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Title

Biosynthesis of jasmonic acid in a plant pathogenic fungus, *Lasiodiplodia theobromae*

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Abstract

Jasmonic acid (JA) is a plant hormone that plays an important role in a wide variety of plant physiological processes. The plant pathogenic fungus, *Lasiodiplodia theobromae* also produces JA; however, the biosynthesis of JA in this fungus has yet to be explored. A feeding experiment incorporating ^{13}C-labeled sodium acetate—[1-^{13}C] and [2-^{13}C]—into *L. theobromae* revealed that JA in this fungus originates from a fatty acid synthetic pathway. The methyl ester of 12-oxo-phytodienoic acid (OPDA) was detected in the culture extracts of *L. theobromae* by GC-MS analysis. This finding indicates the presence of OPDA (a known intermediate of JA biosynthesis in plants) in *L. theobromae*. ^{2}H-NMR spectral data of JA produced by *L. theobromae* with the incorporation of [9,10,12,13,15,16-$^{2}H_6$]linolenic acid showed that five deuterium atoms remained intact. In plants, this is speculated to arise from JA being produced by the octadecanoid pathway. However, the observed stereoselectivity of the cyclopentenone olefin reduction in *L. theobromae* was contrary to that observed in plants. These data suggest that JA biosynthesis in *L. theobromae* is similar to that in plants, but differing in the facial selectivity of the enone reduction.

Key words

Lasiodiplodia theobromae; jasmonic acid; biosynthesis; octadecanoid pathway

1. Introduction

Lasiodiplodia theobromae is a pathogenic fungus that infects plants in tropical and subtropical regions of the world, causing considerable damage to crops during storage. This fungus is known to produce a variety of bioactive compounds (Aldridge et al., 1971). Previous studies in our laboratory have demonstrated the presence of various potato-tuber-inducing substances in a culture of *L. theobromae*: (–)-JA, theobroxide (and related compounds), and lasiodiplodins (Nakamori et al., 1994; Matsuura et al., 1998a; Matsuura et al., 1998b; Yang et al., 2000; Li et al., 2006; Takei et al., 2006). Among these compounds, theobroxide exhibits the most interesting biological activity, which effects not only potato-tuber-inducing activity in potato (*Solanum tuberosum*), but also induces flower bud formation in morning glory (*Pharbitis nil*) (Yoshihara et al., 2000). Encouraged by the unique biological activities that these compounds display, we have elucidated the biosynthesis of theobroxide (Li et al., 2006) and lasiodiplodins (Kashima et al., 2009a; Kashima et al., 2009b). Among the compounds described above, (–)-JA was first isolated from the culture of *L. theobromae* as a substance used to accelerate plant senescence in the 1970s (Aldridge et al., 1971). It has since been established that JA is a plant hormone that controls responses to environmental stresses and developmental events in flowering plants. JA plays an important role in coordinating plant defense responses with physiological stresses associated with herbivores and microbial pathogens. JA biosynthesis and its regulatory mechanism, has been thoroughly investigated (Wasternack, 2007). Recently, the presence of JA and the activity of allene oxide synthase (AOS), with respect to JA biosynthesis, was demonstrated in a model moss *Physcomitrella patens* (Oliver et al., 2009; Bandara et al., 2009).

In plants, JA is biosynthetically produced by the octadecanoid pathway as shown in Fig. 1 (Schaller and Stinzi, 2009). The first step in the octadecanoid pathway is the lipoxygenase (LOX)-catalyzed oxygenation of α-linolenic acid. In the specific case of JA biosynthesis, the hydroperoxidation takes place at C-13 of α-linolenic acid, and is effected by 13-LOX. The resulting hydroperoxide [13(*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT)] can be metabolized by AOS into an unstable allene oxide [12,13(*S*)-epoxyoctadecatrienoic acid (12,13-EOT)], in which cyclization is facilitated by allene oxide cyclase (AOC) to provide 12-oxo-phytodienoic acid (OPDA). OPDA is reduced by OPDA reductase 3 (OPR3) to yield 3-oxo-2-[(*Z*)-pent-2-enyl]-cyclopentane-1-octanoic acid (OPC-8:0). Three subsequent β-oxidation steps afford (+)-*iso*-JA [(3*R*,7*R*)-configuration], which is further epimerized at C-7 to provide (−)-JA [(3*R*,7*S*)-configuration]. Due to keto-enol interconversion, the *cis*-isomer, (+)-*iso*-JA, is easily converted into more stable *trans*-isomer, (−)-JA, for steric reasons.

