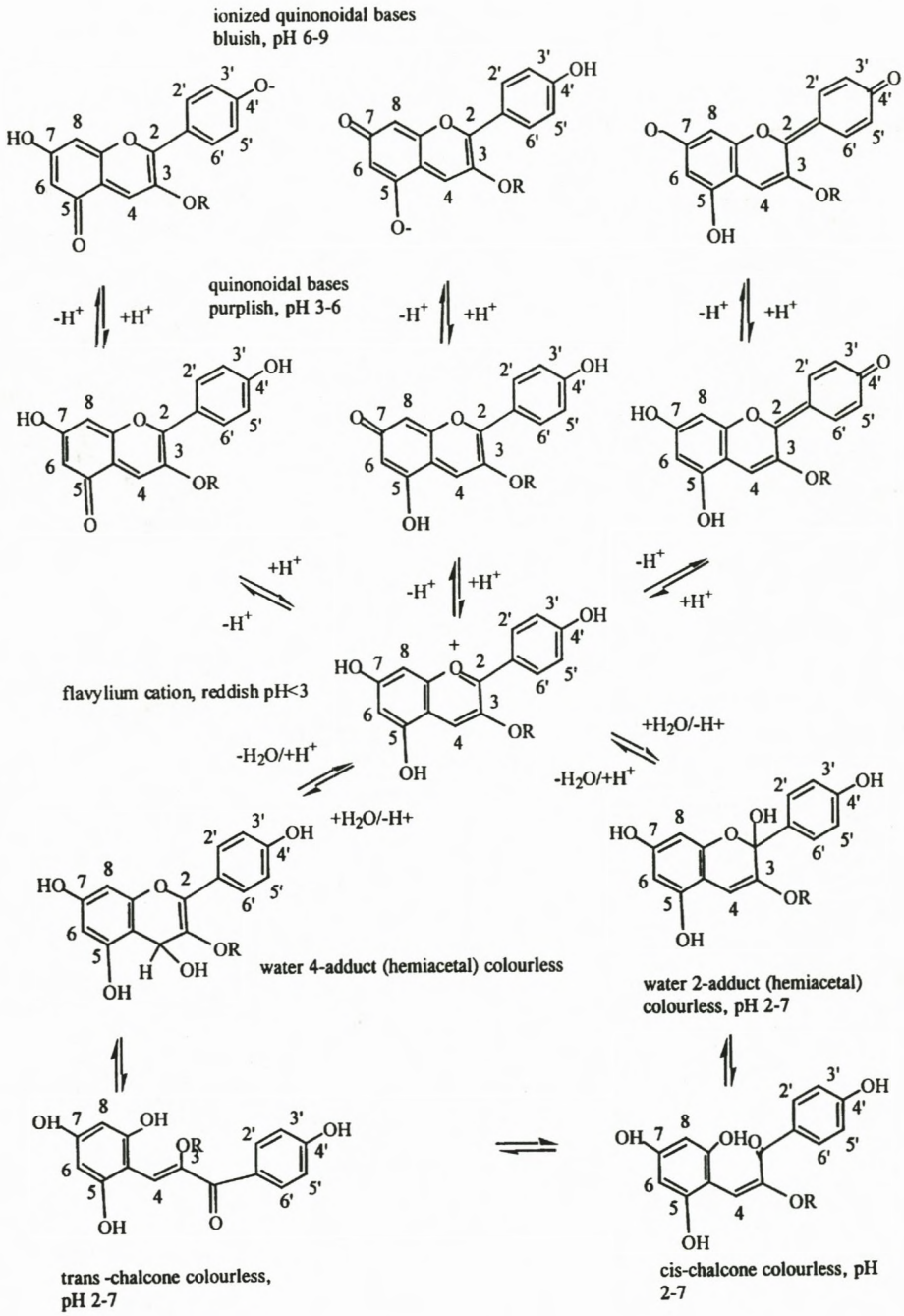


Fig 5.7 Structural transformations of anthocyanins in water [19]

5.4 Selfassociation, intra- and intermolecular copigmentation of anthocyanins

The pH of the cell sap is usually in the region of 4-6. Most anthocyanins *in vitro* are virtually without colour in this region since they exist mainly as colourless carbinol bases before they are transformed to chalcone forms (Fig. 5.7) [21].

The presence of aromatic acyl groups in the anthocyanin molecule seems to hinder hydrolysis of the red flavylium cationic form to the colourless carbinol base, allowing preferential formation of the blue quinonoidal bases and their anions [18, 20]. This intramolecular copigmentation thus implies more bluish colours of high intensity in mildly acidic or neutral media.

A similar mechanism involving stacked complexes is proposed for intermolecular copigmentation of anthocyanins with other flavonoids and related compounds. This stacking produces an increase in colour intensity and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic shift) giving purple to blue colours [18, 21, 22]. Some documented copigments are, flavonol *O*- and *C*-glycosides [18, 21].

When pigment concentrations become relatively high, anthocyanins themselves may act as copigments and participate in self-association reactions. The colour intensity (absorbance) will then increase more than proportionally to anthocyanin concentration [18, 21, 23].

When the anthocyanins take form as a pigment-metal complex, as in the blue flowers of *Commelina communis* they are also stabilised and the blue colour does not change during a pH shift from 7 to 1 [24, 25].

5.5 Functions of anthocyanins

The most significant function of anthocyanins are their ability to impart colour to the plant or plant products in which they occur [26]. The presence of colours in flowers and fruits are believed to ensure pollination and seed dispersal by animals. The colour of anthocyanins attracts pollinators. Bees prefer blue, yellow and ultraviolet colours, whilst humming birds like orange, scarlet and bright red. An evolutionary trend towards blue flowers is reflected in the frequency of blue-coloured species in temperate members of for instance Labiatae, Polemoniaceae and Boraginaceae [27].

In leaves, the presence of anthocyanins is to act as light screen against damaging UV radiation [28].

Anthocyanins have also been associated with resistance to pathogens in species of *Brassica* [29] and *Sunflower* [30].

6. Introduction to classification of *Crocus* and *Lilium*

Liliales comprises eight families; Colchicaceae, Iridaceae, Alstroemeriaceae, Liliaceae, Melanthiaceae, Uvulariaceae, Calochortaceae and Gerosiridaceae [31].

6.1 The genus *Crocus*

Crocus belongs to Iridaceae. Taxonomically the family is closely related to the Liliaceae [32]. Iridaceae has been divided into three subfamilies; the Isophysidoideae, the Iridoideae and the Ixioideae [33]. *Crocus* belongs to Ixioideae among other genera such as *Crocosmia*, *Freesia*, *Gladiolus* and *Romulea*.

The history and taxonomy has been represented in two comprehensive monographs by Maw [34] and Mathew [1]. The genus has about 80 species, which are classified into two subgenera. Subgenus *Crocus* contains about 79 species and is further divided into two sections. Section A contains 6 series (a-f) and section B 9 series (g-o), Subgenus *Crociris* has only one species, *C. banaticus* Gay, which differs from other species in several aspects. Mathew's linear constellation [1] describes the genus *Crocus* thoroughly by morphological characters, cytological data and geographical distribution, and the classification is as follows;

I. Subgenus *Crocus*
- A. Section *Crocus*
  - (a) Series *Verni*
  - (b) Series *Scardici*
  - (c) Series *Versicolores*
  - (d) Series *Longiflori*
  - (e) Series *Kotschyani*
  - (f) Series *Crocus*
- B. Section *Nudiscapus*
  - (g) Series *reticulati*
  - *(h)* Series *Biflori*
  - (i) Series *Orientales*
  - *(j)* Series *Flavi*
  - (k) Series *Aleppici*
  - *(l)* Series *Carpetani*
  - (m)Series *Intertexti*
  - (n) Series *Speciosi*
  - *(o)* Series *Laevigati*

II. Subgenus *Crociris*

In Table 6, Paper 6 an author list of *Crocus* is presented. The perianth of *Crocus* consists of a long tube with six perianth segments divided into two whorls of three, one inner set and one outer set, to which the three stamens are joined. There is a great variation in the colour, size and shape of these segments and their overall appearance obviously has the greatest impact when first viewing a *Crocus* specimen.

Focusing on the *Crocus chrysanthus-biflorus* complex, the two species are shortly described.

Crocus chrysanthus is distributed from Italy, through former Yugoslavia over the Balkans to western and central Turkey. The perianth is generally yellow, but there are some populations in which forms with a more or less distinct brown suffusion or feathered stripes on the outer perianth segments appear. These colour variations are not recognised taxonomically [2].

Crocus biflorus is distributed from Italy through former Yugoslavia over the Balkans, Crimea, Caucasus, Turkey and western Iran. There is a wide colour variation in the flowers of *C. biflorus*.

Except for a few cases, it is not yet possible, to determine the origin of the cultivars, or to what extent they are hybrids between *C. chrysanthus* and *C. biflorus*.

However, *C. chrysanthus* is yellow flowered, sometimes with purple markings on the outer perianth segments, whereas *C. biflorus* generally is white to blue, often with more or less purple coloured stripes on the outer perianth segments. A few of the cultivars fit well as being selections of either of the two species while most of them probably are hybrids between the two [2].

6.2 The genus *Lilium*

The true lily, belonging to the genus *Lilium*, is a member of the family of Liliaceae. The genus comprises more than 80 species, and is classified into seven sections [35], modified slightly by de Jong [36]. The lilies were classified according to growth patterns, flowers, seed, type of germination, arrangement of the leaves, the form and growth habit of the bulb, and the geographical distribution.

1. Martagon section: *L. distichum*, *L. hansonii*, *L. martagon*, *L. medeoloides*, *L. tsingtauense*
2. American section:

2a. *L. bolanderi*, *L. columbianum*, *L. kelloggii*, *L. humboldtii*, *L. rubescens*, *L. washingtonianum*

2b. *L. maritimum*, *L. nevadense*, *L. occidentale*, *L. pardalinum*, *L. paryi*, *L. parvum*, *L. roezlii*

2c. *L. canadense*, *L. grayi*, *L. iridollae*, *L. michauxiii*, *L. michiganense*, *L. superbum*

2d. *L. catesbaei*, *L. philadelpicum*

3. Candidum section: *L. bulbiferum, L. candidum, L. carniolicum, L. chalcedonicum, L. monadelphum, L. polyphyllum, L. pomponium, L. pyrenaicum*
4. Oriental section: *L. auratum, L. brownii, L. japonicum, L. nobilissimum, L. rubellum, L. speciosum*
5. Asiatic section:

5a. *L. davidii, L. duchartrei, L. henryi, L. lancifolium, L. lankongense, L. leichtlinii, L. papilliferum*

5b. *L. amabile, L. callosum, L. cernuum, L. concolor, L. pumilum*

5c. *L. bakerianum, L. macklinia*e, *L. nepalense, L. ochraceum, L. sempervivoideum, L. taliense, L. wardii*

6. Trumpet section:

6a. *L. leucanthum, L. regale, L. sargentiae, L. sulphureum*

6b. *L. formosanum, L. longiflorum, L. neilgherrense, L. philippinense, L. wallichianum*

7. Dauricum section: *L. dauricum, L. maculatum*

7. Introduction to the chemotaxonomy of *Crocus* and *Lilium*

7.1 Flavonols and anthocyanins previously isolated from *Crocus*

Several flavonoids have been isolated from leaves or pollen of *Crocus* (Table 7.1). The data of flavonoid aglycones are based on identification in leaf tissue after acid hydrolysis by direct comparison with authentic markers. The taxa in Table 7.1 has been arranged by Mathew's linear constellation.

The aglycones kaempferol, quercetin and myricetin have been detected in perianth segments of the genus (Table 7.2). They were glycosylated but the carbohydrate moieties have only been identified by chromatographic studies. Acylated flavonoids have not been detected earlier in *Crocus*, although they seem to occur regularly in Iridaceae [44, 45].

Crocus differs from many other Monocotyledon genera in that no red flowers occur and previously the cyanic colours (purple to lilac, mauve and blue) were reported to be delphinidin-petunidin- and malvidin based (Table 7.2). Only one glycoside, delphinidin 3,5-diglucoside has been thoroughly identified [43].

7.2 Flavonols and anthocyanins previously isolated from *Lilium*

Flavonol glycosides have been isolated from petal extracts of *Lilium* species; 3-glucosides and 3-glucoside-7-rhamnosides of kaempferol and isorhamnetin; 3-rutinosides of isorhamnetin and quercetin; 7-rhamnoside and 3-diglucoside of kaempferol and isorhamnetin 3-rutinoside-7-rhamnoside [46, 47]. An acylated kaempferol derivative was identified from the aerial part of *Lilium candidum*; 3,5,7,4'-tetrahydroxy-8-(3'-methylsuccinoyl)-flavone [48].

Only two anthocyanidins, cyanidin and petunidin have been detected in perianth segments and attached sugars were not identified [40].

		leaf constituents																		
Species of *Crocus*	**Ref.**	**Fla C-glc**	**Tri ***	**Ac ***	**Ma ***	**Sco rh glc**	**My ***	**Qu ***	**Ka ***	**Ka 3 so**	**6-OH Lu 7 glc**	**6-OH Lu 7 Me 6 glc**	**6-OH Lu 7 rh glc**	**Scu 7 glc**	**Scu 7 Me 6 glc**	**Ap ***	**Caf**	**pC**	**S**	**F**
C. etruscus (series a)	33, 37,38									+							+	+	+	+
C. heuffelianus (series a)	33	+	+																	
C. kosaninii (series a)	37								+								+	+	+	+
C. tommasinianus cv. 'Whitewell Purple' (series a)	33,37	+	+					+	+							+	+	+	+	+
C. vernus (series a)	33	+																		
C. vernus var. albiflorus (series a)	33	+							+											
C. vernus as *C. purpureus* (series a)	37							+	+								+	+	+	+
C. vernus as *C. coeruleus* (series a)	37							(+)	(+)								+	+	+	+
C. cambessedesii (series c)	37																	+		+
C. corsicus (series c)	33, 37,38	+						+	+	+		+			+		+	+	+	+
C. imperati (series c)	37																+		(+)	(+)
C. minimus (series c)	33,37, 38							+	+			+	+		+	+	+	+	+	

		leaf constituents																		
Species of *Crocus*	**Ref.**	**Fla C-glc**	**Tri ***	**Ac ***	**Ma ***	**Sco rh glc**	**My ***	**Qu ***	**Ka ***	**Ka 3 so**	**6-OH Lu 7 glc**	**6-OH Lu 7 Me 6 glc**	**6-OH Lu 7 rh glc**	**Scu 7 glc**	**Scu 7 Me 6 glc**	**Ap ***	**Caf**	**pC**	**S**	**F**
C. versicolor (series c)	33,37, 38							+	+	+							+	+	+	+
C. clusii (series d)	37								+								+	+	+	+
C. goulimyi (series d)	33,37, 38	+						(+)	+								+	+	+	(+)
C. longiflorus (series d)	37							+	+								+	+	+	+
C. medius (series d)	37							+	+								+			(+)
C. niveus (series d)	33,37	+							+								+	+	(+)	
C. nudiflorus (series d)	37																(+)	(+)	+	+
C. serotinus ssp. *salzmanii* (series d).	37																(+)	+	+	+
C. kotschyanus (series e)	37							+	+								+	(+)		(+)
C. ochroleucus (series e)	37								+								+	(+)		
C. vallicola (series e)	37							+	+								+	(+)	(+)	(+)
C. sativus (series f)	33,37	+							+								+	(+)		(+)
C. sativus as *cashmirianus* (series f)	37								+								+	(+)		(+)
C. ancyrensis(series g)	37								(+)								+	+		(+)
C. cancellatus (series g)	33, 37	+						+	(+)								(+)	(+)		
C. reticulatus (series g)	39					+														

Species of *Crocus*	Ref.	leaf constituents																		
		Fla C-glc	Tri *	Ac *	Ma *	Sco rh glc	My *	Qu *	Ka *	Ka 3 so	6-OH Lu 7 glc	6-OH Lu 7 Me 6 glc	6-OH Lu 7 rh glc	Scu 7 glc	Scu 7 Me 6 glc	Ap *	Caf	pC	S	F
C. sieberi (series g)	33, 37	+							+								+	+	+	+
C. stellaris (series g x j)	37				+		+	+	+								+	+	+	(+)
C. angustifolius (series g)	37							+	+								+	+	(+)	+
C. veluchensis (series g)	37							(+)	(+)								+	+	+	+
C. aerius (series h)	37							(+)	(+)								(+)	+	+	+
C. biflorus (series h)	37																+	(+)	+	(+)
C. biflorus ssp. *nubigena* (series h)	37							+	+								(+)	(+)	(+)	(+)
C. chrysanthus cv. 'Cream Beauty' (series h)	33,38	(+)									+			+						
C. chrysanthus (series h)	37																	+	+	
C. danfordiae (series h)	37																		(+)	(+)
C. korolkowii (series i)	33,37, 38	+	+					+	+	+								+	(+)	+
C. candidus (series j)	37						+	(+)	(+)								+	+		+
C. flavus ssp.flavus (series j)	33,37, 38	(+)			+		+	+	+								+	+	(+)	+
C. graveolens (series j)	37		+					+	+									+	+	+
C. hyemalis (series j)	37							+									+	+	+	

Species of *Crocus*	Ref.	leaf constituents																		
		Fla C-glc	Tri *	Ac *	Ma *	Sco rh glc	My *	Qu *	Ka *	Ka 3 so	6-OH Lu 7 glc	6-OH Lu 7 Me 6 glc	6-OH Lu 7 rh glc	Scu 7 glc	Scu 7 Me 6 glc	Ap *	Caf	pC	S	F
C. olivieri (series j)	37						+	(+)	(+)								+	+	(+)	(+)
C. carpetanus (series l)	37							+	+								+	+	+	+
C. nevadensis (series l)	37							+									+	+	+	+
C. fleischeri (series m)	33,37, 38							+	+								+	+		+
C. pulchellus (series n)	37							(+)	+								+	+	+	+
C. speciosus (series n)	37							+	+								+	+	+	(+)
C. laevigatus (series o)	33,37, 38			+						+							+	+	+	
C. tournefortii (series o)	33,37, 38	+						+	+								+	+		
C. banaticus(Crociris)	37								+								+	+		+

Table 7.1 Leaf constituents in *Crocus* arranged by Mathew's linear constellation.

Key: Fla-C-glc = flavone C-glycoside, Tri = tricin, Ac = acacetin, Ma = mangiferin, Sco-rhglc = scoparin O-rhamnosylglucoside (scoparin=chrysoeriol 8-*C*-glucosyl), My = myricetin, Qu = quercetin, Ka = kaempferol, Ka3so = kaempferol 3-sophoroside, 6-OHLu7glc = 6-hydroxyluteolin 7-glucoside, 6-OHLu7Me6glc = 6-hydroxyluteolin 7-methyl ether-6-glucoside, 6-OHLu7rhglc = 6-hydroxy-luteolin-7-rutinoside, Scu7glc = scutellarein 7-glucoside, Scu7Me6glc = scutellarein 7methylether-6-glucoside, Ap = apigenin, Caf = caffeic acid, pC = p-coumaric acid, S = sinapic acid, F = ferulic acid.

* The data refer to flavonoid aglycones identified in leaf tissue after acid hydrolysis by direct comparison with authentic markers.

		Perianth constituents									
Species of *Crocus*	**Ref.**	**Ka ***	**Qu ***	**My ***	**Ka 3 so**	**Ka 3 rg 7 glc**	**Cr**	**Dp 35 glc**	**Cy 35 glc**	**Ma 35 glc**	**Pet di glc**
C. etruscus (series a)	38				+	+	+	+			+
C. vernus (series a)	40							+			
C. vernus ssp. *albiflorus* (series a)	41							+		+	
C. corsicus (series c)	38				+			+			+
C. minimus (series c)	38				+	+		+	+	+	+
C. versicolor (series c)	38				+					+	+
C. longiflorus (series d)	40							+			
C. nudiflorus (series d)	40							+			
C. serotinus ssp. *salzmanii*.(series d)	39, 40	+						+			
C.hadriaticus (series f)	40							+			
C. sativus (series f)	40-42	+	+	+				+			+
C. reticulatus (series g)	41							+		+	
C. chrysanthus (series h)	38					+	(+)	(+)			+
C. weldenii (series h)	41							+		+	
C. korolkowii (series I)	38				+		+	+			+
C. flavus ssp. *flavus* (series j)	38				+		+				
C. fleischeri (series m)	38					+					
C. pulchellus (series n)	40									+	
C. speciosus (series n)	39,40	+								+	
C. laevigatus (series o)	38				+		+	+			

Table 5.2 Perianth constituents of the genus *Crocus* arranged by Mathew's linear constellation.

Key: Ka = kaempferol, Qu = quercetin, My = myricetin, Ka3so = kaempferol 3-sophoroside, Ka3rg7glc = kaempferol 3-rhamnoglucoside-7-glucoside, Cr = crocetin, Dp35glc = delphinidin 3,5-di-glucoside, Cy35glc = cyanidin 3,5-di-glucoside, Ma 35glc= malvidin 3,5-di-glucoside, Petdiglc= petunidin-diglucoside.

* The data refer to flavonoid aglycones identified after acid hydrolysis by direct comparison with authentic markers.

7.3 Chemosystematics in *Crocus*

From the viewpoint of chemotaxonomy the major surveys focused on flavonoids in *Crocus* have been that of Bate Smith who examined hydrolysed leaf extracts of 49 ssp. [37] and that of Harborne and Williams who by chromatography have analysed leaves and petals of nine commercially available species [38] (Table 7.1, 7.2). Lawrence et al. has focused on the contents of anthocyanidins in perianth segments of *Crocus* [40] and Harborne and Williams confirmed the presence of delphinidin, petunidin but not cyanidin and malvidin [38] (Table 7.2).

Furthermore, the major flavonol glycosides present in flowers of cultivated species were identified as kaempferol 3-sophoroside and kaempferol 3-rutinoside-7-glucoside (Table 7.2). Other flavonoid constituents were tricin, acacetin, mangiferin and scoparin O-rhamnosylglucoside (the structure of these compounds are shown in Fig. 5.2).

The results from Table 7.1 and 7.2 established a complex flavonoid and anthocyanin pattern within the species of *Crocus* partly because of missing details of the glucosidic variation [38]. The aglycones were glycosylated but the carbohydrate moieties were either decomposed during acid hydrolysis or have only been identified by chromatographic studies.

The only approximately relation was between following taxa from Series j: *C. flavus* ssp. *flavus*, *C. stellaris* (Series g x j), *C. candidus* and *C. olivieri* on the basis of the presence of myricetin in them all (Table 7.1).

7.4 Chemosystematics in *Lilium*

No documented results have been found.

8. Results based on papers 1-4

The isolation, purification and structure determination of anthocyanins and other flavonoids is a relatively cumbersome process. In addition, anthocyanins and acylated flavonoids are prone to be unstable and must therefore be handled with precaution. The procedure for obtaining pure flavonoids and anthocyanins consists of several steps, starting with an extraction of the plant material followed by a preliminary purification and a fractionation and isolation of pure pigments. Finally, characterisation and identification follows.

8.1 Extraction and purification of flavonols, flavone and anthocyanins from perianth segments of *Crocus*

Field grown *C. antalyensis*, *C. speciosus*, *C. chysanthus* 'Skyline', *C. chrysanthus* 'Eye-catcher' and *C. sieberi* ssp. *sublimis* 'Tricolor'were collected in Noordwijk, Holland in March 1996.

A screening of the extracted samples by analytical HPLC using a gradient developed for flavonoids [Paper 1-2] showed at least 18 different peaks by detection at 280/360 nm (monitoring on a 3D diodearray detector). The flavonoids showed characteristic flavonols and flavone UV-visible spectra.

Optimizing the elution profile for measuring anthocyanins from the above extracts revealed nine detectable peaks [Papers 3-4] . Their UV-visible spectra (monitoring on a 3D diodearray detector) were in accordance with anthocyanin pigments.

About 50 g of freeze-dried perianth segments of each taxa were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 hr. An acidified acetonitril extraction was used because of high diffusivity and high flavonoid-anthocyanin solubility in this solvent. Acid was used to keep anthocyanins in their most stable equilibrium form, the flavylium ion. However, the anthocyanins could be acylated and therefore minimum amounts of an organic acid (0.1-0.5%trifluoroacetic acid (TFA)) was added in order to avoid hydrolysis of potential esterbonds.

The first step in the purification procedure was column chromatography using XAD-7 Amberlite (adsorption chromatography) to remove free sugars and acids. The extract was dissolved in an acidified aqueous solution containing a low acetonitril content, adsorbed on top of a XAD-7 column, washed with water containing 0.5% TFA and eluted stepwise with increasing proportions of acidified acetonitril in water.

The last step was purification of the crude fraction from XAD-7 column using HPLC (High Performance Liquid Chromatography). The crude flavonoids / anthocyanins were applied to prep. ODS-HPLC (20ϕ X 250 mm, Develosil ODS-HG-5, Nomura Chemicals) and eluted stepwise with acidified acetonitril and water. After evaporation *in vacuo* the compounds were stored at -80°C.

8.2 Characterisation and identification of flavonol, flavone and anthocyanin structures from *Crocus*

Information from chromatographic, spectroscopic and chemical methods were gathered. HPLC, UV-vis and Fast Atom Bombardement (FABMS) provided important structural clues.

Structural determination by Nuclear Magnetic Resonance (NMR) included i) aglycone, ii) sugar, and iii) acyl identification, as well as iv) determination of linkage positions between the different units.

The ^{1}H NMR spectrum provided informations on the nature of aglycone and type and number of sugars and acyl substituents.

In solving the sugar structures, it was helpful with 1D-HOHAHA and ^{1}H-^{1}H-COSY spectra . 1D-HOHAHA (homonuclear Hartman-Hahn spectroscopy) was used to clarify the connectivity due to the spin coupling of the sugar units. By irradiation of the anomeric protons separately the complex region of sugar signals was clarified so that only signals from one sugar unit appeared at a time. ^{1}H-^{1}H-COSY spectra gave informations on the through-bond correlations.

Difference NOE or ^{1}H-^{1}H-NOESY spectra gave information on the correlation due to the Nuclear Overhauser Effect (NOE). The position of the sugar units on the chromophore was determined by this method. If the sugar units were biosides or triosides NOE to the neighbouring sugars appeared in the spectrum.

A ^{13}C NMR spectrum also provided information about the chemical structures. Most informative was the 2D spectra where ^{13}C and ^{1}H nuclei were correlated.

^{1}H-^{13}C-HMBC and ^{1}H-^{13}C-FG-HMBC ((Field Gradient) Heteronuclear Multiple Bond Correlation) spectra showed the correlation due to long-range J-coupling between ^{1}H and ^{13}C nuclei. Cross-peaks detection was optimized for three and two bond distance connections. Correlation between quaternary carbons and ^{1}H nuclei could also be observed.

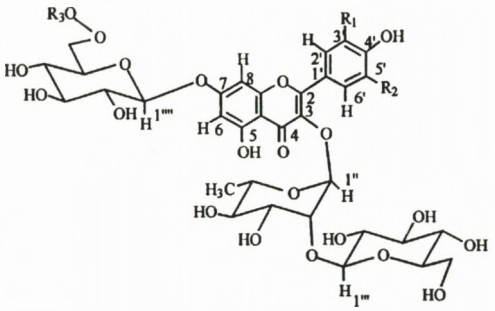
^{1}H-^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra gave information on the correlation between the directly J-coupled ^{1}H and ^{13}C nuclei which was helpful in the assignment of the carbon skeleton of aglycone, sugar and acyl groups. Obviously, quarternary carbons could not be assigned this way.

Concerning 2D techniques, the f_2 axis represented ^{1}H chemical shifts and the f_1 axis ^{13}C chemical shifts. Correlation signals due to long-range J-coupling (HMBC) and to directly J-coupling (HSQC) between ^{1}H and ^{13}C appeared at the positions where the perpendicular lines of the f_2 and f_1 axes crossed each other (Fig. 8.11 and 8.12).

Eighteen flavonoids including six new compounds were reported. The six new flavonoid structures were identified as 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucosides of kaempferol, quercetin and myricetin; kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-*O*-malonyl-β–Dglucoside); kaempferol 3-*O*-α-L-(2-

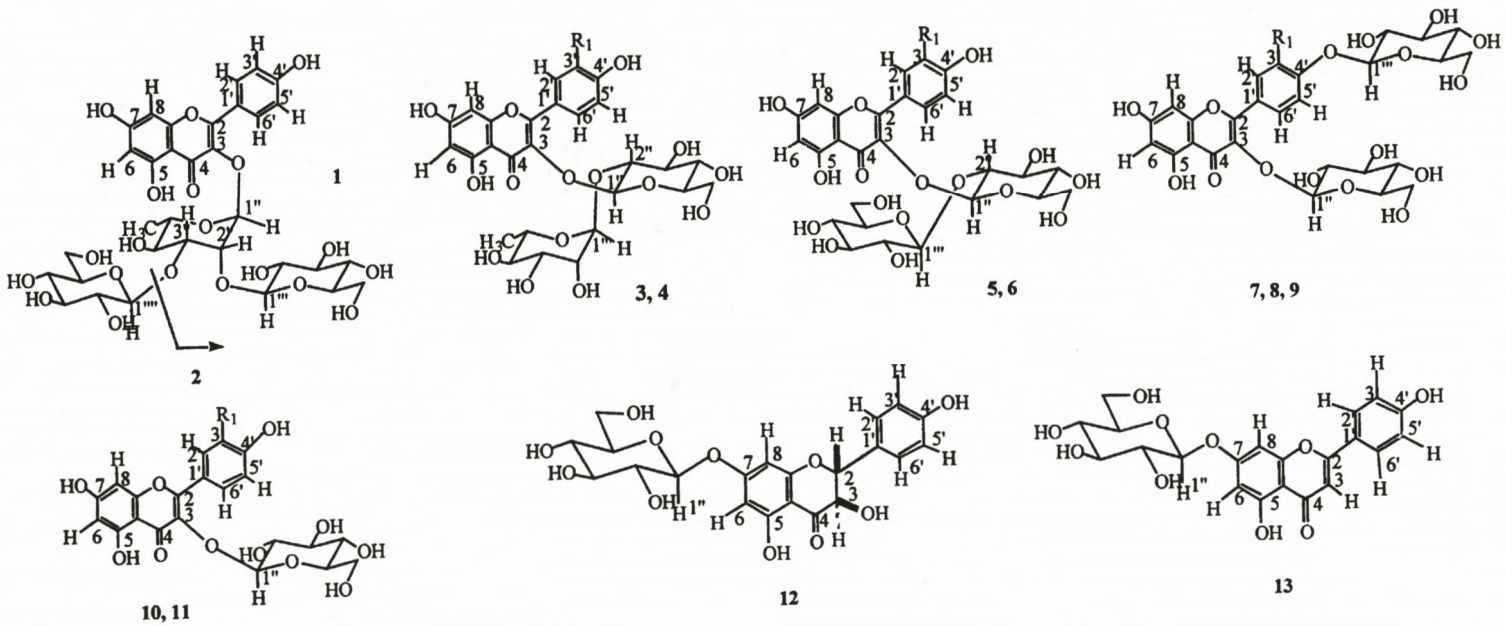
O-*β*-D-glucosyl)rhamnoside-7-*O*-(6-*O*-acetyl-*β*–D-glucoside) and kaempferol 3-*O*-*α* L-(2,3-*di*-*O*-*β*–D-glucosyl)rhamnoside. They ocurred together with kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside; 3-*O*-*β*-D-(2-*O*-*α*-rhamnosyl)glucosides of isorhamnetin and kaempferol; 3-*O*-*β*-D-sophorosides of kaempferol and quercetin; 3, 4'-*di*-*O*-*β*-D-glucosides of kaempferol, quercetin and isorhamnetin; kaempferol 3-*O*-*β*-D-glucoside; apigenin 7-*O*-*β*-D-glucoside and 2,3 *di*-hydrokaempferol 7-*O*-*β*-D-glucoside (Fig. 8.1). Quercetin 3-*O*-*β*-D-glucoside was identified by co-chromatography and UV-vis spectra.

Nine anthocyanins were reported, including four novel compounds identified as; petunidin 3-*O*-(6-*O*-malonyl-*β*-D-glucoside)-7-*O*-(6-*O*-malonyl-*β*-D-glucoside), malvidin 3-*O*-(6-*O*-malonyl-*β*-D-glucoside)-7-*O*-(6-*O*-malonyl-*β*-D-glucoside), delphinidin 3-*O*-(*β*-D-glucoside)-5-*O*-(6-*O*-malonyl-*β*-D-glucoside) and petunidin 3,7-*di*-*O*-*β*-D-glucoside. The new anthocyanins occurred together with 3-O-*β*–D-rutinosides and 3, 5-*di*-*O*-*β*-D-glucosides of delphinidin as well as petunidin and delphinidin 3,7-*di*-*O*-*β*-D-glucoside (Fig. 8.2).



	R_1	R_2	R_3	
1	H	H	H	kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7*O*-*β*-D-glucoside
2	OH	H	H	quercetin 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7*O*-*β*-D-glucoside
3	OH	OH	H	myricetin 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7*O*-*β*-D-glucoside
4	H	H	malonyl	kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7*O*-(6-*O*-malonyl-*β*-D-glucoside)
5	H	H	acetyl	kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7*O*-(6-*O*-acetyl-*β*-D-glucoside)

Fig. 8.1 New flavonol glycosides from*Crocus* [Paper 2]



1: kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside (new compound)

2: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside

3 : R_1= H, kaempferol 3-*O*-β-D-(2-*O*-α-rhamnosyl)glucoside

4 : R_1= OCH_3, isorhamnetin 3-*O*-β-D-(2-*O*-α-rhamnosyl)glucoside

5 : R_1= H, kaempferol 3-*O*-β-D-(2-*O*-β-D-glucosyl)glucoside

6 : R_1= OH, quercetin 3-*O*-β-D-(2-*O*-β-D-glucosyl)glucoside

7 : R_1= H, kaempferol 3,4'-*di*-*O*-β-D-glucoside

8 : R_1= OH, quercetin 3,4'-*di*-*O*-β-D-glucoside

9 : R_1= OCH_3, isorhamnetin 3,4'-*di*-*O*-β-D-glucoside

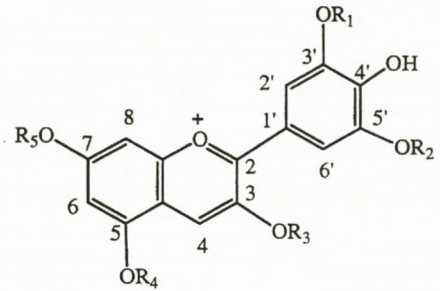
10 : R_1= H, kaempferol 3-*O*-β-D-glucoside

11 : R_1= OH, quercetin 3-*O*-β-D-glucoside

12: dihydrokaempferol 7-*O*-β-D-glucoside

13: apigenin 7-*O*-β-D-glucoside

Fig. 8.1 continued. Flavols and flavones from*Crocus* [Paper 1, 2]



	R_1	R_2	R_3	R_4	R_5	
1	H	H	β-glucoside	H	β-glucoside	delphinidin 3,7-*di*-*O*-β-D-glucoside
2	CH_3	H	β-glucoside	H	β-glucoside	petunidin 3,7-*di*-*O*-β-D-glucoside
3	H	H	β-glucoside	β-glucoside	H	delphinidin 3,5-*di*-*O*-β-D-glucoside
4	CH_3	H	β-glucoside	β-glucoside	H	petunidin 3,5-*di*-*O*-β-D-glucoside
5	H	H	β-rutinoside	H	H	delphinidin 3-*O*-β-D-rutinoside
6	H	H	β-glucoside	β-(6-*O*-malonyl-glucoside)	H	delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside)
7	CH_3	H	β-(6-*O*-malonyl-glucoside)	H	β-(6-*O*-malonyl-glucoside)	petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside)
8	CH_3	H	β-rutinoside	H	H	petunidin 3-*O*-β-D-rutinoside
9	CH_3	CH_3	β-(6-*O*-malonyl-glucoside)	H	β-(6-*O*-malonyl-glucoside)	malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside)

Fig. 8.2 Anthocyanins from *Crocus* [Paper 3-4].

