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Stilbenoids from *Tragopogon orientalis*

Christian Zidorn [a], Sandra Grass [a], Ernst P. Ellmerer [b], Karl-Hans Ongania [b], Hermann Stuppner [a,*]

[a] *Institut für Pharmazie der Universität Innsbruck, Josef-Moeller-Haus, Innrain 52, A-6020 Innsbruck, Austria*
[b] *Institut für Organische Chemie der Universität Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria*

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Abstract

A phytochemical investigation of *Tragopogon orientalis* L. (Asteraceae, Cichorieae) yielded the natural products 6″-*O*-(7,8-dihydrocaffeoyl)-α,β-dihydrorhaponticin, 3′-*O*-methyl-α,β-dihydrorhaponticin, and (*S*)-3-(4-β-glucopyranosyloxybenzyl)-7-hydroxy-5-methoxyphtalide as well as known compounds α,β-dihydrorhaponticin, 3-(4-methoxybenzyl)-5,7-dimethoxyphthalide, *p*-dihydrocoumaric acid methyl ester, and 1-hydroxypinoresinol-1-*O*-β-glucopyranoside. The structures were established by HR mass spectrometry, extensive 1D and 2D NMR spectroscopy, and CD spectroscopy. Moreover, the radical scavenging activities of the major compounds were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The chemosystematic impact of the occurrence of stilbene derivatives in *T. orientalis* is discussed.

Keywords: *Tragopogon*; Asteraceae; Tribe Cichorieae; Subtribe Scorzonerinae; Chemosystematics; Stilbenoids

1. Introduction

Tragopogon orientalis L. (synonym: *Tragopogon pratensis* L. subsp. *orientalis* Čelak.) is an annual to perennial herb of 30–70 cm height, golden yellow ligules and a cylindrical rootstock. The taxon is native to Eastern and Central Europe and Western Siberia. *T. orientalis* is common in Austria; formerly the rootstocks were used as a vegetable similar to black salsify and the leaves as a vegetable similar to spinach (Richardson, 1976; Wagenitz, 1987; Fischer et al., 2005; Jäger and Werner, 2005). Previous phytochemical studies of *T. orientalis* and *T. pratensis* yielded flavonoids, phenolic acids, sterols, triterpenes, triterpene glycosides, and triterpene saponins (Krzaczek and Smolarz, 1988; Smolarz and Krzaczek, 1988; Miyase et al., 1992).

Recently, a number of stilbenoids and biogenetically related dihydroisocoumarins have been isolated from *T. porrifolius* L. subsp. *porrifolius* (Zidorn et al., 2005a). The closely related genus *Scorzonera*, which like *Tragopogon* is a member of the subtribe Scorzonerinae, also yielded a number of biogenetically related dihydroisocoumarins, benzophenone, and dihydrostilbene derivatives. These compounds were reported from *Scorzonera cretica* Willd. (Paraschos et al., 2001) and *Scorzonera humilis* L. (Zidorn et al., 2000, 2002, 2003).

The present communication reports about new (**2**–**3**) and known (**1**) dihydrostilbenes, a new (**4**) and a known (**5**) benzylphtalide, a phenylpropanoid (**6**), and a lignan (**7**) from rootstocks of *T. orientalis* (see Fig. 1).

2. Results and discussion

Compounds **1**–**7** were isolated from rootstocks of *T. orientalis* collected in May 2004 near Innsbruck/Tyrol/Austria. The structures were assigned using NMR, MS, UV, optical rotation, and CD data.

In detail, HRMS of compound **1** in the positive mode showed a $[M+H]^+$ signal at $m/z = 423.1631$ (calculated

* Corresponding author. Tel.: +43 512 507 5300; fax: +43 512 507 2939.
E-mail address: Hermann.Stuppner@uibk.ac.at (H. Stuppner).