As referred to above, the detailed mechanism of JA signaling and biosynthesis in plants has been elucidated. However, there are a little information about biosynthetic pathway and functions of JA in *L. theobromae*. Thakkar et al. reported that development of systemic acquired resistance (SAR) was restricted in the plants infected with *L. theobormae* due to deficiency of salicylic acid (SA) (2004). Inhibition of SA biosynthesis in the infected plants, which was caused by JA released from *L. theobromae*, might contribute the infection of this fungus to plants. In the case of *Pseudomonas syringae*, coronatine, a phytotoxin functioning like JA, was shown to induce suppression of SA-mediated defense system, and disease development in tomato (Uppalapati et al.,

2007)

In this work, we demonstrate that JA is synthesized *via* a fatty acid synthetic pathway in *L. theobromae*, which is supported by a ^{13}C labeling experiment. The incorporation of synthetic ^{2}H-labeled linolenic acid into *iso*-JA indicates that JA biosynthesis in *L. theobromae* is similar to that of plants, differing only in the facial selectivity of the cyclopentenone reduction (i.e., α- vs. β-hydride attack); the facial selectivity observed in plants is posited on the basis of the X-ray crystal structure data of tomato OPR3 (Breithaupt et al., 2006).

2. Results and Discussion

Lasiodiplodia theobromae was statically incubated in 1% potato-glucose medium at 29 °C for 7 days. ^{13}C-labeled sodium acetate ([1-^{13}C] and [2-^{13}C]) was administered to the culture at a concentration of 10 mM. The culture was filtered to separate the mycelia and supernatant after an additional 10 days of incubation. The aqueous layer was extracted with ethyl acetate (150 mL) and subsequently purified by chromatography to provide 3.9 mg/150 mL of [1-^{13}C]acetate-derived JA, and 2.8 mg/150 mL of [2-^{13}C]acetate-derived JA, respectively.

The $^{13}C\{^{1}H\}$-NMR spectrum of [1-^{13}C]acetate-derived JA showed enhanced signals at C-1, -3, -5, -7, -9, and -11. Meanwhile, the $^{13}C\{^{1}H\}$-NMR spectrum of JA derived from [2-^{13}C]acetate revealed intensified signals at C-2, -4, -6, -8, -10, and -12 (Table 1 and Fig. 2). Biosynthetically ^{13}C-labeled JA was converted to the methyl ester with ethereal diazomethane, and then the specific incorporation ratio was calculated by the relative intensity of the methyl ester signal normalized to 1.11% (natural abundance). The specific incorporation of ^{13}C generally observed was 1.3 to 5.7 atom% for [1-^{13}C]acetate-derived carbons and 4.5

to 7.8 atom% for [2-^{13}C]acetate-derived carbons. These data provide clear evidence that JA is produced through a fatty acid biosynthetic pathway in *L. theobromae*.

