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Accumulation of phthalides in elicitor-treated cell suspension cultures of *Petroselinum crispum*

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Abstract

The present study describes the effect of a *Phytophthora sojae* 25-amino acid oligopeptide (Pep25) elicitor on the secondary metabolism of parsley cell cultures (*Petroselinum crispum* L.). HPLC analysis of the accumulated compounds in the elicitor-treated cultures revealed the expected accumulation of furanocoumarins (e.g. marmesin and bergapten) as well as various non-coumarin compounds which have not been described previously to occur in this cell culture. These compounds were isolated by preparative HPLC and identified by spectroscopic methods (MS, NMR) as 5-hydroxy- and 7-hydroxy-3-butylidenephthalides including two novel conjugates of the 7-hydroxy derivative, i.e. 7-*O*-glucoside and 7-*O*-(6′-malonylglucoside). © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Petroselinum crispum*; Apiaceae; Cell culture; Elicitation; Butylidenephthalides

1. Introduction

Secondary metabolism plays an important role in plant defense responses upon infection by microbial pathogens. Secondary products may be involved in signal transduction (e.g. salicylic acid), formation of physical barriers (e.g. lignin) or in mechanisms which might directly adversely affect pathogens (e.g. phytoalexins) (Kombrink & Somssich, 1995).

Parsley plants and cell cultures are well established systems for studying non-host plant/pathogen interactions, particularly with regard to the stimulation of phenylpropanoid metabolism (Hahlbrock & Scheel, 1989) leading to furanocoumarins as the major soluble constituents considered to be potent phytoalexins (Scheel, Hauffe, Jahnen, & Hahlbrock, 1986; Matern, 1991). In detailed studies of the molecular mechanisms underlying elicitor recognition and signal transduction in the parsley/*Phytophthora* system, a fungal 42 kDa surface glycoprotein was isolated whose elicitor activity resides in the protein moiety (Parker, Schulte, Hahlbrock, & Scheel, 1991). It was found that a 13-amino acid peptide fragment (Pep13) of this glycoprotein as well as a larger fragment exceeding Pep13 by 7 (*N*-terminus) and 5 amino acids (*C*-terminus), respectively, (Pep25) were shown to act as potent elicitors in parsley (Nürnberger et al., 1994). The response of parsley cells to either peptide mimics the defense response of intact plants to the fungus (Hahlbrock et al., 1995).

We now extended these earlier studies on the Pep25-stimulated accumulation of secondary compounds in parsley cell cultures and have identified, besides two of the previously reported furanocoumarins, marmesin and bergapten, various non-coumarin compounds. These were isolated by preparative HPLC and identified by spectroscopic methods (MS, NMR) as butylidenephthalide derivatives.

