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The configuration of methyl jasmonate affects paclitaxel and baccatin III production in *Taxus* cells

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Abstract

All stereoisomers of methyl jasmonate (MJA) were prepared, and their effects on cell yield and promotion of paclitaxel (Taxol™) and baccatin III production investigated in cell suspension cultures of *Taxus media*. (3*R*,7*S*)-MJA showed the strongest cell growth inhibition, followed by (3*R*,7*R*)-MJA. In contrast, (3*S*,7*R*)- and (3*S*,7*S*)-MJA had very low inhibitory effects, indicating that this inhibition depends largely on the (3*R*)-configuration. In terms of the promotion of paclitaxel and baccatin III production, (3*R*,7*R*)-MJA had the highest activity. Although it showed considerable activity at low concentration, at higher concentrations the activity was decreased due to strong inhibition of cell growth. Interestingly, paclitaxel and baccatin III contents increased even at a high (3*S*,7*R*)-MJA concentration, whereas the other isomers had the opposite effects. These findings are interpreted to suggest that the optimum configuration is (3*R*,7*R*), the (3*R*)-configuration not being indispensable, and that the (7*R*)-configuration is suitable for the promotion of paclitaxel and baccatin III production.

Keywords: *Taxus media*; Yew; Paclitaxel (taxol); Baccatin III; Cell suspension culture; Methyl jasmonate; Stereochemistry; Structure-activity relationships

1. Introduction

Cultured cells of *Taxus* species accumulate paclitaxel (Taxol™) and related taxanes, all of which have received considerable attention owing to their potential anti-tumor activities against a variety of solid tumors. Several recent reports, including one of ours, show that methyl jasmonate (MJA) strongly promotes taxane biosynthesis (Mirjalili and Linden, 1996; Yukimune et al., 1996; Menhard et al., 1998; Ketchum et al., 1999). These findings, however, were obtained using synthetic MJA, which contains a mixture of stereoisomers. MJA contains two asymmetric centers at C-3 and C-7, which can have either *R* or *S* configuration, resulting in four possible stereoisomers. Commercially available MJA is a mixture of about 5% each of the (3*R*,7*S*)- and (3*S*,7*R*)-*cis* isomers and 45% each of the (3*R*,7*R*)- and (3*S*,7*S*)-*trans* isomers (Helmchen et al., 1991). The absolute configuration of naturally occurring jasmonic acid (JA) is considered to be (3*R*,7*S*) (Vick and Zimmerman, 1984). Various biological activities of the pure stereoisomers of JA and MJA, as well as growth inhibition, senescence promotion (Miersch et al., 1986; Koda et al., 1992), and potato tuber-induction (Koda et al., 1992), have been compared. Results show that naturally occurring (3*R*,7*S*)-MJA was the most active in each assay system, but that the requirements of the absolute configurations of the two side chains differ. We examined

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the stereogenic effect of MJA on cell yield and the promotion of paclitaxel and baccatin III production in cell suspension cultures of *T. media* using the four stereoisomers of MJA: (3*R*,7*S*)-MJA (**1**), (3*S*,7*R*)-MJA (**2**), (3*R*,7*R*)-MJA (**3**), and (3*S*,7*S*)-MJA (**4**).

2. Results

The four stereoisomers of MJA: (3*R*,7*S*)-MJA (**1**), (3*S*,7*R*)-MJA (**2**), (3*R*,7*R*)-MJA (**3**), and (3*S*,7*S*)-MJA (**4**), were obtained by HPLC separation from a 46:54 mixture of 7-*epi*-MJA and MJA. Separation was performed in two steps: the mixture was first separated using silica column chromatography into 7-*epi*-MJA and MJA, which then were individually resolved, respectively, to offer **1** and **2**, and **3** and **4** by chiral column chromatography. Since compounds **1** and **2**, which have the *cis*-side chains, are easily epimerized to the respective *trans*-isomers (**3** and **4**), they were stored at −70°C and used immediately after dilution with MeOH.

The four stereoisomers of MJA were added individually to separate *T. media* cultures, and the cell yield and activity for promoting paclitaxel and baccatin III production in the cells investigated. Compound **1** inhibited cell growth the most, followed by **3**, whereas **2** and **4** both gave very low inhibitions. The respective inhibition of cell growth for **1**, **2**, **3**, and **4** relative to that of the control cultures at 300 μM were 58.8, 18.0, 53.3, and 9.1% (Fig. 1). A two-way analysis of variance (ANOVA) showed that two variables, MJA isomer and concentration of MJA isomer, were both significant at the 1% level, and that there was an interaction between them at the 1% level (data not shown). Bonferroni's multiple comparison test was also performed at 300 μM for each MJA isomer. Compounds **1** and **4** were again significant at the 1% level, whereas, 1 and 2, and 3 and 4 were significant at the 5% level, respectively (data not shown).