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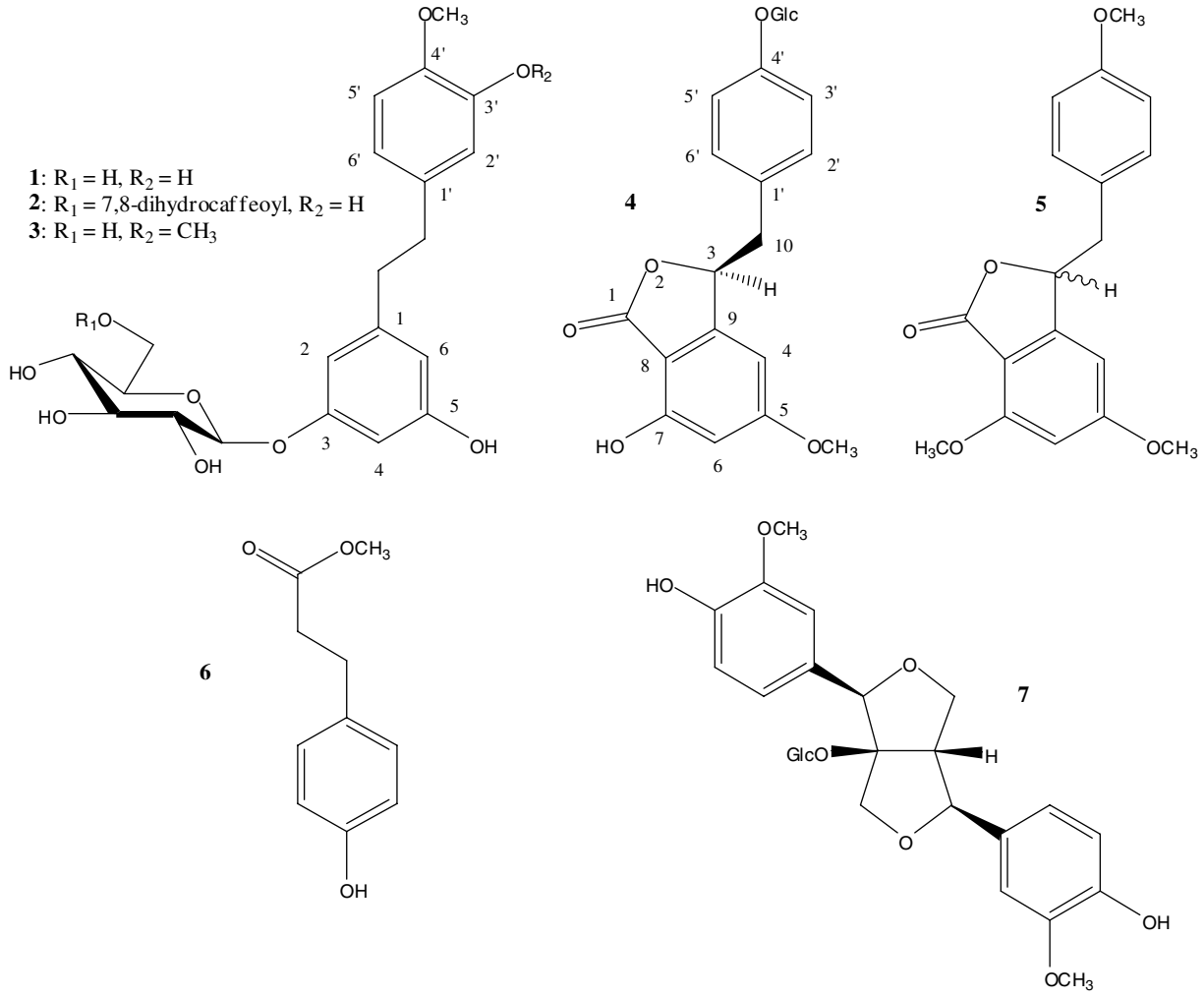

Fig. 1. Structures of new (**2**–**4**) and known (**1** and **5**–**7**) natural products from *Tragopogon orientalis*.

for $C_{21}H_{27}O_9$, $m/z = 423.1655$), which indicated a molecular formula of $C_{21}H_{26}O_9$. ^{1}H NMR (Table 1) and ^{13}C NMR (Table 2) data included signals assignable to a 3,5,3′,4′-tetra-substituted bibenzyl moiety, a β-glucose moiety, and a methoxy-group (Biondi et al., 2005). HMBC correlations indicated that the methoxy group was situated in position 4′ and that the glucose moiety was attached to one of the two oxygens of the other aromatic subunit (position 3). Conclusively, **1** is α,β-dihydrorhaponticin, a natural product, which was recently isolated from *Glycrrhiza glabra* L. leaves (Biondi et al., 2005). The aglycone of α,β-dihydrorhaponticin (**1a**) was synthesized from **1** by enzymatic hydrolysis. Identification was accomplished by mass spectrometry and NMR spectroscopy and by comparison of the obtained data with literature data (Matsuda et al., 2001; Hernandez-Romero et al., 2004; Biondi et al., 2005).