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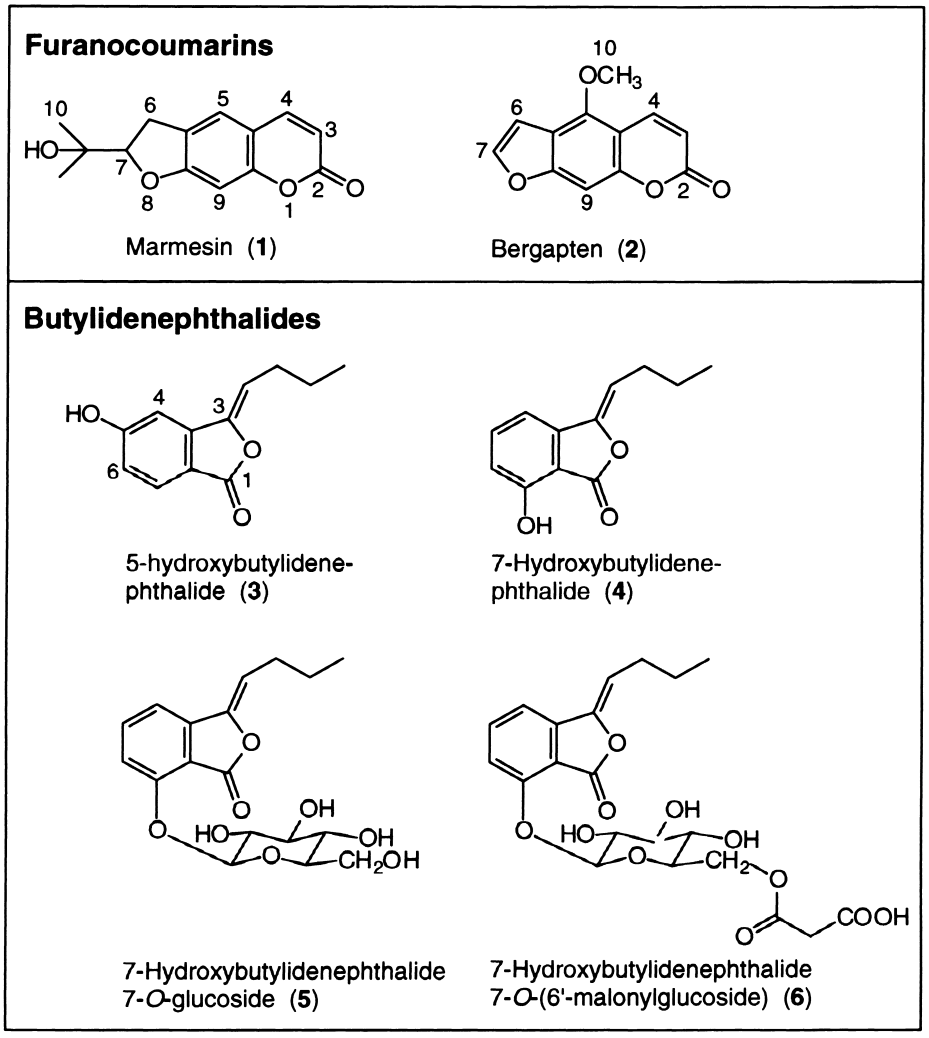
2. Results and discussion

2.1. Identification of compounds

Methanol extracts from elicitor-treated parsley cells were analyzed first by HPLC and then by a combination of MS and NMR. Among the compounds identified were the two reference furanocoumarins marmesin (**1**) and bergapten (**2**) and the four structurally related phthalides 5-hydroxy-3-butylidenephthalide (**3**), 7-hydroxy-3-butylidenephthalide (**4**), 7-hydroxy-3-butylidenephthalide 7-*O*-glucoside (**5**) and 7-hydroxy-3-butylidenephthalide 7-*O*-(6′-malonylglucoside) (**6**) (Scheme 1).

An initial series of GC–MS analyses, combined with data bank searches, allowed a number of structure proposals to be made for compounds **1** and **2**. In the other cases no identity was found although fragmentation patterns and molecular ions were identified. The structures of the former two compounds were confirmed to be identical with those of marmesin (Murray, Sutcliffe, & McCabe, 1971) and bergapten (Harkar, Razdan, & Waight, 1984; Agrawal, Siddiqui, & Singh, 1989; Kuster, Bernardo, Da Silva, Parente, & Mors, 1994) from the ^{1}H NMR data. Thus **1** displayed a M^+ ion at *m/z* 244 and a base peak at *m/z* 187 in the EIMS. It showed characteristic spin systems in which the 1,4-orientation of the aromatic protons were apparent. The assignment of these protons was clear from the long-range coupling between H-5 and H-6 in the 2D COSY spectrum. Similarly **2** (M^+, *m/z* 216) was readily identified as bergapten from the long-range coupling between H-6 and H-9.

The ^{1}H spectrum of compound **4** was relatively simple and showed the presence of three aromatic protons in a 1,2,3-disposition and a $CH_3CH_2CH_2CH$- chain in which the last proton is part of an olefinic system. From the molecular weight of 204 the compound must contain a number of quaternary carbon atoms. The amounts of material available precluded



Scheme 1.

the direct observation of ^{13}C spectra but a ^{1}H-detected long-range ^{13}C-^{1}H correlation afforded a considerable number of correlations (Table 1) that allowed a partial structure to be deduced and an assignment of the indirectly detected ^{13}C shifts. In particular two 3-bond couplings, which are larger than 2-bond couplings in such systems, were observed for each of the aromatic protons. From these data the relative positions of the aromatic carbons were unambiguously identified and the olefinic chain incorporating the quaternary carbon C-3 must be attached to C-3a which is in an *ortho* position to H-4. From the molecular mass there must be 3 oxygen atoms present one of which is part of a carbonyl substitutent whose carbon atom shows no correlations in the long-range correlation. This can only be positioned at C-7a and consequently the low field ^{13}C shifts of C-7 and C-3 were due to the presence of oxygen substituents. The carbonyl group must be part of a cyclic lactone system for which only the five membered ring system attached to the oxygen of C-3 is feasible.

