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Accumulation of apocarotenoids in mycorrhizal roots of *Ornithogalum umbellatum*

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Abstract

Colonization of roots of *Ornithogalum umbellatum* by the arbuscular mycorrhizal fungus *Glomus intraradices* induced the accumulation of different types of apocarotenoids. In addition to the mycorrhiza-specific occurrence of cyclohexenone derivatives and the "yellow pigment" described earlier, free mycorradicin and numerous mycorradicin derivatives were detected in a complex apocarotenoid mixture for the first time. From the accumulation pattern of the mycorradicin derivatives their possible integration into the continuously accumulating "yellow pigment" is suggested. Structure analyses of the cyclohexenone derivatives by MS and NMR revealed that they are mono-, di- and branched triglycosides of blumenol C, 13-hydroxyblumenol C, and 13-nor-5-carboxy-blumenol C, some of which contain terminal rhamnose as sugar moiety.

Keywords: *Ornithogalum umbellatum*; Hyacinthaceae; Star of Bethlehem; Arbuscular mycorrhiza; Apocarotenoids; C_{13} cyclohexenone derivatives

1. Introduction

Mycorrhizas are mutualistic associations between soil-borne fungi and plant roots. One of the most widespread types of these associations is the arbuscular mycorrhiza (AM) (Smith and Read, 1997) formed by fungi of the phylum Glomeromycota (Schüßler et al., 2001) and the roots of the majority of land plants. The obligately biotrophic AM fungi facilitate the uptake of mineral nutrients, particularly phosphate, and water by colonizing root cortical cells forming highly branched structures, named arbuscules. The plants, in return, provide the fungi with carbohydrates. This intimate interaction supporting plant growth (Van der Heijden et al., 1998; Hartwig et al., 2002) and increasing plant stress tolerance (Cordier et al., 1998) is accompanied by alterations in root secondary metabolism. The formation of yellow colored roots upon colonization by AM fungi has been described by Jones already in 1924. The "yellow pigment" was shown to be a mixture of mycorradicin-derived compounds in AM roots of maize (Fester et al., 2002) after characterizing the chromophore from these roots as a C_{14} polyenic acid (10,10′-diapocarotene-10,10′-dioic acid) (Klingner et al., 1995a). Due to structural similarities of mycorradicin with azafrin (Eschenmoser and Eugster, 1975) and with other recently found C_{27} apocarotenoids (Cooper et al., 2003), its formation by oxidative cleavage of two terminal C_{13} units from a C_{40} carotenoid precursor has been suggested (Klingner et al., 1995a,b). At the same time the structure of a C_{13} cyclohexenone derivative (blumenin) occurring specifically in mycorrhizal cereal roots

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was elucidated. Blumenin levels correlated with the degree of mycorrhization (Maier et al., 1995). Retrobiosynthetic studies provided evidence that this C_{13} compound is formed via the plastid-located 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Maier et al., 1998). Due to the occurrence of both C_{13}- and C_{14}-apocarotenoids (cyclohexenone and mycorradicin derivatives) in mycorrhizal roots, their formation by oxidative cleavage of a carotenoid precursor (xanthophyll derivative) was proposed (Klingner et al., 1995a; Walter et al., 2000). Mycorrhiza-induced cyclohexenone derivatives were found not only in roots of Poaceae (Maier et al., 1997), but also in solanaceous plants (tobacco, tomato; Maier et al., 1999, 2000; see review Strack et al., 2003). Very recently it was shown that mycorrhizal roots of *Lotus japonicus* (Leguminosae) contain a set of similar cyclohexenone derivatives from which three were structurally elucidated (Fester et al., 2005). Fester et al. (2002) screened mycorrhizal roots of a number of plant species for the occurrence of the "yellow pigment" by mycorradicin detection and quantification after alkaline hydrolysis of root extracts. The presence of mycorradicin was detected in AM roots of 23 of the 46 analyzed plant species. Whereas the formation of C_{13} cyclohexenone derivatives can be deduced from their structures, the fate of the primary C_{14} apocarotenoid and its integration into the "yellow pigment", presumably a mixture of mycorradicin conjugates, is unknown. Hydrolysis of the "yellow pigment" from maize resulted in various compounds from which mycorradicin isomers and a cyclohexenone derivative (blumenol C 9-*O*-cellobioside) were identified (Fester et al., 2002). As extracts from roots of *Ornithogalum umbellatum* colonized by *Glomus intraradices* yielded a markedly high mycorradicin content after alkaline treatment (Fester et al., 2002), this plant was chosen to study the time-dependent apocarotenoid accumulation during mycorrhization.

2. Results and discussion

2.1. Kinetics of accumulation of yellow apocarotenoids and the "yellow pigment"

Star of Bethlehem (*O. umbellatum* L., Hyacinthaceae) is native to the Mediterranean having grass-like leaves and a cluster of star-shaped white flowers. Their bulbs are toxic due to the presence of cardenolides (Ferth et al., 2001; Burrows and Tyrl, 2001). Cytological studies revealed the presence of globular chromoplasts in yellow colored mycorrhizal roots (Bonfante and Scannerini, 1977; Scannerini and Bonfante-Fasolo, 1977). AM roots of *O. umbellatum* show a bright yellow coloration when compared to nonmycorrhizal ones. Epifluorescence microscopy of root cross sections reveals intensely fluorescing yellow material in the inner part of the root cortex of colonized roots correlating with the site of most symbiotic structures (Fig. 1). HPLC analysis of an extract from *O. umbellatum* AM roots revealed striking differences to the known HPLC pattern of C_{13} cyclohexenone derivatives and the "yellow pigment" of AM roots from other plants (Fester et al., 2002). Numerous apocarotenoids with UV–Vis spectra similar to mycorradicin (yellow apocarotenoids) eluted with shorter retention times than the "yellow pigment" (Fig. 2, Table 1). Further analyses focused on the accumulation of these compounds during the time course of mycorrhization. Four weeks after inoculation with *G. intraradices*, yellow apocarotenoids were already detectable, whereas in contrast the "yellow pigment" was not found (Fig. 3). After nine weeks the "yellow pigment" started to accumulate concomitant with the increase in levels of the yellow apocarotenoids. The kinetics of the accumulation of the major cyclohexenone derivatives and the major yellow apocarotenoids as well as the "yellow pigment" is illustrated in Fig. 4. The accumulation of the yellow apocarotenoids is attenuated after 13 weeks and partially reduced levels in

