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Metabolism of 5′α,8′-cycloabscisic acid, a highly potent and long-lasting abscisic acid analogue, in radish seedlings

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Abstract—We synthesized 5′α,8′-cycloabscisic acid (CycloABA), a highly potent and long-lasting abscisic acid (ABA) analogue, by a different method from that reported before. CycloABA fed to radish seedlings had more metabolic tolerance than ABA. The major metabolite of CycloABA was the glucose conjugate, which was the minor metabolite of ABA. The 8′-hydroxylated metabolite and its cyclized isomer, which were major metabolites of ABA, were not found as metabolites of CycloABA. The present results suggest that the highly potent and long-lasting activity of CycloABA is caused by resistance to ABA 8′-hydroxylase, and that CycloABA is partially metabolized to the glucose conjugate by ABA glucosyltransferase.

1. Introduction

Abscisic acid [(1*S*)-(+)-ABA] is an essential mediator in triggering plant responses to environmental stresses such as desiccation and freezing.[1–4] Biosynthesis, transportation, and metabolic inactivation of ABA have to be appropriately controlled in order to produce adaptive responses to changes in temperature and moisture. The mechanism of ABA metabolism remains obscure, particularly at the molecular level. Several metabolites of ABA have been isolated from various plant materials, and feeding experiments using labeled ABA have uncovered the metabolic pathways involving ABA in plants.[1–4] There are two major pathways, the oxidation and conjugation pathways, in ABA metabolism in plants (Fig. 1).[1–4] The oxidation pathway, which is initiated by the 8′-hydroxylation of ABA, is more intrinsic to plant metabolism than the conjugation pathway, which seems to be a reversible inactivation reaction sequence used to regenerate free ABA. The ABA 8′-hydroxylase is believed to be a microsomal cytochrome P-450 monooxygenase,[5] although no protein or genetic information about the 8′-hydroxylase has ever been reported. The first oxidative metabolite 8′-hydroxy-ABA (8′-HOABA) is so unstable that it can only be isolated as a borate complex,[6] which spontaneously isomerizes in vitro to a more stable tautomer, phaseic acid [PA].[7] The half-life of 8′-HOABA was 4 h at pH 7 and 25 °C. At equilibrium, the 8′-HOABA/PA ratio was 2:98 at all pH levels.[7] The biological activity of 8′-HOABA seems to be more than 1/10 that of ABA,[6,8] whereas the activity of PA is less than 1/10–1/100 that of ABA in most of the assays.[1–4] This data indicates that the isomerization of 8-HOABA to PA is as significant as the 8-hydroxylation of ABA in effecting ABA inactivation. The glucosyl conjugation of ABA is another major ABA metabolic pathway in plants. Since free ABA can be regenerated from its conjugates, this may be the transported form, acting as a root-to-shoot signal that releases free ABA to the target cells.[9] Recently, Xu et al. reported cloning and characterizing the ABA-specific glucosyltransferase gene from UDP-glucosyltransferase homologsue obtained from adzuki bean seedlings.[10] The oxidation/conjugation ratio in ABA metabolism varies amongst species.[11]

ABA analogues that we designed to resist or inhibit ABA metabolism were developed as potent regulators of plant growth, and for use as probes to investigate the molecular mechanism of ABA metabolism.[12] 8′-Trifluoro-ABA and 8′-difluoro-ABA were developed as long-lasting analogues that can resist 8′-hydroxylation.[13,14] 8′-Methoxy-ABA and 3′-fluoro-ABA were

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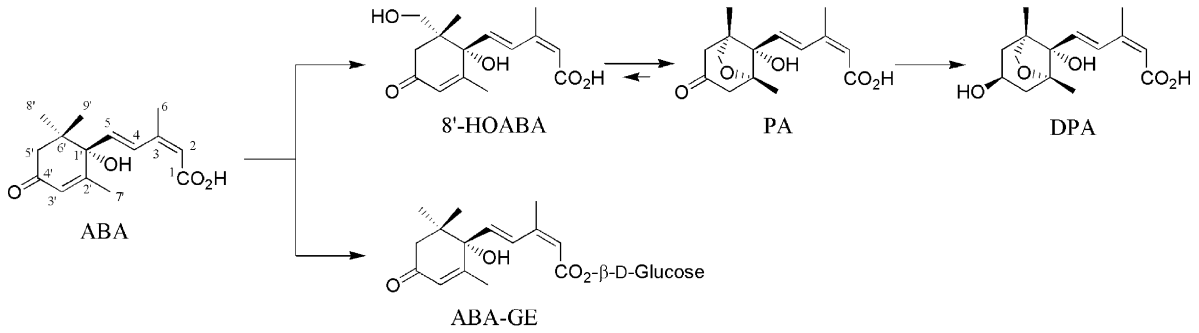

Figure 1. Major metabolic pathways involving ABA in plants.

designed as analogues which resist isomerization of 8′-HOABA to PA.[15,16] 8′-Methylene-ABA and 8′-methylidyne-ABA were synthesized as suicide inhibitors of 8′-hydroxylase.[17,18] Most of these analogues showed stronger activity than ABA. In the rice seedling elongation inhibition assay, 8′-trifluoro-ABA was especially potent, exhibiting stronger and longer-lasting activity than ABA, by a factor of 40. This suggested that 8′-trifluoro-ABA is a potent agonist for ABA receptors, as well as a metabolically stable analogue of ABA. We can use 8′-trifluoro-ABA for physiological, molecular biological, and biochemical studies of ABA metabolism. However, since the synthesis of 8′-trifluoro-ABA is not as easy as that of other analogues, it is difficult for suppliers to provide enough of it.