8.3 Structure determination of a flavonol from *Crocus* (an example) [Paper 2]

The new flavonol glycoside, kaempferol 3-*O*-α-L (2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside has been chosen for illustration of the flavonoid identification work.

The compound showed λ_{max} at 265, 310sh, 345 nm in 0.1% HCl MeOH. Adding shift reagents the compound showed the characteristics of flavonol glycosides.

In FAB-MS the mass peak at 757 corresponded to $C_{30}O_{20}H_{40}$ $[M]^+$. The fragment peaks were observed at m/z 449 [M-307 (glucosylrhamnosyl)]$^+$ and m/z 287 [aglycone].

In figure 8.3 the coupling patterns in an aromatic ring and in two sugar moieties are illustrated. The coupling constants (J_{12} = 7.5 and $J_{2,3} = J_{3,4} = J_{4,5}$ = 9-9.5 Hz) show the sugar is β-glucopyranosyl because the J-values 7.5-9.5 Hz indicates the transdiaxial arrangement of the protons. The rhamnosyl moiety has a characteristic CH_3 group with a chemical shift at about 0.7-1.0 ppm. However, the chemical shift value responds to solvent and measurement temperature and is therefore varying.

In general two solvents were used in the measurements of flavonoids, C_5D_5N-d_5 and DMSO-d_6. The flavonoids were not soluble in H_2O, CD_3OD and $CDCl_3$ at room temperature but very soluble in C_5D_5N-d_5. However, in DMSO-d_6 the sugar part region was more widely spread compared with C_5D_5N-d_5, the exchangable phenolic proton signals were remained and strong negative NOE was observed.

In the ^{1}H-NMR spectrum (Fig. 8.4 & 8.5) the chemical shifts characteristic for aromatic rings at δ_H (7.97) 7.84 (*d*, *J*=8.4) and δ_H (7.22) 6.98 (*d*, *J*=8.4) corresponded to H-2' (=H-6') and H-3' (=H-5') and at δ_H (6.98) 6.80 (*d*, *J*=1.8) and δ_H (6.78) 6.51 (*d*, *J*=1.2) to H-8 and H-6, respectively. This indicated the presence of a kaempferol nucleus. The measurements were in DMSO-d_6-10% TFA-*d* and C_5D_5N-d_5 (shown in the parentheses).

To make the NMR data of the sugar moieties clear figure 8.3 illustrates the coupling patterns of every proton in glucose and rhamnose, respectively.

From 1D-HOHAHA and ^{1}H-^{1}H-COSY spectra the compound was found to contain two glucosyl and one rhamnosyl units. 1D-HOHAHA spectra showed the signals from each of the respective sugar units separately. The coupling pattern of the protons in each sugar unit clearly appeared (Fig. 8.6-8.8).

Combining with a ^{1}H-^{1}H-COSY spectrum, the protons were systematically assigned. Starting with the anomeric proton a cross peak signal to the neigbour protons on C-2 appeared and further H-2 correlated with H-3 and so on (Fig. 8.9).

The signals at δ_H (1.18) 0.84 (*d*, *J* = 6.0 Hz, H-6''), δ_H (6.28) 5.65 (*br s*, H-1''), δ_H (5.03) 4.16 (*m*, H-2''), δ_H (4.56) 3.52 (*dd*, *J* = 3.6 and 9.0 Hz, H-3''), δ_H (4.18) 3.30 (*m*, H-5'') and δ_H (4.40) 3.10 (*t*, *J* = 9.6 Hz, H-4'') indicated the presence of a rhamnopyranosyl unit. The signals of two other sugars appeared at δ_H (5.28) 4.31

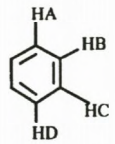
(*d*, J = 7.8 Hz, H-1''') and δ_H (5.81) 5.10 (*d*, J = 7.2 Hz, H-1''''), and δ_H (4.05-4.38) 3.05-3.31 ($J_{2,3} = J_{3,4} = J_{4,5}$ = ca 9.0 Hz) (Fig. 8.3). Thus, the remaining two sugars were β-D-glucopyranose.

The positions of the glycosidic linkages were determined by ^{1}H-^{1}H-NOESY (Fig. 8.10) and Difference NOE spectra (not shown). A strong NOE was observed between H-1''' of glucosyl and H-2'' of rhamnosyl. This indicated that a glucosyl unit was linked to OH-2'' (shown in Fig 8.4 by black arrows).The (1-2) linkage was also confirmed by the lowfield-shift of H-1'' by ca 0.4 ppm more than for other glucorhamnosyl units [49]. A weak NOE between H-6' and H-2'' indicated that rhamnosyl was linked to OH-3 of kaempferol. The linkage of the last glucosyl unit was assigned by strong negative NOEs between H-1'''' and H-6 and H-8, respectively. This indicated that the glucosyl unit was attached on OH-7 of the aglycone.

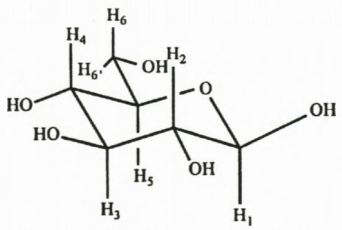
After assignment of ^{13}C signals by a ^{1}H-^{13}C-HSQC spectrum (Fig. 8.11) it was possible to use a ^{1}H-^{13}C-HMBC spectrum for finding three and two bond distance connections. In figure 8.4 the important correlations indicating the glycosidic linkages are shown by dotted arrows; C-3 of kaempferol and H-1'' of rhamnosyl, C-1'''' of 7-glucosyl and H-8 of kaempferol, C-2'' of rhamnosyl to H-1''' of glucosyl. The HMBC spectrum is shown in Fig. 8.12.

Thus, the compound was identified as kaempferol 3-*O*-α-L (2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside.

One of the major flavonol glycosides in the petals of *Crocus* has earlier been characterised as kaempferol 3-rutinoside-7-glucoside by chromatographical procedures [38]. However, a revision of the structure into the major component kaempferol 3-glucorhamnoside-7- glucoside is more likely. No trace of flavonol 3-rutinosides have been found in our survey.



JAB = 6-10 Hz
JAC = 1-3 Hz
JAD = 0-1 Hz



β-D-glucose

H-1, *d*, 7.5 Hz

H-2, *t*, 9.0 Hz

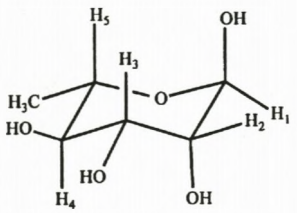
H-3, *t*, 9.0 Hz

H-4, *t*, 9.0Hz

H-5, *m*, 0-2.5, 6.0 & 9.0 Hz

H-6, *dd*, 0-2.5 & 12.0 Hz

H-6', *dd*, 6.0 & 12.0 Hz



α-L-rhamnose

H-1, *d*, 1.5 Hz

H-2, *dd*, 1.5 & 3.0 Hz

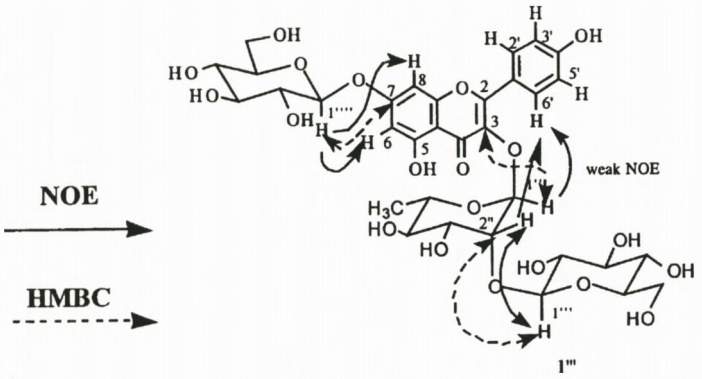
H-3, *dd*, 3.0 & 9.0 Hz

H-4, *t*, 9.0 Hz

H-5, *m*,

H-6, *d*, 6.0 Hz

Figure 8.3. Illustration of the coupling pattern in an aromatic ring and in *β*-D-glucose and *α*-L-rhamnose.



aglycone (H)		rhamnoside(H'')		glucoside (H''')		7-glucoside (H'''')	
6	(6.78) 6.51 *d*(1.2)	1	(6.28) 5.65 *br s*	1	(5.28) 4.31 *d*(7.8)	1	(5.81) 5.10 *d*(7.2)
8	(6.98) 6.80 *d*(1.8)	2	(5.03) 4.16 *m*	2	(4.05) 3.22 *t*(9.0)	2	(4.33) 3.31 *t*(9.0)
2'	(7.97) 7.84 *d*(8.4)	3	(4.56) 3.52 *dd* (3.6; 9.0)	3	(4.22) 3.19 *t*(9.0)	3	(4.15) 3.27 *t*(9.0)
3'	(7.22) 6.98 *d*(8.4)	4	(4.40) 3.10 *t*(9.6)	4	(4.28)3.05 *t*(9.6)	4	(4.38) 3.18 *t*(9.0)
5'	(7.22) 6.98 *d*(8.4)	5	(4.18) 3.30 *m*	5	(3.93) 3.07 *m*	5	(3.92) 3.43 *m*
6'	(7.97) 7.84 *d*(8.4)	6	(1.18) 0.84 *d*(6.0)	6	(4.44) 3.52 *m* (4.55) 3.52 *m*	6	(4.46) 3.47 *dd*(4.8; 11.7) (4.55) 3.70 *m*

Flavonol C		Sugar at C-3		Sugar at C-7	
		1''	102.4	1''''	101.5
2	156.9	2''	82.5	2''''	74.6
3	136.0	3''	72.1	3''''	78.4
4	178.7	4''	71.6	4''''	70.9
5	161.7	5''	73.4	5''''	78.2
6	100.3	6''	18.1	6''''	62.1
7	163.9				
8	95.0	1'''	106.8		
9	156.9	2'''	75.6		
10	107.6	3'''	79.0		
1'	121.2	4'''	70.9		
2'	131.2	5'''	78.4		
3'	116.2	6'''	62.2		
4'	158.3				
5'	116.2				
6'	131.2				

Fig. 8.4 ^{1}H and ^{13}C NMR data of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in DMSO-d_6-10% TFA-*d*. The chemical shifts in C_5D_5N-d_5 are shown in the parentheses.

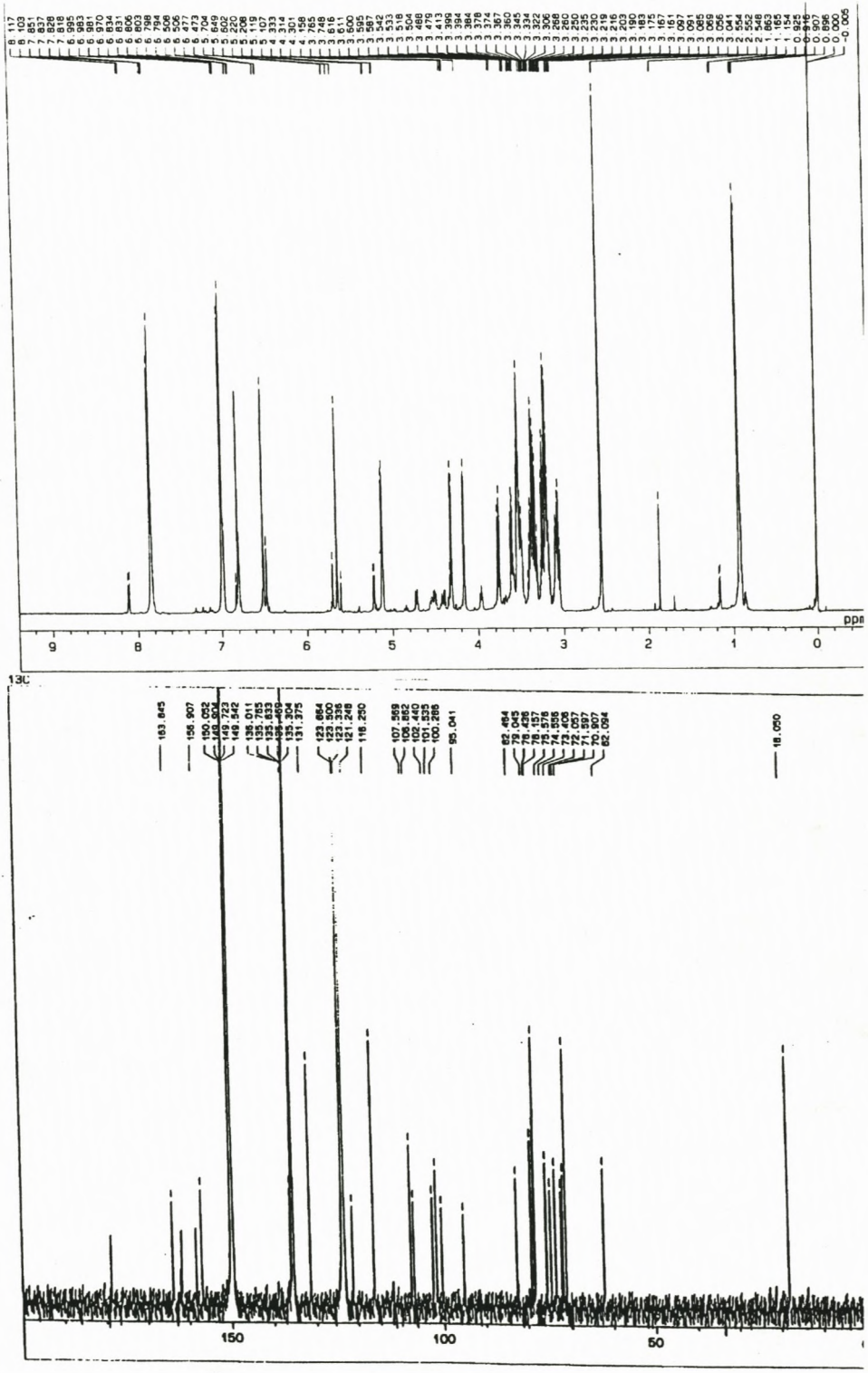


Fig. 8.5 ^{1}H and ^{13}C NMR spectra of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in DMSO-d_6-10% TFA-*d* and C_5D_5N-d_5 , respectively.

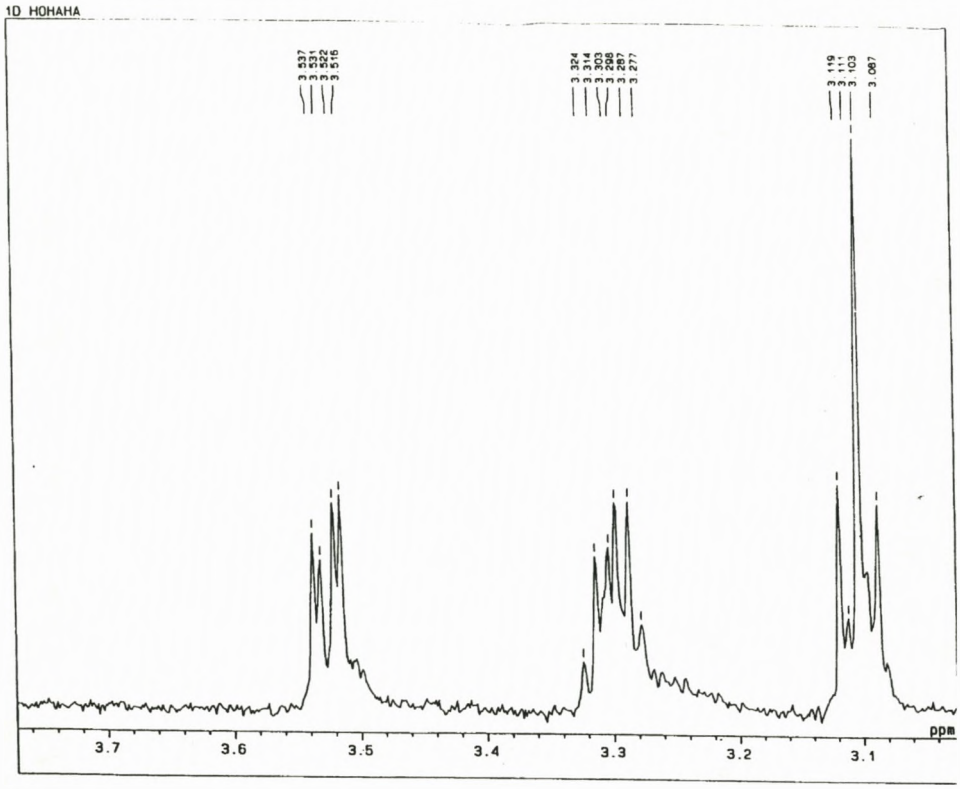


Fig. 8.6 1D-HOHAHA spectrum of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in DMSO-d_6-10% TFA-*d*. Irradiation of the CH_3- group at δ 0.84 (*d*, *J*= 6.0 Hz, H-6'') revealed a spectrum of a rhamnosyl unit (only H-3''(δ_H 3.52), H-4'' (δ_H 3.10) and H-5'' (δ_H 3.30) are shown).

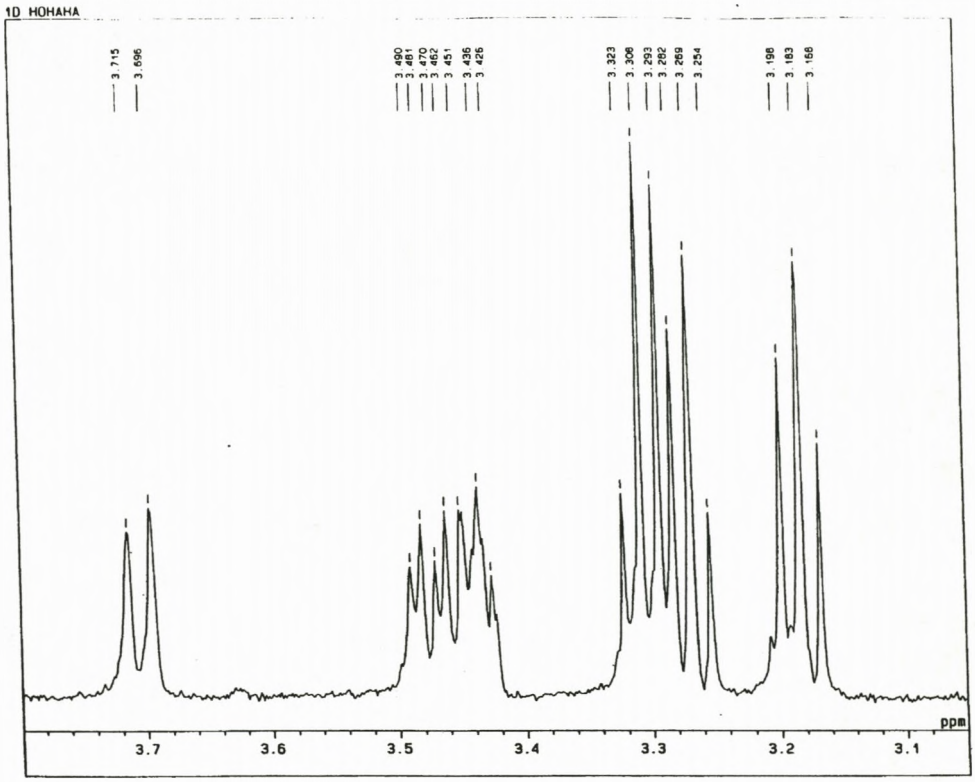


Fig. 8.7 1D-HOHAHA spectrum of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in DMSO-d_6-10% TFA-*d*. Irradiation of the anomeric proton at δ_H 5.10 (*d*, *J*= 7.2 Hz, H-1'''') revealed a spectrum of a glucosyl unit (H-2''''(δ_H 3.31), H-3'''' (δ_H 3.27), H-4'''' (δ_H 3.18), H-5'''' (δ_H 3.43), H-6'''' (δ_H 3.47 & 3.70), anomeric proton not shown).

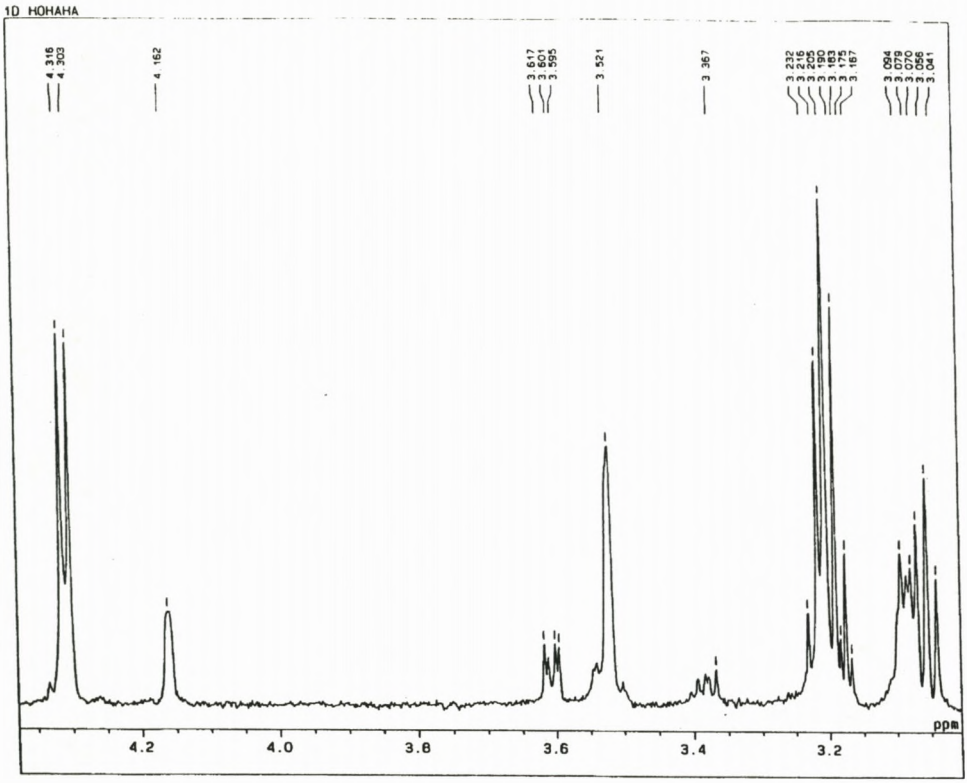


Fig. 8.8 1D-HOHAHA spectrum of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in DMSO-d_6-10% TFA-*d*. Irradiation of the anomeric proton at δ_H 4.31 (*d*, *J*= 7.8 Hz, H-1''') revealed a spectrum of a glucosyl unit (H-2'''(δ_H 3.22), H-3''' (δ_H 3.19), H-4''' (δ_H 3.05), H-5''' (δ_H 3.07), H-6''' (δ_H 3.52).

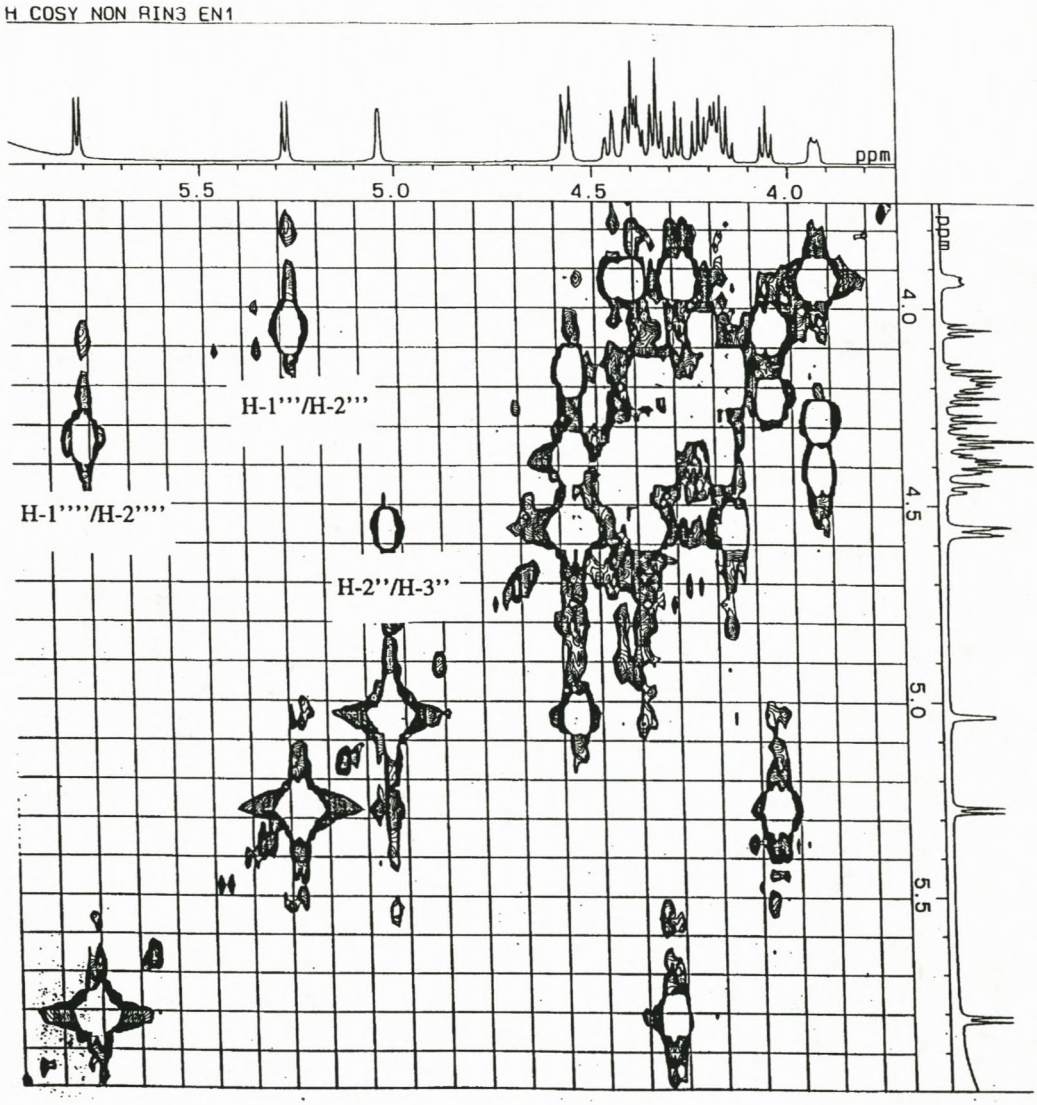


Fig. 8.9 ^{1}H-^{1}H-COSY spectrum of kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7-*O*-*β*-D-glucoside measured in C_5D_5N-d_5.
Through bond correlation signals between H-1'''' (δ_H 5.81) & H-2'''' (δ_H 4.33), H-1''' (δ_H 5.28) & H-2''' (δ_H 4.05), H-2'' (δ_H 5.03) & H-3'' (δ_H 4.56) are marked.

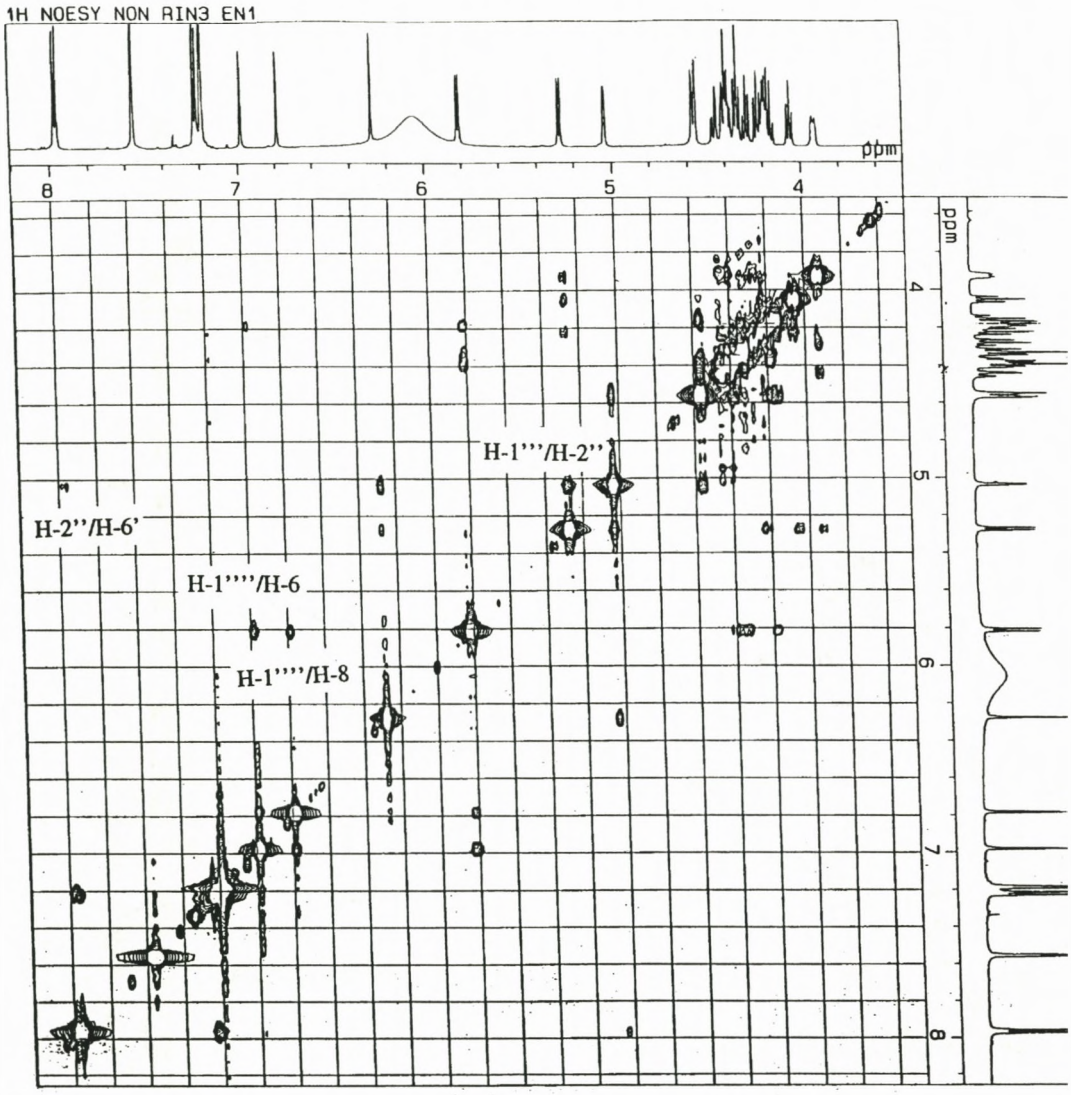


Fig. 8.10 ^{1}H-^{1}H-NOESY spectrum of kaempferol 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7-*O*-*β*-D-glucoside measured in C_5D_5N-d_5.
Appreciable NOE signals between H-6' (δ_H 7.97) & H-2'' (δ_H 5.03), H-8 (δ_H 6.98) & H-1'''' (δ_H 5.81), H-6 (δ_H 6.78) & H-1'''' (δ_H 5.81) and H-1''' (δ_H 5.28) & H-2'' (δ_H 5.03) are shown.

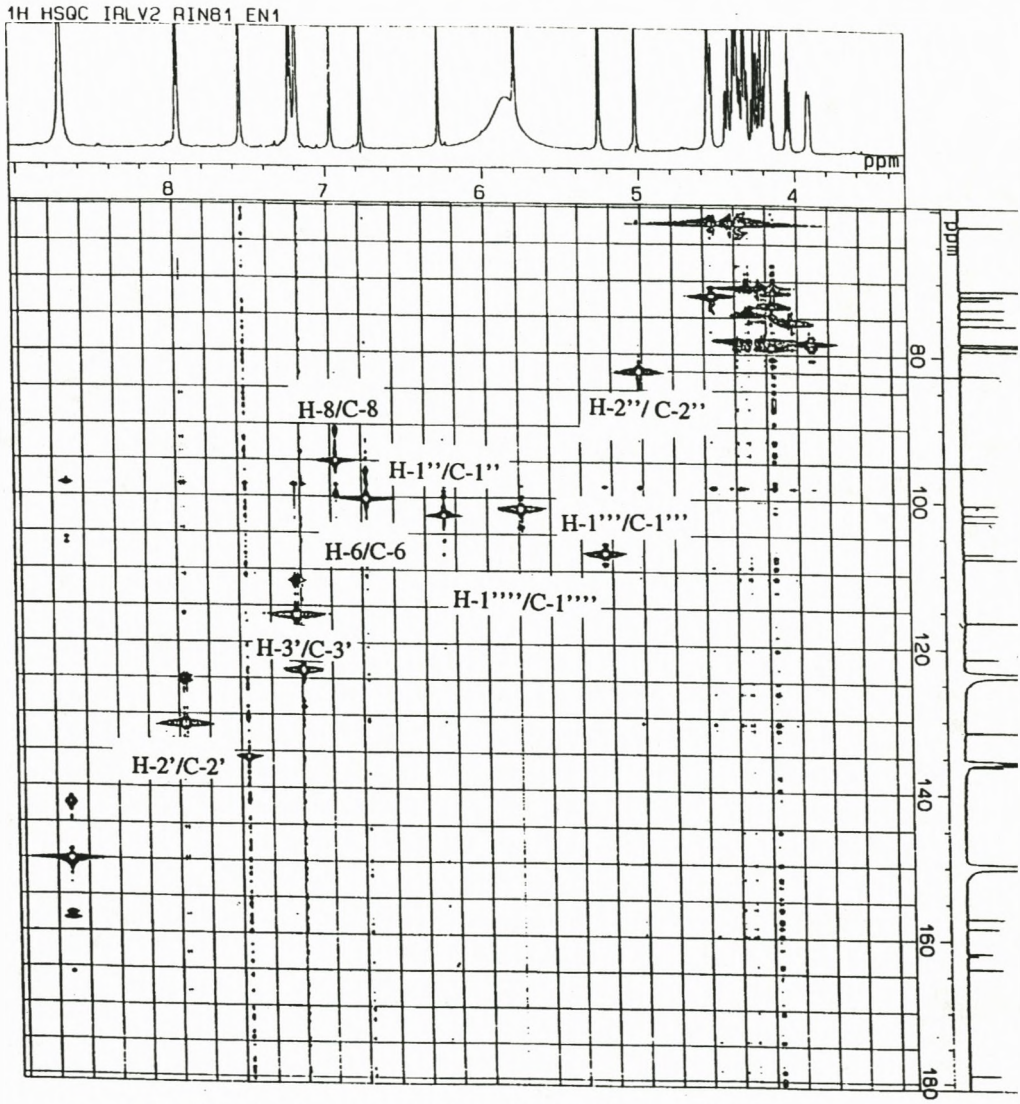


Fig. 8.11 ^{1}H-^{13}C-HSQC spectrum of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in C_5D_5N-d_5.

Some of the shift correlations were; H-2' (=H-6') (δ_H 7.97) & C-2' (=C-6') (δ_c 131.2), H-3' (=H-5') (δ_H 7.22) & C-3' (=C-5') (δ_c 116.2), H-8 (δ_H 6.98) & C-8 (δ_c 95.0), H-6 (δ_H 6.78) & C-6 (δ_c 100.3), H-1'' (δ_H 6.28) & C-1'' (δ_c 102.4), H-1'''' (δ_H 5.81) & C-1'''' (δ_c 101.5), H-1''' (δ_H 5.28) & C-1''' (δ_c 106.8) and H-2'' (δ_H 5.03) & C-2'' (δ_c 82.5).

