Flavone 8-*C*-Glycosides from *Haberlea rhodopensis* FRIV. (Gesneriaceae)

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Phytochemical profiling of a MeOH extract from *Haberlea rhodopensis* by a combination of liquid/liquid extraction, and preparative and semi-preparative HPLC afforded three new flavone *C*-glycosides, hispidulin-8-*C*-(2″-*O*-syringoyl)-*β*-glucopyranoside (**3**), hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside) (**4**), and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside) (**5**), along with two known phenolic glycosides, myconoside (**1**) and paucifloside (**2**). The structures were established by extensive spectroscopic analyses including 1D- and 2D-NMR (COSY, HSQC, and HMBC), and HR-ESI-TOF-MS, and by comparison with published data.

Introduction. – *Haberlea rhodopensis* FRIV. (Gesneriaceae) is a rare perennial herbaceous plant native of the Balkans. In Bulgaria, it can still be found in natural habitats. *H. rhodopensis* is a poikilohydric species which is highly desiccation-tolerant and able to revive upon rehydration of vegetative tissues even after prolonged periods of complete dehydration. Its behavior under dehydration and rehydration has been the subject of several photosynthetic and metabolic studies [1]. The genus *Haberlea* counts only two species [1][2], *H. rhodopensis* FRIV. and *H. ferdinandi-coburgii* URUM., the latter being very close to and eventually conspecific with the first [3].

The chemistry of the family Gesneriaceae is poorly known. Flavonoids, anthocyanins, caffeoyl phenylethanoid glycosides, tannins, zeaxanthin, and ascorbate have been reported from various genera of the family [2][4–8]. *H. rhodopensis* has been studied so far only in a phytochemical survey of the family Gesneriaceae, whereby the occurrence of myconoside, a caffeoyl phenylethanoid glucoside, was reported [5].

In this article, we describe the isolation and structure elucidation of three new flavone *C*-glycosides from the leaves of *Haberlea rhodopensis*, hispidulin 8-*C*-(6-*O*-acetyl-*β*-glucopyranoside)[1]) (**4**), hispidulin 8-*C*-(2-*O*-syringoyl-*β*-glucopyranoside)[1]) (**3**), and hispidulin 8-*C*-(6-*O*-acetyl-2-*O*-syringoyl-*β*-glucopyranoside)[1]) (**5**), along with two known caffeoyl phenylethanoid glucosides (CPGs), myconoside (**1**) and paucifloside (**2**) (*Fig. 1*).

[1]) For systematic names, see *Exper. Part*.

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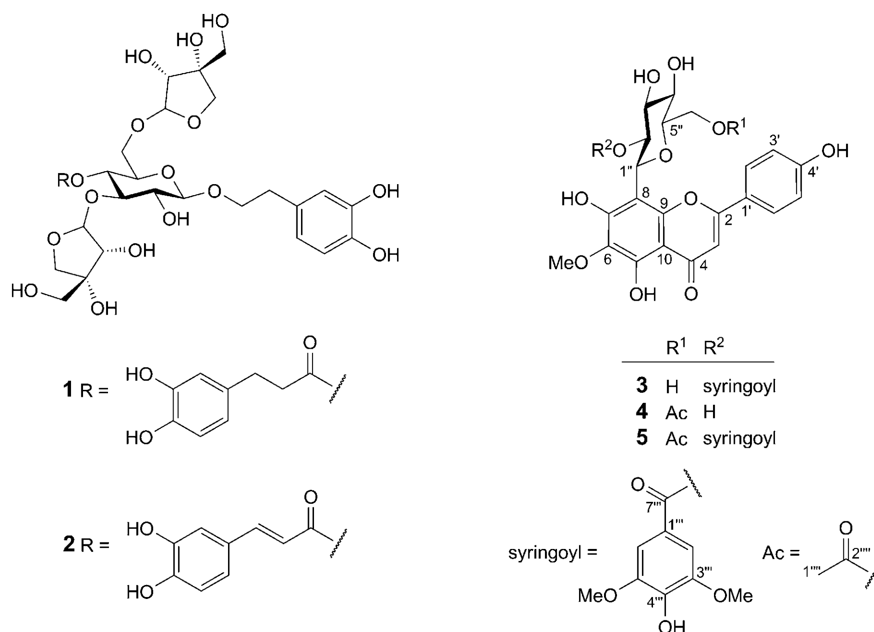

