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ORIGINAL RESEARCH

LC/MS/MS identification of glycosides produced by biotransformation of cinnamyl alcohol in *Rhodiola rosea* compact callus aggregates

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ABSTRACT: Cinnamyl alcohol was added to the media of compact callus aggregates (CCA) of *Rhodiola rosea* for stimulating the production of cinnamyl glycosides. The biotransformation reaction produced high amounts of rosin, while only a very low amount of rosavin was produced. As the consumption rate of cinnamyl alcohol was much higher than production of rosin, the aqueous methanol extracts of compact callus aggregates were studied by liquid chromatography–mass spectrometric methods and four new unexpected biotransformation products of cinnamyl alcohol were identified. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: *Rhodiola rosea* L.; cell culture, cinnamyl alcohol, biotransformation

INTRODUCTION

Golden root (Roseroot, *Rhodiola rosea* L., *Crassulaceae*) has been connected to a number of biological activities, such as effects on prolyl endopeptidase inhibition (Fan *et al*., 1999), antiallergic effects (Yoshikawa *et al*., 1996, 1997), effects on memory and learning (Petkov *et al*., 1996), antidepressant and anti-inflammatory effects (Maslova *et al*., 1994) and effects in cancer therapy (Bocharova *et al*., 1995; Udintsev and Shakov, 1991a,b; Razina *et al*., 2000). The main compounds responsible for these activities are believed to be phenylpropanoid tyrosol (**1**), its glucoside salidroside (*p*-hydroxyphenylethyl-*O*-β-D-glucopyranoside) (**2**) and cinnamyl alcohol glycosides rosin (cinnamyl-*O*-β-D-glucopyranoside) (**3**), rosarin (cinnamyl-(6′-*O*-α-L-arabinofuranosyl)-*O*-β-D-glucopyranoside) (**4**) and rosavin [cinnamyl-(6′-*O*-α-L-arabinopyranosyl)-*O*-β-D-glucopyranoside] (**5**). Other *Rhodiola* species contain only salidroside (Ramazanov and Mar Bernal Suarez, 1999), so the beneficial effects of this plant are probably due to the presence of rosavin or to the synergetic effect of rosavin and salidroside.

Whereas it takes several years to obtain satisfactory content of these compounds in the plant using the traditional field cultivation (Furmanova *et al*., 1995), the use of cell cultures offers an alternative and faster method for their production. In cell suspensions, the salidroside and rosavin are produced effectively when feeding the suspensions with their precursor compounds, such as cinnamyl alcohol (**6**). Amounts up to 95% of added precursors are reported to transform into other constituents, mainly to salidroside and rosavin, by several different studies (Furmanova *et al.*, 1999, 2002; Xu *et al*., 1998a,b). Recently, we reported the formation of rosin (**3**) and minor amounts of rosavin (**5**) through biotransformation reaction from cinnamyl alcohol in cultures of compact callus aggregates (György *et al*., 2004). In the study, the consumption rate of cinnamyl alcohol was found to be much higher than the production of the known secondary metabolites of the plant. To explain this phenomenon, and to analyze if any other biotransformation products were present, a more detailed study of the biotransformation samples was carried out here with liquid chromatography–mass spectrometry, and four new biotransformation products **7**–**10** of cinnamyl alcohol were identified (Fig. 1).

EXPERIMENTAL

Reagents and materials. Salidroside and rosavin standards were purchased from the Anti Aging Center Inc. (Budapest, Hungary) and cinnamyl alcohol standard from Fluka (Buchs, Switzerland). Internal standard salicin (**11**), HPLC-grade acetonitrile and methanol were purchased from

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Abbreviations used: CCA, compact callus aggregates; NAA, naphthalene acetic acid.

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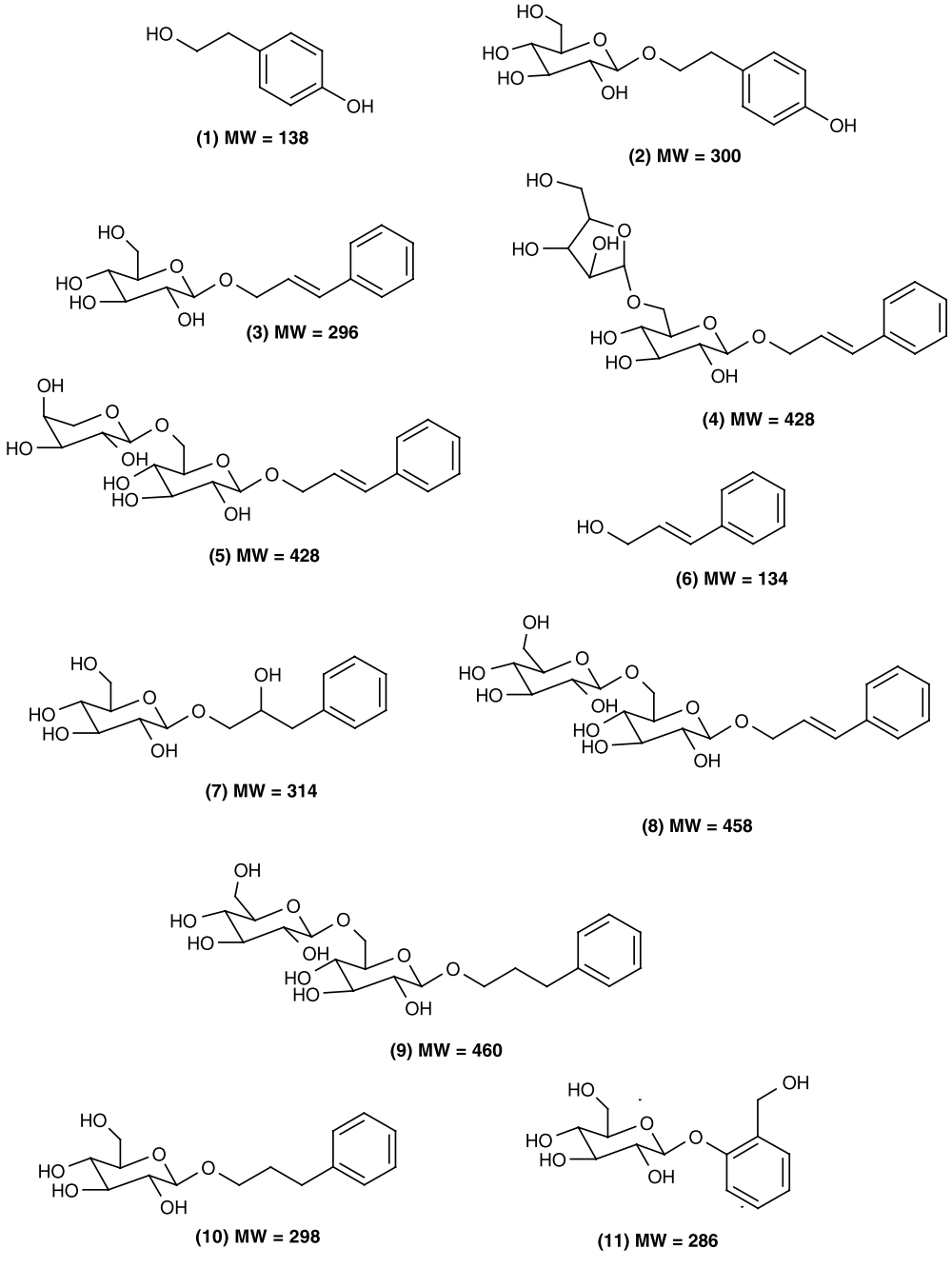

