Natural Product Research: Formerly Natural Product Letters
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

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Published online: 13 Jan 2011.

To cite this article: Ahmed M.A. Abd El-Mawla , Salwa F. Farag & Till Beuerle (2011): Cinnamyl alcohols and methyl esters of fatty acids from Wedelia prostrata callus cultures, Natural Product Research: Formerly Natural Product Letters, 25:1, 45-52

To link to this article: http://dx.doi.org/10.1080/14786419.2010.482937

Natural Product Research
Vol. 25, No. 1, January 2011, 45–52

Taylor & Francis
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Cinnamyl alcohols and methyl esters of fatty acids from *Wedelia prostrata* callus cultures

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(*Received 9 March 2010; final version received 31 March 2010*)

Two methyl esters of fatty acids, namely octadecanoic acid methyl ester (methyl stearate) (**1**) and hexadecanoic acid methyl ester (methyl palmitate) (**2**), in addition to four cinnamyl alcohol derivatives, sinapyl alcohol (**3**), coniferyl alcohol (**4**), *p*-coumaryl alcohol (**5**) and coniferyl alcohol 4-*O*-glucoside (coniferin) (**6**), were isolated from callus cultures of *Wedelia prostrata*. The structure of coniferin was established by spectroscopic and chemical methods, while the other compounds were identified by gas chromatography–mass spectrometry and thin layer chromatography in comparison with standards.

Keywords: *Wedelia prostrata*; MS medium; callus cultures; GC-MS; coniferin

1. Introduction

Wedelia prostrata Hook. et Arn. (Hemsl.) (Asteraceae, tribe Heliantheae) (Benson, 1976; Lawrence, 1968) is cultivated in Egypt as an ornamental plant. The genus *Wedelia* comprises about 70 species, native to the warmer regions of the world (Bailey, 1963). Economically, the plants of family Asteraceae are of considerable importance. They comprise numerous food and drug plants, of which many are ornamentals and several are noxious weeds (Core, 1962). Many species of *Wedelia* have valuable biological activities (Gopalakrishnan, Sadique, & Chandra, 1989; Perry & Metzger, 1980; Watt & Breyer-Brandwijk, 1962). The isolated kaurene diterpenes have shown antibiotic activity and thus justify the utilisation of *Wedelia* species as antitussive agents (Roque, Giannella, Giesbrecht, & Barbosa, 1987).

Previous phytochemical investigations of *W. prostrata* have led to the isolation and identification of eudesmanolides, chalcones, phenylpropanoids (3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid), a sesquiterpene, kaurenoids, monoterpenes, stigmasterol, stigmasterol glucoside and flavonoids (Farag, 2001; Farag, El-Emary, Makboul, Hassanean, & Niwa, 1997; Farag, El-Emary, Makboul, Hassanean, & Niwa, 1998; Farag, El-Emary, & Niwa, 1996).

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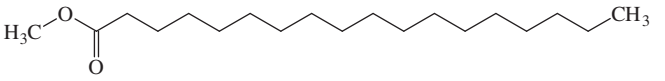
ISSN 1478–6419 print/ISSN 1029–2349 online

DOI: 10.1080/14786419.2010.482937
http://www.informaworld.com



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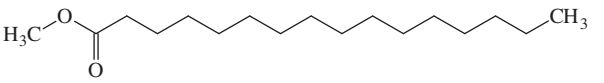

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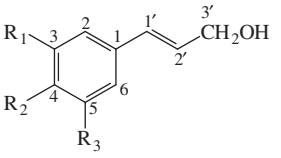

Compounds	R_1	R_2	R_3
3	OCH_3	OH	OCH_3
4	OCH_3	OH	H
5	H	OH	H
6	OCH_3	O-glucose	H

Figure 1. The chemical structures of the isolated compounds.

In this article, we describe the isolation and structural characterisations of six compounds (**1**–**6**) that have been isolated for the first time from the titled plant.