Fig. 1. *Structures of compounds* **1**–**5**

Results and Discussion. – The main chemical constituents of a MeOH extract of *H. rhodopensis* leaves were investigated with a combination of LC-PDA/ESI-MS, HR-ESI-TOF-MS, and microprobe NMR. The extract was partitioned into $CHCl_3$-, BuOH-, and H_2O-soluble fractions. Analysis of the BuOH portion by reversed-phase (RP) HPLC revealed the presence of five main peaks with t_R of 13.7, 14.5, 18.3, 19.6, and 21.1 min, respectively (*Fig. 2*). The target peaks were subsequently collected by preparative HPLC and subjected to semi-preparative HPLC for final purification. The two phenylethanoids myconoside (**1**) and paucifloside (**2**) were identified by comparison with published data [6][9].

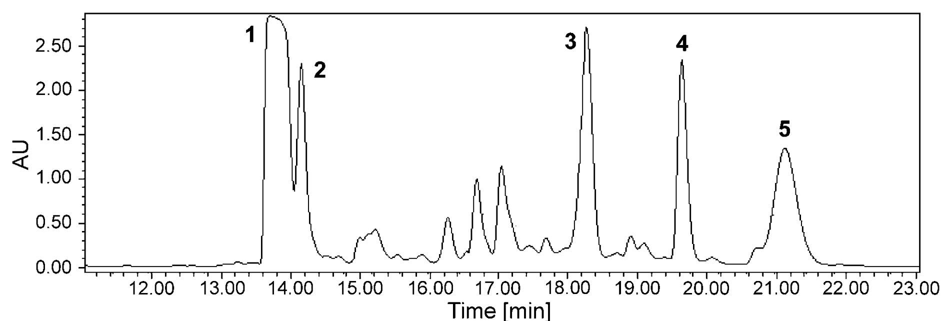

Fig. 2. *HPLC of the BuOH portion obtained by partitioning of MeOH extract of* H. rhodopensis. Detection at 275 nm. Peak numbers correspond to compounds **1**–**5**.

The molecular formula of **4** was established as $C_{24}H_{24}O_{12}$ by ESI-TOF-MS ($[M+Na]^+$ peak at *m/z* 527.1219). The UV spectrum in MeOH showed absorption maxima at 277 and 338 nm, indicative of a flavone. With the aid of UV/VIS shift reagents, phenolic groups at C(5), C(7), and C(4′) were identified [10][11]. The ^{13}C-NMR spectrum showed 16 signals for sp^2-C-atoms, which could be assigned to two aromatic rings, a CO group, an AcO moiety, and an additional O-bearing C-atom. Other signals included that of a MeO group and six resonances indicative of a hexosyl moiety. In the ^{1}H-NMR spectrum, a *singlet* at δ(H) 6.82 was indicative of H–C(3) of a flavone. Resonances of an $AA'BB'$ system at δ(H) 8.29 (2 H, $J=8.5$) and δ(H) 6.90 (2 H, $J=8.5$) suggested a *para*-substitution of ring *B* (*Table*). The sugar moiety was identified by a careful analysis of vicinal coupling patterns as β-glucopyranose, with a J(1″,2″) value of 10.1 Hz confirming β-linkage. The chemical shift of C(1″) (δ(C) 74.5) was indicative of a *C*-glycosidic linkage [4][12]. Analysis of ^{1}H-NMR spectra confirmed that ring *A* was fully substituted (*Table*). The position of the glucosyl moiety was established with the aid of an HMBC experiment, in which long-range correlations were observed between H–C(1″) (δ(H) 4.73), and C(7), C(8), and C(9) (δ(C) 155.6, 104.3, and 151.8 ppm, resp.; *Supplementary Material*[2])). The MeO group at C(6) was confirmed by a correlation between the resonance at δ(H) 3.79 and the quaternary C-atom signal at δ(C) 129.7. Finally, the attachment of the AcO moiety at C(6″) was established *via* three-bond correlations between CH_2(6″) (δ(H) 4.09 and 4.29) and the ester CO (δ(C) 170.8 (C(2‴))). The *Table* compiles NMR chemical-shift assignments for **4** which are similar to literature values for the structurally related flavone hispidulin *C*-glycoside [13][14]. Thus, compound **4** was identified as hispidulin 8-*C*-(6-*O*-acetyl-β-glucopyranoside).