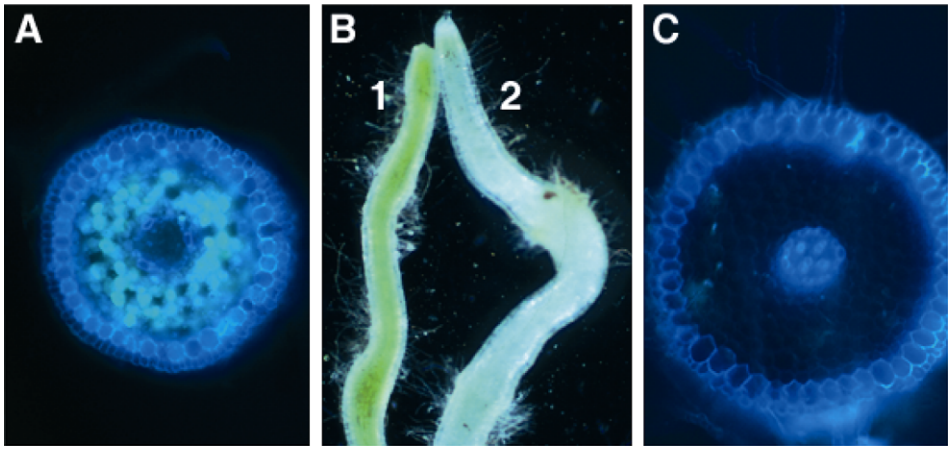

Fig. 1. Micrographs of cross sections (A,C) and whole root segments (B) of mycorrhizal (A,B1) and nonmycorrhizal (B2,C) *O. umbellatum* (6 months old) recorded using epifluorescence microscopy (A,C) or a bright field stereomicroscope (B). Yellow coloration (B1) and yellow fluorescence (A) of accumulating mycorradicin derivatives including the "yellow pigment" in AM roots are visible.

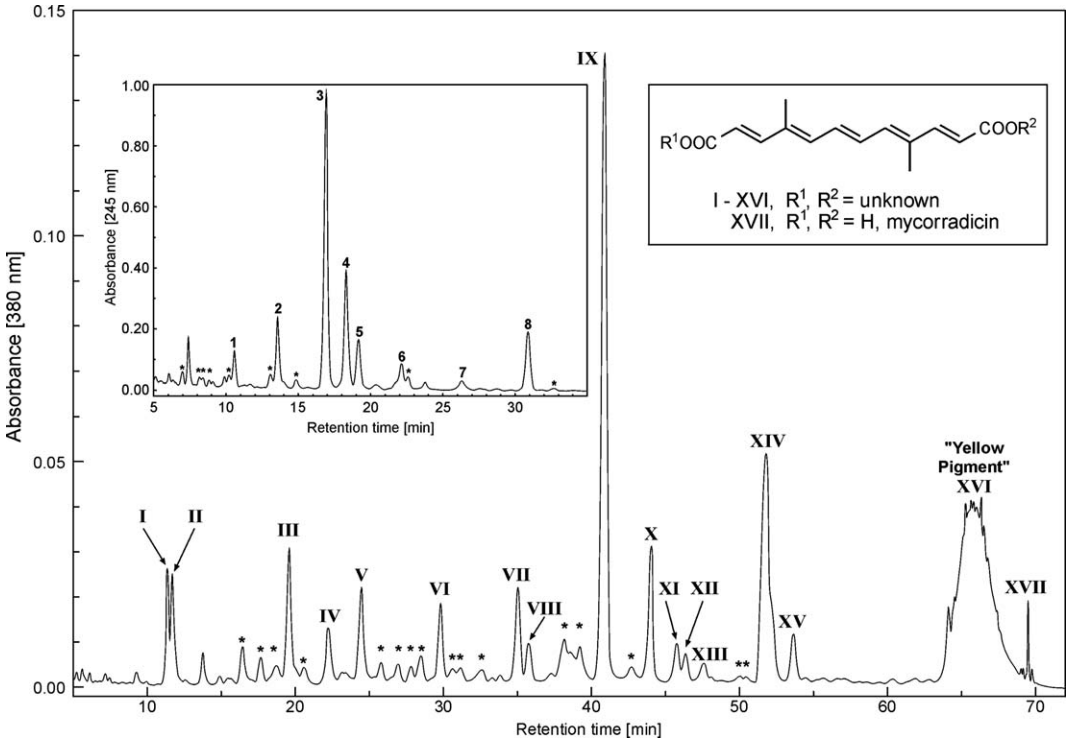

Fig. 2. HPLC elution profile (solvent system 2) of C_{13} cyclohexenone derivatives (left inset) and yellow apocarotenoids including the ''yellow pigment'' (PDA-detection at 245 and 380 nm, respectively) from mycorrhizal roots of *O. umbellatum* (16 weeks after inoculation). Peak numbers of mycorradicin derivatives **I**–**XVII** correspond to the numbers in Table 1. Peak numbers of cyclohexenone derivatives **1**–**8** correspond to compound numbers in Table 2. The inset show the structure of mycorradicin and its derivatives. Additional compounds with UV spectra characteristic for cyclohexenone and mycorradicin derivatives are marked with asterisks.

Table 1
Retention times and HPLC-PDA data of mycorradicin and its derivatives in solvent system 2 from mycorrhizal roots of *O. umbellatum* (16 weeks after inoculation)

Compound	R_t (min)	HPLC-PDA λ_{max} (nm)
I	10.7	216/231/260/380
II	11.0	221/241/257sh/368/385sh
III	19.4	220sh/365sh/391/411
IV	22.1	220sh/243/390
V	24.4	220/230sh/246/365sh/390/410
VI	29.8	221/230sh/282/365sh/388/405sh
VII	35.0	221/241/360sh/386/405
VIII	35.7	220/288/365sh/387/405sh
IX	40.8	226/280/360sh/386/405
X	44.0	26/230sh/284/385
XI	45.8	222/230sh/241/283/360sh/382/405sh
XII	46.4	225sh/231/250sh/285sh/352/385sh/400sh
XIII	47.6	217/225sh/240sh/274/360sh/385/404
XIV	51.9	235sh/283/360sh/382/400sh
XV	53.8	217/236/279/360sh/384/405sh
XVI	64–69	225sh/236/274/360sh/384/405sh
XVII	69.6	225sh/235sh/274/360sh/379/399

Compound numbers correspond to peak numbers in Fig. 2. Mycorradicin derivatives, **I**–**XV**; ''Yellow pigment'', **XVI**; Mycorradicin, **XVII**.

