# NEW CONJUGATED METABOLITES OF ABSCISIC ACID OCCURRING IN FRUITS

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CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| MeOH | Methanol |
| EtOH | Ethanol |
| ABA | Abscisic acid |
| *t*-ABA | *trans*-Abscisic acid |
| HOABA | Hydroxyabscisic acid (6'-hydroxymethyl-abscisic acid) |
| PA | Phaseic acid |
| DPA | Dihydrophaseic acid |
| epi-DPA | epi-Dihydrophaseic acid |
| HMG-HOABA | β-Hydroxy-β-methylglutarylhydroxyabscisic acid |
| Me- | Methyl ester |
| Et- | Ethyl ester |
| Ac- | Acetate |
| -GS | Glucoside |
| -GE | Glucosyl ester |
| -ME | Maltosyl ester |
| $A^+$ | Aglycon ion |
| $S^+$ | Sugar ion |
| TLC | Thin layer chromatography |
| GC | Gas chromatography |
| HPLC | High performance liquid chromatography |
| FID | Flame ionization detector |
| ECD | Electron capture detector |

CHAPTER I

INTRODUCTION

Plant growth phenomenon is under the control of plant hormones. The actual beginning of the hormone concept was made in 1928 when Went successfully demonstrated the existence of auxins in oat seedlings [1]. Today, in addition to auxins, gibberellins, cytokinins, ethylene and abscisic acid are regarded as the principal plant hormones of the higher plants [2].

The growth inhibitor, abscisic acid, is the most recently discovered plant hormone. Work in the 1950s and early '60s by three groups, working on apparently unrelated problems, ultimately resulted in the isolation and identification of abscisic acid in each case [3-6]. The search for an abscission-promoting hormone by Addicott and associates led to the isolation of an active compound in crystalline form, called abscisin II, from young cotton fruit [7,8]. The structure was proposed by Ohkuma in 1965 [9] and Cornforth *et al.* confirmed this by synthesis [10]. A dormancy-inducing substance, called dormin, isolated from sycamore leaves [11] and a lupin-abscission factor from yellow lupin fruit [12] were shown to be the same compound as abscisin II [13-16]. Abscisic acid (ABA) was later proposed as the common name for abscisin II [17]. Since then it has become clear that ABA is widely distributed in higher plants, and that the most active inhibitor of coleoptile elongation in many plants is ABA.

ABA plays a part in the regulation of the abscission of leaves and young fruit, the dormancy of buds and seeds, the ripening of fruit, the flowering, plus the growth and the geotropism of roots [2,6,19]. Apart from these physiological roles, another of its most important roles is its ability to cause the closure of stomata [20,21]. Recently this has come to be regarded as the primary physiological role of ABA.

Some of the physiological roles of ABA are demonstrated by circumstantial evidence indicating that there is a close correlation between changes in ABA and physiological phenomenon. The ABA content of developing cotton fruit is shown in Figure I-1 [22]. High levels of ABA occur in correlation with abortion and abscission of young fruit, and with senescence and dehiscenece of mature fruit. A close correlation between the ABA levels in the leaves and the stomatal aperture is observed throughout the process of wilting and recovery. When cut shoots of bean wilt, the ABA content increases 40-fold, as shown in Figure I-2 [23]. Following recovery from water stress the ABA levels decline rapidly. ABA levels in an organ are controlled by biosynthesis and metabolism according to the physiological state of the plant [4,6,24]. Hence the biosynthesis and metabolism of ABA are important in order to gain a more precise understanding of the relation between ABA and physiological phenomenon.