In order to investigate how the conformational behavior of the cyclohexenone ring in ABA affects the biological activity of ABA, 5′α,8′-cycloabscisic acid (CycloABA, Fig. 2) was developed as a mimic of a conformer of ABA.[19,20] Its bioactivity was close to that of 8′-trifluoro-ABA, suggesting strongly that CycloABA is a metabolically stable ABA analogue. The synthetic yield of CycloABA was very low since we prepared it from 8′-fluoro-ABA, which we synthesized in an earlier investigation.[21] However, knowing the structure of CycloABA should enable us to develop a new pathway which will be easier than the one involving 8′-trifluoro-ABA. CycloABA is physicochemically extremely close to ABA because no heavy atom is added to or subtracted from the ABA structure; therefore, our discussion about the effect of CycloABA can focus on the spatial and chemical properties of C-8′, which is oxidized by ABA 8′-hydroxylase. Metabolism of CycloABA in plants has never been investigated; however, its metabolic profile is necessary in order to investigate the mechanism of ABA metabolism using CycloABA as a molecular probe. In the present study, we developed a fast and easy ABA metabolism assay system using radish seedlings, and examined the metabolism of CycloABA using this system.

2. Results and discussion

2.1. Synthesis of CycloABA

We previously reported the synthesis of CycloABA via 8′-fluoroabscisic acid, which required many steps for preparation, and only provided a low yield (0.02% from an initial precursor).[19] Therefore, we reconsidered the synthetic route in order to increase the yield; the new route is shown in Figure 3. Dimethylbenzoquinone was converted to the monoacetal **1**, which was treated with trimethylsulfoxonium iodide and sodium hydride to give the cyclopropanoid **2**. The reaction of **2** with alkynyl lithium gave **3** whose relative configuration is (2*RS*,1′*S**,5′*R**,6′*S**) and its diastereomer whose relative configuration is (2*RS*,1′*S**,5′*S**,6′*R**), in a ratio of 1:2. Reduction, de-protection and oxidation of a mixture of **3** gave the diketone **4**. A Wittig reaction with **4** afforded the methyl ester **5**. The basic hydrolysis of **5** gave racemic CycloABA, which was optically purified by chiral HPLC to give CycloABA in a 1.4% yield from **1**.

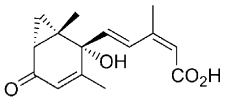

Figure 2. Structure of CycloABA.

2.2. ABA metabolism assay system

In previous studies, the metabolism of ABA was examined using plants (bean shoots,[22] tomato shoots,[23] and duckweed[24]) and using cell-suspension cultures (rice[25] and maize[5]). The metabolism assays using plant materials are of great advantage in a discussion about the results obtained in relation to the physiology of plants. However, it is inferior to the assay system using cells in constructing a stable system, in feeding test compounds, and in extracting and purifying their metabolites. We constructed a new assay system with radish (*Raphanus sativus*) seedlings in order to rapidly and easily investigate the metabolism of ABA analogues. Thirty radish seedlings (7-day-old, 5–6 g fresh weight) were used; they efficiently absorbed ABA (1.0 mg) from an aqueous solution (10 mL) via their roots over 1 day, and then effectively converted it to metabolites over 2 more days, producing an absorption ratio of 86%. The metabolites and unmetabolized ABA were obtained from methanol extracts of the radish seedlings. Acidic compounds were extracted with EtOAc from acidic aqueous solutions of methanol extracts, while glucose conjugates were analyzed as tetraacetates from acetylated methanol extracts. Detection and quantification of compounds was performed using HPLC with an ODS column (Table 1). The amount of in vivo ABA in radish seedlings without

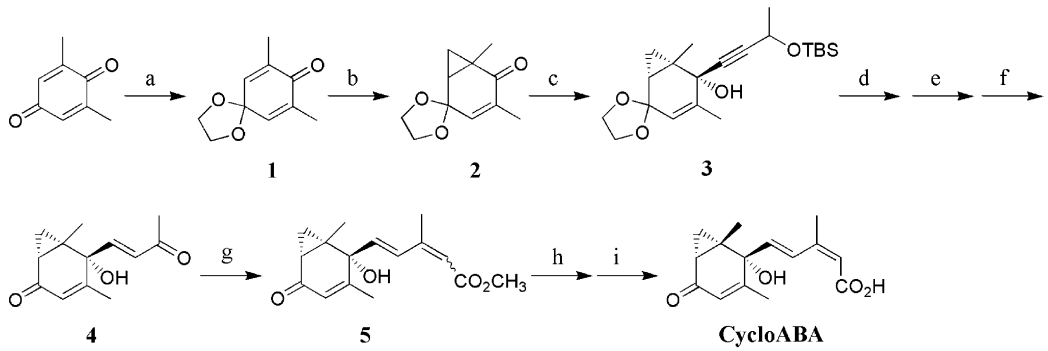

Figure 3. Synthetic route for CycloABA production. Reagents: (a) $HOCH_2CH_2OH$, $HC(OCH_3)_3$, *p*-TsOH; (b) $(CH_3)_3S(O)I$, NaH; (c) 1-methyl-2-propynyl TBS ether, *n*-BuLi; (d) $Na(CH_3OCH_2CH_2O)_2AlH_2$; (e) AcOH, THF, H_2O; (f) MnO_2; (g) $(CF_3CH_2O)_2P(O)CH_2CO_2CH_3$, $KN(TMS)_2$; (h) NaOH in MeOH; (i) HPLC with Chiralpak AD-H. Compound **3** was obtained as a diastereomeric mixture, and **4** and **5** were obtained as a racemic mixture.