Figure 1. The compounds studied.

Merck (LiChrosolv GG, Darmstadt, Germany). Ammonium acetate was purchased from BDH Laboratory Supplies (Poole, UK). Laboratory water was distilled and purified with a Simplicity 185 water purifier (Millipore, Molsheim, France). Purities of the standards were determined by HPLC/PDA using a max plot at range 190–800 nm and were found to be 96–98%.

Initiation and maintenance of CCA cultures. The callus of *R. rosea* was established from *in vitro* grown plants. Leaves of the intact seedlings were transferred onto MS media supplemented with 1.5 mg L^{-1} 6-benzylaminopurine (BAP), 0.5 mg L^{-1} naphthalene acetic acid (NAA), 30 g L^{-1} sucrose and 0.7% agar. The developing calli were transferred onto new media of the same composition.

In order to establish a suspension culture of compact callus aggregates of *R. rosea*, calli from the solid media were freed from media pieces and gently broken using forceps. About 10 g of fresh weight of these small pieces were transferred into 100 mL liquid medium of the same composition (MS-Rh) into 250 mL Erlenmeyer flasks and shaken at 135 rpm. Subcultures were carried out in every 8–10 days by decanting all medium from the flask and adding fresh medium to the cultures.

Biotransformation experiments. The experiment was performed in 100 mL shake flasks containing 33 mL MS-Rh media. Two grams (fresh weight) CCA were inoculated into each flask. Cinnamyl alcohol was added to the media so that it was dissolved in ethanol (0.1 g in 1 mL EtOH) and 4 mL of water, and sterilized by passing through a 0.2 nm filter. The experiment was made in triplicate. Three flasks were harvested every day during 10 days for the determination of fresh and dry weight, viability and for the chemical analysis. The samples were dried in an oven at 40°C for 24 h and then the samples were stored in brown paper bags in a desiccator until the chemical analyses (HPLC/MS) were performed as in earlier study (György *et al*., 2003). After the analyses, the extracts of the samples were stored at –70°C.

LC/MS experiments. The semi-quantitative analyses of the unknown biotransformation product compounds were performed using high performance liquid chromatography with mass spectrometric detection (LC/MS), using the same chromatographic conditions as in the earlier study (György *et al*., 2003). About 50 mg of dried callus samples were extracted for 30 min in a sonicator with 1.5 mL 60% aqueous methanol containing 400 ng/mL of the internal standard salicin (**11**), diluted with 1:999 with 6% aqueous methanol containing the same concentration of the internal standard as the extraction solvent, filtered with 13 mm Gelman GHP Acrodisc 0.45 µm (Gelman Sciences, Ann Arbor, MI, USA) before the injection to the chromatographic system. The media samples were diluted and filtered similarly to the callus extracts before the LC/MS analysis. The chromatography was performed using Waters 2690 Alliance HPLC system (Waters Corp., Milford, USA) and a Waters Polarity 2.1 × 50 mm column with 3.0 µm particle size (Waters Corp., Milford, USA) together with a Phenomenex Max-RP 2.0 × 4.0 mm precolumn (Phenomenex, Torrance, CA, USA). The temperature of the column oven was 35°C and the injection volume was 5 µL. The eluent flow rate was 0.3 mL/min. In the gradient elution solvent A was pure water, solvent B was acetonitrile and solvent C was methanol. In the gradient elution the initial conditions were 96% A:2% B:2% C, kept isocratic for 2 min and then chanced linearly to 68% A:16% B:16% C during the next 14 min. The column was washed for 1 min with acetonitrile and equilibrated for 6 min with initial conditions before the next injection. The mass spectrometric detection was performed using Micromass LCT (Wythenshawe, UK) time-of-flight mass spectrometer with electrospray ionization source at positive ionization mode at mass range 100–600 amu. Nitrogen was used as a nebulizer and a desolvatation gas at 150 and 340°C, respectively. The cone voltage used was 26 V and the capillary voltage was 3.3 kV. Retention times in experiments were 4.3 min for the internal standard salicin (**11**), 8.2 min for **7**, 14.1 min for **8**, 14.4 min for **9**, 15.2 min for **3**, 15.4 min for **5**, 15.6 min for **10** and 16.8 min for **6**. The ions used for extraction of ion chromatograms from total ion chromatograms were sodium adducts $[M+Na]^+$ at *m*/*z* 309 for salicin, *m*/*z* 337 for **7**, *m*/*z* 481 for **8**, 483 *m*/*z* for **9**, *m*/*z* 319 for **3**, *m*/*z* 451 for **5** and *m*/*z* 321 for **10**, while the $[M+H-H_2O]^+$ ion at *m*/*z* 117 was used for **6**.