the root system were observed, whereas the amounts of the ''yellow pigment'' and the major cyclohexenone derivatives steadily increased until 25 weeks after inoculation. From these results it may be assumed that the yellow apocarotenoid accumulation precedes the accumulation of the compounds comprising the ''yellow pigment'' and that the yellow apocarotenoids are either transformed into colorless products or integrated into the ''yellow pigment'' complex. To get an impression of the homogeneity of this complex, a shallow HPLC gradient was applied to separate the ''yellow pigment'' complex partially into 4 major and 30 minor yellow apocarotenoids (data not shown). The peaks eluting after the ''yellow pigment'' area (Fig. 2) were shown to be identical with mycorradicin isomers regarding retention time, UV–Vis spectra and co-chromatography with a standard compound. To provide evidence that both the yellow apocarotenoids and the ''yellow pigment'' are composed of mycorradicin derivatives, alkaline hydrolysis of these components was performed yielding in each case mixtures of mycorradicin isomers. These experiments confirmed our assumption, but gave no details regarding the nature of the conjugating partners. Unfortunately we were also unable to deduce definitive structural information from partially purified components analyzed by LC/ESI-MS, MALDI, and NMR spectroscopy. An additional peculiarity observed is the occurrence of a yellow apocarotenoid (at R_t 18 min, Fig. 3) in extracts from nonmycorrhizal roots, a phenomenon which is in accordance with previous results (Fester et al., 2002) demonstrating the presence of minute amounts of mycorradicin liberated by alkaline treatment of extracts from control roots (0.2% of the amount found

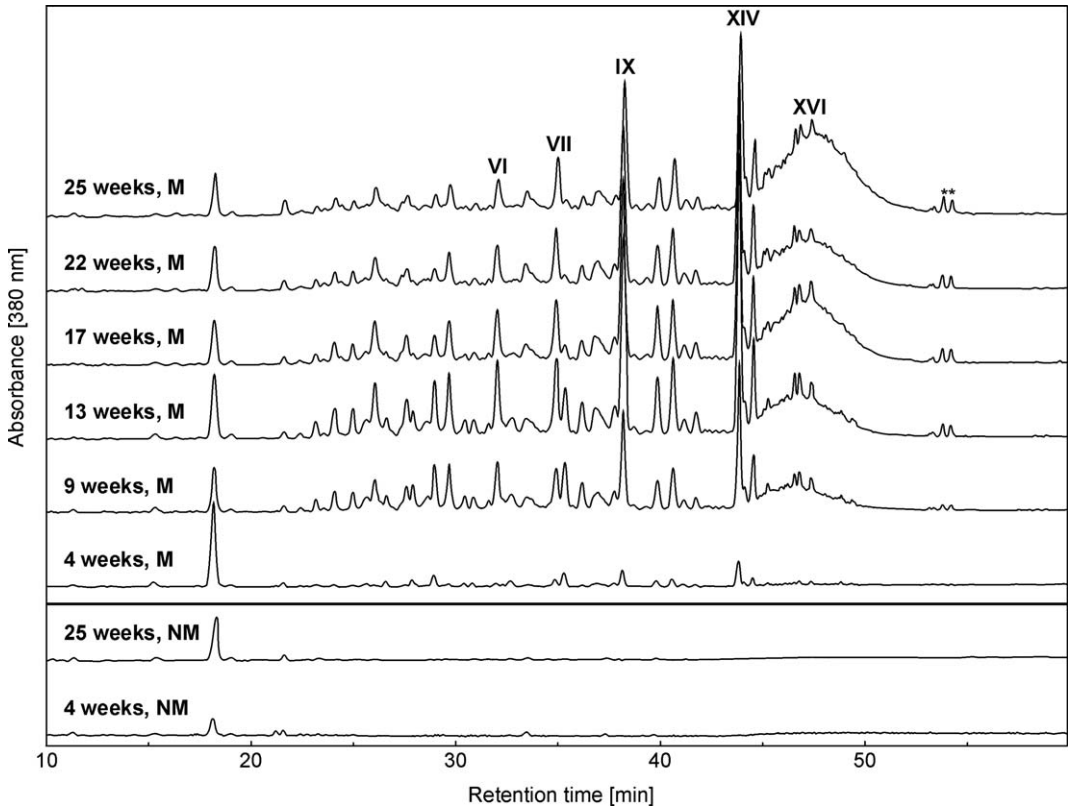

Fig. 3. HPLC elution profiles (solvent system 1) of yellow apocarotenoids (mycorradicin derivatives including the "yellow pigment") (PDA-detection at 380 nm) from roots of *O. umbellatum* harvested at different times of root mycorrhization. (NM, nonmycorrhizal controls; M, mycorrhizal roots). The asterisks indicate free mycorradicin isomers. The absorbance at 380 nm of all HPLC traces are autoscaled to 0.060.

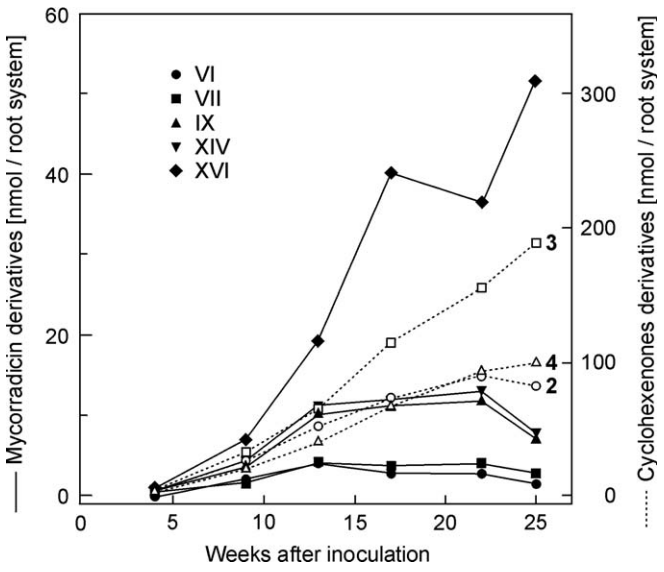

Fig. 4. Time course of accumulation of major cyclohexenone derivatives (**2**, **3**, **4**) and major yellow apocarotenoids (**VI**, **VII**, **IX**, **XIV**) as well as the "yellow pigment" (**XVI**) in mycorrhizal roots of *O. umbellatum*. Peak numbers correspond to the numbers in Figs. 2 and 3.

in extracts from AM roots). The occurrence of such minute amounts of mycorradicin derivatives in nonmycorrhizal root extracts is not a rare phenomenon as it was detected in 7 of 46 plant species analyzed (Fester et al., 2002).