Table 1. The recovery of ABA and its metabolites from radish seedlings and media

	Amount (mg)				
	ABA	PA	DPA	ABA-GE	Total
Seedlings	0.24±0.02	0.31±0.05	0.12±0.02	0.15±0.02	0.82
Medium	0.14±0.03	0.09±0.03	Trace	Trace	0.23
Total	0.38	0.40 (0.42)[a]	0.12 (0.13)[a]	0.15 (0.07)[a]	1.05 (1.00)[a]

[a] ABA equivalent.

ABA application was 0.2 μg per gram of fresh weight, which is negligible compared to that achieved by the application of 1 mg of ABA, ca. 200 μg per gram of fresh weight. Therefore, in this investigation we did not use a radiolabeled compound, which would usually be employed for examining metabolism at a physiological concentration and for seeking very minor metabolites. Figure 4 shows the ratios of ABA-related compounds found in radish seedlings fed 1.0 mg of ABA: PA, 50%; DPA, 16%; ABA-GE, 9%; and unmetabolized ABA, 25%. This result means that the conversion ratio of the applied ABA to the metabolites was 75%, and that the main metabolic pathway was the oxidation pathway. In this assay, 30 μg or more of ABA was incorporated into each radish seedling (ca. 0.2 g fresh weight). Although this is an extraordinary quantity for a radish seedling, 75% of the ABA incorporated was metabolized over 3 days and the appearance of the plant was normal, like those cultivated in media free of ABA; the one exception being their slower transpiration. This suggests that the ABA 8′-hydroxylase and glucosyltransferase are highly activated in a radish seedling, or strongly induced by ABA, and that susceptibility of the seedling to ABA as a growth inhibitor is relatively low, being consistent with the fact that the accumulation of ABI5, which is the bZIP transcription factor in ABA signaling and is necessary to inducing ABA activity, was induced when ABA and drought- or salt stress were applied within a narrow developmental time interval following germination.[26] Seven-day-old radish seedlings should be past the initial stages of seedling establishment during which high endogenous ABA levels and strong ABA responses of plant cells induce developmental arrest under unfavorable environmental conditions. Thus, our assay system would enable us to examine metabolism of ABA analogues with little influence by ABA activity, and with the advantage of using intact plants that are easily cultivated and handled.

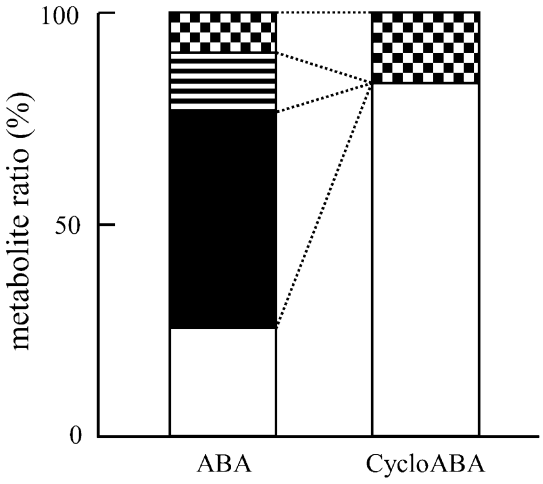

Figure 4. Metabolites profiles of ABA and CycloABA in radish seedlings: unmetabolized ABA (open), PA (closed), DPA (stripes), ABA-GE (dots) in the left bar; unmetabolized CycloABA (open), and CycloABA-GE (dots) in the right bar.

2.3. Biological activity of CycloABA in radish seeds and seedlings

We examined the inhibitory effect of CycloABA on radish seed germination and seedling growth, in addition to reexamining these factors in rice seedling elongation and lettuce seed germination assays (Table 2). In the rice assay, the IC_{50} value of CycloABA was 0.1 μM, whereas that of ABA was 3.8 μM. In the lettuce assay, the IC_{50} value of CycloABA was 1.2 μM, whereas that of ABA was 4.3 μM. These agreed with the previous results;[13] CycloABA was more effective in rice seedlings than in lettuce seeds. We considered whether this resulted from the difference in the main metabolic pathway of ABA in lettuce seeds versus rice seedlings. In fact, the main pathway in lettuce seeds was reported to be the conjugation pathway which CycloABA cannot resist.[27] In the radish seed germination assay, the IC_{50} value of CycloABA was 10 μM, while that of ABA was 30 μM;

this ratio is similar to that observed in the lettuce germination assay. On the other hand, the inhibitory effect of CycloABA on the growth of 5-day-old radish seedlings was very weak, although still stronger than that of ABA (data not shown). These results suggest that the main ABA metabolism of radish seeds may be the conjugation pathway like the lettuce seeds, and that radish seedlings may be insensitive to ABA as a growth inhibitor, as mentioned above.