The *Z* stereochemistry of the olefinic bond was determined from the observation of a NOE between H-4 upon irradiation of H-8 and vice-versa. This observation together with the observation of a correlation between H-8 and the aromatic carbon C-3a precludes alternative arrangements of the substituents. Thus **4** is 7-hydroxy-3-butylidenephthalide. This compound has been isolated by Kobayashi, Fujita, & Mitsuhashi (1984) and Pushan et al. (1984) who have reported ^{1}H NMR data, which is at variance with the data presented here. Although the shifts of the butylidene sidechain are similar, those of the aromatic ring are different. We are confident that the extra heteronuclear long-range correlations presented here unambiguously establish the correct proton assignments. In addition the ^{13}C shift of C-8 (109.5 ppm) is compatible with that reported for the *Z* isomer (Gijbels, Scheffer, & Baerheim Svendsen, 1980; Gijbels, Scheffer, & Baerheim Svendsen, 1982; Puech-Baronnat, Kaouadji, & Mariotte, 1984) of 3-butylidenephthalide (109.4 ppm) in contrast to the *E* isomer (113.9 ppm) (Gijbels et al., 1980; Kobayashi et al., 1984) and the calculated shifts for the introduction of a 7-hydroxyl substituent into the 3-butylidenephthalide system are similar to our experimentally observed data. The opening of the phthalide ring system can be excluded in our system from the MS data.

Table 1
Correlations observed in the ^{1}H-detected multiple-bond ^{13}C–^{1}H correlation of compound *4* in CD_3OD

Proton	Correlated carbons from high to low field[a]
H-4	C-7a, C6
H-5	C-5 (1-bond), C-3a, C-7
H-6	C-7a,C-4, C-6 (1-bond)
H-8	C-10, C-9, C-8 (1-bond), C-3a, C-3
H-9	C-11, C-10, C-9 (1-bond), C-8, C-3
H-10	C-11, C-10 (1-bond), C-9, C-8
H-11	C-11 (1-bond), C10, C-9

[a] Only the larger 3-bond long-range couplings were observed for the aromatic protons.

Compound **3** was related to **4** in having the same molecular weight but was different in showing a 1,2,4-trisubstituted aromatic ring system, together with the butylidene side chain, in the ^{1}H NMR spectrum. Clearly the hydroxyl group was now positioned at either C-5 or C-6. Comparison with the literature indicated that this compound was *Z*-5-hydroxy-3-butylidenephthalide (Puech-Baronnat et al., 1984).

Compound **5** showed a $[M+Na]^+$ ion at *m/z* 389 in the positive ES–MS. The loss of 162 mass units under collision induced dissociation leading to an ion at *m/z* 227 indicates the presence of a hexose moiety. The ^{1}H NMR spectrum was closely related to **4** in showing the same aromatic substitution pattern and butylidene side chain. Additional signals for a β-glucopyranosyl moiety were identified from the cross peaks in the ^{1}H COSY spectrum and the magnitude of the vicinial coupling constants. This can only be attached to the oxygen on C-7.

Compound **6** showed a similar set of signals to that of **5**. The only difference being the low field shift of H-6′A and H-6′B from 3.93 and 3.73 in **5** to 4.58 and 4.26 in **6** which was a clear indication of acylation at C-6′ of the sugar system. Although the acyl system could not be unambiguously identified from the NMR data the presence of a malonyl group was confirmed from the MS data, where the positive ion ESI-MS showed $[M+Na]^+$ at *m/z* 475 and collision induced fragment ions at *m/z* 431 ($[M+Na–CO_2]^+$, *m/z* 389 ([M-malonyl]$^+$) and *m/z* 227 (aglycone + Na]$^+$). This is supported by the negative ion ES–MS which showed ions at *m/z* 451 ($[M–H]^-$), 407 ($[M–H–CO_2]^-$) and 203 ([aglycone–H]$^-$, base peak). Such acyl substituents are often difficult to find in the NMR spectrum because of deuterium exchange of the respective methylene group. To the best of our knowledge, **5** and **6** are new natural compounds.