2.2. C_{13} cyclohexenone derivatives

During the analysis of the yellow apocarotenoids by HPLC the complementary C_{13} apocarotenoids showing characteristic UV absorption maxima at 244 nm were simultaneously detected (Fig. 2) and showed a time-dependent increase (Fig. 4). According to previous results these compounds were expected to be various glycosylated C_{13} cyclohexenone derivatives of the blumenol C type. After isolation of eight major compounds (**1**–**8**) by preparative HPLC their structures were elucidated by LC/ESI-MS, high resolution ESI-FT-ICRMS and NMR spectroscopy (Fig. 5). Prior to these analyses aliquots of the purified cyclohexenone derivatives were treated with a glycosidase-containing cellulase to liberate the aglycones, which were subsequently analyzed by HPLC (Table 2). These experiments revealed that compounds **1**, **2**, and **3** are derived from 13-hydroxyblumenol C, compounds **4** and **5** from 13-nor-5-carboxy-blumenol C, whereas compounds **6**, **7**, and **8** have blumenol C as aglycone as subsequently confirmed by MS and NMR spectra. From the protonated molecular ion of compound **1** at *m*/*z* 551 and from the respective fragmentation pattern the presence of two hexoses bound to 13-hydroxyblumenol C (*m*/*z* 227) was deduced. ^{1}H NMR data indicated that compound **1** is the 9-*O*-gentiobiosyl derivative of 13-hydroxyblumenol C, previously isolated from mycorrhizal tobacco roots and

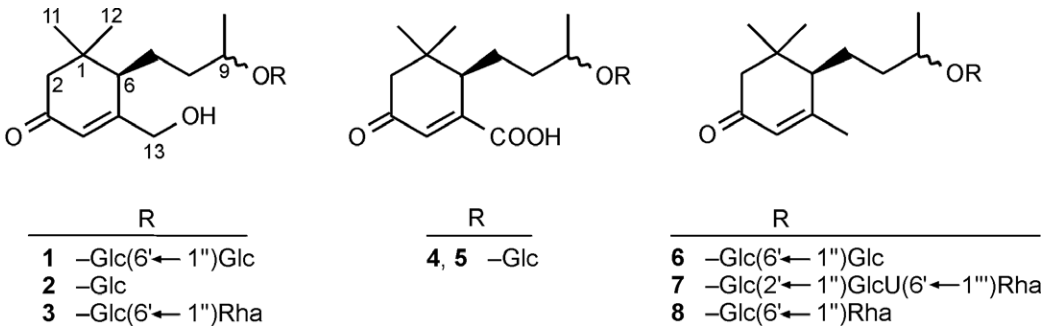

Fig. 5. Structure scheme of C_{13} cyclohexenone derivatives isolated from mycorrhizal roots of *O. umbellatum*.

Table 2
Retention time, HPLC-PDA (solvent system 2), and MS data of cyclohexenone derivatives from mycorrhizal roots of *O. umbellatum*

Compound	R_t (min)	HPLC-PDA λ_{max} (nm)	Hydrolysis product R_t (min)	LC/MS (*m/z*)		Structural assignment
				$[A+H]^{+a}$	$[M+H]^+$	
1	10.6	215/226/243	18.9	227	551	13-Hydroxyblumenol C 9-*O*-β-gentiobioside (Nicoblumin)
2	13.6	216/226/243	18.9	227	389	13-Hydroxyblumenol C 9-*O*-β-glucoside
3	16.9	215/226sh/243	18.9	227	535	13-Hydroxyblumenol C 9-*O*-β-(6′-*O*-rhamnosylglucoside)
4	18.3	230sh/246	25.5	241	403	13-Nor-5-carboxyblumenol C 9-*O*-β-glucoside
5	19.2	214/230sh/246	24.5	241	403	Stereoisomer of **4** at *O*-9
6	22.2	216/245	42.2	211	535	Blumenol C 9-*O*-β-gentiobioside
7	26.4	216/230sh/245	–[b]	211	695	Blumenol C 9-*O*-β-(6′-*O*-rhamnosyl-2′-*O*-β-glucuronosylglucoside)
8	30.9	216/246	42.2	211	519	Blumenol C 9-*O*-β-(6′-*O*-rhamnosyl)glucoside

Compound numbers correspond to peak numbers in Fig. 2.
[a] A, aglycone.
[b] Incomplete hydrolysis or too low concentration.

named nicoblumin (Maier et al., 1999). Similarly, compound **2** was identified as the already described 13-hydroxyblumenol C 9-*O*-β-glucoside (Peipp et al., 1997), probably the precursor of compound **1**. From the protonated molecular ion of compound **3** at *m/z* 535 and from the fragmentation pattern 13-hydroxyblumenol C (*m/z* 227) linked with a hexose and a deoxyhexose was deduced. This compound was identified finally as 13-hydroxyblumenol C 9-*O*-β-(6′-*O*-rhamnosylglucoside), presumably a new natural compound, by means of 1D and 2D ^{1}H as well as ^{13}C NMR data. The very similar compounds **4** and **5** gave *m/z* 241 for the protonated aglycone indicating the replacement of a methyl group by a carboxyl residue and the protonated molecular ion at *m/z* 403 indicates the presence of a hexosyl moiety. 1D and 2D ROESY ^{1}H NMR data demonstrated that compound **4** is a β-glucoside of 13-nor-5-carboxyblumenol C and appears to be identical with compound **5** reported in Maier et al. (2000). Furthermore, a similar NMR data set for compound **5** from *O. umbellatum* is indicative for an isomer of **4**, most probably at the C-9 position as already discussed for isomers from tobacco roots (Maier et al., 2000). From the protonated molecular ion (*m/z* 535) and the fragmentation pattern (*m/z* 211, $[aglycone+H]^+$) compound **6** was shown to be a dihexosyl derivative of blumenol C. ^{1}H NMR data demonstrated that compound **6** is blumenol C 9-*O*-gentiobioside and is identical with the major isomer of the cyclohexenone derivatives **6** and **7**, isolated from mycorrhizal tobacco roots (Maier et al., 2000). By comparing the ESI-MS data of compound **3** (*m/z* 535, $[M+H]^+$) with those of compound **8** (*m/z* 519, $[M+H]^+$), it was suggested that **8** has similar sugar moieties but lacks the 13-hydroxy function found in **3**. 1D and 2D COSY ^{1}H NMR data confirmed that **8** is blumenol C 9-*O*-β-(6′-*O*-rhamnosylglucoside), presumably a new natural compound. Compound **7**, isolated only in minute amounts, showed a protonated molecular ion at *m/z* 695 that is indicative for an additional hexuronosyl moiety attached to the glucose. Signals for blumenol C together with characteristic ^{1}H signals for glucose and rhamnose were observed after long accumulation times (at 600 MHz). A shift of H-1 of glucose compared with **8** suggested the additional residue found by MS is at C-2′ of glucose. This was confirmed by a MS/MS investigation of the permethylated sample which is compatible with the linkage of hexuronic acid at C-2′ and rhamnose at C-6′. In addition to an intense fragment ion at *m/z* 651 [Hex-HexUdeoxyHex+Na]$^+$ confirming the presence of a trisaccharide carbohydrate moiety, fragments characteristic for a terminal deoxyhexose (*m/z* 229) as well as a terminal hexuronic acid (HexU) residue (*m/z* 273) were detected indicating a branched oligosaccharide structure with a hexose as the central residue. Inner-ring fragments of the ^{0,2}A2 (*m/z* 315) and ^{1,3}A2 (*m/z* 329) type according to the nomenclature of Domon and Costello (1988) indicate a linkage of

the hexuronic acid residue to C-2′ of the glucose residue, whereas an inner-ring fragment of the ^{3,5}A2 type was detected at *m*/*z* 299 incorporating the rhamnose residue, limiting its substitution positions to C-4′ or C-6′ of the glucose residue. Therefore, the most probable structure of this trace compound **7** is blumenol C 9-*O*-β-(2′-*O*-glucuronosyl-6′-*O*-rhamnosylglucoside). In blumenin (Maier et al., 1995), as well as in the corresponding 13-hydroxy derivative, isolated from mycorrhizal roots of *L. japonicus* (Fester et al., 2005), a glucuronic acid is also attached to the C-2′-position of glucose.