Compound **3** had a molecular formula $C_{31}H_{30}O_{15}$ as deduced from ESI-TOF-MS ($[M+H]^+$, 643.1697). The UV spectrum was similar to that of **4**, with absorption maxima at 275 and 335 nm, and behavior with UV/VIS shift reagents was also comparable. The ^{13}C-NMR spectrum showed 22 signals for sp^2-C-atoms, which could be assigned to three aromatic rings, a quaternary O-bearing, a CH, and two CO C-atoms. In addition, resonances indicative of a hexosyl moiety and three aromatic MeO groups were present. A more detailed analysis of ^{1}H- and ^{13}C-NMR data confirmed a hispidulin 8-*C*-β-glucopyranoside core structure (*Table*). The acyl residue in **3** was identified as a syringoyl moiety (δ(H) 7.00 (*s*, H–C(2‴), H–C(6‴)), 3.77 (*s*, MeO–C(2), MeO–C(6)); δ(C) 164.3 (C(7‴)), 147.1 (C(3″), C(5″)), 140.0 (C(4″)), 119.6 (C(1″)), and 106.4 (C(2″), C(6″)). The position of the ester linkage was established *via* a HMBC cross-peak between H–C(2″) (δ(H) 5.82) of the glucosyl moiety and the ester CO C(7‴) (δ(C) 164.3). Full NMR assignments are available in the *Supplementary Material*[2]). ESI-MS^n Analysis was carried out in negative-ion mode to corroborate the structure. In MS^2, an ion at *m/z* 442 corresponded to the loss of a 198 fragment ($[M-\text{syringoyl}-H]^-$), and in MS^3, a fragment ion at *m/z* 323 resulted from the cleavage of the sugar moiety. On the basis of the above data, compound **3** was characterized as hispidulin 8-*C*-(2″-*O*-syringoyl-β-glucopyranoside).

Compound **5** was obtained as a yellow powder. The ESI-TOF-MS exhibited a molecular ion at *m/z* 685.1769 ($[M+H]^+$) corresponding to the molecular formula

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[2]) The *Supplementary Material* is available from the corresponding author.

Table. *^{13}C- and ^{1}H-NMR Spectroscopic Data for* **3**–**5**. Recorded in (D_6)DMSO; δ in ppm, J in Hz.