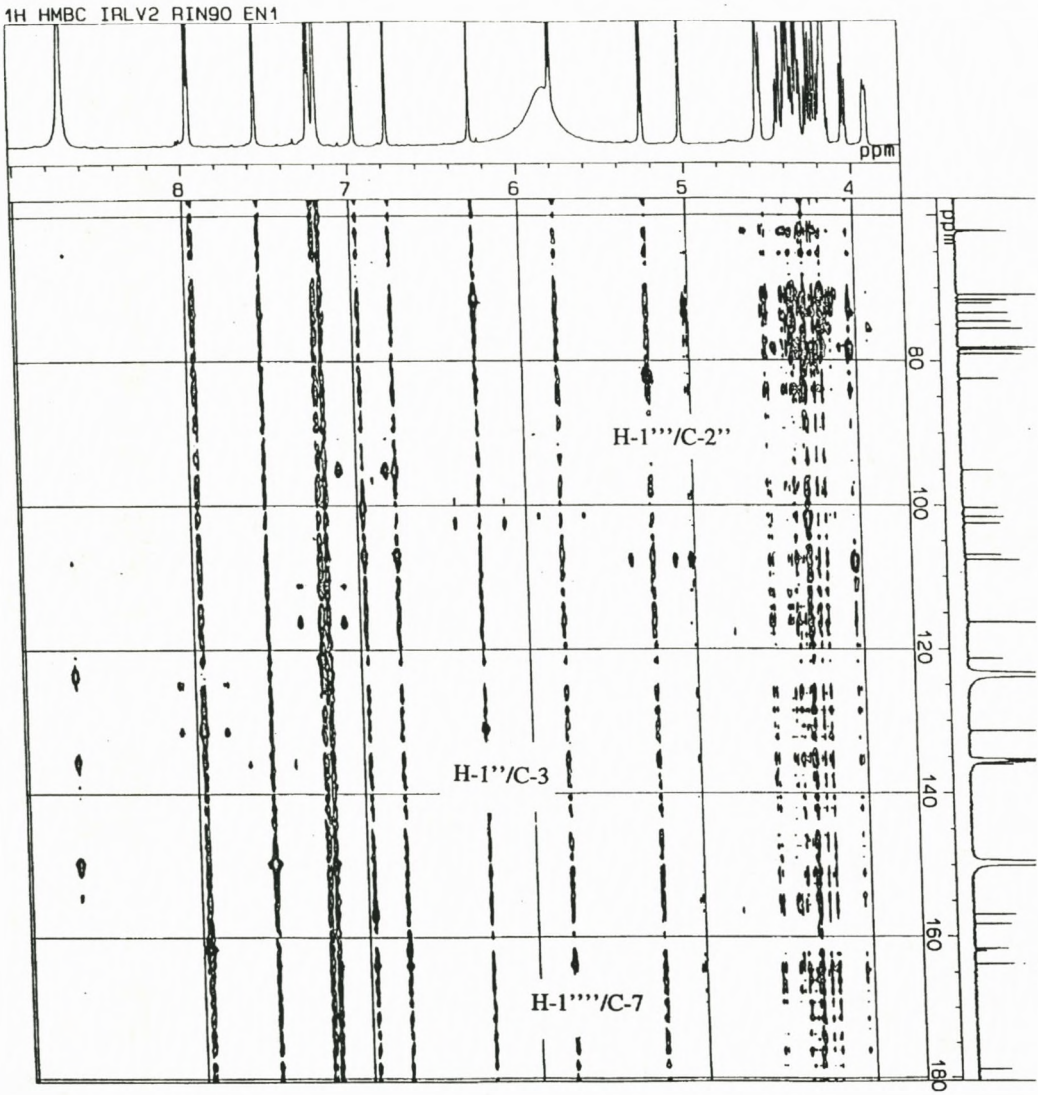


Fig. 8.12 ^{1}H-^{13}C-HMBC spectrum of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucoside measured in C_5D_5N-d_5.
Appreciable shift correlations were observed; H-1'' (δ_H 6.28) & C-3 (δ_c 136.0), H-1'''' (δ_H 5.81) & C-7 (δ_c 163.9) and H-1''' (δ_H 5.28) & C-2'' (δ_c 82.5).

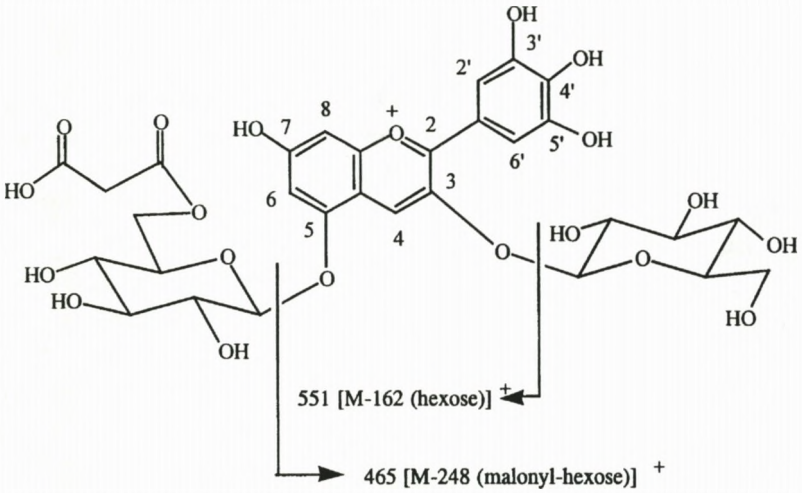
8.4 Structure determination of a malonated anthocyanin from *Crocus* (an example) [Paper 4]

Focusing on the new compound delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside) the procedure of identification was described.
The UV-vis-spectrum (0.1% HCl-MeOH) showed λ_{max} 271 and 538 nm. FAB-MS gave $[M]^+$ at m/z 713, in good agreement with the mass calculated for $C_{30}O_{20}H_{33}^+$. The fragment peaks were observed at m/z 551 [M-162 (hexose)]$^+$, 465 [M-248 (malonyl-hexose)]$^+$ and 303 [aglycone]$^+$, indicating **1** to be comprised of delphinidin, hexosyl and malonylhexosyl (Fig. 8.13). Acylation was confirmed by the characteristically increase in retention time (5) compared with the original delphinidin 3,5-*di*-*O*-*β*-glucoside (3) (Fig. 8.14).

A ^{1}H NMR spectrum confirmed the presence of delphinidin (Fig. 8.15 and 8.16). Two equivalent aromatic protons appeared at δ_H 7.83 (*br s*, H-2'=H-6'). The singlet H-4 signal characteristic for anthocyanins was found at δ_H 9.07 and signals at δ_H 7.00 (*d*, *J*=1.8) and δ_H 7.06 (*d*, *J*=1.8) were assigned as H-6 and H-8, respectively. Furthermore the ^{1}H NMR spectrum confirmed the presence of two glucosyl residues, one of them acylated with malonic acid although H-4 of glucosyl B and the malonyl protons, observed at δ_H 3.39-3.36 were somewhat superimposed (Fig. 8.15).

The assignments of the two glucosyl units were carried out by using 1D-HOHAHA spectra (Fig. 8.17 and 8.18) and a ^{1}H-^{1}H COSY spectrum (not shown). All vicinal coupling constants of both sugars were between 7.8-9.6 Hz including two anomeric protons at δ_H 5.31 (*d*, *J*=7.8 Hz, glucosyl A) and δ_H 5.16 (*d*, *J*=7.8 Hz, glucosyl B). Therefore both sugar units must be *β*-D-glucopyranosyl units. The 6-methylene protons of glucosyl B at δ_H 4.55 and 4.34 were lowfield shifted by ca. 0.6 ppm compared to those in delphinidin 3,5-*di*-*O*-*β*-D-glucoside [Paper 3] indicating acylation with the malonyl group at the 6-OH.

The positions of the glucosidic linkages were determined from NOE difference spectra. A negative NOE was observed at H-4 (δ_H 9.07) of the delphinidin nucleus by irradiation of the anomeric proton (δ_H 5.31) of glucosyl A (Fig. 8.19, shown with arrows in Fig. 8.16). Glucosyl B was deduced to be attached at the 5-OH of delphinidin through a glucosidic bond, because of the presence of a strong NOE between H-6 (δ_H 6.98) of delphinidin and the anomeric proton of glucosyl B (δ_H 5.16) (Fig. 8.20, NOE shown with arrows in Fig. 8.16). Thus, the compound was delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside).



$C_{30}O_{20}H_{33}^{+}$
Mol. Wt. : 713.57

Fig. 8.13 FAB-mass fragments of delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside)

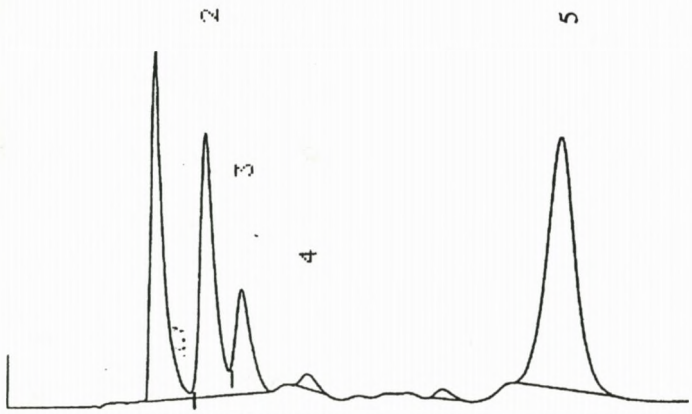


Fig. 8.14 The HPLC profile of extracted perianth segments of *Crocus antalyensis* showed 3,7-*di*-*O*-glucosides of delphinidin (25%) (1) and petunidin (21%) (2) and 3,5-*di*-*O*-glucosides of delphinidin (7%) (3) and petunidin (5%) (4) and delphinidin 3-*O*-glucoside-5-*O*-(6-*O*-malonyl-glucoside (42%) (5).

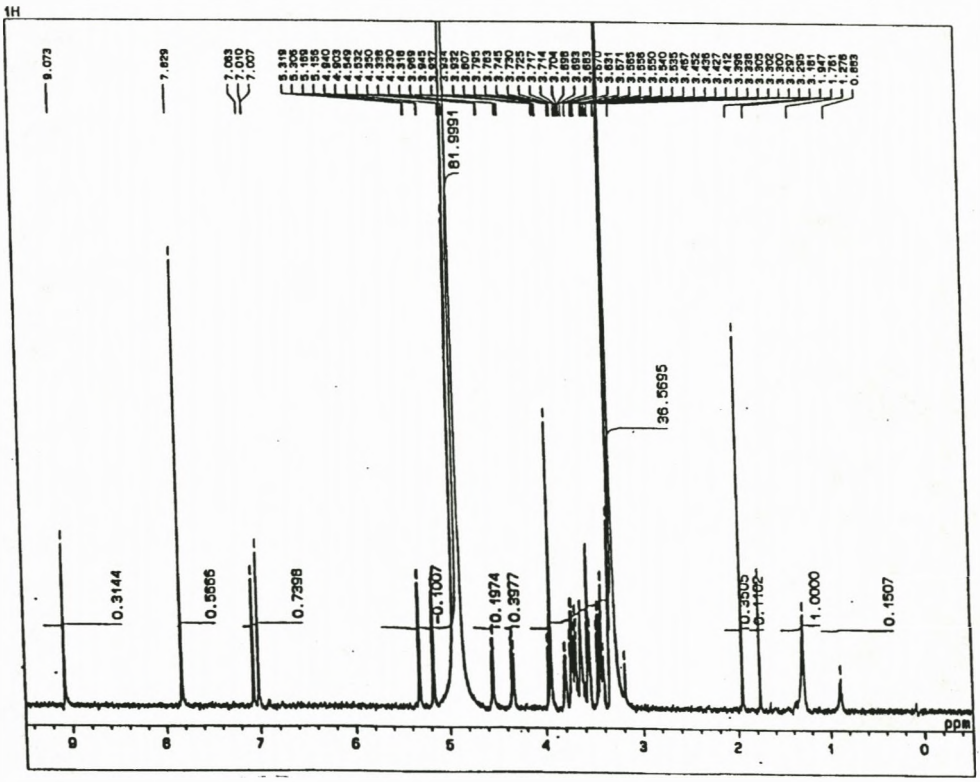
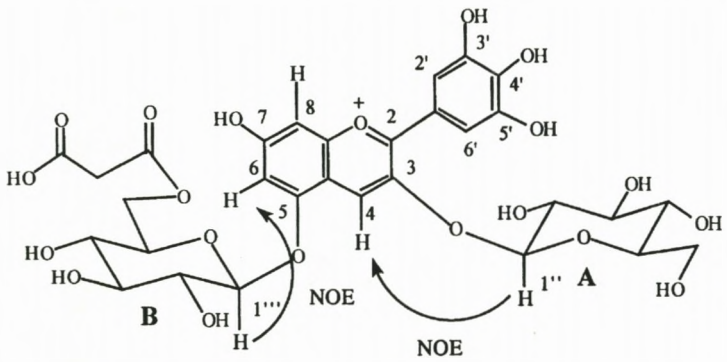


Fig. 8.15 ^{1}H NMR of delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside in CD_3OD, containing 10% TFA-*d*.



H-No. Aglycone

4	9.07 *br s*
6	7.00 *d*(1.8)
8	7.06 *d*(1.8)
2'	7.83 *br s*
6'	7.83 *br s*

H-No. Glucosyl A		H-No. Glucosyl B	
1	5.31 *d*(7.8)	1	5.16 *d*(7.8)
2	3.71 *t*(9.6)	2	3.70 *t*(9.0)
3	3.54 *t*(9.6)	3	3.57 *t*(9.6)
4	3.39 *t*(9.0)	4	3.47 *t*(9.6)
5	3.64 *m*	5	3.80 *m*
6	3.95 *m*	6	4.55 *dd*(6.6; 12.6)
	3.70 *dd*(6.4; 12.7)		4.34 *dd*(6.6; 12.6)

malonic moiety
3.39-3.36

Fig. 8.16 ^{1}H NMR spectral data delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside) in CD_3OD, containing 10% TFA-*d*.

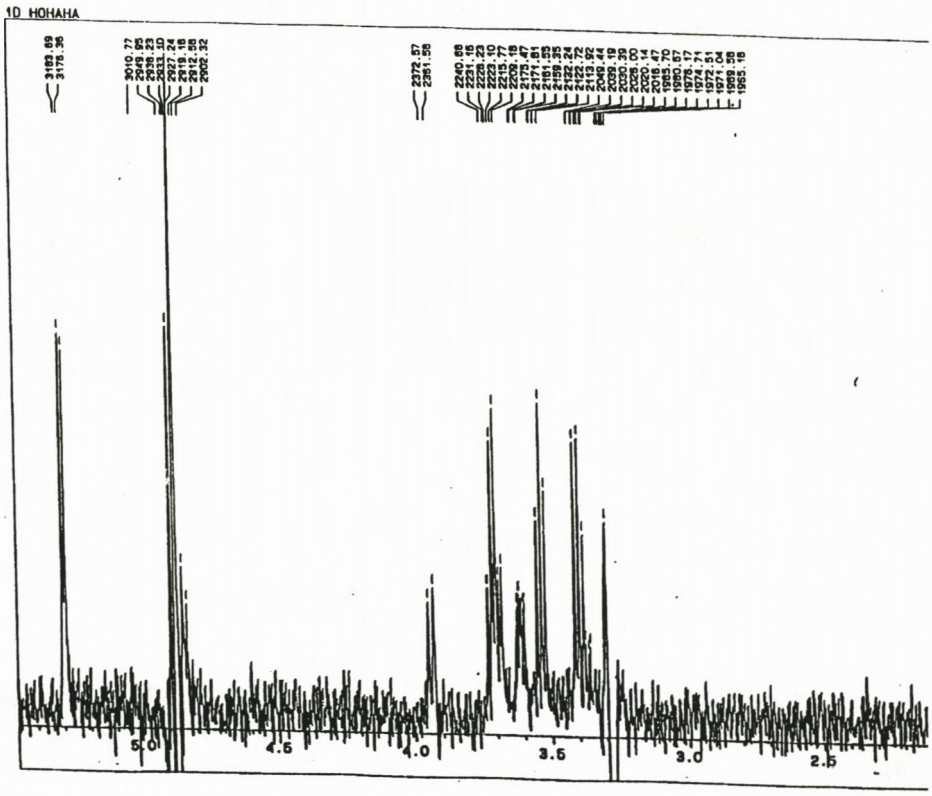


Fig. 8.17 1D-HOHAHA spectrum of delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside in CD_3OD, containing 10% TFA-*d*.
Irradiation of the anomeric proton at δ_H 5.31 (3179 Hz) (glucosyl A) revealed a spectrum of an unacylated glucosyl unit. H-2'' (δ_H 3.71), H-3'' (δ_H 3.54), H-4'' (δ_H 3.39), H-5'' (δ_H 3.64), H-6'' (δ_H 3.70 & 3.95).

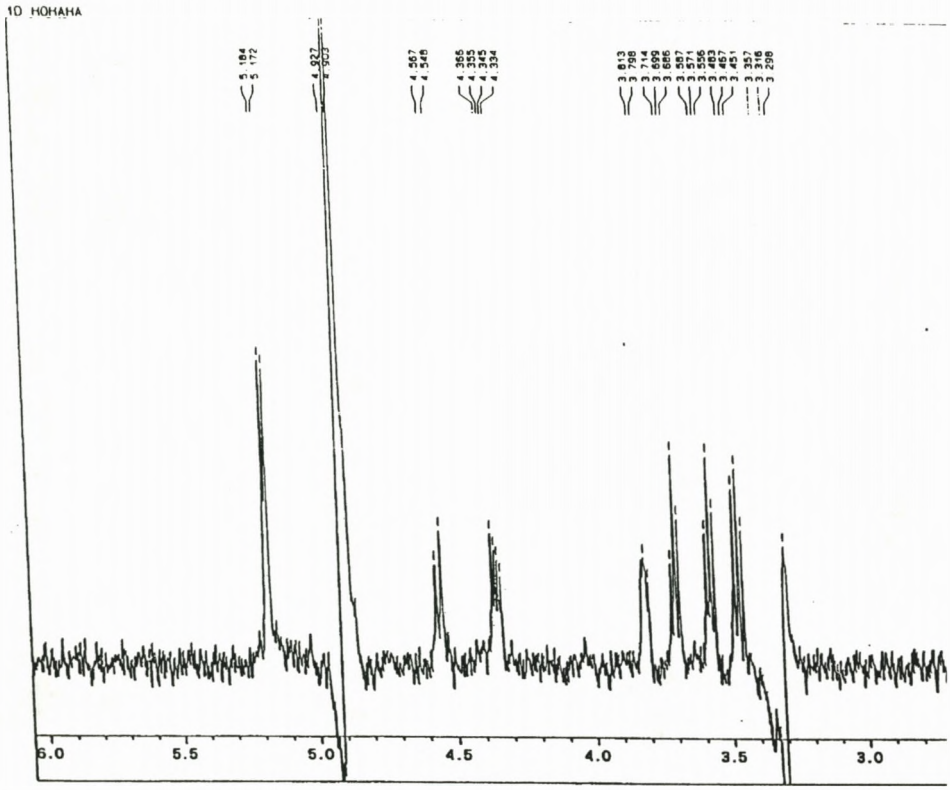


Fig. 8.18 1D-HOHAHA spectrum of delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside in CD_3OD, containing 10% TFA-*d*.

Irradiation of the anomeric proton at δ_H 5.16 (glucosyl B) revealed the presence of lowfield shifted H-6''' protons at δ_H 4.55 & 4.34. This indicated that the malonyl group was attached to OH-6 of glucosyl B. Other signals were H-2''' (δ_H 3.70), H-3''' (δ_H 3.57), H-4''' (δ_H 3.47) and H-5''' (δ_H 3.80).

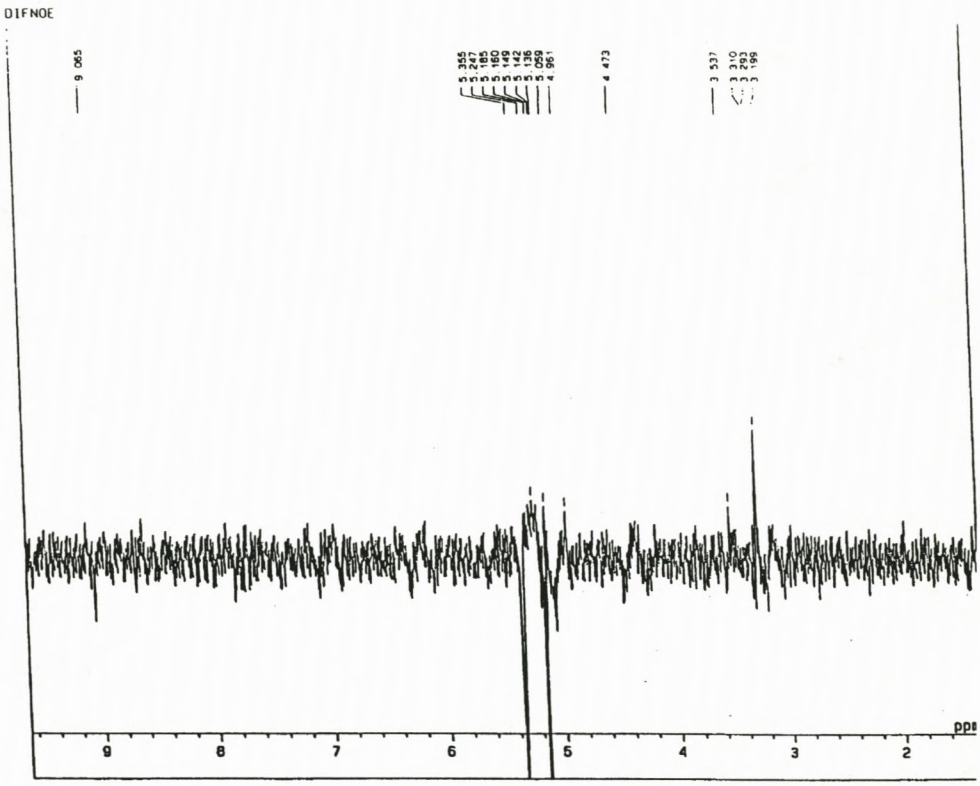


Fig. 8.19 Difference NOE spectrum of delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside in CD_3OD, containing 10% TFA-*d*.
Negative NOE was observed at H-4 (δ_H 9.07) by irradiation of the anomeric proton at δ_H 5.31 (glucosyl A). Thus, glucosyl A was connected to OH-3 of delphinidin.

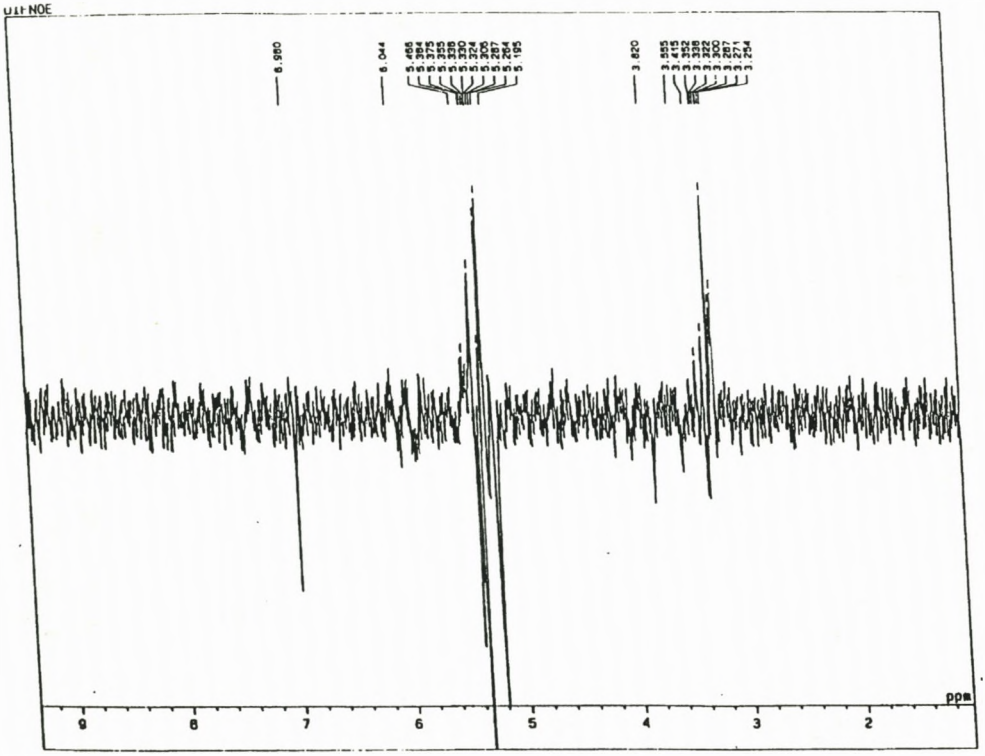


Fig. 8.20 Difference NOE spectrum of delphinidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-malonyl-*β*-D-glucoside in CD_3OD, containing 10% TFA-*d*.
The presence of a strong NOE between H-6 (δ_H 6.98) of delphinidin and the anomeric proton of glucosyl B (δ_H 5.16) indicated that glucosyl B was attached to OH-5 of the chromophore.

9. Results based on Paper 5

9.1 Structure determination of anthocyanins from perianth segments of *Lilium* and a presentation of the anthocyanin contents in Asiatic and Oriental hybrids

The red colours and spots in *Lilium* petals were due to the anthocyanins, cyanidin 3-*O*-*β*-D-rutinoside (2) being the major component and the novel compound, cyanidin 3-*O*-*β*-D-rutinoside–7-*O*-*β*-D-glucoside (1), being present in small amounts or non-detectable.

The isolation work was performed as previous described for *Crocus*.

Compound **1** showed λ_{max} at 280 and 525 nm in 0.1% HCl MeOH and FAB-MS established [M]+ at m/z 757, supporting the molecular formula $C_{33}O_{20}H_{41}^{+}$ with fragments corresponding to cyanidin 3-rutinoside (m/z 595), cyanidin 3-glucoside (m/z 449) and cyanidin (m/z 287).

The proton signals of cyanidin, the aglycone of both **1** and **2**, were assigned using information regarding coupling constants and chemical shifts and the assignment of sugar protons was carried out by using 1D HOHAHA and ^{1}H-^{1}H-COSY spectra.

Focusing on the novel anthocyanin, the 3-*O*-*β*-D-rutinoside–7-*O*-*β*-D-glucosyl moiety has only been identified in another genus as a part of a delphinidin derivative [50]. The determination of the linkage positions of the sugar moieties are described.

The chemical shifts of the two glucosidic anomeric protons were at δ_H 5.34 (*d*, *J*= 8.0 Hz, H-1'') and δ_H 5.22 (*d*, *J*= 7.3 Hz, H 1''''), while the anomeric proton of rhamnosyl (H-1''') was observed at δ_H 4.66 (*br s*) (Fig 9.1).

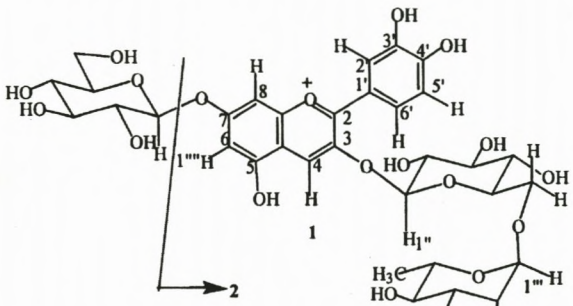
By irradiation of H-1''' of rhamnosyl (δ_H 4.66) NOE was observed to H-6''of glucosyl (δ_H 4.07; 3.75) indicating rutinosyl (Fig. 9.2). Weaker NOE signals to other protons on the neigbour glucosyl unit were observed. The linkage at 3-OH was determined from the presence of a strong negative NOE at H-4 (δ_H 8.87) of the cyanidin nucleus by irradiation of the anomeric proton (H-1'') (δ_H 5.34) (Fig. 9.3). Negative NOEs appeared at H-6 (δ_H 6.87) and H-8 (δ_H 7.21) by irradiation of (H-1'''') (δ_H 5.22) (Fig. 9.4). Thus, **1** was cyanidin 3-*O*-*β*-D-rutinoside-7-*O*-*β*-D-glucoside.

Within both Asiatic and Oriental hybrids, cultivars with or without the novel anthocyanin were found, whereas the known anthocyanin was always present in none-white genotypes (Table 5 in Paper 5).

Although the new anthocyanin was not detected in all the cultivars, it could be present but the contents may be below the detection limit.

However, all cultivars could posses the enzymes involved in 7-glycosylation but the enzyme activity in some genotypes may be too low for cyanidin 3-*O*-*β*-D-rutinoside-7-*O*-*β*-D-glucoside to be detected.

The novel anthocyanin can be characteristic for the genus *Lilium* but the variation within the genus was too small to use this compound as a chemotaxonomical marker. To find out more about the genus, more information could be gathered from using other flavonoid groups.



1: cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-glucoside

2: cyanidin 3-*O*-*β*-rutinoside

	H No.	cyanidin (ppm)	H No.	3-glucoside (H'') (ppm)	rhamnoside (H''') (ppm)	7-glucoside (H'''') (ppm)
1	4	8.85 *br s*	1	5.34 *d*(8.0)	4.66 *br s*	5.22 *d*(7.3)
	6	6.82 *d*(2.2)	2	3.70 *t*(9.0)	3.78 *m*	3.60 *t*(9.6)
	8	7.24 *d*(2.2)	3	3.59 *t*(8.4)	3.61 *dd*(3.6,10.2)	3.57 *t*(9.0)
	2'	8.02 *d*(2.2)	4	3.43 *t*(8.4)	3.30 *t*(9.0)	3.44 *t*(8.4)
	5'	6.97 *d*(8.8)	5	3.60 *m*	3.53 *m*	3.66 *m*
	6'	8.30 *dd*(3.0, 9.0)	6	3.75 *m*	1.15 *d*(6.6)	3.76 *dd*(4.8,12.0)
				4.07 *m*		4.00 *m*
2	4	8.91 *br s*	1	5.27 *d*(8.4)	4.65 *br s*	
	6	6.67 *d*(1.8)	2	3.68 *t*(9.0)	3.78 *m*	
	8	6.88 *d*(1.8)	3	3.58 *t*(9.0)	3.60 *dd*(3.6,10.2)	
	2'	8.01 *d*(2.4)	4	3.43 *t*(9.6)	3.30 *t*(9.0)	
	5'	7.01 *d*(9.0)	5	3.73 *m*	3.53 *m*	
	6'	8.24 *dd*(3.0, 9.0)	6	3.59 *dd*(6.6, 11.4)	1.15 *d*(6.0)	
				4.07 *m*		

Fig. 9.1 Anthocyanins from perianth segments of *Lilium* 'Holean' [Paper 5].

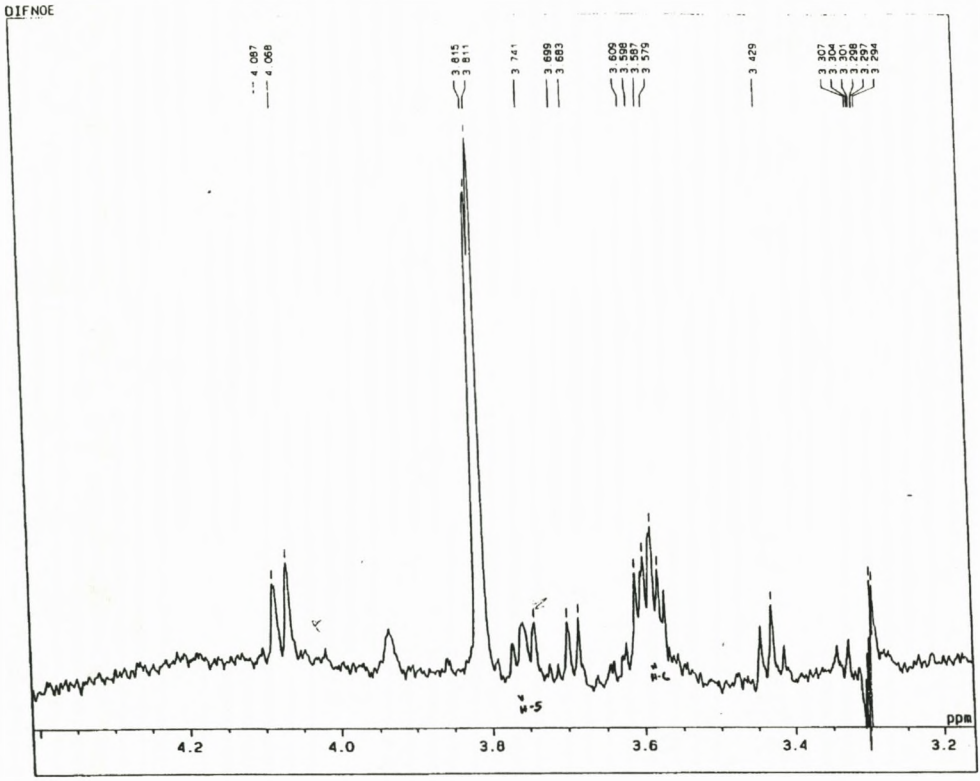


Fig. 9.2 Difference NOE spectrum of cyanidin 3-*O*-β-D-rutinoside-7-*O*-β-D-glucoside (1) in CD_3OD containing 10% TFA-*d*.

By irradiation of H-1''' of rhamnosyl (δ_H 4.66) relatively strong NOE was observed to H-6'' of glucosyl (δ_H 3.74, 4.07) indicating rutinose. Other signals were H-2''' (δ_H 3.81), H-3''' (δ_H 3.60), H-2'' (δ_H 3.69) and H-4'' (δ_H 3.43).

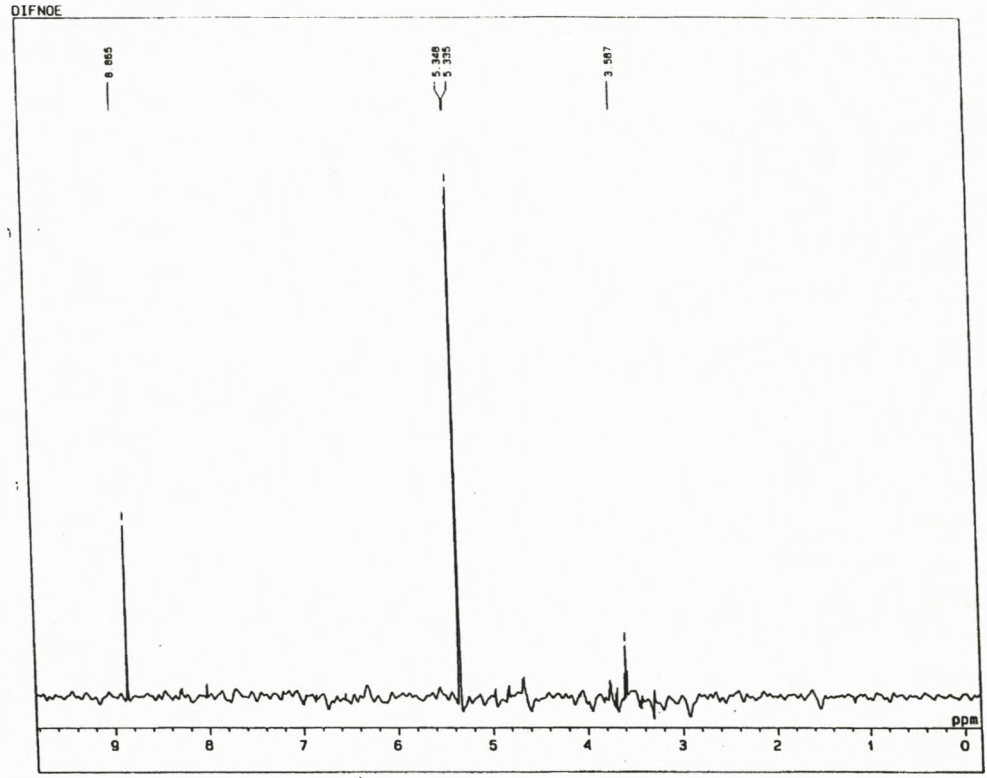


Fig. 9.3 Difference NOE spectrum of cyanidin 3-*O*-*β*-D-rutinoside-7-*O*-*β*-D-glucoside (1) in CD_3OD containing 10% TFA-*d*.
Irradiation of the anomeric proton at δ_H 5.34 gave a strong negative NOE to H-4 (δ_H 8.87) of the cyanidin nucleus indicating the linkage at 3-OH.