HRMS of compound **2** in the positive mode showed a $[M + H]^+$ signal at $m/z = 587.2160$ (calculated for $C_{30}H_{35}O_{12}$, $m/z = 587.2129$), which indicated a molecular formula of $C_{30}H_{34}O_{12}$. ^{1}H NMR (Table 1) and ^{13}C NMR data (Table 2) were similar to those of compound **1**, but showed additional signals assignable to a 7,8-dihydrocaffeoyl moiety (Zidorn et al., 2005b). Downfield shifts of the signals assignable to the protons in position C-6″ of the glucose moiety in comparison to compound **1** ($\delta_H = 4.42$ and 4.18 versus $\delta_H = 3.88$ and 3.70) indicated that the 7,8-dihydrocaffeoyl moiety was linked via this position with the rest of the molecule. This assumption was proven by HMBC experiments, which showed cross-peaks from the signals assignable to the protons at C-6″ to the carbonyl signal of the acyl moiety. Thus, compound **2** was identified as 6″-*O*-(7,8-dihydrocaffeoyl)-α,β-dihydrorhaponticin, which represents a new natural product.

HRMS of compound **3** in the positive mode showed a $[M + H]^+$ signal at $m/z = 437.1788$ (calculated for $C_{22}H_{29}O_9$, $m/z = 437.1812$), which indicated a molecular formula of $C_{22}H_{28}O_9$. ^{1}H NMR (Table 1) and ^{13}C NMR spectra (Table 2) were nearly identical to those of compound **1** but showed additional signals for a second methoxy group. HMBC experiments showed that this group was linked via position C-3′ with the molecule. Conclusively, compound **3** was established as 3′-*O*-methyl-α,

Table 1
^{1}H NMR data of stilbenoids **1**–**3**[a]

Position	1	2	3
Stilbenoid moiety			
1			
2	6.39 1H, *m*	6.33 1H, *t* (2.0)	6.40 1H, *t* (2.0)
3			
4	6.38 1H, *m*	6.35 1H, *t* (2.0)	6.38 1H, *t* (2.0)
5			
6	6.30 1H, *dd* (2.0, 1.5)	6.30 1H, *t* (2.0)	6.29 1H, *t* (2.0)
α	2.74 2H, *m*[b]	2.72 2H, *m*[b]	2.84 2H, *m*[b]
β	2.74 2H, *m*[b]	2.72 2H, *m*[b]	2.79 4H, *m*[b]
1′			
2′	6.65 1H, *d* (2.0)	6.62 1H, *d* (2.5)	6.71 1H, *dd* (2.0)
3′			
4′			
5′	6.79 1H, *d* (8.0)	6.75 1H, *d* (8.0)	6.84 1H,*d* (8.0)
6′	6.58 1H, *dd* (8.0, 2.0)	6.52 1H, *dd* (8.0, 2.5)	6.69 1H, *dd* (8.0, 2.0)
3′ –OCH_3			3.79 3H, *s*
4′ –OCH_3	3.81 3H, *s*	3.80 3H, *s*	3.77 3H, *s*
Glucose moiety			
1″	4.78 1H, *d* (7.5)	4.73 1H, *d* (7.5)	4.78 1H, *d* (7.0)
2″	3.42 1H, *m*[b]	3.41 1H, *m*[b]	3.41 1H, *m*[b]
3″	3.44 1H, *m*[b]	3.43 1H, *m*[b]	3.43 1H, *m*[b]
4″	3.41 1H, *m*[b]	3.32 1H, *m*[b]	3.38 1H, *m*[b]
5″	3.41 1H, *m*[b]	3.55 1H, *m*[b]	3.38 1H, *m*[b]
6″	3.88 1H, *dd* (12.0, 1.5)	4.42 1H, *dd* (12.0, 2.0)	3.88 1H, *dd* (12.0, 2.0)
	3.70 1H, *dd* (12.0, 5.0)	4.18 1H, *dd* (12.0, 7.0)	3.71 1H, *dd* (12.0, 5.0)
7,8-Dihydrocaffeoyl moiety			
1‴			
2‴		6.62 1H, *d* (2.0)	
3‴			
4‴			
5‴		6.63 1H, *d* (8.0)	
6‴		6.47 1H, *dd* (8.0, 2.0)	
7‴		2.73 2H, *m*	
8‴		2.57 2H, *m*	
9‴			

[a] Measured in methanol-d_4 at 300 MHz (**1**–**2**) and 500 MHz (**3**), respectively. Spectra were referenced to solvent residual at $\delta_H = 3.31$ ppm. ^{13}C NMR shift values of compound **3** were derived from HSQC and HMBC spectra.
[b] Overlapping signals.

