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Preparation and Biological Activity of Molecular Probes to Identify and Analyze Jasmonic Acid-binding Proteins

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Several types of jasomonic acid (JA) derivatives, including JA–amino acid conjugates, a JA–biotin conjugate, a JA–dexamethasone heterodimer, and a JA–fluoresceine conjugate, were prepared as candidates for molecular probes to identify JA-binding proteins. These JA derivatives, excepting the JA–fluoresceine conjugate, exhibited significant biological activities in a rice seedling assay, a rice phytoalexin-inducing assay, and/or a soybean phenylalanine ammonia-lyase-inducing assay. These JA derivatives could therefore be useful probes for identifying JA-binding proteins. The activity spectra of the prepared compounds were different from each other, suggesting that different types of JA receptors were involved in the perception of JA derivatives in the respective bioassays.

Key words: jasmonic acid derivative; jasmonic acid-binding protein; rice seedling assay; phenylalanine ammonia-lyase-inducing assay; rice phytoalexin-inducing assay

Jasmonic acid (JA) has been shown to be involved in the plant responses to many types of biotic and abiotic stress.[1)] JA also affects such diverse processes as fruit ripening, production of viable pollens, tuber formation, and root growth.[2)] These JA actions are considered to start with the recognition of JA by specific receptors which are likely to be proteins. However, there is little information on JA receptors. To identify JA receptors, we need to isolate the JA-binding proteins as candidates for JA receptors, and to analyze their functions in detail. Since it is useful to develop molecular probes for JA-binding proteins, we prepared several types of JA derivatives: JA–amino acid conjugates, a JA–dexamethasone (Dex) heterodimer, a JA–biotin conjugate, and a JA–fluoresceine isothiocyanate (FITC) conjugate.

JA–amino acid conjugates are candidates for useful molecular probes, because it is possible to prepare [^{3}H]JA–amino acid conjugates with high specific radioactivity in few steps by using commercially available [^{3}H]amino acids.[3)] These radioactive probes would be useful to monitor JA-binding proteins and determine their JA-binding activity. A JA–Dex heterodimer would be useful as bait in a yeast three-hybrid system for detecting JA-binding proteins. The feasibility of the yeast three-hybrid system for detecting small ligand–protein receptor interaction has been demonstrated by Licitra and Liu.[4)] A JA–biotin conjugate would be a useful tool for the purification of JA-binding proteins, because a convenient purification system can be constructed by utilizing the interaction between the biotin moiety of a JA–biotin conjugate and avidine.[5)] A JA–FITC conjugate would also be useful to identify and analyze the function of JA-binding proteins.[6)] This will make it possible to observe the binding between this type of compound and JA-binding proteins.

If the prepared compounds were to exhibit JA-like activity in a bioassay system, they would possibly function as mimics of JA in the system. This means that biologically active synthesized compounds could be useful molecular probes for isolating and/or characterizing JA-binding proteins. We report here the preparation and biological activities of potential molecular probes for JA-binding proteins. Of the JA derivatives synthesized in this study, (−)-JA–leucine and (−)-JA–isoleucine conjugates have been reported to be bioactive in the induction of sakuranetin, one of major rice phytoalexins, and in the inhibition of rice shoot

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Abbreviations: JA, jasmonic acid; Dex, dexamethasone; FITC, fluoresceine isothyocyanate; CD, circular dichroism; JA-Me, methyl jasmonate; MeCN, acetonitrile; MeOH, methanol; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*′-dimethylformamide; PAL, phenylalanine ammonia-lyase; EtOH, ethanol

growth.[7] However, no extensive studies on the biological activities of JA derivatives have been reported.

Materials and Methods

Instruments. NMR spectra were determined by using tetramethylsilane as an internal standard with an AC-P 300 spectrometer (300 MHz, Bruker). Mass spectra of the synthesized compounds were determined with an API 3000 mass detector (Applied Biosystems Instruments, Foster City, CA, USA), a JMS SX 102 EI MS detector (JEOL, Tokyo, Japan), or a Voyager DE-STR MALDI-TOF mass detector (Applied Biosystems Instruments). Circular dichroism (CD) spectra of the synthesized compounds were determined with a J-720 spectropolarimeter (JASCO, Tokyo, Japan).