Although in cyclohexenone derivatives from mycorrhizal roots isomers at C-9 were observed by HPLC and NMR spectroscopy (Maier et al., 2000), no attempts were known to assign the configuration at C-6 of 9-hydroxy-7,8-dihydro-9-apo-ε-caroten-3-one (7,8-dihydro-3-oxo-α-ionol, blumenol C). CD spectra of two blumenin isomers (retention time: 21.7 and 22.8 min, respectively) were found to be very similar and showed positive Cotton effects ($[\Theta]_{M239} + 7{,}189$ and $[\Theta]_{M241} + 5{,}801$, respectively) corresponding to data described for blumenol C 9-*O*-β-glucoside ($[\Theta]_{M241} + 14{,}800$) which has the 6*R*-configuration (Miyase et al., 1988). For the major compound **3**, a blumenol C derivative hydroxylated at the enone chromophore, a strong positive Cotton effect ($[\Theta]_{M215} + 14{,}254$) was found indicating an identical configuration. The assignment of the 6*R*-configuration of blumenol C in blumenin isomers is in accordance with previous blumenol C reports (Aasen et al., 1974; Siddiqui et al., 2003; D'Abrosca et al., 2004; DellaGreca et al., 2004; Park et al., 2004; De Marino et al., 2005).

By external standardization (see Section 3) the amount of accumulating cyclohexenone and mycorradicin derivatives in mycorrhizal roots (25 month after inoculation) were determined to be 289.5 nmol and 49.8 nmol per g fr. wt, respectively. This is a ratio of 5.8:1 for the two apocarotenoid groups, strongly deviating from the 2:1 ratio expected from the biosynthetic formation. The content for the unique mycorradicin derivative occurring in nonmycorrhizal roots (1.5 nmol/g fr. wt) was found to be almost identical as in mycorrhizal roots. The set of cyclohexenone derivatives identified comprises mono-, di- and branched triglycosides from already known aglycones (blumenol C, 13-hydroxyblumenol C, 13-nor-5-carboxyblumenol C) containing terminal rhamnose as a so far unreported sugar in cyclohexenone glycosides. In summary, the most complex pattern of AM fungus-induced apocarotenoids described so far were found in *O. umbellatum* roots. For the first time free mycorradicin and a series of its derivatives were detected, along with the "yellow pigment". The confirmation of a possible precursor–product relationship indicated by the different accumulation pattern of the yellow apocarotenoids and the "yellow pigment" during the process of mycorrhization awaits further studies. These results strengthen even more the need for elucidating the role of apocarotenoids or their precursors in AM roots.

3. Experimental

3.1. Plant material and mycorrhization experiments

Bulbs of *O. umbellatum* L. (Küpper, Blumenzwiebeln and Saaten GmbH, Eschwege, Germany) were placed in 500-ml plastic pots filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay, Pinneberg, Germany) containing 10% of an AM fungus inoculate (*G. intraradices* Schenk and Smith, isolate 49, provided by H. von Alten, University Hannover, Germany), enriched by previous co-cultivation with leek (*Allium porrum* L.) in expanded clay and were grown in a green house with a 16-h daily light period. The inoculated plants as well as the control plants (without fungal inoculate) were watered three times per weeks and fertilized once per week with Long Ashton nutrition solution (5 ml per pot) (Hewitt, 1966) with reduced phosphate (20%) content.

3.2. Harvesting and extraction

At the harvest times (4, 9, 13, 17, 22, and 25 weeks after inoculation) the roots of two parallels were cleaned from Lecaton, shock frozen with liquid nitrogen and homogenized in a mortar followed by extraction with 80% aq. MeOH (0.5 g ml^{-1}). To remove interfering mucilaginous constituents from the extracts 200 µl aliquots were treated with 600 µl EtOH, incubated for 30 min at room temperature and centrifuged (5 min, 16,000*g*). The supernatants were concentrated to dryness using a Speedvac, the residues dissolved in 50 µl 80% aq. MeOH for HPLC analysis. A second kinetic mycorrhization experiment (harvest times at 2, 4, 6, 8, 12, and 16 weeks after inoculation) was performed with similar results (data not shown).

3.3. Microscopical methods

Using a Nikon (Tokyo, Japan) SMZ-U stereomicroscope thin needles and razor blades, the freshly harvested roots of nonmycorrhizal and mycorrhizal *O. umbellatum* (6 month old) were cut on glass slides in 50 mM HEPES (pH 7.0), 125 mM sorbitol, 2.5 mM β-mercaptoethanol, and 2.5 mM EDTA in order to generate thin cross sections. Epifluorescence microscopy was performed using a Nikon Optiphot-2 with the following filter settings for epifluorescence: EX330–380, DM400, BA435. Photographs were taken using Fujifilm (Tokyo, Japan) Sensia II. The slides were scanned and processed using the programme PhotoPaint 7.0 (Corel Corporation, Ottawa, Canada).

3.4. HPLC

Analytical and semi-prep. HPLC was performed with a system from Waters (Milford, USA), including the separation module 2690. The liquid chromatograph was equipped with a 5 µm Nucleosil C_{18} column (250 × 4 mm i.d.; Macherey–Nagel, Düren, Germany). The following

analytical solvent and gradient systems were used: solvent system 1 (for the separation of both apocarotenoid groups in mycorrhizal kinetics) A, 1.5% aq. H_3PO_4; B, acetonitrile; constant gradient from 5% B to 25% B in (A + B) within 40 min followed by 25% B to 80% B in 20 min; solvent system 2 (for the separation of the cyclohexenone conjugates and yellow apocarotenoids as well as their hydrolysis products) A, 2% aq. HCO_2H; B, acetonitrile; constant gradient from 10% B to 25% B within 60 min and then 25% B to 80% B in additional 10 min; the flow rate was 1 ml min^{-1} each. The compounds were photometrically detected (at 245 and 380 nm; maxplot between 210 and 500 nm) by a Waters 2996 photodiode array detector (injection volume: 20 μl). Whereas the cyclohexenone derivatives were quantified by external standardization based on abscisic acid having the identical 1,1,5-trimethyl-cyclohex-4-en-3-one moiety, the levels of mycorradicin derivatives were calculated using mycorradicin dimethyl ester as standard. The results were expressed as nmol ABA or mycorradicin dimethyl ester equivalents/g fr. wt as well as nmol/root system (deviation of the individual peak areas of the cyclohexenone and mycorradicin derivatives of the duplicates were <20%).