In plants, α-linolenic acid is transformed into JA through the octadecanoid pathway. We have been interested in whether OPDA—a key intermediate of the octadecanoid pathway—is present in the culture of *L. theobromae*; however, the presence of OPDA in this fungus has not been reported. In an attempt to test this hypothesis, the acetone extract of mycelia of *L. theobromae* was purified roughly by preparative TLC, and subsequently subjected to GC-MS analysis. In conclusion, OPDA was not detected as a free form, however the OPDA methyl ester was observed with the selected ion mode monitored at *m/z* 306 ($[M]^+$), 275 ($[M\text{-}OCH_3]^+$), and 238 (Fig. 3) (Laudert et al., 1996). The retention time associated with all of the selected ion peaks in this analysis was 87.2 min, which was same as that of the molecular ion peaks of an authentic (+)-*trans*-OPDA methyl ester prepared (i.e.; CH_2N_2, Et_2O) from natural OPDA. These data strongly suggested that *L. theobromae* produces natural occurring OPDA. JA production by way of a fatty acid synthetic pathway, and the presence of OPDA in *L. theobromae*, suggest that this fungus produces JA *via* OPDA. As previously stated, OPDA is an intermediate derived from α-linolenic acid by way of the known plant pathway.

Next, we attempted to demonstrate whether α-linolenic acid is also a precursor of JA in *L. theobromae*; therefore, [9,10,12,13,15,16-$^2H_6$]linolenic acid ([$^2H_6$]-LA) was synthesized according to Hungerford et al. (1998). [$^2H_6$]-LA was supplemented to a 7-day-old culture of *L. theobromae*, reaching a final concentration of 1 mM, and the fungus was incubated for an additional 10 days. Chromatographic separation of a residue from the culture filtrate of *L.*

theobromae gave *iso*-JA incorporating [$^2H_6$]-LA (1.5 mg). After methyl ester formation, the ^{2}H-NMR spectrum of the resulting *iso*-JA methyl ester was immediately taken. The ^{2}H-NMR data revealed the presence of five ^{2}H signals corresponding to H-3, -4α, -7, -9, and -10 (Fig. 4) (Seto et al., 1999). Analysis of the EI-MS data by comparison of $[M+4]^+$ ion peak (*m/z* = 228, [$^2H_4$]-JA) and $[M+5]^+$ ion peak (*m/z* = 229, [$^2H_5$]*iso*-JA) with the methyl ester of biosynthetically labeled and natural JA showed that 6.98% and 2.96% of the peak intensities were enhanced, respectively. Considering the isomerization, 9.94% of ^{2}H was enriched in the *iso*-JA derived from [$^2H_6$]-LA. This incorporation demonstrates that α-linolenic acid is a precursor in JA biosynthesis of *L. theobromae* (Fig. 5). The presence of *iso*-JA suggests that *iso*-JA is produced first and subsequently isomerized to JA in *L. theobromae*. Moreover, it is interesting that the positions of ^{2}H incorporation in [$^2H_6$]-LA derived *iso*-JA are in accordance with the positions speculated by the cyclization mechanism involved in reactions mediated by AOS and AOC in plants (Schaller and Stinzi, 2009). Accordingly, OPDA is most likely metabolized to JA through the reduction of the cyclopentenone moiety and subsequent β-oxidation of the side chain. While the reduction occurs prior to the β-oxidation in plants, the order of events (i.e., reduction or β-oxidation) are unknown in *L. theobromae*.

From a stereochemical perspective, the reductase could facilitate hydride attack from either the α- or β-face, which would determine the configuration at C-4 in [$^2H_6$]-LA derived *iso*-JA. In plants, the X-ray crystal structural data of tomato OPR3 suggests that the reduced flavin cofactor is placed on the opposite side of the OPDA side chain (i.e., α-face) (Breithaupt et al., 2006). The attack of a hydride from the α-face would result in the original proton attached at C-10 in OPC-8:0 to be in

the β-orientation. On the contrary, the ^{2}H-NMR spectrum of the [$^{2}H_{6}$]-LA derived *iso*-JA methyl ester indicated an α-orientation of ^{2}H-4. This supports the contention that the hydride attack from the reductase occurs on the β-face of the cyclopentenone plane at C-4; this is in contrast to that in plants. These data suggest that the cyclopentenone reduction mechanism in *L. theobromae* is different than that in plants.

