PERGAMON

Phytochemistry 50 (1999) 1147–1152

PHYTOCHEMISTRY

Microbial conversion of jasmonates-hydroxylations by *Aspergillus niger*

Otto Miersch*, Andrea Porzel, Claus Wasternack

Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle/Saale, Germany

Received 15 September 1998; revised 13 October 1998

Abstract

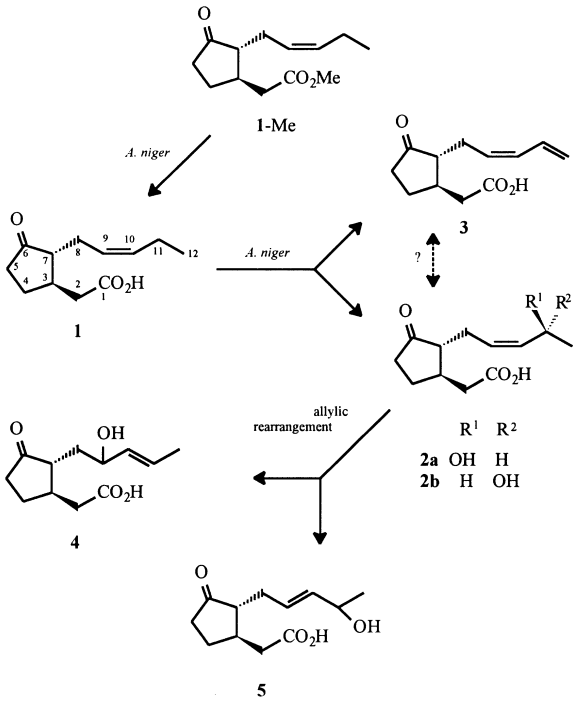
Aspergillus niger is able to hydroxylate the pentenyl side chain of (−)-jasmonic acid (JA) leading to (11*S*)-(−)-hydroxy-JA/(11*R*)-(−)-hydroxy-JA (2:1) and (−)-11,12-didehydro-JA. Methyl (−)-jasmonate (JA-Me) is converted upon hydrolysis. During prolonged cultivation or at non-optimized isolation procedures, the 11-hydroxy-(9*Z*)-pentenyl side chain may isomerize to (10*E*)-9-hydroxy- and (9*E*)-11-hydroxy-compounds by allylic rearrangement. The fungus hydroxylates (±)-9,10-dihydro-JA at position C-11 into 11ξ-hydroxy-9,10-dihydro-JA. As JA-Me, the methyl dihydro-JA is hydroxylated only upon hydrolysis into the free acid.

Keywords: *Aspergillus niger*; Euascomycetidae; fungal hydroxylation; (−)-jasmonic acid; methyl (−)-jasmonate; (11*S*)-(−)-hydroxyjasmonic acid; (11*R*)-(−)-hydroxyjasmonic acid; (−)-11,12-didehydrojasmonic acid; (10*E*)-9-hydroxyjasmonic acid; (9*E*)-11-hydroxyjasmonic acid; (±)-9,10-dihydrojasmonic acid; (±)-11ξ-hydroxy-9,10-dihydrojasmonic acid

1. Introduction

Jasmonates represent a group of plant growth regulators which are involved in various developmentally and environmentally regulated processes such as wounding, osmotic stress, salt stress, touch, elicitation or pathogen attack (Wasternack & Parthier, 1997; Creelman & Mullet, 1997). The free jasmonic acid (JA, **1**) and its methyl ester (JA-Me, **1**-Me) were found in numerous higher plants (Sembdner & Parthier, 1993; Creelman & Mullet, 1997). Beside these main components accumulating upon stress (Gundlach, Müller, Kutchan, & Zenk, 1992; Kramell et al., 1995), numerous metabolites formed by hydroxylation, *O*-glycosylation or conjugation were identified as minor components in higher plants and lower eukaryotes (Sembdner, Atzorn, & Schneider, 1994). Mainly fungi are known to form numerous metabolites within the same species. Recently, we could identify 22 jasmonates and related compounds in the culture filtrate of *Fusarium oxysporum* f sp *matthiolae*, a pathogenic fungus (Miersch, Bohlmann, & Wasternack, 1999).

Jasmonates which are hydroxylated in the pentenyl side chain were frequently detected in fungi (Miersch, Schneider, & Sembdner, 1991; Miersch, Günther, Fritsche, & Sembdner, 1993), and higher plants (Yoshihara et al., 1989; Helder, Miersch, Vreugdenhil, & Sembdner, 1993). They are major metabolites of JA (**1**) or 9,10-dihydro-JA (**9**) in *Hordeum vulgare* (Sembdner,



* Corresponding author. E-mail: omiersch@ipb.uni-halle.de.

