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Secondary products in mycorrhizal roots of tobacco and tomato

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Abstract

Colonization of the roots of various tobacco species and cultivars (*Nicotiana glauca* Grah., *N. longiflora* Cav., *N. rustica* L., *N. tabacum* L., *N. tabacum* L. cv. Samsun NN, *N. sanderae* hort. Sander ex Wats.) as well as tomato plants (*Lycopersicon esculentum* L. cv. Moneymaker) by the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck and Smith resulted in the accumulation of several glycosylated C_{13} cyclohexenone derivatives. Eight derivatives were isolated from the mycorrhizal roots by preparative high performance liquid chromatography (HPLC) and spectroscopically identified (MS and NMR) as mono-, di- and triglucosides of 6-(9-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one and monoglucosides of 6-(9-hydroxybutyl)-1,5-dimethyl-4-cyclohexen-3-one-1-carboxylic acid and 6-(9-hydroxybutyl)-1,1-dimethyl-4-cyclohexen-3-one-5-carboxylic acid. In contrast to the induced cyclohexenone derivatives, accumulation of the coumarins scopoletin and its glucoside (scopolin) in roots of *N. glauca* Grah. and *N. tabacum* L. cv. Samsun NN, was markedly suppressed.

Keywords: Tobacco; Tomato; Solanaceae; *Glomus intraradices*; Arbuscular mycorrhiza; Cyclohexenone derivatives; Isoprenoids; Coumarins

1. Introduction

Colonization of plant roots by arbuscular mycorrhizal fungi induces the methylerythritol phosphate (MEP) pathway (Maier et al., 1998; Walter et al., 2000), resulting in the accumulation of various glycosylated C_{13} cyclohexenone derivatives (Maier et al., 1997, 1999; Vierheilig et al., 2000). First detected in cereal roots (Maier et al., 1995) and various other grass species (Maier et al., 1997), this phenomenon has also been found in mycorrhizal tobacco (Maier et al., 1999). Furthermore, it seems to be independent of the colonizing fungus, since it has been observed in roots colonized by *Glomus intraradices, G. mosseae* and *Gigaspora rosea* (Vierheilig et al., 2000). The induced appearance of cyclohexenone derivatives are accompanied by the accumulation of an acyclic C_{14} polyene (Walter et al., 2000), called mycorradicin (Klingner et al., 1995), a major component of the so-called "yellow pigment" (Jones, 1924). The cyclohexenone derivatives and mycorradicin both seem to be apocarotenoids, products from an oxidative cleavage of a precursor carotenoid (Bothe et al., 1994; Walter et al., 2000).

Recently we identified a major cyclohexenone derivative accumulating in mycorrhizal roots of *Nicotiana tabacum* L. cv. Samsun NN and tentatively described the structures of five minor ones (Maier et al., 1999). We extended our studies on various tobacco species and cultivars as well as on tomato and report here the complete spectroscopic identification of a further six glycosylated C_{13} cyclohexenone derivatives from tobacco and two from tomato, along with the coumarins scopoletin and its glucoside (scopolin) in tobacco roots.

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2. Results and discussion

The roots of various tobacco species and cultivars as well as of tomato plants were inoculated and colonized by the arbuscular mycorrhizal fungus *Glomus intraradices*. High-performance liquid chromatography (HPLC) of methanolic extracts from six-week-old mycorrhizal roots showed distinct patterns of UV-absorbing compounds. Figs. 1 and 2 show HPLC traces of non-mycorrhizal and mycorrhizal roots of *Nicotiana tabacum* 'Samsun' and *N. rustica* as well as of *Lycopersicon esculentum*. The numbered compounds were isolated as individual compounds (**1**, **2**, **4**, **5**) or as mixtures (**6** and **7**, **3** and **8**) by preparative HPLC and identified by spectroscopic methods as glycosylated C_{13} cyclohexenone derivatives, i.e. mono-, di- and triglucosides of 6-(9-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one and monoglucosides of 6-(9-hydroxybutyl)-1,5-dimethyl-4-cyclohexen-3-one-1-carboxylic acid and 6-(9-hydroxybutyl)-1,1-dimethyl-4-cyclohexen-3-one-5-carboxylic acid as well as the coumarins scopoletin (**II**) and its glucoside (**I**, scopolin) (see structure scheme). Another major component, detected in HPLC of all extracts, was identified as tryptophan (**Trp**) by its HPLC retention time and UV spectroscopy as compared to a standard compound. The amount of this amino acid did not significantly differ between mycorrhizal and non-mycorrhizal roots.