2. Results and discussion

The acetone extract of *W. prostrata* callus cultures afforded two methyl esters of fatty acids (**1** and **2**) and four cinnamyl alcohol derivatives (**3**–**6**) (Figure 1). The retention time (R_t), MS fragmentation pattern and rate of flow (R_f) for compounds **1–5** were identical to those of the relevant standards. They were identified as octadecanoic acid methyl ester (methyl stearate) and hexadecanoic acid methyl ester (methyl palmitate), sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol, respectively.

The ESI mass spectrum of compound **6** showed a molecular ion peak at m/z 341 $[M-1]^-$, consistent with the molecular formula $C_{16}H_{22}O_8$. Its UV spectrum showed absorptions at λ_{max} 228 and 280 nm, indicating its phenolic nature. The ^{1}H-NMR spectrum exhibited one methoxyl group at δ 3.78 (3H, s), methylene protons at δ 4.09 (2H, br t, $J = 5.0$ Hz), and *trans*-olefinic protons at δ 6.25 (1H, dt, $J = 15.7$, 5.0 Hz) and 6.50 (1H, d, $J = 15.7$ Hz). In addition, it displayed three aromatic protons at δ 6.89 (1H, dd, $J = 2.2$, 8.0 Hz), 7.03 (1H, d, $J = 8.0$ Hz) and 7.05 (1H, d, $J = 2.2$ Hz). A characteristic doublet of an anomeric proton at δ 5.43 ($J = 7.3$ Hz) indicated the presence of a β-D-glucopyranosyl moiety (Agrawal, 1992; Falshaw, Ormand, Mongkolsuk, & Podimuang, 1969). The spectral data of compound **6** were in good agreement with those reported for coniferin (Sugiyama, Nagayama, & Kikuchi, 1993). Furthermore, acid hydrolysis of compound **6** gave glucose and coniferyl alcohol, which was detected by TLC comparison with an authentic sample.

Coniferyl alcohol, which is the aglycone of coniferin, is known to be a biosynthetic precursor of important biologically active compounds (Dewick, 2002). Two coniferyl alcohol moieties dimerise in early steps in lignan (Broomhead & Dewick, 1990; Molog et al., 2001; Seidel et al., 2002; Umezawa, Davin, & Lewis, 1991), e.g. podophyllotoxin. Flavonolignans, e.g. silymarin, arise from a combination of a flavonoid and a phenylpropanoid, usually coniferyl alcohol (Dewick, 2002; Wenzig et al., 2005). Coumarinolignans, e.g. silybin, are a combination of a coumarin with a cinnamyl alcohol, usually coniferyl alcohol (Dewick, 2002). Also, coniferyl alcohol is a precursor to a number of flavour components of spices, e.g. eugenol and isoeugenol (Wang, Dudareva, Bhakta, Raguso, & Pichersky, 1997).

The isolated phenylpropanoids (**3**–**6**) are pharmacologically active compounds. (*E*)-*p*-coumaryl alcohol has shown antioxidative activity (Ly, Shimoyamada, Kato, & Yamauchi, 2003). Sinapyl alcohol exhibited *in vitro* anti-inflammatory and antinociceptive effects; in addition, some sinapyl alcohol derivatives were found to be cytotoxic agents (Choi et al., 2004; Zhao et al., 2002). Coniferin was shown to have *in vitro* anti-inflammatory activity (Diaz Lanza et al., 2001).

The previous phytochemical studies of *W. prostrata* resulted in the identification of two phenylpropanoids, 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid, from the aerial parts and roots (Farag et al., 1997, 1998; Farag, 2001).

In this article, we report the occurrence of four cinnamyl alcohol derivatives: sinapyl alcohol, coniferyl alcohol, *p*-coumaryl alcohol and coniferin.