The biosynthesis and metabolism of ABA are summarized in

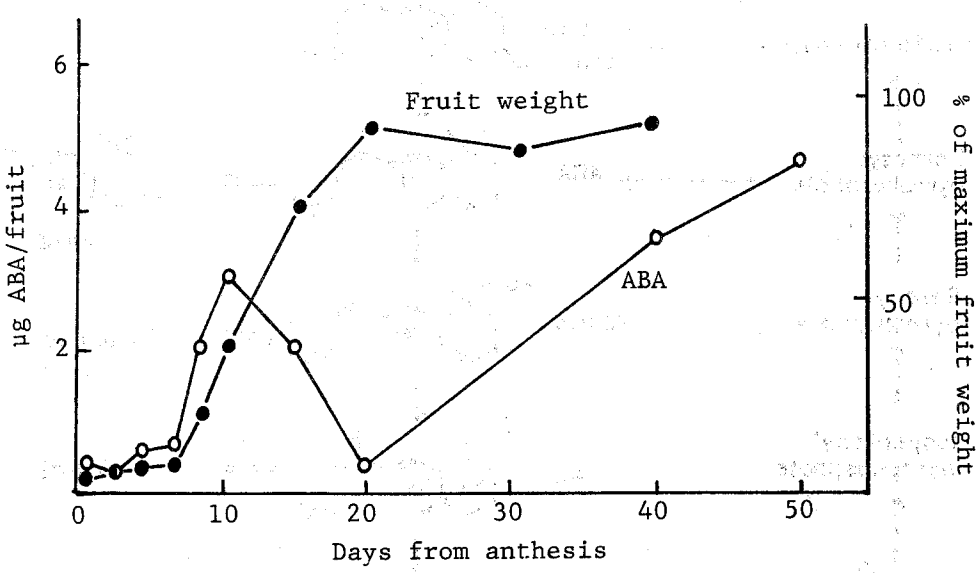


Figure I-1 The abscisic acid content of developing cotton fruit. Data redrawn from Davis and Addicott (1972).

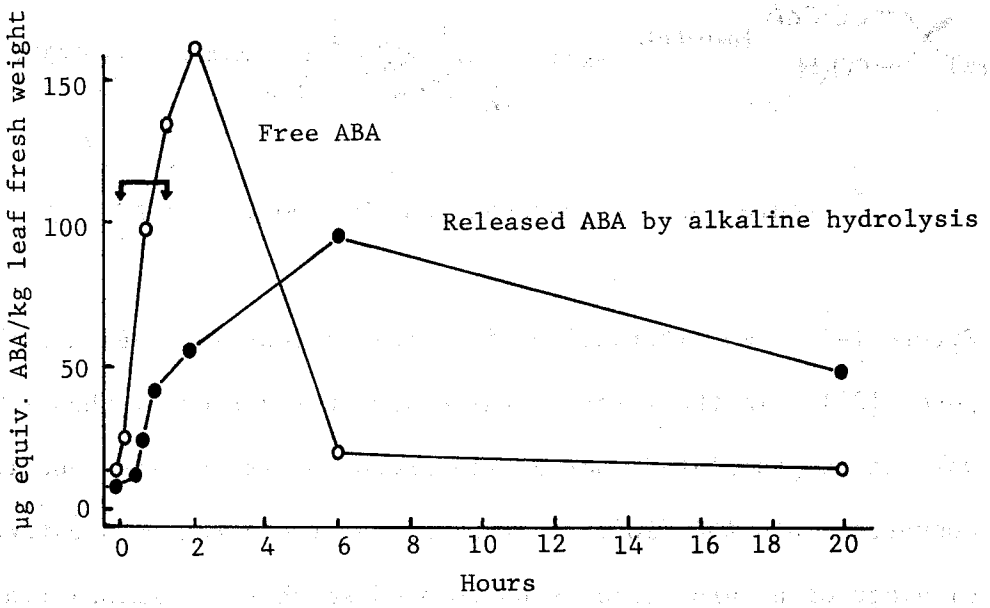


Figure I-2 The abscisic acid content of dwarf bean during a wilt-recovery cycle. The seedlings were stressed for 90 min by exposure to a draught of warm air (between arrows). They were then allowed to recover. Data redrawn from Hiron and Wright (1973).