2.4. Metabolism of CycloABA

Metabolism of CycloABA was examined by the above ABA metabolism assay system with radish seedlings (Table 3). CycloABA was incorporated into the seedlings at a rate of 64% of the applied amount (1 mg), which was less than that of ABA (86%). This may have been caused by the stronger inhibition of transpiration by CycloABA compared to that by ABA. Significant oxidative products of CycloABA were not found using HPLC with an UV-detector, whereas the glucose conjugate of CycloABA (CycloABA-GE) was found as a major metabolite. Since the sum of the unmetabolized CycloABA and CycloABA-GE was nearly equal to the amount of CycloABA incorporated, the other metabolites should be a negligible amount. Figure 4 shows a CycloABA metabolism profile: CycloABA-GE, 14%; and unmetabolized CycloABA, 86%. This means that CycloABA is more difficult to metabolize than ABA, and that it was mainly converted to CycloABA-GE. The net amount of CycloABA-GE was equivalent to that of ABA-GE (Tables 1 and 2), although incorporation of CycloABA into the seedlings was less than that of ABA, meaning that the ABA glucosyltransferase activity increased to inactivate CycloABA that was not inactivated by ABA 8′-hydroxylase. The complete resistance of CycloABA to 8′-hydroxylation suggests that the biological activity of CycloABA depends on the ABA glucosyltransferase activity of a tested plant.

There are at least two possible explanations for the resistance of CycloABA to 8′-hydroxylation. First, either 8′-hydroxylase has no affinity for CycloABA, or the C-8′ part of CycloABA is not enclosed within the catalytic site of 8′-hydroxylase owing to the different conformation of ABA from CycloABA. In a previous investigation, we designed CycloABA as a mimic of a conformer of ABA in order to investigate the ring conformation requirement of ABA for inducing biological activity.[19] A cyclopropane ring was fused to the cyclohexenone ring of ABA in order to fix the orientation of the geminal methyl groups, C-8′ and C-9′, in ABA. ABA has two preferred half-chair conformers; the more preferred half-chair is that with the axial side chain, axial C-8′ and equatorial C-9′ (Fig. 5), and the less preferred half-chair is that with the equatorial side chain, equatorial C-8′ and axial C-9′. The most preferred conformer of CycloABA is a boat form with the axial-like side-chain, the axial-like C-8′ and equatorial-like C-9′, although it has the axial-like C-8′ and equatorial-like C-9′ independent of the side-chain orientation owing to the fused cyclopropane ring (Fig. 5). The orientation of the C-8′ is a little different in CycloABA from that in ABA. The fact that CycloABA showed the same or stronger activity than ABA in all of the bioassays tested means that CycloABA is an agonist for the ABA receptor. This suggests that the fixed orientation of C-8′ and C-9′ in CycloABA is nearly equal to the orientation of those of ABA in an ABA–receptor complex. If the substrate specificity of 8′-hydroxylase is close to that of the ABA receptor, CycloABA will bind to the active site of 8′-hydroxylase. In this case we require the other reason for the oxidative stability of C-8′ in CycloABA.

The second possibility is that the 8′-C-H bond of CycloABA is more difficult to cleave than that of ABA. Although the mechanism of hydroxylation of P-450 monooxygenases remains unclear in detail, the radical cleavage of the C–H bond is believed to be the rate-determining step in the rebound mechanism.[28] This means that the resistance to radical cleavage results in resistance to the hydroxylation. The C atoms in C–H bond formation in cyclopropane are considered to have an sp^2 hybrid orbital character rather than an sp^3.[29] This suggests that the C–H bond in cyclopropane is stronger than that in other alkanes.[29,30] In fact, the C–H bond dissociation energy of cyclopropane was estimated to be higher than that of ethane by ca. 2 kcal mol^{-1} from experimental kinetic data.[31] We examined theoretically the C–H bond dissociation enthalpies in cyclopropane and ethane (Table 4). The C–H bond

Table 2. The IC_{50} values of ABA and CycloABA in bioassays

	IC_{50} in assay (μM)		
	Radish seed germination	Lettuce seed germination	Rice seedling elongation
ABA	30	4.3	3.8
CycloABA	10	1.2	0.1

Table 3. The recovery of CycloABA and its metabolite from radish seedlings and media

	Amount (mg)		
	CycloABA	CycloABA-GE	Total
Seedlings	0.53 ± 0.05	0.20 ± 0.00	0.73
Medium	0.36 ± 0.05	Trace	0.36
Total	0.89	0.20 (0.09)[a]	1.09 (0.98)[a]

[a] CycloABA equivalent.

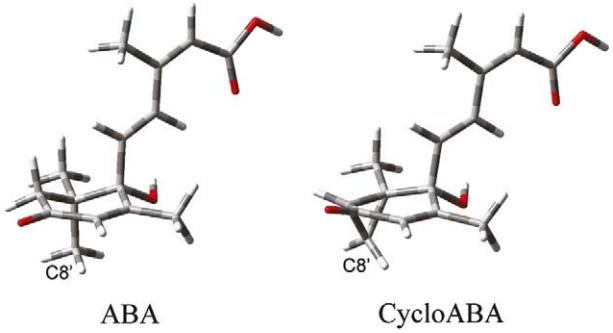

Figure 5. The most preferred conformer of ABA and CycloABA.

