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# Accumulation of apocarotenoids in mycorrhizal roots of leek (*Allium porrum*)

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**Abstract**

Colonization of the roots of leek (*Allium porrum* L.) by the arbuscular mycorrhizal fungus *Glomus intraradices* induced the formation of apocarotenoids, whose accumulation has been studied over a period of 25 weeks. Whereas the increase in the levels of the dominating cyclohexenone derivatives resembles the enhancement of root length colonization, the content of mycorradicin derivatives remains relatively low throughout. Structural analysis of the cyclohexenone derivatives by mass spectrometry and NMR spectroscopy showed that they are mono- and diglycosides of 13-hydroxyblumenol C and blumenol C acylated with 3-hydroxy-3-methyl-glutaric and/or malonic acid. Along with the isolation of three known compounds five others are shown to be hitherto unknown members of the fast-growing family of mycorrhiza-induced cyclohexenone conjugates.


*Keywords:* *Allium porrum* L.; Alliaceae; *Glomus intraradices*; Arbuscular mycorrhiza; Apocarotenoid accumulation; $C_{13}$ cyclohexenone derivatives

## 1. Introduction

Mycorrhizas are highly evolved mutualistic associations between soil fungi and plant roots. The most common form of symbiosis involves arbuscular mycorrhizal (AM) fungi (Smith and Read, 1997) of the phylum *Glomeromycota* (Schüßler et al., 2001). The obligate biotrophic AM fungi facilitate the uptake of mineral nutrients, especially phosphate and nitrogen, and water from the soil by colonizing root cortical cells forming highly branched arbuscules. The plants, in return, provide the fungi with carbohydrates. This intimate interaction, often supporting plant growth (van der Heijden et al., 1998; Hartwig et al., 2002) and increasing resistance against abiotic stress and diseases (Cordier et al., 1998; Liu et al., 2007), is accompanied by alterations of the morphology of roots and their enzyme and metabolite patterns. Characteristic, not only for mycorrhizal roots of leek, is the accumulation of palmitvaccenic acid in lipids, originating from AM fungi (Grandmougin-Ferjani et al., 1995; Olsson et al., 2005).

A general phenomenon in the secondary metabolism of AM roots is the mycorrhiza-specific formation of two types of apocarotenoids, the $C_{13}$ cyclohexenone and the $C_{14}$ mycorradicin derivatives (Strack and Fester, 2006). The latter are responsible for an often observed yellow coloration of AM roots. The chromophore of this "yellow pigment" was isolated from mycorrhizal maize roots and identified as a linear carotenoid cleavage product ($C_{14}$), the 10,10′-diapocarotene-10,10′-dioic acid, named mycorradicin (Bothe et al., 1994; Klingner et al., 1995a). Because of structural similarities of mycorradicin with the $C_{27}$ apocarotenoid azafrin (Eschenmoser and Eugster, 1975), it has been suggested that it arises from a $C_{40}$ carotenoid precursor from which two $C_{13}$-units are cleaved (Klingner et al., 1995a,b). At about the same time when the structure of mycorradicin was established, the nature of a glycosidic

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$C_{13}$ cyclohexenone derivative (blumenin), occurring specifically in mycorrhizal cereal roots, was elucidated (Maier et al., 1995). That blumenin is also derived from carotenoids involving the methylerythritol pathway in early steps of its biosynthesis was shown by retrobiosynthetic studies (Maier et al., 1998). It was recognized that this type of compound may be the missing link ($C_{13}$ apocarotenoid) between $C_{40}$ carotenoids and mycorradicin ($C_{14}$ apocarotenoid). Consequently, the formation of both groups of apocarotenoids by oxidative cleavage of a common carotenoid precursor having one or two cyclized end groups was proposed (Walter et al., 2000).

Leek has been studied here due to its importance in mycorrhiza research comprising cytological (Smith and Smith, 1997; Dickson and Kolesik, 1999; Cavagnaro et al., 2001; Dickson, 2004; van Aarle et al., 2005), morphological (Garriock et al., 1989; Berta et al., 1990) and physiological (Snellgrove et al., 1982; Amijee et al., 1993a,b; Shachar-Hill et al., 1995; Dickson et al., 1999; Augé, 2001) investigations, and the fact that mycorrhiza-induced cyclohexenone derivatives have not been reported as yet in members of the Alliaceae family. A further reason was that AM leek roots accumulate very low amounts of mycorradicin derivatives in contrast to AM roots of *Ornithogalum umbellatum* (Fester et al., 2002b; Schliemann et al., 2006b). Here we report the identification and the time-dependent accumulation of apocarotenoids in leek roots during mycorrhization.

## 2. Results and discussion

### 2.1. Accumulation of mycorrhiza-induced apocarotenoids

The formation of the so-called "yellow pigment", a complex mixture of mycorradin derivatives, causes the roots of leguminous and gramineous plants to turn yellow upon colonization with AM fungi (Jones, 1924; Klingner et al., 1995a,b). Recently, the accumulation of the "yellow pigment" and further mycorradicin derivatives has been studied in depth in *O. umbellatum* (Hyacinthaceae) (Schliemann et al., 2006b) and *Medicago truncatula* (Leguminosae) (Schliemann et al., 2008). In contrast, previous work has reported that AM roots of onion and leek become yellow, although extracts showed absorption spectra distinct from those of the "yellow pigment" (Klingner et al., 1995b) and alkaline hydrolysis of extracts of 6-week-old AM leek roots contained only very low amounts of mycorradicin isomers (Fester et al., 2002a). These results were confirmed by the accumulation kinetics found here (Fig. 2). No peaks in the HPLC with absorption maxima around 380–400 nm, corresponding to the "yellow pigment", could be detected in AM roots. Instead an intense peak with optical characteristics ($\lambda_{max}$ 216, 239, 315 nm) compatible with phenolic compounds occurred obscuring any possibly traces of the "yellow pigment". Only after alkaline treatment of the extracts we were able to quantify low levels of mycorradicin isomers. External standardization (see Section 3) indicated the amount of mycorradicin derivatives in mycorrhizal roots (10 weeks after inoculation) was 0.3 nmol/root, whereas the level of cyclohexenone derivatives was 8.5 nmol/root. This ratio of 1:27.4 for the two apocarotenoid groups, strongly deviates from a 1:1 or 1:2 ratio expected from the cleavage of a carotenoid precursor having one or two cyclic end groups. Any direct conclusion from the apocarotenoid ratio on the nature of the putative precursor is therefore not possible. Only a strong catabolism of the $C_{14}$ cleavage product can be concluded.