With regard to the promotion of paclitaxel production, **3** had the greatest activity (Fig. 2). Although **1** showed considerable activity at 30 μM, at higher concentrations, activity decreased owing to the strong inhibition of cell growth. Compound **4**, on the other hand, had very low activity. Interestingly, the paclitaxel content increased even at a high concentration of **2**, whereas there was an opposite effect with isomers **3** and **4**. The highest paclitaxel productivities obtained with **1** at 30 μM, **2** at 300 μM, **3** at 100 μM, and **4** at 100 μM, respectively, were 0.62, 0.69, 0.72, and 0.39% (w/w) (126, 152, 151, and 90 mg/l/2 weeks). A two-way ANOVA showed that two variables, MJA isomer and concentration of MJA isomer, were both significant at the 1% level, and that there was an interaction between them at the 1% level (data not shown). Bonferroni's multiple comparison test was also performed at the optimum points mentioned above. Compounds **2** and **4**, and **3** and **4** were significant at the 5% level, respectively (data not shown).

Similar results were obtained for baccatin III production, but the optimum concentrations of the MJA isomers were lower than those for paclitaxel production (Fig. 3). The highest baccatin III productivities obtained with **1** at 3 μM, **2** at 300 μM, **3** at 30 μM, and **4** at 100 μM, respectively, were 0.30, 0.39, 0.36, and 0.19% (w/w) (68, 85, 81, and 43 mg/l/2 weeks). A two-way ANOVA showed that two variables, MJA isomer and concentration of MJA isomer, were both significant at the 1% level, and that there was an interaction between them at the 1% level (data not shown). Bonferroni's multiple comparison test was also performed at the optimum points mentioned above. Compounds **2** and **4**, and **3** and **4** were significant at the 1% level, respectively, whereas **1** and **4** were significant at the 5% level (data not shown).

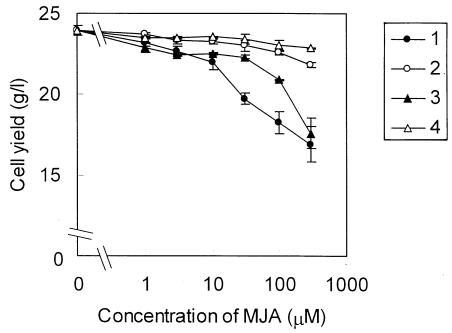


Fig. 1. Effects of MJA-isomers (**1**, **2**, **3**, and **4**) on cell yield in suspension cultures of *T. media*. Various concentrations of MJA were added to cultures in 2.5 μl of methanol per ml of culture medium just after cell inoculation. A 50 μl volume of methanol was added to each culture, after which the cultures were incubated for 14 days. Data are the means of four determinations. Vertical bars represent standard errors.

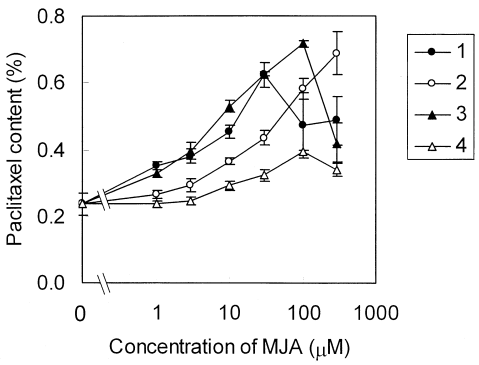


Fig. 2. Effects of MJA-isomers (**1**, **2**, **3**, and **4**) on paclitaxel production in suspension cultures of *T. media*. Culture conditions were the same as in Fig. 1. Data are the means of four determinations. Vertical bars represent standard errors.

