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Biotechnological production of two new 8,4′-oxynorneolignans by elicitation of *Echinacea purpurea* cell cultures

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Abstract—Two new 8,4′-oxynorneolignans were produced by elicitation of *Echinacea purpurea* cell suspension cultures with yeast elicitor. Their structures with an unusual (hydroxy)acetyl group were elucidated by spectroscopic methods including gas chromatography (GC) and electrospray ionization (ESI) mass spectrometry.
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Echinacea purpurea is an old well known medicinal plant and is still very popular in North America and Europe. Herbal products containing extracts of this plant are used for the treatment of common cold and as immunostimulants. Extensive phytochemical and pharmacological studies have disclosed their main and bioactive constituents as caffeic acid derivatives, alkylamides, polyacetylenes and polysaccharides.[1]

Plant cell suspension cultures show great advantages for producing secondary and possibly bioactive natural products.[2] Previously *E. purpurea* cell cultures were used to produce immunologically active polysaccharides,[3] however, no further secondary metabolites were investigated. Elicitation of plants and their cell cultures is a method of studying the function and metabolism of phytoalexins.[4] Recently, it has become a popular technique to increase production rates of known compounds or to find new natural products for biotechnological application[5] and drug discovery.[6] In our laboratory *E. purpurea* cell cultures have been established[7] for the production of potential bioactive compounds using elicitation technique.

Cell suspension cultures of *E. purpurea* were grown in modified LS-medium[8] with thiamine, 2,4-diphenoxyacetic acid, 1-naphthylacetic acid and myo-inositol as organic growth factors. Transfer of 3 g cells into the new medium was carried out every 14 days. The suspension cultures were grown in the dark at 25 °C. The 200 mL flasks containing 40 mL medium were shaken at 120 rpm. A yeast elicitor containing polysaccharides prepared as described[9] (1.0 mg/mL) was added to the five-day-old cell suspension cultures in linear growth phase. Equivalent amounts of distilled water were added to the control cultures. In the nonelicited control medium of cell cultures only few phenolics were found by analytical reverse-phase high performance liquid chromatography (HPLC) monitored at 280 nm, however, in yeast elicitor-treated cell cultures a number of phenolics including compounds **1** and **2** were newly formed and accumulated in the medium to a maximum level at 36 h post-elicitation.

In order to identify them, 540 mL of yeast elicitor-treated medium from 30 flasks for cell cultures was collected and centrifuged at 3000*g* for 10 min. The supernatants were adjusted to pH 2 and extracted using ethyl acetate. The further isolation of **1** and **2** was performed by means of semi-preparative HPLC using a Waters chromatography attached to a photodiode array detector for recording UV spectra with a LiChrosorb RP-18 column (Merck, 10 × 250 mm, 7 μm) and a flow rate of 4 mL/min. The solvents were CH_3CN azeotrope containing 15% H_2O (A) and H_2O (B). For elution of the compounds a linear gradient of 5% A–40% A in 55 min was applied. Compound **1** (≈230 μg) and **2** (<100 μg) (Fig. 1) were obtained.

Keywords: *Echinacea purpurea*; Cell suspension cultures; Elicitation; Norneolignan.
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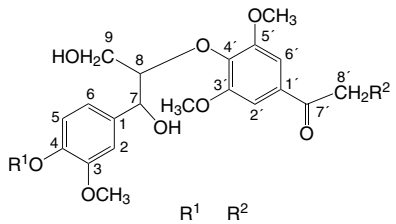

	R^1	R^2
1	H	OH
2	H	H
3	Glc	H

Figure 1. Structures of neolignans **1**–**3** from cell suspension cultures of *E. purpurea* (Glc: β-D-glucopyranosyl).