Conclusions

This study demonstrates that JA biosynthesis in *L. theobromae* is similar to that in plants (e.g., cyclization). It is interesting that the cyclization mechanism in *L. theobromae* appears to be identical to that in plants, which was supported by feeding experiments of stable isotope-labeled compounds. Therefore, *L. theobromae* potentially has proteins, which have similar enzymatic activities to 13-LOX, AOS, and AOC. In stark contrast, the reduction of the cyclopentenone moiety differed between *L. theobromae* and plants. Highlighted by the case of gibberellin biosynthesis, there are large differences between plant and fungal genes involved in gibberellin biosynthesis. Fungi evolved a gibberellin biosynthetic pathway independently (Bömke et al., 2009). Considering the clear difference between the observed facial selectivity of the cyclopentenone reduction that was observed in *L. theobromae* and that in plants, it can be suggested that this fungus has an independently evolved JA biosynthesis pathway. The detailed mechanism of JA biosynthesis in *L. theobromae* requires further investigation. Additional studies aimed at determining the mechanism and specific enzymes involved for each biosynthetic step are required to fully define the JA biosynthetic route in *L. theobromae*. In addition, these studies could lend new insight into plant-microbe interactions on a fundamental level.

4.Experimental

4.1. General

Data were obtained with the following instruments: ^{1}H-NMR (270 MHz) and ^{13}C-NMR (67.8 MHz), Jeol JMN-EX270 FT-NMR spectrometer; ^{2}H-NMR (76.5 MHz), Bruker AMX500 FT-NMR spectrometer; EI-MS, Jeol JMS-T100GCV mass spectrometer; GC-MS, Varian 1200L GC/MS/MS system. The NMR shift values were referenced to residual solvent signals as follows; ^{1}H-NMR, $CDCl_3$ (δ_{1H} = 7.24 ppm); ^{2}H-NMR, $CHCl_3$ (δ_{2H} = 7.24 ppm); ^{13}C-NMR, $CDCl_3$ (δ_{13C} = 77.0 ppm).

4.2. Administration of ^{13}C-labeled sodium acetate in L. theobromae

Spores of *L. theobromae* were maintained on 1% potato-glucose agar medium at 30 °C and transferred at intervals of 6 months. A piece (approximately 1 cm^2) of agar bearing the spore-formed culture of *L. theobromae* was inoculated into a 500-ml Erlenmeyer flask containing 150 ml of a 1% potato-glucose medium. Each medium was statically incubated at 29 °C in the dark. After 7 days of incubation, [1-^{13}C]NaOAc acetate and [2-^{13}C]NaOAc (99 atom%, respectively) were dissolved in 1 ml of H_2O, which was supplemented to give a final concentration of 10 mM, and then the incubation continued for an additional 10 days. The 150 ml of 17-day-old culture in each administration experiment was filtered, made alkaline by the addition of 5% aqueous $NaHCO_3$, and extracted with 250 mL of EtOAc. The resultant aqueous layer was acidified by the addition of 30 ml of 6 M HCl, and then extracted with 300 ml of EtOAc. After the EtOAc extract was concentrated *in vacuo*, the resultant residue was purified by preparative SiO_2 gel TLC (*n*-hexane:EtOAc: acetic acid = 6:4:1, Merck, USA), and subsequently isolated by HPLC (TSK gel

ODS80Ts,TOSOH, 20 mm i.d. × 250 mm, 80% MeOH aqueous solution, 5.0 ml/min, retention time = 16.2 min). For the calculation of incorporated ratio, JA was converted to the methyl ester with ethereal diazomethane.

4.3. Identification of OPDA methyl ester in L. theobromae

Lasiodiplodia theobromae was statically cultured in a 500-ml Erlenmeyer flask containing 150 ml of a 1% potato-glucose medium at 29 °C for 17 days in the dark. The mycelia were soaked in acetone, and the extract was concentrated *in vacuo*. The resulting aqueous solution was extracted with 20 ml of EtOAc. After concentration, EtOAc extract (23 mg) was loaded onto silica gel TLC plate (Merck, USA), which was developed with $CHCl_3$. A band (Rf ≈ 0.3-0.4) was scraped, extracted with a mixed solution of MeOH and $CHCl_3$ (3:7), and concentrated *in vacuo*. The residue (1 mg) was dissolved in 100 µl of $CHCl_3$, and 1 µl of the solution was analyzed by GC-MS spectrometry.

