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# Occurrence and Identification of Jasmonic Acid and Its Amino Acid Conjugates Induced by Osmotic Stress in Barley Leaf Tissue

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**Abstract.** The effect of osmotically active substances on the alteration of endogenous jasmonates was studied in barley (*Hordeum vulgare* L. cv. Salome) leaf tissue. Leaf segments were subjected to solutions of D-sorbitol, D-mannitol, polyethylene glycol 6000, sodium chloride, or water as a control. Alterations of endogenous jasmonates were monitored qualitatively and quantitatively using immunoassays. The structures of jasmonates isolated were determined on the basis of authentic substances by capillary gas chromatography-mass spectrometry. The stereochemistry of the conjugates was confirmed by high performance liquid chromatography with diastereoisomeric references.

In barley leaves, jasmonic acid and its amino acid conjugates, for example, with valine, leucine, and isoleucine, are naturally occurring jasmonates. In untreated leaf segments, only low levels of these native jasmonates were found. After treatment of the leaf tissues with sorbitol, mannitol, as well as with polyethylene glycol, an increase of both jasmonic acid and its conjugates could be observed, depending on the stress conditions used. In contrast, salt stress was without any stimulating effect on the levels of endogenous jasmonates.

From barley leaf segments exposed to sorbitol (1 M) for 24 h, jasmonic acid was identified as the major accumulating compound. Jasmonic acid-amino acid conjugates increased likewise upon stress treatment.

**Abbreviations:** JM, methyl jasmonate; JA, jasmonic acid; JIP(s), jasmonate-induced protein(s); PEG, polyethylene glycol; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; $R_t$, retention time; IAA, indole-3-acetic acid.

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Since methyl jasmonate (JM) was isolated from *Artemisia absinthium* L. and identified to be a senescence-promoting factor (Ueda and Kato 1980), many reports have dealt with the occurrence, metabolism, and biologic activities of jasmonic acid (JA) and/or its methyl ester. Jasmonates are widely distributed in plants and represent new putative plant growth regulators possessing distinct physiologic potencies (for review see Sembdner and Parthier 1993). JA and related compounds, including conjugates, seem to be involved in different physiologic and developmental processes, for example, induction of potato plant tuberization (Koda et al. 1991), induction of defensive proteinase inhibitors (Farmer et al. 1992), increase of vegetative storage protein levels (Anderson 1989, Mason et al. 1992, Staswick et al. 1991), or elicitation of secondary substances such as alkaloids (Gundlach et al. 1992).

Barley leaf tissue responds to JM treatment with alterations of gene expression and accumulation of new abundant polypeptides (Herrmann et al. 1989, Müller-Uri et al. 1988, Weidhase et al. 1987). The molecular masses of the most prominent jasmonate-induced proteins (JIPs) were determined to be 6, 23, 37, 60 (66), and 100 kDa, respectively.

Preliminary experiments have already shown that not only JA and JM, but also amino acid conjugates of JA were able to induce JIPs (Herrmann et al. 1987). It was likewise demonstrated in our laboratory that sorbitol and other stress-inducing substances provoked a marked increase of endogenous jasmonates including JA as well as amino acid conjugates (Lehmann et al. 1994, Parthier et al. 1992), and kinetic experiments showed a direct correlation between this effect and the appearance of JIPs.

So far, not much is known about the occurrence of amino acid conjugates of JA in barley. Investigations on *Vicia faba* L. indicated that such metabo-

lites were native constituents (Brückner et al. 1986, 1988, Schmidt et al. 1990).

Therefore, it was of interest to investigate which kinds of endogenous jasmonates appear in barley leaf segments, which have been treated with compounds known as osmotic stress factors, for example, sugar alcohols, polyethylene glycol (PEG), or NaCl.

## Materials and Methods

### *Chemicals*

(−)-JA in this enantiomeric form was isolated from the fungus *Botryodiplodia theobromae* (Miersch et al. 1987). The JA metabolites were prepared by derivatization of (±)-JA via its carboxy group with the amino acids valine, leucine, and isoleucine, respectively (Kramell et al. 1988, Schneider et al. 1989). (±)-JA was available by alkaline hydrolysis of JM purchased from Firmenich (Geneva, Switzerland). [$^3$H]-(−)-JM (specific activity 3.15 TBq · mol$^{-1}$) was a gift from Prof. E. W. Weiler (Bochum, Germany). [$^3$H]-(−)-JA-*S*-Ile (specific activity 13 TBq) was synthesized from (−)-JA and [$^3$H]-*S*-Ile (Amersham-Buchler, Braunschweig, Germany). All other reagents and solvents used were obtained from Merck (Darmstadt, Germany).