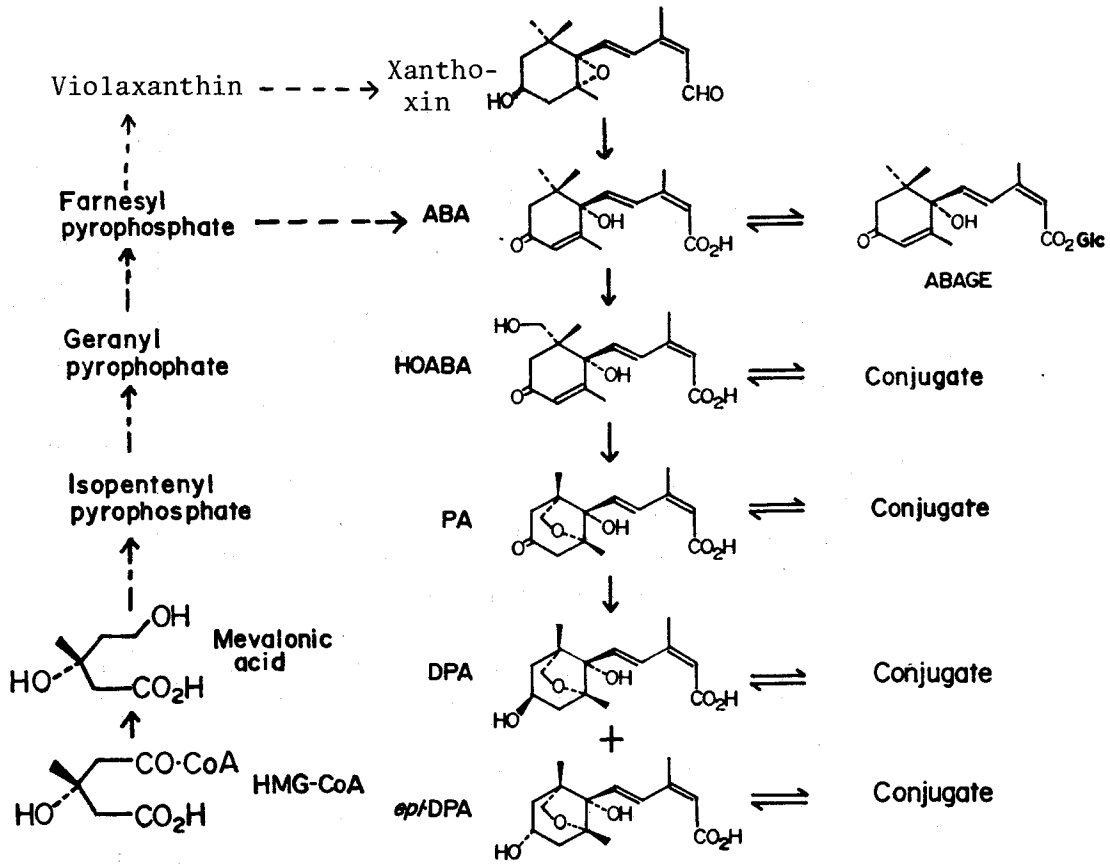


Figure I-3 Biosynthesis and Metabolism of ABA.

Figure I-3. Two alternative biosynthetic routes of ABA are proposed [25]. In the first of these *i.e.* the direct synthesis pathway, it is postulated that a C-15 precursor is cyclized and then converted into ABA [26]. In the second, the carotenoid pathway, a cleavage of a carotenoid is postulated so that a terminal ring and six carbon atoms of the backbone are liberated as a C-15 moiety with the carbon skeleton of ABA [27,28]. However the importance

of the biosynthetic route to ABA *via* the carotenoid in normal biosynthesis remains to be established, and most of the evidence favors the direct synthesis route *via* a C-15 precursor [6].

Labelling studies in higher plants have suggested that exogenous ABA is metabolized to phaseic acid (PA) *via* hydroxyabscisic acid (HOABA)* which has been called "Metabolite C", and the resulting PA is converted to dihydrophaseic acid (DPA) and its epimer (epi-DPA) [29,30,31,33]. It has also been suggested that the conjugate is formed from free acids at each metabolic step [33,34]. Among the free acids, PA, DPA and epi-DPA have been identified as endogeneous metabolites of ABA in the seeds of some plants [35,36]. HOABA has been shown to be an unstable metabolite in the feeding experiment [30]. But its natural occurrence has not yet been confirmed probably because of its easy conversion to PA. On the other hand, the only naturally occurring conjugate known so far is glucosyl-ABA (ABAGE) which was isolated from immature seeds of yellow lupin [37].