Table 2
^{13}C NMR data of stilbenoids **1**–**3**[a]

Position	1	2	3
Stilbenoid moiety			
1	145.5	145.5	145.1
2	109.3	109.3	109.3
3	160.1	160.0	160.5
4	102.7[b]	103.0	102.4
5	159.2	159.2	159.8
6	110.7[b]	110.9	110.5
α	39.2	39.2	39.2
β	38.1	38.0	37.6
1′	136.1	136.0	136.0
2′	116.6[c]	116.6	113.7
3′	147.2	147.2	150.0
4′	147.2	147.2	148.4
5′	112.8[c]	112.8	112.4
6′	120.7	120.8	121.8
3′ –OCH_3			56.3
4′ –OCH_3	56.5	56.5	56.0
Glucose moiety			
1″	102.3	102.3	101.8
2″	74.9	74.8	75.0
3″	78.0	77.8	78.0
4″	71.4	71.8	71.2
5″	78.0	75.4	78.0
6″	62.5	64.8	62.4
7,8-Dihydrocaffeoyl moiety			
1‴		133.6	
2‴		116.5	
3‴		146.1	
4‴		144.6	
5‴		116.4	
6‴		120.6	
7‴		31.3	
8‴		37.2	
9‴		174.8	

[a] Measured in methanol-d_4 at 75 MHz (**1**–**2**) and 125 MHz (**3**), respectively. Spectra were referenced to solvent signals at $\delta_C = 49.0$ ppm. ^{13}C NMR shift values of compound **3** were derived from HSQC and HMBC spectra.
[b] Signal assignments for these two signals were erroneously interchanged by Biondi et al. (2005).
[c] Signal assignments for these two signals were erroneously interchanged by Biondi et al. (2005).

β-dihydrorhaponticin, which represents another new natural product. The aglycon of compound **3** was recently isolated as vittarin-A from *Vittaria anguste-elongata* Hayata (Wu et al., 2005).

HRMS of compound **4** in the positive mode showed a $[M + Na]^+$ signal at $m/z = 471.1233$ (calculated for $C_{22}H_{24}O_{10}Na$, $m/z = 471.1267$), which indicated a molecular formula of $C_{22}H_{24}O_{10}$. ^{1}H NMR and ^{13}C NMR data (Table 3) displayed signals assignable to a *para* substituted aromatic moiety, a tetrasubstituted aromatic moiety with the two protons in *meta* position to each other, a glucose moiety, a methine, and a methylene group. HMBC correlations indicated that compound **4** was a 3-benzylphtalide, i.e. a dihydrostilbene derivative, with a five-ring lactone from a carbonyl moiety connected to one of the aromatic systems to a hydroxy group in α-position to this system. The biogenetically related dihydroisocoumarins are dihydrostilbenes featuring a six-ring lactone, which is formed from a carbonyl moiety connected to one of the aromatic systems to a hydroxy group in β-position to this system. HMBC experiments also indicated that the glucose moiety was attached to the phenolic hydroxy-group of the *para* substituted aromatic system and that the methoxy group was linked to the one of the two phenolic hydroxy groups situated between the two aromatic protons of that system. Conclusively, compound **4** was established as 3-(4-β-glucopyranosyloxybenzyl)-7-hydroxy-5-methoxyphtalide. The configuration at C-3 was deduced from CD-spectra, which showed positive Cotton effects at 240 and 290 nm as well as a negative peak at 255 nm. As no CD spectra of