Table 4. The C–H bond dissociation enthalpies at 298 K in cyclopropane and ethane calculated by the QCISD(T)/6-311++G(2d,p)//B3LYP/6-311G(d,p)

	Cyclopropane	Ethane	Δ
Energy (kcal mol^{-1})	105.6	98.0	7.6

dissociation enthalpy at 298.15 K in the QCISD(T)/6-311++G(2d,p)//B3LYP/6-311G(d,p) level was calculated to be 98.0 kcal mol^{-1}, which agrees with the experimental value at 298 K, 98.2 ± 1 kcal mol^{-1}.[32] The C–H bond dissociation enthalpy in cyclopropane was calculated to be 105.6 kcal mol^{-1} by the same method. Our calculation shows that the C-H bond in cyclopropane is stronger than that in ethane by 7.6 kcal mol^{-1}. Hata et al. theoretically estimated that the activation energy of hydrogen atom abstraction by compound I, an active species of P-450, is ca. 15 kcal mol^{-1}, and that the structure obtained with a substrate radical is less stable than the initial structure by ca. 4 kcal mol^{-1}. Therefore, the negative contribution of 2–7 kcal mol^{-1} to the hydrogen atom abstraction reaction may have a relatively large effect on the hydroxylation by P-450. Dalton et al. reported that cyclopropane was oxidized to cyclopropanol by a P-450 monooxygenase from *Methylococcus capsulatus* while methylcyclopropane was oxidized to cyclopropylmethanol, not methylcyclopropanol, by the same enzyme.[33] These findings may imply that cyclopropane is a worse substrate for monooxygenases than other alkanes.

In this study, we have investigated the metabolic profile of CycloABA, the highly potent long-lasting analogue of ABA, and we uncovered the complete resistance of CycloABA to 8′-hydroxylation, which is the most significant reaction in ABA metabolism. The present finding suggests that CycloABA is a useful tool for studying plant physiology in which ABA metabolism involved.

3. Experimental

3.1. Chemistry

3.1.1. General. ^{1}H NMR spectra were recorded in $CDCl_3$ with TMS as the internal standard using a JEOL JNM-EX 270 NMR spectrometer (270 MHz). For clarity, the atoms of all of the compounds with the carbon skeleton of ABA were numbered as in ABA in the assignment of peaks. Mass spectra were obtained with a JEOL JMS-DX 303HF mass spectrometer. Column chromatography was performed on silica gel (Wakogel® C-200, Wako).

3.1.2. 4,4-Ethylenedioxy-2,6-dimethyl-2,5-cyclohexadien-1-one (1). A solution of 2,6-dimethylbenzoquinone (5 g, 37 mmol), ethylene glycol (21 mL, 0.37 mmol) and trimethyl orthoformate (8.0 mL, 73 mmol) in dry ether (250 mL) was prepared, *p*-TsOH (630 mg, 3.3 mmol) was added, and the mixture was stirred at 4 °C for 5 days. Saturated $NaHCO_3$ was added to the mixture, which was then extracted with EtOAc (50 mL × 4). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with hexane–EtOAc (9:1–4:1) to give **1** (6.7 g, 95% yield) as a pale yellow oil. The ^{1}H NMR and mass spectral data were consistent with those reported previously.[19]

3.1.3. (±)-4,4-Ethylenedioxy-5,6-methano-2,6-dimethyl-2-cyclohexen-1-one (2). A solution of NaH (60% in oil, 0.62 g, 16 mmol) in dry DMSO (10 mL) was stirred as trimethylsulfoxonium iodide (3.2 g, 15 mmol) was added at room temperature under Ar. The mixture was stirred for 1 h. A solution of **1** (1.8 g, 9.9 mmol) in dry DMSO (10 mL) was added with stirring and the mixture was stirred for 15 min. After cooling and adding H_2O, the mixture was extracted with EtOAc (40 mL × 4). The organic layer was washed with saturated $NaHCO_3$ and H_2O, dried over Na_2SO_4, and concentrated. The residual oil was purified by column chromatography with hexane–EtOAc (9:1–7:3) to give **2** (1.8 g, 95% yield) as a colorless oil. ^{1}H NMR: δ 1.07 (1H, dd, J = 8.6 and 5.0 Hz, 5-CH_2-6), 1.19 (1H, dd, J = 5.0 and 5.0 Hz, 5-CH_2-6), 1.32 (3H, s, Me-6), 1.79 (4H, m, H-5 and H_3-7), 4.03–4.16 (4H, m, OCH_2CH_2O), 5.96 (1H, s, H-3); EIMS m/z (rel. int.): 194 $[M]^+$ (20), 179 (5), 166 (17), 151 (28), 138 (8), 126 (29), 122 (15), 111 (9), 107 (29), 41 (100); HREIMS: $[M]^+$ at m/z 194.0948 (calcd for $C_{11}H_{14}O_3$, m/z 194.0943).