The GC-MS spectrometer was a 1200L GC/MS/MS system (Varian, USA) equipped with a β-DEX fused silica capillary column (0.25 mm × 30 m, 0.2 mm film thickness, Supelco, USA). Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. The temperature of the ion source and vaporizing chamber were heated to 200 °C. The ionization voltage was 70 eV. The temperature gradient was initiated at 50 °C, was held isothermal for 1 min, and subsequently raised at a 10 °C/min to a final temperature of 190 °C, which was held for 80 min. The retention times of (+) and (−)-*trans*-OPDA methyl esters were 87.2 min and 88.8 min, respectively. Standard racemic *trans*-OPDA methyl ester was prepared according to the method of Bandara et al. (2009).

4.4. Administration of ^2H-labeled linolenic acid in L. theobromae

[9,10,12,13,15,16-$^{2}H_6$]linolenic acid was synthesized according to the method of Hungerford et al. (1998). One hundred mL of a 1% potato-glucose medium was prepared in a 300-mL Erlenmeyer flask. Incubation of *L. theobromae* was carried out as described above. After 7 days of incubation, [9,10,12,13,15,16-$^{2}H_6$]linolenic acid was dissolved in 500 μl of a 2 mM NH_4OH aqueous solution, which was supplemented to give a final concentration of 1 mM. The incubation was continued for an additional 10 days. The filtrate of the culture, in which an equal volume of 2 M HCl was added, was extracted with 300 ml of EtOAc. After concentration, 120 mg of the extract was subjected to preparative TLC (SiO_2 gel, *n*-hexane:EtOAc: acetic acid=60:40:1, Merck, USA). The resultant residue (8.9 mg) was purified by HPLC (TSK gel ODS80Ts,TOSOH, 20 mm i.d. × 250 mm, 80% MeOH aqueous solution, 5.0 ml/min, retention time = 16.2 min) to give 1.5 mg of pure *iso*-JA. After methyl ester formation with ethereal diazomethane, the ^{2}H-NMR of the methyl ester of *iso*-JA was taken immediately. The intensities of $[M+4]^+$ ion peak (*m/z* = 228, [$^{2}H_4$]-JA) and $[M+5]^+$ ion peak (*m/z* = 229, [$^{2}H_5$]*iso*-JA) with the methyl ester of biosynthetically labeled JA were given as the percent relative to the intensity of the molecular ion peak of natural JA (*m/z* =224) as 100 % in EI-MS data.

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Table 1

Incorporation of ^{13}C-labeled sodium acetate ([1-^{13}C] and [2-^{13}C]) into jasmonic acid.

Position	δ_c (ppm)	^{13}C-atom%	
		[1-^{13}C]acetate	[2-^{13}C]acetate
1	172.4	2.41	1.80
2	38.8	0.69	5.49
3	38.0	2.63	1.73
4	25.5	0.86	4.62
5	37.7	3.00	1.19
6	219.0	0.65	7.81
7	54.0	1.30	1.17
8	27.2	1.06	5.89
9	124.9	2.16	0.73
10	134.1	0.63	4.45
11	20.6	5.68	1.79
12	14.1	1.27	5.70
OMe	51.6	1.11	1.11

Figure legends

Fig. 1. The octadecanoid pathway.

Fig. 2. Labeling patterns of jasmonic acid following incorporation of ^{13}C-labeled sodium acetate into *L. theobromae*.

Filled square and circle symbols represent [1-^{13}C] and [2-^{13}C] in ^{13}C-labeled sodium acetate, respectively.