Table 3
NMR data of compound **4**

Position	^{1}H NMR	^{13}C NMR
Phtalide moiety		
1		171.6
3	5.59 1H, *t* (6.0)	81.8
4	6.36 1H, *s*	99.9
5		167.5
6	6.30 1H, *s*	102.4
7		161.0
8		106.0
9		154.8
10	3.21 1H, *dd* (14.5, 5.5)	40.3
	3.09 1H, *dd* (14.5, 6.0)	
5 –OCH_3	3.80 3H, *s*	56.2
Benzyl moiety		
1′		129.9
2′	7.09 1H, *d* (8.0)	131.8
3′	6.79 1H, *d* (8.0)	117.4
4′		157.7
5′	6.79 1H, *d* (8.0)	117.4
6′	7.09 1H, *d* (8.0)	131.8
Glucose moiety		
1″	4.86 1H [b]	101.8
2″	3.43 1H, *m*[c]	74.3
3″	3.42 1H, *m*[c]	78.0
4″	3.38 1H, *m*[c]	71.2
5″	3.40 1H, *m*[c]	77.4
6″	3.89 1H, *dd* (12.0, 2.0)	62.4
	3.69 1H, *dd*(12.0, 5.0)	

[a] Measured in methanol-d_4 at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR), respectively. Spectra were referenced to solvent residual and solvent signals at $\delta_H = 3.31$ ppm and $\delta_C = 49.0$ ppm, respectively. ^{13}C NMR shift values were derived from HSQC and HMBC spectra.
[b] ^{1}H NMR signal covered by signal of residual water.
[c] Overlapping signal.

3-benzylphtalides were available for comparison, the CD spectra of **4** were interpreted in comparison with 3-(4,7-dimethyl-1-naphthyl)phtalide (Pirkle et al., 1998), 6-hydroxymellein (Krohn et al., 1997), and 3-phenyldihydrosiocoumarins (Paraschos et al., 2001; Zidorn et al., 2005a). Compound **4** showed a CD-spectrum similar to (*R*)-3-(4,7-dimethyl-1-naphthyl)phtalide (Pirkle et al., 1998) and mirror-inverted to that of (*R*)-6-hydroxymellein (Krohn et al., 1997). Conclusively, (*S*)-configuration for position 3 was supposed and compound **4** was elucidated as (*S*)-3-(4-β-glucopyranosyloxybenzyl)-7-hydroxy-5-methoxyphtalide. Compound **4** represents a new natural product. 3-Benzylphtalides are a rare group of natural products; compounds similar to the aglycon of **4** have been reported from the liverwort genera *Balanteopsis* and *Frullania* (Asakawa et al., 1986, 1987, 2003; Kraut et al., 1994). To the best of our knowledge, compound **4** represents the first naturally occurring glucoside of a benzylphtalide.

Known compounds **5**–**7** were identified on the basis of their mass spectra and NMR as 3-(4-methoxybenzyl)-5,7-dimethoxyphthalide (**5**), a natural product known from *Frullania falciloba* Tayl. ex Lehm. (Asakawa et al., 1987, 2003; Mali et al., 2001), as *p*-dihydrocoumaric acid methyl ester (**6**) (Dorrestein et al., 2003), and as 1-hydroxypinoresinol-1-*O*-β-glucopyranoside (**7**) (Wang et al., 1993), respectively.

Compounds **1**, **1a**, and **2** were assessed (in triplicate) for their radical scavenging activity in comparison to the reference compound ascorbic acid. The following IC_{50}values in μg/ml and μmol/ml, respectively, were established for the reduction of 40 mg/l of the DPPH radical (values in brackets indicate standard deviations): IC_{50} (μg/ml): ascorbic acid, 2.29 (0.06); **1** 8.98 (0.52); **1a** 4.82 (0.31); **2** (9.04 (0.22). IC_{50} (μmol/ml): ascorbic acid, 13.0 (0.36); **1** 21.3 (1.24); **1a** 18.6 (1.20); **2** 15.4 (0.37). The differences in IC_{50} values of compounds **1**, **1a**, and **2** given in μmol/ml are statistically significant (Student–Newman–Keuls test; $p < 0.05$). Thus, compound **2**, which has three free phenolic hydroxy groups, two of them in *ortho*-position, is the best radical scavenger on a molar basis. The second best radical scavenging activity on a molar basis is displayed by compound **1a**, which also features three phenolic hydroxy groups, however none of them combined to an *ortho*-dihydroxy moiety. Compound **1**, with only two free phenolic hydroxy groups has the weakest radical scavenging activity of the three compounds analyzed. These results are in accord with the structure activity relationships for phenolic radical scavenging compounds as discussed by Rice-Evans et al. (1996).