For the calibration of the method, eight concentration levels of standards was prepared from rosavin, each of which were injected in triplicate. A good linearity of detection with correlation coefficient 0.992 was obtained for range 0.5–200 ng/mL. Rosavin was used as a standard for all compounds and salicin as an internal standard.

For identification of the compounds the exact mass measurements were performed from LC/MS runs with the same instrumentation as described above. A reference mass compound raffinose was delivered into the flow in water: acetonitrile solution with a syringe pump and T-mode splitter after the column with a flow rate of 15 µL/min, so that the observed amount of lock mass ions per scan was about 500. The potassium adduct of raffinose $[C_{18}H_{32}O_{16}K]^+$ at *m*/*z* 543.1327 was used as a lock mass.

The tandem mass spectrometric experiments (MS/MS) to obtain structural data were performed from LC/MS/MS

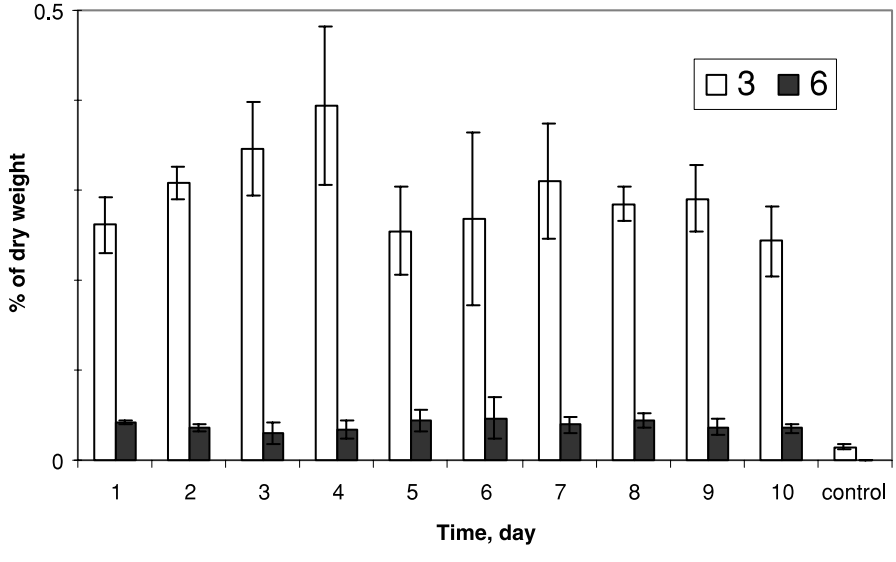

Figure 2. The amounts of rosin (**3**) and cinnamyl alcohol (**6**) in fresh samples.

runs with Micromass Quattro II triple quadrupole mass spectrometer (Altrincham, UK), using both positive and negative ion electrospray mode. The same chromatographic system as described above was used with the exception that the aqueous phase A in the gradient elution was changed to 10 mM aqueous ammonium acetate. This did not have a noteworthy effect on to the retention times of the analytes. The $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions observed (at *m*/*z* M+18, *m*/*z* M+23 and *m*/*z* M+39, respectively) in the positive ion mode were chosen for collision-induced dissociation (CID) in collision cell due to collision with argon gas, while in the negative ion mode $[M-H]^-$ ions (at *m*/*z* M–1) were collided for all analytes. Collision energies used varied from 14 to 30 eV at both positive and negative ion mode. The collision gas (argon) pressure used was $1.4–1.8 \times 10^{-3}$ mbar. In the negative ion mode also the in-source generated fragment ions at *m*/*z* 323 for **8** and at *m*/*z* 297 for **9** were chosen for collision. For generation of these fragment ions, cone voltages up to 65 V were used.

RESULTS AND DISCUSSION

Biotransformation

In our previous study the LC/MS method utilized selective ion monitoring (SIM) detection (with quadrupole mass spectrometer) and, because of that, only the previously known metabolites for which the detection was specified were detected in chromatograms and biotransformation products with any other molecular weight than expected were not detected. In this study the CCA extracts were at the beginning screened with LC/MS using wide spectral range with a time-of-flight (TOF) instrument to find out if any compounds with other molecular weights than those known earlier were present and if their amounts were dependent on the amount of cinnamyl alcohol added.

The rosin was clearly the main biotransformation product found in the samples. In the earlier study with fresh samples and more quantitative detection method (György *et al*., 2003) than described here, the rosin content increased until the fourth day, reaching 0.4% of the dry weight (Fig. 2). Then it decreased to 0.25% of the dry weight, still much higher than the concentration in the control samples. The cinnamyl alcohol content stayed below 0.05% of dry weight. Some rosavin was also observed in the samples, but the content was negligible.

After a few weeks of storage the sample analyses were carried out as described here, giving the results shown in Fig. 3. In addition to rosin and rosavin, four new compounds were found.

Amounts of compound **10** were increasing until the third day and then slightly decreased. However, on the seventh day its amount started to increase again, so that its maximum was in the eighth day's sample, reaching 0.45% of the dry weight.