### *Plant Material and Stress Treatment*

Barley (*Hordeum vulgare* L. cv. Salome) seedlings were grown in a greenhouse under a light regime of 16 h of light and 8 h of darkness at 22–25 °C for 7 days. Primary leaves were detached, and segments of 5-cm length, without the 1-cm apical region, were floated under continuous light and 25 °C as described earlier (Weidhase et al. 1987), on following solutions: D-(−)-sorbitol (1 M), D-(−)-mannitol (1 M), PEG 6000 (25%, w/v), sodium chloride (1 M), and water (control).

### *Immunoassay of Jasmonates*

The jasmonates were detected using either RIA or ELISA, both based on an antiserum raised in rabbits against native (−)-JA linked to hemocyanin. The polyclonal antibodies exhibit a high affinity for (−)-JM and (−)-JA-amino acid conjugates. Extract aliquots were methylated prior to jasmonate analysis. The RIA was performed as described by Knöfel et al. (1990). The ELISA exhibits a detection limit of 10 pg and a linear measuring range between 0.01 and 25 ng. It was performed according to the procedure described by Weiler (1986) for ELISAs utilizing polyclonal antisera.

### *Extraction and Isolation of Jasmonates*

Barley leaf segments (1–20 g fresh weight, depending on experimental conditions) were floated on 1 M sorbitol for 24 h. The plant material was homogenized and extracted twice with appropriate amounts of 80% aqueous methanol.

For immunoassay analyses, extracts were passed through $C_{18}$ reversed phase cartridges (Baker, Groβ-Gerau, Germany) preequilibrated with 70% methanol, concentrated to dryness by vacuum evaporation, and resuspended in water, adjusted to pH 3 with glacial acetic acid. Thereafter, extracts were partitioned three times with equal volumes of ethyl acetate, concentrated to dryness, and stored at −20 °C prior to HPLC separation or analysis by immunoassay.

For HPLC, solvents were delivered at a flow rate of 1 ml · min$^{-1}$ by a Knauer (Berlin, Germany) liquid chromatograph. Reversed phase HPLC utilized a $C_{18}$ Eurochrome column (Knauer) eluted with 45% methanol in 0.2% glacial acetic acid. Fractions of 1 ml each were reduced to dryness by a Speed-Vac concentrator, methylated with ethereal diazomethane, and aliquots of appropriate amounts were analyzed by enzyme immunoassay. JA equivalents of regions coeluting with (−)-JA-amino acid conjugates occurring in barley [(−)-JA-*S*-Val, (−)-JA-*S*-Leu, (−)-JA-*S*-Ile, fractions 17–23] were corrected by corresponding cross-reactivities.

Recoveries were estimated in the following ways. To representative series of samples, appropriate amounts of [$^3$H]-(−)-JA-*S*-Ile were added before passing extracts to Sep-Pak cartridges. Prior to HPLC or methylation, aliquots were estimated by liquid scintillation counting. Average rates of recoveries at this stage were 79 ± 3%. Because of the relatively high volatility of JM, additional recoveries during the methylation procedure were measured to be 71 ± 4% by adding [$^3$H]-(−)-JM according to Albrecht et al. (1993). All jasmonate concentrations quantified by immunoassay were corrected by these values.

For structural elucidation by GC-MS, methanolic extracts were evaporated; thereafter the aqueous phase was frozen, thawed, and centrifuged. The supernatant adjusted to pH 3–4 was partitioned with ethyl acetate. The combined organic extract was separated on a DEAE-Sephadex A-25 column using a discontinuous gradient of acetic acid in methanol according to Gräbner et al. (1976). Aliquots of the eluates were immunologically checked (see Fig. 3).