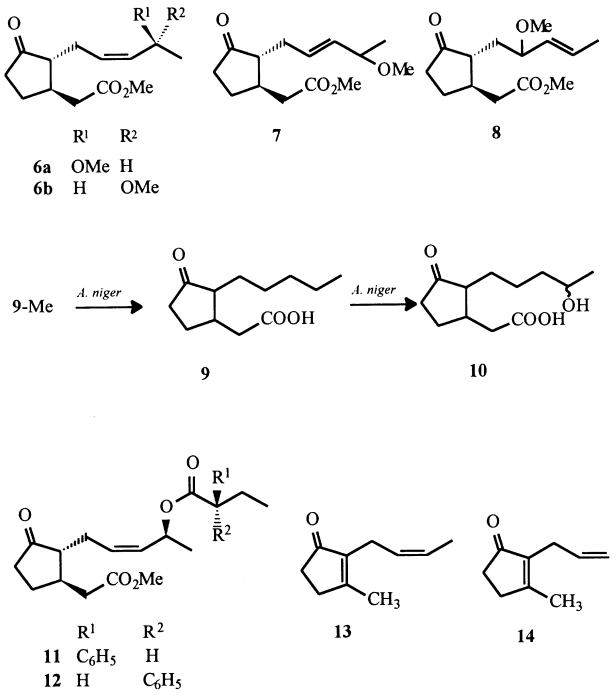
0031-9422/99/$ - see front matter
PII: S0031-9422(98)00698-0

Meyer, Miersch, & Brückner, 1988), in cell cultures of *Lycopersicon peruvianum*, or *Solanum tuberosum* (Kehlen, 1991) and *Eschscholtzia* (Xia & Zenk, 1993). So far, a biological activity is only reported for 12-hydroxy-**1** and its *O*-*β*-D-glucoside, both of them showing high tuber-inducing activity (Koda et al., 1991).

In a large-scale analysis of structure-activity-relationships of jasmonates in barley (Miersch, Kramell, Parthier, & Wasternack, 1999), we were interested to test the endogenously occurring (11*S*)-hydroxy-JA (**2a**). Isolation, however, from plant species containing hydroxylated jasmonates was inefficient due to the low content of these compounds. Partial chemical synthesis was an unsuitable approach since chemical hydroxylation of the JA molecule always led only to the hydroxylated jasmonates **4** and **5**.

Interestingly, the fungus *Aspergillus niger* carries high hydroxylating activities. *A. niger* was used to hydroxylate cinerone (**13**) or allethrone (**14**), compounds which contain jasmonate-like structures (Tabenkin, LeMahieu, Berger, & Kierstead, 1969; LeMahieu, Tabenkin, Berger, & Kierstead, 1970). To be in our knowledge, there is no information on the synthesis of compounds **2a** and **2b**. The fungus contains hydrolytic activities which hydrolyze methyl jasmonate (**1**-Me) into the free acid **1** (Dart, Kerry, & Marples, 1992). In an initial experiment we noted that *A. niger* incubated with **1** forms two metabolites.

Here we show metabolic transformations of (−)-JA (**1**), **1**-Me, (±)-9,10-dihydro-JA (**9**) and **9**-Me by a strain of *A. niger*, which is not able to form jasmonates itself.



2. Results and discussion

Aspergillus niger was precultured on malt agar and transferred to a liquid medium. After three days jasmonates **1**, **1**-Me, **9** or **9**-Me were added and kept for 36 h. Metabolites extracted from the culture filtrate and purified were identified by GC–MS, ^{1}H NMR and ^{13}C NMR using authentic compounds for comparison. Initial studies on fermentation of **1**-Me revealed the formation of JA (**1**), 11-(−)-hydroxy-JA (**2a**, **2b**) and (−)-11,12-didehydro-JA (**3**). Methylated products of **2a**, **2b** and **3** could not be found after 3 days of fermentation. Compounds **2a**, **2b** and **3** were always found together. This suggests hydrolysis of **1**-Me into the free acid (**1**) and a subsequent 11-hydroxylation and an 11,12-dehydration.