Compounds **1** and **3** were previously identified, **1** from tobacco (*Nicotiana tabacum* L. cv. Samsun) (Maier et al., 1999) and **3** from barley (*Hordeum vulgare* L. cv. Salome) (Peipp et al., 1997). Present spectroscopic data of these compounds (not shown) were identical with those obtained in the earlier studies. The structures of the unknown compounds (compounds **2**, **4**–**8**) were elucidated by a combination of MS and multidimensional NMR techniques. Since there is extensive literature on the spectroscopic identification of C_{13} cyclohexenone derivatives (Aasen et al., 1972; Galbraith and Horn, 1972; Kodama et al., 1981, 1984; Winterhalter and Schreier, 1995; Maier et al., 1995, 1999; Peipp et al., 1997; Mohamed et al., 1999) we only briefly comment on the present data. In each case ^{1}H 1D and 2D COSY spectra allowed ready identifi-

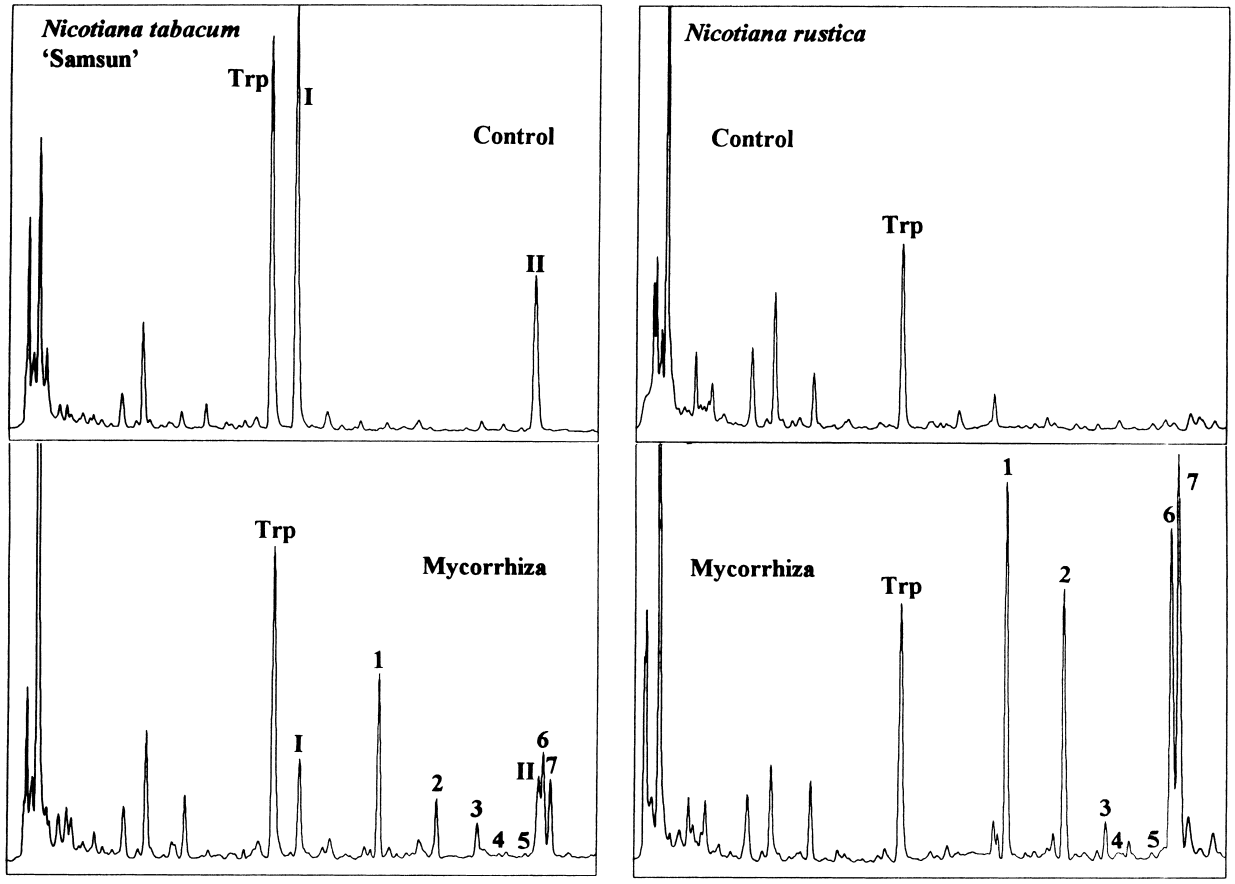


Fig. 1. HPLC traces (35 min) of methanolic extracts from six-week-old non-colonized roots (Control) of *Nicotiana tabacum* 'Samsun' and *N. rustica* and the respective roots colonized by *Glomus intraradices* (Mycorrhiza). Compounds were traced by maxplot detection between 210 and 400 nm (0.5 full-scale absorbance). For peak identification see structure scheme.