	3		**4**		**5**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
C(2)	164.9		164.4		164.2	
H–C(3)	102.2	6.73 (*s*)	102.1	6.82 (*s*)	101.5	6.73 (*s*)
C(4)	182.1		183.2		182.3	
C(5)	151.8		151.8		151.9	
C(6)	130.3		131.1		130.2	
C(7)	155.9		155.6		155.6	
C(8)	102.9		104.3		102.5	
C(9)	151.9		151.8		151.7	
C(10)	103.6		104.8		103.7	
C(1′)	121.0		121.2		120.9	
H–C(2′,6′)	129.8	8.37 (*d*, J = 8.6)	129.7	8.29 (*d*, J = 8.6)	129.7	8.34 (*d*, J = 8.6)
H–C(3′,5′)	115.8	6.93 (*d*, J = 8.6)	116.5	6.90 (*d*, J = 8.6)	115.9	6.91 (*d*, J = 8.6)
C(4′)	161.1		161.5		161.2	
H–C(1″)	71.9	5.08 (*d*, J = 10.1)	74.5	4.73 (*d*, J = 10.1)	71.7	5.14 (*d*, J = 10.1)
H–C(2″)	71.5	5.82 (*dd*, J = 10.1, 10.3)	68.3	4.23 (*dd*, J = 10.1, 9.1)	71.1	5.80 (*dd*, J = 10.1, 10.3)
H–C(3″)	72.7	3.90 (*dd*, J = 10,3, 7.6)	75.3	3.48 (*dd*, J = 9.1, 7.5)	72.2	3.92 (*dd*, J = 10.3, 7.6)
H–C(4″)	69.4	4.02 (*dd*, unres.)	69.8	3.79 (*dd*, unres.)	69.7	3.98 (*dd*, unres.)
H–C(5″)	80.8	3.72 (*m*)	77.2	3.91 (*m*)	77.1	4.00 (*m*)
CH_2(6″)	61.2	3.71 (*dd*, J = 11.5, 7.6), 3.67 (*dd*, J = 11.5, 4.5)	65.2	4.29 (*dd*, J = 11.5, 4.5), 4.09 (*dd*, J = 11.5, 7.6)	64.8	4.32 (*dd*, J = 11.5, 3.5), 4.18 (*dd*, J = 11.5, 7.2)
C(1‴)	119.6				119.4	
H–C(2‴,6‴)	106.4	7.00 (*s*)			106.4	7.00 (*s*)
C(3‴,5‴)	147.1				147.2	
C(4‴)	140.0				140.1	
C(7‴)	164.3				164.9	
Me(1⁗)			21.2	1.98 (*s*)	20.7	2.00 (*s*)
C(2⁗)			170.8		170.4	
MeO–C(6)	59.6	3.49 (*s*)	60.4	3.79 (*s*)	59.7	3.48 (*s*)
MeO–C(3‴,5‴)	55.9	3.77 (*s*)			55.9	3.77 (*s*)
HO–C(5)		13.39 (br. *s*)		13.33 (br. *s*)		13.44 (*s*)

$C_{33}H_{32}O_{16}$. The UV spectrum and the behavior with the usual UV/VIS shift reagents corresponded to those of **3** and **4**. The ^{13}C-NMR spectrum (*Table*) revealed signals which suggested the presence of a hispidulin 8-*C*-β-glucoside, and an AcO and a syringoyl moiety. The core structure and the attachment positions for the two CO moieties were confirmed by a detailed analysis of the HMBC correlations (*Fig. 3* and *Supplementary Material*). Thus, the AcO rest was at C(6″), and the syringoyl moiety at C(2″). The position was further corroborated by upfield shifts of C(1″) (δ(C) 71.7) and C(3″) (δ(C) 72.2) when compared with the corresponding resonances in **3**. ESI-MS^n Analysis of the molecular ion with the peak at m/z 683 ($[M - H]^-$) gave a fragment-ion peak at m/z 485 in MS^2 corresponding to the loss of the syringoyl residue, and to an ion peak at m/z 323 in MS^3 due to fragmentation of the sugar moiety (*Fig. 4*). The fragmentation pattern is comparable to other flavone *C*-glycosides [14][15].

Up to now, myconoside (**1**) was the only secondary metabolite known in the genus *Haberlea* [5]. This phenylethanoid glycoside has been reported in Gesneriaceae from

