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JASMONATES FROM PINE POLLEN

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Key Word Index—*Pinus mugo*; Pinaceae; pollen; jasmonates; *N*[(−)-jasmonoyl]-(*S*)-isoleucine; *N*-[7-iso-cucurbinoyl]-(*S*)-isoleucine.

Abstract—Two jasmonates, *N*-[(−)-jasmonoyl]-(*S*)-isoleucine and *N*-[7-iso-cucurbinoyl]-(*S*)-isoleucine, were identified from pollen of *Pinus mugo*.

INTRODUCTION

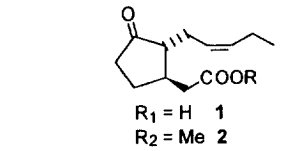
(−)-Jasmonic acid (JA, **1**) and structurally related compounds, designated as jasmonates, are known to be widely distributed within the plant kingdom. They exert different physiological effects when exogenously applied to plants and are believed to be involved in stress signal transduction chains by activating or expressing specific response genes (for review see [1]).

The occurrence of **1** and its methyl ester (JAMe, **2**) in pollen and anthers was described for three *Camellia* species. Owing to its inhibitory effect on pollen germination, JA was suggested to be an endogenous regulator of pollen germination; JAMe does not influence this process [2]. No other jasmonates are as yet known to occur in pollen. The present paper deals with the isolation and structural elucidation of *N*-[(−)-jasmonoyl]-(*S*)-isoleucine (**3**) as the main jasmonate of pollen of *Pinus mugo* T. As a minor component a conjugate (**4**) of 7-iso-cucurbic acid with isoleucine was detected for the first time.

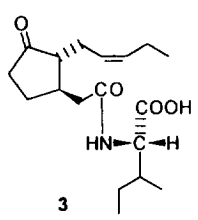
RESULTS AND DISCUSSION

Pollen grains of *P. mugo* were extracted with aqueous methanol. Conventional procedures gave an acidic extract which was separated by ion exchange chromatography on DEAE-Sephadex A25 [3]. This fractionation and the following ones were monitored by enzyme immunoassays based on polyclonal [4] and monoclonal [5] antibodies towards **2**. The polyclonals preferentially recognize jasmonate amides and to a lesser extent **2**, whereas the monoclonals are specific to **2** and show high sensitivity. Immunoreactive substances could only be eluted by 0.75 M acetic acid in methanol, indicative of the elution pattern of amino acid conjugates of **1** [6]. Further purification by repeated preparative HPLC on reversed phase silica gel gave an immunoreactive fraction (IF1) composed of two substances which were not completely separable by this method. GC-MS analysis, after methylation, showed the main component to be identical with a conjugate of **1** with isoleucine. Thus the GC and mass spectral data agreed with those of an authentic synthetic sample [7]. The stereochemistry of the JA moiety was established to be (3*R*,7*R*) by its cross-reactivity with the antibodies recognizing preferentially the (−)-enantiomer [4], and by its negative CD value. The separation of diastereomeric JA–amino acid conjugates by HPLC enables the complete stereochemistry of a conjugate to be assigned if the chirality of one component is known [8]. Therefore, from the HPLC retention time the adjacent amino acid could be assigned to be the (*S*)-enantiomer. Thus, the main component of IF1 was determined to be (−)-JA(*S*)Ile (**3**). This is the first isolation of a JA conjugate from the male gametophyte. Its inhibition of pollen germination (Knöfel, unpublished results) suggests an important role for it in plant reproduction. Compound **3** is known to occur in apical leaves of the broad bean (*Vicia faba* L.) [7, 8] and has been isolated from the fungus *Gibberella fujikuroi* (Sans.) [9].

The minor component of IF1 was shown to be **4** by means of GC-MS. The mass spectrum of the methyl ester



R_1 = H **1**
R_2 = Me **2**



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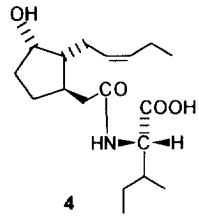

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(based on ref. [7]) showed typical key ions at *m/z* 128, 131 and 146 and ion abundances of the isoleucine moiety. The molecular ion at *m/z* 411 of **4**-Me-TMSi, formed by silylation of **4**-Me, indicated the presence of a hydroxyl group in **4**. Further key ions, *m/z* 342 formed by the loss of the pentenyl side chain at C-7 and *m/z* 225 formed by cleavage of the side chain at C-3, suggested that the hydroxyl group was located on the cyclopentane ring. This requirement is fulfilled by a cucurbic acid (CA) like structure with a hydroxyl group at C-6. In order to prove this possibility, synthetic **3** was reduced by $NaBH_4$ yielding the Ile conjugates 7-iso-CA and 6-epi-7-iso-CA. GC-MS analysis, after methylation and silylation of these products, showed that the endogenous pollen substance was identical with **4**. For GC monitoring, (−)-JA-(*S*)-AlaMe was used as a reference. This is the first evidence for a 7-iso-cucurbic acid conjugate from plant material. Compound **4** might be formed in pollen by a stereospecific enzyme-mediated reduction of **3** or its non-conjugated precursor **1**. There are no indications of the occurrence of **1** and **2** in pine pollen.