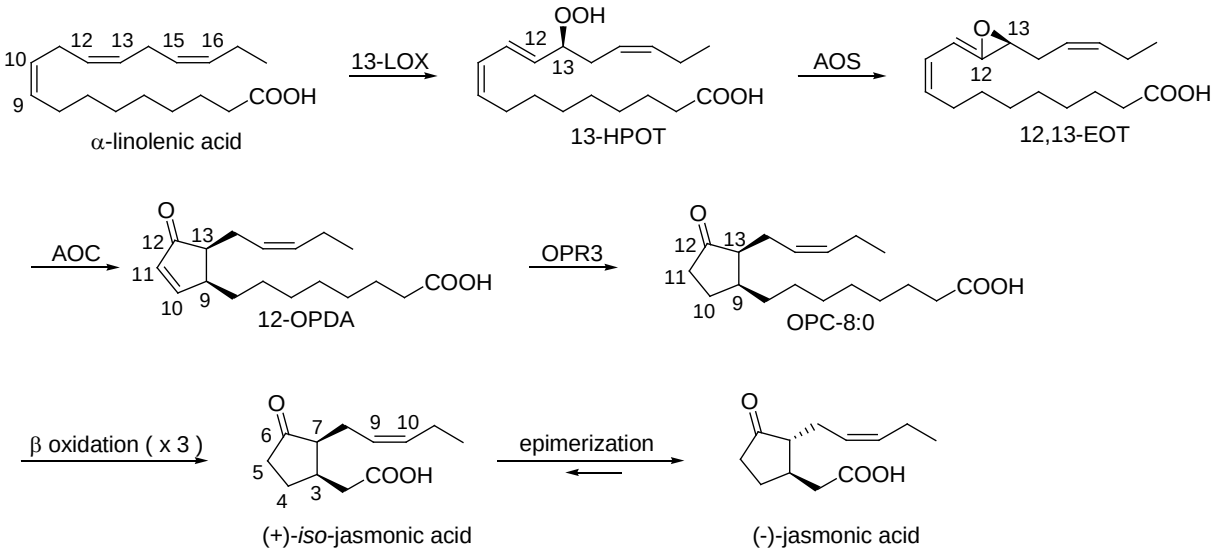
Fig. 3. Segment of selected ion monitored chromatograms of GC-MS spectral data of OPDA methyl ester produced by *L. theobromae*.

Column; a chiral β-DEX fused silica capillary column (0.25 mm i.d. × 30 m)

Fig. 4. ^{1}H-NMR spectrum of natural jasmonic acid (A) and ^{2}H-NMR spectrum of biosynthetically labeled *iso*-jasmonic acid derived from [9,10,12,13,15,16-$^{2}H_6$]linolenic acid (B).

Fig. 5. The presumed jasmonic acid biosynthetic pathway in *L. theobromae*.

Fig. 1. Tsukada et al.



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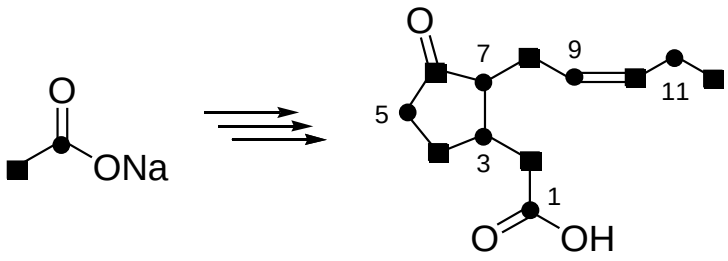