### 2.2. $C_{13}$ cyclohexenone derivatives

HPLC of root extracts prepared at selected harvest times (5, 10, 15, 20, and 25 weeks after inoculation) showed the occurrence of a remarkably complex pattern of $C_{13}$ apocarotenoids with characteristic UV absorption maxima at 244 nm (Fig. 1). The total amount of the cyclohexenone derivatives showed a time-dependent increase comparable with the time course of root length colonization (Fig. 2). According to previous results these compounds were expected to be various glycosylated $C_{13}$ cyclohexenone derivatives of the blumenol C type.

The cyclohexenone derivatives (**1**–**11**) were isolated by preparative HPLC and enzymatic hydrolysis of each indicated, after HPLC analysis, that they were either blumenol C or 13-hydroxyblumenol C conjugates. Subsequently their structures were elucidated by a combination of LC/ESI-MS, high resolution ESI-FTICR-MS and NMR spectroscopy as summarized in Fig. 3. In each case characteristic signals in the 1D $^1$H NMR spectra allowed ready distinction between blumenol C and 13-hydroxyblumenol C conjugates, and the distinct patterns of cross peaks in the 2D $^1$H COSY spectra identified the sugar units present in all compounds. Acylation of the β-glucopyranose unit was evident from the downfield shifts of the attached protons at the acylation site (**3**, **8**–**11**) and its attachment to C-9 of the aglycone was confirmed in those cases (**1, 6**–**9**) where a HMBC spectrum was recorded from the three-bond correlations of the anomeric proton with C-9 and, vice versa, H-9 with the anomeric carbon, C-1.

The ESI-HRMS of the sodiated molecular ion and ESI-MS/MS fragmentation of the protonated molecular ion of **1** suggested the presence of two hexose moieties that were both β-glucopyranose units from the NMR data and sugar analysis. The correlations in the HMBC spectrum indicated that these were attached to C-9 and C-13 of 13-hydroxyblumenol C. These data clearly indicated the structure of **1** is 13-hydroxyblumenol C di-9,13-*O*-β-glucoside, a new natural product, which is isomeric with the 9-*O*-gentiobiosyl derivative of 13-hydroxyblumenol C, previously isolated from mycorrhizal tobacco roots and named nicoblumin (Maier et al., 1999). In contrast, the major compound **2** was identical in all respects (HRMS, NMR) with the known 13-hydroxyblumenol C 9-*O*-β-glucoside, originally described by Peipp et al. (1997) from mycorrhizal

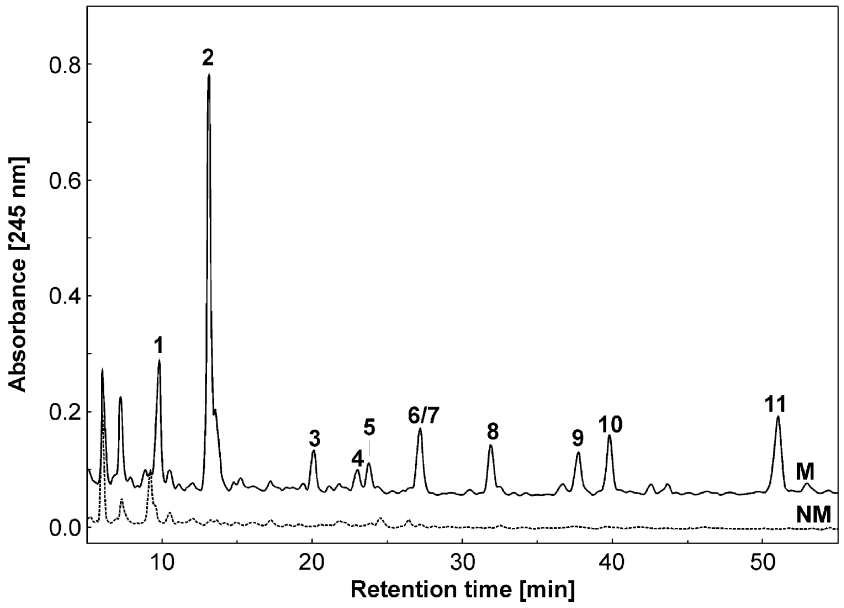



Fig. 1. HPLC elution profile (solvent system 1) of C cyclohexenone derivatives (PDA-detection at 245 nm) from mycorrhizal leek roots (25 weeks after inoculation, M) compared to the corresponding extract from nonmycorrhizal roots (NM). Peak numbers of cyclohexenone derivatives **1**–**11** correspond to compound numbers in Table 1.

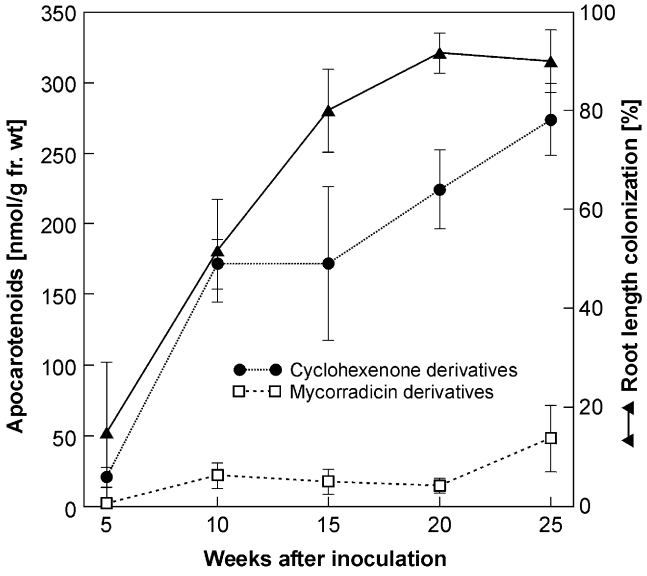
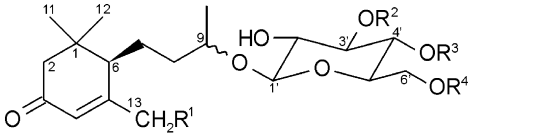