For prep. HPLC the Waters Delta 600 liquid chromatograph was equipped with a VP 250/40 Nucleosil 100–10 C_{18} column (Macherey-Nagel, Düren, Germany) and the following solvent and gradient system was used: A, 1% aq. HOAc; B, acetonitrile; constant gradient from 10% B to 35% B within 220 min. The compounds were photometrically detected as in the analytical HPLC at a flow rate of 20 ml min^{-1} (injection volume: 5 ml). Data acquisition and evaluation was performed using Empower 5.0. The preparative separation started with an extract derived from 63 mycorrhizal root systems (100 g fr. wt, 25 month after inoculation).

3.5. Mass spectrometry, NMR, and optical measurement

Positive ion electrospray ionisation mass spectra were recorded on a Finnigan MAT TSQ 7000 instrument and high resolution ESI mass spectra were obtained on a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, USA) as detailed recently (Schliemann et al., 2006). Standard micro-methylation techniques were applied for the permethylation of the samples. Carbohydrate compositional analyses were performed on a Finnigan GCQ GC–MS as described previously (Nimtz et al., 1996).

1D and 2D (COSY and ROESY) ^1H and ^{13}C (1D) NMR spectra were recorded at 300 K on Bruker AVANCE DMX 600 spectrometer locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are reported in ppm relative to TMS and coupling constants in Hz.

The CD spectrum (200–500 nm) was recorded in MeOH on a Jasco J710 spectropolarimeter.

3.6. Hydrolyses

The yellow apocarotenoids (**I**–**XVII**) were isolated by HPLC (solvent system 2), dissolved in 27 μl 80% aq. MeOH and treated with 3 μl 10 N KOH for 3 h at 30 °C. Subsequently 20 μl were analyzed by HPLC (solvent system 2) to detect hydrolysis products. For enzymic hydrolysis of cyclohexenone derivatives aliquots were treated with 50 μl dialyzed cellulase in 0.1 M citrate/phosphate buffer, pH 5.2 (128 μg protein ml^{-1}, Onozuka SS, Yakult Biochemicals, Nishinomiya, Japan) for 24 h at 30 °C. The protein was precipitated by addition of 50 μl MeOH, the supernatant was taken to dryness in a Speedvac, the residues dissolved in 20 μl MeOH and 10 μl analyzed by HPLC (solvent system 2).

3.7. NMR, mass spectral, and optical data of cyclohexenone derivatives

3.7.1. 13-Hydroxyblumenol C 9-O-β-gentiobioside (*1*)

ESI-HRMS *m/z* 573.2514 ($[M+Na]^+$, $C_{25}H_{42}O_{13}Na$, calc. 573.2518). ESI-MS/MS of *m/z* 551: 551 $[M+H]^+$, 389 $[(M+H)\text{-Glc}]^+$, 227 [13-hydroxyblumenol C+H]$^+$, 209 $[(13\text{-hydroxyblumenol C+H})\text{-}H_2O]^+$. ^1H NMR (600 MHz, CD_3OD): δ 6.10 (1 H, *bs*, H-4), 4.44 (1H, *d*, H-1″, $J_{1'',2''} = 7.8$ Hz), 4.36 (1H, *d*, H-1′, $J_{1',2'} = 7.9$ Hz), 4.36 (1H, *dd*, H-13α, $J_{13\alpha,4} = 1.5$ Hz, $J_{13\alpha,13\beta} = 17.7$ Hz), 4.20 (1H, *dd*, H-13β, $J_{13\beta,4} = 1.7$ Hz), 4.15 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 2.1$ Hz, $J_{6'\alpha,6'\beta} = 11.7$ Hz), 3.90 (1H, *dd*, H-6″α,$J_{6''\alpha,5''} = 2.2$ Hz, $J_{6''\alpha,6''\beta} = 11.9$ Hz), 3.87 (1H, *m*, H-9), 3.83 (1H, *dd*, H-6′β, $J_{6'\beta,5'} = 5.7$ Hz), 3.70 (1H, *dd*, H-6″β, $J_{6''\beta,5'} = 5.4$ Hz), 3.48 (1H, *ddd*, H-5′, $J_{5',4'} = 9.4$ Hz), 3.39–3.31 (4H, *m*, H-3′, H-4′, H-3″, H-4″), 3.29 (1H, *m*, H-5″), 3.24 (1H, *dd*, H-2″, $J_{2'',3''} = 9.0$ Hz), 3.19 (1H, *dd*, H-2′, $J_{2',3'} = 9.0$ Hz), 2.63 (1H, *d*, H-2α, $J_{2\alpha,2\beta} = 17.6$ Hz), 2.06 (1H, *d*, H-2β), 1.96 (1H, *t*, H-6, $J_{6,7\alpha,\beta} = 5.0$ Hz), 1.86 (1H, *m*, H-7α), 1.74-1.61 (3H, *m*, H-7β, H-8α, H-8β), 1.28 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.15 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

3.7.2. 13-Hydroxyblumenol C 9-O-β-glucoside (*2*)

ESI-HRMS *m/z* 411.1988 ($[M+Na]^+$, $C_{19}H_{32}O_8Na$, calc. 411.1989). ESI-MS/MS of *m/z* 389: 389 $[M+H]^+$, 227 [13-hydroxyblumenol C+H]$^+$, 209 $[(13\text{-hydroxyblumenol C+H})\text{-}H_2O]^+$. ^1H NMR (600 MHz, CD_3OD): δ 6.10 (1H, *bs*, H-4), 4.36 (1H, *dd*, H-13α, $J_{13\alpha,4} = 1.4$ Hz, $J_{13\alpha,13\beta} = 17.7$ Hz), 4.35 (1H, *d*, H-1′, $J_{1',2'} = 7.8$ Hz), 4.20 (1H, *dd*, H-13β, $J_{13\beta,4} = 1.6$ Hz), 3.89 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 2.1$ Hz, $J_{6'\alpha,6'\beta} = 12.0$ Hz), 3.86 (1H, *m*, H-9), 3.68 (1H, *dd*, H-6′β, $J_{6'\beta,5} = 5.3$ Hz), 3.35–3.26 (3H, *m*, H-3′, H-4′, H-5′), 3.18 (1H, *dd*, H-2′, $J_{2',3'} = 9.1$ Hz), 2.63 (1H, *d*, H-2α , $J_{2\alpha,2\beta} = 17.6$ Hz), 2.06 (1H, *d*, H-2β), 1.96 (1H, *t*, H-6, $J_{6,7\alpha\beta} = 5.4$ Hz), 1.86 (1H, *m*, H-7α), 1.75-1.58 (3H, *m*, H-7β, H-8αβ), 1.29 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.16 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