EXPERIMENTAL

Chromatographic methods. Anal. and prep. TLC: aluminium sheets silica gel 60 with concentrating zone (Merck), $CHCl_3$–EtOAc HOAc (14:6:1), detection by anisaldehyde reagent and heating for 2 min at 120° [10]; HPLC: Lichrospher RP18 (anal.: 250 × 4 mm, prep.: 250 × 10 mm), elution with MeOH/0.2% HOAc in H_2O (3:2), flow rate 1 ml min^{-1} (anal.) 6 ml min^{-1} (prep.), UV detector 228 nm, R_t (min) (anal.) **1** 7.7, **3** 16.2, **4** 16.3; GC-MS: 80 eV (MD 860, Fisons) with a fused silica capillary column (15 m × i.d. 0.32 mm) DB-5 MS, film thickness 0.25 μm. He, 1.3 ml min^{-1}, temp. progr.: 1 min to 60°, 25° min^{-1} to 110°, 10° min^{-1} to 260°, RR_t [relative to (−)-JA(*S*)-AlaMe] **3**-Me-TMSi 1.12, **4**-Me-TMSi 1.13, 6-epi-7-iso-CAMe-TMSi 1.11.

Plant material. Pollen was collected in 1992 and 1993 from two *Pinus mugo* T. trees near the institute. The pollen was dried over P_2O_5 for 24 hr and stored in absolute EtOH at −20°.

Extraction and isolation. The EtOH extract from 100 g pollen and three further extracts made from this pollen with 80% MeOH were combined and evapd to the aq. phase. This phase was partitioned at pH 8.5 and 3.0 with $CHCl_3$ to give an alkaline and acid fr. The latter fr. was chromatographed on DEAE-Sephadex A25 (acetate form, 45 ml gel) with a gradient of HOAc in 80% MeOH according to ref. [3]. Aliquots of the frs, after methylation with ethereal CH_2N_2, were monitored by enzyme immunoassays and combined immunoreactive frs were further purified on prep. HPLC (2 ×) yielding at R_t 16.5 min a mixture of **3** and **4**.

Enzyme immunoassays (EIA). EIA I using monoclonal antibodies directed towards **2** was performed as described in ref. [5] and was used to look for the occurrence of **1** and **2**. EIA II based on polyclonal antibodies (PAs) was used to recognize jasmonate amides, e.g. **3**. PAs were prepared by a conventional method from a characterized rabbit antiserum described in ref. [4]. For tracer synthesis, **3** was bound by the common carbodiimide method (EDC) to alkaline phosphatase. The tracer was finally dialysed against 0.1 M Tris–HCl, pH 7.8, and stored as a 1:1 mixture with glycerol at −20°. EIA II protocol: wells of microtietrplates, precoated with antibodies by incubation for 24 hr at 4° with an antibody soln (15 μg ml^{-1}) in 0.05 M Na_2CO_3 buffer, pH 9.6, were H_2O washed, incubated for 4 hr at 4° with standard (0.5–32 pmol **1**/150 μl 0.1 M Tris-buffered saline (TBS) at pH 7.8, or 0.15–10 pmol **3**/150 μl TBS) or the appropriate sample and 50 μl (125 ng) tracer soln. The amount of bound enzyme was estimated after a 1 hr incubation with *p*-nitrophenyl phosphate as substrate in 0.1 M diethanolamine buffer, pH 9.5, at 37°. The absorbance of each well was measured at 405 nm using a computerized plate reader (EAR 400 AT, SLT Lab instruments).

(−)*JA*(S)*Ile* [N-(−)-*jasmonoyl*]-(S)-*isoleucine*, N-[(1R,2R)-3-*oxo*-2-(2Z-*pentenyl*)-*cyclopentane*-1-*acetyl*]-(S)-*isoleucine* (**3**). R_t 16.5 min (**3**/**4** 23:2 mixture by GC). GC-MS (80 eV) *m/z* (rel. int.) of **3**-Me: 337 $[M]^+$ (3), 319 $[M - H_2O]^+$ (6), 269 $[M - C_5H_8]^+$ (4), 187 $[-CH_2-CO-Ile-Me + H]^+$ (19), 146 $[IleMe + H]^+$ (53), 131 (26), 128 (66), 86 $[C_5H_{12}N]^+$ (100); identical with synthetic **3**-Me [7].

7-*iso-CA*-(S)*Ile* N-[7-*iso-cucurbinoyl*]-(S)-*isoleucine*, N-[(1R,2R,3S)-3-*hydroxy*-2-(2Z-*pentenyl*)-*cyclopentane*-1-*acetyl*]-(S)-*isoleucine* (**4**). GC-MS (80 eV) *m/z* (rel. int.) of **4**-Me-TMSi: 411 $[M]^+$ (22), 396 $[M - Me]^+$ (3), 342 $[M - C_5H_9]^+$ (4), 321 $[M - TMSiOH]^+$ (20). 262 $[321 - CO_2Me]^+$ (4), 225 $[M - CH_2 - CO\text{-}IleMe]^+$ (10), 187 $[-CH_2-CO-IleMe + H]^+$ (18), 155 $[225 - C_5H_{10}]^+$ (21), 146 $[IleMe + H]^+$ (62), 135 [225 − TMSiOH] (16), 134 $[135 - H]^+$ (30), 131 $[187 - C_4H_8] +$ (23), 128 $[187 - CO_2Me]^+$ (52), 86 $[C_5H_{12}N]^+$ (100); identical with synthetic **4**-Me-TMSi, prepared by $NaBH_4$ reduction of **3** in 80% EtOH, prep. TLC for the sepn of 6α,β-hydroxy-isomers and derivatization. CD **3** synthetic: $\Delta\varepsilon_{297.2} = -2.39$ (MeOH, 1.43 × 10^{-3} M); **3** endogenous: $\Delta\varepsilon_{298} = -2.17$ (MeOH, 0.92 × 10^{-3} M).

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