3.1.4. (2*RS*,1′*S,5′*R**,6′*S**)-(±)-4-(1′-Hydroxy-4′,4′-ethylenedioxy-5′,6′-methano-2′,6′-dimethyl-2′-cyclohexen-1′-yl)-3-but-yn-2-ol-TBS ether (3).** A 1.6 M solution of *n*-BuLi in hexane (6.5 mL, 10 mmol) was added dropwise to a stirred solution of 1-methyl-2-propynyl TBS ether (2.7 g, 15 mmol) in dry THF (100 mL) at −78 °C under Ar. After being stirred for 1 h, a solution of **2** (1.0 g, 5.4 mmol) in dry THF (25 mL) was added dropwise to the stirred mixture. The mixture was stirred for 5 h at −78 to 20 °C. After being quenched with H_2O, the mixture was extracted with EtOAc (50 mL × 4). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with hexane–EtOAc (9:1–4:1) to give a mixture of **3** (0.57 g, 28% yield) and its diastereomer (1.18 g), as pale yellow oils. ^{1}H NMR: δ 0.11 (6H, s, $Si(CH_3)_2$), 0.58 (1H, dd, J = 8.6 and 5.0 Hz, H-8′), 0.69 (1H, dd, J = 5.0 and 5.0 Hz, H-8′), 0.90 (9H, s, $SiC(CH_3)_3$), 1.34–1.43 (7H, m, H-5′, H_3-9′ and H_3-1), 1.86 (3H, s, H_3-7′), 3.94–4.05 (4H, m, OCH_2CH_2O), 4.57 (1H, q, J = 6.6 Hz, H-2), 5.17 (1H, s, H-3′); EIMS m/z (rel. int.): 378 $[M]^+$ (6), 360 $[M-H_2O]^+$ (10), 348 (6), 321 (106), 309 (13), 291 (38), 277 (51), 259 (43), 247 (82), 233 (64), 217 (52), 203 (48), 179 (39), 159 (90), 143 (45), 128 (51), 119 (115), 57 (100); FABHRMS: $[M+H]^+$ at m/z 379.2307 (calcd for $C_{21}H_{35}O_4Si$, m/z 379.2305).

3.1.5. (1′*S,5′*R**,6′*S**)-(±)-(3*E*)-4-(1′-hydroxy-5′,6′-methano-2′,6′-dimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-buten-2-one (4).** Sodium bis(2-methoxyethoxy)aluminum hydride (330 μL, 1.1 mmol) was added to a solution of **3** (96 mg, 0.25 mmol) in dry THF (3 mL) at 0 °C, and stirred for 4 h at room temperature under Ar. After cooling, and adding H_2O and saturated $NaHCO_3$, the

resulting mixture was extracted with EtOAc (4 mL × 8). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. The crude oil was dissolved in THF (0.5 mL), and then AcOH (1.5 mL) and H_2O (0.5 mL) were added. The mixture was stirred for 17 h at room temperature, H_2O was added, and the solution was extracted with EtOAc (7 mL × 5). The organic layer was washed with 1 M NaOH and H_2O, dried over Na_2SO_4 and concentrated. Column chromatography with CH_2Cl_2–acetone (9:1–4:1) gave the enone alcohol (33 mg) as a colorless oil. MnO_2 (activated, 0.27 g) was added to a solution of the enone alcohol (33 mg) in CH_2Cl_2 (15 mL), and stirred for 2 h at room temperature. The reaction mixture was filtered, and the resulting cake of MnO_2 was washed with CH_2Cl_2, acetone and MeOH. After being concentrated, the residual oil was purified by column chromatography with CH_2Cl_2–acetone (9:1–4:1) to obtain **4** (30 mg, 54% yield) as a colorless oil. ^{1}H NMR: δ 1.13 (1H, dd, J = 8.9 and 4.3 Hz, H-8′), 1.21 (3H, s, H_3-9′), 1.25 (1H, dd, J = 4.3 and 4.3 Hz, H-8′), 1.82 (3H, d, J = 1.3 Hz, H_3-7′), 1.95 (1H, ddd, J = 8.9, 4.3 and 1.7 Hz, H-5′), 2.29 (3H, s, H_3-1), 5.68 (1H, dq, J = 1.7 and 1.3 Hz, H-3′), 6.49 (2H, s, H-3 and H-4); EIMS m/z (rel. int.): 220 $[M]^+$ (13), 205 $[M\text{-}Me]^+$ (23), 202 $[M\text{-}H_2O]^+$ (15), 192 (17), 177 (100), 162 (59), 159 (55), 149 (70), 135 (62), 123 (59), 109 (78); HREIMS: $[M]^+$ at m/z 220.1082 (calcd for $C_{13}H_{16}O_3$, m/z 220.1099).