The MS spectra of the methyl esters (**2a**-Me/**2b**-Me) indicated a pentanone ring moiety, an intact acetate side chain, and a modified pentenyl side chain as described for *Botryodiplodia theobromae* (Miersch, Schneider, & Sembdner, 1991). The structure of the hydroxylated pentenyl side chain is clearly indicated by ^{1}H,^{1}H COSY correlation and by the ^{1}HNMR spectra including the coupling patterns and constants for selected hydrogens H-9, H-10, [(9*Z*)-double bond, $^{3}J_{\text{H-9/H-10}}$ 10.4 Hz] and H-11 and H-12 for the position of the hydroxyl groups in **2a** and **2b** (cf. Table 1).

Table 1
Selected ^{1}H NMR data of compounds **2a**, **2b**, **7** and **8** ($CDCl_3$, ^{1}H,^{1}H coupling constants (Hz) in parentheses)

Position	**2a**/**2b**	**7**	**8**
H-9	5.225 ddd (10.4/10.4/5.4)/ 5.385 ddd (10.4/10.4/5.4)	5.532 dt (15.3/7.4)/ 5.517 dt (15.2/7.1)	ca. 3.73 m/ca. 3.73 m
H-10	ca. 5.49 dd (10.4/10.4)/ ca. 5.49 dd (10.4/10.4)	5.412 ddt (15.3/7.6/1.2)/ 5.387 ddt (15.2/.6/1.2)	5.257 ddq (15.3/8.2/1.6)/ 5.195 ddq (15.3/8.4/1.7)
H-11	ca. 3.72 m/ca. 3.72 m	ca. 3.67 m/ca. 3.67 m	5.664 dqd (15.3/6.5/0.6)/ 5.656 dqd (15.3/6.5/0.6)
H-12	1.256 d(6.4)/1.246 d (6.4)	1.208 d (6.3)/1.201 d (6.3)	1.71 d (6.5)/1.71 d (6.5)
COOMe	–	3.240 s/3.228 s	3.199 s/3.185 s
OMe	–	3.700 s/3.700 s	3.706 s/3.701 s

The mixture of compounds **2a** and **2b** can be converted into **3** by heat treatment (Miersch et al., 1991). Using the method of Horeau–Brooks (Brooks & Gilbert, 1973; Horeau, 1961) we determined the configuration at C-11 of **2a** and **2b** to be predominantly in (11*S*). The same result could be obtained by GC analysis of the 2-phenylbutyryloxy-derivatives **11** and **12** (coproducts of the method). From the ^{1}H NMR spectra and together with the determination of the predominant configuration at C-11 we estimated the ratio between (11*S*)-hydroxy-JA (**2a**) and (11*R*)-hydroxy-JA (**2b**) to be 2:1.

Compound **3** carries the more stable diene system. It is probable that the fungus can form such a structure directly by action of a desaturase or *via* loss of H_2O from the hydroxylated jasmonates **2a** and **2b**.

(−)-11,12-didehydro-JA (**3**) was identified after methylation by GC–MS. Retention time and fragmentation pattern where identical to those of authentic **3** previously isolated from *Botryodiplodia theobromae* (Miersch et al., 1987) and synthetic (±)-**3**. A mass peak of *m*/*z* 222, a loss of the methyl acetate side chain (149 $[M–CH_2–COOCH_3]^+$), an additional loss of water (130 $[149\text{-}H_2O]^+$) and a base peak at *m*/*z* 79 were the characteristic mass fragments.

In the extract traces of two compounds, **4** and **5** occurred which were identified to be artefacts. This is indicated by the facts that the double bound is (*E*)-configurated and is shifted to position C-10. Both events can be explained only by allylic rearrangement. Compound **4** seems to be racemic with respect to the OH-group in C-11, due to properties of compound **8** which is derived from **4** (see below). The amounts of both compounds increased during a longer fermentation time, and both of them can be formed non-enzymatically by a rearrangement of the mixture **2a**/**2b** during treatment with hydrochloric acid. Methylation with diazomethane for more than 1 h leads to the stable *O*-methylated compounds **7** and **8**. The mass spectrum of **7** is identical to **6**.

Methylation of **2** by diazomethane for 1 h gave the more stable methoxylated products **6a**/**6b** and methyl esters of **2a** and **2b**. A mass fragment of *m*/*z* 98 indicates the presence of the *O*-methylated side chain $[CH_2CH\text{-}CHC(OMe)CH_3]^+$, whereas *m*/*z* 149 is indicative for the loss of CH_3OH and methyl acetate from **6a** and **6b**.