2.2. Elicitor-induced accumulation

Three of the identified phthalides were among the major compounds whose accumulation was induced by treatment with the Pep25 elicitor, thus expanding the spectrum of elicitor-induced phenolic compounds in parsley by a whole new class. Fig. 1 shows the accumulation patterns of the two furanocoumarins (**1**, **2**) and four phthalides (**4**–**6**) in elicited parsley cultures. These compounds could not be detected in non-elicited control cultures. Whereas **1** accumulated mainly in the

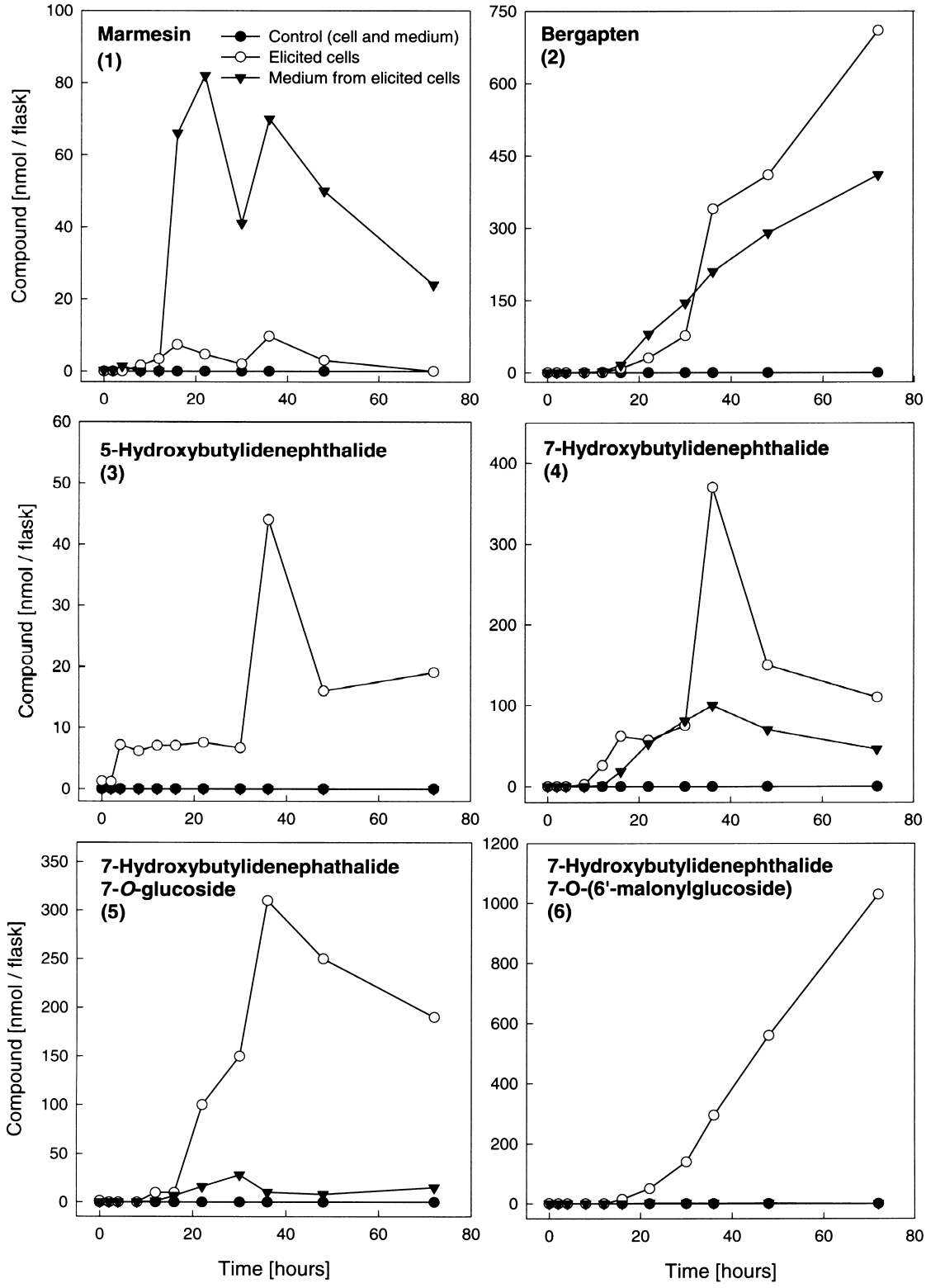


Fig. 1. Accumulation of furanocoumarins (**1**, **2**) and phthalides (**3**–**6**) in parsley cell cultures after Pep25 elicitation as indicated.

culture medium and **2** in both the medium and the cells, the phthalides were found almost exclusively in the cells. In contrast to phthalide **3** that was detected as a minor component, the phthalides **4**, **5** and **6** accumulated as major components and reflect a precursor-product relationship. The plausible pathway proceeds from the aglycone **4** to its glucoside **5** (glucosyl transfer) and the final product **6**, the malonylglucoside (malonyl transfer). The latter reached highest concentrations at about 1 μmol per flask 72 h after elicitation (Fig. 2).