3. Discussion

Jasmonates inhibit the growth of seedings (Dathe et al., 1981; Yamane et al., 1980), roots (Yamane et al., 1981), and cell division (Miersch et al., 1986; Koda et al., 1992; Ueda and Kato, 1982). Koda et al. (1992) compared inhibition activities of the four MJA stereoisomers on the growth of soybean callus induced by zeatin riboside, and showed that it was inhibited strongly by (3*R*,7*S*)- and (3*R*,7*R*)-MJA, but not by either (3*S*,7*S*)- or (3*S*,7*R*)-MJA. Our findings from experiments on *T. media* cultures are consistent with their results, indicative that their reported inhibition of cell growth was strongly dependent on the (3*R*)-configuration. Abe et al. (1990) reported that MJA disrupted cortical microtubules in cultured tobacco cells in the S phase of the cell cycle. As Koda et al. (1996) suggested, growth inhibition by MJA appears to be caused mainly by the disruption of cortical microtubules, a phenomenon ubiquitous in plants.

We earlier reported that paclitaxel is accumulated dose dependently, with maximum induction at 100 μM of MJA (Yukimune et al., 1996). Those experiments were done on a mixture of the four stereoisomers. In the present study, we found that the individual isomers have different optimum concentrations for the maximum promotion of paclitaxel and baccatin III production. Furthermore, unlike growth inhibitory activity, the highest productivity obtained by the addition of the individual isomers differed, (3*S*,7*R*)-MJA (**2**) and (3*R*,7*R*)-MJA (**3**) having the highest productivity.

Koda et al. (1992) suggest that in the various biological activities, different receptors trigger reactions leading to the requirement of the absolute configurations of the two side chains with respect to the plane of the cyclopentanone ring. Weiler et al. (1993) also speculated that a hypothetic receptor family exists for the specific recognition of different jasmonates. Although it is not clear whether there are different receptors for jasmonates in *Taxus* cells, our findings indicate that growth inhibition and the promotion of paclitaxel and baccatin III production are regulated differently by different methyl jasmonates.

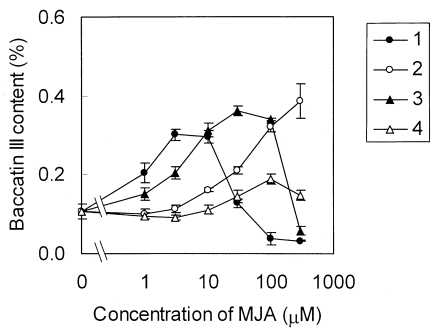
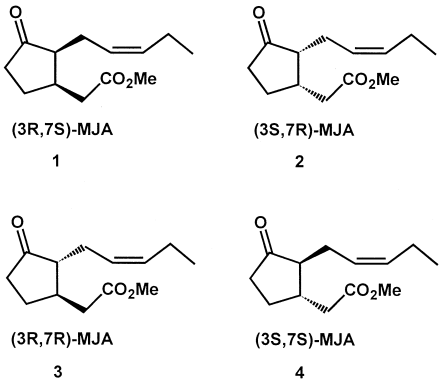


Fig. 3. Effects of MJA-isomers (**1**, **2**, **3**, and **4**) on baccatin III production in suspension cultures of *T. media*. Culture conditions were the same as in Fig. 1. Data are the means of four determinations. Vertical bars represent standard errors.

Hezari et al. (1997) suggest that the rate-limiting steps for paclitaxel production are located farther down the pathway than the cyclization step of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene. Srinivasan et al. (1996) speculate that baccatin III need not be a direct precursor of paclitaxel and that pacilitaxel production is limited by the ability of the cells to convert phenylalanine into phenylisoserine. Although the rate-limiting steps for baccatin III biosynthesis are not known, our results suggest that MJA isomers act similarly for both paclitaxel and baccatin III biosynthesis.

Vick and Zimmerman (1984) suggest that thermodynamically *epi*-jasmonates are less stable than jasmonates owing to a higher steric hindrance between the *cis* side chains and that they are easily epimerized to the *trans*-isomers. MJA may undergo epimerization under the physiological conditions present in the cells. For example, the weak activity of **4** for paclitaxel and baccatin III production may be due to its conversion to **2** within cells. To eliminate the possibility of epimerization, several research groups have synthesized stereochemically-locked derivatives of MJA by introduing the fluoro-group at C-7 (Taapken et al., 1994), the methyl-group at C-7 (Koda et al., 1995; Holbrook et al., 1997), and the methyl-group at C-3 (Ward et al., 1997), and have examined the effects of these compounds on various biological activities. Introduction of the methyl- and the even smaller fluoro-group, however, caused loss of activity, which suggests that stringent steric demands prevail in jasmonate-receptor interactions. In our experiments, it was not clear which C-7 isomer was responsible for the observed activity. Since the stereochemistry at C-3 remains unchanged in this epimerization and exogenously applied MJA was easily metabolized in plant cells (Sembdner and Parthier, 1993, and our unpublished results), we conclude that the optimum configuration is (3*R*,7*R*) and that, although the (3*R*)-configuration is not indispensable, the (7*R*)-configuration is more suitable for the promotion of both paclitaxel and baccatin III production, when exogenous MJA isomers are added to *T. media* cultures. This is the first report to show that the unnatural isomer (3*S*,7*R*)-MJA (**2**) has considerable promoting activity for both paclitaxel and baccatin III production.