Fig. 2. Time course of root length colonization and accumulation of cyclohexenone and mycorradicin derivatives in mycorrhizal roots of leek (*Allium porrum*).



| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **1** | OGlc | H | H | H |
| **2** | OH | H | H | H |
| **3** | OH | –COCH$_2$C(OH)(CH$_3$)–CH$_2$CO$_2$H | H | H |
| **6** | H | H | H | Ara |
| **7** | H | H | Glc | H |
| **8** | OH | –COCH$_2$C(OH)(CH$_3$)–CH$_2$CO$_2$H | H | –COCH$_2$CO$_2$H |
| **9** | H | –COCH$_2$C(OH)(CH$_3$)–CH$_2$CO$_2$H | H | H |
| **10** | H | H | H | –COCH$_2$CO$_2$H |

Fig. 3. Structures of $C_{13}$ cyclohexenone derivatives isolated from mycorrhizal roots of leek (*Allium porrum*).

barley roots, but recently found both in mycorrhizal roots of *O. umbellatum* (Schliemann et al., 2006b) and *M. truncatula* (Schliemann et al., 2008).

The $[M+H]^+$ ion of **3** (*m/z* 533), also derived from 13-hydroxyblumenol C, showed a mass difference of 144 amu compared to **2** (*m/z* 389) which indicates the attachment of a $C_6H_8O_4$ unit from the ESI-HRMS. This extra unit was readily identified in the $^1$H NMR spectrum from the small number of characteristic signals of a 3-hydroxy-3-methylglutaric acid (HMG) moiety, which is attached to *O*-3′ of the β-glucopyranose from the low field shift of H-3′. Although we have not detected this conjugating moiety previously in connection with the apocarotenoids in mycorrhizal roots, it occurs quite often in other secondary metabolites of plants and fungi (Tanaka et al.,

Table 1
Retention time, HPLC-PDA (solvent system 1), and MS data of cyclohexenone derivatives from mycorrhizal roots of leek (*A. porrum*)

| Compound | *R* (min) | HPLC-PDA$\lambda$ (nm) | ESI-MS (*m/z*) | | Structural assignment |
|---|---|---|---|---|---|
| | | | [[M+H]$^+$ | [A+H]$^{+a}$ | |
| **1** | 9.8 | 225sh/243 | 551 | 227 | 13-Hydroxyblumenol C di-9,13-*O*-β-glucopyranoside |
| **2** | 13.1 | 225sh/245 | 389 | 227 | 13-Hydroxyblumenol C 9-*O*-β-glucopyranoside |
| **3** | 20.1 | 215sh/225sh/243 | 533 | 227 | 13-Hydroxyblumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methylglutaryl)-β-glucopyranoside] |
| **4** | 23.0 | 230sh/244 | 533 | 227 | 13-Hydroxyblumenol C derivative |
| **5** | 23.8 | 225sh/241 | 533 | 227 | Mixture of 13-hydroxyblumenol C and blumenol C derivatives |
| **6** | 27.2 | 230sh/244 | 505 | 211 | Blumenol C 9-*O*-(6′-*O*-α-arabinopyranosyl-β-glucopyranoside) |
| **7** | 27.2 | 230sh/244 | 535 | 211 | Blumenol C 9-*O*-(4′-*O*-glucosyl-β-glucopyranoside) |
| **8** | 31.9 | 220sh/230sh/242 | 619 | 227 | 13-Hydroxyblumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methylglutaryl)-6′-*O*-malonyl-β-glucopyranoside] |
| **9** | 37.7 | 220sh/230sh/245 | 517 | 211 | Blumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methylglutaryl)-β-glucopyranoside] |
| **10** | 39.8 | 220sh/230sh/245 | 459 | 211 | Blumenol C 9-*O*-(6′-*O*-malonyl-β-glucopyranoside) |
| **11** | 51.0 | 230sh/245 | 517 | 211 | Isomer of **9** |

Compound numbers correspond to peak numbers in Fig. 1.
[a] A: aglycone.

1992). Acylation of quercetin/apigenin glycosides (Wald et al., 1986; Liu et al., 1994; Jung et al., 1993; Wiesen et al., 1994; Kim et al., 1994) as well as of betacyanins (Minale et al., 1966; Cai et al., 2001; Wybraniec et al., 2001) by HMG was found to take place exclusively at the 6-*O*-position of the sugars, whereas in **3** acylation in the 3-*O*-position was observed for the first time. It is interesting to note that in **3** an intermediate of the cytosolic mevalonate pathway (HMG) is covalently attached to a cyclohexenone glycoside originating from the plastidial methylerythritol phosphate pathway.

The minor compounds **4** and **5**, which seemed at first to be isomeric to **3** from the ESI-MS, could not be unambiguously characterized by NMR spectroscopy as both were only available in small amounts and were mixtures of a number of compounds. The major component of **4** was a 13-hydroxyblumenol C derivative with a C-9-*O*-(6′-*O*-acyl)-β-glucopyranosyl moiety. The nature of the acyl group could not be unambiguously established as signals of a HMG residue were not apparent. Similarly only signals of a blumenol C derivative were apparent in the NMR spectrum of **5**, although 13-hydroxyblumenol C was also present from the ESI-MS analysis.