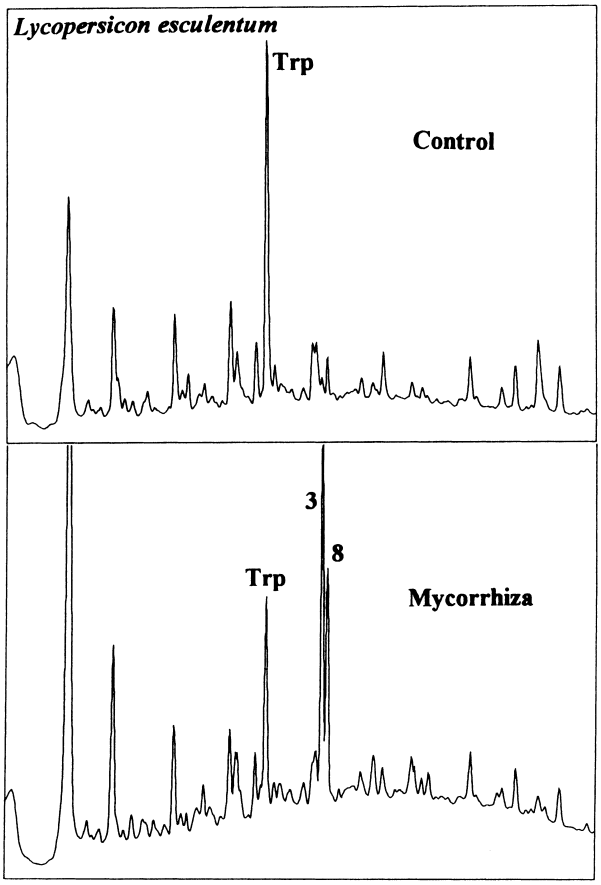
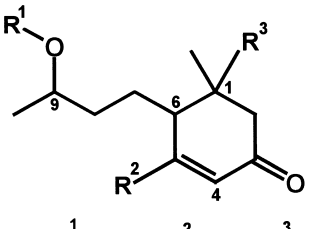


Fig. 2. HPLC traces (22 min) of methanolic extracts from 6-week-old non-colonized tomato roots (Control) and the respective roots colonized by *Glomus intraradices* (Mycorrhiza). Compounds were traced by maxplot detection between 210 and 400 nm (0.1 full-scale absorbance). For peak identification see structure scheme. (Note that compared to HPLC of the tobacco compounds a different gradient elution system and time trace was used.)

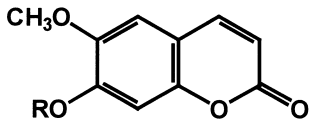
cation of the 6-(9-hydroxybutyl)-4-cyclohexen-3-one and sugar moieties. For the former, the lowest field signal belongs to the olefinic proton, H-4, and this showed in the appropriate molecules allylic coupling to H-13, detected in the COSY spectra. Similarly, the low field doublet (H-2A) of the AB system characteristic of the methylene group at C-2 always showed long-range coupling to one of the adjacent methyl groups (H-12) on C-1 indicating the axial dispositions of both H-2A and H-12. A similar correlation of H-2B and H-4 indicated the equatorial position of the former and planar W-relationship of both protons. Of particular usefulness is the chemical shift of H-4 which is sensitive to the nature of the C-13 substituent and occurs at ca. 6.5 ppm for $-CO_2H$, ca. 6.1 ppm for $-CH_2OH$ and ca. 5.8 ppm for $-CH_3OH$.

The number, nature and anomeric configuration of the sugar units present were evident from both the ^1H and ^{13}C data. Characteristic chemical shifts proved the presence of glucose for all the sugar units present and in each case the vicinal ^1H–^1H coupling to the anomeric proton, H-1′, of ca. 7.8 Hz established the β-configuration. In several cases, ^{13}C chemical shifts were assigned indirectly from 2D heteronuclear ^1H-detected long-range ^{13}C–^1H correlations (HMBC), which also provided confirmation of the intramolecular connections within the various moieties and unambiguous information regarding the inter-fragment connections. In particular, the first glucose unit was always attached to C-9 and the position of the two other sugar units in compound **4** were established by the appropriate heteronuclear three-bond correlations. In all multiple sugar-containing compounds, sugar methylation analysis either confirmed (compounds **6**, **7** and **4**) or established (compound **2**) the sugar linkages.

The relative configurations of the asymmetric sites in the molecules at C-6 and C-9, and C-1 in compound **8**, could not be established from the NMR data. However, it is clear that **6** and **7** are an isomeric mixture of compounds. Comparison of the respective ^{13}C chemical shifts, which could be readily assigned from the different signal intensities, shows that the largest shift differences ($\geq$ 1.9 ppm) occur for C-9, C-10 and C-1′ (all other differences are $\leq$ 0.3 ppm) which is indicative of the presence of *R*- and *S*-isomers at C-9. A similar situation has been observed for the related monosaccharide derivative



	R^1	R^2	R^3
1	Glc-(6)-Glc	CH_2OH	CH_3
2	Glc-(6)-Glc	COOH	CH_3
3	Glc	CH_2OH	CH_3
4	Glc-(2,6)-Glc	CH_3	CH_3
5	Glc	COOH	CH_3
6 + 7	Glc-(6)-Glc	CH_3	CH_3
8	Glc	CH_3	COOH



I R = Glc
II R = H

Structure scheme.