4. Experimental

4.1. Chemicals

Preparation of a 46:54 mixture of 7-*epi*-MJA and MJA from commercially available MJA by C-7 epimerization with a base, and subsequent fractional distillation were reported previously (Seto et al., 1999). The four stereoisomers of MJA were separated as follows: A 46:54 mixture of 7-*epi*-MJA and MJA was subjected to HPLC in a silica column (Senshu Pak Silica-5251-N, 250 × 20 mm, Senshu Scientific). The eluent system was hexane:AcOEt (10:1) at a flow rate of 8 ml/min. Peaks were detected with a refractive index detector. Elution at the retention time (R_t) of 48 min afforded MJA as a colorless oil, and at R_t of 55 min gave 7-*epi*-MJA as a colorless oil. 7-*epi*-MJA and MJA were then individually resolved optically by HPLC, according to Okamoto and Nakazawa (1992). Separation was done on an optical column (Chiralpak AS, 250 × 10 mm, Daicel Chemical) with an eluent system of hexane:2-propanol (93:7) at a flow rate of 2 ml/min. Peaks were detected with a refractive index detector. Racemic 7-*epi*-MJA gave (3*R*,7*S*)-MJA [(+)-*epi*-MJA] (**1**) with the natural configuration at R_t = 19.0 min and (3*S*,7*R*)-MJA [(−)-*epi*-MJA] (**2**) at 30.8 min. Racemic MJA gave (3*S*,7*S*)-MJA [(+)-MJA] (**4**) at 18.4 min and (3*R*,7*R*)-MJA [(−)-MJA] (**3**) with the natural configuration at 22.8 min. The ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of the optically active compounds agree with previously published data (Kitahara et al., 1991; Nishida et al., 1985).

4.2. Cell culture conditions

Cell culture was done as reported elsewhere (Yukimune et al., 1996). Suspension cultures of *T. media* were established from embryo-derived callus tissue grown on WPM solid medium. Cultures were maintained in WPM liquid medium supplemented with 3% sucrose and 10 μM α-naphthylacetic acid. An ≈1 g fresh weight sample was inoculated to 20 ml of fresh WPM medium in a 100-ml Erlenmeyer flask and subcultured at 14-day intervals. Cultures were shaken on a gyratory shaker at 25°C in the dark at an agitation speed of 100 rpm. In the elicitation experiments, an approximately 2.5 g fresh weight (about 12 g/l calculated dry weight) sample was inoculated to 20 ml of fresh WPM medium supplemented with 3% sucrose and 10 μM α-naphthylacetic acid. The individual stereoisomers of MJA were added to separate cultures in 2.5 μl of methanol per ml of culture medium just after cell inoculation. An equal volume of methanol (50 μl) was added to control culture. Cells were harvested 14 days after treatment.

4.3. Extraction and analysis of taxanes

These were done in essentially the same manner as reported previously (Yukimune et al., 1996). Harvested cells were freeze-dried, powdered, then ultrasonicated for 30 min in MeOH. After centrifugation at 1700 × *g* for 10 min, a portion of the MeOH extract partitioned between CH_2Cl_2 and H_2O gave the CH_2Cl_2-soluble fraction. The extract was evaporated to dryness at 25°C, the residue then dissolved in *n*-hexane/acetone, 1:1 (vol/vol), and applied to a silica gel column with *n*-Hexane/acetone, 1:1 (vol/vol), as eluent. The eluted sample was evaporated to dryness at 25°C, then dissolved in 1 ml of MeOH; and an aliquot (40 μl) analyzed by HPLC.