Lavelling studies are not always useful for finding unknown metabolites because of small quantities of radioactive compounds and don't mean that the same metabolic pathway as in the vivo system works when exogenous ABA is fed to cut plants or plant tissues. Recently Tietz and co-workers have reported that a new metabolite

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* The 6'-hydroxymethyl-ABA is named "hydroxyabscisic acid" (HOABA) in this thesis.

was isolated from wilted pea seedlings which have been supplied $^{14}$C-ABA, and identified as 4'-deoxy-ABA [38]. However 4'-deoxy-ABA can not be regarded as a natural metabolite since its natural occurrence has not been certified and its position in the metabolic pathway is obscure. The author investigated natural metabolites of ABA in plants using the following method. Since the biological activities of ABA metabolites are weak [30], not only the rice seedling assay but also chemical properties [6] such as the ultraviolet absorption due to the conjugated dienecarboxyl group were used for the detection and isolation of metabolites.

This thesis describes the structure elucidation of two conjugated metabolites of ABA whose occurrence in the fruit of *Robinia pseudacacia* and avocado *(Persea americana)* was demonstrated by screening.

CHAPTER II

# A NOVEL CONJUGATE OF HYDROXYABSCISIC ACID FROM FRUIT OF *ROBINIA PSEUDACACIA* L.

Several reports have suggested that ABA plays an important role in the physiological phenomenon of Leguminosae as well as other plants [39,40,41]. However ABA metabolites of Leguminosae are less studied in trees than in herbs. In an investigation of natural metabolites of ABA in fruits of *Robinia pseudacacia* L., *Sophora japonica* L., *Cercis chinensis* Bunge and *Acacia Baileyana* F. Mueller, which are trees belonging to Leguminosae, the author has found a novel conjugate of hydroxyabscisic acid along with its monomethyl ester, ABA and PA.

All steps of the isolation were monitored by UV absorption, a colour reaction and fluorescence under UV-light on TLC together with the inhibitory activity of the fractions on the growth of rice seedlings. The purification procedure of ABA metabolites is summarized in Figure II-1. Fresh fruit (19 kg) of *R. pseudacacia* were extracted with methanol. The acidic ethyl acetate-soluble part of the methanol extract was chromatographed successively on granular charcoal and Celite impregnated with phosphate buffer. Further purification of the fractions eluted with 5, 10 and 30-40% *n*-butanol in benzene by rechromatography afforded four ABA-like inhibitors, 1 (5.2 mg), 2 (100 mg), 3 (9.6 mg) and 4 (95 mg).