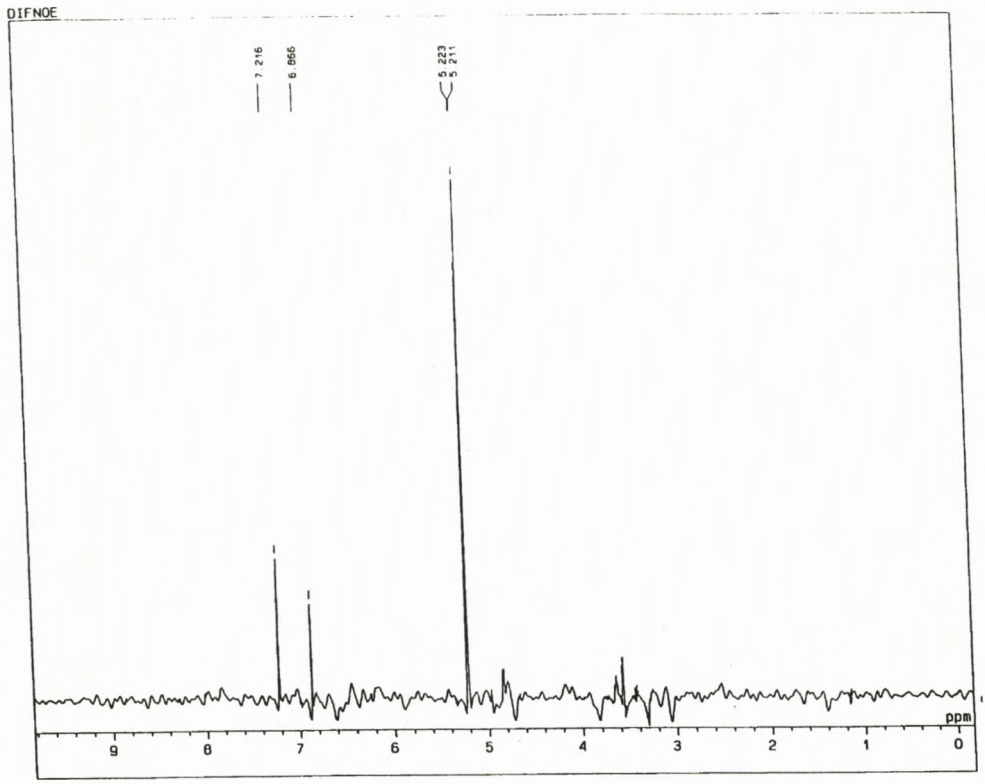


Fig. 9.4 Difference NOE spectrum of cyanidin 3-*O*-β-D-rutinoside-7-*O*-β-D-glucoside (1) in CD_3OD containing 10% TFA-*d*.
Negative NOE's appeared at H-6 (δ_H 6.87) and H-8 (δ_H 7.21) by irradiation of H-1'''' (δ_H 5.22) indicating linkage at 7-OH.

10. Results based on paper 6

10.1 Preliminary analytical results to a chemotaxonomical investigation

A survey of floral anthocyanins and other flavonoids by analytical HPLC among 87 species, 36 cultivars and 6 artificial hybrids was performed.

The perianth segments have been frozen, freezedried, extracted and analysed by analytical HPLC monitoring on a 3D diode-array detector.

Nine anthocyanins were detected and they were identified by their retention times and UV spectra in accordance with the above isolated pigments.

Five chemotypes were defined by the contents of 3-*O*-*β*-D-rutinosides (1), 3,5-*di*-*O*-*β*–D-glucosides (2), 3,5- and 3,7-*di*-*O*–*β*-D-glucosides (3), 3-*O*-*β*-D-rutinosides and 3,5- and 3,7-*di*-*O*–*β*-D-glucosides (4) of delphinidin and petunidin. The fifth chemotype contained anthocyanins from chemotypes 1-4 in combination with delphinidin 3-*O*-*β*-D-glucoside-5-*O*-*β*–D-(6-*O*-malonyl)glucoside (**6**) , petunidin 3,7-*di*-*O*-(6-*O*-malonyl-*β*–D-glucoside) (**7**) and malvidin 3,7-*di*-*O*-(6-*O*-malonyl-*β*–Dglucoside) (**9**).

The same eighteen flavonoids were detected in every taxon corresponding to the structures in Fig. 8.1.

Only quantitative flavonoid differences were noted between the taxa. The major enzymatic reactions involved in the biosynthesis were categorised from the presence of flavonoids with different glycosylation patterns. Based on these, four flavonoid groups were defined by their major composition of 3-*O*-*β*-D-(2-*O*-*α*-L-rhamnosyl)glucosides (I) (>20%); 3-*O*-*β*-D-sophorosides (II) (>30%); 3,4-*di*-*O*-*β*-D-glucosides (III) (>20%) and 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)rhamnoside-7-*O*-*β*-D-glucosides (including compounds acylated at 6-OH of 7-*O*-*β*-D-glucosides), 3-*O*-*α*-L-(2,3-*di*-*O*-*β*-D-glucosyl)rhamnoside and 3-*O*-*α*-L- (2-*O*-*β*-D-glucosyl)rhamnosides (IV) (>60%).

In every genotype, 7-*O*–*β*–D-glucosides of dihydrokaempferol and apigenin appeared as minor components and 3-*O*-*β*-D-glucosides of kaempferol and quercetin as major components. No considerable variation of the amounts of these compounds were found and they were not treated any further because of no chemotaxonomical value.

In Table 2, Paper 6 information about the anthocyanin contents in species of *Crocus* is shown. The species were classified according to the subdivision proposed by Mathew[1]. The chemotypes of anthocyanins in species of Series (h) were shown again in Table 3 together with their cultivars and hybrids.

10.2 The use of flavonol, flavone and anthocyanin structures as chemotaxonomical markers in *Crocus*.

As an introduction to the evaluation of anthocyanin chemotypes and flavonoid groups in *Crocus* some general aspects are presented.

A selection pressure for flower colour could have influence on the contents of 3,7-*di*-*O*-malonylglucosides of petunidin and malvidin (**7** and **9**) and delphinidin 3-*O*-glucoside-5-*O*-malonylglucoside (**6**) in *Crocus.*

It was likely that the presence of two dominant genes with alleles that promote methoxylation in the 2' and 4' positions of the chromophore and malonylation on 6-OH position of the glucoside moieties, made the perianth segments more blue [4].

The presence of a selectory pressure was indicated by the frequent co-occurrence of the two characteristics. Out of 14 species containing malonated anthocyanins 9 also contained malvidin (**9**).

The acylated anthocyanins **6**, **7** and **9** included in chemotype 5 can not be considered as neutral markers since these compounds can appear as a result of selection for blue colour hue. These malonated anthocyanins appear in many different series of *Crocus* (Series c, d, f, g, h, j, and n) as expected.

However, it is likely that selection for colour do not have an affect on compounds **1-5**, **7** because the glucosyl- and rutinosyl-moieties do not increase the intensity of the blue petal colour so these compound can have a considerable chemotaxonomic importance [4].

In general, the chemical data supported the morphological data, only raising some doubt about the placement of seven species.

The major anthocyanins in species belonging to Series d were 3,5-*di*-*O*-*β*-D-glucosides of delphinidin and petunidin. The presence of 3-*O*-*β*-D-rutinosides of delphinidin and petunidin in *C. medius* made this species different from the others (Table 2, Paper 6). Also the placement in flavonoid group (I) deviated from the other species in the series and the data indicated that *C. medius* should be further investigated as to whether it may belong to Series c.

C. kotchyanus and *C. vallicola* from Series e differed in both anthocyanin and flavonoid contents so the data did not by themselves supported their inclusion in the same series.

In Series f, misplacement of four species can be discussed. Both the anthocyanin chemotypes and flavonoid groups were varying in this series. However, *C. asumaniae*, *C. hadriaticus* and *C. oreocreticus* resemble both the anthocyanin chemotypes and flavonoid groups of Series c better than f .

Another species in Series f was *C. mathewii* which may show relation to e.g. Series g.

Analyses of the *C. chrysanthus-biflorus* complex in Series h showed a wide range of anthocyanin chemotypes, which supports the earlier view that the *C. biflorus* subspecies actually comprises several different species.

No systematic correspondances were found between what was known earlier from chemical surveys described in 7.1 and the above results.

11. Discussion and perspectives of the work

The isolation and identification of anthocyanins and flavonoids from perianth segments of *Crocus* revealed ten structures not found in the plant kingdom before. In addition eighteen known compounds were isolated.

Five novel flavonols all had a unique 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucosyl moiety. The compounds were 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucosides of kaempferol, quercetin and myricetin; kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-O-(6-*O*-malonyl-β-D-glucoside) and kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-O-(6-*O*-acetyl-β-D-glucoside). Another novel compound was kaempferol 3-*O*-α L-(2,3-*di*-*O*-β–D-glucosyl)rhamnoside.

Other combination of flavonoid sugar structures were; 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnosyl; 3-*O*-β-D-(2-*O*-α-rhamnosyl)glucosyl; 3-*O*-β-D-sophorosyl; 3, 4'-*di*-*O*-β-D-glucosyl; 3-*O*-β-D-glucosyl and 7-*O*-β-D-glucosyl of common flavonols and flavone.

With regard to flavonoid aglycones, kaempferol dominates, as kaempferol glycosides constituted between 70 and 90 % of the total contents of flavonoids in the flowers of the genus. The contents of quercetin glycosides varied from 5 to 10 % in all taxa and glycosides of dihydrokaempferol, isorhamnetin, myricetin and apigenin were only minor components.

The identification of anthocyanins also revealed a great diversity in structures. Four novel ones were, petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside), malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside), delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside) and petunidin 3,7-*di*-*O*-β-D-glucoside. They co-orcurred with common 3-O-β–D-rutinosides, 3, 5-*di*-*O*-β-D-glucosides of delphinidin and petunidin and delphinidin 3,7-*di*-*O*-β-D-glucoside.

The differences in the sugar structures were determined by different NMR techniques.

The (1-2) linkages in 3-*O*-(2-*O*-glucosyl)rhamnosyl, 3-*O*-(2-*O*-rhamnosyl)glucosyl and 3-*O*-sophorosyl were distinguished by Difference NOE spectra. Likewise, the (1-3) linkage in 3-*O*-(2,3-*di*-*O*-glucosyl)rhamnosyl and the (1-6) linkage in rutinosyl were determined using the same technique.

The aim has been to provide new characters and evaluate whether they were useful to resolve ambiguities in the existing classification scheme of *Crocus*

The three novel malonated anthocyanins and six flavonols, seem to be unique for *Crocus*. The compounds were widely distributed within the genus, the six flavonols occurred in every taxon examined, and so can be used as distinguishing markers for this family.

Within the genus, the evaluation of anthocyanin chemotypes and flavonoid groups each generally supported the classification of Mathew, and using the two types of compounds the results reinforced each other. For all series except Series h the chemical data were very similar for all subspecies or accessions within a species, and anthocyanin chemotypes and flavonoid groups within a series were more similar than across series.

Analyses of the *C. chrysanthus-biflorus* complex in series h showed a wide range of anthocyanin chemotypes, which supports the earlier view that the *C. biflorus* subspecies actually comprise several different species.

Regarding the other series, the analyses suggest that seven other species should be further investigated using other methods, to evaluate whether it can be relevant to move them into other series.

From the chemical data it is not possible to suggest an evolutionary direction (primitive and advanced characters) because there seem to be a pressure of selection on colour hue.

It can be concluded that the chemotaxonomical markers as additional independent characters did supplement the existing traits in the genus *Crocus*. A perspective could be to continue the *in-situ* hybridization of the species pointed out to deviate from their series.

The anthocyanin structures in perianth segments of *Lilium* were cyanidin 3-*O*-*β*-D-rutinoside being the major component and the novel compound, cyanidin 3-*O*-*β*-D-rutinoside–7-*O*-*β*-D-glucoside. The anthocyanins were widely distributed within the genus and can be characteristic for the genus *Lilium*. To find out more about the genus, more information could be gathered from using other flavonoid groups.

12. References

1. Mathew, B. (1982) *The Crocus*. B. T. Batsford. Ltd.
2. Jacobsen, N., Van Scheepen, J. & Ørgaard, M. (1997) The *Crocus chrysanthus-biflorus* cultivars. *The New Plantsman* **4**, 6.
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Flavonol glycosides from flowers of *Crocus speciosus* and *C. antalyensis*

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Abstract

From the flower extracts of *Crocus speciosus* and *C. antalyensis* nine flavonol glycosides have been isolated. One of these products is a new flavonol glycoside identified as kaempferol 3-*O*-α-(2,3-*di*-*O*-β-D-glucopyranosyl)rhamnopyranoside by UV, mass and NMR spectroscopy. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Crocus speciosus*; *C. antalyensis*; Iridaceae; Flavonol glycosides; Kaempferol 3-*O*-α-(2,3-*di*-*O*-β-D-glucopyranosyl)rhamnopyranoside; ^{1}H; ^{13}C; 2D NMR

1. Introduction

In a chemotaxonomic study on the genus *Crocus*, we have isolated five new flavonol 3-*O*-α-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucosides (Nørbæk, Nielsen, & Kondo, in press). We now report on identification of a new modified kaempferol 3-*O*-α-(2-*O*-β-D-glucosyl)rhamnoside, which was linked by glucose at OH-3 of the rhamnose unit. Other investigations on the flavonoids of *Crocus* have been restricted to chromatographic methods. From *Crocus laevigatus*, *C. heuffelianus* and *C. aureus* some flavone and flavonol glycosides based on 6-hydroxyluteolin, scutellarein, scutellarein 7-methyl ether and kaempferol have been isolated; in addition the aglycones acacetin and tricin have been identified (Harborne & Williams, 1984). Isorhamnetin 3,4′-diglucoside has been isolated from *Crocus cv Sir John Bright* (Kuhn & Low, 1944) and a C-glycosylflavone has been isolated from *Crocus reticulatus* (Sergeyeva, 1977).

There is no evidence that the present nine flavonoids contribute to flower color.

It has previously been suggested that flavone and flavonol glycosides from three *Crocus* species give no contribution to yellow petal color which is probably based on carotenoids (Harborne & Williams, 1984). In addition anthocyanins have been found in *Crocus* species (Price, Robinson, & Robinson, 1938; Nørbæk & Kondo, 1998; Nørbæk & Kondo, 1999).

2. Results and discussion

Perianth segments of *Crocus speciosus* and *C. antalyensis* were extracted with aqueous acetonitrile containing 0.5% trifluoroacetic acid. Nine flavonoids were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. A new flavonol glycoside, kaempferol 3-*O*-α-(2,3-di-*O*-β-D-glucopyranosyl)rhamnopyranoside (**1**) was isolated, together with eight known flavonol glycosides, kaempferol 3-*O*-α-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside (**2**) (Markham, Geiger, & Jaggy, 1992), 3-*O*-β-D-(2-*O*-α-rhamnopyranosyl)glucopyranosides of kaempferol (**3**) (Esperanza et al., 1998; Carotenuto et al., 1997) and

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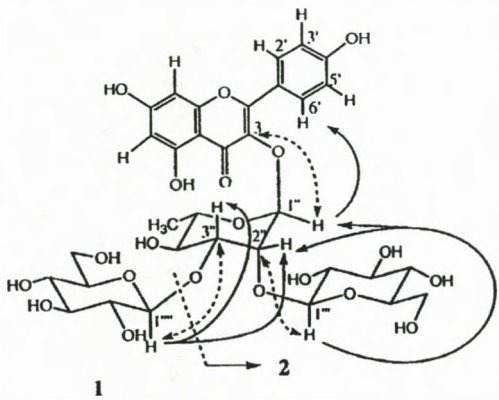
Table 1
Analytical HPLC retention times (R_t) and FAB–MS of **1**–**9**. FAB–MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix

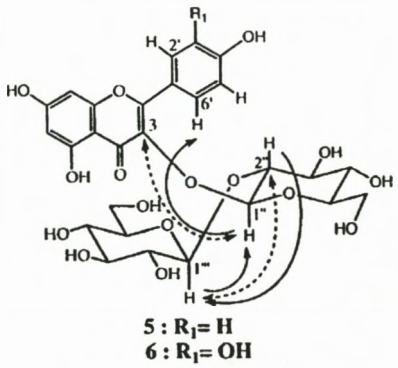
Flavonoid	HPLC (R_t (min))	FAB–MS ($[M]^+$, fragment ions)
1	29.5	757 $[C_{33}H_{41}O_{20}]^+$, 595, 433, 287
2	31.9	595 $[C_{27}H_{31}O_{15}]^+$, 433, 287
3	24.7	595 $[C_{27}H_{31}O_{15}]^+$, 449, 287
4	25.2	625 $[C_{28}H_{33}O_{16}]^+$, 479, 317
5	21.6	611 $[C_{27}H_{31}O_{16}]^+$, 449, 287
6	15.6	627 $[C_{27}H_{31}O_{17}]^+$, 465, 303
7	19.0	611 $[C_{27}H_{31}O_{16}]^+$, 449, 287
8	17.0	627 $[C_{27}H_{31}O_{17}]^+$, 465, 303
9	44.8	449 $[C_{21}H_{21}O_{11}]^+$, 287

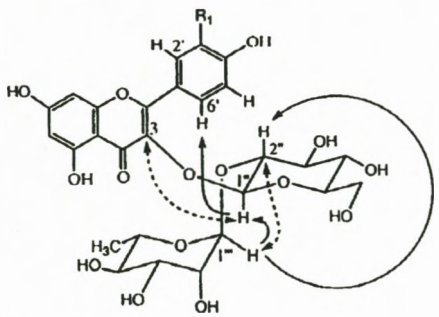
isorhamnetin (**4**) (Dandapani & Nagarajan, 1989; Vidal-Ollivier et al., 1989), 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl)glucopyranosides of kaempferol (**5**) (Budzianowski, 1990) and quercetin (**6**) (Harborne, 1963), 3,4′-*di*-*O*-β-D-glucopyranosides of kaempferol (**7**) (Stein & Zinsmeister, 1990) and quercetin (**8**) (Krauze-Baranowska & Cisowski, 1996) and kaempferol 3-*O*-β-D-glucopyranoside (**9**) (Chaurasia & Wichtl, 1987; Stein & Zinsmeister, 1990).

The FAB (positive-ion mode) mass spectrum of **1** showed a quasi molecular ion peak at *m/z* 757 $[M+H]^+$ (Table 1). The fragments at *m/z* 595 $[(M+H)\text{-}162]^+$, *m/z* 433 $[(M+H)\text{-}162\text{-}162]^+$ and *m/z* 287 $[(M+H)\text{-}162\text{-}162\text{-}146]^+$ correspond to the subsequent losses of two hexose units and one deoxyhexose unit. Fragment at *m/z* 287 corresponds to kaempferol and this observation was supported by UV, ^1H and ^{13}C NMR spectra. The UV spectral analysis in the presence of customary shift reagents (see Section 3) showed the presence of free hydroxyl groups at position 5, 7 and 4′ of kaempferol (Mabry, Markham, & Thomas, 1970); hence a trisaccharide must be attached to hydroxyl group at position 3 of kaempferol.

^1H-NMR and/or ^{13}C-NMR spectra assigned by







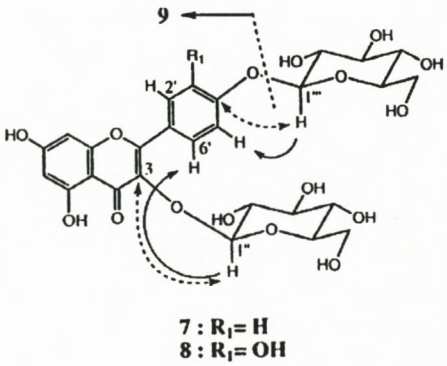


Fig. 1. ← NOE, ←- - -→ HMBC. Only important correlations was described.

Table 2
^{1}H-NMR spectral data of carbohydrate moieties of **1–9** in DMSO-d_6–10% TFA-*d*. Coupling constants (J in Hz) given in parentheses

	H	Sugar units at C-3		Sugar unit at C-4′	
		3-Glc	(2→1)Glc	(3→1)Glc	4′Glc
1	1	5.65 br s	4.51 d(7.8)	4.42 d(7.2)	
	2	4.46 m	3.17 t(9.0)	3.15 t(9.6)	
	3	3.71 m	3.21 t(9.0)	3.27 t(9.0)	
	4	3.39 t(9.0)	3.01 t(9.0)	3.09 t(9.0)	
	5	3.41 m	3.09 m	3.32 m	
	6	0.93 d(6.0)	3.53 m	3.58 dd(6.0;11.4)	
			3.53 m	3.79 m	
2	1	5.63 br s	4.31 d(7.8)		
	2	4.14 m	3.22 t(9.0)		
	3	3.60 dd(3.6;9.9)	3.20 t(9.0)		
	4	3.17 t(9.0)	3.06 t(9.0)		
	5	3.35 m	3.08 m		
	6	0.91 d(6.6)	3.53 m		
			3.53 m		
		3-Glc	(2→1)Rha		
3	1	5.65 d(7.8)	5.07 br s		
	2	3.42 t(9.0)	3.72 dd(4.2;9.6)		
	3	3.37 t(9.0)	3.46 dd(3.0; 9.6)		
	4	3.08 t(9.0)	3.12 t(9.0)		
	5	3.07 m	3.71 m		
	6	3.27 m	0.76 d(6.0)		
		3.53 m			
4	1	5.75 d(7.2)	5.03 br s		
	2	3.48 t(9.0)	3.71 dd(4.2; 9.6)		
	3	3.36 t(9.0)	3.42 dd(3.0; 9.3)		
	4	3.09 t(9.6)	3.12 t(9.0)		
	5	3.12 m	3.67 m		
	6	3.28 dd(4.8; 11.7)	0.65 d(6.6)		
		3.56 m			
		3-Glc	(2→1)Glc		
5	1	5.58 d(7.3)	4.62 d(7.3)		
	2	3.52 t(8.8)	3.21 t(8.8)		
	3	3.51 t(8.8)	3.25 t(8.8)		
	4	3.19 t(8.8)	3.12 t(8.8)		
	5	3.10 m	3.16 m		
	6	3.48 m	3.60 m		
		3.32 dd(4.4; 12.1)	3.49 dd(4.4; 12.1)		
6	1	5.67 d(7.2)	4.60 d(7.2)		
	2	3.52 t(8.4)	3.17 t(8.4)		
	3	3.49 t(8.4)	3.20 t(8.4)		
	4	3.15 t(8.4)	3.10 t(8.4)		
	5	3.08 m	3.14 m		
	6	3.28 m	3.54 m		
		3.29 dd(6.0; 12.0)	3.47 m		
7	1	5.47 d(7.2)			5.00 d(7.2)
	2	3.23 t(9.0)			3.25 t(9.0)
	3	3.18 t (9.6)			3.29 t(9.0)
	4	3.09 t (9.6)			3.18 t(9.0)
	5	3.10 m			3.37 m
	6	3.56 m			3.67 m
		3.33 m			3.46 dd(4.8; 11.7
8	1	5.48 d(7.2)			4.84 d(7.8)

(continued on next page

Table 2 (*continued*)

	H	Sugar units at C-3		Sugar unit at C-4′	
		3-Glc	(2→1)Glc	(3→1)Glc	4′Glc
	2	3.23 t(9.0)			3.29 t(9.0)
	3	3.22 t(9.6)			3.31 t(9.6)
	4	3.10 t(9.0)			3.17 t(9.0)
	5	3.11 m			3.36 m
	6	3.59 m			3.68 dd(6.0; 11.7)
		3.35 m			3.45 dd(6.0; 11.7)
9	1	5.39 d(7.2)			
	2	3.10 t(9.0)			
	3	3.21 t(9.0)			
	4	3.13 t(9.0)			
	5	3.24 m			
	6	3.54 m			
		3.34 dd(4.8; 12.0)			

HSQC and HMBC confirmed the aglycone of **1** as kaempferol (Section 3 and Tables 2 and 3). 1D-HOHAHA, ^{1}H–^{1}H-COSY and homodecoupling spectra of **1** showed the presence of two hexose units and one deoxyhexose unit. The signals at δ_H 5.65 (br s, H-1″), 4.46 (m, H-2″), 3.71 (m, H-3″), 3.39 (t, J=9.0 Hz, H-4″), 3.41 (m, H-5″) and 0.93 (d, J=6.0 Hz, H-6″) indicate existence of a rhamnopyranosyl unit in the trisaccharide. The signals of two other sugar units appeared at δ_H 4.51 (d, J=7.8 Hz, H-1‴) and 4.42 (d, J=7.2 Hz, H-1⁗), and 3.0–3.7 ($J_{2,3}=J_{3,4}=J_{4,5}$=ca 9.0 Hz). Thus, the remaining two sugar units must be β-D-glucopyranose.

The glycosidic linkages of **1** were determined by NOE difference spectra. By irradiation of H-1″, strong negative NOE was observed on H-1‴ and weaker on H-6′, but not on H-3″ and H-5″, indicating that rhamnose is directly linked to OH-3 of the flavonol and in α-configuration. By irradiation of H-1‴, strong negative NOEs appeared to H-2″ and H-1″ indicating that a glucosyl unit is linked to OH-2″. The β 1→3 linkage was confirmed by irradiation of H-1⁗ which showed a strong NOE effect on H-3″ and H-2″ (Fig. 1). Furthermore, C-3″ was lowfield-shifted by ca 10 ppm more than other glucosylrhamnosyl units (Table 3) (Markham et al., 1992). Finally the linkages of the sugar units were directly confirmed by HMBC since correlations were observed between C-3(δ_c 134.0) and H-1″(δ_H 5.65), C-2″(δ_c 78.6) and H-1‴(δ_H 4.51) and between C-3″(δ_c 80.5) and H-1⁗(δ_H 4.42) (Fig. 1 and Table 3). Thus, **1** were identified as kaempferol 3-*O*-α-(2,3-*di*-*O*-β-D-glucopyranosyl)rhamno-pyranoside, a new natural product.

FAB–MS of **2** and **3** showed $[M+H]^+$ at m/z 595 supporting the molecular formula $C_{27}H_{31}O_{15}$ but with different fragments corresponding to kaempferol 3-rhamnoside (m/z 433) and kaempferol 3-glucoside (m/z 449), respectively. In addition **2** and **3** showed different HPLC retention times (Table 1). ^{1}H NMR signals of **2** and **3** were assigned by using 1D-HOHAHA and ^{1}H–^{1}H-COSY. The anomeric proton signals of 3-*O*-glucosylrhamnosyl moiety of **2** appeared at δ_H 5.63 (br s, H-1″ of rhamnosyl) and δ_H 4.31 (d, J=7.8 Hz, H-1‴ of glucosyl) while the corresponding anomeric proton of glucosyl unit in the 3-*O*-rhamnosylglucoside moiety of **3** appeared at δ_H 5.65 (d, J=7.8 Hz, H-1″ of glucosyl) and H-1‴ of rhamnosyl appeared at δ_H 5.07. This indicates the difference in sugar sequence (Markham et al., 1992) and was confirmed by HSQC and HMBC (Fig. 1). The structures were established as kaempferol 3-*O*-α-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside (**2**) and kaempferol 3-*O*-β-D-(2-*O*-α-rhamnopyranosyl)glucopyranoside (**3**), respectively (Fig. 1). All data were consistent with those previously reported (Markham et al., 1992; Carotenuto et al., 1997; Esperanza et al., 1998) (Tables 2 and 3).

Compound **4** gave the characteristic UV spectrum of isorhamnetin (Dandapani & Nagarajan, 1989). The $[M+H]^+$ was 30 mass units higher than that of **3**. Fragments were observed at m/z 479 [(M+H)-rhamnosyl]$^+$ and 317 [isorhamnetin]$^+$. The ^{1}H-NMR spectrum showed the expected signals of isorhamnetin in the aromatic region; the methoxy protons appeared at δ_H 3.85 (br s) (Section 3).

The ^{1}H and ^{13}C NMR spectra of the sugar units were almost identical to that of **3** and the structure was confirmed using our NMR methods (Tables 2 and 3). Thus, **4** is isorhamnetin 3-*O*-β-D-(2-*O*-α-rhamnopyranosyl)glucopyranoside (Fig. 1).

UV spectra and FAB–MS (Harborne, 1963; Budzianowski, 1990) of **5** and **6** suggested that these flavonoids may be diglucosides of kaempferol and quercetin, respectively (Table 1). ^{1}H NMR showed that all glucose units were β-linked to the other glucose unit or to the aglycone since $J_{1,2}$ was ca 7 Hz. The anomeric protons of **5** and **6** at δ 5.58 and 5.6

Table 3
^{13}C-NMR spectral data of **1**–**7** and **9** in DMSO-d_6–10% TFA-*d*. Assignments have been confirmed by 2D techniques (^{1}H–^{1}H COSY, HSQC or HMBC), but carbons having almost the same chemical shifts may be reversed. ^{13}C-NMR of **8** has not been measured because of limited amounts

C	1	2	3	4	5	6	7	9
2	157.0	157.5	156.5	156.4	156.3	156.7	156.0	156.4
3	134.0	135.0	133.2	132.9	132.8	133.5	134.2	133.3
4	177.6	178.1	177.7	177.6	177.5	177.9	177.9	177.4
5	161.0	161.5	161.4	161.3	160.9	161.6	161.4	161.0
6	98.6	99.0	98.9	98.9	98.4	98.5	100.3	98.4
7	164.0	164.5	164.3	164.3	163.8	164.4	164.5	163.9
8	93.6	94.1	94.0	93.9	93.3	93.8	94.0	93.4
9	156.5	157.0	156.7	156.7	156.1	156.0	156.9	156.4
10	104.5	104.6	104.5	104.4	103.9	104.5	104.5	104.0
1′	120.4	120.9	121.3	121.5	120.9	122.2	124.1	120.9
2′	130.5	131.0	131.1	113.8	130.6	116.5	130.9	130.7
3′	115.3	116.2	115.3	147.1	115.0	145.1	118.1	117.5
4′	160.0	160.4	160.2	149.5	159.6	148.8	159.6	159.8
5′	115.3	116.2	115.3	115.4	115.0	115.8	118.1	117.5
6′	130.5	131.0	131.1	122.1	130.6	121.6	130.9	130.7
OMe				55.9				
Sugar at C-3								
1″	100.4	101.3	98.7	98.7	98.4	99.0	101.2	101.2
2″	78.6	81.7	78.0	78.0	81.8	83.1	73.5	74.0
3″	80.5	70.5	77.7	77.6	76.0	76.9	76.6	76.1
4″	69.4	72.0	70.9	70.8	69.4	69.9	69.8	69.6
5″	69.3	70.8	77.5	77.3	76.5	77.8	77.4	77.0
6″	17.2	17.6	61.1	60.7	60.5	61.0	60.9	60.6
1‴	104.5	106.5	101.0	101.0	103.5	104.4		
2‴	69.7	69.6	70.5	70.3	73.9	74.6		
3‴	75.7	76.5	70.8	70.8	76.0	76.8		
4‴	73.7	74.1	72.2	72.0	69.1	69.8		
5‴	76.4	76.9	68.7	68.6	76.4	77.1		
6‴	60.5	60.8	17.5	17.2	60.2	60.9		
1⁗	104.1							
2⁗	73.7							
3⁗	76.4							
4⁗	69.9							
5⁗	76.8							
6⁗	60.8							
Sugar at C-4′								
1‴							99.0	
2‴							74.4	
3‴							76.7	
4‴							70.1	
5‴							77.8	
6‴							61.1	

were shift-correlated with signals of C-3 at δ 132.8 and 133.5, respectively. The anomeric protons signals at δ 4.62 and 4.60 correlated unambiguously with C-2″ at δ 81.8 and 83.1, respectively, indicating β 1 → 2 linkage. These results were consistent with NOE difference spectra, since NOEs between H-1″ and H-6′ and between H-1″ and H-1‴, were observed (Fig. 1). Thus, **5** and **6** are 3-*O*-β-D-(2-*O*-β-D-glucopyranosyl)glucopyranosides of kaempferol and quercetin, respectively (Fig. 1).

FAB–MS suggested that **7** and **8** were diglucosides of kaempferol and quercetin, respectively (Table 1). ^{1}H NMR showed that the hexoses were β-linked glucopyranose because all vicinal coupling constants were 7.2–9.6 Hz. Connection was directly determined by NOE difference and by HSQC and HMBC (compound **7** only). UV data were similar to those reported (Stein & Zinsmeister, 1990; Krauze-Baranowska & Cisowski, 1996). Thus, **7** and **8** were determined to be 3,4′-di-*O*-β-D-glucopyranosides of kaempferol and quercetin, respectively (Fig. 1).

Compound **9** was kaempferol 3-*O*-β-D-glucopyranoside according to UV (Vidal-Ollivier et al., 1989; Harborne, 1963), FAB–MS, ^{1}H NMR and ^{13}C NMR data (Tables 1–3). Correlations found by NOE difference, HSQC and HMBC spectra are shown in Fig. 1.

3. Experimental

3.1. Plant material

Field grown flowers of the *Crocus speciosus* and *C. antalyensis* were collected in Noordwijk, Holland, in March 1996 and identified by Professor N. Jacobsen, Royal Veterinary and Agricultural University, Copenhagen.

3.2. Isolation of flavonoids

Freeze-dried perianth segments of *speciosus* (50 g) and *C. antalyensis* (40 g) were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 h. The conc extracts were adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 4 to 20% aq. CH_3CN containing 0.5% TFA. The flavonoids were further purified by prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system; flow rate 7 ml min^{-1}; monitoring at 280 nm. Evaporation of solvents *in vacuo* gave TFA salts which were stored at −80°. From *C. speciosus* **1** (15 mg) and **2** (15 mg) were obtained; from *C. antalyensis* **3** (15 mg), **4** (10 mg), **5** (10 mg), **6** (60 mg), **7** (17 mg), **8** (7 mg) and **9** (10 mg) were isolated.

3.3. Analysis of flavonoids

About 1 g of the freeze-dried perianth segments of *Crocus speciosus* and *C. antalyensis* were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA. After filtration the extract was analyzed by ODS HPLC (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°, monitoring on a 3D diode array detector at 280–360 nm. Solvent A (H_2O–TFA 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1) were used in elution profile: 0 min 16% B, 3 min 38%

B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40–50 min 100% B; flow rate 1.5 ml min^{-1}.