3.1.6. (±)-5′α,8′-Cyclo-ABA. $KN(TMS)_2$ (15% in toluene, 2.5 mL, 1.9 mmol) was added to a solution of bis(2,2,2 - trifluoroethyl)(methoxycarbonylmethyl)phosphonate (0.39 g, 1.2 mmol) in dry THF (10 mL), and the mixture was stirred for 1 h at 0 °C under Ar. A solution of **4** (0.18 g, 0.84 mmol) in dry THF (3 mL) was then added and the resulting mixture was stirred for 7 h at room temperature. After being quenched with saturated NH_4Cl, the mixture was extracted with ether (15 mL × 4). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. Column chromatography with hexane–EtOAc (10:3–5:2) gave a 2Z/2E mixture of **5** (0.11 g) as a pale yellow oil. A solution of 1 M NaOH (4 mL, 4 mmol) was added to **5** in MeOH (2 mL), and stirred for 18 h at room temperature in the dark. H_2O (50 mL) was added to the resulting mixture. The solution was extracted with hexane and the aqueous layer was extracted with EtOAc (10 mL × 8) after being acidified with 1 M HCl to pH 2. The organic layer was washed with brine and H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with CH_2Cl_2–acetone–AcOH (190:10:1–180:20:1) to give (±)-5′α,8′-cycloABA (0.43 g, 20% yield) as a white amorphous solid. The ^{1}H NMR and mass spectral data were consistent with those reported previously.[19]

3.1.7. Optical resolution of (±)-5′α,8′-cyclo-ABA. A Chiralpak AD-H HPLC column (250 mm × 4.6 mm i.d., Daicel; solvent, 8% i-PrOH in hexane containing 0.1% TFA; flow rate, 1.5 mL min^{-1}; detection, 254 nm) was injected with (±)-5′α,8′-cyclo-ABA. The materials with retention times of 14.1 and 17.4 min were collected, providing 17.3 mg of (+)-5′α,8′-cyclo-ABA (CycloABA) and 17.1 mg of (−)-enantiomer, as white amorphous powders with optical purities of 99.8 and 99.7%, respectively, measured by HPLC on the same column. The optical rotation and CD data were consistent with those reported previously.[19]

3.1.8. ABA, PA, and DPA. ABA (natural type) was a gift from Toray Industries Inc., Tokyo, Japan. PA[34] and DPA[35] were prepared as reported previously.

3.1.9. ABA-GE tetraacetate. Acetobromo-α-D-glucose (20 mg, 48 μmol) and Ag_2CO_3 (40 mg, 150 μmol) were added to a solution of ABA (10 mg, 38 μmol) in ether (4 mL) at room temperature, and stirred for 5 h under Ar. Afterwards, ether (30 mL) was added, the mixture was washed with brine, dried over Na_2SO_4, and concentrated. The residual oil was purified by column chromatography with hexane–EtOAc (9:1–2:3) and by using HPLC with an ODS column (AQ 311, 150 × 20 mm, YMC; solvent, 60% MeOH in H_2O; flow rate, 9.9 mL min^{-1}; detection, 254 nm) to obtain ABA-GE tetraacetate (0.54 mg, 2% yield) as a white amorphous solid. The ^{1}H NMR and mass spectral data were consistent with those already reported.[36]

3.1.10. CycloABA-GE tetraacetate. CycloABA-GE tetraacetate was synthesized from CycloABA (5 mg, 19 μmol) in the same manner as was used to produce ABA-GE tetraacetate; the process gave CycloABA-GE tetraacetate (0.45 mg, 4% yield) as a white amorphous solid. ^{1}H NMR: δ 1.12 (1H, dd, J = 8.6 and 4.3 Hz, H-8′), 1.20 (3H, s, H_3-9′), 1.27 (1H, dd, J = 4.3 and 4.3 Hz, H-8′), 1.86 (3H, d, J = 1.3 Hz, H_3-7′), 1.87 (1H, m, H-5′), 2.01 (3H, s, OAc), 2.02 (3H, s, OAc), 2.03 (3H, s, OAc), 2.04 (3H, s, OAc), 2.09 (3H, s, H_3-6), 3.83 (1H, m, H-5″), 4.11 (1H, dd, J = 12.6 and 1.9 Hz, H-6″), 4.28 (1H, dd, J = 12.6 and 4.6 Hz, H-6″), 5.12 (1H, t, J = 9.1 Hz, H-4″), 5.17 (1H, t, J = 9.1 Hz, H-2″), 5.22 (1H, t, J = 9.1 Hz, H-3″), 5.63 (1H, s, H-3′), 5.74 (1H, s, H-2), 5.78 (1H, d, J = 8.1 Hz, H-1″), 5.92 (1H, d, J = 15.7 and Hz, H-5), 7.87 (1H, d, J = 15.7 Hz, H-4); FABHRMS (NBA): $[M+H]^+$ at m/z 593.2241 (calcd for $C_{29}H_{37}O_{13}$, m/z 593.2234).

3.1.11. Calculations. The minimum-energy conformers of ABA and CycloABA were minimized using the Becke three parameter functional (B3LYP) method with the 6-31G(d) basis set in Gaussian 03.[37] The C–H bond energies of cyclopropane and propane were estimated by calculating the energies of these compounds, their dehydrogenated radicals, and a hydrogen atom using the restricted (for ethane and cyclopropane), or unrestricted (for their radicals), Quadratic CI [QCISD(T)] with the 6-311++G(2d,p) basis set. Zero-point and thermal corrections (298.15 K) were calculated using unrestricted B3LYP with a 6-311G(d,p) basis set.

3.2. Biology

3.2.1. Plant material. Seeds of radish [*R. sativus* L. var. *raphanistroides* (Makino) *Sinsk.*] were sown on a stainless mesh in a glass dish (60 mm × 110 mm i.d.), soaked in water at 25 °C in the dark for a day, and grown at

25 °C in continuous light for 6 days. Seven-day-old seedlings were used for the subsequent experiments, which were conducted at least three times.