Inhibitor 1 gave colourless needles, mp 163.5-164.2°, $[\alpha]_D^{20}$

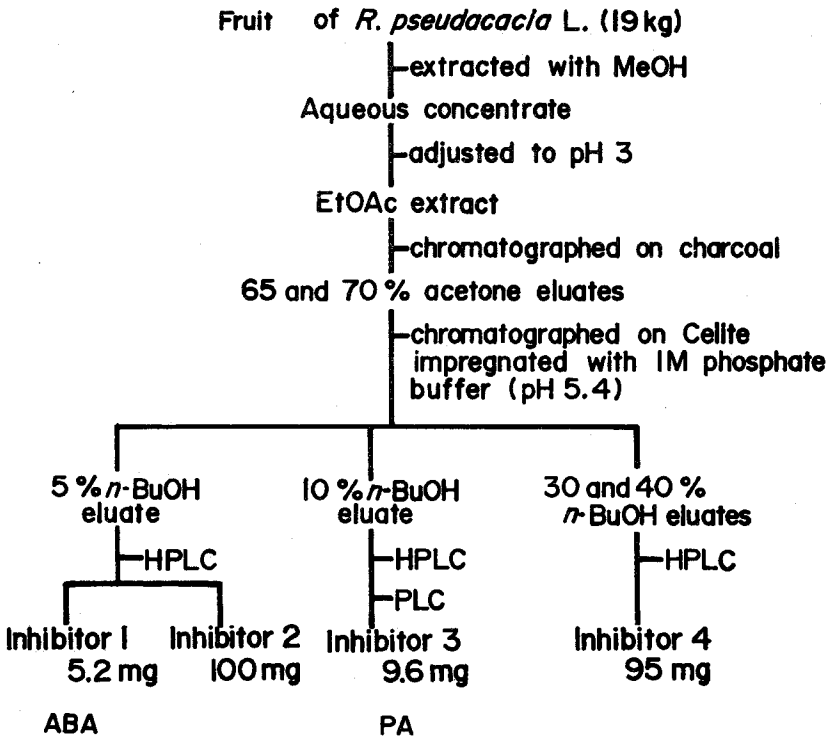


Figure II-1 Purification procedure of ABA-like inhibitors 1, 2, 3 and 4 from fruit of *R. pseudacacia*.

+425° (EtOH; *c* 0.200), and was identified as (+)-ABA by spectral comparison ($^1$HNMR and ORD) with reference data [25].

Inhibitor 3 showed a negative plain curve in the ORD spectrum and its methyl ester gave colourless needles, mp 156.7-157.5°. The $^1$HNMR and MS spectra of methyl ester of 3 revealed that 3 was PA. Identity was confirmed by spectral comparison with reference

data of methyl phaseate (MePA) [25,42].

Inhibitor 4, $[\alpha]_D^{20}$ +560° (EtOH; *c* 0.013), showed a yellow-green fluorescent spot under UV at $R_f$ 0.24 on silica gel TLC after spraying with 5% $H_2SO_4$ in ethanol followed by heating. This colouration was not distinguishable from that of ABA [43], but its $R_f$ value ($R^*_{ABA}$: 0.28) showed that 4 was more polar than ABA.

The IR spectrum of 4 indicated the presence of hydroxyls (3400 $cm^{-1}$, br), an ester carbonyl and carboxyls (1740-1700 $cm^{-1}$), a conjugated carbonyl (1655 $cm^{-1}$), and double bonds (1600 $cm^{-1}$). Inhibitor 4 showed an UV absorption maximum at 258 nm (EtOH; ε 18800) and a very intense positive Cotton effect with extrema at 286 ($[\alpha]^{20}$ +13800°) and 245 nm ($[\alpha]^{20}$ -35900°) (EtOH; *c* 0.0015) in the ORD curve, which are characteristic of ABA. In the $^1$HNMR spectrum of 4, as shown in Figure II-2, signals due to the protons of ABA lacking one of the 6'-geminal-methyl groups were observed. In addition, the $^1$HNMR spectrum indicated a singlet at δ 1.37 (3H) due to a tertiary methyl group attached to a hydroxyl bearing carbon, a broad singlet at δ 2.67 (4H) assignable to two methylenes adjacent to carboxyls, and an AB-quartet at δ 4.15 and 4.19 (1H each, *J*=17 Hz) attributable to acylated carbinol protons. These $^1$HNMR data provided strong evidence that the inhibitor 4 was an ABA derivatives modified at C-6'.

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* $R_{ABA}$ = $R_f$ value of 4 ÷ $R_f$ value of ABA