The discovery of further dihydrostilbenes in *T. orientalis* corroborates the finding that taxa from the tribe Scorzonerinae are a rich source of this class of natural compounds (Paraschos et al., 2001; Zidorn et al., 2000, 2002, 2003, 2005a). Dihydrostilbenes are not occurring ubiquitously. However, dihydrostilbene derivatives are occurring in various only loosely related plant taxa, including the primitive and phylogentically old liverworts (Yoshida et al., 1996). Moreover, the distribution of dihydrostilbene derivatives in the higher plants (Trachaeophyta) is rather erratic. For example dihydrostilbenoids have been reported from ferns (Wollenweber et al., 1993), monocots (Adesanya et al., 1989), and Rosids (Biondi et al., 2005), including occasional reports from the Asteraceae family (Braca et al., 1999). Conclusively, dihydrostilbenoids are not employable as chemosystematic markers for higher categories (family level or above). Moreover, it is plausible to assume that the common ancestor of the Trachaeophyta already possessed the genetic and enzymatic requirements for the biosynthesis of these natural products. In many taxa of contemporary higher plants this system is either lost or switched off. The observed frequency and high structural diversity of dihydrostilbenes observed in the subtribe Scorzonerinae might be of pronounced chemosystematic interest for the phenetic characterization of this taxon, which due to recent (macro-)molecular investigations is monophyletic (Mavrodiev et al., 2004). The distribution of benzylphtalides, which are biogenetically closely related to dihydrostilbenes, follows the same erratic pattern; benzylphtalides have been reported from liverworts (Asakawa et al., 1986, 1987) as well as various families of

angiosperms [monocots e.g. Shode et al. (2002), Rosids e.g. Yoshikawa et al. (1992)].

3. Experimental

3.1. Plant material

T. orientalis was collected between Völs and Kematen W of Innsbruck/Tyrol/Austria [coordinates (WGS84): N 47°15′40″ E 11°19′01″; alt.: 590 m a.m.s.l.] on the 24th of May 2004. A voucher specimen (SG20040524-A1) was deposited in the herbarium of the Institut für Pharmazie/Innsbruck.

3.2. Extraction and isolation

3.2.1. Isolation of compounds 1–7

Air-dried ground rootstocks (240 g) of *T. orientalis* were exhaustively extracted with MeOH. The crude extract was dried *in vacuo* to give 54.0 g of residue. The residue was redissolved in a mixture of MeOH and H_2O and successively partitioned with petrol ether and EtOAc. The EtOAc layer was dried *in vacuo* yielding 2.50 g. The EtOAc layer was fractionated by silica gel column chromatography (CC) employing a gradient of CH_2Cl_2 and MeOH. Fractions were combined based on their TLC profiles (mobile phases: various mixtures of CH_2Cl_2 and MeOH; detection: UV/VIS, spraying with vanillin/H_2SO_4) resulting in 21 combined fractions (CF) of increasing polarity.

A mixture (3/2, 9.7 mg) of compounds **5** and **6** was isolated from CF-4 [values given in brackets indicate the mass of the respective fraction and the ratio of the solvent mixture during elution] (68.3 mg; CH_2Cl_2/MeOH, 9/1, v/v) by Sephadex LH-20 CC using MeOH as the eluant. Fractions CF-12 (57.7 mg; CH_2Cl_2/MeOH, 8/2, v/v), CF-13 (43.8 mg; CH_2Cl_2/MeOH, 7/3, v/v), and CF-14 (347 mg; CH_2Cl_2/MeOH, 7/3, v/v) were repeatedly fractionated by Sephadex LH-20 CC (eluant: MeOH). Sephadex LH-20 of CF-14 yielded 39.8 mg of compound **1**. Fractions of CF-14 enriched in compounds **2**, **3**, and **7** (31.5 mg) were combined with CF-13 and fractionated by Sephadex LH-20 to yield fractions further enriched in **2** (28.5 mg) and **3** and **7** (14.1 mg). Compound **2** (13.0 mg) was finally purified by Sephadex LH-20 CC. Sephadex LH-20 of these fractions enriched in **3** and **7** yielded 6.8 mg of a mixture of both compounds. This was combined with subfractions of CF-12 also enriched in **3** and **7** (5.2 mg) and subjected to semi-preparative RP-HPL chromatography using a gradient of H_2O and MeCN to yield 0.9 mg of compound **3** and 1.3 mg of compound **7**. Compound **4** was isolated from CF-15 (105 mg; CH_2Cl_2/MeOH, 5/5, v/v) and CF-16 (142 mg; CH_2Cl_2/MeOH, 5/5, v/v) by repeated Sephadex LH-20 CC and successive semi-preparative RP-HPL chromatography using a gradient of H_2O and MeCN to yield 2.2 mg of compound **4**.