Preparation of (±)-JA. To a solution of (±)-methyl jasmonate (JA-Me; 5.2 g, 24 mmol) in methanol (MeOH; 50 ml), a 5 M KOH aqueous solution (7.5 ml) was added while stirring. Stirring was continued at room temperature for 8 h, before the reaction mixture was neutralized with 6 M aqueous HCl and concentrated *in vacuo*. The residue was dissolved in H_2O (50 ml), and the solution adjusted to pH 2–3 with 6 M aqueous HCl, before being extracted with ethyl acetate (50 ml, 3 times). The combined organic layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was chromatographed in a column of silica gel (150 g of Wako C-300 gel; Wako Pure Chemical Industries Ltd., Tokyo, Japan), using a mixture of *n*-hexane–ethyl acetate–acetic acid (14:6:1, v/v/v) as an eluent, to give (±)-JA (3.8 g, 18 mmol, 75% yield).

*Preparation of a (±)-JA–*N*-hydroxysuccinimide ester.* To a mixture of (±)-JA (1 g, 4.8 mmol) in acetonitrile (MeCN; 10 ml) and *N*-hydroxysuccinimide (1.5 g, 13 mmol) in *N*,*N*′-dimethylformamide (DMF; 7.5 ml), dicyclohexylcarbodiimide (DCC; 1.25 g, 6.1 mmol) in MeCN (5 ml) was added while stirring. Stirring was continued at room temperature for 48 h, before water (20 ml) was added to decompose the excess DCC, and the reaction mixture was filtered to remove the dicyclohexylurea. The filtrate was concentrated *in vacuo* and purified in a column of silica gel (100 g of Wako C-300 gel), using a mixture of *n*-hexane–ethyl acetate (1:3, v/v) as an eluent, to give the [(±)-JA]–*N*-hydroxysuccinimide ester (1.2 g, 3.9 mmol, 81% yield).