Compound **1** showed UV absorptions at 220 and 284 nm indicating the presence of a benzenoid moiety. The molecular weight of **1** was deduced as 408 from the positive and negative electrospray ionization (ESI) mass spectra. The positive high resolution (HR)-ESIMS (found 431.1324, calcd 431.1318, $C_{20}H_{24}O_9Na$) determined its molecular formula as $C_{20}H_{24}O_9$. The ^1H NMR (600 MHz, CD_3OD) spectrum of **1** showed the presence of 1,3,4-trisubstituted phenyl aromatic protons at δ 6.95 (1H, d, $J = 1.8$ Hz), 6.70 (1H, d, $J = 8.4$ Hz), 6.77 (1H, dd, $J = 8.4$, 1.8 Hz) and one pair of equivalent aromatic protons at 7.22 (2H, s); three methoxy groups at 3.80 (3H, s) and 3.86 (6H, s); a hydroxyacetyl group at 4.58 (2H, s); 1-phenyl-2-aryloxypropane-1,3-diol moiety at 4.86 (1H, overlapped), 4.46 (1H, m), 3.90 (1H, dd, $J = 12.0$, 5.4 Hz) and 3.67 (1H, dd, $J = 12.0$, 4.0 Hz). These structural moieties were further supported by the ^1H–^1H COSY spectrum with the following correlations: 6.95–6.77; 6.70–6.77; 4.86–4.46; 4.46–3.90 and 3.90–3.67. The ^1H NMR data for parts A and B of **1** were similar as those of 1-(4-hydroxy-3-methoxyphenyl)-2-{2-methoxy-4-[1-(*E*)-propen-3-ol]-phenoxy}-propane-1,3-diol[10,11] and 2,4′-dihydroxy-3′,5′-dimethoxyacetophenone (α-hydroxyacetosyringone),[12,13] respectively, which were also isolated as elicitor-induced products.

Trimethylsilyl (TMS) derivative of **1** was prepared with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), which converts all hydroxy groups as well as ketones and aldehydes into corresponding TMS ether and enol ether groups.[14,15] Figure 2A shows the gas chromatography (GC) electron impact (EI) mass spectrum and analysis of fragmentations of the TMS derivative of **1**. The molecular ion at *m*/*z* 768 [M+72(TMS)×5] indicated that it contained five TMS groups derived from a ketone and four hydroxy groups. The ion at *m*/*z* 103 ($CH_2{=}O^+TMS$) indicated a trimethylsilyloxymethyl group; the ion at *m*/*z* 209 indicated the presence of 3-methoxy-4-trimethylsilyloxyphenyl ring, and the ion at *m*/*z* 297 indicated the