3.4. Kaempferol 3-O-α-L-(2,3-di-O-β-D-glucopyranosyl)rhamnopyranoside (1)

UV λ_{max} (nm): 268, 315 sh, 347; +NaOH: 272, 326, 409; +$AlCl_3$: 269, 305 sh, 348, 397; +$AlCl_3$+HCl: 269, 302 sh, 349, 392; +NaOAc: 279, 398; +NaOAc+H_3BO_3: 268, 352. ^{1}H NMR (aglycone): δ 7.83 (2H, d, J=9.0 Hz, H-2′, H-6′), 6.97 (2H, d, J=9.0 Hz, H-3′, H-5′), 6.46 (1H, d, J=1.8 Hz, H-8), 6.26 (1H, d, J=2.4 Hz, H-6).

3.5. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside (2)

UV data similar to that of **1**. ^{1}H NMR (aglycone): δ 7.81 (2H, d, J=8.4 Hz, H-2′, H-6′), 6.97 (2H, d, J=8.4 Hz, H-3′, H-5′), 6.46 (1H, d, J=1.8 Hz, H-8), 6.26 (1H, d, J=2.4 Hz, H-6).

3.6. Kaempferol 3-O-β-D-(2-O-α-rhamnopyranosyl)glucopyranoside (3)

UV data similar to that of **1**. ^{1}H NMR (aglycone): δ 8.01 (2H, d, J=9.0 Hz, H-2′, H-6′), 6.86 (2H, d, J=9.0 Hz, H-3′, H-5′), 6.41 (1H, d, J=2.4 Hz, H-8), 6.19 (1H, d, J=2.4 Hz, H-6).

3.7. Isorhamnetin 3-O-β-D-(2-O-α-rhamnopyranosyl)glucopyranoside (4)

^{1}H NMR (aglycone): δ 7.95 (1H, d, J=2.4 Hz, H-2′), 7.47 (1H, dd, J=2.4; 9.0 Hz, H-6′), 6.89 (1H, d, J=9.0 Hz, H-5′), 6.43 (1H, d, J=2.4 Hz, H-8), 6.20 (1H, d, J=1.8 Hz, H-6), 3.85 (3H, br s, OMe).

3.8. Kaempferol 3-O-β-D-(2-O-β-D-glucopyranosyl)glucopyranoside (5)

^{1}H NMR (aglycone): δ 7.99 (2H, d, J=8.8 Hz, H-2′, H-6′), 6.88 (2H, d, J=8.8 Hz, H-3′, H-5′), 6.19 (1H, d, J=2.2 Hz, H-8), 6.16 (1H, d, J=2.2 Hz, H-6).

3.9. Quercetin 3-O-β-D-(2-O-β-D-glucopyranosyl)glucopyranoside (6)

^{1}H NMR (aglycone): δ 7.58 (1H, d, J=2.3 Hz, H-2′), 7.45 (1H, m, H-6′), 6.87 (1H, d, J=7.8 Hz, H-5′), 6.39 (1H, d, J=1.8 Hz, H-8), 6.17 (1H, d, J=2.4 Hz, H-6).

3.10. Kaempferol 3,4′-di-O-β-D-glucopyranoside (7)

^{1}H NMR (aglycone): δ 8.10 (2H, d, J=9.0 Hz, H-2′, H-6′), 7.14 (2H, d, J=9.0 Hz, H-3′, H-5′), 6.44 (1H, d, J=1.8 Hz, H-8), 6.21 (1H, d, J=1.8 Hz, H-6).

3.11. Quercetin 3,4′-di-O-β-D-glucopyranoside (8)

^{1}H NMR (aglycone): δ 7.64 (1H, d, J=1.8 Hz, H-2′), 7.60 (1H, dd, J=1.8; 10.8, H-6′), 7.19 (1H, d, J=9.0 Hz, H-5′), 6.43 (1H, d, J=1.8 Hz, H-8), 6.20 (1H, d, J=2.4 Hz, H-6).

3.12. Kaempferol 3-O-β-glucopyranoside (9)

^{1}H NMR (aglycone): δ 7.98 (2H, d, J=9.0 Hz, H-2′, H-6′), 6.85 (2H, d, J=9.0 Hz, H-3′, H-5′), 6.38 (1H, d, J=1.8 Hz, H-8), 6.18 (1H, d, J=2.4 Hz, H-6).

FAB–MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix.

NMR: 600 MHz (JNM alpha 600, JEOL) (^{1}H, ^{1}H–^{1}H-COSY, 1D-HOHAHA, homodecoupling, NOE difference, ^{13}C, HSQC and HMBC) in DMSO-d_6–10%TFA-d; CD_2HOD (3.326 ppm) int. standard. 1D HOHAHA, homodecoupling and 2D spectra were obtained using a pulse sequence supplied from JEOL.

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PERGAMON

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PHYTOCHEMISTRY

Flavonoids from flowers of two *Crocus chrysanthus-biflorus* cultivars: "Eye-catcher" and "Spring Pearl" (Iridaceae)

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Abstract

Eight flavonoids, of which five are new flavonols, were isolated from perianth segments of two *Crocus chrysanthus-biflorus* cultivars. The new flavonols were identified as 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranosides of kaempferol, quercetin and myricetin, and two were the corresponding modified kaempferol triglycosides acylated at OH-6 of the 7-glucoside with malonic acid or acetic acid. The flavonols coexist with the known 7-*O*-β-D-glucosides of dihydrokaempferol and apigenin and with isorhamnetin 3,4′-di-*O*-β-D-glucoside. Complete structural determination of all compounds was achieved using 1-D and 2-D NMR techniques and other spectral evidence. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Crocus*; Iridaceae; Flower pigments; Flavonoids; Malonic acid; Acetic acid; HPLC

1. Introduction

Several aglycones have been isolated from leaves or pollen of *Crocus* (Iridaceae) (Kuhn & Low, 1944; Bate-Smith, 1968; Harborne & Williams, 1983; Williams, Harborne, & Goldblatt, 1986). However, only kaempferol, quercetin and myricetin have been detected in perianth segments of the genus (Price, Robinson, & Robinson, 1939; Bate-Smith, 1968; Harborne & Williams, 1983). They were glycosylated but the carbohydrate moieties have only been identified by chromatographic studies (Harborne & Williams, 1983). Acylated flavonoids have not been detected earlier in *Crocus*, although they seem to occur regularly in Iridaceae (Pryakhina, Sheichenko, & Blinova, 1984; Kachroo et al., 1990; Abdul & Kumar, 1992).

More than 100 cultivars of *Crocus* are known today. They are derived from selection within and hybridization between relatively few species. Various characters including distribution pattern, habitat and morphological trait of the naturally occurring ca. 80 species have been described (Harborne, 1967; Mathew, 1982). However, about *Crocus chrysanthus-biflorus* cultivars, it is not possible to say to what extent they are hybrids between *C. chrysanthus* and *C. biflorus* (Jacobsen, Van Scheepen, & Ørgaard, 1997). We hope to elucidate the problems by using chemical markers in combination with cytological results (Ørgaard & Heslop-Harrison, 1994; Ørgaard, Jacobsen, & Heslop-Harrison, 1995aØrgaard, Jacobsen, & Heslop-Harrison, 1995b).

As a part of ongoing chemotaxonomic investigations of the genus, we report here on the identification of flavonoids from perianth segments of "Spring Pearl" and "Eye-catcher".

2. Results and discussion

Perianth segments of "Spring Pearl" and "Eye catcher" were extracted with aqueous acetonitril con

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Table 1
Analytical HPLC retention times (R_t) and FAB-MS of flavonoids found in two *Crocus chrysanthus-biflorus* cultivars. **1**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **2**: quercetin 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **3**: myricetin 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **4**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-(6-*O*-malonyl-β-D-glucopyranoside), **5**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-(6-*O*-acetyl-β-D-glucopyranoside), **6**: dihydrokaempferol 7-*O*-β-D-glucopyranoside, **7**: isorhamnetin 3,4′-di-*O*-β-D-glucopyranoside, **8**: apigenin 7-*O*-β-D-glucopyranoside

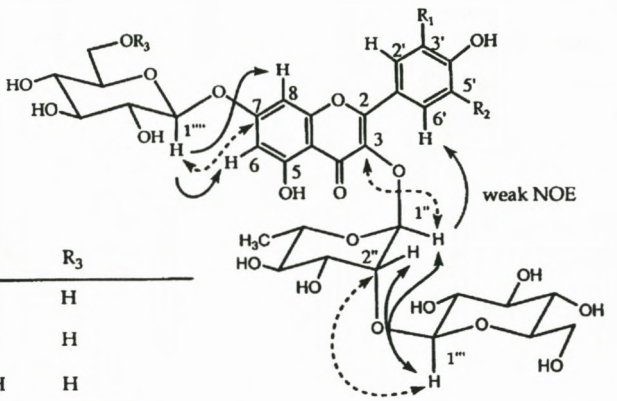
Flavonoid	Anal. HPLC R_t (min)	FAB-MS $[M]^+$, fragment ions
1	15.0	757 $[C_{33}H_{40}O_{20}]^+$, 595, 449, 287
2	12.2	773 $[C_{33}H_{40}O_{21}]^+$, 611, 465, 303
3	9.7	789 $[C_{33}H_{40}O_{22}]^+$, 627, 481, 319
4	27.7	843 $[C_{36}H_{42}O_{23}]^+$, 535, 287
5	30.2	799 $[C_{35}H_{42}O_{21}]^+$, 595, 491, 287
6	8.4	451 $[C_{21}H_{22}O_{11}]^+$, 289
7	21.6	641 $[C_{28}H_{32}O_{17}]^+$, 479, 317
8	30.7	433 $[C_{21}H_{20}O_{10}]^+$, 271

taining 0.5% trifluoroacetic acid (TFA). Purification of eight flavonoids was achieved by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. A new series of flavonol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranosides (**1–5**) was isolated, together with dihydrokaempferol 7-*O*-β-D-glucopyranoside (**6**) (Slimestad, Andersen, & Francis, 1994), isorhamnetin 3,4′-di-*O*-β-D-glucopyranoside (**7**) (Tsushida & Suzuki, 1995; Krauze-Baranowska & Cisowski, 1996) and apigenin 7-*O*-β-D-glucopyranoside (**8**) (Hmamouchi, Es-safi, Lahrichi, Fruchier, & Essassi, 1996; Skaltsa, Lazari, Loukis, & Harvala, 1996).

UV spectra of all compounds except **6** and **8** showed the characteristics of flavonol glycosides. Positive FAB-MS suggested that **1–3** had identical triglycosyl moieties and **4–5** were the malonate and the acetate of **1**, respectively (Table 1). The observed *m/z* 287 of **1**, **4** and **5** corresponded to kaempferol, while $[aglycone]^+$ of **2** and **3** were 16 and 32 mass units larger than **1**, indicating that **2** and **3** are quercetin and myricetin derivatives, respectively. This suggestion was supported by the reversed-phase HPLC retention times (**3** > **2** > **1** in order, Table 1).

^1H NMR and/or ^{13}C NMR spectra assigned by HSQC and HMBC confirmed the identity of the aglycones of **1**, **2** and **3** as kaempferol, quercetin and myricetin, respectively (Tables 2 and 3). The aromatic signals of **4** and **5** were identical with those of **1**, indicating that **4** and **5** are kaempferol glycosides.

^1H NMR signals of the sugar parts of **1–5** were identical (Table 2) except for a lowfield-shift of a signal corresponding to methylene group in **4** and **5**. By assignment using 1-D-HOHAHA, ^1H–^1H-COSY and homo-decoupling spectra, **1–5** were found to contain three hexose units. The signals at δ_H 0.84 (d, J = 6.0 Hz, H-6″), 5.65 (br s, H-1″), 3.52 (dd, J = 3.6 and 9.0



	R_1	R_2	R_3
1	H	H	H
2	OH	H	H
3	OH	OH	H
4	H	H	malonyl
5	H	H	acetyl

Fig. 1. ← NOE (negative), ↔ HMBC.

Table 2
^{1}H NMR spectral data of five new flavonoids from two *Crocus chrysanthus-biflorus* cultivars. The assignment of the sugar protons were carried out by 1-D-HOHAHA. By irradiation of H-1″ of rhamnoside, a negative NOE was observed at H-6′ of the nucleus. A negative NOE was also observed between the same anomeric proton and H-1‴. A strong negative NOE observed at H-6 and H-8 of the nucleus appeared by irradiation of H-1⁗ of the glucoside. Coupling constants J (in Hz) in parentheses. Columns with two sets of ppm data show values measured in C_5D_5N-d_5 in parentheses. The remaining data are in DMSO-d_6–10% TFA-d

Compound	1	2	3[a]	4	5
Aglycone					
6	(6.78) 6.51 d(1.2)	(6.78) 6.50 d(1.2)	6.75 d(1.2)	6.49 d(2.4)	6.43 d(2.4)
8	(6.98) 6.80 d(1.8)	(6.98) 6.78 d(1.8)	6.82 d(1.2)	6.78 d(2.4)	6.72 d(2.4)
2′	(7.97) 7.84 d(8.4)	(7.97) 7.58 br s	7.62 br s	7.82 d(8.4)	7.77 d(9.0)
3′	(7.22) 6.98 d(8.4)			6.97 d(8.4)	6.92 d(9.0)
5′	(7.22) 6.98 d(8.4)	(7.22) 6.95 d(8.4)		6.97 d(8.4)	6.92 d(9.0)
6′	(7.97) 7.84 d(8.4)	(7.97) 7.35 m	7.62 br s	7.82 d(8.4)	7.77 d(9.0)
Rhamnoside (H″)					
1	(6.28) 5.65 br s	(6.28) 5.56 br s	6.21 br s	5.65 br s	5.59 br s
2	(5.03) 4.16 m	(5.03) 4.18 dd(3.0;8.7)	4.99 m	4.15 m	4.09 m
3	(4.56) 3.52 dd (3.6; 9.0)	(4.58) 3.60 m	4.57 dd(2.4; 10)	3.58 dd(3.6; 10.2)	3.53 m
4	(4.40) 3.10 t(9.6)	(4.39) 3.14 t(9.0)	4.40 t(9.0)	3.17 t(9.6)	3.11 t(9.0)
5	(4.18) 3.30 m	(4.18) 3.53 m	4.15 m	3.34 m	3.31 m
6	(1.18) 0.84 d(6.0)	(1.18) 0.90 d(6.0)	1.18 d(6.0)	0.91 d(6.0)	0.86 d(6.0)
Glucoside (H‴)					
1	(5.28) 4.31 d(7.8)	(5.28) 4.28 d(7.8)	5.26 d(8.4)	4.30 d(7.2)	4.23 d(7.8)
2	(4.05) 3.22 t(9.0)	(4.05) 3.13 t(9.0)	4.03 t(9.0)	3.15 t(8.4)	3.16 t(9.0)
3	(4.22) 3.19 t(9.0)	(4.20) 3.12 t(9.0)	4.19 t(9.0)	3.12 t(9.0)	3.15 t(9.0)
4	(4.28) 3.05 t(9.6)	(4.28) 2.96 t(9.6)	4.30 t(9.0)	2.98 t(9.0)	3.01 t(9.0)
5	(3.93) 3.07 m	(3.94) 2.99 m	3.96 m	3.02 m	3.03 m
6	(4.44) 3.52 m	(4.39) 3.33 m	4.36 dd(5.4; 12.6)	3.46 m	3.48 m
	(4.55) 3.52 m	(4.43) 3.45 m	4.44 m	3.46 m	3.48 m
7-Glucoside (H⁗)					
1	(5.81) 5.10 d(7.2)	(5.81) 5.12 d(7.8)	5.75 d(7.2)	5.14 d(7.2)	5.07 d(7.2)
2	(4.33) 3.31 t(9.0)	(4.33) 3.30 t(8.4)	4.32 t(9.0)	3.30 t(9.0)	3.29 t(8.4)
3	(4.15) 3.27 t(9.0)	(4.18) 3.26 t(9.6)	4.15 t(9.0)	3.27 t(9.0)	3.27 t(8.4)
4	(4.38) 3.18 t(9.0)	(4.38) 3.18 t(9.6)	4.37 t(9.0)	3.18 t(9.0)	3.15 t(9.6)
5	(3.92) 3.43 m	(3.94) 3.43 m	3.95 m	3.72 m	3.69 m
6	(4.46) 3.47 dd(4.8; 11.7)	(4.42) 3.47 m	4.46 m	4.12 dd(6.6;12.0)	4.03 dd(6.6;11.4
	(4.55) 3.70 m	(4.55) 3.70 m	4.51 dd(2.4; 12.6)	4.37 m	4.33 m
Acid				3.41–3.42	2.02 s

[a] Only measured in C_5D_5N-d_5.

Hz, H-3″) and 3.10 (t, J=9.6 Hz, H-4″) indicate the presence of a rhamnopyranosyl unit. The signals of two other sugars appeared at 4.31 (d, J=7.8 Hz, H-1‴) and 5.10 (d, J=7.2 Hz, H-1⁗) and 3.0–3.7 ($J_{2,3}=J_{3,4}=J_{4,5}$=ca. 9.0 Hz). Thus, the remaining two sugars must be β-D-glucopyranose.

The positions of the glycosidic linkages of **1–5** were determined by NOE difference spectra. By irradiation of H-1″, strong negative NOE was observed on H-1‴ and weaker on H-6′, but not on H-3″ and H-5″, indicating that rhamnose is directly linked to OH-3 of the flavonol and in α-configuration. By irradiation of H-1‴ glucosyl unit, strong negative NOEs appeared to H-2″ and H-1″ indicating that a glucosyl unit is linked to OH-2″ by the (β1–2) linkage. The (1–2) linkage was also confirmed by the lowfield-shift of H-1″ by ca. 0.4 ppm more than for other glucorhamnosyl units (Tabl 2) (Markham, Geiger, & Jaggy, 1992). Strong negativ NOEs to H-6 and H-8 on A-ring of the chromophor were also observed by irradiation of H-1⁗, indicatin that the other glucosyl unit is attached on OH-7 Finally, the glycosidic linkages were confirmed b HMBC (Table 3). Thus, **1–3** were identified as 3-*O*-α L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D glucopyranosides of kaempferol, quercetin and myrice tin, respectively. The glucosyl(β1-2)rhamnosyl moiet has been found in a few other flavonoids (Oshima Okamoto, & Hikino, 1987; Fukai & Nomura, 198 Mizuno et al., 1990; Markham et al., 1992).

Comparison of H-6⁗ (-CH_2-) signals of **4** and with that of **1**, showed a lowfield-shift of ca. 0.65 an 0.56 ppm, respectively (Liu, Shi-Lin, Roberts, *

Table 3
^{13}C NMR spectral data of flavonoids. Assignments of carbons having almost the same chemical shifts may be reversed

Flavonol	C	1[a,b]	2[a]	3[a]	5[b]	6[b]	7[b]
	2	156.9	156.9	156.9	158.2	83.4	156.1
	3	136.0	135.9	138.9	135.3	71.9	133.9
	4	178.7	178.4	178.9	178.3	198.9	177.8
	5	161.7	162.2	162.3	161.1	162.8	161.5
	6	100.3	100.3	100.2	99.7	97.1	99.0
	7	163.9	163.9	163.8	163.1	165.7	164.5
	8	95.0	95.1	95.1	95.0	95.7	94.1
	9	156.9	156.9	156.9	156.5	158.6	156.9
	10	107.6	107.7	107.9	106.2	102.4	104.6
	1′	121.2	122.1	120.9	120.6	127.7	124.1
	2′	131.2	117.0	109.4	130.9	129.8	113.9
	3′	116.2	147.1	162.1	115.6	115.2	148.5
	4′	158.3	158.8	158.8	160.5	158.6	148.9
	5′	116.2	116.5	162.1	115.6	115.2	115.0
	6′	131.2	121.7	109.4	130.9	129.8	121.8
	OMe						56.0
Sugar at C-3	1″	102.4	102.6	102.8	101.2		101.2
	2″	82.5	82.7	82.9	81.6		74.6
	3″	72.1	72.2	72.2	70.0		76.6
	4″	71.6	71.7	71.7	71.9		70.1
	5″	73.4	73.7	73.7	70.7		77.8
	6″	18.1	18.1	18.2	17.6		60.9
	1‴	106.8	106.9	106.8	106.5		
	2‴	75.6	75.7	75.7	70.4		
	3‴	79.0	78.4	78.3	76.4		
	4‴	70.9	71.2	70.9	74.3		
	5‴	78.4	79.0	79.1	77.0		
	6‴	62.2	62.3	62.3	60.7		
Sugar at C-7	1⁗	101.5	101.6	101.6	100.0	100.0	
	2⁗	74.6	74.7	74.6	73.2	73.2	
	3⁗	78.4	78.3	78.2	76.5	76.5	
	4⁗	70.9	70.9	70.7	69.5	69.8	
	5⁗	78.2	78.5	78.4	74.1	77.4	
	6⁗	62.1	62.2	62.2	63.7	60.8	
Sugar at C-4′	1⁗						100.0
	2⁗						73.4
	3⁗						77.0
	4⁗						69.4
	5⁗						77.4
	6⁗						60.4
OCOMe					170.5		
OCO*Me*					20.7		

[a] Compounds measured in pyridine-d_5. Remaining compounds measured in DMSO-d_6–10% TFA-d.
[b] Assignments have been confirmed by 2-D techniques (^1H–^1H COSY, HSQC or HMBC). In the HMBC spectra of **1** and **5**, appreciable shi correlations were observed; correlations of **1** in pyridine-d_5 were C-7(δ_C 163.9)-H-1⁗(δ_H 5.81), C-3(δ_C 136.0)-H-1″(δ_H 6.28), and C-2″(δ_C 82.5)-H 1‴(δ_H 5.28); these of **5** in DMSO-d_6-10% TFA-d., C-7(δ_C 163.1)-H-1⁗(δ_H 5.07), C-3(δ_C 135.3)-H-1″(δ_H 5.59) and C-2″(δ_C 81.6)-H-1‴(δ_H 4.23).

Phillipson, 1989; Nørbæk & Kondo, 1998) (Table 2). From MS data, **4** must be malonated, and the methylene of malonate appeared at δ_H 3.41–3.42. Thus, **4** is kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-(6-*O*-malonyl)glucopyranoside. In **5** additional CH_3CO-signals (δ_H 2.02 and δ_C 170.5, 20.7 ppm) was observed Liu et al., 1989; Allais et al 1991; Allais, Chulia, Kaouadji, Simon, & Delage 1995). The lowfield-shift of C-6⁗ by ca. 3 ppm and th smaller highfield-shift of the neighboring carbon (C 5⁗) indicates that the acetyl group was attached to th 6-OH (Liu et al., 1989) (Table 3). This result was cor

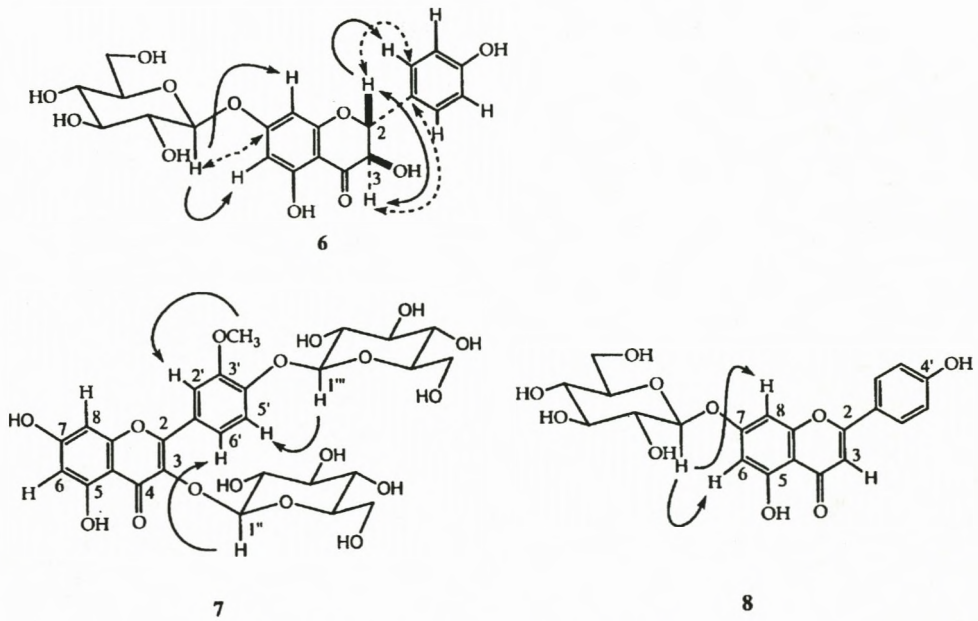


Fig. 2. ← NOE, ↔ HMBC. Only important correlation was described.

sistent with the HMBC spectrum, showing shift-correlation between ^{13}C of carbonyl and H-6''''. Thus, **5** is OH-6''''-*O*-acetate of **1** (Fig. 1).

From "Spring Pearl" **6–8** also were isolated. Compound **6** was eluted earlier and its UV spectrum showed similarity with dihydrokaempferol (λ 284 and 335 sh) (Markham, 1982) and the molecular ion *m/z* 451 (Table 1) suggested a dihydroflavonoid monoglucoside (Slimestad et al., 1994). ^1H and ^{13}C NMR signals were assigned by using 1-D-HOHAHA and ^1H–^1H-COSY. Compound **6** had two aromatic systems linked through -CH–CH–CO-, and one β-D-glucopyranosyl unit. Connection of the components was directly determined by NOE difference, HSQC and HMBC (as shown in Fig. 2). All data were consistent with those previously reported (Slimestad et al., 1994) (Tables 3 and 4). Thus, **6** was determined to be dihydrokaempferol 7-*O*-β-D-glucopyranoside (Fig. 2).

Compound **7** gave the characteristic UV spectrum of isorhamnetin and a free 7-OH group (Markham, 1982) and the FAB-MS fragmentation pattern *m/z* 479 (M-162), 317 (M-324 M- 2 × hexose, isorhamnetin + 1) (Table 1). The ^1H NMR confirmed the presence of one isorhamnetin nucleus since methoxy (δ_H 3.89) and two aromatic spin systems, δ_H 7.58 (J = 1.8, 9.0), 7.26 (J = 9.0) and 8.01 (J = 2.4 Hz) corresponded to H-6', H-5' and H-2', respectively, and δ_H 6.51 (J = 2.4) and δ 6.27 (J = 1.8) to H-8 and H-6, respectively (Table 4). *J*-values of all vicinal couplings in the sugar regions were 7.8–9.6 Hz, indicating that the two sugars must be β-D-glucopyranose. Thus, **7** is isorhamnetin 3,4'-di-*O*-β-D-glucopyranoside (Tsushida & Suzuki, 1995; Krauze-Baranowska & Cisowski, 1996) (Fig. 2).

UV of **8** was identical with a flavone monoglucoside (Markham, 1982) which was supported by the FAB-MS, showing peaks of *m/z* 433 $[M+H]^+$ and 271 [M-hexose, apigenin] (Table 1). The structure was identified by ^1H NMR. A characteristic signal appeared at δ_H 7.28 (s), corresponding to H-3, indicating the chromophore is a flavone, apigenin (Table 4). The hexose was a β-linked glucopyranoside because all vicinal coupling constants were 7.2–9.6 Hz. From the glycosidic linkage determination by NOE difference, **8** is apigenin 7-*O*-β-D-glucopyranoside, previously isolated from other plants (Hmamouchi et al., 1996; Skaltsa et al., 1996) (Fig. 2).

3. Experimental

3.1. Plant material

Field grown flowers of the *Crocus chrysanthus biflorus* cultivars "Eye-catcher" and "Spring Pearl" were collected in Noordwijk, Holland, in March 1996. The identity was verified by Professor N. Jacobsen

Table 4
^{1}H NMR spectral data of three known flavonoids from perianth segments of *Crocus chrysanthus-biflorus* "Spring Pearl" in DMSO-d_6–10% TFA-d. Coupling constants J (in Hz) in parentheses

	H No.	Aglycone (ppm)	H No.	3-Glucoside (H″) (ppm)	4′-Glucoside (H‴) (ppm)	7-Glucoside (H⁗) (ppm)
6	2	5.15 d(12.0)	1			5.00 d(7.8)
	3	4.67 d(10.8)	2			3.30 t(9.0)
	6	6.18 br s	3			3.25 t(9.0)
	8	6.21 br s	4			3.19 t(9.0)
	2′	7.36 d(9.0)	5			3.42 m
	3′	6.84 d(7.8)	6			3.47 dd(5.4,11.7)
	5′	6.84 d(7.8)				3.69 m
	6′	7.36 dd(9.0)				
7[a]	6	6.27 d(1.8)	1	5.62 d(8.4)	5.10 d(7.8)	
	8	6.51 d(2.4)	2	3.31 t(9.0)	3.36 t(9.6)	
	2′	8.01 d(2.4)	3	3.27 t(9.6)	3.34 t(8.4)	
	5′	7.26 d(9.0)	4	3.16 t(9.6)	3.25 t(9.6)	
	6′	7.58 dd(1.8, 9.0)	5	3.17 m	3.41 m	
	OMe	3.89 s	6	3.44 m	3.51 dd(4.8;11.7)	
				3.63 m	3.72 m	
8	3	7.28 br s	1			5.10 d(7.2)
	6	6.82 d(1.2)	2			3.30 d(8.4)
	8	6.46 d(1.8)	3			3.25 t(8.4)
	2′	8.10 d(8.4)	4			3.18 t(9.6)
	3′	6.97 d(8.4)	5			3.43 m
	5′	6.97 d(8.4)	6			3.46 m
	6′	8.10 d(8.4)				3.73 m

[a] Negative difference NOE measured at 10°.

Royal Veterinary and Agricultural University, Copenhagen.

3.2. Isolation of flavonoids

Freeze-dried perianth segments of "Eye-catcher" (40 g) and "Spring Pearl" (50 g) were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 h. The conc extracts were adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 4 to 20% aq. CH_3CN containing 0.5% TFA. The flavonoids were further purified by prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system; flow rate of 7 ml min^{-1}; monitoring at 280 nm. The pure fractions were conc to dryness in vacuo and stored at −80°. From "Eye-catcher" **1** (50 mg) and **2** (15 mg), from "Spring Pearl" **1** (30 mg), **2** (10 mg), **3** (10 mg), **4** (6 mg), **5** (17 mg), **6** (15 mg), **7** (8 mg) and **8** (10 mg) were isolated.

3.3. Analysis of flavonoids

Analytical HPLC was carried out on a ODS-HPLC column (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) using an elution profile as follows 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 mir 50% B, 25 min 67% B, 40–50 min 100% B; solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA 60:140:1); flow rate 1.5 ml min^{-1}.

3.4. Spectral measurements

3.4.1. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (1)

UV λ_{max}(nm): 265, 310sh, 345; +NaOH: 269, 305sh 335sh, 379; +$AlCl_3$: 269, 302sh, 348, 395 +$AlCl_3$+HCl: 269, 302sh, 348, 395; +NaOAc: 267 385; +NaOAc+H_3BO_3: 267, 347.

3.4.2. Quercetin 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (2)

UV λ_{max} (nm): 255, 268sh, 301sh, 352; +NaOH 272, 326, 399 (dec.); +$AlCl_3$: 272, 299sh, 325sh, 43⁴ +$AlCl_3$+HCl: 270, 299sh, 362, 398; +NaOAc: 26² 296sh, 374, 410sh; +NaOAc+H_3BO_3: 261, 295sh 370.

3.4.3. Myricetin 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (3)

UV λ_{max} (nm): 295, 358; +NaOH: 295, 398 (dec.); +$AlCl_3$: 280, 310, 425; +$AlCl_3$+HCl: 308, 365, 402; +NaOAc: 300, 408; +NaOAc+H_3BO_3: 378.

3.4.4. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-(6-O-malonyl)glucopyranoside (4)

UV λ_{max} (nm): 266, 301sh, 315sh, 345; + NaOH: 265, 295, 340sh, 384; +$AlCl_3$: 267, 299, 346, 398; +$AlCl_3$+HCl: 269, 297, 342, 397; +NaOAc: 267, 290, 362, 398sh; +NaOAc+H_3BO_3: 267, 292, 315sh, 346.

3.4.5. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-(6-O-acetyl)glucopyranoside (5)

UV λ_{max} (nm): 265, 290sh, 315sh, 345; +NaOH: 265, 295, 340sh, 381; +$AlCl_3$: 267, 299, 346, 398; +$AlCl_3$+HCl: 267, 297, 342, 397; +NaOAc: 267, 290, 362, 400sh; +NaOAc+H_3BO_3: 267, 292, 315sh, 346.

3.4.6. Dihydrokaempferol 7-O-β-D-glucopyranoside (6)

UV λ_{max} (nm): 284, 335sh; +NaOH: 244, 289, 360; +$AlCl_3$: 315, 367; +$AlCl_3$+HCl: 284, 315, 362; +NaOAc: 283, 330sh; +NaOAc+H_3BO_3: 283, 330sh.

3.4.7. Isorhamnetin 3,4'-di-O-β-D-glucopyranoside (7)

UV λ_{max} (nm): 268, 300sh, 351; +NaOH: 260, 301, 403; +$AlCl_3$: 266, 304sh, 352sh, 402; +$AlCl_3$+HCl: 267, 309sh, 351sh, 402; +NaOAc: 280, 319, 375; +NaOAc+H_3BO_3: 280, 350.

3.4.8. Apigenin 7-O-β-D-glucopyranoside (8)

UV λ_{max} (nm): 270, 323sh, 425; +NaOH: 269, 335, 421; +$AlCl_3$: 276, 304, 354, 423; +$AlCl_3$+HCl: 276, 304, 354, 423; +NaOAc: 268, 320sh, 398; +NaOAc+H_3BO_3: 268, 365.

FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix.

Using a 600-MHz instrument (JNM alpha 600, JEOL) (^{1}H, ^{1}H–^{1}H-COSY, 1-D-HOHAHA, homodecoupling, NOE difference, ^{13}C, HSQC and HMBC), spectra were measured in DMSO-d_6 containing 10% TFA-d with internal standard CD_2HOD (3.326 ppm). Exceptionally pyridine-d_5 was used as solvent. 1-D HOHAHA, homodecoupling and 2-D spectra were obtained using a pulse sequence supplied from JEOL.

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ANTHOCYANINS FROM FLOWERS OF *CROCUS* (IRIDACEAE)

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Key Word Index—*Crocus*; Iridaceae; flower pigments; malonated anthocyanins; HPLC.

Abstract—The perianth segments of three cultivars of *Crocus* were investigated by HPLC for their content of anthocyanins. The investigation revealed the presence of four known and two new anthocyanins. The novel anthocyanins were isolated from the blue flowers of *C. chrysanthus* 'Skyline', and identified as petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) and malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside). The anthocyanins, isolated from the blue flowers of *C. sieberi* ssp. *sublimis* 'Tricolor', were identified as 3,5-β-D-diglucosides of delphinidin and petunidin, and from *C. chrysanthus* 'Eyecatcher' were as their 3-β-rutinosides. The complete structural determination of each compound was achieved by use of 1D and 2D NMR techniques and other spectral evidence.

INTRODUCTION

The flowers of *Crocus* (Iridaceae) vary in colour from white, yellow, pale-brown, purple to lilac, mauve and blue. The genus differs from many other monocotyledonous genera in that no red flowers occur. Previously the cyanic colours (lilac, mauve and blue) were reported to be delphinidin-based; only delphinidin 3,5-diglucoside has been identified, and traces of petunidin have been found on the basis of simple colour reactions and chromatographic studies [1–3]. Traces of malvidin have also been detected [4] but no acylated anthocyanins have been reported earlier in *Crocus*, even though acylated anthocyanins seem to occur regularly in Iridaceae [5–9].