The base peak ion at *m/z* 211 in the MS fragmentation of **6** clearly indicates the presence of the protonated ion of the blumenol C aglycone, whereas the protonated molecular ion at *m/z* 505 and its fragmentation indicates the presence of a pentose (increment 132 amu) attached to an inner hexose (increment 162 amu). These were identified as arabinose and glucose as these were the only sugars found in the sugar analysis. Although there was considerable overlap of the signals in the $^1$H NMR spectrum, the shifts of the additional sugar unit were identified from a combination of data from the 2D COSY, TOCSY and HMQC spectra. Although a full complement of couplings could not be determined, their magnitudes were evident from the 1D and 2D data and suggested an α-arabinopyranosyl system which, from the HMBC data, was attached to *O*-6′ of the inner β-glucopyranosyl unit. Therefore, **6** is blumenol C 9-*O*-(6′-*O*-α-arabinopyranosyl)-β-glucoside. Furthermore, a second component with *m/z* 535 [M+H]$^+$ was present in this fraction pointing to blumenol C conjugated with two hexoses. A full $^1$H and $^{13}$C assignment indicated that this compound **7** was identical with blumenol C 9-*O*-(4′-*O*-β-glucopyranosyl)-β-glucopyranoside, reported previously as a hydrolysis product of the "yellow pigment" isolated from mycorrhizal maize roots (Fester et al., 2002a).

The [M+H]$^+$ ion (*m/z* 619) of **8** showed a mass difference of 86 amu to that of **3** suggesting malonyl was the additional moiety present. The $^1$H NMR data confirmed the similarity to **3.** The HMBC spectrum again confirmed that the HMG unit was attached to *O*-3′ through the correlation of H-3′ of the glucose with C-1″ of HMG. Hence, the low field shifts of H-6′A and B could only be explained by attachment of a malonyl group to *O*-6′. As is usual for the malonyl moiety no signals could be detected in the $^1$H NMR spectrum presumably due to H/D exchange.

The ESI-HRMS data and fragmentation pattern of **9** revealed that it is a blumenol C derivative carrying a hexose acylated with HMG. A full complement of NMR data unambiguously identified it as blumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-β-glucopyranoside], the 13-deoxy analog of **3**. Similarly, the MS and NMR data of **10** were identical in all respects with blumenol C 9-*O*-(6′-*O*-malonyl-β-glucopyranoside), recently identified from mycorrhizal roots of *M. truncatula* (Schliemann et al., 2008).

Finally **11**, available only in small amounts for structure analysis, appears to be an isomer of **9** as it exhibited a MS fragmentation identical with **9** and $^1$H NMR data indicating the presence of a 3′-*O*-(3″-hydroxy-3″-methylglutaryl)-β-glucopyranosyl moiety. The scarcity of material did not allow a decision to be made whether **11** is a possible 9-enantiomer of **9**.

On the basis of CD spectra of blumenin isomers (Schliemann et al., 2006b) and comparison of the molar ellipticity data with the literature (Miyase et al., 1988), a 6*R*-configu-

ration has been assigned for the major blumenol C isomer. This was also confirmed for the main cyclohexenone derivative isolated from mycorrhizal roots of *O. umbellatum* (Schliemann et al., 2006b), suggesting this configuration for all mycorrhiza-induced cyclohexenone derivatives. As only cyclohexenone derivatives of the α-ionone type have been isolated from AM roots to date, it may be hypothesized that the corresponding carotenoid precursor only contains this cyclic end group.

In summary, the most complex pattern of AM fungus-induced cyclohexenone derivatives so far described were found in leek roots. Eleven compounds were isolated, comprising derivatives of 13-hydroxyblumenol C and of blumenol C, of which eight have been unambiguously identified as mono- or diglycosides, in some cases acylated with 3-hydroxy-3-methylglutaric and/or malonic acid. Compounds **1**, **3**, **6**, **8**, and **9** are new natural products whereas **2**, **7**, and **10** were found to be identical with previously isolated mycorrhiza-specific cyclohexenone derivatives (Peipp et al., 1997; Fester et al., 2002a; Schliemann et al., 2006b, 2008). While the accumulation of the cyclohexenone derivatives correlated with the increase of root length colonization, the corresponding levels of mycorradicin derivatives remained low, pointing to an intensive catabolism of the primary $C_{14}$ cleavage product of precursor carotenoids. The stimulation of carotenoid biosynthesis in AM roots appears to be a general prerequisite for the formation of apocarotenoids (Fester et al., 2002a, 2005), but the rationale for the formation of both groups of apocarotenoids and their possible function in mycorrhiza development remains unclear (Strack and Fester, 2006; Walter et al., 2007; Akiyama, 2007). These results shed light again on the different metabolism and catabolism of the primary cleavage products of mycorrhiza-induced carotenoids: although the role of the still unknown precursor(s) and of their derived apocarotenoids needs to be clarified.

## 3. Experimental

### 3.1. Plant material and mycorrhization experiments

About 30 seeds of *Allium porrum* L. cv. Elefant (N.L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany) were placed in 500-ml plastic pots filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay, Pinneberg, Germany) containing 30% of an AM fungus inoculate (*Glomus intraradices* Schenk and Smith, isolate 49, provided by H. von Alten, University Hannover, Germany), enriched by previous co-cultivation with leek 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 week and fertilized once per week with 10 × Long Ashton nutrition solution (5 ml per pot) (Hewitt, 1966) with reduced phosphate (20%) content.

### 3.2. Harvesting, extraction and determination of mycorrhization

At various times after inoculation (5, 10, 15, 20, and 25 weeks) the roots of three parallel pots were cleaned from Lecaton and the fresh weights noted. The material was shock frozen with liquid nitrogen and homogenized separately in a mortar followed by extraction with 80% aq. MeOH (0.5 g $ml^{-1}$). To remove interfering mucilaginous constituents and to concentrate the extracts, 350 µl aliquots were taken to dryness using a SpeedVac, the residues suspended in 35 µl 80% aq. MeOH and the supernatant used for HPLC analysis. Root samples of the same age were stained with ink according to Vierheilig et al. (1998) for the estimation of the approximate root length colonization.

### 3.3. HPLC

Analytical and semi-preparative 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 the cyclohexenone conjugates as well as their hydrolysis products) A: 2% aq. $HCO_2H$; B: acetonitrile; constant gradient from 10% B to 25% B within 60 min, followed by 25% B to 80% B in additional 10 min; solvent system 2 (for the separation of mycorradicin isomers) A: 1.5% aq. $H_3PO_4$; B: acetonitrile; constant gradient from 0% B to 100% B within 35 min (Fester et al., 2002a); the flow rate was 1 ml $min^{-1}$ in both solvent systems. 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 ABA acid which has the identical 1,1,5-trimethylcyclohex-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 per g fr. wt as well as nmol per root (deviation of the individual peak areas of the cyclohexenone and mycorradicin derivatives of the triplicates were <20%).