Beside the mass spectrum that showed a methoxylated side chain in compound **7**, the structure of the pentenyl-side chain was further specified by ^{1}H NMR, ^{13}C NMR and 2D COSY spectra verifying a (9*E*)-double bond ($^3H_{H\text{-}9/H\text{-}10}$ 15 Hz) and an 11-hydroxyl group (cf. Tables 1–2).

Compound **8** was identified by its mass spectrum and ^{1}H NMR. Typical mass fragments are *m*/*z* 254 M^+, *m*/*z* 181 (loss of the methyl acetate group), *m*/*z* 99 $[CH_2CH\text{-}(OMe)CHCHCH_3]^+$ (loss of the methoxylated pentenyl side chain) and the base peak *m*/*z* 85. ^{1}H NMR, ^{13}C NMR and 2D COSY spectra, gave clear evidence for a (10*E*)-double bond ($^3J_{H\text{-}10/H\text{-}11}$ 15 Hz) and a 9-methoxyl group (cf. Tables 1–2). For **7** as well as for **8** two sets of ^{1}H and ^{13}C NMR signals were found in a ratio of 1:1 indicating that both compounds **7** and **8** are racemic.

Table 2
^{13}C chemical shifts of compounds **7** and **8** ($CDCl_3$)

Pos.	**7**	**8**
1	172.4	172.8/172.6
2	38.5	38.6/38.5
3	37.6	39.0/38.4
4	27.1	27.4/27.2
5	37.7	37.5/37.4
6	218.4	219.3
7	53.8/53.7	50.9/50.8
8	30.5/30.4	34.4/33.9
9	128.7/128.5	79.8/79.6
10	135.0/134.9	131.4/131.2
11	77.7/77.6	129.9/129.2
12	21.4/21.3	17.6
COOMe	51.6	51.5
OMe	55.8	55.7/55.5

Preliminary experiments without kinetic analysis revealed, that *A. niger* can hydroxylate 9,10-dihydro-jasmonic acid (**9**) to 11-hydroxy-9,10-dihydro-jasmonic acid (**10**). Compound **10** is identical with a metabolite found after feeding of barley with **9** (Meyer et al., 1989), however, the configuration at C-11 remains unclear.

The mass spectrum of **10**-Me indicated the molecular mass at *m*/*z* 242, loss of water (*m*/*z* 224 $[M–H_2O]^+$) as well as of the methyl acetate moiety (*m*/*z* 151 $[M–CH_2CO_2Me]^+$) and the *m*/*z* 169 ($[M–CH_2CO_2Me]^+$). These fragments are characteristic for a hydroxylated pentyl side chain, whereas the loss of the hydroxypentyl side chain led to *m*/*z* 156, which is a typical fragment in the spectrum of **1**. As expected, elimination of both side chains led to the fragment *m*/*z* 83 $[C_5H_7O]^+$ indicating an intact cyclopentanone ring in the jasmonate molecule.

During the fermentation of 9,10-dihydro-JA (**9**), no compound could be found by GC–MS which was indicative for the introduction of a double bond or dehydratization of the hydroxyjasmonate **10**. Similar to the fermentation of methyl jasmonate (**1**-Me), growth of the fungus on methyl 9,10-dihydro-JA (**9**-Me) led first to the free acid **9** and the subsequent formation of **10**.

The data presented here suggest *A. niger* as a useful tool for the synthesis of 11-hydroxy-JA, even in a preparative scale (**2**). The fungus hydroxylates jasmonic acid (**1**), preferring the (*S*)-configuration. However, at present we can not exclude an altered *R*/*S* ratio during time course of fermentation. The (*S*)-configuration is formed by the fungus, since the (*Z*)-double bond is present and the (*E*)-double bond is undetectable Table 1. However, it can not be excluded that *A. niger* is able to epimerize enzymatically a hydroxy group. The allylic hydrogens seem

to be the energetically favored position for hydroxylation, and no hydroxylation of C-8 was found. Beside ring hydroxylation, cinerone (**13**), a structural related compound missing one carbon atom in the side chain, and possessing a methyl group instead of an acetic acid side chain (compared to **1**), is microbially transformed to a compound hydroxylated in the allylic position (Tabenkin et al., 1969), that corresponds to the hydroxylation of the pentenyl side chain of **1** in position C-11, shown here. Allethrone (**14**) lacking one carbon atom of the side chain as compared to cinerone (**13**) is hydroxylated in the allylic position (C-8 as compared to compound **1**) (LeMahieu et al., 1970). In *A. niger* the allylic position is favored through the more electrophilic C-11. Among higher plants barley leaves are able to metabolize 9,10-dihydro-JA (**9**) into 12- and 11-hydroxy-9,10-dihydro-JA (**10**) (Meyer et al., 1989). Similarly to the allylic hydroxylation of prostaglandines, the hydroxylating activity of *A. niger* seems to be specific for position C-11 of jasmonates. No other hydroxylated products could be found.