It has been suggested that phenolic compounds have been formed in the callus cultures as a response to vitamins and hormones present in the MS medium and as a response to external stress in the entire plant (Hart, 1981).

L-Phenylalanine and L-tyrosine, as C_6C_3 building blocks, are precursors for a wide range of natural products. In plants, a frequent first step is the elimination of ammonia from the side-chain by specific lyases to generate the appropriate *trans* (*E*) cinnamic acid. In the case of phenylalanine, this would give cinnamic acid, whilst tyrosine could yield 4-coumaric (*p*-coumaric) acid; other cinnamic acids are obtained by further hydroxylation and methylation reactions, sequentially building up substitution patterns typical of shikimate pathway metabolites, i.e. an *ortho* oxygenation pattern. Some of the more common natural cinnamic acids are 4-coumaric, caffeic, ferulic and sinapic acids. These can be found in plants in free form and in a range of esterified forms, e.g. with quinic acid, as in chlorogenic acid (5-*O*-caffeoylquinic acid, 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid) (Dewick, 2002).

3. Experimental

3.1. *General procedures*

Gas chromatographic–mass spectrometric (GC-MS) analyses were carried out with a Finnigan TSQ 700 triple-stage quadrupole mass spectrometer (Thermo Quest, Bremen, Germany) coupled to a Hewlett Packard 5890A gas chromatograph (Hewlett-Packard, Waldbronn, Germany). The GC was equipped with a 3 m fused silica guard column (deactivated, Phenomenex, 0.32 mm ID) and a 30 m analytical column (ZB 1, Phenomenex, 0.32 mm ID, 0.25 μm film thickness).

The ^{1}H-NMR spectrum was measured on a Bruker AM-400 MHz instrument (Bruker, Germany) with tetramethylsilane (TMS) as an internal standard. UV spectra were recorded on an Ultrospec 1000, UV-vis spectrophotometer (Pharmacia, Biotech., England). The negative-ion electrospray ionisation (ESI) mass spectrum was performed on a MAT 95 XL Trap hybrid tandem mass spectrometer (ThermoFinnigan MAT, Bremen, Germany). Column chromatographic separation was performed with silica gel (60–200 mesh, Merck).

Preparative thin layer chromatography (TLC) was conducted on silica gel layers using silica gel 60 F_{254} (Merck). TLC analyses were carried out on a pre-coated silica gel 60 F_{254} aluminium sheets (Merck).

The following solvent systems were used for TLC:

(1) *n*-Hexane–ethyl acetate (85 : 15).
(2) Chloroform–methanol (70 : 30).
(3) *n*-Butanol–acetone–formic acid–water (60 : 17 : 8 : 15).

Compounds were detected by spraying with 10% H_2SO_4 followed by heating at 110°C for 2 min.

Melting points were determined by Electrothermal 9100 digital melting point instrument (Electrothermal Engineering Ltd, UK) and are uncorrected.

3.2. *Chemicals*

All the media components were purchased from Merck (Darmstadt, Germany). The authentic compounds methyl stearate, methyl palmitate, sinapyl alcohol, coniferyl alcohol and *p*-coumaryl alcohol were provided by Professor Dr L. Beerhues, Institute for Pharmaceutical Biology, Braunschweig University of Technology, Germany.

3.3. *Plant material*

The fresh young shoots of *W. prostrata* Hook. et Arn. (Hemsl.) were obtained from the plants cultivated in the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Assiut University, Assiut, Egypt. The identification of the plant was confirmed by Professor Dr Abdel-Aziz Fayed (Professor of Plant Taxonomy, Faculty of Science, Assiut University, Assiut, Egypt). A voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.4. *Callus induction*

Callus cultures were initiated from the fresh *W. prostrata* young shoots cultivated in Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 4.5 μM of 1-naphthaleneacetic acid, 4.5 μM of 6-benzylaminopurine and 1 g of casamino acid. The calli were maintained in the dark at a temperature of 25 ± 2°C and subcultured to a new MS medium (50 mL) every four weeks.