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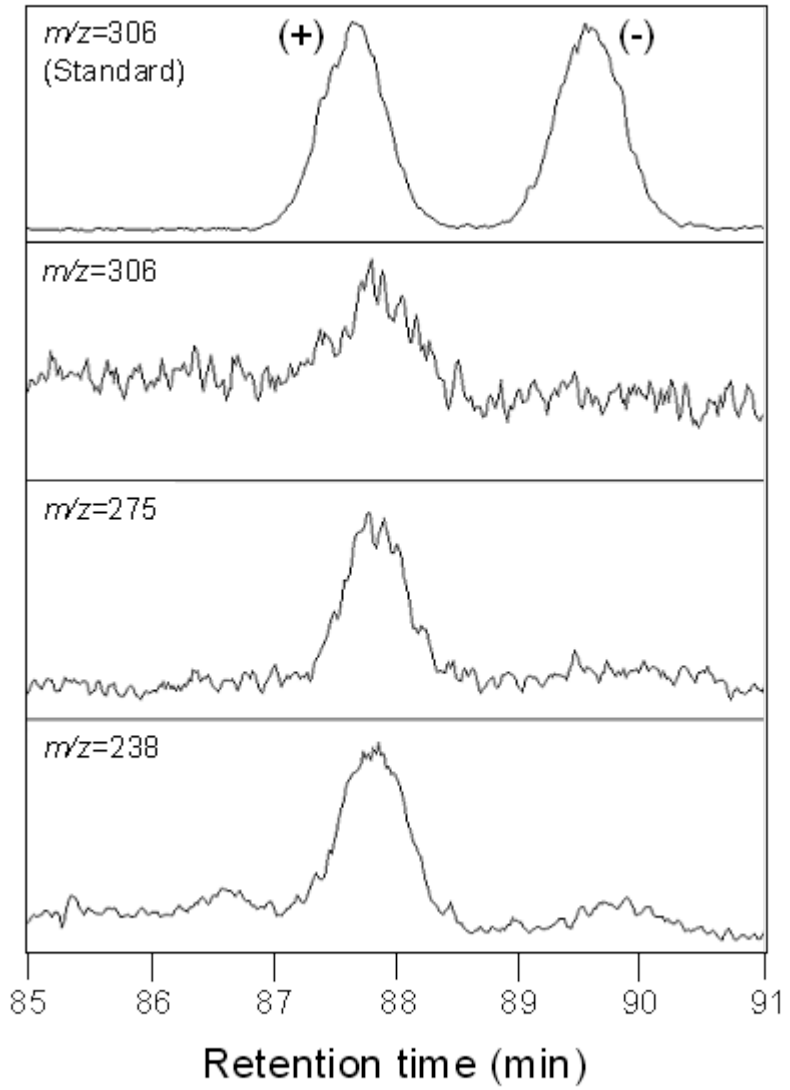