As a part of an ongoing chemotaxonomic work, structural determination of six anthocyanins is reported, including two novel compounds, that are present in the blue flowers of *Crocus* cultivars.

RESULTS AND DISCUSSION

Six anthocyanins (**1**–**6**) were detected and isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. The UV-VIS and FAB mass spectra of all six compounds are shown in Table 1.

Compounds **1** and **2** were isolated from perianth segments of *C. chrysanthus* 'Skyline'. The FAB mass spectrum of **1** showed a $[M]^+$ at *m/z* 813, in good agreement with the mass calculated for $C_{34}O_{23}H_{37}^+$. Fragment peaks were observed at *m/z* 565 [M-248 (malonylhexose)]$^+$ and 317 [aglycone]$^+$, indicating **1** to be comprised of petunidin, and two malonylhexoses.

Analysis of the ^{1}H NMR spectrum of **1** revealed the presence of petunidin and two glucose residues, both acylated with malonic acid (Table 2). Although H-4 of the glucosides and the malonyl protons, observed at δ 3.44–3.37, were somewhat superimposed, the assignments of the two hexoses were carried out by 1D-HOHAHA spectra and ^{1}H-^{1}H-COSY. All vicinal coupling constants of both sugars were at 7.8–9.6 Hz including two anomeric protons at δ 5.37 (*d*, *J* = 7.8 Hz, glucoside A) and δ 5.23 (*d*, *J* = 7.8 Hz, glucoside B). Therefore, both sugar units must be β-D-glucopyranoside. The 6-methylene protons of glucose A and B are lowfield shifted by *ca* 0.6 ppm more than **3** and **4**. Thus, the malonyl groups were attached to the 6-OH of each glucoside [10]. The positions of the glucosidic linkages were determined by NOE difference spectra. A strong negative NOE was observed at H-4 (δ 8.92) of the petunidin nucleus by irradiation of the anomeric proton (δ 5.37) of glucoside A and strong

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Table 1. Analytical HPLC R_ts and spectral properties of anthocyanins found in *Crocus*

Anthocyanin	R_t* (min)	R_t† (min)	UV-VIS (0.1% HCl–MeOH) λ_{max} (nm)		FAB mass spectra $[M]^+$, fragment ions
1	16.1	11.9	269	536	813 [M], 565, 317
2	17.5	16.7	278	536	827 [M], 579, 331
3	9.2	4.5	269	538	627 [M], 465, 303
4	12.4	6.4	269	537	641 [M], 479, 317
5	13.5	7.5	276	543	611 [M], 303
6	16.6	13.2	275	542	625 [M], 317

* R_t on HPLC, linear gradient elution for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA.
† R_t on HPLC, isocratic solvent system with 14% aq. CH_3CN containing 0.5% TFA.

Table 2. ^1H NMR spectral data of anthocyanins from 3 *Crocus* cultivars (in CD_3OD, containing 10% TFA-*d*)

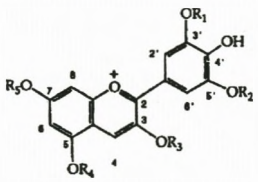
	1*	2*	3†	4†	5‡	6‡
Aglycone						
4	8.92 *br s*	8.98 *br s*	9.04 *br s*	9.09 *br s*	8.86 *br s*	8.88 *br s*
6	6.85 *d* (1.8)	6.86 *br s*	7.04 *d* (2.4)	7.05 *d* (2.4)	6.66 *d* (2.0)	6.66 *d* (2.2)
8	7.26 *d* (1.8)	7.35 *br s*	7.02 *d* (1.8)	7.06 *d* (2.4)	6.85 *d* (2.0)	6.87 *d* (2.2)
2′	8.04 *d* (2.4)	8.09 *br s*	7.76 *br s*	7.98 *d* (1.8)	7.75 *br s*	7.91 *d* (2.2)
6′	7.87 *d* (1.8)	8.09 *br s*	7.76 *br s*	7.80 *d* (1.8)	7.75 *br s*	7.75 *d* (2.2)
OMe	4.00 *br s*	4.01 *br s*		3.98 *br s*		3.97 *br s*
Glucoside A						
1	5.37 *d* (7.8)	5.38 *d* (7.2)	5.31 *d* (7.2)	5.32 *d* (7.2)	5.29 *d* (7.8)	5.31 *d* (7.8)
2	3.71 *t* (9.6)	3.68 *t* (9.0)	3.74 *t* (8.4)	3.69 *t* (9.6)	3.68 *t* (9.5)	3.66 *t* (9.5)
3	3.58 *t* (9.6)	3.57 *t* (9.0)	3.58 *t* (9.6)	3.57 *t* (9.6)	3.56 *t* (9.5)	3.55 *t* (9.5)
4	3.43 *t* (9.6)	3.42 *t* (9.0)	3.44 *t* (9.6)	3.42 *t* (9.6)	3.43 *t* (9.5)	3.41 *t* (9.5)
5	3.87 *m*	3.87 *m*	3.64 *m*	3.63 *m*	3.72 *m*	3.73 *m*
6	4.54 *dd* (6.6; 12.3)	4.52 *dd* (6.6; 12.3)	3.97 *m*	3.97 *m*	4.06 *dd* (1.5; 11.3)	4.07 *m*
	4.31 *dd* (6.6; 12.3)	4.31 *dd* (6.6; 12.3)	3.73 *dd* (3.0; 12.3)	3.72 *dd* (4.8; 12.0)	3.59 *dd* (1.5; 11.5)	3.57 *m*
Glucoside B						
1	5.23 *d* (7.8)	5.26 *d* (7.2)	5.15 *d* (7.2)	5.14 *d* (7.2)		
2	3.56 *t* (9.0)	3.54 *t* (9.0)	3.68 *t* (8.4)	3.66 *t* (9.6)		
3	3.54 *t* (9.0)	3.53 *t* (9.0)	3.56 *t* (9.6)	3.56 *t* (9.6)		
4	3.44 *t* (9.0)	3.44 *t* (9.0)	3.47 *t* (9.0)	3.47 *t* (9.6)		
5	3.85 *m*	3.85 *m*	3.57 *m*	3.54 *m*		
6	4.51 *dd* (6.6; 12.3)	4.49 *dd* (6.0; 12.3)	3.93 *m*	3.93 *m*		
	4.36 *dd* (6.6; 12.3)	4.35 *dd* (6.0; 12.3)	3.75 *dd* (4.8; 11.7)	3.76 *dd* (4.8; 12.0)		
Rhamnoside						
1					4.65 *br s*	4.65 *br s*
2					3.80 *m*	3.79 *m*
3					3.64 *dd* (3.3; 9.5)	3.61 *dd* (3.6; 9.5)
4					3.32 *t* (9.5)	3.17 *t* (9.5)
5					3.54 *m*	3.54 *m*
6					1.15 *d* (6.0)	1.15 *d* (6.6)
malonic moiety						
	3.42–3.37	3.42–3.37				

Coupling constants *J* (in Hz) in parentheses.

* By irradiation of H-1 of glucoside A in DMSO-d_6 containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of the nucleus, and strong negative NOEs of H-6 and H-8 of the nucleus appeared by irradiation of H-1 of glucoside B.

† On ^1H-^1H NOESY a NOE in DMSO-d_6 containing 10% TFA-*d* was observed between H-4 of the nucleus and H-1 of glucoside A and a NOE between H-6 of the nucleus and H-1 of glucoside B. A weak negative NOE was also observed between H-4 of the nucleus and H-1 of glucoside B.

‡ On ^1H-^1H-NOESY a NOE in CD_3OD, containing 10% TFA-*d* was observed between H-4 of the nucleus and H-1 of glucoside. A weak negative NOE was also observed between H-6 of glucoside and H-1 of rhamnoside.



	R_1	R_2	R_3	R_4	R_5
1	CH_3	H	β-(6-*O*-malonyl-glucoside) (A)	H	β-(6-*O*-malonyl-glucoside) (B)
2	CH_3	CH_3	β-(6-*O*-malonyl-glucoside) (A)	H	β-(6-*O*-malonyl-glucoside) (B)
3	H	H	β-glucoside (A)	β-glucoside (B)	H
4	CH_3	H	β-glucoside (A)	β-glucoside (B)	H
5	H	H	β-rutinoside	H	H
6	CH_3	H	β-rutinoside	H	H

Fig. 1.

negative NOEs at H-6 (δ 6.85) and H-8 (δ 7.26) by irradiation of that of glucoside B (δ 5.23). Thus, **1** is petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) (Fig. 1).

The $[M]^+$ of **2** was 14 mass units larger than that of **1** (Table 1). The ^1H NMR spectrum of **2** showed two equivalent aromatic protons on the B-ring and two CH_3- (δ 4.01), thus the aglycone of **2** is malvidin. ^1H spectrum of the sugar moieties were almost identical to that of **1** and also the assignment was confirmed by 1D HOHAHA, ^1H-^1H-COSY and NOE difference spectrum (Table 2). Thus **2** is malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) (Fig. 1).

Compounds **3** and **4** were found in flowers of *C. sieberi* ssp. *sublimis* 'Tricolor' while **5** and **6** were isolated from *C. chrysanthus* 'Eyecatcher' (Fig. 1). Also in flowers of *C. chrysanthus* 'Skyline' **5** and **6** were detected. These compounds are very common in plant species of Iridaceae [5–8, 11–13]. The structures of **3–6** were determined by FAB mass spectrometry (Table 1) and finally elucidated by the complete assignments of the ^1H NMR signals deduced with 1D and 2D techniques (Table 2).

By inspection of ^1H-^1H-NOESY the linkage position of glucoside B in **3** and **4** was found to be different from the position in **1** and **2**. Glucoside B was deduced to be attached at the OH-5 of delphinidin of **3** through a glucosidic bond, because of the presence of a strong NOE between H-6 (δ 7.04) of delphinidin and H-1 of glucoside B (δ 5.15) and a weak NOE between same anomeric proton and H-4 (δ 9.04) of delphinidin. This linkage position of glucose B was also obtained for **4**.

On ^1H-^1H-NOESY a NOE was observed between H-4 (δ 8.88) of petunidin and H-1 of glucoside (δ 5.31) of **6**. A weak negative NOE in DMSO-d_6 containing 10% TFA-*d* was also observed between H-6 of glucoside (δ 3.57; 4.07) and H-1 of rhamnoside (δ 4.65). Therefore, **6** is petunidin 3-β-rutinoside. The linkage of rutinose was further confirmed by H_2O_2 oxidation of **6**, followed by positive FAB mass spectrometry (327 $[M+1]^+$.

EXPERIMENTAL

Plant material. Fieldgrown flowers of *Crocus* were collected in Noordwijk, Holland, in March 1996.

Isolation of anthocyanins. Freeze-dried perianth segments of *C. chrysanthus* 'Skyline' (50 g), *C. sieberi* ssp. *sublimis* 'Tricolor' (50 g) and *C. chrysanthus* 'Eyecatcher' (40 g) were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 hr. The concd extracts were adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 4 to 20% aq. CH_3CN containing 0.5% TFA. For further purification, the crude anthocyanins were applied to prep. ODS-HPLC (20 $\phi \times 250$ mm, Develosil ODS-HG-5, Nomura Chemicals) by monitoring at 280 nm. The elution was carried out stepwise with 5 to 16% aq. CH_3CN containing 0.5% TFA (at a flow rate of 7 ml min^{-1}, at 40°, eluent

solvents were distilled just before use). The pigment frs were concd to dryness *in vacuo* and stored at −80°. From *C. chrysanthus* 'Skyline' **1** (5 mg) and **2** (4.5 mg), from *C. sieberi* ssp. *sublimis* 'Tricolor' **3** (80 mg) and **4** (18 mg) and from *C. chrysanthus* 'Eyecatcher' **5** (19 mg) and **6** (15 mg) were isolated as pure TFA salts.

Analysis of anthocyanin. Identification of the anthocyanins in *Crocus* cultivars was performed by analytical HPLC using two solvent systems (Table 1). About 1 g of the freeze-dried perianth segments was extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extract was analysed by ODS-HPLC (4.6 ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°, at a flow rate of 1 ml min^{-1}, monitoring on a 3D diode-array detector at 260–530 nm. In the first, a linear gradient elution for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA was carried out, and in the second, an isocratic solvent system with 14% aq. CH_3CN containing 0.5% TFA for 30 min.

Spectroscopic analysis. UV-visible spectra of the isolated anthocyanins were recorded in MeOH containing 0.1% HCl. FABMS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix. ^1H NMR and other NMR spectra were measured in CD_3OD containing 10% TFA-*d* by 600 MHz (JNM alpha 600, JEOL) with internal standard $CD_2\underline{H}OD$ (3.326 ppm). Exceptionally DMSO (TMS as internal standard) containing 10% TFA-*d* was used as solvent. 1D HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

H_2O_2 degradation. The H_2O_2 degradation of **6** was carried out according to [10, 14]. To a soln of petunidin 3-β-rutinoside (7 mg) in H_2O (0.5 ml) and CH_3CN (0.2 ml) was added 30% aq. H_2O_2 (0.6 ml). After 4 hr 10% palladium on charcoal (10 mg) was added to the colourless solr. and the mixt. was allowed to stand for 3 hr. The catalyst was removed by filtration and the filtrate was evapd to dryness. The residue was treated with 28% aq. NH_3 (4 ml) and EtOH (9 ml) at room temp. for 14 hr. The product was partitioned between H_2O and Et_2O and the aq. layer was dried to give rutinose, detected by FABMS using NBA as the matrix (*m/z* 327 $[M+1]^+$ corresponding to rutinose and *m/z* 479 $[M+NBA]^+$.

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PERGAMON

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PHYTOCHEMISTRY

Further anthocyanins from flowers of *Crocus antalyensis* (Iridaceae)

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Abstract

As a part of a continuing chemotaxonomic survey of pigments in *Crocus* two new and three known anthocyanins have been isolated from the blue perianth segments of *Crocus antalyensis* [Mathew, B., *The Crocus*. B.T. Batsford, London]. The novel anthocyanins were identified as delphinidin 3-*O*-(*β*-D-glucopyranoside)-5-*O*-(6-*O*-malonyl-*β*-D-glucopyranoside and petudin 3,7-di-*O*-(*β*-D-glucopyranoside. Further 3,7-di-*O*-*β*-D-glucoside of delphinidin was isolated together with two minor components 3,5-di-*O*-*β*-D-glucosides of delphinidin and petunidin. The complete structural determination of the compounds was achieved by use of 1D and 2D NMR techniques and other spectral evidence. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Crocus antalyensis*; Iridaceae; Flower pigments; Malonated anthocyanin; HPLC

1. Introduction

The flower pigments causing cyanic colors in *Crocus* (Iridaceae) have partly been identified by simple color reactions and chromatographic studies (Harborne & Williams, 1984; Garrido, Diez De Bethencourt, & Revilla, 1987; Hayashi, 1960; Lokar & Poldini, 1977). Recently we have isolated two new anthocyanins, 3,7-di-(6-malonyl)glucosides of petunidin and malvidin as well as 3-rutinosides and 3,5-diglucosides of delphinidin and petunidin from blue perianth segments of *Crocus* (Nørbæk & Kondo, 1998). Further studies on anthocyanin occurrence in *Crocus* proved another novel malonated anthocyanin along with the 3,7-diglucosides of petunidin and delphinidin. Delphinidin 3,7-diglucosides had already been isolated from maqui berries but was identified only by chromatographic methods (Diaz, Rosende, & Antunez, 1985). This is the first report on petunidin 3,7-diglucoside.

2. Results and discussion

Blue perianth segments of *Crocus antalyensis* were extracted with aqueous acetonitrile containing 0.5% TFA. HPLC chromatograms detected in the visible region of the crude anthocyanin extract showed the presence of three major anthocyanins as well as two minor pigments. The anthocyanins were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. The anthocyanins were identified as delphinidin 3-glucoside-5-(6-malonyl)glucoside (**1**), the 3,7-diglucosides of delphinidin (**2**) and petunidin (**3**) and as the 3,5-diglucosides of delphinidin (**4**) and petunidin (**5**). R_t's, UV-VIS and FAB mass spectra are shown in Table 1.

FAB-MS of **1** gave $[M]^+$ at m/z 713, in good agreement with the mass calculated for $C_{30}O_{20}H_{33}^+$. The fragment peaks were observed at m/z 551 [M −162 (hexose)]$^+$, 465 [M−248 (malonyl–hexose)]$^+$ and 303 [aglycone]$^+$, indicating **1** to be comprised of delphinidin, hexose and malonylhexoside. Acylation was also confirmed by the characteristically increased in retention time (R_t) of **1** compared with the original delphinidin 3,5-di-*O*-(*β*-D-glucopyranoside) (**4**) (Table 1).

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Table 1
Analytical HPLC retention times and spectral properties of anthocyanins found in blue perianth segments of *Crocus antalyensis*

Anthocyanin	R_t[a] (min)	R_t[b] (min)	UV-VIS (0.1% HCl–MeOH)		FAB-mass spectra
			λ_{max}	(nm)	$[M]^+$, fragment ions
1	14.3	17.4	271	538	713 [M], 551, 465, 303
2[c]	5.7	8.0	280	537	627 [M], 465, 303
3	8.2	9.4	279	537	641 [M], 479, 317
4[c]	9.2	10.8	269	538	627 [M], 465, 303
5	12.4	14.6	269	537	641 [M], 479, 317

[a]Retention time (R_t) on HPLC, linear gradient elution for 30 min using from 0–30% aq. CH_3CN containing 0.5% TFA.
[b]R_t on HPLC, elution profile see Section 3.
[c]FAB-mass spectrum obtained in a positive mode using NBA (1 drop of HCl aq. was added) as a matrix.

NMR data of compounds **1**–**3** are shown in Table 2, while data of the minor components have been described previously (Nørbæk & Kondo, 1998). The proton signals of delphinidin, the aglycone of both **1** and **2**, were assigned (Table 2) using information regarding coupling constants and chemical shifts. The ^1H NMR spectrum of **1** also revealed the presence of two glucose residues, one of them acylated with

Table 2
^1H-NMR spectral data of three new anthocyanins from *Crocus antalyensis* (in CD_3OD, containing 10% TFA-d)

	1[a]	**2**[b]	**3**[b]
Aglycone			
4	9.07 *br s*	8.89 *br s*	8.97 *br s*
6	7.00 *d* (1.8)	7.17 *d* (1.8)	6.85 *d* (1.8)
8	7.06 *d* (1.8)	7.26 *d* (1.8)	7.28 *d* (1.8)
2′	7.83 *br s*	7.76 *br s*	8.00 *br s*
6′	7.83 *br s*	7.76 *br s*	7.86 *d* (2.4)
OMe			3.98 *br s*
Glucoside A			
1	5.31 *d* (7.8)	5.37 *d* (7.8)	5.40 *d* (7.8)
2	3.71 *t* (9.6)	3.76 *t* (9.0)	3.71 *t* (9.0)
3	3.54 *t* (9.6)	3.61 *t* (9.0)	3.59 *t* (9.0)
4	3.39 *t* (9.0)	3.50 *t* (9.0)	3.47 *t* (9.6)
5	3.64 *m*	3.60 *m*	3.60 *m*
6	3.95 *m*	3.95 *dd* (6.6; 12.3)	3.95 *m*
	3.70 *dd* (6.4; 12.7)	3.76 *dd* (6.6; 12.3)	3.72 *m*
Glucoside B			
1	5.16 *d* (7.8)	5.21 *d* (7.8)	5.20 *d* (7.8)
2	3.70 *t* (9.0)	3.58 *t* (9.0)	3.56 *t* (9.0)
3	3.57 *t* (9.6)	3.57 *t* (9.0)	3.54 *t* (9.0)
4	3.47 *t* (9.6)	3.44 *t* (9.0)	3.41 *t* (9.0)
5	3.80 *m*	3.66 *m*	3.63 *m*
6	4.55 *dd* (6.6; 12.6)	4.00 *dd* (6.0; 12.3)	3.98 *m*
	4.34 *dd* (6.6; 12.6)	3.75 *dd* (6.0; 12.3)	3.73 *m*
Malonic moiety	3.39–3.36		

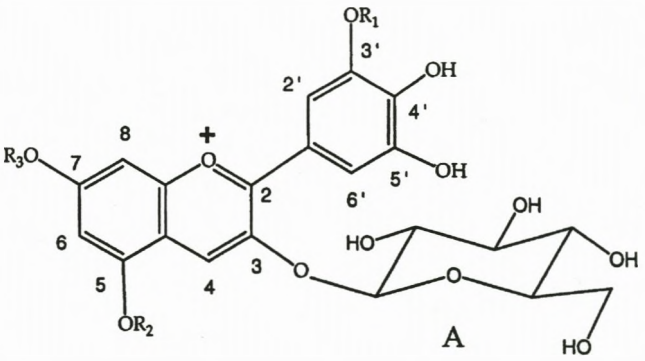
Coupling constants *J* (in Hz) in parentheses.
[a]By irradiation of H-1 of glucoside A in CD_3OD-d_6 containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of the nucleus. By irradiation of H-1 of glucoside B, a strong negative NOE was observed at H-6 of the nucleus. A weak negative NOE was also observed between the same anomeric proton and H-4.
[b]By irradiation of H-1 of glucoside A in CD_3OD-d_6 containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of the nucleus, and strong negative NOEs of H-6 and H-8 of the nucleus appeared by irradiation of H-1 of glucoside B. Concerning **3** a strong negative NOE was observed at H-2′ of the nucleus by irradiation of –OMe.

malonic acid, although H-4 of glucoside B and the malonyl protons, observed at δ3.39–3.36, were somewhat superimposed. The assignments of the two hexoses were perfectly carried out by 1D-HOHAHA spectra and ^{1}H–^{1}H-COSY. All vicinal coupling constants of both sugars were between 7.8–9.6 Hz including two anomeric protons at δ 5.31 (d, J = 7.8 Hz, glucoside A) and δ 5.16 (d, J = 7.8 Hz, glucoside B). Therefore, both sugar units must be β-D-glucopyranoside. The 6-methylene protons of glucoside B are lowfield shifted by ca 0.6 ppm compared to those in delphinidin 3,5-di-*O*-β-glucopyranoside (Nørbæk & Kondo, 1998) indicating acylation with the malonyl group at the 6-OH (Kondo, Ueda, & Goto, 1990). The positions of the glucosidic linkages were determined by NOE difference spectra (Goto & Kondo, 1991). A strong negative NOE was observed at H-4 (δ 9.07) of the delphinidin nucleus by irradiation of the anomeric proton (δ 5.31) of glucoside A. Glucoside B was deduced to be attached at the 5-OH of delphinidin through a glucosidic bond, because of the presence of a strong NOE between H-6 (δ 7.00) of delphinidin and the anomeric proton of glucoside B (δ 5.16) and a weak NOE between the same anomeric proton and H-4 (δ 9.07). Thus, **1** is delphinidin 3-*O*-(β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Scheme 1).

FAB-MS of **2** and **4** both established [M$^+$] at m/z 627 supporting the molecular formula $C_{27}O_{17}H_{31}^+$ by two fragments corresponding to delphinidin 3-glucoside (m/z 465) and delphinidin (m/z 303). The diversity of **2** and **4** was found in the different chromatographic profiles, **2** having lower R_t values than **4** (Table 1).

The ^{1}H spectrum of the sugar moieties of **2** was almost identical to that of delphinidin 3,5-di-*O*-β-glucoside (Nørbæk & Kondo, 1998), but the glucosidic linkages determined by NOE difference spectra were 3,7-positions instead of 3,5-positions. A strong negative NOE was observed at H-4 (δ 8.89) of the delphinidin nucleus by irradiation of the anomeric proton (δ 5.37) of glucoside A and strong negative NOEs at H-6 (δ 7.17) and H-8 (δ 7.26) by irradiation of the anomeric proton of glucoside B (δ 5.21) (Table 2). Thus **2** is delphinidin 3,7-di-*O*-(β-D-glucopyranoside) (Fig. 1).

Same diversities and similarities were found between **3** and **5**. The retention times on anal. HPLC were different, **3** being eluted faster than **5**. The [M]$^+$ of **3** and **5** were 14 mass units larger than that of **2** and **4**



	R_1	R_2	R_3
1	H	(6-*O*-malonyl)-β-D-glucopyranoside (B)	H
2	H	H	β-D-glucopyranoside (B)
3	CH_3	H	β-D-glucopyranoside (B)
4	H	β-D-glucopyranoside (B)	H
5	CH_3	β-D-glucopyranoside (B)	H

Scheme 1.

(Table 1) consistent with ^{1}H-NMR which revealed the presence of an aglycone containing –OMe (δ 3.98). By irradiation of the –OMe group a strong NOE was observed at H-2′ (δ 8.00) indicating petunidin. The ^{1}H spectrum of the sugar moieties of **3** was almost identical to that of petunidin 3,5-di-*O*-β-glucoside (Nørbæk & Kondo, 1998). However NOE spectra showed that glucoside B of **3** was attached at the 7-OH of petunidin, because of a strong NOE between H-6 (δ 6.85) and H-8 (δ 7.28) of petunidin and the anomeric proton of glucoside B (δ 5.20). The linkage position of glucoside A was not changed. Therefore, **3** is petunidin 3,7-di-*O*-(β-D-glucopyranoside) (Fig. 1).

3. Experimental

3.1. Plant material

Fieldgrown flowers of *Crocus antalyensis* were collected in Noordwijk, Holland, in March 1996.

3.2. Isolation of anthocyanins

Freeze-dried perianth segments of *C. antalyensis* (50 g) were extracted with 50% aq. CH_3CN containing 0.5% trifluoroacetic acid (TFA) at room temp. for 1 h. The conc. extract was adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln. and then eluted stepwise from 4 to 16% aq. CH_3CN containing 0.5% TFA. For further purification, the crude anthocyanins were sepd. on prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) using isocratic stepwise elution (5–16% aq. CH_3CN containing 0.5% TFA) at a flow rate of 7 ml min^{-1}. The pigment frs. were concd. to dryness in vacuo and stored at –80° as pure TFA salts; 10 mg (**1**), 20 mg (**2**), 10 mg (**3**), 5 mg (**4**) and 5 mg (**5**).

3.3. Analysis of anthocyanin

The relatively concentrations of delphinidin 3-glucoside-5-(6-malonyl)glucoside (**1**) (42%), the 3,7-diglucosides of delphinidin (**2**) (25%) and petunidin (**3**) (21%) and the 3,5-diglucosides of delphinidin (**4**) (7%) and petunidin (**5**) (5%), were determined by anal. HPLC. About 1 g of the freeze-dried perianth segments were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extract was analyzed by ODS-HPLC (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°, monitoring on a 3D diode-array detector at 260–530 nm. Two gradient systems were used to verify the presence of the anthocyanins. One was, a linear gradient from 0–30% aq. CH_3CN containing 0.5% TFA during 30 min with a flowrate of 1 ml min^{-1}. The second elution profile was as follows: 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40–50 min 100% B using solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1), with a flowrate of 1.5 ml min^{-1}.

3.4. Spectroscopic analysis

UV-visible spectra were recorded in MeOH containing 0.1% HCl. FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix. Exceptionally NBA was used as a matrix. ^{1}H NMR and other NMR-spectra were measured in CD_3OD containing 10% TFA*d* by 600 MHz (JNM alpha 600, JEOL) with internal standard CD_2HOD (3.326 ppm). 1D HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

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Anthocyanins from flowers of *Lilium* (Liliaceae)

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Abstract

The perianth segments of 10 cultivars including Asiatic and Oriental hybrids and one species of *Lilium*, were investigated by HPLC for their content of anthocyanins. The investigation revealed the presence of one new and one known anthocyanin. The nove anthocyanin, cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-glucoside and cyanidin 3-*O*-*β*-rutinoside were both isolated from the red flowers o *Lilium* 'Holean'. Within both Asiatic and Oriental hybrids, cultivars with or without the novel anthocyanin were found, whereas th known anthocyanin was always present in none-white genotypes. The structural determination of the compounds was achieved b 1D and 2D NMR techniques and other spectral evidence. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Lilium*; Liliaceae; Flower pigments; Anthocyanins; HPLC

1. Introduction

Lilies are popular ornamental plants with red, pink, orange, yellow or white flowers, often with dark red spots. The lilies are grouped in sections. Two major sections are the Asiatic and Oriental hybrids, both interspecific hybrids, including different species. Chromatographic studies of anthocyanins in flowers of *Lilium* showed the presence of two aglycones, cyanidin and petunidin, but attached sugars were not identified (Lawrence, Price, Robinson, & Robinson, 1938). However, the 3-*O*-*β*-rutinosyl-7-*O*-*β*-glucosyl moiety has earlier been identified in another genus as a part of a delphinidin derivative (Brandt, Kondo, Aoki, & Goto, 1993). Anthocyanin content in extracts of freeze-dried perianth segments of selected cultivars and one species of *Lilium* were measured by analytical HPLC. The red colours and spots are due to the anthocyanins, cyanidin 3-*O*-*β*-rutinoside being the major component and the novel compound, cyanidin 3-*O*-*β*-rutinoside-7-*O*-*β*-glucoside, being present in small amounts or non-detectable. Petunidin glycosides were not found.

2. Results and discussion

HPLC chromatograms detected in the visible spectra region of the crude anthocyanin extract from periant segments, occasionally showed one minor component (1 while the major anthocyanin (**2**) was always preser (Table 1), with the exception of the white flowered *l longiflorum*, that did not contain anthocyanins (Table 1 The anthocyanins were isolated by column chroma tography on Amberlite XAD-7 with subsequent pr parative HPLC.

Compound **1** showed λ_{max} at 280 and 525 nm in 0.1% HCl–MeOH and FAB-MS established $[M]^+$ at *m/z* 75 supporting the molecular formula $C_{33}O_{20}H_{41}^+$ with fra ments corresponding to cyanidin 3-rutinoside (*m/z* 595 cyanidin 3-glucoside (*m/z* 449) and cyanidin (*m/z* 287 The proton signals of cyanidin, the aglycone of both **1** an **2**, were assigned (Table 2) using information regardir coupling constants and chemical shifts. The assignme of the sugar protons was carried out by 1D-HOHAH spectra and ^1H–^1H-COSY (Table 2) and the position the glucosidic linkages determined by NOE differen spectra (Goto & Kondo, 1991). The chemical shifts the two glucosidic anomeric protons of **1** appeared at 5.34 (*d*, *J*=8.0 Hz, H-1″) and *δ* 5.22 (*d*, *J*=7.3 Hz, H 1‴), while the anomeric proton of rhamnosyl (H-1 was observed at *δ* 4.66 (*br s*). By irradiation of H-1‴ rhamnosyl (*δ* 4.66) NOE was observed to H-6″ of glucos (*δ* 4.07; 3.75) indicating rutinose. The linkage at 3-O

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Table 1
Distribution of anthocyanins in flower extracts from cultivars and one species of *Lilium*

Cultivars/species	Approximate concentrations (μM)		Flower colour
	Cy-3-Ru-7-Glu	Cy-3-Ru	
Asiatic hybrids			
L. 'Holean'	20	130	lavender red
L. 'Red Carpet'	5	80	lavender red
L. 'Hollandicum'	5	40	red
L. 'Monte Negro'	bd	80	lavender red
L. 'Montreux'	bd	40	red
L. 'Avignon'	bd	50	reddish orange
L. 'Compas'	bd	10	orange with spots
L. 'Las Vegas'	bd	10	yellow with spots
Oriental hybrids			
L. 'Star Gazer'	5	30	white with red patches
L. 'Le Reve'	bd	10	pink
L. longiflorum	bd	bd	white

Cy-3-Ru-7-Glu: cyanidin 3-*O*-β-rutinoside-7-O-β-*D*-glucoside and Cy-3-Ru: cyanidin 3-*O*-β-rutinoside. Concentrations were calculated from the HPLC chromatograms by using the extinction coefficient of cyanidin 3-glucoside log ε = 4.47 (Boyles & Wrolstad, 1993).bd: below detection limit.

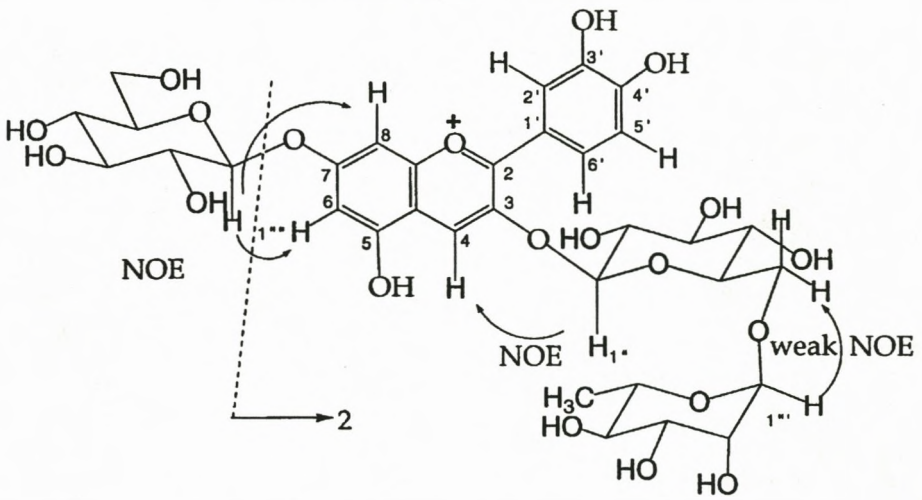
was determined from the presence of a strong negative NOE at H-4 (δ 8.87) of the cyanidin nucleus by irradiation of the anomeric proton (H-1″) (δ 5.34). Negative NOEs appeared at H-6 (δ 6.87) and H-8 (δ 7.21) by irradiation of (H-1⁗) (δ 5.22). Thus, **1** is cyanidin 3-*O*-β-rutinoside-7-*O*-β-D-glucoside (Fig. 1).

The FAB-MS spectrum of **2** showed $[M]^+$ at *m/z* 595, in good agreement with the mass calculated for $C_{33}O_{20}H_{41}^+$ with same fragmentation as **1** corresponding to cyanidin 3-glucoside and the aglycone. A strong negative NOE was observed at H-4 (δ 8.91) of the nucleus b irradiation of H-1″ of glucosyl (δ 5.27) indicating that thi sugar moiety is linked to 3-OH of cyanidin. Furthermor there was a weak negative NOE between H-6 of glucos (δ 4.07; 3.59) and H-1‴ of rhamnosyl (δ 4.65) indicatin a 1→6 linkage (Fig. 1). The spectral data of **2** was foun to be in accordance with data found in literature of cyar idin 3-*O*-β-rutinoside (Saito, Yokoi, Ogawa, Kamijo, Honda, 1988; Nørbæk, Christensen, Bojesen, & Brand 1996).