*General procedure for preparing JA–*L*-amino acid conjugates.* [(±)-JA]–*N*-hydroxysuccinimide ester (200 mg, 0.65 mmol) in MeCN (10 ml) was mixed with a solution of 1 mmol amino acid (glycine, *β*-alanine, L-alanine, L-valine, L-leucine, or L-isoleucine) in H_2O (10 ml). To this mixture, triethylamine (1 ml, 8.5 mmol) was added while stirring. Stirring was continued overnight at room temperature, the resulting reaction mixture then being concentrated *in vacuo*. The concentrate was dissolved in 0.1 M aqueous HCl (50 ml) and extracted with ethyl acetate (50 ml, three times). The combined ethyl acetate layers were dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was chromatographed in a column of silica gel (10 g of Wako C-300 gel), using a mixture of ethyl acetate–acetic acid (99:1, v/v) as an eluent, to give the [(±)-JA]–L-amino acid conjugate. The reaction of the [(±)-JA]–*N*-hydroxysuccinimide ester with L-alanine, L-valine, L-leucine and L-isoleucine afforded a mixture of diastereoisomeric *N*-jasmonoyl–L-amino acid conjugates, [(+)-JA–L-amino acid and (−)-JA–L-amino acid]. The respective diastereomers were separated by high-performance liquid chromatography (HPLC) in an ODS 4253D column (250 mm long, 10 mm internal diameter; Senshu Scientific, Inc., Tokyo Japan), elution being performed with 42.5% (JA–alanine), 50% (JA–valine), or 57.5% (JA–leucine and JA–isoleucine) aqueous MeOH containing 0.1% acetic acid at a flow rate of 3 ml min^{-1}. The products were monitored at 210 nm, their retention times being as follows: [(+)-JA]–L-alanine, 28.0 min; [(−)-JA]–L-alanine, 34.5 min; [(+)-JA]–L-valine, 28.8 min; [(−)-JA]–L-valine, 39.2 min; [(+)-JA]–L-leucine, 20.0 min; [(−)-JA]–L-leucine, 25.3 min; [(+)-JA]–L-isoleucine, 19.9 min; and [(−)-JA]–L-isoleucine, 24.8 min. The stereochemistry of the JA–L-amino acid conjugates was determined by analyzing the CD data shown next. The [(+)-JA]– and [(−)-JA]–L-amino acid conjugates showed positive and negative Cotton effects, respectively.[3] The yields and analytical data for the reaction products were as follows. [(±)-JA]–glycine (182 mg, 0.45 mmol, 69% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.49 (1H, m), 5.49 (1H, m), 5.30 (1H, m), 4.10 (2H, d, $J = 3.9$ Hz), 0.95 (3H, t, $J = 7.5$ Hz); MS (API 3000) m/z: 266.1 $[M - H]^-$. [(±)-JA]–*β*-alanine (132 mg, 0.31 mmol, 47% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.49 (1H, m), 5.30 (1H, m), 3.49 (2H, d, $J = 3.6$ Hz), 1.59 (3H, t, $J = 7.5$ Hz) 0.95 (3H, t, $J = 7.5$ Hz); MS (API 3000) m/z: 280.4 $[M - H]^-$. [(+)-JA]–L-alanine (155 mg, 0.36 mmol, 55% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.18 (1H, m), 5.48 (1H, m), 5.29 (1H, m), 4.63 (1H, m), 1.59 (3H, d, $J = 7.9$ Hz), 0.95 (3H, t, $J = 7.5$ Hz); MS (API 3000) m/z: 280.4 $[M - H]^-$; CD (c 0.0281, MeOH) $\Delta\varepsilon_{max}$ (nm): +2.28 (296). [(−)-JA]–L-alanine (169 mg, 0.39 mmol, 60% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.35 (1H, m), 5.46 (1H, m), 5.28 (1H, m), 4.63 (1H, m), 1.59 (3H, d, $J = 7.9$ Hz), 0.95 (3H, t, $J = 7.5$ Hz); MS (API 3000) m/z: 280.4 $[M - H]^-$; CD (c 0.0281, MeOH) $\Delta\varepsilon_{max}$ (nm): −1.78 (299). [(+)-JA]–L-valine (223 mg, 0.47 mmol, 72% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.10 (1H, m), 5.47 (1H, m), 5.26 (1H, m), 4.62 (1H, m), 1.60 (1H, m), 0.88–1.09 (9H); MS (API 3000) m/z: 308.4 $[M - H]^-$; CD (c 0.0309, MeOH) $\Delta\varepsilon_{max}$ (nm): +2.39 (298). [(−)-JA]–L-valine (158 mg, 0.33 mmol, 51% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.08 (1H, m), 5.51 (1H, m), 5.40 (1H, m), 4.63 (1H, m), 1.63 (1H, m), 0.91–1.07 (9H); MS (API 3000) m/z: 308.4

$[M-H]^-$; CD (*c* 0.0309, MeOH) $\Delta\varepsilon_{max}$ (nm): −2.53 (296). [(+)-JA]–L-leucine (171 mg, 0.34 mmol, 53% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.95 (1H, m), 5.47 (1H, m), 5.29 (1H, m), 4.69 (1H, m), 0.95–1.08 (9H); MS (API 3000) *m/z*: 322.4 $[M-H]^-$; CD (*c* 0.0323, MeOH) $\Delta\varepsilon_{max}$ (nm): +2.49 (297). [(−)-JA]–L-leucine (168 mg, 0.34 mmol, 52% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.86 (1H, m), 5.47 (1H, m), 5.35 (1H, m), 4.64 (1H, m), 0.91–1.03 (9H); MS (API 3000) *m/z*: 322.4 $[M-H]^-$; CD (*c* 0.0323, MeOH) $\Delta\varepsilon_{max}$ (nm): −2.03 (297). [(+)-JA]–L-isoleucine (178 mg, 0.36mmol, 55% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.13 (1H, m), 5.46 (1H, m), 5.29 (1H, m), 4.66 (1H, m), 0.90–1.05 (9H); MS (API 3000) *m/z*: 322.4 $[M-H]^-$; CD (*c* 0.0323, MeOH) $\Delta\varepsilon_{max}$ (nm): +0.19 (298). [(−)-JA]–L-isoleucine (184 mg, 0.37 mmol, 57% yield): ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.13 (1H, m), 5.47 (1H, m), 5.30 (1H, m), 4.66 (1H, m), 0.91–1.01 (9H); MS (API 3000) *m/z*: 322.4 $[M-H]^-$; CD (*c* 0.0323, MeOH) $\Delta\varepsilon_{max}$ (nm): −0.43 (299).