3. Experimental

3.1. Chemicals

(±)-9,10-dihydro-JA (**9**) was obtained by alkaline hydrolysis of (±)-methyl 9,10-dihydrojasmonate (**9**-Me) (Firmenich S.A., Switzerland). (−)-JA (**1**) was obtained by isomerization of (+)-7-*iso*-JA isolated from culture filtrates of *Botryodiplodia theobromae* Pat. (Miersch et al., 1987). (±)-11,12-Didehydro-JA (**3**) was synthesized by Wittig-reaction according to (Kitahara et al., 1984) using methyl 3-oxo-2-formyl-ethyl-cyclopentane-1-acetate and allylidene-triphenylphosphoran followed by alkaline hydrolysis of the methyl ester.

3.2. Plant material

Aspergillus niger v.T., strain i-400, was obtained from the Culture Collection of the Friedrich-Schiller-University Jena, Weimar.

3.3. Fermentation

The fungus were precultured on malt agar 2 weeks at 22°C. Two mycelium disks (8 mm diameter) of the preculture were homogenized with H_2O and used for inoculation of production medium (100 ml).

The fungi were grown in flasks (400 ml) containing 100 ml of Czapek-medium at 28°C on a rotary shaker. After 3 days, 20 mg of compounds **1**, **1**-Me, **9** or **9**-Me dissolved in 1 ml EtOH were added to each flask. Incubation was continued for 1.5 days and 5 days. Flasks with addition of 1 ml EtOH only were incubated as control.

3.4. Chromatographic methods

TLC (silica gel HF_{254}, precoated Merck) (a) $CHCl_3$–EtOAc–HOAc (5:4:1) R_f values: **1** 0.34, **1**-Me 0.66, **2a**/**2b** 0.11, **2a**-Me/**2b**-Me 0.29, **3** 0.33, **3**-Me 0.64, **4** 0.11, **4**-Me 0.28, **5** 0.10, **5**-Me 0.24, **6a**/**6b** 0.53, **7** 0.53, **8** 0,59, **9** 0.37, **9**-Me 0.71, **10** 0.12, (b) *n*-hexane–EtOAc–HOAc (60:40:1) R_f values: **1** 0.27, **1**-Me 0.56, **2a**/**2b** 0.04, **2b**-Me/**2b**-Me 0.12, **3** 0.22, **3**-Me 0.5, **4** 0.05, **4**-Me 0.18, **5** 0.03, **5**-Me 0.10, **6a**/**6b** 0.34, **7** 0.34, **8** 0.49, **9** 0.6, **9**-Me 0.14, **10** 0.05; detection by anisaldehyde reagent and heating for 5–10 min at 120°C (Stahl & Glatz, 1982).

HPLC: Eurospher 100 C18, (10 μm, 250 × 4 mm) elution with MeOH-0.1% H_3PO_4 in H_2O (c) (45:55), (d) (60:40), flow rate 1 ml min^{-1}, UV detector of 210 nm. R_t (min): (c) **2a**/**2b** 4.8, **2a**-Me/**2b**-Me 8.0, **10** 7.6; (d) **1** 10.2, **1**-Me 24.5, **2a**/**2b** 2.9, **2a**-Me/**2b**-Me 4.4, **3** 6.9, **3**-Me 15.5, **4** 3.1, **4**-Me 6.9, **5** 2.9, **5**-Me 4.4, **6a**/**6b** 9.2, **7** 9.2, **8** 14.2, **9** 16.5, **9**-Me 42.0, **10** 4.6.

3.5. Isolation procedure

The content of a flask was filtered and the aqueous phase extracted with EtOAc (3 × 20 ml), dried with Na_2SO_4 and evapd. The mycelium was homogenized and ultrasonified in 50 ml EtOAc, filtered and the organic phase elaborated as described before. Both extracts were separated with TLC (solvent system b). Positive layers were scrapped off, extracted with EtOAc and the solvent evaporated and extracts separated with TLC (solvent system a). Further purification was done with HPLC (solvent system c) for **2** and **10** and HPLC (solvent system d) for **3**, substances were recovered with $CHCl_3$ and prepared for structural identification to be:

(11*S*)-(−)-*Hydroxy-JA* [(1*R*,2*R*)-2(4*S*-hydroxy-2*Z*-pentenyl)cyclopentane-1-acetic acid] (**2a**)/(11*R*)-(−)-*hydroxy-JA* [(1*R*,2*R*)-2(4*R*-hydroxy-2*Z*-pentenyl)cyclopentane-1-acetic acid] (**2b**). 12.2 mg; CD (MeOH; c 0.02): $\Delta\varepsilon$ −1.82; ^{1}H NMR cf. Table 1; MS 70 eV, *m*/*z* (rel. int.) of **2a**-Me/**2b**-Me: 222 $[M–H_2O]^+$ (19), 207 (3), 193 (23), 167 $[M–CH_2CO_2Me]^+$ (15), 156 $[M–C_5H_8O]^+$ (6), 149 $[M–H_2O–CH_2CO_2Me]^+$ (30), 131 (20), 107 (24), 84 $[C_5H_8O]^+$ (43), 83 $[C_5H_7O]^+$ (100).

(−)-11,12-*Didehydro-JA* [(1*R*,2*R*)-3-oxo-2-(2*Z*,4-pentadienyl)cyclopentane-1-acetic acid] (**3**). 3 mg; ^{1}HNMR identical with (Miersch et al., 1987). MS 70 eV, *m*/*z* (rel. int.) of **3**-Me: 222 M^+ (7), 204 $[M–H_2O]^+$ (8), 193 (13), 167 (10), 155 (14), 149 $[M–CH_2CO_2Me]^+$ (87), 130 $[M–CH_3CO_2Me–H_2O]^+$ (94), 119 (27), 107 (78), 105 (64), 91 (80), 79 (100), 67 $[C_5H_7]^+$ (99).

(10E)-9-*Hydroxy-JA* [(1*R*,2*R*)-3-oxo-2-(2-hydroxy-3*E*-pentenyl)cyclopentane-1-acetic acid] (**4**). Traces; MS 70 eV, *m*/*z* (rel. int.) of **4**-Me: 222 $[M–H_2O]^+$ (88), 207 $[M–H_2O–CH_3]^+$ (14), 193 (81), 167 (81), 167 $[M–CH_2CO_2Me]^+$ (39), 156 $[M–C_5H_8O]^+$ (19), 149 $[M–CH_2CO_2Me–H_2O]^+$ (66), 131 $[M–CH_2CO_2Me–2H_2O]^+$

(34), 119 (19), 107 (37), 105 (34), 91 (44), 83 $(C_5H_7O]^+$ (100).

(9E)-11-*Hydroxy-JA* [(1*R*,2*R*)-3-oxo-2-(11-hydroxy-2*E*-pentenyl)cyclopentane-1-acetic acid] (**5**). Traces; MS 70 eV, *m*/*z* (rel. int.) of **5**-Me: identical with MS of compound **2**-Me. Longer methylation of compounds **2a/2b** gave:

Methyl (11S), (9Z)-*methoxyjasmonate* [methyl (1*R*, 2*R*)-3-oxo-2-(4*S*-methoxy-2*Z*-pentenyl)cyclopentane-1-acetate] (**6a**)/*methyl* (11R), (9Z)-*methoxyjasmonate* [methyl (1*R*,2*R*)-3-oxo-2-(4*R*-methoxy-2*Z*-pentenyl)-cyclopentane-1-acetate] (**6b**). **6a**/**6b** = 2:1; MS 70 eV, *m*/*z* (rel. int.) of **6a**/**6b**: 222 $[M–MeOH]^+$ (17), 207 $[M–MeOH–Me]^+$ (5), 193 (9), 167 (6), 149 $[M–CH_2CO_2Me–MeOH]^+$ (16), 133 (19), 131 $[M–CH_2CO_2Me–MeOH–H_2O]^+$ (13), 119 (7), 105 (16), 98 $[CH_2CHCHC(CH_3)–OMe]^+$ (100).

Rearrangement of **2a**, **2b**, **2a**-*Me or* **2b**-*Me*: 5 mg of compounds **5** or **6a**/**6b** was heated with 1 N HCl/1 ml) for 1 h at 30°C, compounds extracted with 0.5 ml $CHCl_3$, evaporated and methylated with ethereal diazomethane for 30 min giving a mixture of **4**-Me, **5**-Me, **7** and **8**. Separation using TLC (solvent mixture a) and HPLC (method d) gave:

Methyl (9E)-11-*methoxyjasmonate* [methyl (1*R*,2*R*)-3-oxo-2-(4-methoxy-2*E*-pentenyl)-cyclopentane-1-acetate (**7**). 2.4 mg; ^{1}H NMR cf. Table 1; ^{13}C NMR cf. Table 2; MS 70 eV, identical with compound **6a**/**6b**.