For preparative 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 0% B to 35% B within 120 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 were performed using Empower 5.0. The preparative separation started with an extract derived from 125 g AM leek roots (24 months after inoculation with *G. intraradices*).

### 3.4. Hydrolyses

For the detection of mycorradicin derivatives 90 μl of the crude extract were treated with 10 μl 10 N KOH for 1 h at room temperature and subsequently analyzed by HPLC (solvent system 2) to detect liberated mycorradicin. For enzymatic 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 1).

### 3.5. Mass spectrometry and NMR measurement

The positive ion electrospray ionization (ESI) mass spectra and ESI-MS/MS data (collision energies: 10 eV and/or 15 eV, respectively) were recorded on a Thermofinnigan TSQ Quantum AM instrument coupled with a Surveyor microHPLC system. High resolution ESI mass spectra were recorded on a Bruker Apex III Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, USA) as detailed recently (Schliemann et al., 2006a). Standard micro-methylation techniques were applied for the permethylation of the samples. Carbohydrate compositional analysis were performed on a Finnigan GCQ GC–MS as described previously (Nimtz et al., 1996).

1D ($^1$H and $^{13}$C) and 2D ($^1$H: COSY and TOCSY; $^{13}$C: HMQC and HMBC) NMR spectra were recorded at 300 K on a 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.

### 3.6. Mass spectral and NMR data of cyclohexenone derivatives

#### 3.6.1. 13-Hydroxyblumenol C di-9,13-O-β-glucopyranoside (1)

ESI-HRMS *m/z* 573.2524 ($[M+Na]^+$, $C_{25}H_{42}O_{13}Na^+$, calc. 573.2518). ESI-MS/MS (10 eV) of $[M+H]^+$ (*m/z* 551) (rel. int.): 551 $[M+H]^+$ (100), 389 $[(M+H)-Glc]^+$ (95), 371 $[(M+H)-Glc-H_2O]^+$ (12), 227 [13-hydroxyblumenol C+H]$^+$ (76), 209 [(13-hydroxyblumenol C+H)$-H_2O]^+$ (86), 191 [(13-hydroxyblumenol C+H)$-2H_2O]^+$ (4)). $^1$H NMR (600 MHz, $CD_3OD$): δ 6.21 (1 H, *bs*, H-4), 4.58 (1H, *dd*, $J_{13A,4} = 1.6$ Hz, $J_{13A,13B} = 16.7$ Hz, H-13A), 4.44 (1H, *dd*, $J_{13B,4} = 1.1$ Hz, H-13B), 4.39 (1H, *d*, $J_{1'',2''} = 7.8$ Hz, H-1″), 4.36 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 3.90 (2H, *m*, H-6′A, H-6″A), 3.85 (1H, *m*, H-9), 3.70 (2H, *m*, H-6′B, H-6″B), 3.42–3.33 (*m*, H-3′, H-3″, H-4′, H-4″, H-5′, H-5″), 3.30 (1H, *dd*, $J_{2'',3''} = 9.0$ Hz, H-2″), 3.19 (1H, *dd*, $J_{2',3'} = 9.1$ Hz, H-2′), 2.62 (1H, *d*, $J_{2A,2B} = 17.5$ Hz, H-2A), 2.06 (1H, *d*, H-2B), 2.05 (1H, *t*, $J_{6,7} = 4.9$ Hz, H-6), 1.87 (1H, *m*, H-7A), 1.77–1.70 (2H, *m*, H-8AB), 1.67 (1H, *m*, H-7B), 1.29 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.15 (3H, *s*, H-11), 1.07 (3H, *s*, H-12). $^{13}$C NMR (150 MHz, $CD_3OD$): δ 202.3 (*s*, C-3), 168.0 (*s*, C-5), 123.1 (*d*, C-4), 104.0, 103.6 (*d* × 2, C-1′, C-1″), 78.2, 78.1 × 2, 77.8 (*d* × 4, C-3′, C-3″, C-5′, C-5″), 77.6 (*d*, C-9), 75.4, 75.1 (*d* × 2, C-2′, C-2″), 71.7 (*d* × 2, C-4′, C-4″), 62.8 (*t* × 2, C-6′, C-6″), 48.7 (*t*, C-6), 47.9 (*d*, C-2), 37.3 (*t*, C-8), 37.3 (*s*, C-1), 28.9 (*q*, C-12), 27.7 (*q*, C-11), 26.9 (*t*, C-7), 22.0 (*q*, C-10). Glucose unit mark ″ is bound to *O*-13 and ′ to *O*-9.

#### 3.6.2. 13-Hydroxyblumenol C 9-O-β-glucopyranoside (2)

ESI-HRMS, ESI-MS/MS, and $^1$H NMR data were identical with those published recently (Schliemann et al., 2006b, 2008).