Compound **7** slightly increased and reached its maximum in the ninth day's sample by 0.079% of the dry weight. Compound **9** in the samples of the third to sixth days was around 0.04% of the dry weight. Then it increased, reaching its maximum in the eighth day's sample by 0.14% of the dry weight, and than it decreased. Compound **8** did not exceed 0.1% of the dry weight in any sample. These figures also show a clear decrease in rosin (**3**) concentration in samples during the storage. The formation of compounds **7**–**10** already in the biotransformation phase, and not as a degradation

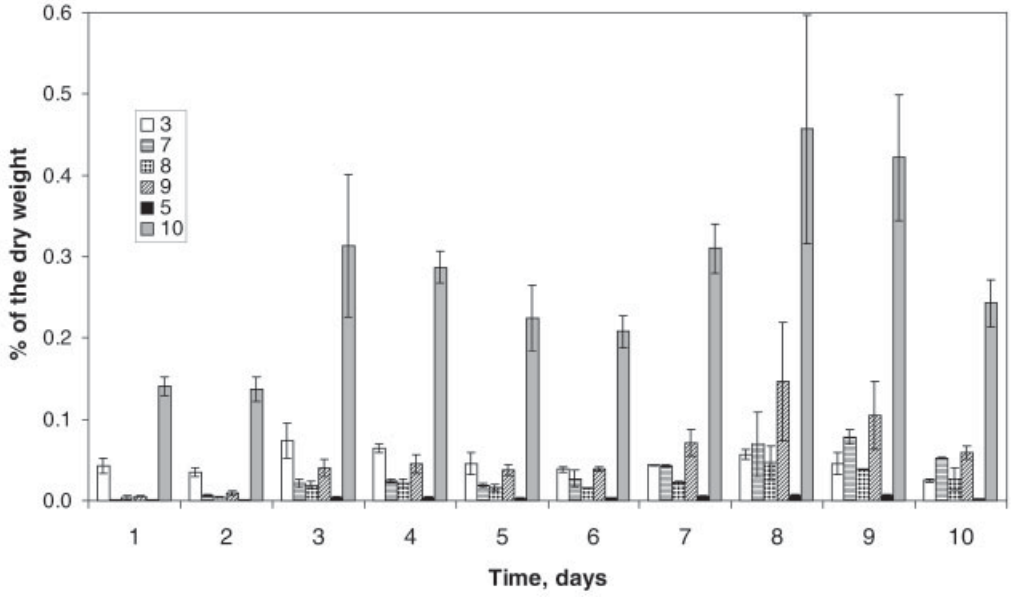

Figure 3. The semi-quantitative amounts of compounds **3**, **5** and **7**–**10** in samples after storage in freezer at –70°C for 3 weeks.

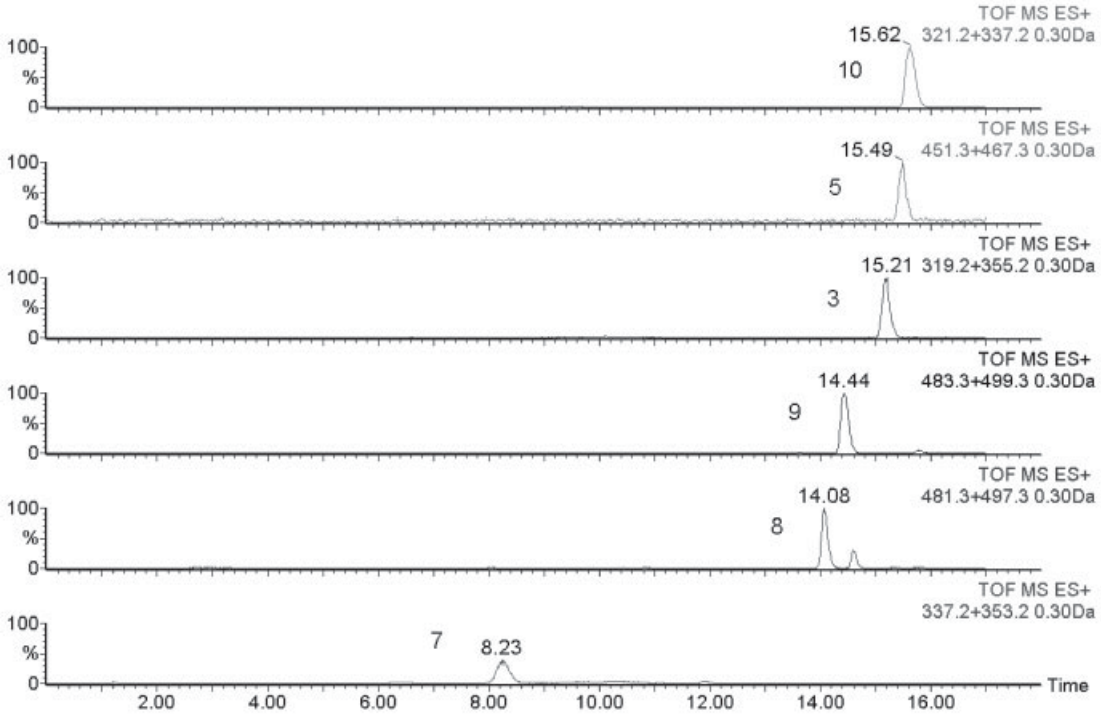

Figure 4. Extracted ion chromatograms for the compounds **3**, **5** and **7–10**. The $[M+Na]^+$ ions at *m/z* M+23 and $[M+K]^+$ ions at *m/z* M+39 used for extraction are shown in each trace.

product of **3** during the storage, was therefore ensured by preparing in a similar way few fresh CCA samples, in which compounds **7–10** were also detected.

IDENTIFICATION OF THE COMPOUNDS

Compounds **1–6** were identified according to their retention times and mass spectrometric data, as shown earlier (György *et al*., 2003; Tolonen *et al*., 2003a,b). Extracted ion chromatograms for compounds **3**, **5** and **7–10** are shown in Fig. 4.