The immunoreactive material of fractions 4 and 5 (0.25 M HOAc) was pooled to yield free JAs (group A). After evaporation, the residue was further purified on LiChroprep RP-18, giving the active material with 55–75% methanol in 0.2% acetic acid. The jasmonates of group A were finally rechromatographed by reversed phase HPLC. The preparative chromatography was carried out on a Knauer HPLC set fitted with an Eurospher 100 RP-18 column (4 × 250 mm, 5 μm) using a mobile phase MeOH/0.2% aqueous HOAc (60:40, v/v) with a flow rate of 1 ml · min$^{-1}$; UV detection at 210 nm. Each fraction was monitored by immunoassay. (−)-JA was eluted at 7–8 ml.

The immunoreactive fractions 7 and 8 from DEAE-Sephadex (0.5 M/0.75 M HOAc) were combined as group B (JA conjugated with amino acids) and subjected to chromatography on LiChroprep RP-18. The metabolites were recovered with 55–75% acidic methanol (0.1% HOAc) and subsequently purified by reversed phase HPLC. The conjugated jasmonates (group B) were separated into B1 (JA-Val) = 10–11 ml; B2/B3 (JA-Leu/JA-Ile) = 16–19 ml (see Fig. 4). After partitioning with chloroform, the organic fractions were subjected to GC-MS.

### *Identification of Stress-Induced Jasmonates*

*GC-MS.* Prior to capillary GC-MS, the compounds were esterified by treatment with ethereal diazomethane. The GC-MS system was equipped with a HP 9000/300-9133 computer set. Anal-

yses were done with a Hewlett Packard quadrupole mass spectrometer (model 5970B) combined with a HP 5890 gas chromatograph. GC was performed on a 25-m × 0.2-mm inner diameter cross-linked methyl silicon-fused silica column, film thickness 0.11 μm, helium as carrier gas (2.5 ml · $min^{-1}$) and splitless injection. Temperatures: injection, 275 °C; direct inlet interface, 230 °C; ion resource, 250 °C. The temperature program was as follows: from 60 °C (1 min) to 180 °C (30 °C $min^{-1}$); from 180 °C (1 min) to 200 °C (10 °C $min^{-1}$); from 200 °C (1 min) to 270 °C (5 °C $min^{-1}$). The electron impact energy was 70 eV.

The average mass spectra of the methylated compounds were background corrected. Retentions of the methyl esters: A-Me, $R_t$ = 9.29 min; B1-Me, $R_t$ = 11.44 min; B2-Me, $R_t$ = 12.32 min; B3-Me, $R_t$ = 12.73 min.

The retention times were checked by coinjection with standards. The GC spectra of (−)-JA (A-Me) and its conjugates (B1-Me, B2-Me, B3-Me) are accompanied by signals caused by the stereoisomer (+)-7-iso-JA ($R_t$ = 9.59 min) and its metabolites with Val ($R_t$ = 11.85 min) and Ile ($R_t$ = 13.15 min).

The MS data coincide with those described for synthetic references (Schmidt et al. 1990).

*Analytical HPLC.* The isolated conjugates were stereochemically identified by cochromatography with synthetic references (Schneider et al. 1989). The compounds were subjected to reversed phase HPLC on LiChrospher 100 RP-18 column (5 μm, 4 × 125 mm); eluent: MeOH/HOAc (0.2%) = 50:50 (v/v); flow rate: 1 ml · $min^{-1}$; UV detection at 210 nm. The isolated jasmonates showed the following retention properties: A [(−)-JA], $R_t$ = 5.33 min; B1 [(−)-JA-(*S*)-Val], $R_t$ = 8.21 min; B2 [(−)-JA-(*S*)-Leu], $R_t$ = 15.26 min; B3 [(−)-JA-(*S*)-Ile], $R_t$ = 14.23 min.

## Results

### *Alterations of Endogenous Jasmonates in Barley Leaf Segments in Response to Osmotic Stress*

The level of total native jasmonates in control leaf segments was found to be low compared with sorbitol-stressed plant material (Fig. 1C). The controls show that the jasmonate content did not significantly change during the floating time up to 24 h.

Treatment of barley leaf segments with 1 M sorbitol resulted in a marked increase of endogenous jasmonates (Fig. 1). The time course demonstrates that the naturally occurring jasmonates increased slightly within the first 4 h starting from stress initiation. Thereafter, jasmonates increased steadily up to 24 h. The kinetics of the formation of JA and its amino acid conjugates yield similar curves, suggesting equal increases of both jasmonate types, but their absolute values are very different (Fig. 1, A and B).