3.2.2. Semi-preparative HPLC

Semi-preparative HPLC was performed using a Dionex P580 system with ASI-100 automated sample injector and an UVD17OU detector; fractions were collected employing a Gilson Abimed 206 fraction collector. For both separations a X-terra prep MS C18 7.8×100 mm column with 5 μm particle size was used. Compounds **3** and **7** were separated using a flow rate of 1.5 ml/min and a gradient from 20% MeCN to 40% MeCN in 20 min. Compounds **3** and **7** were collected in the intervals between 12.4 and 13.5 min and 8.5 and 9.5 min, respectively. Compound **4** was purified using a flow rate of 2.0 ml/min and a gradient 10–40% MeCN in 20 min. Compound **4** was collected in the interval between 12.2 and 13.0 min.

3.2.3. Enzymatic preparation of compound 1a from 1

Fractions enriched in compound **1** (30.6 mg) were dissolved in 1.3 ml of H_2O (adjusted to pH 5), mixed with 0.5 ml of H_2O (adjusted to pH 5) containing 30.6 mg of cellulase (Sigma, St. Louis, USA). The mixture was kept for 2 days at 37 °C. Compound **1a** (0.6 mg) was purified by partitioning with CH_2Cl_2 and Sephadex LH-20 CC of the CH_2Cl_2 layer.

3.3. Physical data of new compounds

3.3.1. 6″-O-(7,8-Dihydrocaffeoyl)-α,β-dihydrorhaponticin (2).

Yellow crystals, m.p. 106 °C; $[a]_D^{20}$ −28° (*c* 0.69, MeOH); FTIR υ_{max}^{ZnSe} cm^{-1}: 3394 (br), 2930, 2860, 1715, 1601, 1514, 1450, 1414, 1359, 1276, 1174, 1127, 1114, 1077, 1028; ^{1}H NMR: see Table 1; ^{13}C NMR: see Table 2.

3.3.2. 3′-O-Methyl-α,β-dihydrorhaponticin (3)

Yellow amorphous solid, glass transition 100–105 °C; $[a]_D^{20}$ −16.49° (*c* 0.10, MeOH); FTIR υ_{max}^{ZnSe} cm^{-1}: 3370 (br), 2922, 2858, 1596, 1515, 1461, 1420, 1331, 1302, 1260, 1233, 1172, 1156, 1136, 1075, 1027, 999; ^{1}H NMR: see Table 1; ^{13}C NMR: see Table 2.

3.3.3. (S)-3-(4-β-Glucopyranosyloxybenzyl)-7-hydroxy-5-methoxyphtalide (4)

Colorless crystals, m.p. 119 °C; $[a]_D^{20}$ −36° (*c* 0.19, MeOH); FTIR υ_{max}^{ZnSe} cm^{-1}: 3373 (br), 2921, 1730, 1686, 1613, 1512, 1457, 1438, 1379, 1320, 1226, 1194, 1161, 1074, 1038; ^{1}H NMR and ^{13}C NMR: see Table 3.

3.4. Radical scavenging activity

Methanolic solutions of test compounds were mixed with a methanolic solution of DPPH (Sigma–Aldrich, Steinheim, Germany). The final DPPH concentration was 40 mg/l. Compounds **1**, **1a**, and **2** were tested in final concentrations of 1, 2, 5, 10, and 20 μg/ml, respectively. After incubation in 96 well-plates, the reaction mixture (250 μl) was kept in the dark at ambient temperature (25 °C) for 30 min. Then, the optical density of the test mixtures in

comparison to DPPH and pure methanol was measured using a Hidex Chameleon plate reader at 515 nm. IC_{50} values for each replicate were calculated using the following formula: $IC_{50} = [(50 - LP)/(HP - LP) * (HC - LC)] + LC$. LP = low percentage, i.e. highest percent inhibition less than 50%; HP = high percentage, i.e. lowest percent inhibition greater than 50%; HC = high concentration, i.e. concentration of test substance at the high percentage, LC = low concentration, i.e. concentration of test substance at the low percentage. All compounds and concentrations were assayed in triplicate and mean values were calculated for each compound. Ascorbic acid and DPPH were obtained from Merck (Darmstadt, Germany) and Sigma–Aldrich (Steinheim, Germany), respectively.

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