(Strack and Wray, unpublished results). There is no evidence of such a variation at C-6.

Compounds **2**, **5** and **8** arose from a different structure of the cyclohexenone moiety, bearing a carboxyl group. These compounds were readily identified from the 1D and 2D NMR data. Again, however, each asymmetric centre is enantiomerically pure although the relative configuration could not be established. Compounds **2** and **4–8** are new structures, among which **6** and **7**, bearing a 1,6-diglucose moiety, are similar to the previously identified 1,2-diglucoside from barley (Peipp et al., 1997).

Tobacco is one of the prime candidates for the formation of complex patterns of C_{13} cyclohexenone derivatives, since structures related to the present ones accumulate in leaves of various tobacco species as so-called 'aroma constituents', which are presumed to be liberated from their glucosides (Kodama et al., 1984). The same phenomenon has been described in an extensive study on fruits from starfruit (Winterhalter and Schreier, 1995). Table 1 summarizes the results obtained from six tobacco species and cultivars, along with tomato, and shows considerable variation in the patterns of cyclohexenone derivatives. This is illustrated in Fig. 1, which compares *N. tabacum* 'Samsun' with *N. rustica*. These structures, along with the acyclic C_{14} polyene mycorradicin (Klingner et al., 1995; Walter et al., 2000), seem to be apocarotenoids, products of an oxidative cleavage of a precursor carotenoid (Bothe et al., 1994; Walter et al., 2000), formed via the methylerythritol phosphate (MEP) pathway (Maier et al., 1998; Walter et al., 2000). With regard to their possible function, it has only been shown so far that exogenous blumenin [9-*O*-(2-*O*-β-glucuronosyl)-β-glucopyranoside of 6-(9-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one] applied to barley roots strongly inhibits early fungal colonization and arbuscule formation (Fester et al., 1999). Whether or not mycorrhizal roots also evolve apocarotenoid aglycones as volatiles, which might be involved in the 'molecular dialogue' between plant roots and arbuscular mycorrhizal fungi in the plant rhizosphere, awaits further studies.

Compounds **I** and **II** were identified from the roots of some of the tobacco species by MS and co-chromatography (HPLC) with authentic samples. This supports the finding of Sheen (1969), who reported a high proportion of these coumarins in the root of *Nicotiana tabacum*. Their accumulation in *N. tabacum* L. cv. 'Samsun', however, is markedly suppressed in the mycorrhizal roots. Control roots accumulated 277 nmol scopoletin and 748 nmol scopolin per 1 g root fresh weight, whereas the respective compounds in mycorrhizal roots accumulated to 164 and 213 nmol, respectively. A similar phenomenon was observed with *N. glauca*, where the amount of scopolin was reduced from 364 to 195 nmol. Roots of the other tobacco species and cultivars showed — if at all — trace amounts of these coumarins (not documented). It is known that scopoletin exhibits biological activities associated with disease resistance (Ahl Goy et al., 1993, and literature cited therein). Thus, it is tempting to assume that in those tobacco species which constitutively accumulate these coumarins, the AM fungi suppresses their accumulation to prevent being adversely affected during root colonization.

3. Experimental

3.1. Plant material and fungal inoculation

Tobacco seeds came from the Botanical Garden of the University in Halle and tomato seeds from Hild Samen GmbH (Marbach, Germany). Tobacco (*N. glauca* Grah., *N. longiflora* Cav., *N. rustica* L., *N. tabacum* L., *N. tabacum* L. cv. 'Samsun' NN, *N. x sanderae* hort. Sander ex Wats.) and tomato plants (*Lycopersicon esculentum* L. cv. Moneymaker) were cultivated in plastic pots (3 tobacco or 10 tomato plants per pot) filled with expanded clay and inoculated with the AM fungus *Glomus intraradices* Schenck and Smith by ap-

Table 1
Occurrence of cyclohexenone derivatives (see structure scheme) in mycorrhizal roots of tobacco (*Nicotiana*) species and cultivars as well as of tomato (*Lycopersicon esculentum*) (–, not detected; tr, trace amount). Values [nmol (± s.d.) g^{-1} root fresh weight] are the mean of three independent experiments

Mycorrhizal plant	Cyclohexenone derivative							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
N. glauca	63 ± 11	76 ± 21	tr	tr	tr	103 ± 19	104 ± 30	–
N. longiflora	339 ± 25	119 ± 14	80 ± 23	–	–	311 ± 106	138 ± 2	–
N. rustica	701 ± 89	579 ± 43	64 ± 12	20 ± 5	15 ± 2	698 ± 73	849 ± 131	–
N. tabacum	264 ± 45	77 ± 18	tr	tr	85 ± 52	46 ± 11	212 ± 32	–
N. tabacum 'Samsun'	344 ± 123	114 ± 44	53 ± 21	tr	tr	246 ± 53	139 ± 64	–
N. x *sanderae*	235 ± 23	32 ± 10	45 ± 18	–	–	53 ± 8	43 ± 7	–
L. esculentum	–	–	198 ± 29	–	–	–	–	140 ± 4

plication of propagules in expanded clay (isolate 49). Mycorrhization was achieved by cultivating the plants in expanded clay, mixed with 10% (v/v) of the fungal inoculum, reaching 75–90% colonization along the root lengths. Details of plant growth conditions have been published previously (Maier et al., 1995). The approximate percentage values of mycorrhization were estimated microscopically by counting the frequency of colonization from 30 root pieces (2 cm) after staining with trypan blue in lactophenol according to a procedure described by Phillips and Hayman (1970).