Preparation of the JA–Dex heterodimer. Dex primary amine (8.5 mg, 0.016 mmol) prepared as described previously[4] and [(±)-JA]–*N*-hydroxysuccinimide ester (5 mg, 0.016 mmol) were dissolved in ethanol (EtOH; 10 ml). To this solution, triethylamine (0.1 ml, 0.85 mmol) was added while stirring, stirring being continued at room temperature for 48 h. The resulting reaction mixture was concentrated *in vacuo* and purified in a column of silica gel (1 g of Wako C-300 gel), using a mixture of *n*-hexane–ethyl acetate (1:2, v/v) as the eluent, to give a mixture of diastereomeric JA–Dex heterodimers (7.8 mg, 0.011 mmol, 67% yield), [(+)-JA]–Dex and [(−)-JA]–Dex. Since separation of these diastereomers by reverse-phase HPLC was not successful, the mixture of diastereomeric heterodimers was used in the subsequent bioassays without further purification. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 6.16 (1H, m), 6.11 (1H, br. s), 5.43 (1H, m), 5.24 (1H, m), 1.05 (3H, t, $J = 7.5$ Hz); MS (API 3000) *m/z*: 728.3 $[M+H]^+$.

Preparation of the JA–biotin conjugate. 6-(biotinoylamino)hexanoic acid hydrazide (102 mg, 0.20 mmol) and [(±)-JA]–*N*-hydroxysuccinimide ester (108 mg, 0.30 mmol) were dissolved in dry DMF (10 ml). To this solution, triethylamine (0.1 ml, 0.85 mmol) was added while stirring, stirring being continued at room temperature for 48 h. The resulting reaction mixture was concentrated *in vacuo* and purified in a column of silica gel (600 mg of Wako C-300 gel), using a mixture of chloroform–MeOH (15:1, v/v) as an eluent, to give a mixture of diastereomeric JA–biotin conjugates (89 mg, 0.1 mmol, 49% yield), [(+)-JA]–biotin and [(−)-JA]–biotin. Since separation of the diastereomers by reverse-phase HPLC was not successful, this mixture of the diastereomeric isomers was used in the subsequent bioassays without further purification. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 5.45 (1H, m) 5.57 (1H, m), 4.27 (1H, m), 4.48 (1H, m), 0.99 (3H, t, $J = 7.5$ Hz); MS (MALDI-TOF) *m/z*: 564.3 $[M+H]^+$.

Preparation of the JA–FITC conjugate. FITC (312 mg, 0.80 mmol) and 1,5-diaminopentane (106 mg, 1.5 mmol) were dissolved in dry DMF (20 ml). To this solution, triethylamine (1 ml, 8.5 mmol) was added while stirring, stirring being continued at room temperature for 20 min. The resulting reaction mixture was concentrated *in vacuo*, and purified in a column of silica gel (15 g of Wako C-300 gel), using a mixture of chloroform–MeOH–acetic acid (50:49:1, v/v/v) as an eluent, to give 1-amino-5-(*N*-fluoresceine thiocarbamoyl)-pentane (362 mg, 0.87 mmol). 1-Amino-5-(*N*-fluoresceine thiocarbamoyl)-pentane (180 mg, 0.47 mmol) and [(±)-JA]–*N*-hydroxysuccinimide ester (150 mg, 0.48 mmol) were dissolved in MeOH (10 ml). To this solution, triethylamine (1 ml, 8.5 mmol) was added while stirring, stirring being continued at room temperature for 48 h. The resulting reaction mixture was concentrated *in vacuo* and purified on a column of silica gel (30 g of Wako C-300 gel), using a mixture of chloroform–MeOH–acetic acid (80:19:1, v/v/v) as an eluent, to give the [(±)-JA]–FITC conjugate (285 mg, 0.44 mmol, 95% yield). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: 7.88 (1H, s), 6.35 (1H, m), 5.19 (1H, m) 5.06 (1H, m) 0.73 (3H, t, $J = 7.5$ Hz). MS (MALDI-TOF) *m/z*: 684.0 $[M+H]^-$.