*3.7.3. 13-Hydroxyblumenol C 9-O-β-(6′-O-rhamnosylglucoside) (**3**)*

CD (MeOH) $[\Theta]_{M215}$ +14,254, $[\Theta]_{M255}$ −1843, $[\Theta]_{M329}$ +2,467 deg cm^2 dmol^{-1}. ESI-HRMS *m/z* 557.2563 ($[M+Na]^+$, $C_{25}H_{42}O_{12}Na$, calc. 557.2568). ESI-MS/MS of *m/z* 535: 535 $[M+H]^+$, 389 $[(M+H)\text{-Rha}]^+$, 227 [13-hydroxyblumenol C+H]$^+$, 209 [(13-hydroxyblumenol C+H)-H_2O]$^+$. ^{1}H NMR (600 MHz, CD_3OD): δ 6.10 (1H, *bs*, H-4), 4.80 (1H, *d*, H-1″), 4.36 (1H, *dd*, H-13α, $J_{13\alpha,4} = 1.6$ Hz, $J_{13\alpha,13\beta} = 17.6$ Hz), 4.34 (1H, *d*, H-1′, $J_{1',2'} = 7.7$ Hz), 4.20 (1H, *dd*, H-13β, $J_{13\beta,4} = 1.7$ Hz), 4.00 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 1.9$ Hz, $J_{6'\alpha,6'\beta} = 11.2$ Hz), 3.86 (1H, *dd*, H-2″, $J_{1'',2''} = 1.6$ Hz, $J_{2'',3''} = 3.4$ Hz), 3.80 (1H, *m*, H-9), 3.69 (1H, *m*, H-5″), 3.69 (1H, *dd*, H-3″, $J_{3'',4''} = 9.4$ Hz), 3.63 (1H, *dd*, H-6′β, $J_{6'\beta,5} = 6.4$ Hz), 3.41 (1H, *m*, H-5′), 3.40 (1H, *t*, H-3′, $J_{3',2'} = 9.5$ Hz, $J_{3',4'} = 9.5$ Hz), 3.40 (1H, *m*, H-4″), 3.29 (1H, *t*, H-4′, $J_{4',5'} = 9.2$ Hz), 3.18 (1H, *dd*, H-2′), 2.62 (1H, *d*, H-2α, $J_{2\alpha,2\beta}$= 17.6 Hz), 2.06 (1H, *d*, H-2β), 1.97 (1H, *t*, H-6, $J_{6,7\alpha\beta} = 5.6$ Hz), 1.85 (1H, *m*, H-7α), 1.76–1.58 (3H, *m*, H-7β, H-8αβ), 1.29 (3H, *d*, H-6″, $J_{6'',5''} = 6.2$ Hz), 1.29 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.15 (3H, *s*, H-11), 1.06 (3H, *s*, H-12). In the ROESY spectrum sequential correlations were found between H-1′ and H-9, H-1′ and H-10, H-1″and H-6′α, and H-1″ and H-6′β. ^{13}C NMR (150 MHz, CD_3OD): δ 202.4 (*s*, C-3), 172.4 (*s*, C-5), 121.5 (*d*, C-4), 104.4 (*d*, C-1′), 102.3 (*d*, C-1″), 78.2 (*d* ×2, C-9, C-3′), 76.7 (*d*, C-5′), 75.3 (*d*, C-2′), 74.0 (*d*, C-4″), 72.4, 72.2 (*d* ×2, C-2″, C-3″), 71.8 (*d*, C-4′), 69.8 (*d*, C-5″), 68.3 (*t*, C-6′), 65.1 (*t*, C-13), 48.7 (*t*, C-2), 48.1 (*d*, C-6), 37.6 (*t*, C-8), 37.4 (*s*, C-1), 28.8 (*q*, C-12), 27.7 (*q*, C-11), 27.2 (*t*, C-7), 22.1 (*q*, C-10), 18.1 (*q*, C-6″).

*3.7.4. 13-Nor-5-carboxyblumenol C 9-O-β-glucoside (**4**)*

ESI-HRMS *m/z* 425.1784 ($[M+Na]^+$, $C_{19}H_{30}O_9Na$, calc. 425.1782). ESI-MS/MS of *m/z* 403: 403 $[M+H]^+$, 241 [13-nor-5-carboxyblumenol C+H]$^+$, 223 [(13-nor-5-carboxyblumenol C+H)-H_2O]$^+$, 195 [(13-nor-5-carboxyblumenol C+H)-HCO_2H]$^+$.

^{1}H NMR (600 MHz, CD_3OD): δ 6.46 (1H, *bs*, H-4), 4.35 (1H, *d*, H-1′, $J_{1',2'} = 7.8$ Hz), 3.88 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 2.1$ Hz, $J_{6'\alpha,6'\beta} = 12.0$ Hz), 3.86 (1H, *m*, H-9), 3.70 (1H, *dd*, H-6′β, $J_{6'\beta,5'} = 5.3$ Hz,), 3.42-3.28 (3H, *m*, H-3′, H-4′, H-5′), 3.18 (1H, *dd*, H-2′, $J_{2',3'}$ 8.9), 2.68 (1H, *m*, H-6), 2.64 (1H, *d*, H-2α, $J_{2\alpha,2\beta} = 17.5$ Hz), 2.08 (1H, *d*, H-2β), 1.94 (1H, *m*, H-7α), 1.72-1.60 (3H, *m*, H-7β, H-8αβ), 1.26 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.17 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

*3.7.5. Compound (**5**) (stereoisomer of **4** at O-9)*

ESI-HRMS *m/z* 425.1790 ($[M+Na]^+$, $C_{19}H_{30}O_9Na$, calc. 425.1782). ESI-MS/MS of *m/z* 403: 403 $[M+H]^+$, 241 [13-nor-5-carboxyblumenol C+H]$^+$, 223 [(13-nor-5-carboxyblumenol C+H)-H_2O]$^+$, 195 [(13-nor-5-carboxyblumenol C+H)-HCO_2H]$^+$. ^{1}H NMR (600 MHz, CD_3OD): δ 6.33 (1H, *bs*, H-4), 4.33 (1H, *d*, H-1′, $J_{1',2'} = 7.8$ Hz), 3.89 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 1.8$ Hz), 3.88 (1H, *m*, H-9), 3.70 (1H, *dd*, H-6′β, $J_{6'\beta,5'} = 5.4$ Hz, $J_{6'\beta,6'\alpha} = 11.9$ Hz), 3.37 (1H, *t*, H-3′, $J_{3',2'} = 9.1$ Hz, $J_{3',4'} = 9.4$ Hz), 3.29 (1H, *t*, H-4′, $J_{4',5'} = 9.7$ Hz), 3.27 (1H, *m*, H-5′), 3.17 (1H, *dd*, H-2′), 2.68 (1H, *dd*, H-6, $J_{6,7\alpha\beta} = 14.7$, 7.9 Hz), 2.59 (1H, *d*, H-2α, $J_{2\alpha,2\beta} = 17.2$ Hz), 2.05 (1H, *d*, H-2β), 2.03 (1H, *m*, H-7α), 1.74 (1H, *m*, H-8α), 1.66 (1H, *m*, H-8β), 1.48 (1H, *m*, H-7β), 1.19 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.15 (3H, *s*, H-11), 1.07 (3H, *s*, H-12). In the ROESY spectrum sequential correlations were found between H-1′ and H-9, and H-1′ and H-10.