3.5. *Extraction and isolation of the constituents*

About 100 g of fresh *W. prostrata* cultured cells were ground in 700 mL acetone and the homogenate was filtered. The residue was extracted twice with 500 mL acetone. The acetone phases were combined and evaporated under reduced pressure to dryness (Beerhues & Berger, 1995). The solvent-free residue (10 g) was partitioned between distilled water and *n*-hexane (3×100 mL), EtOAc (3×100 mL) and *n*-BuOH (3×100 mL) to give the corresponding solubles (2.0 g), (4.0 g), (1.5 g) and aqueous (2.5 g), respectively. The obtained soluble fractions were separately concentrated under reduced pressure and screened for different constituents by TLC.

3.5.1. *n-Hexane fraction*

About 2.0 g of the *n*-hexane soluble fraction was chromatographed on silica gel column (60 g, 30×2 cm) using *n*-hexane–ethyl acetate step gradients. Fractions of 30 mL were collected, concentrated under reduced pressure and monitored by TLC using solvent system I. Similar fractions were combined. The fractions eluted with *n*-hexane–ethyl acetate (90 : 10) were concentrated to give an oily residue (50 mg). The oily residue contained a crude mixture of compounds **1** and **2**. This was subjected to GC-MS analysis.

3.5.2. *Ethyl acetate fraction*

About 4.0 g of the ethyl acetate soluble fraction was subjected to column chromatography on silica gel (120 g, 75×3 cm) with chloroform containing gradually increasing amounts of methanol. Fractions of 30 mL were collected, concentrated under reduced pressure and monitored by TLC using solvent system II. Similar fractions were grouped together to obtain five main groups, A (800 mg, eluted with chloroform), B (500 mg, eluted with chloroform–methanol 90 : 10), C (1 g, eluted with chloroform–methanol 85 : 15), D (700 mg, eluted with chloroform−methanol 80 : 20) and E (900 mg, eluted with chloroform–methanol 70 : 30). Both groups B and C were purified separately by preparative TLC on silica gel layers with solvent system II to obtain pure compounds **3** (15 mg) and **4** (50 mg), respectively. Group D contained a crude mixture of compounds **5** and **6**. It was subjected to preparative TLC on silica gel layers using solvent system II to afford pure compounds **5** (15 mg) and **6** (20 mg).

3.6. *GC-MS analysis*

The column oven temperature was rapidly raised at the rate of 40°C min^{-1} to 190°C and held at this temperature for 2 min. The temperature was then programmed to increase linearly at the rate of 5°C min^{-1} to 320°C, where it was maintained for 5 min. The temperature for the injector was 280°C. The injected volume was 1 μL. The carrier gas used was helium with a split ratio of 1 : 20 and 1.6 mL min^{-1} constant flow. Mass spectra were obtained in electron impact (EI) mode with an electron energy of 70 eV. Ion source and transfer-line temperatures were 180°C and 280°C, respectively. The mass spectrometer was equipped with a digital alpha station computer data system. The identification of compounds **1**–**5** was based on sample

retention time data, comparison with the relevant standards and electron impact–mass spectrometry (EI-MS) data.

3.7. *Acid hydrolysis*

Compound **6** (5 mg) was dissolved in 5 mL methanol to which an equal volume of N/2 methanolic sulphuric acid was added. The mixture was refluxed on a boiling water bath for 3 h and cooled; the aglycone was extracted with chloroform, purified and subjected to TLC (using solvent system II). The resultant sugar was identified by TLC using solvent system III.

3.8. *Spectral data of compounds*

Octadecanoic acid methyl ester (methyl stearate) (**1**): $R_f = 0.75$ (solvent system I); $R_t = 18.75$ min; EI-MS (70 eV) m/z (relative intensity): 298 $[M]^+$ for $C_{19}H_{38}O_2$ (45), 267 (10), 255 (15), 241 (4), 227 (14), 213 (6), 199 (13), 185 (15), 171 (5), 157 (13), 143 (44), 129 (18), 115 (15), 97 (35), 87 (95), 74 (100), 69 (52), 55 (90), 43 (90).