For compound **7** the high-resolution ESI/TOF mass spectrum showed $[M+K]^+$ at *m/z* 353.1027, which was consistent with molecular formula $C_{15}H_{22}O_7$ (calculated mass for $C_{15}H_{22}O_7K$ 353.1005 amu). In the positive ion mode electrospray mass spectrum the $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were observed at *m/z* 332, *m/z* 337 and *m/z* 353 (Fig. 5). The fragment ions observed were at *m/z* 297, which is due to $[M-17]^+$ ion and is typical for CID spectrum of ammoniated glucoside without double bond next to bridging oxygen (Tolonen *et al*., 2003a,b), at *m/z* 191 due to the loss of hexose sugar from potassium adduct (i.e. $[M+K-162]^+$), at 179 due to $[GluO]^+$ ion, at *m/z* 163 due to $[Glu]^+$, at *m/z* 136 due to $[AglH]^+$ ion, and at *m/z* 117 that could be due to simultaneous losses of glucose moiety of the molecule (together with the bridging oxygen and the charging cation) and water from the aglycone part that is left. Also small intensity peak was detected at *m/z* 122, which could be due to loss of CH_2 from ion 136.

In CID experiments of the $[M+NH_4]^+$ the ion at *m/z* 297 was seen again, together with peaks for ions at *m/z* 117 and at *m/z* 91 that are commonly observed for $C_6H_5CH_2$ structures (Fig. 6). Peaks at *m/z* 179, *m/z* 133 and *m/z* 119 due to fragmentation of glucose ring were also observed. In the CID spectrum of potassium adduct the main fragment ions detected were at *m/z* 217 and at *m/z* 191 due to losses of aglycone with proton and glucose, respectively ($[M+K-136]^+$ and $[M+K-162]^+$). Small peaks were also detected at *m/z* 293 and at *m/z* 263 for the glucose ring fragmentation, i.e. losses of two and three CHOH parts of the ring from the $[M+K]^+$. However, the crucial ion detected was at the *m/z* 91, which is due to the same benzyl ion as in CID of $[M+NH_4]^+$. Together with peaks at *m/z* 136, and at *m/z* 122 seen in other spectra suggests that the hydroxyl group is attached to the C-8 of phenylpropyl structure in aglycone, i.e. the carbon in the middle of the propyl chain. The ion at *m/z* 91 may be due to loss of CH_2OH from the ion at *m/z* 122, which is for its part due to loss of CH_2 from the ion at *m/z* 136. At negative ion mode the $[M-H]^-$ ion was seen at *m/z* 313, and with higher cone voltage values also fragment ions at *m/z* 161, *m/z* 113 and *m/z* 101 were observed, all of which are very typical for glucosides (Mulroney *et al*., 1995). In the CID spectrum of $[M-H]^-$ all common glucose ring

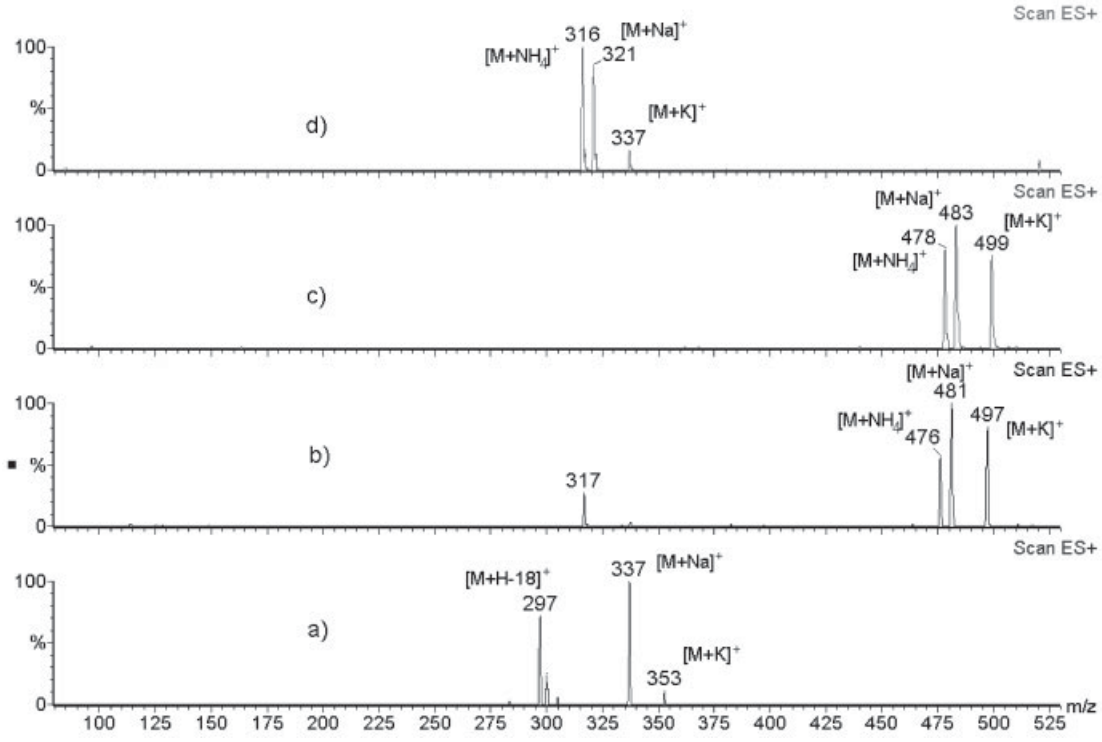

Figure 5. Positive ion mode ESI spectra for compounds **7**–**10**. (a) **7**, (b) **8**, (c) **9** and (d) **10**.