Increase of the jasmonate concentration was likewise detected using 1 M mannitol (Fig. 2). The exposition of leaf tissues to solutions (25%, w/v) of PEG 6000 led also to an accumulation of jasmonates. In contrast to sorbitol or mannitol, sodium chloride did not stimulate jasmonate accumulation (Fig. 2). Therefore, in the case of the salt stress, there was no stress-induced accumulation of jasmonates in barley leaves (Lehmann et al. 1994, Parthier et al. 1992).

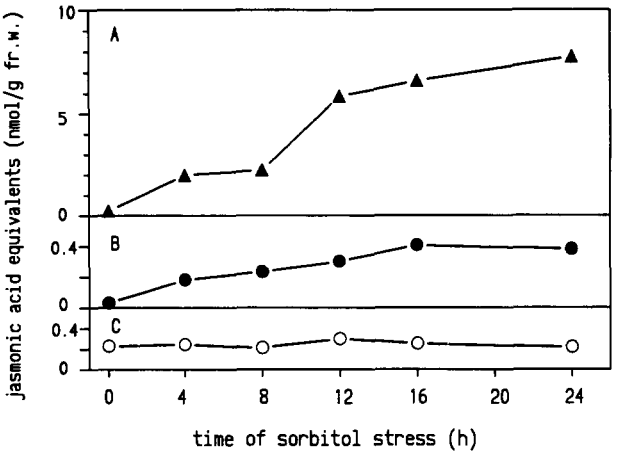


**Fig. 1.** Time courses of free (A) and conjugated (B) jasmonates in sorbitol-stressed barley leaf segments compared with total jasmonates in water controls (C). Selective quantifications of JA and amino acid conjugates were performed by ELISA after reversed phase HPLC separation.

To study the sorbitol-induced jasmonate pool in more detail, the plant material after 24-h sorbitol treatment was extracted with aqueous methanol. The extract was groupwise separated on DEAE-Sephadex A-25 (Fig. 3). The neutral fraction was not yet analyzed, although it contained significant immunoreactivity. The acidic fractions consisted of either free JA (group A) or conjugated JA bound to amino acids (group B). Thus, JA was determined as the main jasmonate isolated from sorbitol-stressed barley leaves.

The amino acid conjugates were found to be the minor metabolites. The conjugate fraction (group B) was isocratically rechromatographed under reversed phase HPLC conditions (Fig. 4). The metabolites were separated into the components B1 (JA-Val) and the mixture of B2 (JA-Leu) and B3 (JA-Ile). The chromatographic data suggest that B2/B3 was the most abundant amino acid conjugate fraction induced by sorbitol stress.

The stereochemistry of the JA conjugates was determined on the basis that the used polyclonal antibodies show only affinity to the conjugates of (−)-JA (Knöfel et al. 1990). In addition, by HPLC it is possible to separate diastereoisomeric amino acid conjugates of (−)JA and its synthetic (+)-enantiomer (Schneider et al. 1989). From both experimental observations the isolated conjugates are:

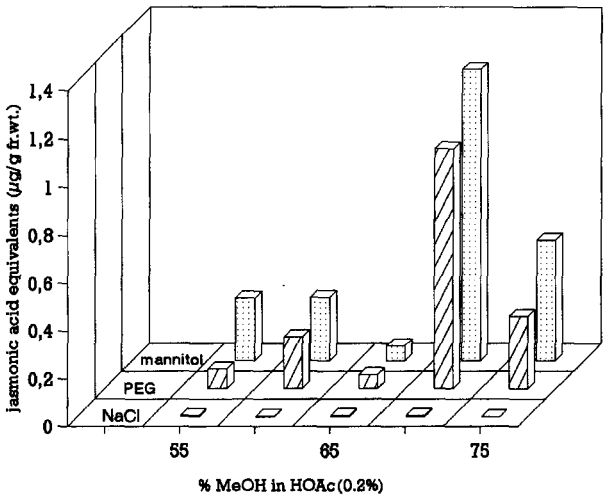


**Fig. 2.** Occurrence of jasmonates in barley leaves (1 g fresh weight) exposed to mannitol and NaCl (1 M) as well as PEG 6000 (25%, w/v) for 24 h. The methanolic leaf extracts were chromatographed on cartridges filled with 0.5 g of LiChroprep RP-18. The jasmonates detected by RIA were recovered with increasing concentrations of MeOH in 0.2% HOAc (5% elution steps).