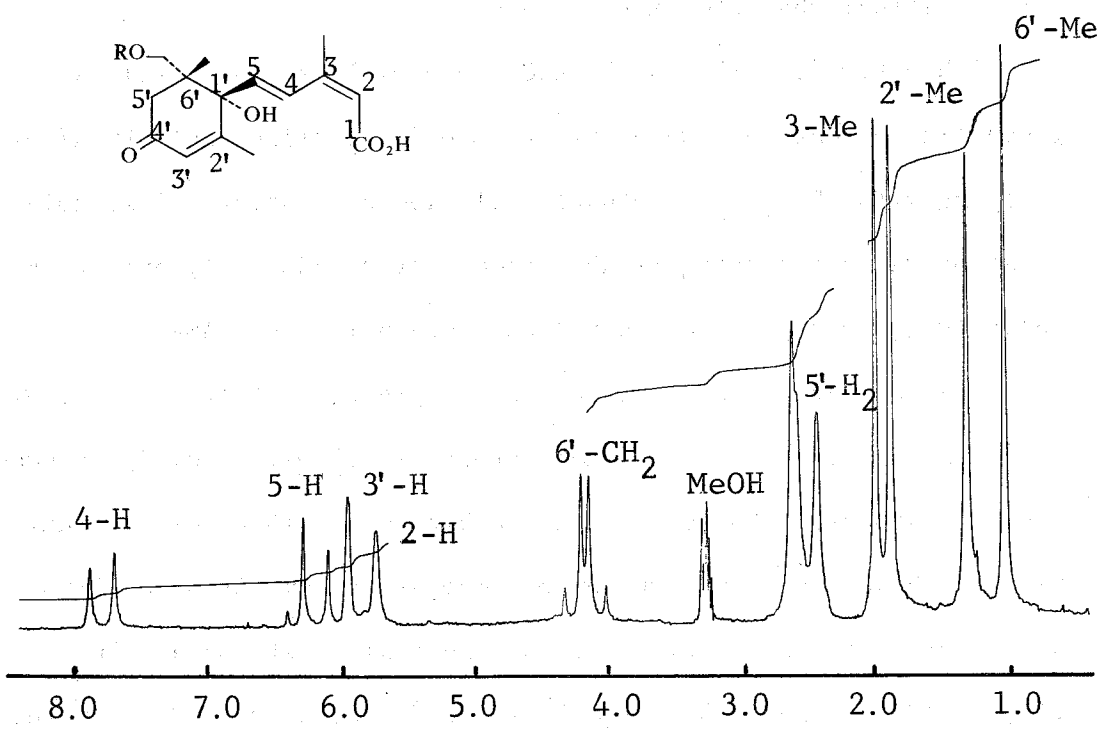


Figure II-2 $^1$HNMR spectrum of inhibitor 4 ($CD_3OD$).

Treatment of 4 with diazomethane gave a dimethylester whose $^1$HNMR spectrum (90 MHz, $CDCl_3$) showed a singlet at $\delta$ 3.72 (6H) assignable to two carbomethoxyls. The parent ion peak at *m/z* 452 in the MS of the dimethyl ester together with the data mentioned above indicated the molecular formular $C_{23}H_{32}O_9$ and consequently the molecular formula $C_{21}H_{28}O_9$ for 4.

Alkaline hydrolysis of 4 followed by treatment with diazomethane gave two methyl esters. On GC-MS, as shown in Figure II-3, one was identified as dimethyl β-hydroxy-β-methylglutarate (MeHMG) by compariosn with the MS of an authentic sample synthesized from