Formation of phthalides along with furanocoumarins has also been found in other systems. Stanjek, Herhaus, Ritgen, Boland, & Städler (1997) described jasmonate-induced changes in the leaf surface chemistry of celery (*Apium graveolens* cv. *secalinum*), including biosynthesis of furanocoumarins and butylphthalides along with the typical constitutive celery fragrance, sedanolide, which represents an important aroma constituent of celery plants (MacLeod & Ames, 1989). With regard to their biological activity Meade, Hare, Midland, Millar, & Sims (1994) report about the phthalide-based host-plant resistance to certain insect larvae.

Phthalides are generally well known flavour components of apiaceous vegetables (MacLeod, MacLeod, & Subramanian, 1988). However, similar structures

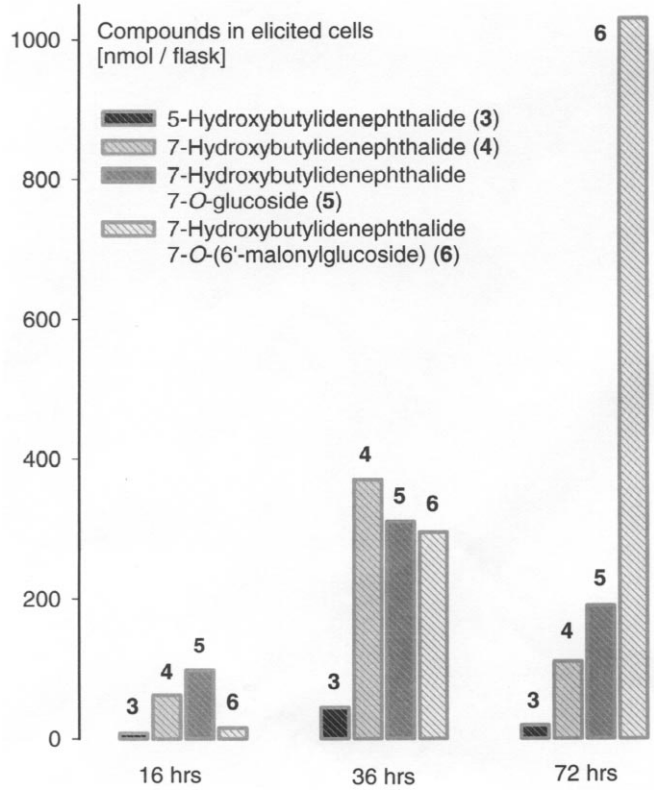


Fig. 2. Patterns of phthalides in parsley cells at 16, 36 and 72 h after Pep25 elicitation.

occur in other plant families. For example, glycosylated derivatives of propylphthalides have been isolated from the leaves of *Gentiana* species (Gentianaceae) (Mpondo Mpondo, Garcia, & Chulia, 1987; Garcia, Mpondo Mpondo, Chulia, Kaouadji, & Cartier, 1989).