B1 = *N*-[(−)-jasmonoyl]-(*S*)-valine; B2 = *N*-[(−)-jasmonoyl]-(*S*)-leucine; B3 = *N*-[(−)-jasmonoyl]-(*S*)-isoleucine.

### *Identification of Sorbitol-induced Jasmonates by GC-MS*

To elucidate the structure of jasmonates contained in the immunoreactive peaks, the respective HPLC fraction was subjected to capillary GC-MS analysis. The MS spectral fragmentations of JA-conjugates with amino acids are shown in Figure 5.

The mass spectrum of the methylated compound A exhibits the molecular ion $[M]^{+\cdot}$ at *m/z* 224. The base peak at *m/z* 83 $[C_5H_7O]^+$ is derived from the cyclopentanone ring. The obtained full scan spectra correspond to that of (−)-JM (Dathe et al. 1981, Ueda et al. 1980).

MS *m/z* (rel. int.): A-Me (JM): 224 $[M]^{+\cdot}$ (35), 206 $[M\text{-}H_2O]^{+\cdot}$ (14), 193 $[M\text{-}OCH_3]^+$ (11), 156 $[M\text{-}C_5H_8]^+$ (26), 150 $[M\text{-}CH_3COOMe]^{+\cdot}$ (21), 135 (15), 133 (28), 121 (19), 95 (57), 83 $[C_5H_7O]^+$ (100).

The methyl ester of component B1 produced a spectrum yielding the molecular ion $[M]^{+\cdot}$ at *m/z* 323 indicating a JA conjugate with the amino acid valine. The spectral behavior is additionally characterized by the appearance of a key ion a at *m/z* 173 originating from the cleavage of the bond at C-3 as well as the formation of abundant fragments at *m/z* 132 and *m/z* 114. The base peak (b-HCOOMe) at *m/z* 72 is formed by the nitrogen-containing ion $[C_4H_{10}N]^+$ of the valine moiety (Fig. 5).

The MS spectra of the methylated cross-reactive peaks B2-Me and B3-Me comprise the molecular ion at $[M]^{+\cdot}$ at *m/z* 337, respectively (Fig. 5). The data suggest derivatives of JA bound to the amino acids leucine and isoleucine supported by the presence of the main fragment $[C_5H_{12}N]^+$ at *m/z* 86. The acylium ion a at *m/z* 187 has been found to be the parent fragment at *m/z* 131 formed by the elimination of the amino acid side chain and at *m/z* 128, yielded by the decarboxymethylation, respectively. The fragmentation patterns of both metabolites are in accordance to data described for synthetic standards (Schmidt et al. 1990).

The GC retention times of the sorbitol-induced JA metabolites allow a differentiation of the amino acid conjugates and correlate with the chromatographic behavior of authentic substances (see the Materials and Methods section). The GC data confirm that the signal of the isolated (−)-JA is accompanied by that of its stereoisomer (+)-7-iso-JA. Similarly, the spectra of the conjugates B1, B2, and B3 also contain traces of the respective metabolites of (+)-7-iso-JA (see the Materials and Methods section).

## Discussion

Our previous studies showed that osmotic stress, that is, treatment of leaf tissue with sorbitol or other sugar alcohols, or desiccation, mimics the effects of exogenously applied jasmonates or abscisic acid in forming specific proteins, JIPs (Lehmann et al. 1994, Parthier et al. 1992, Reinbothe et al. 1992, Weidhase et al. 1987). First estimations of endogenous jasmonates in stressed tissues supported the view of participation of jasmonates in the signal transduction chain between (osmotic) stress and JIP gene expression (Parthier et al. 1992). However, the nature of the endogenous jasmonates involved remained to be identified. This to do was one aim of the present paper.