Rice seedling assay. Dwarf rice seedlings (*Oryza. sativa* L. cv. Tan-ginbouzu) were used for this assay which was carried out according to the micro-drop method at 30 °C under continuous white light (5 W m^{-2}) as reported by Murakami.[8] The second leaf sheath length was measured 4 days after a sample application. Six seedlings were used for each sample.

Momilactone A-inducing assay. This assay was carried out according to the method reported by Rakwal *et al.*[9] with slight modifications. Rice plants (*O. sativa* L. cv. Nipponbare) were grown in a greenhouse, and the immature top leaves of the plants at the fifth or sixth-leaf stage were collected. Leaf disks (6 mm internal diameter) were prepared from these leaves by using a cork borer. Each leaf disk was floated on a solution containing a test sample in distilled water (200 μl) in a well of a 96-well micro-plate (Iwaki, Tokyo, Japan) and incubated at 25 °C under continuous white light (5 W m^{-2}) for 72 h. Five leaf disks were used to estimate the momilactone A-inducing activity of a test sample. Leaf disks that had been treated with the test sample were boiled with 70% aqueous MeOH for 20 min. Each 70% aqueous MeOH extract was directly analyzed by LC-MS-MS with an HP 1100 HPLC instrument (Hewlett Packard, Palo Alto, CA, USA) fitted with a Pegasil ODS column (150 mm long, 4.6 mm internal diameter; Senshu Scientific, Inc.), elution being performed with 80% aqueous MeCN containing 0.1% formic acid at a flow

rate of 1 ml min^{-1}. An API-3000 instrument with APCI inlet system was also used for the LC-MS-MS analysis, momilactone A being analyzed in the positive-ion mode with nitrogen as a collision gas. Momilactone A was detected in combination at m/z 315/271 in the multiple-reaction monitoring mode.

PAL-inducing assay. This assay was carried out by the method reported by Recourt *et al.*[10] with several modifications. Approximately 5-ml aliquots of calli of *Glycine max* were transferred to 300-ml Erlenmeyer flasks containing 90 ml each of a Gamborg B5 medium[11] and cultured on a rotary shaker (79 rpm) at 25 °C in the dark for 1 week. The cell suspension 3 days after being transferred to a fresh medium was used for the subsequent experiments. One-ml aliquots of the cell suspension were transferred to the respective wells of a 24-well micro-plate (Iwaki, Tokyo, Japan) and incubated on a rotary shaker (79 rpm) at 25 °C in the dark. After 48 h, a 10-μl aliquot of the stock solution of a test compound (10 mM in a 10% aqueous MeOH solution) was added to each well. Each reaction mixture was incubated for 72 h under the same conditions. The reaction mixture was then filtered off and the resulting cells were sonicated in 5 ml of a borate buffer (0.1 M borate and 20 mM 2-mercaptoethanol at pH 8.8) on ice. After centrifugation (3000 *g*, 5 min), the supernatant was assayed for PAL activity. To 1.5 ml of the supernatant, 0.5 ml of 7.25 mM of an L-phenylalanine solution was added, and the reaction mixture was incubated at 37 °C for 36 h. The reaction was stopped by adding 100 μl of 2 M aqueous HCl, and 1-ml aliquots of the reaction mixture were extracted with ethyl acetate (0.5 ml, three times). After concentrating the combined ethyl acetate layers *in vacuo*, the residue was analyzed to quantify *trans*-cinnamic acid by HPLC in a Pegasil ODS column (250 mm long, 4.6 mm internal diameter; Senshu Scientific, Inc.), eluting with 62% aqueous MeOH containing 0.1% acetic acid at a flow rate of 1 ml min^{-1}. *Trans*-cinnamic acid was monitored at 280 nm.