Methyl (10E)-*9-methoxyjasmonate* [methyl (1*R*,2*R*)-3-oxo-2-(2-methoxy-3*E*-pentenyl)cyclopentane-1-acetate] (**8**). 1.1 mg; ^{1}H NMR cf. Table 1; ^{13}C NMR cf. Table 1; MS 70 eV, *m*/*z* (rel. int.): 254 M^+ (56), 239 $[M–CH_3]^+$ (35), 223 $[M–OMe]^+$ (39), 207 $[M–CH_3–OMe]^+$ (23), 205 (21), 193 (52), 191 (35), 181 $[M–CH_2CO_2Me]^+$ (65), 99 $[CH_2CH(OMe)CHCHCH_3]^+$ (52), 85 $HC(OMe)–CHCHCH_3]^+$ (100).

(±)-(11ξ)-*Hydroxy*-9,10-*dihydrojasmonic acid* [3-oxo-2-(4-hydroxypentyl)cyclopentane-1-acetate] (**10**). 2.3 mg; ^{1}H NMR identical with (Meyer et al., 1989); MS 70 eV, *m*/*z* (rel. int.): 242 M^+ (1), 224 $[M–H_2O]^+$ (2), 169 $[M–CH_2CO_2Me]^+$ (9), 156 $[M–(CH_2)_3CH(OH)CH_3]^+$ (36), 151 $[M–CH_2CO_2Me–H_20]^+$ (25), 137 (7), 133 (7), 135 (11), 109 (15), 95 (13), 83 $[C_5H_7O]^+$ (100).

3.6. Horeau–Brooks method (Horeau, 1961; Brooks & Gilbert, 1973) for determining the configuration of secondary hydroxyl group

Mixture of compounds **2a**/**2b** (2.8 mg) was methylated with ethereal CH_2N_2 (5 min), dissolved in dry pyridine (12.5 µl) and treated with (±)-2-phenylbutyric anhydride (6.2 mg) in dry pyridine (12.5 µl) and heated in a sealed vial at 40°C for 2 h. Then (+)-(*R*)-α-phenylethylamine (6 µl) was added and mixed for 15 min. The sample was diluted with EtOAc (400 µl) and analyzed by GC–MS on a silicon-fused silica column DB-1 (15 m × 0.25 mm inner diameter, film thickness 0.25 µm), helium as carrier gas (0.8 ml × min^{-1}) with the temperature program from 60°C (1 min) to 110°C (25°C × min^{-1}); from 110 to 270°C (10°C × min^{-1}), injector temperature 250°C. The peak areas of (+)-(*R*)-phenylethylamides (in %) of (−)-(*R*)-2-phenylbutyric acid and (+)-(*S*)-2-phenylbutyric acid (R_t: 10.8 and 11.1 min, respectively) were estimated and substracted from the corresponding value of cyclohexanol giving an increment of +7.0. This implies predominately *S*-configuration at C-11 in the mixture **2a**/**2b**.

3.7. Combined GC–MS analysis

GC–MS was performed with an 70 eV mass spectrometer and a silicon-fused silica column DB-1 as described above. Samples were methylated with ethereal CH_2N_2 for 5 min at 20°C and 30 min for methyl ether formation; R_t (min): **1**-Me 7.7, **2a**-Me/**2b**-Me 9.55, **3**-Me 7.88, **4**-Me 9.15, **5**-Me 9.64, **6a**/**6b** 9.28, **7** 9.37, **8** 8.9, **9**-Me 7.8, **10**-Me 9.7.

3.8. GC–MS of methyl (*R*)-(+)-2-*phenylbutyryloxy*-(11*S*)-(−)-*jasmonate* (**11**) and methyl (*S*)-(−)-2-*phenylbutyryloxy*-(11*S*)-(−)-*jasmonate* (**12**) (*coproducts of* the *Horeau–Brooks method*)

Compounds were analyzed on a silicon-fused column DB-5MS (15 m × 0.32 mm inner diameter, film thickness 0.25 µm), conditions as described above; R_t (min): **1**-Me 6.65, **11** 15.45, and **12** 15.65. MS 70 eV, *m*/*z* (rel. int.) of **11** and **12**: 239 $[M–OCC(C_6H_5)C_2H_5–H]^+$ (18), 222 $[M–(C_6H_5)CH(C_2H_5)CO_2H]^+$ (88), 205 (9), 191 [222-MeOH–H]$^+$ (42), 163 (11), 149 $[222\text{-}CH_2CO_2Me]^+$ (42), 131 (40), 119 $[OCCH(C_6H_5)+H]^+$ (68), 91 $[CH_2(C_6H_5)]^+$ (100).