After 24 h of treatment with 1 M sorbitol, endogenous jasmonates increased markedly (Fig. 1), and chromatographic separation/ELISA elucidated both free JA and in a less amount, amino acid conjugates (Figs. 1 and 4). The kinetics of sorbitol-induced formation of endogenous jasmonates (Fig. 1) suggest a concomitant synthesis of free and conjugated JA rather than a secondary conjugation of preformed JA. As we do not yet have data about the metabolic stability of the conjugates and their compartmentation, we hesitate to make a final conclu-

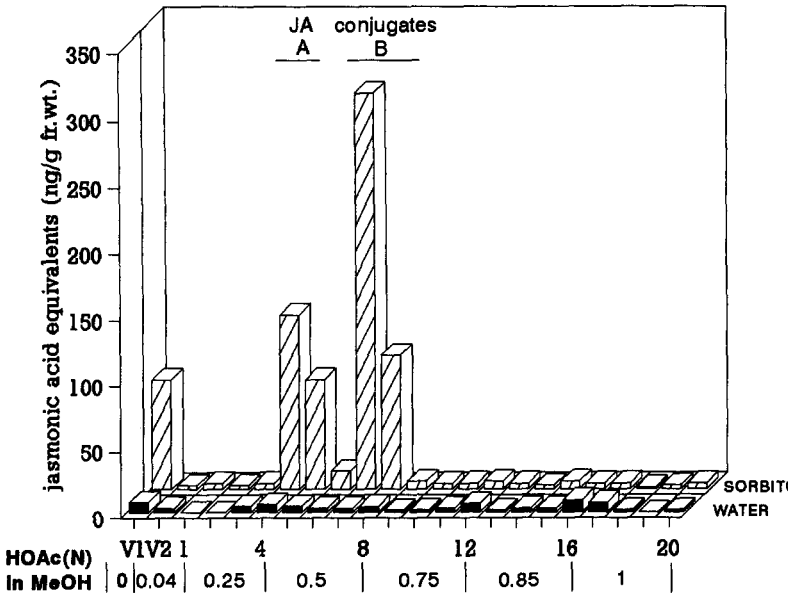


Fig. 3. Separation of endogenous jasmonates isolated from sorbitol-treated (1 M, 24 h) leaves on DEAE-Sephadex A-25 using a discontinuous gradient of HOAc in MeOH. Each fraction was monitored by RIA. The acidic immunomaterials were pooled, resulting in free JAs (**group A**) and JA-amino acid conjugates (**group B**).

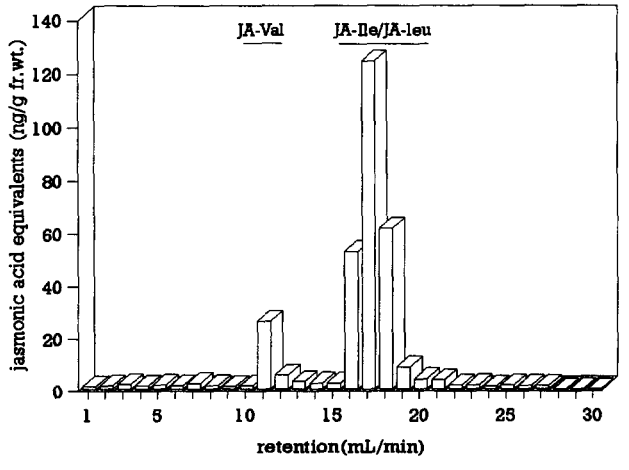


Fig. 4. HPLC separation of sorbitol-induced metabolites (group B) into different amino acid conjugates on Eurospher 100 RP-18 with methanol in acetic acid (60:40, v/v). Immunoreactive areas B1 (10–11 ml) and B2/B3 (16–19 ml) are coeluting with standards JA-Val and JA-Leu/JA-Ile, respectively.