Results and Discussion

Preparation of the JA derivatives

JA–amino acid conjugates were prepared by reacting [(±)-JA]–*N*-hydroxysuccinimide ester with the amino acids, glycine, β-alanine, L-alanine, L-valine, L-leucine, and L-isoleucine, in the presence of triethylamine. The reactions of [(±)-JA]–*N*-hydroxysuccinimide ester with L-alanine, L-valine, L-leucine and L-isoleuscine afforded a diastereomeric mixture of each amino acid conjugate. This diastereomeric mixture was subjected to ODS-HPLC to separate the [(+)-JA]– and [(−)-JA]–amino acid conjugates. Under these conditions, the respective [(+)-JA]–L-amino acid diastereomers were eluted before the corresponding [(−)-JA]–L-amino acid–diastereomers. The JA–Dex heterodimer was prepared by the base-catalyzed reaction of [(±)-JA]–*N*-hydroxysuccinimide ester with Dex primary amine which had been prepared from Dex-acid and 1,10-diaminodecane. The JA–biotin conjugate was prepared by the base-catalyzed reaction of [(±)-JA]–*N*-hydroxysuccinimide ester with 6-(biotinoylamino)hexanoic acid hydrazide. The JA–FITC conjugate was prepared by the base-catalyzed reaction of [(±)-JA]–*N*-hydroxysuccinimide ester with 1-amino-5-(*N*-fluoresceine thiocarbamoyl)-pentane which had been prepared by the reaction of FITC with 1,5-diaminopentane in the presence of triethylamine. The structures of these synthesized JA-derivatives are shown in Fig. 1.

Biological activities of the synthesized JA derivatives

The activities of the JA derivatives were examined by three bioassays, all the results being shown in Table 1. The JA–amino acid conjugates in the rice seedling assay generally showed a clear inhibitory effect on the second leaf sheath elongation at a dosage of 10 nmol $plant^{-1}$. [(±)-JA]–glycine, [(±)-JA]–β-alanine, and [(+)-JA]– and [(−)-JA]–L-valine exhibited almost the same inhibitory effect as that of (±)-JA. [(+)-JA]– and [(−)-JA]–L-alanine, [(−)-JA]–L-alanine, [(+)-JA]– and [(−)-JA]–L-leucine, and [(+)-JA]– and [(−)-JA]–L-isoleucine were slightly less active than (±)-JA. The [(±)-JA]–Dex heterodimer and [(±)-JA]–biotin conjugate revealed slight but significant inhibitory activity at a dosage of 10 nmol $plant^{-1}$. The [(±)-JA]–FITC conjugate did not show any significant inhibitory activity. It is noteworthy that the [(+)-JA]– and [(−)-JA]–L-amino acid conjugates revealed almost the same activity, suggesting that the JA receptor involved in JA-induced growth inhibition similarly perceived both the [(+)-JA]– and [(−)-JA]–L-amino acid conjugates. It was also found that both (+)- and (−)-JA-Me exhibited an inhibitory effect on the second leaf sheath elongation of rice seedlings, the (+)-form being slightly less active than the (−)-form.[12]

The effects of the JA derivatives on the production of the major phytoalexin in rice, momilactone A, were examined by using the immature top leaves of rice plants at the fifth- or sixth-leaf stage. [(−)-JA]–L-alanine, [(−)-JA]–L-valine, and [(−)-JA]–L-leucine were fairly active in the momilactone A-inducing assay, their respective activity being 25%, 70%, and 75% of that of (±)-JA at a concentration of 5×10^{-4} M. The other conjugates did not show any significant momilactone A-inducing activity at a dosage of 5×10^{-4} M. It has been reported that [(−)-JA]–L-valine and [(−)-JA]–L-leucine were identified as natural compounds from rice plants.[13] These results strongly suggest that the JA receptor involved in momilactone A production recognized the stereochemical difference between the (−)- and (+)-jasmonoyl moieties in the JA–L-amino acid conjugates, showing that it preferred the [(−)-JA]–L-amino acid conjugates to the corresponding diastereomeric [(+)-JA]–L-amino acid conjugates.