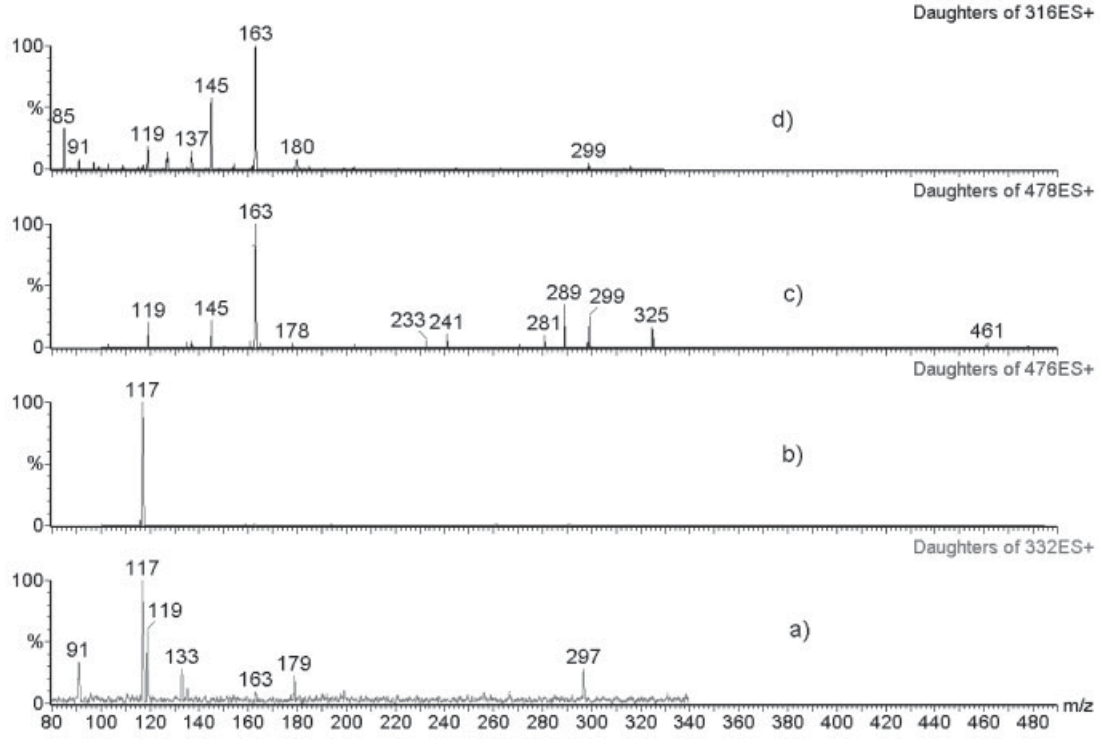

Figure 6. Product ion MS/MS spectra of $[M+NH_4]^+$ ions for compounds **7**–**10**. (a) CID of *m*/*z* 332 for **7**, (b) CID of *m*/*z* 476 for **8**, (c) CID of *m*/*z* 478 for **9** and (d) CID of *m*/*z* 316 for **10**.

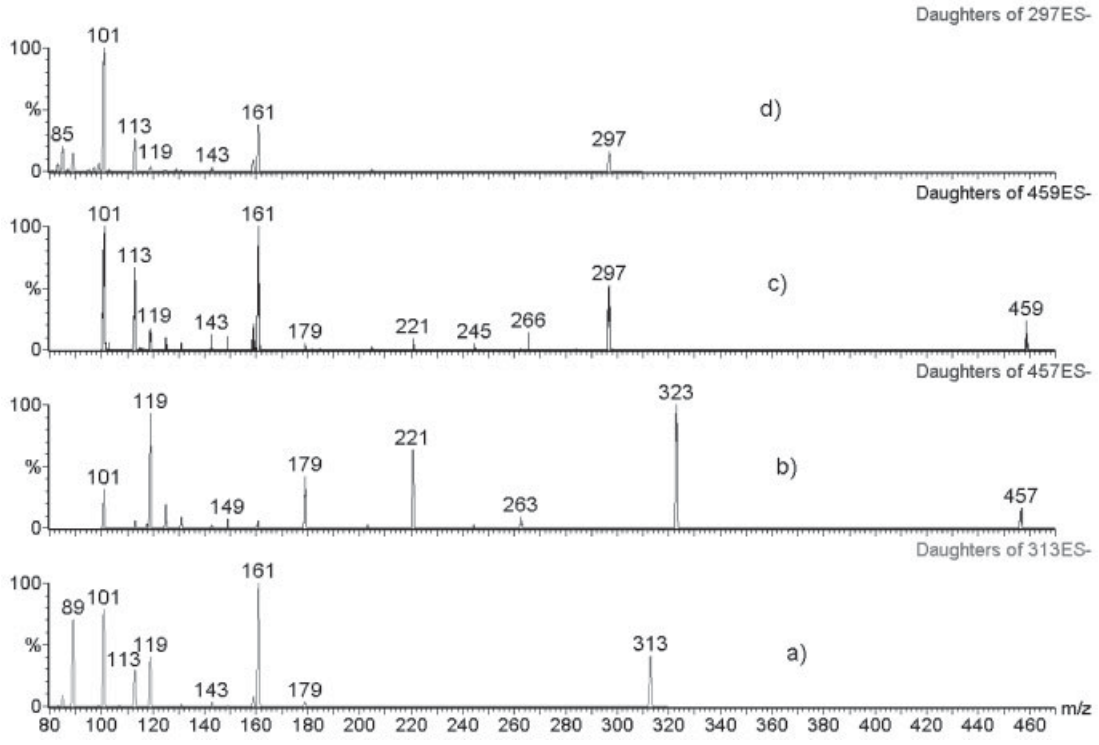

Figure 7. Product ion MS/MS spectra of $[M–H]^-$ ions for compounds **7**–**10**. (a) CID of *m*/*z* 313 for **7**, (b) CID of *m*/*z* 457 for **8**, (c) CID of *m*/*z* 459 for **9** and (d) CID of *m*/*z* 297 for **10**.

fragment ions were seen, i.e. at *m*/*z* ratios 179, 161, 143, 119, 113, 101 and 89 (Fig. 7).