#### 3.6.3. 13-Hydroxyblumenol C 9-O-[3′-O-(3″-hydroxy-3″-methylglutaryl)-β-glucopyrano side] (3)

ESI-HRMS *m/z* 555.2419 ($[M+Na]^+$, $C_{25}H_{40}O_{12}Na^+$, calc. 555.2418). ESI-MS/MS (15 eV) of $[M+H]^+$ (*m/z* 533) (rel. int.): 533 $[M+H]^+$ (6), 307 $[(HMG-Glc)+H]^+$ (12), 289 $[(HMG-Glc+H)-H_2O]^+$ (3), 227 [13-hydroxyblumenol C+H]$^+$ (16), 209 [(13-hydroxyblumenol C+H)$-H_2O]^+$ (100), 191 [(13-hydroxyblumenol C+H)$-2H_2O]^+$ (20)). $^1$H NMR (600 MHz, $CD_3OD$): δ 6.10 (1H, *bs*, H-4), 4.98 (1H, *t*, $J_{2',3'} = J_{3',4'} = 9.5$ Hz, H-3′), 4.45 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 4.35 (1H, *dd*, $J_{13A,4} = 1.6$ Hz, $J_{13A,13B} = 17.7$ Hz, H-13A), 4.19 (1H, *dd*, $J_{13B,4} = 1.7$ Hz, H-13B), 3.89 (1H, *dd*, $J_{6'A,5'} = 2.3$ Hz, $J_{6'A,6'B} = 12.0$ Hz, H-6′A), 3.86 (1H, *m*, H-9), 3.72 (1H, *dd*, $J_{6'A,5'} = 2.3$ Hz, H-6′A), 3.51 (1H, *t*, $J_{4',5'} = 9.5$ Hz, H-4′), 3.37 (1H, *ddd*, H-5′), 3.33 (1H, *dd*, H-2′), 2.82/2.76, 2.71/2.66 (2H, *d* × 4, $J_{A,B} = 14.1$ and 15.3 Hz, respectively, H-2″ and H-4″), 2.62 (1H, *d*, $J_{2A,2B} = 17.6$ Hz, H-2A), 2.06 (1H, *d*, H-2B), 1.96 (1H, *t*, $J_{6,7} = 5.3$ Hz, H-6), 1.85 (1H, *m*, H-7A), 1.73–1.60 (3H, *m*, H-7B, H-8AB), 1.45 (1H, *s*, H-6″), 1.29 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.15 (3H, *s*, H-11), 1.05 (3H, *s*, H-12).

#### 3.6.4. Compound 4 (13-hydroxy blumenol C derivative with a C-9-O-[6′-O-acylated]-β-glucopyranoside)

ESI-MS/MS (15 eV) of $[M+H]^+$ (*m/z* 533) (rel. int.): 533 $[M+H]^+$ (19), 515 $[M+H-H_2O]^+$ (3), 497 $[(M+H)-2H_2O]^+$ (3), 307 (100), 289 (24), 227 [13-hydroxyblumenol C+H]$^+$ (18), 209 [(13-hydroxyblumenol C+H)$-H_2O]^+$ (89), 191 [(13-hydroxyblumenol C+H)$-2H_2O]^+$ (18). $^1$H NMR (600 MHz, $CD_3OD$): δ 6.10 (1H, *bs*, H-4), 4.48 (1H, *dd*, $J_{6'A,5'} = 2.2$ Hz, $J_{6'A,6'B} = 11.9$ Hz, H-6′A), 4.36 (1H, *d*, $J_{13A,13B} = 17.7$ Hz, H-13A), 4.35 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 4.30 (1H, *dd*, $J_{6'B,5'} = 6.3$ Hz, H-6′B), 4.19 (1H, *dd*, $J_{13B,4} = 1.8$ Hz, H-13B), 3.79 (1H, *m*, H-9), 3.50 (1H, *ddd*, $J_{5',4'} = 9.5$ Hz, H-5′), 3.38 (1H, *m*, H-3′), 3.33 (1H, *m*, H-4′), 3.20 (1H, *m*, H-2′), 2.62 (1H, *d*, $J_{2A,2B} = 17.5$ Hz, H-2A), 2.06 (1H, *d*, H-2B), 1.96 (1H, *t*, $J_{6,7} = 5.0$ Hz, H-6), 1.85 (1H, *m*, H-7A), 1.75–1.60 (3H, *m*, H-7B, H-8AB), 1.26 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.15

(3H, *s*, H-11), 1.06 (3H, *s*, H-12). The acyl moiety could not be unambiguously identified, but it was not 3′-hydroxy-3-methylglutaric acid.

### 3.6.5. Compound **5**

ESI-MSMS (15 eV) of $[M+H]^+$ (*m/z* 533) (rel. int.): 533 $[M+H]^+$ (5), 307 (9), 289 (4), 227 [13-hydroxyblumenol C+H]$^+$ (63), 209 [(13-hydroxyblumenol C+H)$-H_2O]^+$ (100), 191 [(13-hydroxyblumenol C+H)$-2H_2O]^+$ (17). $^1$H NMR suggests this is a blumenol C derivative and not an isomer of **3**.

### 3.6.6. Blumenol C 9-*O*-(6′-*O*-α-arabinopyranosyl)-β-glucoside) (**6**)

ESI-HRMS *m/z* 527.2462 ($[M+Na]^+$, $C_{24}H_{40}O_{11}Na^+$, calc. 527.2468), *m/z* 395.2039 ($[(M+Na)-Ara]^+$, $C_{19}H_{32}O_7Na^+$, calc. 395.2046); ESI-MS/MS (10 eV) of $[M+H]^+$ (*m/z* 505) (rel. int.): 505 $[M+H]^+$ (77), 373 $[(M+H)-Ara]^+$ (95), 211 [blumenol C+H]$^+$ (100), 193 [(blumenol C+H)$-H_2O]^+$ (4), 175 [(blumenol C+H)$-2H_2O]^+$ (3). Major component in mixture. $^1$H NMR (600 MHz, $CD_3OD$): $\delta$ 5.84 (1H, *bs*, H-4), 4.36 (1H, *d*, $J_{1'',2''} = 6.7$ Hz, H-1″), 4.36 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 4.10 (1H, *dd*, $J_{6'A,5'} = 2.3$ Hz, $J_{6'A,6'B} = 11.4$ Hz, H-6′A), 3.89 (H-5″A from HMBC), 3.88 (*m*, H-9 from COSY), 3.76 (1H, *dd*, $J_{6'B,5'} = 5.7$ Hz, H-6′B), 3.63 (1H, *dd*, $J_{2'',3''} = 8.7$ Hz, H-2″), 3.57 (H-5″B from HMBC), 3.57 (*m*, H-3″ from COSY, TOCSY shows coupling to H-4″ is small), 3.95–3.50 (rest of pentose system), 3.46 (1H, *m*, H-5′), 3.38 (2H, *m*, H-3′, H-4′), 3.19 (1H, *dd*, $J_{2',3'} = 9.9$ Hz, H-2′), 2.52 (1H, *d*, $J_{2A,2B} = 17.4$ Hz, H-2A), 2.09 (3H, *bs*, H-13), 2.02 (1H, *d*, H-2B), 2.01 (1H, *t*, $J_{6,7} = 4.9$ Hz, H-6), 1.84 (1H, *m*, H-7A), 1.76–1.63 (3H, *m*, H-7B, H-8AB), 1.29 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.14 (3H, *s*, H-11], 1.06 (3H, *s*, H-12). $^{13}$C NMR (150 MHz, $CD_3OD$): $\delta$ 202.1 (*s*, C-3), 169.7 (*s*, C-5), 125.2 (*d*, C-4), 104.8 (*d*, C-1″), 103.9 (*d*, C-1′), 77.8 (*d*, C-3′), 77.5 (*d*, C-9), 76.5 (*d*, C-5′), 75.1 (*d*, C-2′), 74.0 (*d*, C-3″), 72.1 (*d*, C-2″), 71.5 (*d*, C-4′), 69.3 (*t*, C-6′), 66.4 (*t*, C-5″), 52.4 (*t*, C-6), 47.9 (*d*, C-2), 37.3 (*t*, C-8), 37.2 (*s*, C-1), 28.9 (*q*, C-12), 27.7 (*q*, C-11), 26.5 (*t*, C-7), 21.8 (*q*, C-10). C-4″ could not be unambiguously identified in the HMQC or HMBC due to overlap of $^1$H spectrum with the second component.