3.2. Root extraction and high-performance liquid chromatography

Freshly harvested 6-week-old mycorrhizal and non-mycorrhizal whole tobacco roots were washed with H_2O, cut into small pieces, and aliquots (1 g fresh weight) were treated twice for about 1 min with an Ultra Turrax homogenizer in 5 ml 80% aq. MeOH. The extracts were centrifuged and the supernatants used for HPLC analysis (20 μl aliquots).

The HPLC system (Waters 600, Milford, MA, USA), equipped with a 5 μm Nucleosil C_{18} column (25 × 4 mm i.d.; Macherey-Nagel, Düren, Germany), was as described previously (Maier et al., 1995). For separation of the cyclohexenone derivatives from tomato, a linear gradient elution system was used at a flow rate of 1 ml min^{-1} within 30 min from solvent A (1.5% H_3PO_4 in H_2O) to solvent B (MeCN). However, separation of the various tobacco compounds was achieved by a linear gradient system within 30 min from 5 to 20% solvent B in (A + B). Compounds were detected photometrically (maxplot between 210 and 400 nm) by a Waters 996 photodiode array detector. Quantification of the cyclohexenone derivatives was achieved by using abscisic acid as an external standard as described (Maier et al., 1995). Scopoletin and scopolin were quantified using authentic scopoletin (Sigma, Deisenhofen, Germany) as a standard.

3.3. Preparative extraction and isolation

Freshly harvested mycorrhizal *N. tabacum* 'Samsun' and tomato roots (25 g each) were washed with H_2O, cut into small pieces and transferred into 100 ml 80% aq. MeOH. The plant material was treated twice with an Ultra Turrax homogenizer for about 1 min and the homogenates were allowed to stand for 30 min with continuous stirring. The suspension was centrifuged and the pellet re-extracted twice. The combined extracts were evaporated at 40°C (in vacuo) to dryness, the residue redissolved in 8 ml H_2O, centrifuged, and aliquots (1 ml) of the supernatant were subjected to preparative HPLC (Beckman Instruments, München, Germany, System Gold, and a photodiode array detector 168), equipped with a Nucleosil 100–10 C_{18} column (VarioPrep;10 μm, 250 × 40 mmi.d.; Macherey-Nagel, Düren, Germany). The compounds were separated with a flow rate of 11 ml min^{-1} with a linear gradient within 10 min from 30% solvent B (MeOH) in A (0.4% HCO_2H in H_2O) to 50% B followed by isocratic elution.

3.4. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy

The electrospray (ESI) mass spectra were recorded on a Finnigan MAT TSQ 7000 instrument [electrospray voltage 4.5 kV (positive ions), 3.5 kV (negative ions); heated capillary 220 °C; sheath gas nitrogen] coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a C_{18} column (4 μm; 100 mm long, 1 mm i.d.; ULTRASEP). For HPLC, a gradient system starting from H_2O-MeCN at 17:3 (containing 0.2% HOAc) to 1:9 within 15 min at a flow rate of 70 μl min^{-1} was applied.

Sugar methylation analyses were carried out according to Nimtz et al. (1996). The positive ESI mass spectra of the permethylated compounds were recorded on a Finnigan MAT TSQ 7000 (Finnigan, Bremen) using a syringe pump (Harvard Apparatus, South Natick, MA) and 1D as well as 2D homonuclear (^1H–^1H COSY) and heteronuclear (^1H–^{13}C HMBC) NMR spectroscopy (DMX600, ARX400, DPX300, Bruker, Rheinstetten) were performed as described previously (Maier et al., 1995).

Compounds **1** *and* **3**: MS and NMR data were identical with those previously published. **1** in Maier et al. (1999) and **3** in Peipp et al. (1997).