For compound **8** the high-resolution ESI/TOF mass spectrum showed $[M+K]^+$ at *m*/*z* 497.1434, which was consistent with molecular formula $C_{21}H_{30}O_{11}$ (calculated mass for $C_{21}H_{30}O_{11}K$ 497.1425 amu). In the positive ion mode electrospray mass spectrum the $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were observed at *m*/*z* 476, *m*/*z* 481 and *m*/*z* 497 (Fig. 5). The most intense fragment ion was at *m*/*z* 117, due to charged aglycone, i.e. the $[C_6H_5(CH)_2CH_2]^+$ ion. A smaller fragment ion peak was detected at *m*/*z* 185, whcih was due to simultaneous loss of aglycone (with bridging oxygen) and the 'non-reducing' glucose ring from the sodium adduct of the molecule, i.e. $[M+Na–133–163]^+$. This kind of fragmentation is usual for phenylpropanoid diglycosides (Tolonen *et al*., 2003a,b). In the CID spectrum of $[M+Na]^+$ the main fragment ion peaks were at *m*/*z* 363 and *m*/*z* 117, the former being due to loss of neutral aglycone as AglH, i.e. $[M+Na–AglH]^+$, and the latter being the same as the full-scan spectrum described above. A minor fragment ion peak was also observed at *m*/*z* 305, due to $^{0,2}X_{Glu1}$ fragmentation of the first, 'reducing', glucose ring also usually observed for phenylpropanoid diglycosides (Tolonen *et al*., 2003a,b). In the CID spectrum of $[M+NH_4]^+$ only the very intense fragment ion peak at *m*/*z* 117 was observed (Fig. 6).

In negative ion mode electrospray spectrum the $[M–H]^-$ ion was seen at *m*/*z* 457, and when high cone voltage values were used, small fragment ion peaks at *m*/*z* 323, *m*/*z* 305 and *m*/*z* 179 were also observed. The two first of these were due to the loss of aglycone in the form of AglOH from the deprotonated molecule, and the consecutive loss of water from it. The ion at *m*/*z* 179 was due to repulsion of charged hexose sugar. In the CID spectrum of the $[M–H]^-$ ion the same ion at *m*/*z* 323 was the main fragment peak with high intensity (Fig. 7). High intensity fragment peaks due to fragmentation typical for the second, 'non-reducing' glucose ring (Mulroney *et al*., 1995) were present at *m*/*z* ratios 179, 161, 149, 119, 113 and 101. The fragment ion peaks of 'reducing' hexose sugar ring were seen at *m*/*z* 263, *m*/*z* 245, *m*/*z* 221 and *m*/*z* 203, and were all usual peaks in spectra of diglucosides. The ion at *m*/*z* 263 was due to loss of two CHOH-units from ion at *m*/*z* 323 and ion at *m*/*z* 245 due to consecutive loss of water, whereas the ion at *m*/*z* 221 was due to $^{0,4}X_{Glu1}$ fragmentation of the first, 'reducing', glucose ring. All these same peaks were detected also in CID spectrum of ion at *m*/*z* 323 that was first generated with in-source fragmentation (with high cone voltages), and a peak at *m*/*z* 305 due to loss of water from the parent ion was also detected.

For compound **9** the high-resolution ESI/TOF mass spectrum showed $[M+K]^+$ at *m*/*z* 499.1579, consistent with molecular formula $C_{21}H_{32}O_{11}$ (calculated mass for $C_{21}H_{32}O_{11}K$ 499.1582 amu). In the positive ion mode electrospray mass spectrum the $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were observed at *m*/*z* 478, *m*/*z* 483

and *m/z* 499 (Fig. 5). The same fragment ions as for **8** were detected at *m/z* 363 and at *m/z* 185, together with peak at *m/z* 201 due to simultaneous loss of aglycon and one glucose ring from sodium adduct, i.e. $[M+Na–AglH–162]^+$. In CID spectrum of $[M+Na]^+$ only small fragment ion peaks were dected at *m/z* 321 due to loss of hexose sugar, i.e. $[M+Na–162]^+$ and at *m/z* 305 due to $^{0,2}X_{Glu1}$ fragmentation of the first, 'reducing', glucose ring. In CID spectrum of $[M+NH_4]^+$ the most intense fragment ion peaks were due to fragmentation of second glucose ring at *m/z* ratios 163, 145 and 119 (Fig. 6). Other fragment peaks were detected at *m/z* 325 due to $[M+NH_4–AglONH_4]^+$, at *m/z* 298 due to $[M+NH_4–Glu–NH_4]^+$ and at *m/z* 289 that could be due to loss of two water molecules from the ion at *m/z* 325. A small peak at *m/z* 461 was also detected due to $[M+NH_4–17]^+$, as is typical in CID spectrum of ammoniated glycosides with no double bond next to bridging oxygen (between aglycone and sugar part).

In the negative ion mode the $[M–H]^-$ ion was detected at *m/z* 459 and a loss of glucose $[M–H–162]^-$ at *m/z* 297. The same fragment ion was also seen as an intensive peak in CID spectrum of $[M–H]^-$, together with the typical fragment ions due to dissociation of the second 'non-reducing', glucose ring at *m/z* ratios 179, 161, 143, 119, 113 and 101 and small peaks due to fragmentation of the first, 'reducing' glucose ring at *m/z* ratios 263, 245, 233 and 221 (Fig. 7). A small peak at *m/z* 266 was also detected, and could be due to dissociation of the bond between the carbons 5 and 6 of the first glucose ring, leading to ion $[M–H–CH_2OGlu]^-$. In the CID spectrum of ion at *m/z* 297 that was first generated with in-source fragmentation (with high cone voltages) were detected the ions due to the fragmentation of the remaining (the first, reducing) glucose ring at *m/z* ratios 161, 119, 113, 101 and 89.