2.3. Conclusions

The present results are in line with earlier observations indicating extensive reprogramming of both primary and secondary metabolism at the gene expression level in pathogen-infected or elicitor-treated parsley cells (Batz, Logemann, Reinold, & Hahlbrock, 1998; Somssich & Hahlbrock, 1998). Secondary metabolism was greatly stimulated by either treatment, far beyond the previously demonstrated accumulation of furanocoumarins. Our present data suggest that variously substituted butylidenephthalides constitute a second major class of soluble, defense-related phenolics in this system, possibly being products of the polyketide pathway (Mitsuhashi & Nomura, 1966). However, we do not exclude the possibility that their accumulation at infection sites in intact plants is confined to certain tissues or organs. This would be in agreement with preliminary experiments which failed to detect these compounds in fungus-infected parsley leaves (data not shown). In uninfected parsley plants, they have been described as characteristic root constituents (Nitz, Spraul, Drawert, & Spraul, 1992).

More detailed experiments will have to clarify the actual role of phthalides in pathogen defense and other stress-response or developmental programs. Whatever the outcome, we infer from all available data (e.g. Somssich & Hahlbrock, 1998) that the two most abundant classes of soluble phenolics, furanocoumarins and butylidenephthalides, are the most readily detectable ones and will be followed by numerous others, soluble and wall bound, whose functions may at least in part be synergistic (Strauss, 1998) and correspondingly difficult to fully establish.

3. Experimental

3.1. Plant material

Cell suspension cultures of parsley (*Petroselinum crispum* L.) were propagated for 6 days as described previously (Kombrink & Hahlbrock, 1986) and then treated with 1 μg/ml synthetic Pep25 oligopeptide elicitor (Nürnberger et al., 1994). Equivalent amounts of H_2O were added to the control cultures. Cells were harvested at the indicated times, washed twice with H_2O and frozen in liquid nitrogen. The corresponding

media (35 ml) were freeze dried. Cells and media were stored at −80°C.

3.2. Extraction of soluble compounds

All extraction steps were performed twice. Frozen cells were ground to a fine powder in a mortar and pestle, freeze dried and transferred into CH_2Cl_2 (1 g fr. wt per 10 ml), allowed to stand with continuous stirring for 15 min and centrifuged. The remaining pellets were extracted with 80% aq. MeOH. The supernatants were evaporated at 30°C in vacuum to dryness and redissolved in 250 μl CH_2Cl_2 or 80% aq. MeOH, respectively. The freeze-dried media were consecutively redissolved in these solvents. Aliquots (20 μl) were used for HPLC analysis.

3.3. Isolation of compounds

Elicited freeze-dried suspension culture (8 g dry wt.) was suspended in CH_2Cl_2 and treated with an Ultra Turrax homogenizer, allowed to stand for 3 h with continuous stirring and centrifuged. The pellet was extracted three times with MeOH. The CH_2Cl_2 extract was fractionated on a silica gel column (80 × 1.9 i.d.; Merck, Darmstadt) with a stepwise gradient (400 ml each) of *n*-hexane, *n*-hexane:EtOAc (from 9:1 to 1:9), EtOAc and finally MeOH. The MeOH extract was fractionated on a polyamide column (CC6, 30 × 4 cm i.d.; Macherey-Nagel, Düren) with a stepwise gradient of H_2O, 30% aq. MeOH, 60% aq. MeOH, MeOH, 0.1% NH_4OH in MeOH and finally 0.5% NH_4OH in MeOH.

3.4. HPLC

3.4.1. Analytical

HPLC analyses and data processing (Baseline 810 software, Millenium software) were performed with a Waters™ Millipore system (Eschborn, Germany; Multisolvent delivery system 600E, Controller 600, Autosampler 717^{plus}, photodiode array detector 996). The chromatograph was equipped with a 5 μm-Nucleosil C-18 column (250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany). The following solvents were used: solvent A = 1.5% aq. *ortho*-phosphoric acid; solvent B = 80% aq. CH_3CN. Compounds were separated at a flow rate of 1 ml min^{-1} with a linear gradient elution system within 20 min from solvent A to 50% solvent B in (A + B) and within another 10 min to 100% solvent B. Quantifications of the furanocoumarins and phthalides (maxplot detection, 220–400 nm) were achieved by external standardization with bergapten and 4-hydroxybenzoate, respectively.