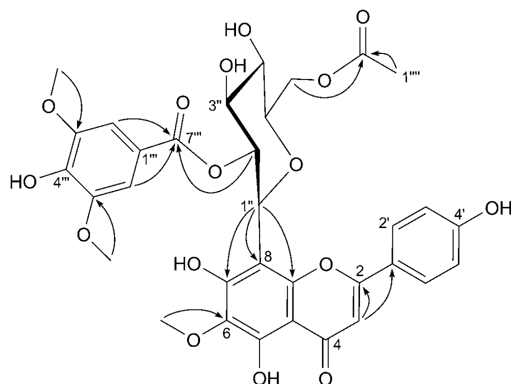

Fig. 3. *Key HMBC correlations for* **5**

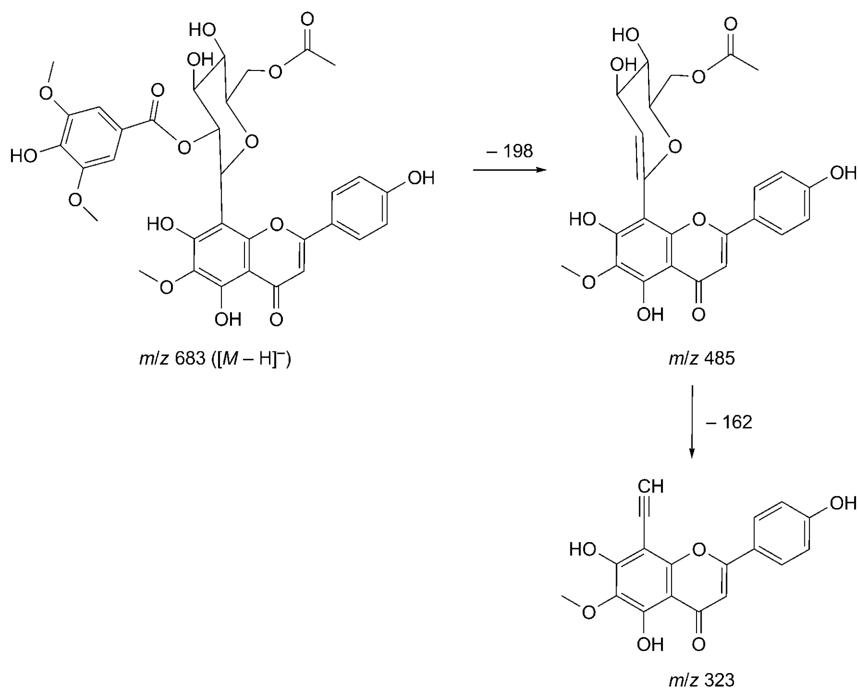

Fig. 4. *Proposed MS*^{n} *fragmentation for* **5**

the genus *Ramonda*, which belongs like *Haberlea* to the Didymocarpae in the subfamily Cyrtandroideae. According to our findings, myconoside is the dominant secondary metabolite in *H. rhodopensis.* We found the structurally closely related paucifloside (**2**) as a minor compound. On the basis of the approach proposed by *Jensen* [5], a possible co-occurrence of the minor metabolite paucifloside in *Ramonda* and other Gesneriaceae would likely not have been detected.

The structural diversity in flavone *C*-glycosides is rather limited, apigenin and luteolin being the two dominant aglycons and an 8-*C*-β-glucopyranosyl residue the most common glycosidic moiety. Hispidulin is much less frequent as aglycone, and hispidulin *C*-β-galactopyranoside was reported as its first *C*-glycoside only quite recently and, interestingly, from a Gesneriaceae [6].

Acylated flavone 8-*C*-glycosides are quite rare. A *SciFinder* search revealed that acyloxy moieties at C(2″) have been reported in 2″-*p*-coumaroyl vitexin from *Mollugo oppositifolia* (Molluginaceae) [16], in luteolin 8-*C*-{2″-*O*-[(*E*)-*p*-coumaroyl]-β-glucopyranoside} and apigenin 8-*C*-{2″-*O*-[(*E*)-*p*-coumaroyl]-β-glucopyranoside} from *Trigonella foenum-graecum* (Fabaceae) [17]. Further apigenin and luteolin 8-*C*-glycosides with 2″-*O*-galloyl residues have been found in *Cladogynos orientalis* (Euphorbiaceae) [18], 2″-*O*-(4-hydroxybenzoyl) esters in *Vitex altissima* (Lamiaceae) [19], 2″-*O*-vanilloyl and 2″-*O*-feruloyl derivatives in *Trollius ledebouri* (Ranunculaceae) [20], and a series of 2″-*O*-galloyl esters in *Geranium reniforme* (Geraniaceae) [14]. To the best of our knowledge, diacyl glycosides of flavone 8-*C*-glycosides have not been reported so far.