Hexadecanoic acid methyl ester (methyl palmitate) (**2**): $R_f = 0.60$ (solvent system I); $R_t = 18.32$ min; EI-MS at (70 eV) m/z (relative intensity): 270 $[M]^+$ for $C_{17}H_{34}O_2$ (40), 239 (10), 227 (14), 213 (2), 199 (4), 185 (5), 171 (5), 157 (2), 143 (17), 129 (8), 115 (2), 97 (5), 87 (73), 83 (6), 74 (100), 69 (12), 55 (15), 43 (15).

Sinapyl alcohol (**3**): colourless gum; $R_f = 0.60$ (solvent system II); $R_t = 18.96$ min; UV λ_{max} (MeOH) nm: 222, 273; EI-MS (70 eV) m/z (relative intensity): 210 $[M]^+$ for $C_{11}H_{14}O_4$ (100), 192 (6), 182 (28), 167 (68), 154 (24), 133 (12), 121 (14), 103 (11), 91 (9), 77 (14), 65 (6), 55 (8), 39 (7).

Coniferyl alcohol (**4**): colourless gum; $R_f = 0.58$ (solvent system II); $R_t = 16.38$ min; UV λ_{max} (MeOH) nm: 229, 280; EI-MS (70 eV) m/z (relative intensity): 180 $[M]^+$ for $C_{10}H_{12}O_3$ (82), 162 (4), 147 (9), 137 (100), 131 (13), 124 (47), 119 (22), 103 (13), 91 (26), 77 (13), 65 (10), 55 (10), 39 (4).

p-Coumaryl alcohol (**5**): colourless gum; $R_f = 0.41$ (solvent system II); $R_t = 18.69$ min; UV λ_{max} (MeOH) nm: 226, 273; EI-MS (70 eV) m/z (relative intensity): 150 $[M]^+$ for $C_9H_{10}O_2$ (100), 132 (10), 121 (15), 103 (9), 91 (6), 77 (10), 65 (5), 55 (6), 39 (4).

Coniferyl alcohol 4-*O*-glucoside (coniferin) (**6**): colourless needles; $R_f = 0.33$ (solvent system II); m.p. 186–188°C; UV λ_{max} (MeOH) nm: 228, 280; ESI–MS at m/z: 341 $[M-1]^-$ for $C_{16}H_{22}O_8$; ^{1}H-NMR spectrum (400 MHz, DMSO-d_6): δ 3.1–3.3 (4H, m, H-2″-5″), 3.52 (1H, dd, $J = 11.7$, 5.5 Hz, H-6″b), 3.72 (1H, dd, $J = 11.7$, 2.0 Hz, H-6″a), 3.78 (3H, s, OMe), 4.09 (2H, br t, $J = 5.0$ Hz, H-3′), 5.43 (1H, d, $J = 7.3$ Hz, H-1″), 6.25 (1H, dt, $J = 15.7$, 5.0 Hz, H-2′), 6.50 (1H, d, $J = 15.7$ Hz, H-1′), 6.89 (1H, dd, $J = 8.0$, 2.2 Hz, H-6), 7.03 (1H, d, $J = 8.0$ Hz, H-5), 7.05 (1H, d, $J = 2.2$ Hz, H-2).

Acknowledgements

The authors are grateful to Prof. Dr L. Beerhues (Institute for Pharmaceutical Biology, Braunschweig University of Technology, Germany) for providing the authentic samples,

and Prof. Dr L. Ernst and Dr U. Papke (Institute for Organic Chemistry, Braunschweig University of Technology, Germany) for NMR and ESI-MS measurements, respectively.

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