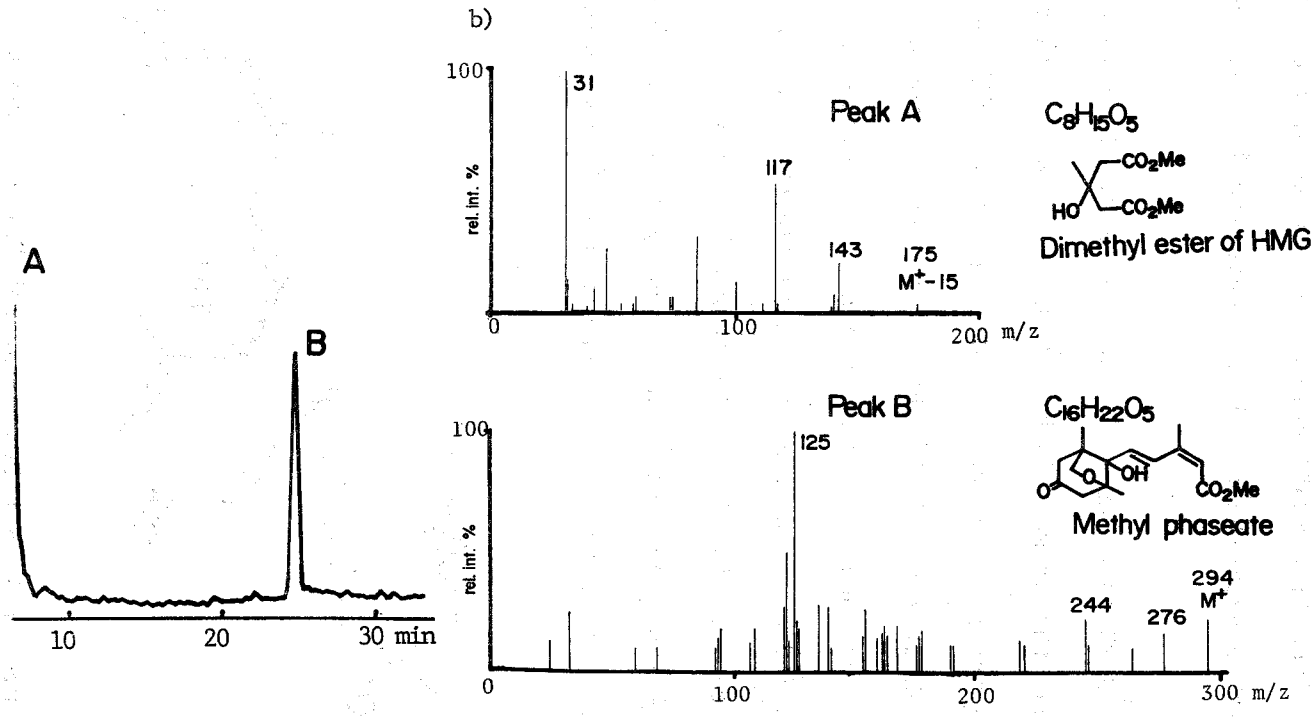


Figure II-3 GC-MS of the methyl esters of inhibitor 4-hydrolyzate.
a) GC-trace; column, 2m x 2mm i.d. 3% OV-1; He flow-rate, 18 ml/min; temperature, 80-180°, 10°/min.
b) Mass spectrum of peak A and B.

methyl 2-bromoacetate and methyl acetoacetate, and the other was shown to be MePA, by MS comparison with a reference sample [42], which was derived from HOABA during the course of the esterification of 4 with diazomethane. Hence conjugate 4 consisted of HOABA and HMG.

In the $^{1}$HNMR spectrum of 4, an AB-quartet (δ 4.15 and 4.19) indicated that the primary hydroxyl of HOABA was acylated with HMG. This was supported by the parent ion peak at *m/z* 452 ($M^{+}$) and the prominent peak at *m/z* 294 ($M^{+}$-158) in the MS of dimethyl ester of 4. The MePA obtained above showed a negative plain ORD curve between 250 and 350 nm which was in good agreement with the data reported by Milborrow [30,44]. The stereochemistry of the C-6' position of 4 was, therefore, the *R* configuration as shown in the structure for 4 (Figure II-4). Consequently 4 was shown to be

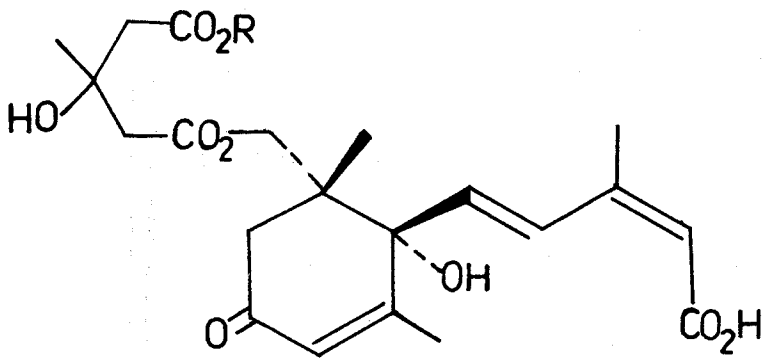


Figure II-4 Structures of inhibitor 2 and 4.

| | R |
|---|---|
| Inhibitor 2 : | Me |
| Inhibitor 4 : | H |

(+)-3-methyl-5-[1(*S*),6(*R*)-2,6-dimethyl-1-hydroxy-6-(3-hydroxy-3-methyl-4-carboxybutanoyloxymethyl)-4-oxo-cyclohex-2-enyl]-2-*Z*-4-*E*-pentadienoic acid, and tentatively named β-hydroxy-β-methylglutaryl-hydroxyabscisic acid (HMG-HOABA).