*3.7.6. Blumenol C 9-O-β-gentiobioside (**6**)*

ESI-HRMS *m/z* 557.2572 ($[M+Na]^+$, $C_{25}H_{42}O_{12}Na$, calc. 557.2568). ESI-MS/MS of *m/z* 535: 535 $[M+H]^+$, 373 $[(M+H)\text{-Glc}]^+$, 211 [blumenol C+H]$^+$, 193 [(blumenol C+H)-H_2O]$^+$. ^{1}H NMR (600 MHz, CD_3OD): δ 5.84 (1H, *bs*, H-4), 4.44 (1H, *d*, H-1″, $J_{1'',2''} = 7.8$ Hz), 4.36 (1H, *d*, H-1′, $J_{1',2'} = 7.8$ Hz), 4.15 (1H, *dd*, H-6′α, $J_{6'\alpha,5'} = 2.0$ Hz, $J_{6'\alpha,6'\beta} = 11.7$ Hz), 3.90 (1H, *dd*, H-6″α, $J_{6''\alpha,5''} = 2.0$ Hz, $J_{6''\alpha,-6''\beta} = 12.0$ Hz), 3.87 (1H, *m*, H-9), 3.83 (1H, *dd*, H-6′β, $J_{6'\beta,5'} = 5.8$ Hz), 3.70 (1H, *dd*, H-6″β, $J_{6''\beta,5''} = 5.4$ Hz), 3.47 (1H, *m*, H-5′), 3.40–3.31 (4H, *m*, H-3′, H-4′, H-3″, H-4″), 3.29 (1H, *m*, H-5″), 3.24 (1H, *dd*, H-2″, $J_{2'',3''} = 9.0$ Hz), 3.19 (1H, *dd*, H-2′, $J_{2',3'} = 9.0$ Hz), 2.52 (1H, *d*, H-2α, $J_{2\alpha,2\beta} = 17.4$ Hz), 2.09 (3H, *d*, H-13, $J_{13,4} = 1.0$ Hz), 2.02 (1H, *d*, H-2β), 2.01 (1H, *t*, H-6, $J_{6,7\alpha\beta} = 5.3$ Hz), 1.85 (1H, *m*, H-7α), 1.76–1.61 (3H, *m*, H-7β, H-8α, H-8β), 1.29 (3H, *d*, H-10, $J_{10,9} = 6.3$ Hz), 1.14 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

*3.7.7. Blumenol C 9-O-β-(6′-O-rhamnosyl-2′-O-β-glucuronosylglucoside) (**7**)*

ESI-HRMS *m/z* 717. 2942 ($[M+Na]^+$, $C_{31}H_{50}O_{17}Na$, calc. 717.2940). ESI-MS/MS of *m/z* 695: 695 $[M+H]^+$, 549 $[(M+H)\text{-Rha}]^+$, 519 $[(M+H)\text{-GlcU}]^+$, 373 $[(M+H)\text{-Rha-GlcU}]^+$, 211 [blumenol C+H]$^+$. Only a limited number of ^{1}H signals could be identified due to the scarcity of available isolated compound. ^{1}H NMR (600 MHz, CD_3OD): δ 5.84 (1H, *bs*, H-4), 4.66 (1H, *d*, H-1′, $J_{1',2'} = 7.6$ Hz), 4.00 (1H, *bd*, H-6′α, $J_{6'\alpha,6'\beta} =$ 11.1 Hz), 2.51 (1H, *d*, H-2α, $J_{2\alpha,2\beta} =$ 17.4 Hz), 2.11 (3H, *bs*, H-13), 2.01 (1H, *d*, H-2β), 2.06 (1H, *bs*, H-6), 1.84 (1H, *m*, H-7α), 1.80–1.60 (3H, *m*, H-7β, H-8αβ), 1.29, 1.28 (6H, *d* ×2, H-10, H-6″′, $J_{9,10}$–$J_{6''',5'''} = 6.2$ Hz), 1.14 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

*3.7.8. Blumenol C 9-O-β-(6′-O-rhamnosylglucoside) (**8**)*

ESI-HRMS *m/z* 541.2610 ($[M+Na]^+$, $C_{25}H_{42}O_{11}Na$, calc. 541.2619). ESI-MS/MS of *m/z* 519: 519 $[M+H]^+$, 373 $[(M+H)\text{-Rha}]^+$, 211 [blumenol C+H]$^+$, 193 [(blumenol C+H)-H_2O. ^{1}H NMR (600 MHz, CD_3OD): δ 5.84 (1H, *bs*, H-4), 4.80 (1H, *d*, H-1″), 4.34 (1H, *d*, H-1′, $J_{1',2'} = 7.9$ Hz), 4.00 (1H, *dd*, H-6′α, $J_{6'\alpha,\ 5'} = 1.8$ Hz, $J_{6'\alpha,6'\beta} = 11.1$ Hz), 3.85 (1H, *dd*, H-2″, $J_{1'',2''} = 1.6$ Hz, $J_{2'',3''} = 3.4$ Hz), 3.81 (1H, *m*, H-9), 3.70 (2H, *m*, H-3″, H-5″), 3.63 (1H, *dd*, H-6′β, $J_{6'\beta,5'} = 6.4$ Hz), 3.43 (1H, *m*, H-5′), 3.40 (1H, *m*, H-4″), 3.37 (1H, *t*, H-3′, $J_{3',2'} = 9.1$ Hz, $J_{3',4'} = 9.1$ Hz), 3.29

(1H, *t*, H-4′, $J_{4',5'} = 9.1$ Hz), 3.18 (1H, *dd*, H-2′), 2.52 (1H, *d*, H-2α, $J_{2\ \alpha,2\beta} = 17.4$ Hz), 2.09 (3H, *d*, H-13, $J_{13,4} = 1.2$ Hz), 2.02 (1H, *d*, H-2B), 2.02 (1H, *t*, H-6, $J_{6,7} = 5.0$ Hz), 1.85 (1H, *m*, H-7α), 1.90-1.60 (3H, *m*, H-7β, H-8αβ), 1.29 (1H, *d*, H-6″, $J_{6'',5''} = 6.2$ Hz), 1.29 (3H, *d*, H-10, $J_{10,9}$ 6.2 Hz), 1.14 (3H, *s*, H-11), 1.06 (3H, *s*, H-12).

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