The flavonoid compound, sakuranetin, is another major phytoalexin in rice. Tamogami *et al.*[7] have

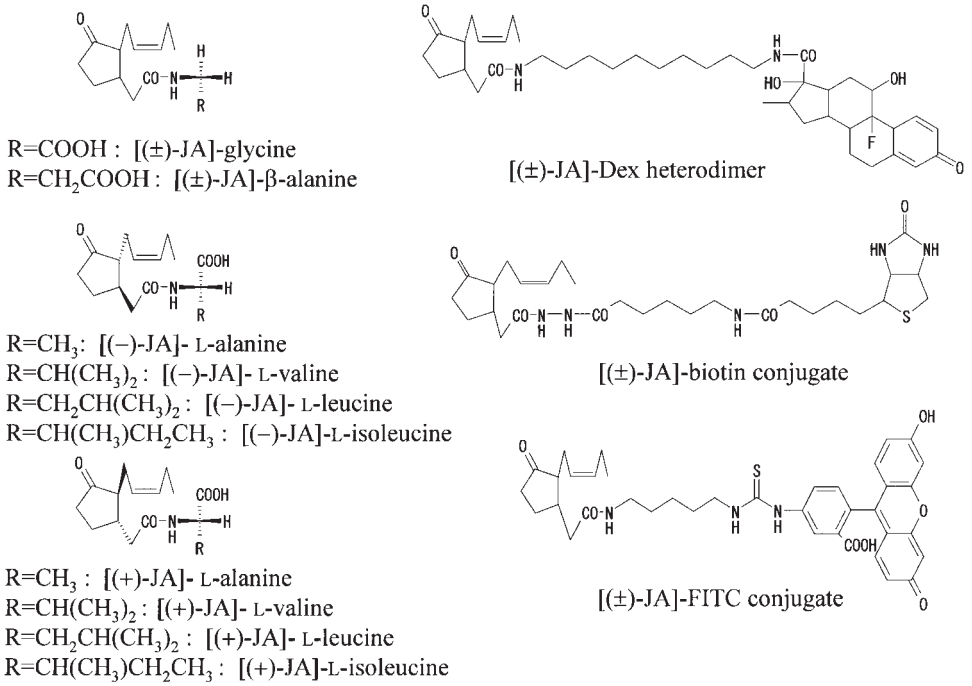

Fig. 1. Structures of the Synthesized JA Derivatives.

Table 1. Effects of the Synthesized JA Derivatives and Their Related Compounds on the Growth of Rice Seedlings, Production of Momilactone A in Immature Rice Leaves, and Production of *trans*-cinnamic Acid in Suspension-cultured *G. max* Cells

Compound	Length of 2nd leaf sheath of rice seedlings (mm)[a]	Production of momilactone A in rice leaves (ng disk^{-1})[b]	Production of *trans*-cinnamic acid in *G. max* cells [ng (mg fr wt)$^{-1}$][c]
Control	12.0 ± 0.4	1.5 ± 0.3	0.4 ± 0.1
(±)-JA	5.0 ± 0.3	60.7 ± 2.2	153.7 ± 20.9
[(±)-JA]–glycine	5.5 ± 0.6	1.1 ± 0.4	109.9 ± 1.6
[(+)-JA]–L-alanine	6.4 ± 0.3	n.d.	58.8 ± 2.0
[(−)-JA]–L-alanine	6.7 ± 0.8	32.7 ± 4.4	143.5 ± 10.4
[(±)-JA]–*β*-alanine	6.5 ± 0.4	n.d.	99.3 ± 9.2
[(+)-JA]–L-valine	4.8 ± 0.5	0.2 ± 0.0	48.8 ± 4.6
[(−)-JA]–L-valine	4.8 ± 0.9	42.7 ± 1.1	231.4 ± 43.1
[(+)-JA]–L-leucine	6.5 ± 0.4	0.1 ± 0.0	35.1 ± 2.5
[(−)-JA]–L-leucine	6.0 ± 0.8	44.2 ± 5.5	100.2 ± 17.1
[(+)-JA]–L-isoleucine	6.5 ± 0.4	n.d.	33.2 ± 7.8
[(−)-JA]–L-isoleucine	6.0 ± 0.6	n.d.	47.3 ± 7.9
Dex	12.0 ± 0.4	n.d.	8.7 ± 2.3
[(±)-JA]–dex	10.0 ± 0.3	n.d.	20.7 ± 2.5
Biotin	13.0 ± 0.5	n.d.	1.6 ± 0.4
[(±)-JA]–biotin	9.0 ± 0.5	n.d.	16.0 ± 2.0
FITC	10.0 ± 0.4	4.7 ± 1.0	34.6 ± 9.2
[(±)-JA]–FITC	12.0 ± 0.7	n.d.	4.8 ± 0.4