Compound **2**: ^1H NMR Spectral data (CD_3OD); δ = 6.53 [*bs*, H-4], 4.44 [*d* H-1′*J*(1′-2′)7.9], 4.35 [*d*, H-1″*J*(1″-2″) 7.7], 4.15 [*dd*, H-6′A, *J*(6′A-6′B) 11.7, *J*(6′A-5′) 2.0], 3.90 [*dd*, H-6″A, *J*(6″A-6″B) 12.0, *J*(6″A-5″) 2.3], 3.86 [*m*, H-9], 3.82 [*dd*, H-6′B, *J*(6′B-5′) 5.6], 3.69 [*dd*, H-6″B, *J*(6″B-5″) 5.1], 3.4-3.28 [*m*, H-3′-H-5′ and H-3″-H-5″], 3.24 [*dd*, H-2′, *J*(2′-3′) 8.8], 3.18 [*dd*, H-2″, *J*(2″-3″) 8.9], 2.67 [*d*, H-2A, *J*(2A-2B) 17.4], 2.09 [*d*, H-2B], 2.07 [*m*, H-6], 1.96 [*m*, H-7A], 1.75-1.6 [*m*, H-7B, H-8AB], 1.26 [*d*, H-10, *J*(10-9) 6.2], 1.18 [*s*, H-11], 1.05 [*s*, H-12]. Long-range couplings were observed between H-4 and H-2B, and H-2A and H-12 in the 2D ^1H–^1H COSY spectrum. Positive ion ESIMS: *m*/*z* 587 ($[M + Na]^+$, 15), 565 ($[M + H]^+$, 5), 403 ($[M + H\text{-}C_6H_{10}O_5]^+$, 40), 241 ([aglycone + H]$^+$, 100), 223 ([aglycone + H-H_2O]$^+$, 57), 195 ([aglycone + H-HCO_2H]$^+$, 21), 177 (aglycone + H-HCO_2H-H_2O]$^+$, 9); negative ion ESIMS: *m*/*z* 563 ([M-H]$^-$, 100); sugar methylation analysis: terminal glucose and 1,6-disubstituted glucose.

Compound **4**: ^1H NMR Spectral data (CD_3OD); δ = 5.86 [*bs*, H-4], 4.58 [*d*, H-1″, *J*(1″-2″) 7.7], 4.52 [*d*, H-1′,

J(1′-2′) 7.7], 4.38 [*d*, H-1‴, J(1‴-2‴) 7.7] 4.16 [*dd*, H-6′A, J(6′A-6′B) 11.7, J(6′A-5′) 1.7], 3.95 [*m*, H-9], 3.91, 3.87 [*dd* x2, H-6″A, H-6‴A, J(6A-6B) 11.6, 11.9, J(6A-5) 2.0, 2.4], 3.78 [*dd*, H-6′B, J(6′B-5′) 5.6], 3.73, 3.70 [*dd* x2, H-6″B, H-6‴B, J(6B-5) 5.0, 5.3], 3.59 [*dd*, H-3′, J(3′-2′) 8.8, J(3′-4′) 8.8], 3.48 [*m*, H-5′], 3.45 [*dd*, H-2′], 3.43-3.28 [*m*, H-4′, H-3″-H-5″, and H-3‴-H-5‴], 3.45 [*dd*, H-2′], 3.28, 3.25 [*dd* x2, H-2″, H-2‴], 2.52 [*d*, H-2A, J(2A-2B) 17.5], 2.10 [*d*, H-13, J(13-4) 1.1], 2.02 [*d*, H-2B], 2.01 [*m*, H-6], 1.74-1.50 [*m*, H-7AB, H-8AB], 1.25 [*d*, H-10, J(10-9) 6.2], 1.14 [*s*, H-11], 1.06 [*s*, H-12]. Long-range couplings were observed between H-4 and H-13 (allylic), H-4 and H-2B, and H-2A and H-12 in the 2D ^{1}H-^{1}H COSY spectrum. A partial assignment of the ^{13}C data was possible from a HMBC spectrum.^{13}C NMR Spectral data (CD_3OD); $\delta = 202.1$ (C-3), 169.7 (C-5), 125.0 (C-4), 82.6 (C-2′), 76.0 (C-9), 69.7 (C-6′), 52.1 (C-6), 47.8 (C-2), 37.5 (C-8), 37.1 (*s*, C-1), 28.7 (*q*, C-12), 27.4 (*q*, C-11), 24.8 (C-13), 19.7 (*q*, C-10). In this spectrum H-1′ correlated with C-9, H-1″ with C-2′ and H-1 with C-6′. Positive ion ESIMS: m/z 697 ($[M+H]^+$, 25), 535 ($[M+H\text{-}C_6H_{10}O_5]^+$, 100), 373 ($>[M+H\text{-}2C_6H_{10}O_5]^+$, 90), 211 ([aglycone + H]$^+$, 40); negative ion ESIMS: m/z 695 ($[M\text{-}H]^-$, 100); sugar methylation analysis: terminal glucose and 1, 2, 6-trisubstituted glucose.