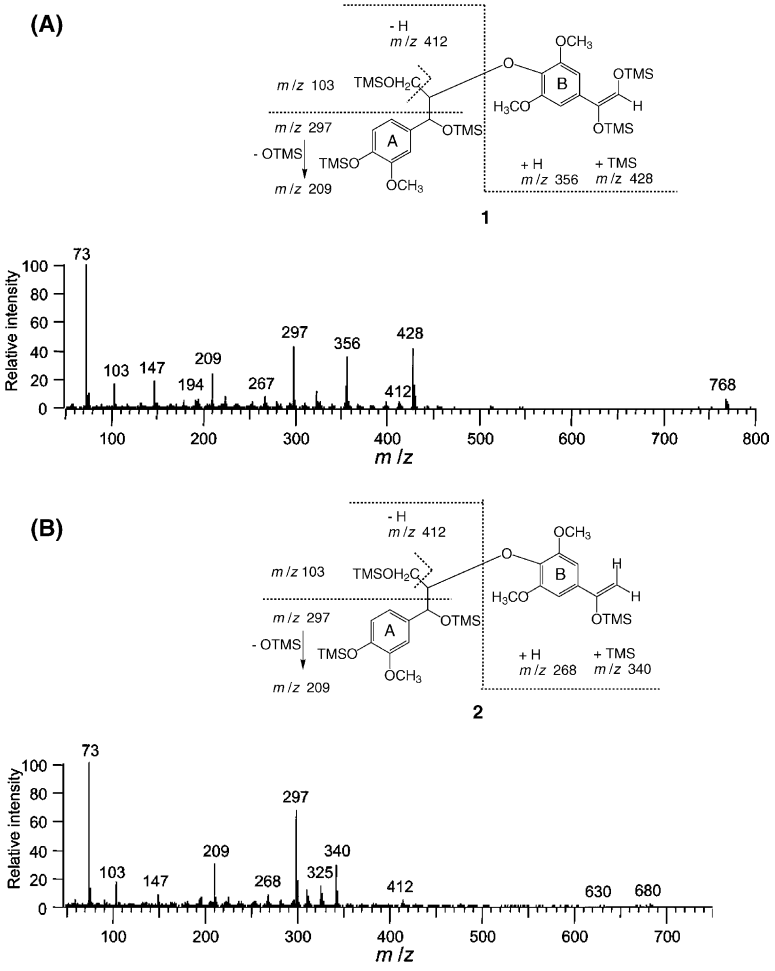

Figure 2. GC–EI mass spectra and fragmentations of TMS derivatives of **1** (A) and **2** (B).

presence of an extra trimethylsilyloxy group at benzylic position of ring A.[15,16] In addition, cleavage of β-aryl-ether bond [C(8)–O(4′)] resulted in the presence of the ion at *m/z* 356 and another ion at *m/z* 428 due to the transfer of a TMS group to part B.

Collision-induced dissociation (CID)–ESIMS of the protonated molecular ion of **1** at *m/z* 409 $[M+H]^+$ (Fig. 3) generated a base ion at *m/z* 213 $[M-196(\text{part A})+H]^+$ and a minor ion at *m/z* 197 $[M-212(\text{part B})+H]^+$ probably via the same C(8)–O(4′) bond cleavage as found for its TMS derivative in GC–MS. Further possible cleavage of the bond [C(1′)–C(7′)] from the ion at *m/z* 213 and the loss of H_2O from the ion at *m/z* 197 generated the ions at *m/z* 155 and *m/z* 179, respectively. These fragment ions in GC–EIMS and CID–ESIMS resulting from part B of **1** were also similar as those of α-hydroxyacetosyringone.[12] All these data suggested **1** to be 4,7,9,8′-tetrahydroxy-3,3′,5′-trimethoxy-8,4′-oxy-9′-norneolignane-7′-one [nomenclature of lignans and neolignans (IUPAC recommendations 2000)][17] [= 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxy-4-hydroxyacetylphenoxy)-propane-1,3-diol].[18] This is a new 8,4′-oxynorneolignan with an unusual hydroxyacetyl group.