[a] The dose of each compound was 10 nmol plant^{-1}. Each value represents the mean ± standard error from 6 replicates.
[b] The dose of each compound was 5×10^{-4} M. Each value represents the mean ± standard error from 5 replicates.
n.d.: not detected.
[c] The concentration of each compound was 10^{-4} M. Each value represents the mean ± standard error from 4 replicates.

reported that, in a sakuranetin-inducing assay with rice leaves, [(−)-JA]–L-leucine, [(−)-JA]–L-isoleucine, and [(−)-JA]–L-phenylalanine were more active than (−)-JA, while the corresponding diastereomeric [(+)-JA]–L-amino acid conjugates were inactive. It was thus suggested that the receptors involved in the production of sakuranetin as well as momilactone A strictly recognized the stereochemical difference between the (−)- and (+)-jasmonoyl moieties in the JA–L-amino acid conjugates.

The effects of the JA derivatives on the PAL-inducing activity in suspension-cultured soybean cells were determined. Such JA–amino acid conjugates as [(±)-JA]–glycine, [(±)-JA]–*β*-alanine, [(−)-JA]–L-alanine,

[(−)-JA]–L-valine, and [(−)-JA]–L-leucine were highly active, their activity being 64.5–150.7% of that of (±)-JA. On the other hand, the activity of [(+)-JA]–L-alanine, [(+)-JA]–L-valine, [(+)-JA]–L-leucine, and [(+)-JA]– and [(−)-JA]–L-isoleucine was 21.4–38.8% of that of (±)-JA. The [(±)-JA]–Dex heterodimer and [(±)-JA]–biotin conjugate were slightly but significantly active, their respective activity being 10.2% and 13.2% of that of (±)-JA. The [(±)-JA]–FITC conjugate was almost inactive. It should be noted that the [(−)-JA]–L-amino acid conjugates were more active than the corresponding [(+)-JA]–L-amino acid conjugates, although the [(+)-JA]–L-amino acid conjugates were still active in the PAL-inducing assay. These results suggest that the receptor involved in JA-induced PAL induction in *G. max* perceived a wide range of JA derivatives, but recognized the stereochemical difference between the (−)- and (+)-jasmonoyl moieties in the JA–L-amino acid conjugates.

It is noteworthy that the [(−)-JA]–L-amino acid conjugates were highly active in these three bioassays. Tamogami *et al.*[7] have also reported that some [(−)-JA]–L-amino acid conjugates were more polar than (−)-JA and therefore seemed to permeate less into plant cells, although the former were more active than (−)-JA in the sakuranetin-inducing assay in rice. These facts suggest that the [(−)-JA]–L-amino acid conjugates were active *per se* in the plant cells; in other words, the biological activity of the [(−)-JA]–L-amino acid conjugate was not due to (−)-JA released by hydrolytic enzymes.

We prepared in this study JA–L-amino acid conjugates, a JA–biotin conjugate, a JA–Dex heterodimer, and a JA–FITC conjugate as candidates for molecular probes to identify JA-binding proteins. These JA derivatives, except the JA–FITC conjugate, exhibited significant biological activities in the rice seedling assay, rice momilactone A-inducing assay, and/or soybean PAL-inducing assay. The activity spectra of the prepared JA derivatives were different from each other, suggesting that different types of JA receptor functioned in the respective bioassays. The prepared compounds with JA-like activity could be useful molecular probes for isolating and analyzing JA-binding proteins.

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