3.2.2. Application of ABA and CycloABA. Thirty seven-day-old seedlings with roots were placed in a 0.4 mM ABA or CycloABA solution (1.0 mg in 10 mL solution). The seedlings were incubated for 3 days at 25 °C in continuous light. Water was never added until the initial sample solution dropped to less than 1 mL.

3.2.3. Extraction. Seedlings which had incorporated ABA or CycloABA were frozen in liquid nitrogen, pulverized, and extracted with MeOH (60 mL) containing 20 μg mL^{-1} BHT for 2 days. After filtration, the tissue residue was further extracted with MeOH (60 mL) for a day, and then filtered. This procedure was repeated one more time. The combined filtrates were divided in two, and each was concentrated in vacuo to give the MeOH extract. One MeOH extract was dissolved in H_2O (30 mL). After lowering the pH to 2 with 1 M HCl, the solution was extracted with EtOAc. The EtOAc layer was washed with H_2O and concentrated in vacuo. The final extract was dissolved in MeOH for HPLC analysis of ABA or CycloABA and their oxidative metabolites. The other MeOH extract was dissolved with pyridine (20 mL) and acetic anhydride (10 mL). The reaction mixture was stirred at room temperature for 24 h before being concentrated in vacuo. The residue was dissolved in EtOAc, washed with H_2O, and concentrated in vacuo. The final acetylated extract was dissolved in MeOH for HPLC analysis of the tetraacetates of ABA-GE or CycloABA-GE. After incubation, the water culture medium was filtered, acidified with 1 M HCl, and extracted with EtOAc. The EtOAc extract was washed with H_2O and concentrated in vacuo. The residue was dissolved in MeOH for HPLC analysis of ABA, PA, or CycloABA.

3.2.4. Detection of ABA and CycloABA, and their metabolites. ABA, PA, DPA, ABA-GE acetate, CycloABA, and CycloABA-GE acetate were detected in the HPLC with an ODS column (Hydrosphere C18, 250-mm × 4.6 mm i.d., YMC; flow rate, 1.0 mL; detection, 254 nm), and quantified by comparison with the authentic samples. The mobile phases and retention times of the first run: 35% MeOH containing 0.1% AcOH (0–20 min) and 45% MeOH containing 0.1% AcOH (20–45 min); ABA, 31.1 min; PA, 15.1 min; and DPA, 9.7 min. The mobile phases and retention times of the second run: 29% MeOH containing 0.1% AcOH (0–20 min) and 43% MeOH containing 0.1% AcOH (20–40 min); CycloABA, 30.1 min. The mobile phases and retention times of the third run: 40% MeOH containing 0.1% AcOH (0–20 min) and 55% MeOH containing 0.1% AcOH (20–45 min); ABA-GE tetraacetate, 41.0 min; and CycloABA-GE tetraacetate, 38.0 min.

3.2.5. Bioassay. Radish seed germination assay was performed as follows. One hundred seed of radish was placed on two sheets of Advantec No.2 filter paper soaked in 3 mL of a test solution in a polystyrene dish (60 mm i.d.) and allowed to germinate in continuous light (6400 lux) at 25 °C. After 19 h, the inhibition ratio was defined as $[(A - B)/A] \times 100$, where A = the number (100) of seeds that germinated when water was used, and B = the number of seeds that germinated when a test compound was used. Radish seedling growth assay was performed as follows. Thirty 5-day-old seedlings with roots were placed in a sample solution (1–100 μM). The seedlings were incubated for 3 days at 25 °C in continuous light. Lettuce seed germination and rice seedling elongation assays were performed as reported previously.[38]

3.2.6. Quantification of endogenous ABA. Thirty seven-day-old seedlings with roots were frozen in liquid nitrogen, pulverized, and extracted with MeOH (60 mL) containing 1.2 mg of BHT and 1.0 μg of [3′,5′,5′,7′,7′,7′-$^2H_6$]ABA in MeOH (100 μL) as an internal standard for 2 days. After filtration, the tissue residue was further extracted with MeOH (60 mL) for a day, and then filtered. This procedure was repeated one more time. The combined filtrates were divided in two, and each was concentrated in vacuo to give the MeOH extract. One MeOH extract was dissolved in H_2O (30 mL). After lowering the pH to 2 with 1 M HCl, the solution was extracted with EtOAc. The EtOAc layer was washed with H_2O and concentrated in vacuo. The final extract was dissolved in 100 μL of MeOH, and methylated with TMS–CHN_2 before analyzed with a gas chromatograph connected with a mass spectrometer (GCMS-QP5000, Shimadzu). A capillary column TC-1 (30 m × 0.25 mm i.d., 0.25 mm thick; GL Sciences) was used under the following condition: initial oven temperature, 130 °C, linear gradient to 200 °C at 5 °C min^{-1}, and then linear gradient to 250 °C at 25 °C min^{-1}; He flow, 1 mL min^{-1}; ionization, EI (70 eV). Selected ion monitoring mode was used to perform quantitative analysis. The ratio of peak areas for m/z 190 (d_0)/194 (d_6) determined an ABA concentration.

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