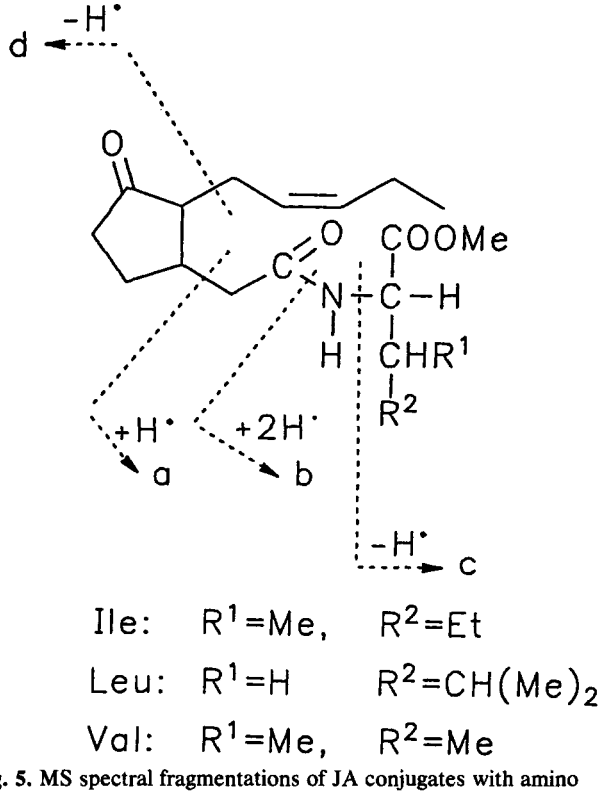


Fig. 5. MS spectral fragmentations of JA conjugates with amino acids as methyl esters.

sion about the function of the amino acid conjugates identified. It is interesting to observe that NaCl was unable to induce an increase of endogenous jasmonates (Fig. 2) and of gene transcripts for JIPs (Lehmann et al. 1994), although inorganic salts are known by its higher potential (Money 1989) compared with the sugar alcohols or PEG, a neutral desiccation agent.

It is known that the classic phytohormones exist as free forms as well as conjugated metabolites. In the case of auxins, certain amino acid conjugates of indole-3-acetic acid (IAA) have been identified such as IAA-aspartate (Cohen 1982) and IAA-glutamate (Epstein et al. 1986). In general, IAA-amino acid conjugates are supposed to act as a storage pool to release free IAA required during the plant development (Bandurski et al. 1987, Cohen et al. 1987, Sembdner et al. 1994).

Similarly, amino acid conjugates of JA and re-

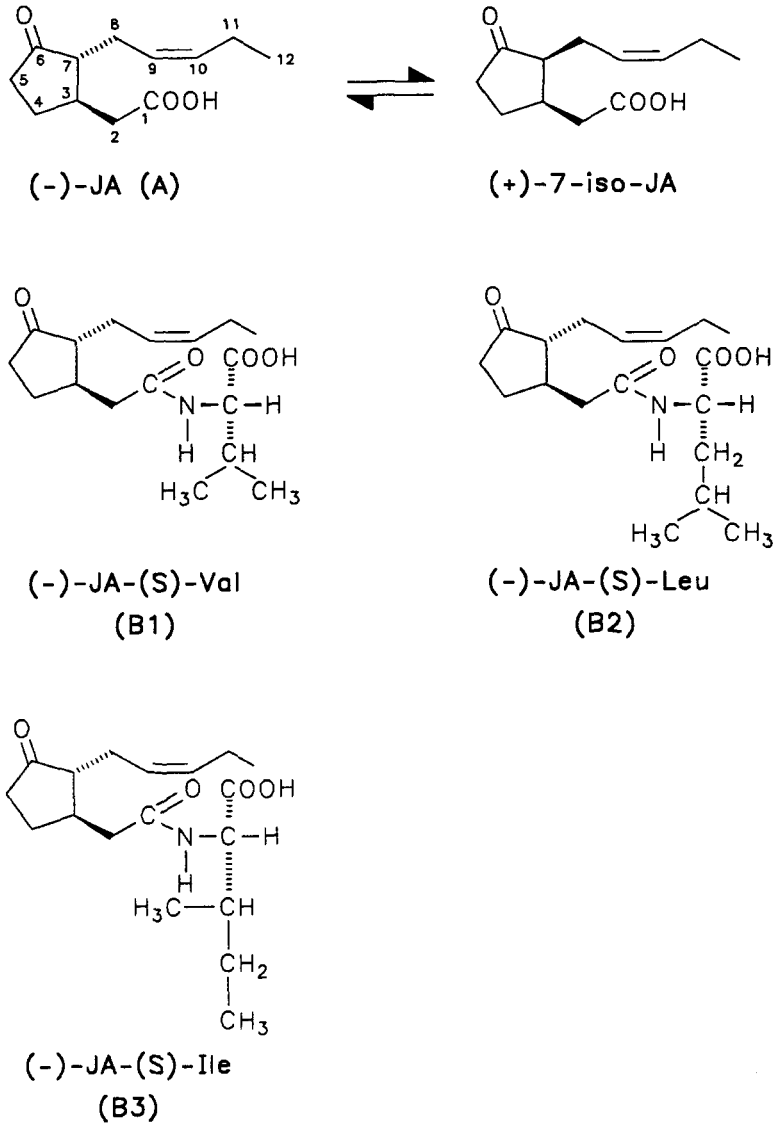


**Fig. 6.** Structures of jasmonates isolated from sorbitol-stressed barley leaf segments. **A**, (−)-JA; **B1**, *N*-[(−)-jasmonoyl]-(*S*)-valine; **B2**, *N*-[(−)-jasmonoyl]-(*S*)-leucine; **B3**, *N*-[(−)-jasmonoyl]-(*S*)-isoleucine.