Table 2
^{1}H-NMR spectral data of anthocyanins (**1**, **2**) from *Lilium* (in CD_3OD, containing 10% TFA-*d*)

H no.	Cyanidin (ppm)	H no.	3-Glucoside (H″) (ppm)	Rhamnoside (H‴) (ppm)	7-Glucoside (H⁗) (ppm)
1					
4	8.87 *br s*	1	5.34 *d* (8.0)	4.66 *br s*	5.22 *d* (7.3)
6	6.87 *d* (2.2)	2	3.70 *t* (9.0)	3.78 *m*	3.60 *t* (9.6)
8	7.21 *d* (2.2)	3	3.59 *t* (8.4)	3.61 *dd* (3.6, 10.2)	3.57 *t* (9.0)
2′	8.02 *d* (2.2)	4	3.43 *t* (8.4)	3.30 *t* (9.0)	3.44 *t* (8.4)
5′	6.97 *d* (8.8)	5	3.60 *m*	3.53 *m*	3.66 *m*
6′	8.30 *dd* (3.0, 9.0)	6	3.75 *m*	1.15 *d* (6.6)	3.76 *dd* (4.8, 12.0)
			4.07 *m*		4.00 *m*
2					
4	8.91 *br s*	1	5.27 *d* (8.4)	4.65 *br s*	
6	6.67 *d* (1.8)	2	3.68 *t* (9.0)	3.78 *m*	
8	6.88 *d* (1.8)	3	3.58 *t* (9.0)	3.60 *dd* (3.6, 10.2)	
2′	8.01 *d* (2.4)	4	3.43 *t* (9.6)	3.30 *t* (9.0)	
5′	7.01 *d* (9.0)	5	3.73 *m*	3.53 *m*	
6′	8.24 *dd* (3.0, 9.0)	6	3.59 *dd* (6.6, 11.4)	1.15 *d* (6.0)	
			4.07 *m*		

Coupling constants *J* (in Hz) in parentheses.



cyanidin 3-*O*-β-rutinoside-7-*O*-β-glucoside (1)

⟶NOE

Fig. 1. By irradiation of H-1″ of the glucosyl of **1** in CD_3OD, containing 10% TFA-d, a strong negative NOE was observed at H-4 of the nucleu and strong negative NOEs of H-6 and H-8 of the nucleus appeared by irradiation of H-1‴ of the glucosyl and H-1‴ of the rhamnosyl. Strong NO was observed at H-4 of the nucleus of **2** by irradiation of H-1″ of the glucosyl in CD_3OD, containing 10% TFA-d. A negative NOE was also observe between H-6″ of the glucosyl and H-1‴ of the rhamnosyl.

The genotypes investigated include Asiatic and Oriental hybrids as well as one species, *L. longiflorum* clone (Table 1). The cultivars cover the shade of flower colours found in *Lilium* (Table 1). Flowers containing high amounts of anthocyanin have a red hue (Table 1) while some of the flowers have yellow or orange colours due to carotenoids (Mummery & Valadon, 1974; Valadon & Mummery, 1977; Toth & Szabolcs, 1981; Marki-Fischer & Eugster, 1985).

Among the investigated Asiatic hybrids *L.* 'Holean' produces significant amounts of cyanidin 3-*O*-β-rutinoside-7-*O*-β-D-glucoside. Both anthocyanins were also detected in *L.* 'Hollandicum' and *L.* 'Red Carpet'. *L.* 'Hollandicum' is believed to be a common ancestor to the Asiatic hybrids (Synge, 1980). In other Asiatic cultivars, like *L.* 'Monte Negro', *L.* 'Montreux', *L.* 'Avignon', *L.* 'Compas' and *L.* 'Las Vegas' the new anthocyanin was not present or in amounts below the detection limit. The Oriental *Lilium* 'Star Gazer' also contained cyanidin 3-*O*-β-rutinoside-7-*O*-β-D-glucoside, whereas in *L.* 'Le Reve' the content was below the detection limit. The ability to produce the novel anthocyanin is thus present in several distantly related species. However, all cultivars may posses the enzymes involved in 7-glycosylation but the enzyme activity in some genotypes may be to low for cyanidin 3-*O*-β-rutinoside-7-*O*-β-D-glucoside to b detected.

3. Experimental

3.1. Plant material

Cutflowers of eight *Lilium* cultivars and one specie were purchased from GASA, Odense (8 Asiatic, 2 Orier tal cultivars and *L. longiflorum*. *L.* 'Red Carpet' and *L* 'Hollandicum' were grown at the Department of Botany Dendrology and Forest Genetics, The Royal Veterinar and Agricultural University, Copenhagen. The identit was verified by Professor N. Jacobsen, Royal Veterinar and Agricultural University, Copenhagen. The inve tigated cultivars cover the colour range present in *Lilium*

3.2. Isolation and quantification of anthocyanins

Freeze-dried perianth segments of *Lilium* 'Holean' (8 g) was extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 h. The conc. extract wa adsorbed on an Amberlite XAD-7 column, washed wit 0.5% TFA aq. soln and then eluted stepwise from 5 t

14% aq. CH_3CN containing 0.5% TFA. The pigments were further purified by prep. ODS-HPLC (20$\phi \times$250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system; flow rate of 7 ml min^{-1}. From *Lilium* 'Holean' 10 mg of **1** and 40 mg of **2** were stored at −80°C as pure TFA salts.

For analytical HPLC about 1 g of the freeze-dried perianth segments were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extracts were analyzed by ODS-HPLC (4.6$\phi \times$250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°C, detection on a 3D diode-array detector at 260–530 nm. Two gradient systems were used to verify the presence of the anthocyanins. One was, a linear gradient elution with a flow rate of 1 ml min^{-1} for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA. Retention times for the anthocyanins were 6.1 (**1**) and 14.4 min (**2**), respectively. The second elution profile was as follows: 0 min, 16% B; 3 min, 38% B; 10 min, 44% B; 20 min, 50% B; 25 min, 67% B and 40–50 min, 100% B using solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1), with a flowrate of 1.5 ml min^{-1}. The anthocyanins were eluted at 9.5 (**1**) and 19.8 min (**2**), respectively. The limit for detection was 1.2 μM for each anthocyanin.

3.3. Spectral analysis

UV–Vis spectra were measured in MeOH containing 0.1% HCl. FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix of the isolated anthocyanins. ^1H NMR and othe NMR-spectra were measured in CD_3OD containing 10% TFA-d by 600 MHz (JNM alpha 600, JEOL) witl internal standard CD_2HOD (3.326 ppm). 1D HOHAH/ and 2D spectra were obtained using a pulse sequenc supplied from JEOL.

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FLOWER PIGMENT COMPOSITION OF *CROCUS* SPECIES AND CULTIVARS USED FOR A CHEMOTAXONOMIC INVESTIGATION.

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Key Word Index—*Crocus*, Iridaceae, flavonoids, anthocyanins, HPLC, chemotaxonomy.

Abstract—A survey of floral anthocyanins and other flavonoids by analytical high-performance liquid chromatography (HPLC) was performed among 87 species, 36 cultivars and 6 artificial hybrids of *Crocus* and was compared with taxonomical divisions established earlier by Mathew (1982).

Nine anthocyanins were detected. The *Crocus* species and cultivars were placed into five chemotypes as to their contents of 3,5-*di*-*O*-, 3,7-*di*-*O*-glucosides or 3-*O*-rutinosides of delphinidin and petunidin and to the presence of 3,7-*di*-*O*-malonyl-glucosides of petunidin and malvidin and delphinidin 3-*O*-glucoside-5-*O*-malonylglucoside. These malonated anthocyanins have only been found in *Crocus* and may be characteristic for this genus.

The same eighteen flavonoids were detected in every taxon. However, quantitative differences were noted and four chemotypes of *Crocus* were defined by their major contents of flavonoids. Six of the flavonoids appeared to be unique for *Crocus*.

The anthocyanin/flavonoid patterns of some of the taxa supplement the taxonomy based on morphological and cytological patterns. Most chemotypes were represented in several series but the chemical data were useful to distinguish between different species.

Introduction —The history and taxonomy of *Crocus* has been dealt with in the two comprehensive monographs by Maw (1886) and Mathew (1982). The genus has about 80 species classified into two subgenera.

Subgenus *Crocus* contains about 79 species and is further divided into two sections. Section A contains 6 series (a-f) and section B 9 series (g-o), Subgenus *Crociris* (Schur.) Mathew has only one species, *Crocus banaticus* Gay, which differs from other species in several aspects.

In some species the taxonomy is rather complicated as the various classification characters, i.e. distribution pattern, habitat, various morphological traits and cytological data, contribute confusing, non-correlating data to the problem of systematic and phylogenetic grouping. So additional independent characters will be useful to supplement existing traits.

More than 100 cultivars of *Crocus* are known today. They are derived from selection within and hybridisation between relatively few species. In the so-called *Crocus chrysanthus-C. biflorus* cultivars, it is difficult to say to what extent they are indeed hybrids between *C. chrysanthus* and *C. biflorus* as the name implies (Jacobsen et al. 1997).

The aim has been to provide new characters and evaluate whether they were useful to resolve ambiguities in the existing classification scheme.Especially the *Crocus chrysanthus-C. biflorus* cultivars have been studied to find out which cultivars fit well being selections of either of the two species or hybrids between them (Jacobsen et al. 1997).

From previous studies it appeared that anthocyanins and carotenoids are the most important pigments determining the colour of *Crocus* flowers (Harborne & Williams, 1984; Nørbæk & Kondo, 1998, 1999a). In the following carotenoid chemistry will not be discussed with respect to the pigment composition.

Recently nine anthocyanins have been reported as responsible for the cyanic colours (lilac, mauve and blue) of *Crocus* flowers. They were identified by modern NMR techniques as 3,7-*di*-*O*-β-D-glucosides, 3,5-*di*-*O*-β-D-glucosides and 3-*O*-β-D-rutinosides of delphinidin and petunidin, respectively, and delphinidin 3-*O*-β–D-glucoside-5-*O*-(6-*O*-malonyl-β-D-glucoside) and 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O* -(6-*O*-malonyl-β-D-glucosides) of petunidin and malvidin (Nørbæk & Kondo, 1998, 1999 a).

The colourless flavonoids in *Crocus* flowers have been reported as 3-*O*-α-L-(2-*O*-β–D-glucosyl)rhamnoside-7-*O*-β-D-glucosides, 3-*O*-α-L-(2-*O*-β–D-glucosyl)rhamnosides, 3-*O*-β-D-(2-*O*-α-L-rhamnosyl)glucosides, 3-*O*-β-D-sophorosides, 3,4-*di*-*O*-β-D-glucosides of different flavonols. Also included were kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β–D-glucosides acylated with malonic acid or acetic acid in the OH-6 position of the 7-glucosides, kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside, kaempferol 3-*O*-β-D-glucoside and 7-*O*-β–D-glucosides of apigenin and dihydrokaempferol (Nørbæk & Kondo, 1999 b; Nørbæk et al. 1999).

This paper is concerned with the analytical contents of the cyanic flower pigments and other flavonoids in 129 taxa of *Crocus* in order to determine their distribution and use as chemotaxonomical markers. Their retention characteristics in a standard HPLC system are given

and the results are compared with cytological and morphological characters (Jacobsen et al., 1997; Ørgaard & Heslop-Harrison, 1994; Ørgaard et al. 1995a,b; Mathew, 1982).

Materials and Methods —**Plant material**. All of the artificial *Crocus* hybrids and most of species and cultivars were grown at The Royal Veterinary and Agricultural University, Copenhagen, while P-numbers were obtained from the Botanical Garden, University of Copenhagen. The fresh perianth segments were frozen (-80°), then freeze-dried and analysed within six months (Table 6).

Analysis of perianth segments— Flower pigments in 87 species, 36 cultivars and 6 produced hybrids of *Crocus* have been analysed. For analytical HPLC about 1 g of the freeze-dried perianth segments were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extracts were analysed by ODS HPLC (4.6ϕ X 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40 °, detection on a 3D diode-array detector 280-530 nm.

Identification of anthocyanins—Two gradient systems were used to verify the presence of the anthocyanins. One was, a linear gradient elution with a flow rate of 1 ml min^{-1} for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA. The second elution profile was as follows: 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40-50 min 100% B using solvent A (H_2O TFA, 99 : 1) and solvent B (CH_3CN-H_2O-TFA, 60 : 140 : 1), with a flowrate of 1.5 ml min^{-1}. The anthocyanins were identified by their retention times and UV-spectra (Table 1) in accordance with Nørbæk & Kondo (1998, 1999a). The limit for detection at 530 nm was 1.2 µM for each anthocyanin in the extract, and injection volume was 50 µl.

Identification of flavonoids—The flavonoids from the previous extracts were chromatographed in solvent A (H_2O-TFA, 99 : 1) and solvent B (CH_3CN-H_2O-TFA, 60 : 140 : 1) by the following elution profile: 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40-50 min 100% B; flow rate 1.5 ml min^{-1}. Detection at 360 nm with injection volume 30 µl. The flavonoids were identified by their retention times and UV-spectra (Table 1) (Nørbæk et al.; 1999; Nørbæk & Kondo, 1999b).

In earlier work the anthocyanins and flavonoids have been identified by fast atom bombardment mass spectrometry (FAB-MS), 1D and 2D nuclear magnetic resonance (NMR) (Nørbæk & Kondo, 1998; Nørbæk & Kondo, 1999 a, b; Nørbæk et al. 1999). Quercetin 3-*O*-glucoside was only identified by co-chromatography and UV.

Data processing—On the HPLC chromatograms for each genotype the peak area of each anthocyanin or flavonoid was expressed as a percentage of the sum of the areas of all anthocyanin or flavonoid peaks, respectively. The ratings used in Table 2 and 3 indicate the relatively percentages of anthocyanin as +++ correspond to > 70%; ++ correspond to >40%; + correspond to > 15% and ± correspond to < 5%.

The chemotypes were defined by the contents of 3-*O*-β-D-rutinosides (1), 3,5-*di*-*O*-β–D-glucosides (2), 3,5- and 3,7-*di*-*O*–β-D-glucosides (3), 3-*O*-β-D-rutinosides and 3,5- and 3,7-*di*-*O*–β-D-glucosides (4) of delphinidin and petunidin. The fifth chemotype contained anthocyanins from chemotypes 1-4 in combination with delphinidin 3-*O*-β-D-glucoside-5-*O*-(6-

O-malonyl-β–D-glucoside) (**6**) , petunidin 3,7-*di*-*O*-(6-*O*-malonyl-β–D-glucoside) (**7**) and malvidin 3,7-*di*-*O*-(6-*O*-malonyl-β–D-glucoside) (**9**).

The major enzymatic reactions involved in the biosynthesis were categorised from the presence of flavonoids with different glycosylation patterns. Based on these, four flavonoid groups were defined by their major composition of 3-*O*-β-D-(2-*O*-α-L-rhamnosyl)glucosides (I) (>20%); 3-*O*-β-D-sophorosides (II) (>30%); 3,4-*di*-*O*-β-D-glucosides (III) (>20%) and 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β-D-glucosides (including acylation of 7-*O*-β-D-glucosides), 3-*O*-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside and 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside (IV) (>60%). The relative amounts of major flavonoids in the groups I-IV are shown in parentheses.

To test if one or two enzymes were involved in 2-glucosylation with rhamnose or glucose the ratio: percentages of 3-*O*-β-D-sophorosides/(percentages 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnosides + 3-*O*-β-D-sophorosides) in every flower were calculated.

Similarly, the ratios; percentages of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-O-malonyl-β-D-glucoside) / (percentages of kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-O-malonyl-β-D-glucoside) + kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-O-acetyl-β-D-glucoside)) were tested.

Results—

The anthocyanins—The HPLC retention characteristics and UV data of the detected pigments, nine anthocyanins and eighteen flavonoids are given in Table 1.

In Table 2 information about the anthocyanin contents in species of *Crocus* is shown. The species are classified according to the subdivision proposed by Mathew (1982). The chemotypes of anthocyanins in species of Series (h) are shown again in Table 3 together with their cultivars and hybrids. In most series there is a great variability hence several chemotypes are represented. The chemotypes 1 to 3 are characterised by their respective contents of 3-*O*-β-D-rutinosides, 3,5- or 3,7-*di*-*O*-β-D-glucosides of delphinidin and petunidin, respectively. Chemotype 4 can contain all three types of delphinidin- and petunidin glycosides. Chemotype 5 includes malonylated anthocyanins. In most cases malvidin 3,7-*di*-*O*-(6-*O*-malonyl-β–D-glucoside) is present which was the reason for including this glucoside of a di-methoxy anthocyanidin in chemotype 5.

Comparing the results with the subgeneric division as proposed by Mathew (1982) Table 2 shows that species/taxa from the same taxonomic series appear in different anthocyanin chemotypes (* represents one taxon). Series a: chemotypes 2*, 3*, 4****, Series b chemotype 4*, Series c: chemotypes 4***, 5**, Series d: chemotypes 2****, 4*, 5*, Series e : chemotypes 2*, 3* Series f: chemotypes 2**, 4****, 5****, Series g: chemotypes 1****, 2*****, 3*, 4*******, Series h: chemotype s1***, 2**, 4***, 5******, Series i: chemotype 1*, Series j: chemotypes 1***, 4**, 5* Series k:-, Series l: chemotype 4*, Series m: chemotype 1*, Series n: chemotypes 1**, 4**, 5* and Series o: chemotype 1*.

In several cases subspecies appear in the same anthocyanin chemotypes, i.e. subspecies of *C. reticulatus* (Series g) in chemotype 1, *C. serotinus* (Series d) in chemotype 2, *C.*

cancellatus (Series g) in chemotype 4, while others, i.e. *C. sieberi* (Series g) appears with subspecies in chemotype 2 and 4 and *C. biflorus* (Series h) with subspecies in chemotype 1, 2, 4 and 5.

None of the taxonomic series correspond exactly to the anthocyanin chemotypes except where only one taxon is involved. E.g. Series a, with five species, is represented in three anthocyanin chemotype.

The flavonoids—Every taxon of *Crocus* contain the 18 detected flavonoids. With regard to flavonoid aglycones, kaempferol dominates, as kaempferol glycosides constitutes between 70 and 90 % of the total contents of flavonoids in the flowers of the genus. The contents of quercetin glycosides varied from 5 to 10 % in all taxa and glycosides of dihydrokaempferol, isorhamnetin, myricetin and apigenin were only minor components.

The taxa in Tables 4 and 5 have been arranged in order to be compared with Tables 2 and 3, respectively. Because of the presence of the eighteen flavonoids in every taxon, the taxa are represented by their major composition of flavonoids. Four flavonoid groups were defined.

Flavonoid group I is characterised by having major flavonol 3-*O*-β-D-(2-*O*-α-L-rhamnosyl)glucosides. The major compounds in flavonoid group II are flavonol 3-*O*-β-D-sophorosides. The non-normal data of percentages of (3-*O*-β-D-sophorosides/(percentages 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnosides + 3-*O*-β-D-sophorosides)) varied prominently from 0-80% in the flowers (Tables 4 and 5).

Flavonoid group III represents taxa with relatively high amounts of flavonol 3,4'-β-D-diglucosides. Finally, flavonoid group IV is characterised by taxa having relatively high amounts of flavonol 3-*O*-α-L-(2-*O*-β-D glucosyl)rhamnoside-7-*O*-β-D-glucosides (including compounds acylated at 6-OH of 7-*O*-β-D-glucoside) and kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside. An insignificant variation in the amounts of different acylated flavonoids was found to be 5 to 20% (Tables 4 and 5).

In every genotype, 7-*O*–β–D-glucosides of dihydrokaempferol and apigenin appeared as minor components and 3-*O*-β-D-glucosides of kaempferol and quercetin as major components. No considerable variation of the amounts of these compounds were found and they were not treated any further because of no chemotaxonomical value.

In comparison to the anthocyanins the flavonoids present a simpler picture, but the overall picture does not become clearer on this basis.

Discussion—

General aspects of the anthocyanins in the genus *Crocus*—The anthocyanins cause the cyanic colours in flowers (Harborne, 1996) and modifications like malonation of the attached glucoside units and hydroxylation or methoxylation on the B-ring of the anthocyanidins will cause a shift to bluish hues (Asen & Griesbach, 1983; Asen, 1984; Arisumi et al., 1985; Cohen et al., 1986; Nørbæk et al., 1998, Yabuya, 1991; Yabuya et al. 1994).

Correlation between anthocyanin type and natural selection for flower colour has been documented (Harborne, 1967) and as an example, an evolutionary trend towards blue flowers

has been reflected in the frequency of blue-coloured species in temperate members of Labiatae, Polemoniaceae and Boraginaceae (Harborne, 1983).

The colour variation caused by anthocyanins in the flowers of *Crocus* ranges from purple or brownish markings or stripes on the outer perianth segments to self coloured lilac mauve or blue flowers.

A selection pressure for flower colour may have influence on the contents of 3,7-*di*-*O*-malonylglucosides of petunidin and malvidin (**7** and **9**) and delphinidin 3-*O*-glucoside-5-*O*-malonylglucoside (**6**) in *Crocus*. It is likely that the presence of two dominant genes with alleles that promote methoxylation in the 2' and 4' positions of the chromophore and malonylation on 6-OH position of the glucoside moieties, make the perianth segments more blue (Nørbæk et al., 1998). The presence of a selectory pressure was indicated by the frequent co-occurrence of the two characteristics. Out of 14 species containing malonated anthocyanins 9 also contained malvidin (**9**).

The acylated anthocyanins **6**, **7** and **9** included in chemotype 5 can not be considered as neutral markers since these compounds may appear as a result of selection for blue colour hue. These malonated anthocyanins appear in many different series of *Crocus* (Series c, d, f, g, h, j, and n) as expected.

From this point of view the ancestor of the genus may have been capable of producing methoxylated and malonylated anthocyanins but these characters were lost during evolution. This possible explanation is supported by the fact that acylated flavonoids and thus the relevant class of enzymes are found in every taxon of *Crocus*.

Furthermore, it is possible that a mutation has occurred so the specificity of substrate for the acyltransferase in *Crocus* has evolved from including only flavonoids to also including anthocyanins.

However, it is likely that selection for colour does not have an affect on compounds **1-5**, **7** because the glucosyl- and rutinosyl-moieties do not increase the intensity of the blue perianth colour and these compounds may have a considerable chemotaxonomic importance (Nørbæk et al., 1998)

Taxa included in chemotype 1 are capable of making 3-O-β-D-rutinosides of delphinidin and petunidin, which are very common pigments in plant species of Iridaceae (Arisumi, 1974; Ashtakala & Forward, 1975; Ishikura, 1980; Ishikura, & Yamamoto, 1978; Williams et al., 1986; Yabuya, 1987; Yabuya, 1991).

The species in chemotype 2 and 3 are not so distantly separated. They both contain the same anthocyanidins only distinguished by the position of *di*-glucosylation, and several plants of chemotype 2 may contain traces of 3,7-*di*-O-β-D-glucosides of delphinidin and petunidin.

The taxa in chemotype 4 are more variable than chemotypes 1-3 since both 3,5- and 3,7-*di*-*O*-β-D-diglucosides and 3-*O*-β-D-rutinosides of delphinidin and petunidin appear in this chemotype.

Chemotype 5 includes the same compounds as chemotype 4 but differs by the presence of malonated anthocyanins, as discussed above.

General aspects of other flavonoids in the genus *Crocus*—The flavonols and flavones, although yellow compounds, often impart no visible colour to the tissues in which they are

found (Harborne, 1996). However, the flavonols and flavones are potential copigments and their sugar units may have importance for the interactions with anthocyanins. Copigments do not by themselves significantly contribute to the colour, but they cause a bathochromic wavelength shift in the visible λ_{max} of natural anthocyanins, which makes them appear bluer and increase absorptivity (Asen et al, 1972, 1975, Scheffeldt and Hrazdina 1978, Kim and Fujieda, 1991). The absorption of anthocyanin divided by the absorption of flavonoids (A) are shown in Table 4 and 5 for every taxa since in earlier studies a linear relation with colour hue was shown in the genus *Alstroemeria* (Nørbæk et al., 1998). However, within a series this relation (A) vary by a factor 100 and no systematic relation with anthocyanin chemotype was evident.

Most of the flavonoids from *Crocus* are of relatively widespread occurrence in the plant kingdom. However, the discovery of flavonol 3-*O*-α–L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-β–D-glucosides occasionally modified with acetic acid and malonic acid and kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside is to our knowledge unique for *Crocus* and separates the genus from other relatives in the Iridaceae. However, since the chemotaxonomy of Liliales is rudimentary, future phytochemical studies may reveal such flavonol glycosides in other relatives also.

Flavonoid group I-III all have high activity of a 3-*O*-glucosyltransferase (FGT), an enzyme earlier described in flowers (Besson et al., 1979; Hrazdina, 1988; Ishikura & Yamamoto, 1990; van Nigtevecht and van Brederode, 1975). In addition, group I is characterised by high activity of a 2-*O*-rhamnosyltransferase (2ORT). Flavonoid group II has an active flavonoid 2-O-glucosyltransferase (2OGT[1]) (van Brederode & van Nigtevecht, 1974) and flavonoid group III represent taxa with high activity of a flavonoid 4'-*O*-glucosyltransferase (F4'OG) (Latchinian-Sadek & Ibrahim, 1991).

Group IV is characterised by taxa having relatively high activity of five enzymes; a flavonoid 2-O-glucosyltransferase (2OGT[2]), a flavonoid 3-O-glucosyltransferase (3OGT), a flavonoid 3-O-rhamnosyltransferase (FRT), a flavonoid 7-O-glucosyltransferase (F7OG) and a acyltransferase (AT).

The stereochemical differentiation between β–D-glucopyranoside and α–L-rhamnopyranoside is prominent and therefore different glucosyltransferases are probably needed for the 2-*O*-glucosylation (2OGT[1], 2OGT[2]) (Figure 1). This is supported by the great variation of the relative amounts of flavonol 3-*O*-β-D-sophorosides and 3-*O*-α–L-(2-*O*-β–D-glucosyl)rhamnosides.

The formation of kaempferol 3-O-α-L-(2,3-*di*-*O*-β-D-glucosyl)rhamnoside is an end product. The only substrate for the glucosyltransferase responsible for the 3-*O*-glucosylation seem to be kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside since no flavonol 3-*O*-α–L-(3-*O*-β–D-glucosyl)rhamnosides were found in the *Crocus* flowers.

Acylation of flavonoid metabolites is generally a late event in flavonoid biosynthesis (Harborne, 1996). An insignificant variation in the ratio of the co-existing kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-*O*-malonyl-β-D-glucoside) and kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl)rhamnoside-7-*O*-(6-*O*-acetyl-β-D-glucoside) was found so the same acyltransferase is probably catalysing both malonylation and acetylation. The results are

consistent with the hypothesis that only one enzyme catalyses both reactions as has been shown earlier for enzymes (Fujiwara, H. et al., 1998).

The anthocyanins and other flavonoids in the *Crocus* species— The survey of anthocyanins shows that in Series a most of the species belong to chemotype 4, only *C. etruscus and C. baytopiorum* being in chemotype 2 and 3, respectively (Table 2). *C. etruscus* does not differ much from the species in chemotype 4, since the major components in both chemotypes are 3,5-*di*-*O*-*β*–D-glucosides of delphinidin and petunidin and the flavonoid pattern matches to most other members of the series.

Both the anthocyanin and flavonoid patterns of *C. baytopiorum* deviate from other members of the series. While this does not by itself discredit earlier interpretations it indicates that the position of this species merits further investigation. The remaining species in Series a showed a uniform flavonoid pattern (Table 4).

Series b is only represented by one taxon, *C. pelistericus* belonging to anthocyanin chemotype 4 and flavonoid flavonoid group III and IV (Tables 2 and 4).

The species represented from Series c, which on morphological grounds seem related, show the same anthocyanin pattern, only *C. versicolor* deviates by the minor contents of malonylated compounds (Table 2). As earlier mentioned, the presence of malonylated compounds may not have an important chemotaxonomic value. The major flavonoid pattern in Series c was identical (Table 4) so the chemotaxonomic evidence clearly substantiates earlier work in this series.

The major anthocyanins in the species belonging to Series d are 3,5-*di*-*O*-*β*–D-glucosides of delphinidin and petunidin. Most species have similar anthocyanins and flavonoids, and only *C. medius* and *C. nudiflorus* deviate. The presence of delphinidin 3-*O*-*β*–D-glucoside-5-*O*-(malonyl-*β*–D-glucoside) in *C. nudiflorus* does not make it so different from the others (Table 2). To some extent the data indicate that among the two neighbouring series (c and d) the species *C. medius* should be further investigated as to whether it may belongs to Series c.

Concerning the flavonoids, Series d showed some diversity but all species contained the major flavonoids of group IV (Table 4).

C. kotchyanus and *C. vallicola* from Series e differ in anthocyanin and flavonoid contents so the chemical data do not by themselves support their inclusion in the same series (Tables 2 and 4).

Series f shows four non-white *C. pallasii* accessions belonging to chemotypes 2 and 4. Other species included are placed both in chemotypes 2 and 5 (Table 2). *C. cartwrightianus* is in chemotype 5 while its white form 'Albus' is in chemotype 2, similar to the white form of *C. pallasii.*

The variation in this series may be explainable as two of the taxa in chemotype 2 are regarded as white forms of otherwise chemotypes 4 and 5 taxa. The apparently simpler anthocyanin pattern may be due to the physical limit of detection. The uniformity of the *C. pallasii* accessions is confirmed by the major flavonoid contents (Table 4). Another noteworthy characteristic is that *C. mathewii* differs from that of the rest of the taxa in Series f (Table 4). This species may show relations to e.g series g.. Three species, *C. asumaniae*, *C. hadriaticus* and *C. oreocreticus* resemble both the anthocyanin and flavonoid patterns of Series c.

In Series g seventeen taxa have been analysed. All five anthocyanin chemotypes are represented and a further subdivision of the series may be suggested. The subspecies *C. reticulatus* and *C. cancellatus* are well defined in chemotypes 1 and 4, respectively. Two accessions of *C. angustifolius* are represented in chemotype 4 and differ only by the trace of 3,7-*di*-*O*-glucosides of delphinidin and petunidin in *C. angustifolius* 'Minor' (Table 2).

C. sieberi is represented by four subspecies, where *C. sieberi* ssp. *atticus* deviates from the others. From the major flavonoid contents It is noteworthy that *C. sieberi* ssp. *atticus* again does not group together with other subspecies (Table 4). However, it is not explainable why the *C. sieberi* ssp. *atticus* is so much different from the other *C. sieberi* subspecies. It is interesting that the morphologically similar *C. veluchensis* differs in both the anthocyanin and flavonoid pattern. This supports that *C. veluchensis* and *C. sieberi* are different species.

Series h is described separately in next section.

Series i is represented by two species, *C. korolkowii* and *C. alatavicus*, both capable of making only 3-*O*-β–D-rutinosides of delphinidin and petunidin (Table 2). However, they differ in contents of major flavonoids (Table 4).

Series j shows some diversity as chemotypes 1, 4 and 5 are found. *Crocus flavus*, *C. olivieri* and *C. vitellinus* are placed in anthocyanin chemotype 1 (Table 2). However, the flavonoid contents do not confirm grouping of these species (Table 4). The systematic position of *C. antalyensis* is interesting when considering anthocyanin and flavonoid patterns. The others are consistent with the grouping.

Series l and m are only represented by one species each. The pigments support a close connection between i, j and m.

In Series n three and two accessions of *C. pulchellus* and *C. speciosus* are included, respectively. In this series the chemical markers are in accordance with a common origin. The flavonoid pattern is uniform and the differences of anthocyanin contents are correlated with the colour and may be caused by selective processes on sub- and species level.

Series o shows that *C. laevigatus* belongs to chemotype 1 and the white flowered *C. boryi* did not contain anthocyanins or at least the contents was below the detection limit (Table 2). However, both species are placed in flavonoid flavonoid group III (Table 4).

The subgenus *Crociris* has only one species, *C. banaticus*, which differs from other species in several morphological traits and also in anthocyanin pattern (Table 2). The species belongs to group IV of the flavonoids (Table 4).

In general, the chemical data support the morphological data, only rising some doubt about the placement of six species. *C. medius* from Series d should be further investigated as to whether it may belongs to Series c. *C. asumaniae*, *C. hadriaticus* and *C. oreocreticus* from Series f match both the anthocyanin and flavonoid patterns of Series c and may be closer to this series. *C. mathewii* in Series f shows similarities to series e.g. g. *C. siberi* ssp. *atticus* in Series g differs from the other subspecies.

The anthocyanins and other flavonoids in species and cultivars of *Crocus* Series Biflori (h)
Series h comprises the annulate species (see also Series n) including the many subspecies of *C. biflorus*. The yellow flowered *C. chrysanthus* is found in anthocyanin chemotype 1 together with both white and yellow flowered *C. danfordiae*. The *C. biflorus* subspecies are found in anthocyanin chemotypes 1, 2, 4, 5 (Table 3). Whether some of the different *C. biflorus* subspecies should have rank as species, is difficult to say but many have previously been regarded as so.

Fourteen cultivars in Series h seem to be *C. chrysanthus* hybrids, as they only posses the same two simple anthocyanins (**5**, **8**) as *C. chrysanthus*. The occurrence of *C. biflorus* ssp. *melantherus* and *C. biflorus* ssp. *crewei* in chemotype 1 could indicate their relations to *C. chrysanthus*. *C. biflorus* ssp. *crewei* only has petunidin 3,5-*O*-*di*-*β*–D-glucoside as a minor component.

Other *C. biflorus* subspecies are characterised by a combination of many compounds, which is an important dissimilarity from the *C. chrysanthus* chemotype.

Two *C. biflorus* subspecies, *C.* 'Zenith' and *C. aerius* are placed in chemotype 4. The only difference between chemotypes 4 and 5 is the presence of malonated anthocyanins which may not be an important chemotaxonomical character. Thus, this places *C.* 'Zenith' and the *C. biflorus* subspecies close to those from chemotype 5.

Within the Series Biflori (h) most accessions fall in flavonoid group IV, both species, subspecies, and cultivars. However, *C. biflorus* ssp. *crewei*, *C. biflorus* ssp. *adamii* 'Serevan' and *C. biflorus* ssp. *taurii* differ from the other subspecies (Table 5).

The pigments indicate that the annulate species in Series n is a well defined series which are separated from the annulate species in Series h. In every other series than h, a relation between the chemical markers and the division of species was found. The diversity of chemotypes in Series h indicates that *C. biflorus* may include different species and a further division may be a suggestion.

The anthocyanins in hybrids —Our own documented artificial hybrids showed the following patterns*: C. reticulatus* has anthocyanins **5** and **8** in equal amounts and *C. angustifolius* has anthocyanins **3**, **4**, **5** (major) and **8**, while the hybrid has the expected **4**, **5**, **8** (major) but also **6** and **7**, the latter as minor components and not found in the parents (Table 2). The minor amounts of anthocyanins **6** and **7** only detected in the hybrid are considered as limitations of detection and thus not important.
The major flavonoids in the parental species were from flavonoid groups III, IV and II, III, IV, respectively, while the hybrid only showed the major flavonoids from group III and IV (Table 4).

In the hybrid *C. chrysanthus* (anthocyanins **5** (major) and **8**) x *C.* 'Major' (anthocyanins **5** (major), **8**) the hybrid has **5** and **8** as major components and insignificant traces of **6** and **7**. The reciprocal hybrid combination showed the same anthocyanin pattern (Table 3). The hybrids both belong to group III and IV and not group I from the parental *C. chrysanthus* (Table 5).

In the hybrid *C.* 'Major' x *C. biflorus* ssp. *crewei* (anthocyanins **4** (trace), **5**, **8**) the hybrid has the parental anthocyanins **5**, **8** as major components and anthocyanins **1-4**, **6** as traces

(Table 3). The hybrid contains flavonoids from group III and IV, corresponding to the parental *C.* 'Major' but *C. biflorus* ssp. *crewei* also contains major flavonoids of character I, which is not expressed in the hybrid (Table 5).

In the hybrid *C. chrysanthus* (anthocyanins **5** and **8**) x *C. biflorus* ssp. *pulchricolor* (anthocyanins **4-9**) the hybrid has the parental anthocyanins. Even though the parental species belong to group I, III, IV and IV, respectively, the hybrid only belongs to flavonoid group IV (Table 5).