Thus, flavones **3**–**5** are the first acylated hispidulin *C*-glycosides, and possess some unique features such as 2″-*O*-syringoyl and/or an 6″-*O*-Ac moieties. Whether such metabolites can be found in other tribes and subfamilies of the Gesneriaceae remains to be investigated. Such an analysis will also clarify the chemotaxonomic significance of flavone *C*-glycosides in this family.

Experimental Part

General. Anal.-grade solvents for extraction and HPLC-grade solvents for chromatography were from *Scharlau* (E-Barcelona). HPLC-Grade H_2O was obtained by an *EASY-pure II* (Barnstead, Dubuque IA, USA) H_2O purification system. Deuterated solvents were purchased from *Armar Chemicals* (CH-Döttingen). Anal. HPLC separations were carried out on a system consisting of a *1100* series binary high-pressure mixing pump with degasser module, column oven and a *1100* series photodiode array (PDA) detector (all *Agilent*, D-Waldbronn). A *Gilson 215* liquid handler with a *Gilson 819* injection module and 50-µl loop was used as autosampler. The HPLC was coupled to an *Esquire 3000 Plus* ion-trap mass spectrometer equipped with an electrospray (ESI) interface (*Bruker Daltonics*, D-Bremen). Data acquisition and processing was performed using HyStar 3.0 software (*Bruker Daltonics*). Semi-prep. HPLC separations were carried out on an *Agilent 1100* series HPLC system consisting of a *1100* series quaternary low-pressure mixing pump with degasser module, column oven, a *1100* series PDA detector, and an autosampler with a 1000-µl loop. The prep. HPLC system consisted of a *Shimadzu SCL-10VP* controller and binary pump (LC-8A), a UV-VIS *SPD-M10A VP* detector and Class-VP 6.12 as software. Optical rotations: *Perkin-Elmer 341* polarimeter. UV/VIS Spectra: *Amersham Ultrospec 3100 pro* spectrophotometer. NMR Spectra: *Avance III* spectrometer, at 500 and 125 MHz for ^{1}H and ^{13}C, resp. (*Bruker Biospin*, CH-Fällanden); a 1-mm *TXI* probe was used, and data processing was performed with Topspin 2.1 (*Bruker*). High-resolution (HR) MS (ESI-TOF): *microTOF II* (*Bruker Daltonics*, D-Bremen).

Plant Material. The plant material originated from cultivated *Haberlea rhodopensis* grown by *Jakob Eschmann* nursery, CH-Emmen. The cultures had been originally established from seeds obtained from the Botanical Garden of Utrecht, the Netherlands. Voucher specimens are deposited with *Induchem AG* (No 12609) and at the Division of Pharmaceutical Biology, University of Basel (No 768).

Extraction and Isolation. Dried leaf material (240 g) was ground with a *ZM 1* ultracentrifugal mill (*Retsch*, D-Haan) equipped with a 0.75-mm *Conidur* ring sieve, and extracted by successive percolation with hexane, AcOEt, and MeOH (2 l each). After evaporation to dryness under reduced pressure, 38.1 g