3.4.2. Preparative

The combined extracts were evaporated at 40°C in vacuum to dryness, the residue redissolved in 8 ml of H_2O, centrifuged and the supernatant separated by preparative HPLC (Beckman Instruments, München, Germany, System Gold), equipped with a Nucleosil 100-10 C_{18} column (VarioPrep; 10 μm, 250 × 40 mm i.d.; Macherey-Nagel, Düren, Germany). The compounds were separated at a flow rate of 10 ml min^{-1} with a linear gradient within 60 min from 50% solvent B (MeOH) to 80% solvent B in solvent A (0.4% formic acid in H_2O) and then within 30 min to 90% solvent B in A. Furanocoumarins were detected at 336 nm and the phthalides at 256 nm.

3.5. NMR and MS

^1H and ^{13}C NMR spectra were recorded at 300 K on Bruker DPX300, ARX400 or DMX600 NMR spectrometers locked to the major deuterium resonance of the solvent, CD_3OD. 1D (^1H and NOE difference) and 2D (homonuclear COSY and heteronuclear ^1H-detected multiple-bond ^{13}C–^1H correlations) data were recorded using standard Bruker software. Chemical shifts are reported in ppm relative to TMS and coupling constants in Hz. The 70 eV electron impact (EI) mass spectra were obtained from a AMD-402 double focusing mass spectrometer. The electrospray (ES) mass spectra and the collision induced dissociation (CID) mass spectra (collision energy 25 eV, collision gas N_2, collision pressure 2 mTorr) were recorded on a Finnigan TSQ 7000 instruments.

3.5.1. Compound **1** (marmesin)

^1H (CD_3OD) $\delta = 7.87$ (d, H-4, J(4-3) 9.5), 7.44 (s, H-5), 6.76 (s, H-9), 6.23 (d, H-3), 4.79 (t, H-7, J(7-6) 8.6), 3.29 (d, H-6), 1.33, 1.27 (s x2, H-10). COSY shows a long-range coupling between H-5 and H-6. EIMS (m/z, rel. int.): 246 (M^+, 38), 228 (4), 213 (15), 188 (76), 187 (100), 175 (18), 160 (24), 131 (14), 59 (26).

3.5.2. Compound **2** (bergapten)

^1H (CD_3OD) $\delta = 8.32$ (d, H-4, J(4-3) 9.7), 7.82 (d, H-7, J(7-6) 2.5), 7.30 (dd, H-6, J(6-9) 0.9), 7.21 (d, H-9), 6.32 (d, H-3), 4.36 (s, H-10). The long-range coupling between H-6 and H-9 was confirmed in the COSY spectrum. EIMS (m/z, rel. int.): 216 (M^+, 100), 201 (33), 188 (13), 173 (25), 145 (12), 89 (7).

3.5.3. Compound **3** (5-hydroxy-3-butylidenephthalide)

^1H (CD_3OD) $\delta = 7.72$ (d, H-7, J(7-6) 8.4), 7.11 (d, H-4, J(4-6) 2.0), 7.01 (dd, H-6), 5.74 (t, H-8, J(8-9) 7.9), 2.45 (dt, H-9, J(9-10) 7.5), 1.61 (hextet, H-10, J(10-11) 7.4), 1.04 (t, H-11). EIMS (m/z, rel. int.): 204 (M^+, 38), 175 (100), 162 (45), 147 (22), 119 (8), 91 (9).