The parents of the yellow-flowered *Crocus* cultivars *C.* 'Golden Yellow' and *C.* 'Stellaris' are reported to be *C. flavus* and *C. angustifolius*, based on morphological traits and by genomic *in situ* hybridisation (Ørgaard et al. 1995b). The diploid hybrid *C.* 'Stellaris' (anthocyanins **1-5**), although **8** was not detected, has most of the parental anthocyanins: *C. flavus* (anthocyanin **5**) and perhaps the other parent is *C. angustifolius* 'Minor' (anthocyanins **1-5**, **8**(trace)) rather than *C. angustifolius* (anthocyanin **3-5**, **8** (low)) (Table 2). The flavonoid patterns of the parental species and the hybrid do not give any informations to the results. Both *C. angustifolius* and *C. angustifolius* 'Minor' belong to flavonoid group II, III and IV, while the hybrid *C.* 'Stellaris' has major flavonoids from group III and IV (Table 4).

The triploid hybrid *C.* 'Golden Yellow' (anthocyanins **2-6**, **8**), seems to have the parental anthocyanins of *C. flavus* (anthocyanin **5**) and C. *angustifolius* 'Minor' (anthocyanins **1-5**, **8**(trace)) rather than *C. angustifolius* (anthocyanin **3-5**, **8** (low)), but has an additional trace of anthocyanin **6** (Table 2). The flavonoid pattern supplemented the results since the hybrid belongs to group III, while the parental *C. flavus* belongs to I and IV and both *C. angustifolius* and *C. angustifolius* 'Minor' are chatagorised in group II, III and IV (Table 4).

A conclusion regarding the hybrids is that their chemical composition is not always a sum of the two parental anthocyanin components i.e. they have the majority, but may lack one or two components. The ability to produce relatively high amounts of flavonol 3-*O*-β-D-(2-*O*-α-L-rhamnosyl)glucosides (group I) is also recessive. However, it seems like the anthocyanin chemotype 1, the ability to make relatively high amounts of anthocyanidin 3-*O*-β-D-rutinosides, is dominant.

The loss of anthocyanins in hybrids may be the explanation for the sometimes diverse anthocyanin patterns found in otherwise morphologically related species and subspecies, i.e. it may be the result of previous hybridisations in the evolutionary history of these plants.

Conclusions—

Three malonated anthocyanins and six flavonol glycosides seem to be unique for *Crocus*. All these compounds were widely distributed within the genus, the six flavonoids occurred in every taxon examined, and so can be used as distinguishing markers for this family.

The diversity of the distribution of the chemical structures within the genus made it possible to use them as chemotaxonomical markers, in particular the anthocyanins. Evaluation of anthocyanin and flavonoid chemotypes, each generally support the classification by Mathew (1982), and using the two types of compounds the results reinforce each other. For all series

except Series h the chemical data were very similar for all subspecies or accessions within a species, and chemotypes within a series were more similar than across series. However, the analyses suggest that for seven species should be further investigated using other methods, to evaluate their relations to other series.

Analyses of the *C. chrysanthus-biflorus* complex in Series h showed a wide range of anthocyanin chemotypes, which could support the earlier view that the *C. biflorus* subspecies actually comprise several different species.

Methoxylated and malonylated anthocyanins are widely distributed in the genus, occurring in several series in both sections, and a few species contain all the nine anthocyanins found. This indicates that the ancestral *Crocus* may have contained all the anthocyanin and flavonoid chemotypes found in the genus today.

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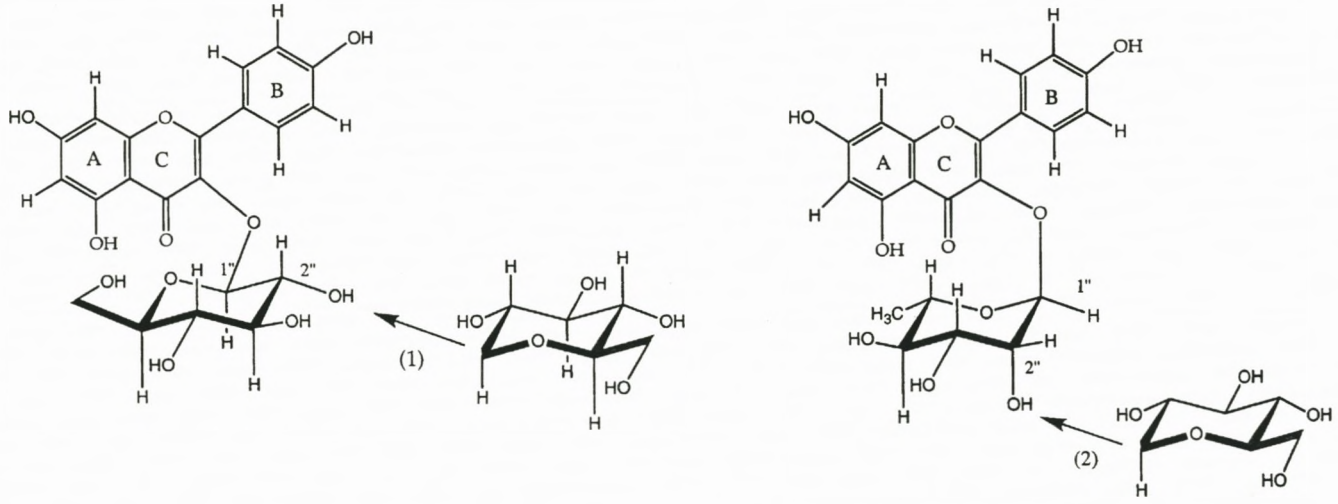

Fig 1. Stereo-structures of kaempferol β-D-glucopyranoside and 3-*O*-α-L-rhamnopyranoside, respectively.
Two different 2-*O*-glucosyltransferases control the formation of flavonol 3-*O*-sophorosides (1) (2OGT[1]) and flavonol 3-*O*-(2-*O*-glucosyl)rhamnosides (2) (2OGT[2]), respectively.

Table 1. Analytical HPLC retention times and λ_{max} of anthocyanins and flavonoids isolated from perianth segments of *Crocus*.

	Systematic names	R^at (min)	λ_{max} (nm)	
	Anthocyanins			
1	delphinidin 3,7-*di*-*O*-*β*-D-glucoside	5.7	280	537
2	petunidin 3,7-*di*-*O*-*β*–D-glucoside	8.2	279	537
3	delphinidin 3,5-*di*-*O*-*β*-D-glucoside	9.2	269	538
4	petunidin 3,5-*di*-*O*-*β*-D-glucoside	12.4	269	537
5	delphinidin 3-*O*-*β*–D-rutinoside	13.5	282	515
6	delphinidin 3-*O*-*β*–D-glucoside-5-*O*-*β*-D-(6-*O*-malonyl)glucoside	14.3	271	538
7	petunidin 3,7-*di*-*O*–*β*–D-(6-*O*-malonyl) glucoside	16.1	269	536
8	petunidin 3-*O*-*β*-D-rutinoside	16.6	275	542
9	malvidin 3,7-*di*-*O*-*β*-D-(6-*O*-malonyl) glucoside	17.5	278	536
	Colourless flavonoids			
10	dihydrokaempferol 7-*O*-*β*–D-glucoside	8.4	284, 335 *sh*	
11	myricetin 3-*O*-*α*–L-(2-*O*-*β*-D-glucosyl)-rhamnoside-7-*O*-*β*–D-glucoside	9.7	295, 358	
12	quercetin 3-*O*-*α*-L-(2-*O*-*β*-D-glucosyl)-rhamnoside-7-*O*-*β*–D-glucoside	12.2	255, 268 *sh*, 301*sh*, 352	

	Systematic names	R[a]t (min)	λ_{max} (nm)
13	kaempferol 3-*O*-α-L-(2-*O*-β–D-glucosyl)-rhamnoside-7-*O*-β-D-glucoside	15.0	265, 310 *sh*, 345
14	quercetin 3-*O*-β-D-sophoroside	15.6	257, 269 *sh*, 299 *sh*, 362
15	quercetin 3,4'-*di*-*O*-β-D-glucoside	17.0	267, 301 *sh*, 354
16	kaempferol 3,4'-*di*-*O*-β-D-glucoside	19.0	265, 302 *sh*, 317 *sh*, 342
17	isorhamnetin 3,4'-*di*-*O*-β-D-glucoside	21.6	268, 300 *sh*, 351
18	kaempferol 3-*O*-β-D-sophoroside	21.6	267, 300 *sh*, 350
19	kaempferol 3-*O*–β–D-(2-*O*-α–L-rhamnosyl)-glucoside	24.7	265, 295 *sh*, 317 *sh*, 347
20	isorhamnetin 3-*O*-β–D-(2-*O*-α– L-rhamnosyl)-glucoside	25.2	253, 265 *sh*, 293 *sh*, 360
21	kaempferol 3-*O*-α–L-(2-*O*-β–D-glucosyl)-rhamnoside-7-*O*-β-D-(6-*O*-malonyl)glucoside	27.7	266, 301 *sh*, 315 *sh*, 345
22	kaempferol 3-*O*-α-L-(2,3-*di*-*O*-β–D-glucosyl) rhamnoside	29.5	268, 315 *sh*, 347
23	kaempferol 3-*O*-α-L-(2-*O*-β-D-glucosyl) rhamnoside-7-*O*-β-D-(6-*O*-acetyl)glucoside	30.2	265, 290 *sh*, 315 *sh*, 345
24	apigenin 7-*O*-β–D-glucoside	30.7	270, 323 *sh*, 425
25	kaempferol 3-*O*-α–L-(2-*O*-β–D-glucosyl)-rhamnoside	31.9	268, 315 *sh*, 347
26	quercetin 3-*O*-D-glucoside*	38.2	270sh, 299sh, 363
27	kaempferol 3-*O*-β-D-glucoside	44.8	266, 301 *sh*, 350

The structures were also determined by FAB-MS, 1D and 2D NMR spectroscopy (Nørbæk & Kondo, 1998, Nørbæk & Kondo, 1999 a, b, Nørbæk et al., 1999).

* only identified by co-chromatography and UV (in accordance with Markham, 1982).

Table 2. Distribution of anthocyanins in *Crocus* species.

Species	1	2	3	4	5	8	6	7	9	C_t†	Chemo types	Series
C. etruscus			++	++						40	2	a
C. baytopiorum	++	++								10	3	a
C. kosaninii			++	++	+					80	4	a
C. vernus scepusiensis 234			++	++	±	±				180	4	a
C. vernus 95-101	±	±	++	++	±	±				50	4	a
C. vernus 95-115			++	+	+	+				50	4	a
C. tommasinianus			++	++	±					130	4	a
C. pelistericus			+	++		++				30	4	b
C. minimus			+	++	+	+				90	4	c
C. corsicus			+	++	+	+				180	4	c
C. imperati			+	++	+	±				60	4	c
C. versicolor			+	++	+	±	±	±		20	5	c
C. longiflorus			++	++						20	2	d
C. niveus			+++	±						10	2	d
C. serotinus ssp. *clusii*			+++	+						70	2	d
C. serotinus ssp. *salzmanii*			++	++						20	2	d
C. medius			++	++	±	±				40	4	d
C. nudiflorus			++	++			+			40	5	d
C. kotchyanus			+++							20	2	e
C. vallicola	++	++								10	3	e
C. cartwrightianus 'Albus'			++	++						10	2	f
C. cartwrightianus			++	+	+	±		+	±	50	5	f
C. pallasii-white 92-6			+++							10	2	f
C. pallasii 90-12			+++							10	2	f
C. pallasii HNL 91-99			++	+	+	±				50	4	f
C. asumaniae			++	++	+					10	4	f
C. hadriaticus			+	+	++	+				10	4	f
C. oreocreticus			++	++	+	±				80	4	f
C. mathewii					+	++	+		+	30	5	f
C. sativus			++	+	+	±	±	+	±	120	5	f
C. ancyrensis					+++					10	1	g
C. cvjicii						+++				10	1	g
C. reticulatus ssp. *hittiticus*					++	++				80	1	g
C. reticulatus ssp. *reticulatus*					+++	+				80	1	g
C. gargaricus			+	+++						10	2	g
C. robertianus			+++							10	2	g
C. sieberi ssp. *nivalis*			+++	+						10	2	g
C. sieberi ssp. *sieberi*			++	++						50	2	g
C. sieberi ssp. *sublimis*			+++	+						40	2	g

Species	1	2	3	4	5	8	6	7	9	C_t†	Chemo types	Series
C. veluchensis	+	++	++	+						30	3	g
C. abantensis				±	++	++				40	4	g
C. angustifolius			+	+	++	+				70	4	g
C. angustifolius 'Minor'	±	±	±	+	+++	±				30	4	g
C. cancellatus ssp. *lycius*			+	+	+	++				10	4	g
C. cancellatus ssp. *mazziaricus*			±	+	++	++				10	4	g
C. sieberi ssp. *atticus*		±	++	++	±	+				20	4	g
C. reticulatus x C. angustifolius				+	+	++	±	±		20	5	gxg
C. chrysanthus 'Mugla'					++	++				30	1	h
C. chrysanthus					++	++				10	1	h
C. danfordiae					+++	+				20	1	h
C. danfordiae-white/blue					+++	+				10	1	h
C. biflorus ssp. *melantherus*					+++	+				20	1	h
C. adanensis			+++	+						30	2	h
C. biflorus ssp. *weldenii*			++	++						10	2	h
C. aerius			++	++	+					20	4	h
C. biflorus ssp. *taurii*	±	±	±	+	++	++				40	4	h
C. biflorus ssp. *crewei*				±	+++	+				120	4	h
C. biflorus ssp. *adamii* 'Serevan'		±	+	+	++	+	±		±	80	5	h
C. biflorus ssp. *isauricus*			±	±	++	+	+	+	±	60	5	h
C. biflorus ssp. *nubigena*					++	+	+	+	±	50	5	h
C. biflorus ssp. *pseudonubigena*					+++	+	+			20	5	h
C. biflorus ssp. *pulchricolor*				+	+	++	+	±	+	30	5	h
C. biflorus ssp. *punctatus*			±	+	+	++	+		±	20	5	h
C. leichtlinii					±	+	±	++	++	10	5	h
C. korolkowii					++	++				30	1	i
C. alatavicus					+++	+				70	1	i
C. flavus					+++					10	1	j
C. olivieri					+++					10	1	j
C. vitellinus					++	++				30	1	j
C. candidus			+	+	++	++				10	4	j
C. graveolens			+		++	+				10	4	j
C. antalyensis	±	+	±	+	+	+	++	±	+	50	5	j
C. 'Stellaris'	±	±	++	++	+					70	4	gxj
C. 'Golden Yellow'		+	++	+	++	+	±			10	5	gxj
C. carpetanus			+	++		++				10	4	l
C. fleicheri					++	++				10	1	m
C. pulchellus 'Albus'					++	++				10	1	n
C. speciosus 'Albus'					+++	+				10	1	n
C. pulchellus				+	±	+++				20	4	n

Species	1	2	3	4	5	8	6	7	9	C_t†	Chemo types	Series
C. pulchellus 'Zephyr'				+	+	+++				10	4	n
C. speciosus			±	±	+	+	±	+	++	70	5	n
C. laevigatus					+++	+				20	1	o
C. boryi										0		o
C. banaticus			±	±	+	+++				50	4	Crociris

Classification and authornames in accordance with Mathew (1982) (Table 6).

Key for Table 2 and 3: Anthocyanin structures of **1-9** see Table 1. Following compounds are included in the respective chemotypes; 1: anthocyanin **5** and **8**; 2 : **3** and **4**; 3: **1-4** ; 4: **1-5**, **8** and 5: **1-9**.

Rating of anthocyanins in relative concentration on HPLC. +++: high, ++: intermediate, +: low, ±: trace.

C_t†: Approximate total anthocyanin concentration in μM. C_t was calculated from the HPLC chromatograms by using the extinction coefficient of malvidin 3,5-diglucoside ($\log \varepsilon = 4.58$) (Wrolstad, 1993).

Table 3. Distribution of anthocyanins in *Crocus* series Biflori (h) their cultivars and hybrids.

Cultivars and produced hybrids	1	2	3	4	5	8	6	7	9	C_i†	Chemo types
C. danfordiae-yellow					+++	+				20	1
C. danfordiae–white/blue					+++	+				10	1
C. chrysanthus					++	++				20	1
C. chrysanthus 'Mugla'					++	++				30	1
C. biflorus ssp. *melantherus*					+++	+				20	1
C. biflorus ssp. *crewei*				(±)	+++	+				120	1
C. 'Ard Schenk'					+++					10	1
C. 'Blue Bird'					+++	+				30	1
C. 'Creme Beauty'					+++	+				10	1
C. 'Elegance'					+++	+				20	1
C. 'Eye-catcher'					+++	+				170	1
C. 'Fairy'					+++					20	1
C. 'Fuscotinctus'					+++					20	1
C. 'Gipsy Girl'					++	++				10	1
C. 'Harlequin'					+++	+				30	1
C. 'Herald'					++	++				60	1
C. 'Jeannine'					+++	+				20	1
C. 'Major'					+++	+				120	1
C. 'Miss Vain'					+++					10	1
C. 'Mrs. Moon'					+++					10	1
C. 'Romance'					+++					10	1
C. 'Saturnus'					+++	+				10	1
C. 'Snowbunting'					+++					10	1
C. 'Spotlight'					+++	+				20	1
C. 'Sulphur'					+++					10	1
C. 'Warley'					+++	+				20	1
C. adanensis			+++	+						30	2

Cultivars and produced hybrids	1	2	3	4	5	8	6	7	9	C_t†	Chemo types
C. biflorus ssp. *weldenii*			++	++						20	2
C. aerius			++	++	+					20	4
C. biflorus ssp. *tauri*	±	±	±	+	++	++				40	4
C. 'Zenith'			±	+	++	++				40	4
C. biflorus ssp. *adamii* 'Serevan'		±	+	+	++	+	±		±	80	5
C. biflorus ssp. *nubigena*					++	+	+	+	±	50	5
C. biflorus ssp. *isauricus*			±	±	++	+	+	+	±	60	5
C. biflorus 'Parkinsonii'					++	++	±	+	+	110	5
C. biflorus ssp. *pseudonubigena*					+++	+	+			20	5
C. biflorus ssp. *pulchricolor*				+	+	++	+	±	+	30	5
C. biflorus ssp. *punctatus*			±	+	+	++	+		±	20	5
C. leichtlinii					±	+	±	++	++	10	5
C. 'Advance'			±	±	++	+	+	+		30	5
C. 'Aubade'					+	+++	+			40	5
C. 'Blue Jay'					+	++	+			20	5
C. 'Blue Pearl'			+	++	++	+	+			20	5
C. 'Blue Peter'			±	±	++	+	+	+	±	40	5
C. 'Brassband'					++	+	+	+	+	30	5
C. 'Brunette'			+	+	++	+	++			20	5
C. 'Goldilocks'			±	±	+++	±	+			10	5
C. 'Ladykiller'					++	++		+		10	5
C. 'Parkinsonii'				+	+	±	+	++	+	30	5
C. 'Prins Claus'			±	±	++	+	+	±	±	10	5
C. 'Prinses Beatrix'					++	+	+	+		20	5
C. 'Skyline'					±	+	+	++	+	70	5
C. 'Spring Pearl'					+	+	+	+	±	40	5
C. 'Zwanenburg Bronze'	±	±	±	±	+++	+	±	±		100	5
C. aerius x *C. adanensis*			±	+	+	++	+	++		20	5
C. chrysanthus x *C. biflorus* ssp. *pulchricolor*				±	++	+	++	+	±	10	5
C. chrysanthus x *C.* 'Major'					++	++	±	±		10	5
C. 'Major' *x C. biflorus* ssp. *crewei*	±	±	±	±	++	++	±			60	5
C. 'Major' x *C. chrysanthus*					++	+	±	±		40	5

Key: see Table 2.

Table 4. Distribution of major flavonoids in *Crocus* species. Grouping in accordance to the major composition of flavonoids.

Crocus species	I	II	III	IV	A	Series
C. etruscus			+	+	0.24	a
C. baytopiorum		+	+		0.007	a
C. kosanini			+	+	0.33	a
C. vernus scepusiensis 234			+	+	0.57	a
C. vernus 95-101			+	+	0.20	a
C. vernus 95-115			+	+	0.090	a
C. tommasinianus			+	+	0.43	a
C. pelistericus			+	+	0.15	b
C. minimus				+	0.17	c
C. corcicus				+	0.53	c
C. imperati				+	0.21	c
C. versicolor				+	0.09	c
C. longiflorus		+	+	+	0.17	d
C. niveus			+	+	0.046	d
C. serotinus ssp. *clusii*		+	+	+	0.083	d
C. serotinus ssp. *salzmanii*		+	+	+	0.053	d
C. medius				+	0.21	d
C. nudiflorus		+		+	0.082	d
C. kotchyanus		+		+	0.058	e
C. vallicola			+		0.007	e
C. cartwrightianus 'Albus'			+	+	0.042	f
C. cartwrightianus			+	+	0.50	f
C. pallasii white 92-6	+		+		0.004	f
C. pallasii 90-12	+		+		0.009	f
C. pallasii HNL 91-99	+		+		0.063	f
C. asumaniae				+	0.019	f
C. hadriaticus				+	0.013	f
C. oreocreticus				+	0.55	f
C. mathewii	+				0.089	f
C. sativus		+		+	0.39	f

Crocus species	I	II	III	IV	A	Series
C. ancyrensis	+		+	+	0.018	g
C. cvicijii			+	+	0.005	g
C. reticulatus ssp. *hittiticus*			+	+	0.31	g
C. reticulatus ssp. *reticulatus*			+	+	0.044	g
C. gargaricus				+	0.016	g
C. robertianus		+	+		0.035	g
C. sieberi ssp. *nivalis*		+		+	0.034	g
C. sieberi ssp. *sieberi*		+		+	0.11	g
C. sieberi ssp. *sublimis*		+		+	0.11	g
C. veluchensis			+		0.14	g
C. abantensis		+		+	0.14	g
C. angustifolius		+	+	+	0.020	g
C. angustifolius 'Minor'		+	+	+	0.16	g
C. cancellatus ssp. *lycius*			+	+	0.038	g
C. cancellatus ssp. *mazziaricus*			+	+	0.014	g
C. sieberi ssp. *atticus*		+			0.048	g
C. reticulatus x *C. angustifolius*			+	+	0.070	gxg
C. chrysanthus 'Mugla'	+		+	+	0.003	h
C. chrysanthus	+		+	+	0.003	h
C. danfordiae				+	0.061	h
C. danfordiae –white/blue				+	0.012	h
C. biflorus ssp. *melantherus*	+			+	0.070	h
C. adanensis		+			0.058	h
C. biflorus ssp. *weldenii*				+	0.086	h
C. aerius				+	0.11	h
C. biflorus ssp. *adamii* 'Serevan'			+		0.51	h
C. biflorus ssp. *tauri*	+		+		0.20	h
C. biflorus ssp. *crewei*	+		+		0.18	h
C. biflorus ssp. *isauricus*				+	0.24	h
C. biflorus ssp. *nubigena*				+	0.28	h
C. biflorus ssp. *pseudonubigena*				+	0.036	h
C. biflorus ssp. *pulchricolor*				+	0.27	h
C. biflorus ssp. *punctatus*				+	0.081	h
C. leichtlinii			+		0.055	h
C. korolkowii	+	+			0.19	i
C. alatavicus				+	0.20	i

Crocus species	I	II	III	IV	A	Series
C. flavus	+			+	0.005	j
C. olievieri	+		+		0.016	j
C. vitellinus	+			+	0.12	j
C. candidus			+		0.004	j
C. graveolens			+	+	0.020	j
C. antalyensis	+	+	+		0.041	j
C. 'Stellaris'			+	+	0.004	g x j
C. 'Golden Yellow'			+		0.004	g x j
C. carpetanus	+		+		0.025	l
C. fleicheri		+		+	0.019	m
C. pulchellus 'Albus'			+	+	0.017	n
C. speciosus 'Albus'			+	+	0.006	n
C. pulchellus			+	+	0.24	n
C. pulchellus 'Zephyr'			+	+	0.011	n
C. speciosus			+	+	0.16	n
C. laevigatus			+		0.10	o
C. boryi			+		0	o
C. banaticus				+	0.12	Crociris

Classification and author names in accordance with Mathew (1982) (Table 6).

Key:

A: absorption of anthocyanin at 535 nm divided by the absorption of flavonoid at 360 nm.

Grouping by contents of flavonoid structures **10**-**27**, see Table 1. Enzymes shown in parantheses.

1: >20% **19**, **20** (FGT, 2ORT)

2: >30% **14**, **18** (FGT, 2OGT^1)

3: >20% **15**, **16**, **17** (FGT,F4'OG)

4: >60% **11**, **12**, **13**, **21**, **22**, **23**, **25**, (FRT, F7OG, 2OGT^2, 3OGT, AT)

FGT, Flavonoid 3-*O*-glucosyltransferase; 2ORT, Flavonoid 2-*O*- rhamnosyl transferase; 2OGT^1, Flavonoid 2-*O*-glucosyltransferase (3-*O*-sophoroside); F4'OG, Flavonoid 4'-*O*-glucosyltransferase; FRT, Flavonoid 3-*O*-rhamnosyltransferase; F7OG, Flavonoid 7-*O*-glucosyltransferase; 2OGT^2, Flavonoid 2-*O*-glucosyltransferase (3-*O*-(2-*O*-glucosyl)rhamnoside); 3OGT, Flavonoid 3-O-glucosyltransferase; AT, acyltransferase.

The minor components; 7-*O*-glucosides of dihydro-kaempferol (**10**) and apigenin (**24**) and and the major components; 3-*O*-glucosides of quercetin (**26**) and kaempferol (**27**) were not included in groupings.

Table 5. Grouping in accordance to the major composition of flavonoids in *Crocus* series (h), their cultivars and hybrids.

Cultivars	I	II	III	IV	A
C. danfordiae–white/blue				+	0.012
C. danfordiae-yellow				+	0.061
C. chrysanthus	+		+	+	0.004
C. chrysanthus 'Mugla'	+		+	+	0.003
C. biflorus ssp. *melantherus*	+			+	0.070
C. biflorus ssp. *creweii*	+		+		0.18
C. 'Ard Schenk'				+	0.0008
C. ' Blue Bird'				+	0.062
C. 'Crème Beauty'			+	+	0.019
C. 'Elegance'			+	+	0.12
C. 'Eye-catcher'			+	+	0.42
C. 'Fairy'			+	+	0.050
C. 'Fuscotinctus'			+	+	0.025
C. 'Gipsy Girl'			+	+	0.01
C. 'Harlequin'		+		+	0.055
C. 'Herald'			+	+	0.087
C. 'Jeannine'				+	0.050
C. 'Major'			+	+	0.28
C. 'Miss Vain'			+	+	0.002
C. 'Mrs. Moon'				+	0.007
C. 'Romance'			+	+	0.01
C. 'Saturnus'				+	0.031
C. 'Snowbunting'			+	+	0.020
C. 'Spotlight'				+	0.035
C. 'Sulphur'				+	0.01
C. 'Warley'				+	0.056
C. adanensis		+			0.058
C. biflorus ssp. *weldenii*				+	0.086
C. aerius				+	0.11
C. biflorus ssp. *tauri*	+		+		0.20
C. biflorus ssp. *adamii* 'Serevan'			+		0.51
C. 'Zenith'			+	+	0.081
C. biflorus ssp. *nubigena*				+	0.28
C. biflorus ssp. *isauricus*				+	0.24
C. biflorus 'Parkinsonii'				+	0.24
C. biflorus ssp. *pseudonubigena*				+	0.036
C. biflorus ssp. *pulchricolor*				+	0.27
C. biflorus ssp. *punctatus*				+	0.081

Cultivars	I	II	III	IV	A
C. leichtlinii			+		0.055
C. 'Advance'				+	0.050
C. 'Aubade'				+	0.076
C. 'Blue Jay'				+	0.036
C. 'Blue Pearl'				+	0.23
C. 'Blue Peter'				+	0.022
C. 'Brassband'			+	+	0.053
C. 'Brunette'			+	+	0.034
C. 'Goldilocks'			+	+	0.002
C. 'Ladykiller'			+	+	0.023
C. 'Parkinsonii'				+	0.23
C. 'Prins Claus'				+	0.069
C. 'Prinsses Beatrix'				+	0.087
C. 'Skyline'			+	+	0.035
C. 'Spring Pearl'			+	+	0.060
C. 'Zwanenburg Bronze'			+	+	0.21
C. aerius x *C. adanensis*		+		+	0.25
C. chrysanthus x *C. biflorus* ssp. *pulchricolor*				+	0.041
C. chrysanthus x C. 'Major'			+	+	0.089
C. 'Major' x *C. biflorus* ssp. *crewei*			+	+	0.23
C. 'Major' x *C. chrysanthus*			+	+	0.067

Key: see Table 4.

Table 6.

Nomenclature and origin of plant material. Voucher specimen are being held at the Royal Veterinary and Agricultural University, Department of Ecology, Botanical Section, and refered to by the number in parentheses.

C. abantensis T. Baytop & B. Mathew (P 1993-5312)
C. adanensis T. Baytop & B. Mathew (P 1993-5235)
C. aerius Herbert (P1993-5313)
C. alatavicus Semenov & Regel (C 107)
C. ancyrensis (Herbert) Maw (C 32)
C. angustifolius Weston (C 116)
C. angustifolius Weston 'Minor' (C 247)
C. antalyensis B. Mathew (92-16)
C. asumaniae B. Mathew & T. Baytop (C 338)
C. banaticus Gay (P 1992-5240)
C. baytopiorum B. Mathew (P 1980-5094)
C. biflorus Miller ssp. *crewei* (Hook) B. Mathew (92-38)
C. biflorus Miller ssp. *isauricus* (Bowles) B. Mathew (90-33)
C. biflorus Miller ssp. *melantherus* (Boiss. & Orph.) B. Mathew (93-26)
C. biflorus Miller ssp. *nubigena* Herbert(92-44)
C. biflorus Miller ssp. *pseudonubigena* B. Mathew (C 339)
C. biflorus Miller ssp. *pulchricolor* (Herbert) B. Mathew (C 100)
C. biflorus Miller ssp. *punctatus* B. Mathew (90-18)
C. biflorus Miller ssp. *adamii* (Gay) B. Mathew 'Serevan' (C 158)
C. biflorus Miler. ssp. *tauri* (Maw)B. Mathew (C 404)
C. biflorus Miller ssp. *weldenii* (Hoppe & Furnrohr) B. Mathew (95-110)
C. boryi Gay (P 25313)
C. cancellatus Herbert ssp. *lycius* B. Mathew (92-7)
C. cancellatus Herbert ssp. *mazziaricus* (93-27)
C. candidus Clarke (P 1992-5261)
C. carpetanus Boiss & Reut. (P 1994-5410)
C. cartwrightianus Herbert (C 236)
C. carwrightianus Herbert 'Albus'(C 253)
C. chrysanthus Herbert (P 1992-5264)
C. chrysanthus (Herbert)Herbert 'Mugla' (92-74)
C. corsicus Maw (C 96)
C. cvjicii Kosanin (93-49)
C. danfordiae Maw–yellow (90-80)
C. danfordiae Maw-white/blue (90-71)
C. etruscus Parl. (C 410)
C. flavus Weston (C 49)
C. fleicheri Gay (90-27)
C. gargaricus Herbert (P 1992-5270)
C. graveolens Boiss. (P 1992-5272)
C. hadriaticus Herbert (P 1979-5459)
C. imperati Ten. (95-54)
C. korolkowii Maw (C 170)
C. kosaninii 'Pulevic' (C 139)
C. kotchyanus K. Koch (C 78)
C. laevigatus Bory & Chaub. (94-20)
C. leichtlinii Bowles (C 351)
C. longiflorus Raf. (P 1992-5293)
C. mathewii Kerndorff & Pasche (92-4B)
C. medius Balb. (C 84)
C. minimus DC. (C 15)

C. niveus Bowles (P 1992-5298)
C. nudiflorus Smith(P 1992-5300)
C. olivieri Gay (G 88-3-2)
C. oreocreticus Burtt(94-26)
C. pallasii Goldb. (90-12)
C. pallasii Goldb.-white (92-6)
C. pallasii Goldb. (HNL 91-99)
C. pallasii Goldb. (90-35)
C. pelistericus Pulevic (C 350)
C. pulchellus Herbert 'Albus'(C 407)
C. pulchellus Herbert (93-41)
C. pulchellus Herbert 'Zephyr'(C 242)
C. reticulatus Adams ssp. *hittiticus* (T. Baytop & B. Mathew) B. Mathew (C 259)
C. reticulatus Adams ssp. *reticulatus* (C 149)
C. robertianus C. Brickell (P 1986-5411)
C. sativus L. (C 340)
C. serotinus Salisb. ssp. *clusii* (Gay) B. Mathew (P 1992-5319)
C. serotinus Salisb. ssp. *salzmanii* (Gay) B. Mathew (C 429)
C. sieberi Gay ssp. *atticus* (Boiss & Orph.) B. Mathew (93-4)
C. sieberi Gay ssp. *nivalis* (Bory & Chaub.) B. Mathew (C 441)
C. sieberi Gay ssp. *sieberi* (90-03-02)
C. sieberi Gay ssp. *sublimis* (Herbert) B. Mathew (93-8)
C. speciosus M. Bieb. 'Albus'(C 11)
C. speciosus M. Bieb. (C 79)
C. tommasinianus Herbert (C 413)
C. vallicola Herbert (C 123)
C. veluchensis Herbert (C s.n.)
C. vernus (L.) Hill (95-101)
C. vernus (L.) Hill (95-115c)
C. vernus (L.) Hill-*scepusiensis* (C 234)
C. versicolor Ker-Gawler (C 446)
C. vitellinus Wahlenb. (P 1992-5340)

C. 'Advance' (C 82)
C. 'Ard Schenk' (C 165)
C. 'Aubade' (C 22)
C. 'Blue Bird' (C 30)
C. 'Blue Jay' (C 391)
C. 'Blue Pearl' (C 24)
C. 'Blue Peter' (C 169)
C. 'Brass band' (C 166)
C. 'Brunette' (C 226)
C. 'Creme Beauty' (C 31)
C. 'Elegance' (C 168)
C. 'Eye-catcher' (C 38)
C. 'Fairy' (C 171)
C. 'Fuscotinctus' (C 35)
C. 'Gipsy Girl'(C 164)
C. 'Golden Yellow' (C 67)
C. 'Goldilocks' (C 167)
C. 'Harlequin' (C 279)
C. 'Herald' (C 163)
C. 'Jeannine' (C 263)
C. 'Ladykiller' (C 62)
C. 'Major' (C 40)
C. 'Miss Vain' (C 128)
C. 'Mrs. Moon' (C 80c)
C. 'Parkinsonii'(C 94)
C. 'Prinses Beatrix' (C 81)
C. 'Prins Claus' (C 85)

C. 'Romance' (C 205)
C. 'Saturnus' (C 45)
C. 'Skyline' (C 37)
C. 'Snow Bunting' (C 161)
C. 'Spotlight'(C 34b)
C. 'Spring Pearl' (C 162)
C. 'Stellaris'(C 246)
C. 'Sulphur' (C 195)
C. 'Warley'(C 70)
C. 'Zenith' (C 160)
C. 'Zwanenburg Bronze' (C 155)
C. aerius x *C. adanensis* (CC 203)
C. 'Major' x *C. creweii* (CC 518)
C. 'Major' x *C. chrysanthus* (CC 197)
C. chrysanthus x C. 'Major (CC 537)
C. chrysanthus x *C. biflorus* ssp. *pulchricolo*r (CC 532)
C. reticulatus x *C. angustifolius* (CC 67)

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