of MeOH extract was obtained. The extract was suspended in dist. H_2O (1 l) and successively partitioned with $CHCl_3$ and BuOH (*Supplementary Material*). An aliquot (1.0 g) of the aq. phase was subjected to gel chromatography on *Sephadex LH-20* (MeOH) to give *myconoside* (**1**; 200 mg). An aliquot (200 mg) of the BuOH portion was dissolved in 2.0 ml of MeOH and separated by prep. HPLC (*SunFire C_{18}*, 5 μm, 30 × 150.0 mm i.d.; *Waters*) with 10–100% MeOH in H_2O (both containing 0.1% HCOOH), over 40 min; flow rate, 20 ml/min; injection volume, 200 μl. Collected peaks from prep. HPLC were evaporated and subjected to semi-prep. HPLC (*SunFire C_{18}*, 5 μm, 150 × 10.0 mm i.d.; *Waters*) with 10–100% MeOH in H_2O (both containing 0.1% HCOOH) over 40 min; flow rate 4.0 ml/min. Several injections yielded **2** (5.5 mg), **3** (4.5 mg), **4** (4.8 mg), and **5** (6.0 mg).

*(1*S*)-6-*O*-Acetyl-1,5-anhydro-1-[5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4-oxo-4*H*-1-benzopyran-8-yl]-*D*-glucitol* (**4**). Yellow powder. $[\alpha]^{20} = -10.5$ ($c = 0.16$, MeOH). UV (MeOH): 223, 277 (6.8), 338 (6.8). UV (MeOH + MeONa): 281, 332, 399. UV (MeOH + $AlCl_3$): 282, 305, 363. UV (MeOH + $AlCl_3$/HCl): 282, 305, 355. UV (MeOH + AcONa): 281, 312, 332, 390. UV (MeOH + AcONa/H_3BO_3): 282, 323, 350. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 505 ($[M+H]^+$). ESI-MS^n (neg.): 503 ($[M-H]^-$), 442 ($[M-61-H]^-$), 323 ($[M-180-H]^-$), 298 ($[M-205-H]^-$). HR-ESI-MS: 527.1219 ($[M+Na]^+$, $C_{24}H_{24}O_{12}^+$; calc. 527.1201).

*(1*S*)-1,5-Anhydro-1-[5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4-oxo-4*H*-1-benzopyran-8-yl]-2-*O*-[(4-hydroxy-3,5-dimethoxyphenyl)carbonyl]-*D*-glucitol* (**3**). Yellow powder. $[\alpha]^{20} = -23.2$ ($c = 0.11$, MeOH). UV (MeOH): 222 (7.2), 277 (6.9), 338 (7.1). UV (MeOH + MeONa): 283, 328, 397. UV (MeOH + $AlCl_3$): 281, 305 (sh), 362. UV (MeOH + $AlCl_3$/HCl): 282, 302, 355. UV (MeOH + AcONa): 280, 315, 332, 391. UV (MeOH + AcONa/H_3BO_3): 282, 322. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos.): 643 ($[M+H]^+$). ESI-MS (neg.): 641 ($[M-H]^-$), 443 ($[M-198-H]^-$), 323 ($[M-318-H]^-$). HR-ESI-MS: 645.1514 ($[M+Na]^+$, $C_{31}H_{30}O_{15}^+$; calc. 665.1482).

*(1*S*)-6-*O*-Acetyl-1,5-anhydro-1-[5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4-oxo-4*H*-1-benzopyran-8-yl]-2-*O*-[(4-hydroxy-3,5-dimethoxyphenyl)carbonyl]-*D*-glucitol* (**5**). Yellow powder. $[\alpha]^{20} = -31.3$ ($c = 0.12$, MeOH). UV (MeOH): 223 (7.2), 277 (7.0), 339 (6.9). UV (MeOH + MeONa): 281, 329, 397. UV (MeOH + $AlCl_3$): 278, 305 (sh), 362. UV (MeOH + $AlCl_3$/HCl): 282, 302, 355. UV (MeOH + AcONa): 280, 335, 395. UV (MeOH + AcONa/H_3BO_3): 282, 322. ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS (pos): 685 ($[M+H]^+$). ESI-MS^n (neg.): 683 ($[M-H]^-$), 485 $[M-198-H]^-$, 323 ($[M-360-H]^-$. HR-ESI-MS: 685.1769 ($[M+H]^+$, $C_{33}H_{32}O_{16}^+$; calc. 685.1769).

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