Compound **5**: ^{1}H NMR Spectral data (CD_3OD); $\delta =$ 6.53 [*bs*, H-4], 4.35 [*d*, H-1′, J(1′-2′) 7.8], 3.88 [*dd*, H-6′A, J(6′A-6′B) 12.0, J(6′A-5′) 2.0], 3.86 [*m*, H-9], 3.69 [*dd*, H-6′B, J(6′B-5′) 6.1], 3.4-3.3 [*m*, H-3′, H-4′], 3.28 [*m*, H-5′], 3.17 [*dd*, H-2′, J(2′-3′) 8.8], 2.68 [*d*, H-2A, J(2A-2B) 17.3], 2.08 [*d*, H-2B], 2.08 [*m*, H-6], 1.96 [*m*, H-7A], 1.7-1.6 [*m*, H-7B, H-8AB], 1.25 [*d*, H-10, J(10-9) 6.3], 1.18 [*s*, H-11], 1.05 [*s*, H-12]. Long-range couplings were observed between H-4 and H-2B, and H-2A and H-12 in the 2D ^{1}H-^{1}H COSY spectrum. ^{13}C NMR Spectral data; (CD_3OD) $\delta = 203.0$ (*s*, C-3), 171.5 (*s*, C-13), 158.1 (*s*, C-5), 130.0 (*d*, C-4), 104.3 (*d*, C-1′), 78.2, 77.8 (*d* x 2, C-3′, C-5′), 77.9 (*d*, C-9), 75.3 (*d*, C-2′), 71.7 (*d*, C-4′), 62.8 (*t*, C-6′), 48.1 (*t*, C-2), 46.2 (*d*, C-6), 37.5 (*s*, C-1), 36.3 (*t*, C-8), 28.6 (*q*, C-12), 27.9 (*t*, C-7), 27.9 (*q*, C-11), 22.1 (*q*, C-10). Assignments were established from a HMBC spectrum. Positive ion ESIMS: m/z 403 ($[M+H]^+$, 15), 241 ([aglycone + H]$^+$, 40), 223 ([aglycone + H-H_2O]$^+$, 100), 195 ([aglycone + H-HCO_2H]$^+$, 32), 177 ([aglycone + H-HCO_2H-H_2O]$^+$, 10); negative ion ESIMS: m/z 401 ($[M\text{-}H]^-$, 100).

Compounds **6** *and* **7**: mixture of *R*- and *S*-forms at C-9. Major isomer (63%): ^{1}H NMR Spectral data (CD_3OD); $\delta = 5.84$ [*bs*, H-4], 4.44 [*d*, H-1′, J(1′-2′) 7.8], 4.36 [*d*, H-1″, J(1″-2″) 7.8], 4.15 [*dd*, H-6′A, J(6′A-6′B) 11.7, J(6′A-5′) 2.0], 3.90 [*dd*, H-6″A, J(6″A-6″B) 11.9, J(6″A-5″) 2.1], 3.88 [*m*, H-9], 3.83 [*dd*, H-6′B, J(6′B-5′) 5.8], 3.70 [*dd*, H-6″B, J(6″B-5″) 5.4], 3.47 [*m*, H-5′], 3.41-3.30 [*m*, H-3′-H-4′ and H-3″-H-4″], 3.29 [*m*, H-5″], 3.24 [*dd*, H-2′, J(2′-3′) 8.8], 3.19 [*m*, H-2″, J(2″-3″) 9], 2.53 [*d*, H-2A, J(2A-2B) 17.4], 2.09 [*d*, H-13, J(13-4) 1.2], 2.02 [*d*, H-2B], 2.02 [*m*, H-6], 1.83 [*m*, H-7A], 1.76-1.50 [*m*, H-7B, H-8AB], 1.29 [*d*, H-10, J(10-9) 6.2], 1.14 [*s*, H-11], 1.06 [*s*, H-12]. Long-range couplings were observed between H-4 and H-13 (allylic), H-4 and H-2B, and H-2A and H-12 in the 2D ^{1}H-^{1}H COSY spectrum. ^{13}C NMR Spectral data (CD_3OD) $\delta = 202.4$ (*s*, C-3), 169.9 (*s*, C-5), 125.5 (*d*, C-4), 104.9 (*d*, C-1″), 104.1 (*d*, C-1′), 78.1 (*d*, C-3′, C-3″, C-5″), 77.7 (*d*, C-9), 77.1 (*d*, C-5′), 75.3 (*d*, C-2′), 75.1 (*d*, C-2″), 71.7 (*d*, C-4″), 71.6 (*d*, C-4′), 69.9 (*t*, C-6′), 62.8 (*t*, C-6″), 52.6 (*d*, C-6), 48.1 (*t*, C-2), 37.6 (*t*, C-8), 37.4 (*s*, C-1), 29.0 (*q*, C-12), 27.5 (*q*, C-11), 26.7 (C-7), 25.0 (*q*, C-13), 22.1 (*q*, C-10). Minor isomer (37%): ^{1}H NMR data (CD_3OD); $\delta = 5.85$[*bs*, H-4], 4.39 [*d*, H-1′, J(1′-2′) 7.8], 4.36 [*d*, H-1″, J(1″-2″) 7.8], 4.16 [*dd*, H-6′A, J(6′A-6′B) 11.6, J(6′A-5′) 2.0], 3.91 [*m*, H-9], 3.90 [*dd*, H-6″A, J(6″A-6″B) 11.9, J(6″A-5″) 2.1], 3.78 [*dd*, H-6′B, J(6′B-5′) 5.8], 3.70 [*dd*, H-6″B, J(6″B-5″) 5.8], 3.47 [*m*, H-5′], 3.41-3.30 [*m*, H-3′-H-4′ and H-3″-H-4″], 3.29 [*m*, H-5″], 3.24 [*dd*, H-2′, J(2′-3′) 8.8], 3.19 [*m*, H-2″, J(2″-3″) 9], 2.51 [*d*, H-2A, J(2A-2B) 17.4], 2.10 [*d*, H-13, J(13-4) 1.2], 2.02 [*d*, H-2B], 2.02 [*m*, H-6], 1.83 [*m*, H-7A], 1.76-1.50 [*m*, H-7B, H-8AB], 1.22 [*d*, H-10, J(10-9) 6.1], 1.14 [*s*, H-11], 1.05 [*s*, H-12]. Long-range couplings were observed between H-4 and H-13 (allylic), H-4 and H-2B, and H-2A and H-12 in the 2D ^{1}H-^{1}H COSY spectrum. ^{13}C NMR Spectral data (CD_3OD); $\delta = 202.5$ (*s*, C-3), 170.2 (*s*, C-5), 125.4 (*d*, C-4), 105.1 (*d*, C-1″), 102.2 (*d*, C-1′), 78.1 (*d*, C-3′, C-3″, C-5″), 77.0 (*d*, C-5′), 75.7 (*d*, C-9), 75.1 (*d*, C-2′), 75.1 (*d*, C-2″), 71.7 (*d*, C-4″), 71.7 (*d*, C-4′), 70.1 (*t*, C-6′), 62.9 (*t*, C-6″), 52.4 (*d*, C-6), 48.2 (*t*, C-2), 37.8 (*t*, C-8), 37.4 (*s*, C-1), 29.1 (*q*, C-12), 27.6 (*q*, C-11), 26.9 (C-7), 25.1 (*q*, C-13), 20.0 (*q*, C-10). Positive ion ESIMS: m/z 535 ($[M+H]^+$, 100), 373 ($[M+H\text{-}C_6H_{10}O_5]^+$, 85), 211 ([aglycone + H]$^+$, 12); negative ion ESIMS: m/z 533 ($[M\text{-}H]^-$, 100); sugar methylation analysis: terminal glucose and 1, 6-disubstituted glucose.