For compound **10** the high-resolution ESI/TOF mass spectrum showed $[M+Na]^+$ at *m/z* 321.1319, consistent with molecular formula $C_{15}H_{22}O_6$ (calculated mass for $C_{15}H_{22}O_6Na$ 321.1314 amu). In positive ion mode electrospray mass spectrum the $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were observed at *m/z* 316, *m/z* 321 and *m/z* 337 (Fig. 5). The only fragment ion observed was at *m/z* 119 due to $[C_6H_5(CH_2)_3]^+$ ion (charged aglycone). In CID experiments the sodium and potassium adducts of the molecule proved to be very stable and fragmentation was not observed. When the $[M+NH_4]^+$ ion was collided in CID experiment, the main fragment ions detected were at *m/z* 163 due to cleavage of the aglycone part of the molecule with the bridging oxygen and ammonium cation (i.e. the charged hexose sugar moiety), at *m/z* 145 due to loss of water from ion at *m/z* 163 and at *m/z* 119 due to charged aglycone part of the molecule (Fig. 6). The two first of these are usually typical for glucosides. Also small intensity peaks for ions at *m/z* 299, *m/z* 180 and *m/z* 85 were detected, the two first being typical for CID spectrum of ammoniated glucoside without double bond next to bridging oxygen (Tolonen *et al*., 2003a,b) ($[M+NH_4–17]^+$ and $[Glu+NH_4]^+$), and the last one a typical ion detected in fragmentation of glucose ring. In the negative ion mode electrospray mass spectrum the deprotonated molecule $[M–H]^-$ was observed at *m/z* 297 and with higher cone voltage values fragment ions at *m/z* 161, *m/z* 113 and *m/z* 101 were also observed, all of which are very typical for glucosides (Mulroney *et al*., 1995). In the CID spectrum of $[M–H]^-$ the same fragment ions were seen together with small peak at *m/z* 143, which is also typical for glucosides and due to loss of water from $[Glu]^-$ ion at *m/z* 161 (Fig. 7). The CID spectrum of $[M–H]^-$ was very similar when compared with that of the ion *m/z* 297 generated with in-source fragmentation of compound **9**, also suggesting that compound **10** is otherwise similar to **9**, except with one sugar ring less.

In all of the compounds identified above, the stereochemistry of the sugar rings is expected to be glucose, although the data obtained does not give the absolutely certainty of the sugar moiety. However, the most typical reactions studied lead to glucose structure in nature when hexose sugars are concerned, especially in the metabolism of the plant studied. To date, at least to our knowledge, no other hexose sugars except glucose have been identified from the secondary metabolites of *R. rosea*. Similarly, the linkage positions between the sugar rings in the identified diglycosides cannot be concluded unambiguously, but the assumption of linkages to be in 1,6-position is made by comparison of fragmentation reactions with those of the compounds with similar aglycone part but one CHOH-unit smaller 'non-reducing' sugar ring, e.g. rosavin, rosarin and cinnamyl-(6′-*O*-*β*-xylopyranosyl)-*O*-*β*-glucopyranoside (data not shown, Tolonen, 2003b). Those all compounds are known to have 1,6-linkage between their sugar moieties, the 'reducing' ring b eing glucopyranoside in all of them and the 'non-reducing' rings being arabinopyranoside, arabinofuranoside and xylopyranoside. In corresponding CID spectra of those compounds, all the same corresponding fragmentation reactions are seen as with the compounds studied here, the only difference being the 30 amu smaller *m/z* ratios for the fragment ions due to the change of the 'non-reducing' sugar ring from glucose to the smaller ones mentioned above. Some articles (Mulroney *et al*., 1995; Hofmeister *et al*., 1991; Asam and Glish, 1997) have been published concerning the determination of the linkage positions in diglucosides by MS/MS, and in those studies a clear difference in presence of certain fragment ions [i.e. *m/z* 221, 251, 263, 281 and 323 in CID of $[M–H]^-$ and *m/z* 347, 305, 275, 245 in CID of $[M+Na]^+$) are obtained for 1,2-, 1,3-, 1,4- and 1,6-linked diglucosides. However,

those studies have been performed using diglucosides without an aglycone part, and if their results are applied to the 1,6-linked diglycosides mentioned above (rosavin etc.), no clear conclusions about the linkage position can be made. Or more exactly, those results would suggest the compounds (rosavin etc.) to have rather a 1,4-linkage than the existing 1,6-linkage. Thus, the presence of aglycone seems to reflect to the formation of sugar moiety fragment ions, probably through a change of the charge position in the molecular ion.

However, if some sort of conclusion with the compounds studied here are tried to make from the earlier studies (Mulroney *et al*., 1995; Hofmeister *et al*., 1991; Asam and Glish, 1997), the presence of the ion at *m*/*z* 305 at CID spectra of [M+Na] for compounds **8** and **9** seems to be diagnostic that the linkage is not in the 1,2- or 1,3-position (Asam and Glish, 1997). Similarly the presence of intensive peak at *m*/*z* 221 due to $^{0,4}X_{Glu1}$ fragmentation of the 'reducing' glucose ring seems to exclude the 1,3-linkage position (Mulroney *et al*., 1995). Finally, the conclusion whether the sugars are 1,4- or 1,6-linked seems to remain somewhat unclear.

CONCLUSIONS

Four new glycosidic products of the biotransformation of cinnamyl alcohol in *Rhodiola rosea* cell suspensions were identified using mass spectrometric methods. The identifications were made using the knowledge of the starting compounds and expected known biotransformation reactions, as well as the knowledge of the mass spectrometric behavior of similar types of compounds.

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