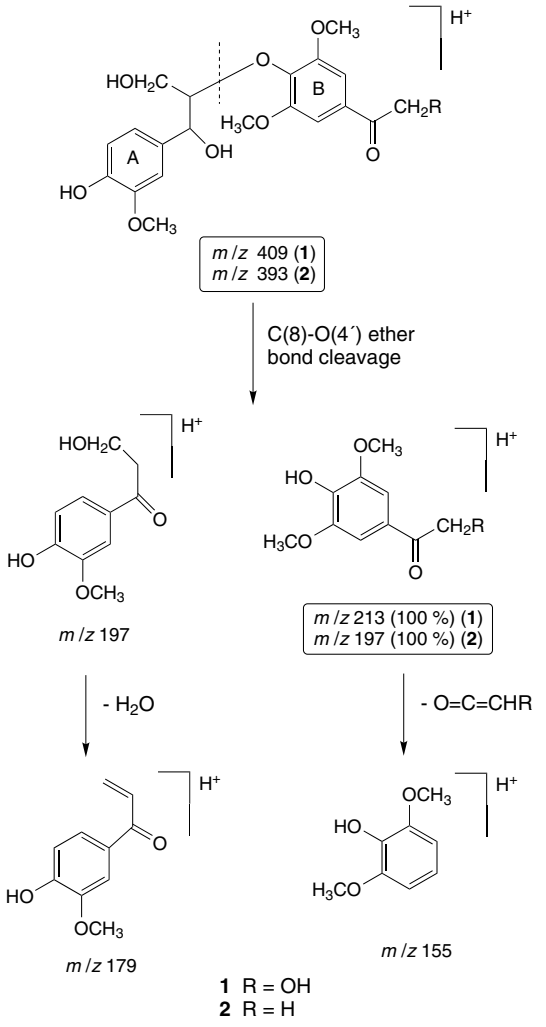

Figure 3. Possible fragmentation pathways of the protonated ions of **1** and **2** in the CID–ESIMS.

Compound **2** showed a very similar UV spectrum as **1** indicating the similarity in their structures. Its molecular weight was determined to be 392 and its molecular formula was deduced to be $C_{20}H_{24}O_8$ by HRESIMS, which differed by only one oxygen from **1**. The GC–EI mass spectrum and possible fragmentations of the TMS derivative of **2** are shown in Figure 2B. The molecular ion at *m/z* 680 [M+72(TMS)×4] suggested the presence of four trimethylsilyloxy groups, one of which was also derived from a ketone group via enolization. The major fragment ions at *m/z* 103, 209 and 297 supported the part A of **2** as that of **1**. The ions at *m/z* 268 and 340 from **2** were generated by the similar cleavage of β -arylether bond, which indicated the loss of one hydroxyl group in the part B. CID–ESIMS of the protonated molecular ion at *m/z* 393 showed a very similar fragmentation pattern as that of **1** (Fig. 3) but with a different base ion at *m/z* 197 $[M-196(\text{part A})+H]^+$, which is 16 Da less than that of **1** indicating again the loss of one oxygen atom in the part B. On the basis of these mass spectroscopic analysis, **2** was tentatively suggested as 4,7,9-trihydroxy-3,3′,5′-trimethoxy-8,4′-oxy-9′-norneolignane-7′-one {= 1-(4-hydroxy-3-methoxyphenyl)-2-(4-acetyl-2,6-dimethoxyphenoxy)-propane-1,3-diol}.[19] This was further supported by a structural elucidation of its glycoside form (**3**) (Fig. 1) isolated from both control and elicitor-treated cell extracts with a relatively more amount (610 μg).

The molecular formula of compound **3** was deduced as $C_{26}H_{34}O_{13}$ by HRESIMS (found 577.1821, calcd 577.1897, $C_{26}H_{34}O_{13}Na$). CID–ESIMS of the sodium cationized molecular ion of **3** at *m/z* 577 $[M+Na]^+$ showed a major fragment ion at *m/z* 415 $[M-162+Na]^+$, which indicated that **3** contained a hexose moiety. After hydrolysis of **3** with β-glucosidase, its aglycone was extracted and determined to be **2** by comparison with their analytical HPLC retention times and on-line UV spectra, ESIMS and GC–MS. ^1H NMR and ^1H–^1H COSY spectra of **3** showed similar as that of **1** except the appearance of signals for a glucopyranosyl moiety and an acetyl group [2.58 (3H, s)] instead of a hydroxyacetyl group in **1**. The signal at 4.82 (1H, d, $J = 7.2$ Hz, H-1″-Glc) and the downfield shift of H-5 and H-6 in the ring A indicated that β-D-glucopyranosyl group was located at C-4[11,20] rather than at C-9 or C-7. ^{13}C NMR (150 MHz) signals for only methyls, methylenes and methines were observed despite recording for as long as 72 h. It showed the carbon signals at 121.1 (C-6), 117.3 (C-5), 112.4 (C-2), 87.1 (C-8), 73.5 (C-7), 62.2 (C-9) and 56.7 (3-OCH_3) for the part A, the signals at 107.1 (C-2′, 6′), 56.8 (3′,5′-OCH_3) and 30.7 (C-8′) for the part B, and the left signals at 78.1 (C-3″), 77.8 (C-5″), 74.9 (C-2″), 71.3 (C-4″) and 62.5 (C-6″) for the β-D-glucopyranosyl moiety, respectively. The ^{13}C NMR signals of **3** were assigned by comparison with those of citrucin B[10] and 3′,5′-dimethoxy-4′-hydroxyacetophenone (acetosyringone)[21] (Aldrich spectral library D13,440-6, CAS[2478-38-8]). Taken these data together, compound **3** was identified as 4-(β-D-glucopyranosyloxy)-7,9-dihydroxy-3,3′,5′-trimethoxy-8,4′-oxy-9′-norneolignane-7′-one {= 1-[4-(β-D-glucopyranosyloxy)-3-methoxyphenyl]-2-(4-acetyl-2,6-dimethoxyphenoxy)-propane-1,3-diol},[22] which consequently confirmed the structure of **2** (Fig. 1). They are