A 1 ml portion of CH_2Cl_2 was added to 1 ml of the medium, and the whole mixed for 10 s, then centrifuged at 1700 × *g* for 10 min to extract the taxanes from the culture medium. The CH_2Cl_2-soluble fraction was collected, evaporated to dryness at 25°C, then dissolved in 1 ml of MeOH; and an aliquot (40 μl) analyzed by HPLC. Analyses were done on a reverse phase column (YMC pack ODS-AM, 5 μm, 300 × 4.6 mm). The eluent system, MeOH–0.1% TFA–MeCN (5.0:50.3:44.7), was run for 40 min and included a 15 min wash in MeOH; the flow rate was 1 ml/min. All chromatograms were plotted at 227 nm. Taxane contents were calculated on a dry weight basis. Paclitaxel and baccatin III were confirmed by FRIT-FAB LC-Mass spectrometry with a JMS-SX102A mass-spectrometer, and ^{1}H- and ^{13}C-NMR analyses in $CDCl_3$, respectively at 400 and 100 MHz, with a JEOL GSX-400 NMR spectrometer.

References

Abe, M., Shibaoka, H., Yamane, H., Takahashi, N., 1990. Protoplasma 156, 1.

Dathe, W., Rönsch, H., Preiss, A., Schade, W., Sembdner, G., Schreiber, K., 1981. Planta 153, 530.

Helmchen, G., Goeke, A., Lauer, G., Urmann, M., Fries, J., 1991. Angew. Chem. 102, 1079.
Hezari, M., Ketchum, R.E.B., Gibson, D.M., Croteau, R., 1997. Arch. Biochem. Biophys. 337, 185.
Holbrook, L., Tung, P., Ward, K., Reid, D.M., Abrams, S., Lamb, N., Quail, J.W., Moloney, M.M., 1997. Plant Physiol. 114, 419.
Ketchum, R.E., Gibson, D.M., Croteau, R.B., Shuler, M.L., 1999. Biotechnol. Bioeng. 62, 97.
Kitahara, T., Nishi, T., Mori, K., 1991. Tetrahedron 47, 6999.
Koda, Y., Kikuta, Y., Kitahara, T., Nishi, T., Mori, K., 1992. Phytochemistry 31, 1111.
Koda, Y., Takahashi, K., Kikuta, Y., Greulich, F., Toshima, H., Ichihara, A., 1996. Phytochemistry 41, 93.
Koda, Y., Ward, J.L., Beale, M.H., 1995. Phytochemistry 38, 821.
Menhard, B., Eisenreich, W., Hylands, P.J., Bacher, A., Zenk, M.H., 1998. Phytochemistry 49, 113.
Miersch, O., Meyer, A., Volkefeld, S., Sembdner, G., 1986. J. Plant Growth Regul. 5, 91.
Mirjalili, N., Linden, J.C., 1996. Biotechnol. Prog. 12, 110.
Nishida, R., Acree, T.E., Fukami, H., 1985. Agric. Biol. Chem. 49, 769.
Okamoto, M., Nakazawa, H., 1992. Biosci. Biotech. Biochem. 56, 1172.
Sembdner, G., Parthier, B., 1993. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 569.
Seto, H., Nomura, E., Fujioka, S., Koshino, H., Suenaga, T., Yoshida, S., 1999. Biosci. Biotech. Biochem. 63, 361.
Srinivasan, V., Ciddi, V., Bringi, V., Shuler, M.L., 1996. Biotechnol. Prog. 12, 457.
Taapken, T., Bleckert, S., Weiler, E.W., Zenk, M.H., 1994. J. Chem. Soc. Perkin. Trans. 1, 1439.
Ueda, J., Kato, J., 1982. Physiol. Plant 54, 249.
Vick, B.A., Zimmerman, D.C., 1984. Plant Physiol. 75, 458.
Ward, J.L., Gaskin, P., Beale, M.H., Sessions, R., Koda, Y., Wasternack, C., 1997. Tetrahedron 53, 8181.
Weiler, E.W., Albrect, T., Groth, B., Xia, Z.-Q., Luxem, M., Liss, H., Andert, L., Spengler, P., 1993. Phytochemistry 32, 591.
Yamane, H., Sugawara, J., Suzuki, Y., Shimamura, E., Takahashi, N., 1980. Agric. Biol. Chem. 44, 2857.
Yamane, H., Takagi, H., Abe, H., Yokota, T., Takahashi, N., 1981. Plant Cell Physiol. 22, 689.
Yukimune, Y., Tabata, H., Higashi, Y., Hara, Y., 1996. Nature Biotech. 14, 1129.