Compound **8**: ^{1}H NMR Spectral data (CD_3OD); $\delta =$ 5.85 [*bs*, H-4], 4.33 [*d*, H-1′, J(1′-2′) 7.8], 3.89 [*m*, H-6′A], 3.83 [*m*, H-9], 3.69 [*m*, H-6′B], 3.42-3.28 [*m*, H-4′], 3.37 [*m*, H-3′], 3.29 [*m*, H-5′], 3.17 [*dd*, H-2′, J(2′-3′) 9.0], 2.97 [*d*, H-2A, J(2A-2B) 18.0], 2.47 [*t*, H-6, J(6-7) 4.9], 2.29 [*d*, H-2B], 2.13 [*d*, H-13, J(13-4) 1.2], 1.9-1.6 [*m*, H-7AB, H-8AB], 1.32 [*s*, H-12], 1.26 [*d*, H-10, J(10-9) 6.5]. The ^{1}H–^{1}H COSY spectrum showed correlations between H-4 and H-2B, H-2A and H-12, and H-4 and H-13. ^{13}C NMR Spectral data (CD_3OD) $\delta = 200.6$ (C-3), 178.9 (C-11), 167.2 (C-5), 125.0 (C-4), 103.6 (C-1′), 77.9, 77.6 (C-3′, C-5′), 77.2 (C-9), 75.1 (C-2′), 71.4 (C-4′), 62.5 (C-6′), 49.5 (C-6), 48.5 (C-1), 42.3 (C-2), 36.7 (C-8), 28.9 (C-7), 24.9 (C-13), 24.5 (C-12), 21.6 (C-10). Positive ion ESIMS: m/z 425

($[M+Na]^+$, 100), 403 ($[M+H]^+$, 13), 241 ([aglycone + H]$^+$, 51), 223 ([aglycone + H-H_2O]$^+$, 55), 195 ([aglycone + H-HCO_2H]$^+$, 43), 177 ([aglycone + H-HCO_2H-H_2O]$^+$, 47); negative ion ESIMS: *m/z* 401 ($[M-H]^-$, 100).

Compounds **I** *and* **II**: scopoletin (**I**): positive ion ESIMS: *m/z* 193 ($[M+H]^+$, 100); scopolin (**II**): negative ion ESIMS: *m/z* 353 ($[M-H]^-$, 100), 191 ([M-H-162]$^-$, 90).

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