3.9. NMR analysis

^{1}H NMR and 2D COSY spectra were recorded on a BRUKER AM500 at 500.13 MHz and on a Varian UNITY500 spectrometer at 499.84 MHz, respectively. ^{13}C NMR and APT spectra were recorded on a BRUKER AC300 operating at 75.5 MHz. $CDCl_3$ was used as solvent. Chemical shifts were referenced to internal TMS ($\delta = 0$, ^{1}H) and $CDCl_3$ ($\delta = 77.0$, ^{13}C), respectively (cf. Tables 1–2).

Acknowledgements

We thank Mrs. A. Kuntzsch for technical assistance and Mrs. C. Kuhnt for GC–MS analyses.

References

Brooks, C. W. R., & Gilbert, J. D. (1973). *Journal of Chemical Society and Chemical Communication*, 194.

Creelman, R. A., & Mullet, J. E. (1997). *Annual Review of Plant Physiology and Plant Molecular Biology*, *48*, 355.
Dart, R. K., Kerry, S., & Marples, B. A. (1992). *Enzyme and Microbial Technology*, *14*, 954.
Gundlach, H., Müller, M.J., Kutchan, T.M., & Zenk, M.H. (1992). *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2389.
Helder, H., Miersch, O., Vreugdenhil, D., & Sembdner, G. (1993). *Physiological Plantarum*, *88*, 647.
Horeau, A. (1961). *Tetrahedron Letters*, 506.
Kehlen, A. (1991). Ph.D. thesis, Universität Halle.
Kitahara, T., Iwamoto, M., Takagi, K., Mori, Y., & Matsui, M. (1984). *Agricultural Biological Chemistry*, *48*, 1731.
Koda, Y., Kikuta, Y., Tazaki, H., Tsujino, Y., Sakamura, S., & Yoshihara, T. (1991). *Phytochemistry*, *30*, 1435.
Kramell, R., Atzorn, R., Schneider, G., Miersch, O., Brückner, C., Schmidt, J., Sembdner, G., & Parthier, B. (1995). *Journal of Plant Growth Regulation*, *14*, 29.
LeMahieu, R. A., Tabenkin, B., Berger, J., & Kierstead, R. W. (1970). *Journal of Organic Chemistry*, *35*, 1687.
Meyer, A., Gross, D., Vorkefeld, S., Kummer, M., Schmidt, J., Sembdner, G., & Schreiber, K. (1989). *Phytochemistry*, *28*, 1007.
Miersch, O., Bohlmann, H., & Wasternack, C. (1999). *Phytochemistry*, (in press).
Miersch, O., Günther, T., Fritsche, W., & Sembdner, G. (1993). *Natural Product Letters*, *2*, 293.
Miersch, O., Kramell, R., Parthier, B., & Wasternack, C. (1999). *Phytochemistry*, (in press).
Miersch, O., Preiss, A., Sembdner, G., & Schreiber, K. (1987). *Phytochemistry*, *26*, 1037.
Miersch, O., Schneider, G., & Sembdner, G. (1991). *Phytochemistry*, *30*, 4049.
Sembdner, G., & Parthier, B. (1993). *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*, 569.
Sembdner, G., Atzorn, R., & Schneider, G. (1994). *Plant Molecular Biology*, *26*, 1459.
Sembdner, G., Meyer, A., Miersch O., & Brückner, C. (1990). In R. P. Pharis, & S. B. Rood (Eds.), *Plant growth substances 1988* (p. 374). Springer Verlag, Berlin, Heidelberg, New York.
Stahl, E., & Glatz, A. (1982). *Journal of Chromatography*, *243*, 139.
Tabenkin, B., LeMahieu, R. A., Berger, J., & Kierstead, R. W. (1969). *Applied Microbiology*, *17*, 714.
Wasternack, C., & Parthier, B. (1997). *Trends in Plant Science*, *2*, 302.
Xia, Z. Q., & Zenk, M. H. (1993). *Planta Medica*, *59*, 575.
Yoshihara, T., Omer, E. A., Koshino, E. A., Sakamura, S., Kikuta, Y., & Koda, Y. (1989). *Agricultural Biological Chemistry*, *53*, 2835.