### 3.6.7. Blumenol C 9-*O*-(4′-*O*-glucosyl-β-glucoside) (**7**)

ESI-HRMS *m/z* 557.2566 ($[M+Na]^+$, $C_{25}H_{42}O_{12}Na^+$, calc. 557.2568; ESI-MS/MS (10 eV) of $[M+H]^+$ (*m/z* 535) (rel. int.): 535 $[M+H]^+$ (56), 373 $[(M+H)-Glc]^+$ (23), 211 [blumenol C+H]$^+$ (100), 193 [(blumenol C+H)$-H_2O]^+$ (5), 175 [(blumenol C+H)$-2H_2O]^+$ (3). The $^1$H and $^{13}$C data of this minor component in the mixture correspond to those given in Fester et al. (2002a).

### 3.6.8. 13-Hydroxyblumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-6′-*O*-malonyl-β-glucopyranoside] (**8**)

ESI-MS *m/z* (rel. int.): 641 $[M+Na]^+$ (84), 619 $[M+H]^+$ (100); ESI-MS of a major degradation product (demalonylation) corresponds completely to **3.** $^1$H NMR (600 MHz, $CD_3OD$): $\delta$ 6.10 (1H, *bs*, H-4), 4.98 (1H, *dd*, $J_{2',3'} = J_{3',4'} = 9.4$ Hz, H-3′), 4.47 (1H, *dd*, $J_{6'A,5'} = 2.1$ Hz, $J_{6'A,6'B} = 11.9$ Hz, H-6′A), 4.45 (1H, *d*, $J_{1',2'} = 7.9$ Hz, H-1′), 4.35 (1H, *dd*, $J_{13A,4} = 1.6$ Hz, $J_{13A,13B} = 17.9$ Hz, H-13A), 4.33 (1H, *dd*, $J_{6'B,5'} = 5.5$ Hz, H-6′B), 4.18 (1H, *dd*, $J_{13B,4} = 1.8$ Hz, H-13B), 3.81 (1H, *m*, H-9), 3.60 (*ddd*, H-5′), 3.52 (1H, *dd*, $J_{4',5'} = 9.4$ Hz, H-4′), 3.33 (1H, *dd*, H-2′), 2.82 (1H, *d*, $J_{2''A,2''B} = 14.2$ Hz, H-2″A), 2.76 (1H, *d*, H-2″B), 2.71 (1H, *d*, $J_{4''A,4''B} = 15.3$ Hz, H-4″A), 2.67 (1H, *d*, H-4″B), 2.62 (1H, *d*, $J_{2A,2B} = 17.7$ Hz, H-2A), 2.06 (1H, *d*, H-2B), 1.96 (1H, *t*, $J_{6,7} = 5.2$ Hz, H-6), 1.84 (1H, *m*, H-7A), 1.74–1.59 (3H, *m*, H-7A, H-8AB), 1.45 (1H, *s*, H-6″), 1.27 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.15 (3H, *s*, H-11), 1.05 (3H, *s*, H-12). The HMBC spectrum showed correlations of H-3′ and H-2″ to same carbonyl signal at 172.0 ppm of C-1″. The malonyl signal was not detected by NMR presumably due to H/D exchange.