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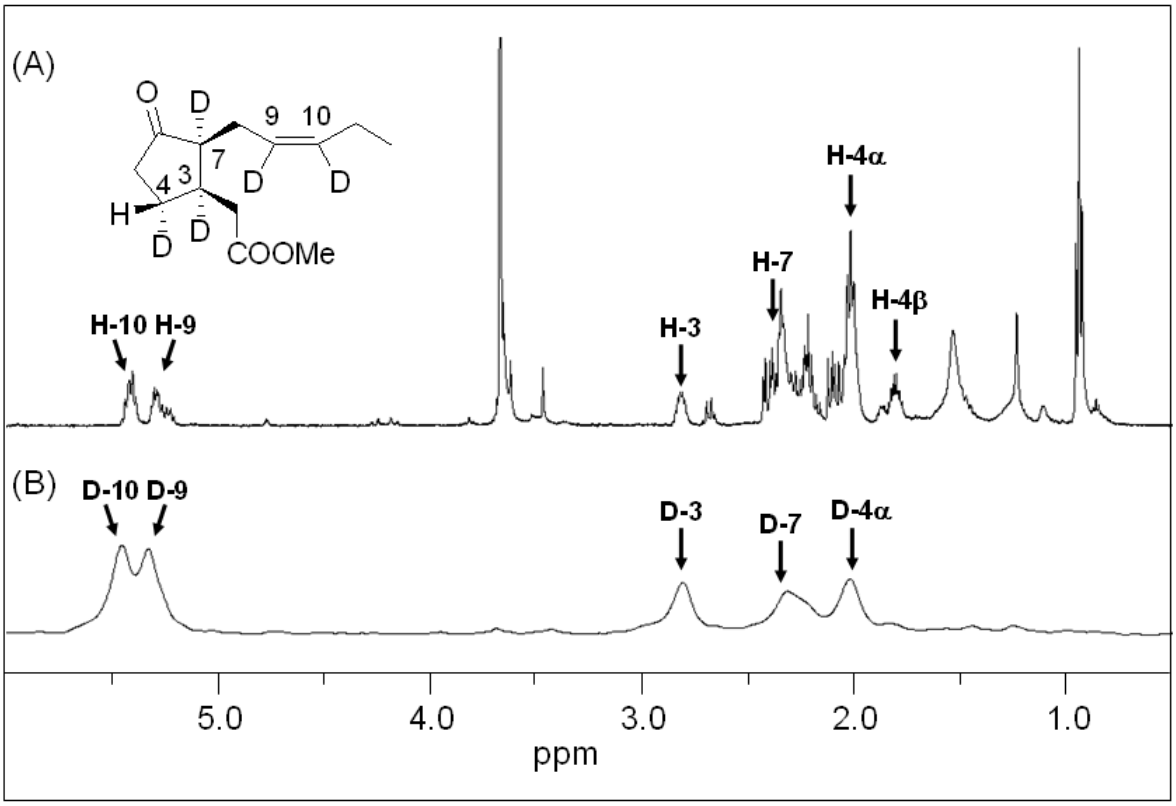
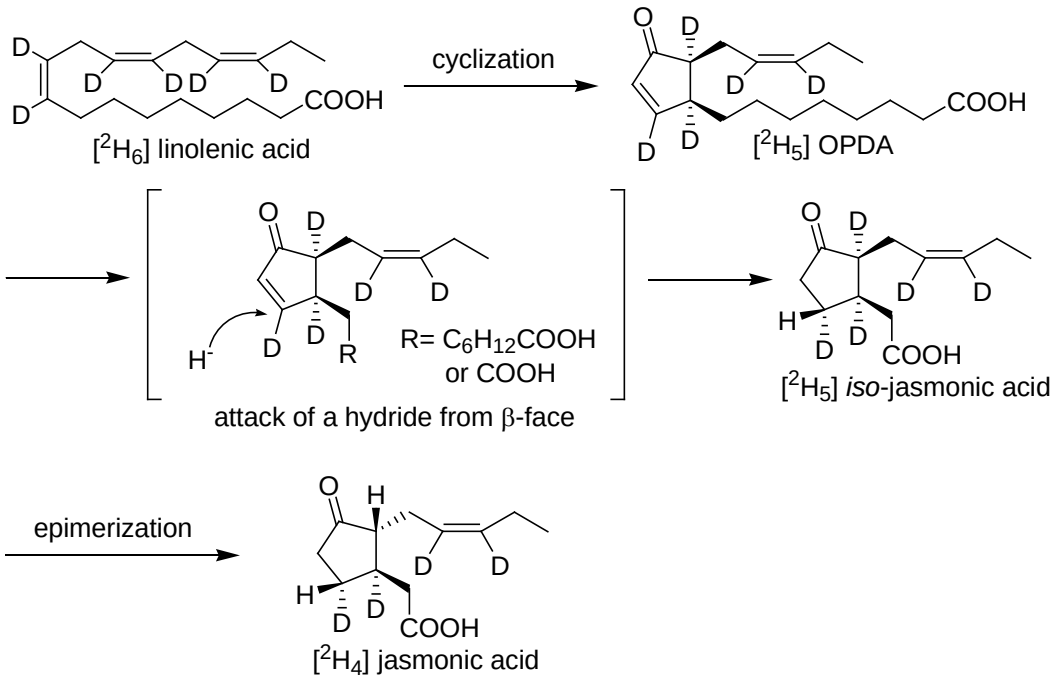
Fig. 5. Tsukada et al.