lated compounds have been isolated from fungi (Cross and Webster 1970) and higher plants (Bohlmann et al. 1984). Investigations on the occurrence of jasmonates in the broad bean plant indicate that the JA conjugation seems to be organ specific in respect to the amino acids. In generative parts such as flowers and fruits, JA conjugates with the aromatic amino acids tyrosine, tryptophan, and phenylalanine are present endogenously (Brückner et al. 1986, 1988, Schmidt et al. 1990). However, JA conjugates with the aliphatic amino acids isoleucine and leucine were found as native components in young leaves of *V. faba* L. (Schmidt et al. 1990).

Here we report the occurrence of endogenous JA conjugated with the amino acids isoleucine, leucine, and valine in barley (*H. vulgare* L.) in connection with osmotically stressed leaf tissues.

After application to barley leaf segments, JA-amino acid conjugates exert activities in chlorophyll degradation and JIP induction similar to those known for JA and JM (Herrmann et al. 1987, Parthier et al. 1992). This experiment points to bi-

**Table 1.** Prominent ions of the methyl esters of sorbitol-induced amino acid conjugates of JA. Results shown are *m/z* abundance, with percentage in parentheses.

| Ion | B1-Me (JA-Val) | B2-Me (JA-Leu) | B3-Me (JA-Ile) |
|---|---|---|---|
| $[M]^{+\cdot}$ | 323 (16) | 337 (24) | 337 (18) |
| $[M\text{-}COOMe]^{+}$ | 264 (12) | 278 (11) | 278 (11) |
| a | 173 (32) | 187 (25) | 187 (26) |
| b | 132 (69) | 146 (85) | 146 (66) |
| (a-$C_4H_8$) | | 131 (82) | 131 (34) |
| (a-COOMe), c | 114 (63) | 128 (36) | 128 (82) |
| (b-HCOOMe) | 72 (100) | 86 (100) | 86 (100) |

ologic activity of either JA conjugates as such, or, more probably, to a fast splitting of the conjugates into free JA and amino acids (unpublished results).

Approaches to cleave the amide structure of the JA conjugates using common peptidases or proteinases were not successful (data not shown). This might indicate a high specificity of naturally occurring enzymes that are able to hydrolyze the amide bond. It was also not possible to synthesize such conjugates in vitro by using crude enzyme preparations. On the other hand, amino acid conjugation of JA seems not to belong to the major metabolic pathways of jasmonates if exogenously applied (Sembdner and Parthier 1993). Feeding experiments with 9,10-dihydrojasmonic acid to barley seedlings resulted in the formation of oxygenated products as the mainly isolated metabolites, whereas only traces of amino acid conjugates could be detected (Meyer et al. 1991, Sembdner et al. 1990).

The jasmonates induced by sorbitol treatment were structurally elucidated by GC-MS data (Fig. 6). The full scan spectra exhibit the respective molecular ion $[M]^{+\cdot}$ and key ions originated from fragments of the amino acid moiety and the jasmonoyl residue (Table 1). The chemical data are in agreement with those described for the synthetic references (Schmidt et al. 1990).

In general, JA is a mixture containing the biogenetic isomer (+)-7-iso-JA and its more stable diastereomer (−)-JA, which can be formed by isomerizations during the isolation procedure (Miersch et al. 1987, Vick and Zimmerman 1983). The GC retentions of the induced (−)-JA and its metabolites are characterized by the occurrence of respective signals of (+)-7-iso forms at low abundance.

In conclusion, JA and its amino acid conjugates seem to be directly involved in the gene expression caused by osmotically active substances. Further investigations are required in attempting to establish both function and position of these jasmonates in the signal transduction chain.

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