3.5.4. Compound **4** (7-hydroxy-3-butylidenephthalide)

^1H (CD_3OD) $\delta = 7.58$ (dd, H-5, J(4-5) 7.5, J(5-6)

8.2), 7.26 (d, H-4), 6.91 (d, H-6), 5.79 (t, H-8, *J*(8-9) 7.8), 2.34 (dt, H-9, *J*(9-10) 7.5), 1.61 (hextet, H-10, *J*(10-11) 7.4), 1.04 (t, H-11). An NOE was observed between H-8 and H-4 upon irradiation of H-4 and vice-versa. ^{13}C from the ^{1}H-detected long-range ^{13}C–^{1}H correlation (CD_3OD) $d = 157.8$ (C-7), 146.9 (C-3), 142.4 (C-3a), 137.4 (C-5), 116.8 (C-6), 111.5 (C-4), 110.9 (C-7a), 109.5 (C-8), 28.5 (C-9), 23.8 (C-10), 13.8 (C-11). Only C-1 could not be detected in this correlation as it possessed no appropriate couplings. EIMS (*m*/*z*, rel. int.): 204 (M^+, 31), 175 (100), 162 (37), 147 (18), 119 (6), 91 (5).

*3.5.5. Compound **5** (7-hydroxy-3-butylidenephthalide 7-O-glucoside)*

^{1}H (CD_3OD) $\delta = 7.74$ (dd, H-5, *J*(4-5) 7.6, *J*(5-6) 8.2), 7.49 (d, H-4), 7.29 (d, H-6), 5.89 (t, H-8, *J*(8-9) 7.8), 5.18 (d, H-1′, *J*(1′–2′) 7.7), 3.93 (dd, H-6′A, *J*(6′A–6′B) 12.1, *J*(6′A–5′) 2.0), 3.73 (dd, H-6′B, *J*(6′B–5′) 5.7), 3.64 (dd, H-2′, *J*(2′–3′) 9.2), 3.55 (dd, H-3′, *J*(3′–4′) 9.2), 3.55 (ddd, H-5′), 3.47 (dd, H-4′, *J*(4′–5′) ~9), 2.47 (dt, H-9, *J*(9–10) 7.4), 1.62 (hextet, H-10, *J*(10–11) 7.3), 1.05 (t, H-11). Positive ES–MS: *m*/*z* 389 ($[M+Na]^+$); positive CID–MS: *m*/*z* 227 (100), 185 (43); EIMS (*m*/*z*, rel. int.): 204 (M^+, 49), 175 (100), 162 (46), 147 (20), 119 (6), 73 (6), 60 (11).

*3.5.6. Compound **6** [7-hydroxy-3-butylidenephthalide 7-O-(6′-malonylglucoside)]*

^{1}H (CD_3OD) $\delta = 7.79$ (dd, H-5, *J*(4–5) 7.7, *J*(5–6) 8.2), 7.49 (d, H-4), 7.26 (d, H-6), 5.89 (t, H-8, *J*(8–9) 8.0), 5.17 (d, H-1′, *J*(1′–2′) 7.6), 4.58 (dd, H-6′A, *J*(6′A–6′B) 11.9, *J*(6′A–5′) 2.0), 4.26 (dd, H-6′B, *J*(6′B–5′) 6.8), 3.79 (ddd, H-5′, *J*(5′–4′) ~9), 3.65 (dd, H-2′, *J*(2′–3′) 9.3), 3.55 (dd, H-3′, *J*(3′–4′) 9.0), 3.45 (dd, H-4′), 2.47 (dt, H-9, *J*(9–10) 7.5), 1.62 (hextet, H-10, *J* (10–11) 7.3), 1.05 (t, H-11). The signal of the methylene group of the malonyl group could not be unambiguously identified but may be the singlet at 3.75 ppm. EIMS (*m*/*z*, rel. int.): 204 (aglycone moiety, 63), 187 (7), 175 ([204-CH_2CH_3], 100), 162 ([204-CH_2=CH–CH_3], 45), 147 (8), 119 (5), 91 (8); positive ion ES–MS: *m*/*z* 475 ($[M+Na]^+$, 100); positive ion CID–ES–MS of *m*/*z* 475: 431 ($[M+Na–CO_2]^+$, 100), 415 (45), 389 ($[M+Na–CO=CH–CO_2H]^+$, 20), 271 ([389-H_2O], 34), 227 ([aglycone + Na], 55); negative ion ES–MS: *m*/*z* 527 (10), 451 ($[M–H]^-$, 6), 407 ([M–H–CO_2]$^-$, 32), 245 (10), 203 ([aglycone–H]$^-$, 100).

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