### 3.6.9. Blumenol C 9-*O*-[3′-*O*-(3″-hydroxy-3″-methyl-glutaryl)-β-glucopyranoside] (**9**)

ESI-HRMS *m/z* 539.2467 ($[M+Na]^+$, $C_{25}H_{40}O_{11}Na^+$, calc. 539.2468). ESI-MS/MS (15 eV) of $[M+H]^+$ (*m/z* 517) (rel. int.): 517 $[M+H]^+$ (5), 307 $[(HMG-Glc)+H]^+$ (3), 211 [blumenol C+H]$^+$ (100), 193 [(blumenol C+H)$-H_2O]^+$ (12), 175 [(blumenol C+H)$-2H_2O]^+$ (8). $^1$H NMR (600 MHz, $CD_3OD$): $\delta$ 5.84 (1H, *bs*, H-4), 4.98 (1H, *dd*, $J_{3',2'} = J_{3',4'} = 9.4$ Hz, H-3′), 4.45 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 3.89 (1H, *dd*, $J_{6'A,5'} = 2.1$ Hz, $J_{6'A,6'B} = 11.9$ Hz, H-6′A), 3.87 (1H, *m*, H-9), 3.72 (1H, *dd*, $J_{6'B,5'} = 5.5$ Hz, H-6′B), 3.51 (1H, *dd*, $J_{4',5'} = 9.5$ Hz, H-4′), 3.38 (1H, *ddd*, H-5′), 3.33 (1H, *dd*, H-2′), 2.82 (1H, *d*, $J_{2''A,2''B} = 14.2$ Hz, H-2″A), 2.77 (1H, *d*, H-2″B), 2.71 (1H, *d*, $J_{4''A,4''B} = 15.4$ Hz, H-4″A), 2.67 (1H, *d*, H-4″B), 2.62 (1H, *d*, $J_{2A,2B} = 17.5$ Hz, H-2A), 2.08 (3H, *s*, $J_{13,4} = 1.1$ Hz, H-13), 2.02 (1H, *d*, H-2B), 2.02 (1H, *t*, $J_{6,7} = 4.9$ Hz, H-6), 1.84 (1H, *m*, H-7A), 1.76–1.68 (2H, *m*, H-8AB), 1.65 (1H, *m*, H-7B), 1.45 (1H, *s*, H-6″), 1.30 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.13 (3H, *s*, H-11), 1.05 (3H, *s*, H-12). $^{13}$C NMR (150 MHz, $CD_3OD$): $\delta$ 202.5 (*s*, C-3), 175.6 (*s*, C-5″), 172.5 (*s*, C-1″), 170.0 (*s*, C-5), 125.5 (*d*, C-4), 104.0 (*d*, C-1′), 79.4 (*d*, C-3′), 78.2 (*d*, C-10), 77.7 (*d*, C-5′), 73.7 (*d*, C-2′), 71.1 (*s*, C-3″), 69.8 (*d*, C-4′), 62.6 (*t*, C-6′), 52.7 (*d*, C-6), 48.3 (*d*, C-2), 47.1 (*t*, C-2″), 46.1 (*t*, C-4″), 37.6 (*t*, C-8), 37.4 (*s*, C-1), 29.1 (*q*, C-12), 27.8 (*q*, C-6″), 27.7 (*q*, C-11), 26.9 (*t*, C-7), 25.1 (*q*, C-13), 22.2 (*q*, C-10).

### 3.6.10. Blumenol C 9-*O*-(6′-*O*-malonyl-β-glucopyranoside) (**10**)

ESI-MS data were identical with those published recently (Schliemann et al., 2008). $^1$H NMR (600 MHz, $CD_3OD$): $\delta$ 5.84 (1H, *bs*, H-4), 4.49 (1H, *dd*, $J_{6'A,5'} = 2.2$ Hz, $J_{6'A,6'B} = 11.8$ Hz, H-6′A), 4.36 (1H, *d*, $J_{1'',2''} = 6.7$ Hz, H-1″), 4.36 (1H, *d*, $J_{1',2'} = 7.9$ Hz, H-1′), 4.30 (1H, *dd*, $J_{6'B,5'} = 6.4$ Hz, H-6′B), 3.80 (1H, *m*, H-9), 3.51 (1H, *ddd*, $J_{5',4'} = 9.7$ Hz, H-5′), 3.37 (1H, *dd*,

$J_{3',2'} = J_{3',4'} = 9.2$ Hz, H-3′), 3.31 (1H, *dd*, H-4′), 3.19 (1H, *dd*, H-2′), 2.52 (1H, *d*, $J_{2A,2B} = 17.4$ Hz, H-2A), 2.08 (3H, *d*, $J_{13,4} = 1.3$ Hz, H-13), 2.02 (1H, *d*, H-2B), 2.01 (1H, *t*, $J_{6,7} = 5.3$ Hz, H-6), 1.83 (1H, *m*, H-7A), 1.77–1.68 (2H, *m*, H-8AB), 1.63 (1H, *m*, H-7B), 1.27 (3H, *d*, $J_{10,9} = 6.3$ Hz, H-10), 1.13 (3H, *s*, H-11), 1.06 (3H, *s*, H-12). The malonyl signal was not detected by $^1$H NMR presumably due to H/D exchange.

### 3.6.11. Compound **11** (isomer of **9**)

ESI-MS: *m/z* (rel. int.): 539 $[M+Na]^+$ (71), 517 $[M+H]^+$ (48), 307 $[(HMG-Glc)+H]^+$ (8), 211 [blumenol C+H]$^+$ (100); ESI-MS/MS (15 eV) of $[M+H]^+$ (*m/z* 517) (rel. int.): 517 $[M+H]^+$ (4), 307 $[(HMG-Glc)+H]^+$ (3), 211 [blumenol C+H]$^+$ (100), 193 $[(\text{blumenol C+H})-H_2O]^+$ (12), 175 $[(\text{blumenol C+H})-2H_2O]^+$ (7). $^1$H NMR (600 MHz, $CD_3OD$): $\delta$ 5.83 (1H, *bs*, H-4), 4.96 (1H, *m*, H-3′), 4.46 (1H, *d*, $J_{1',2'} = 7.8$ Hz, H-1′), 3.90 (1H, *m*, H-6′A), 3.82 (1H, *m*, H-9), 3.71 (1H, *dd*, $J_{6'B,5'} = 5.5$ Hz, $J_{6'B,6'A} = 11.9$ Hz, H-6′B), 3.6–3.3 (2H, *m*, H-4′, H-5′), 3.32 (1H, *m*, H-2′), 2.77 (1H, *d*, $J_{2''A,2''B} \sim 13$ Hz, H-2″A), 2.63 (1H, *d*, H-2″B), 2.56 (1H, *d*, $J_{4''A,4''B} \sim 16.5$ Hz, H-4″A), 2.52 (1H, *d*, $J_{2A,2B} = 17.2$ Hz, H-2A), 2.42 (1H, *d*, H-4″), 2.08 (3H, *s*, $J_{13,4} = 1.1$ Hz,H-13), 2.01 (1H, *d*, H-2B), 2.01 (1H, *t*, $J_{6,7} = 4.9$ Hz, H-6), 1.90–1.60 (3H, *m*, H-7AB, H-8AB), 1.44 (1H, *s*, H-6″), 1.27 (3H, *d*, $J_{10,9} = 6.2$ Hz H-10), 1.13 (3H, *s*, H-11), 1.05 (3H, *s*, H-12). Shifts were assigned from the combined 1D and 2D COSY spectra.

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