also new natural products. The stereochemistry of C-7 and C-8 in **1**–**3** is unknown.

8,4′-Oxynorneoligans with a formyl group were previously found in *Picea excelsa*,[23] *Larix leptolepis*[10] and *Persea obovatifolia*.[24] An *erythro* isomer has also been stereoselectively synthesized.[25] It is the first time that 8,4′-oxynorneoligans containing acetophenonyl moieties have been produced by elicitation of *E. purpurea* cell suspension cultures. Although the biosynthetic pathways for 8,4′-oxyneolignans and acetophenone derivatives are unknown,[21] α-hydroxyacetosyringone might be formed from acetosyringone via the hydroxylation of the α-methyl group. A similar pathway of α-hydroxyacetovanillone was previously found from acetovanillone by a cytochrome P450-dependent monooxygenase.[26] Phenoxy radical couplings of these acetophenone derivatives with monolignol probably generated the new norneolignans with an unusual (hydroxy)acetyl group. These neolignans have not been detected in intact plants so far.[1] Therefore, plant biotechnology like cell cultures and elicitation is a promising way of finding novel compounds, and this offers an opportunity of creating molecular diversity in nature.

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12. α-Hydroxyacetosyringone: HPLC-UV λ_{max} nm: 220, 305. Positive ion ESIMS: *m/z* 213 $[M+H]^+$, 235 $[M+Na]^+$, 447 $[2M+Na]^+$. Positive ion CID–ESIMS of *m/z* 213: 195 $[M-H_2O]^+$, 167, 163, 155, 140, 135, 123, 107. Negative ion CID–ESIMS: *m/z* 211 $[M-H]^-$, 196, 163, 151, 135. GC–MS (tri-TMS) *m/z* (rel. int.): 428 $[M]^+$ (80), 413 (3), 398 $[M-2\times CH_3]^+$ (12), 355 (4), 147 (41), 73 (100). ^{1}H NMR (600 MHz, CD_3OD): δ 7.27 (2H, s, H-2′, 6′), 4.58 (2H, s, H-2), 3.89 (6H, s, $2\times OCH_3$).
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18. Compound **1**: HPLC-UV λ_{max} nm: 220, 284. Positive ion ESIMS: *m/z* 409 $[M+H]^+$, 431 $[M+Na]^+$, 839 $[2M+Na]^+$. Positive ion CID–ESIMS of *m/z* 409: 213 (100), 195, 179, 163, 155 (50). Positive ion CID–ESIMS of *m/z* (rel. int.) 431 (25): 295, 234 (60), 219, 203, 177, 173, 159, 23 (Na, 100). Negative ion ESIMS: *m/z* 407 $[M-H]^-$. Negative ion CID–ESIMS of *m/z* 407: 271, 211, 195, 165, 135. Positive HRESIMS: *m/z* 431.1324 (calcd for $C_{20}H_{24}O_9Na$: 431.1318). EI-MS *m/z* (rel. int.): 408 (1), 360 (1), 313 (2), 283 (3), 272 (2), 238 (30), 207 (48), 181 (72), 153 (37), 137 (38), 136 (38), 121 (100), 93 (64), 65 (62). GC–MS (TMS) *m/z* (rel. int.): 768 $[M]^+$ (6), 428 (50), 412 (5), 356 (40), 323 (3), 310 (3), 297 (47), 267 (8), 253 (4), 223 (8), 209 (25), 194 (7), 147 (20), 103 (18), 73 (100). ^{1}H NMR (600 MHz, CD_3OD): δ 7.22 (2H, s, H-2′, 6′), 6.95 (1H, d, J = 1.8 Hz, H-2), 6.70 (1H, d, J = 8.4 Hz, H-5), 6.77 (1H, dd, J = 8.4, 1.8 Hz, H-6), 4.86 (overlapped, H-7), 4.58 (2H, s, H-8′), 4.46 (1H, m, H-8), 3.90 (1H, dd, J = 12.0, 5.4 Hz, H_a-9), 3.86 (6H, s, $2\times OCH_3$), 3.80 (3H, s, OCH_3), 3.67 (1H, dd, J = 12.0, 4.0 Hz, H_b-9).
19. Compound **2**: HPLC-UV λ_{max} nm: 220, 284. Positive ion ESIMS: *m/z* 393 $[M+H]^+$, 415 $[M+Na]^+$, 431 $[M+K]^+$. Positive ion CID–ESIMS of *m/z* (rel. int.) 393: 197 (100), 179 (2), 155 (20), 43 (20). Positive ion CID–ESIMS of *m/z* 415: 218 $[M-197+Na]^+$ (45). Positive HRESIMS: *m/z* 415.1419 (calcd for $C_{20}H_{24}O_8Na$: 415.1369). GC–MS (TMS) *m/z* (rel. int.): 680 $[M^+]$ (1), 425 (1), 412 (1), 340 (52), 325 (15), 323 (21), 297 (100), 268 (7), 223 (6), 209 (36), 147 (5), 103 (14), 73 (72).
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22. Compound **3**: HPLC-UV λ_{max} nm: 220, 278. Positive ion ESIMS: *m/z* 577 $[M+Na]^+$, 593 $[M+K]^+$. Positive ion CID–ESIMS of *m/z* 577: 415 $[M-162+Na]^+$, 365, 351, 309, 219, 202, 175. Positive HRESIMS: *m/z* 577.1821 (calcd for $C_{26}H_{34}O_{13}Na$: 577.1897). ^{1}H NMR (600 MHz, CD_3OD): δ 7.25 (2H, s, H-2′, 6′), 7.08 (1H, d, J = 8.4 Hz, H-5), 7.04 (1H, d, J = 2.0 Hz, H-2), 6.89 (1H, dd, J = 8.4, 2.0 Hz, H-6), 4.88 (1H, overlapped, H-7), 4.82 (1H, d, J = 7.2 Hz, H-1″-Glc), 4.48 (1H, m, H-8), 3.92 (1H, dd, J = 12.0, 5.4 Hz, H_a-9), 3.85 (6H, s, $2\times OCH_3$), 3.80 (3H, s, OCH_3), 3.69 (1H, m, H_b-9), 3.40–3.88 (H-Glc), 2.58 (3H, s, H-8′). ^{13}C NMR (150 MHz, CD_3OD): δ 121.1 (C-6), 117.3 (C-5), 112.4 (C-2), 107.1 (C-2′, 6′), 87.1 (C-8), 78.1 (C-3″-Glc), 77.8 (C-5″-Glc), 74.9 (C-2″-Glc), 73.5

(C-7), 71.3 (C-4″-Glc), 62.5 (C-6″-Glc), 62.2 (C-9), 56.7 (3-OCH_3), 56.8 (3′,5′-OCH_3), 30.7 (C-8′).
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