HMG-HOABA was stable in methanol, while the dimethyl ester was gradually decomposed into MePA and MeHMG by solvolysis in methanol at room temperature.

The $^{1}$HNMR spectrum of 2 showed a singlet at δ 3.67 (3H) assignable to a carbomethoxyl in addition to all the signals observed in that of HMG-HOABA, indicating that 2 is a monomethyl ester of HMG-HOABA. This was confirmed by MS comparison between methyl ester of 2 and dimethyl ester of HMG-HOABA. Treatment of 2 with diazoethane gave an ethyl ester and its MS was analyzed to elucidate which carboxyl group of HMG-HOABA was methylated. The MS of dimethyl ester of HMG-HOABA showed a MePA ion peak at *m/z* 294 along with a parent ion peak at *m/z* 452, and that of ethyl ester of 2 showed a parent ion peak at *m/z* 466 as well as a prominent peak at *m/z* 308 assignable to the EtPA ion, but no peak corresponding to the MePA ion was observed for the ethyl ester, as shown in Figure II-5. This indicates that the carboxyl group of the HOABA moiety of 2 were esterified with diazoethane.

Thus the inhibitor 2 was elucidated as HMG-HOABA methylated at the carboxyl group of HMG moiety (MeHMG-HOABA) (Figure II-4).

It is known that methyl abscisiate (MeABA) is easily formed

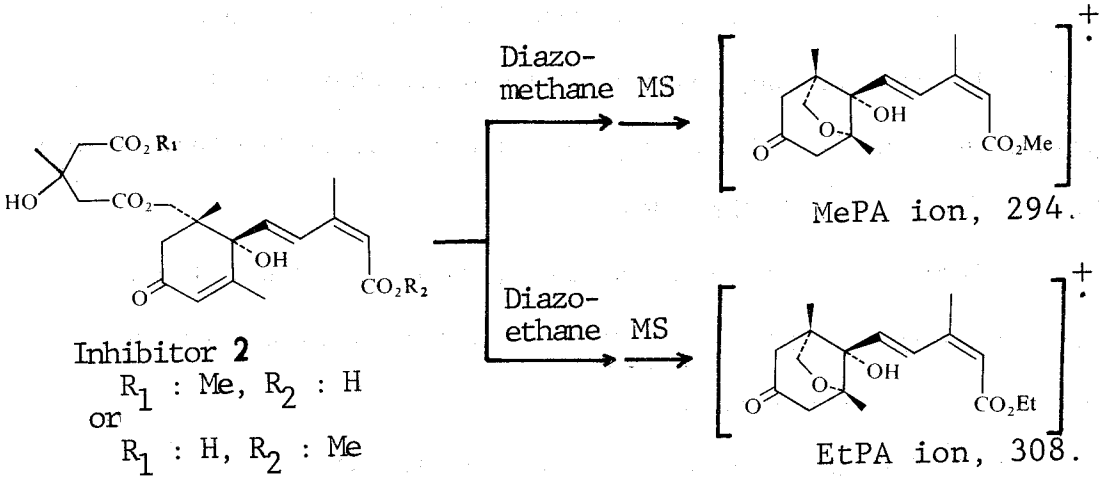


Figure II-5 Mass fragmentation of methyl ester and ethyl ester of inhibitor 2.

from ABAGE through transesterification with methanol [4,45]. The specific methylation occurring in Me-HOABA suggests, therefore, that HMG-HOABA might occur in fruit in its conjugated form which is unstably esterified with glucose or other alcohol.

HMG-HOABA was about 100 times less active than (+)-ABA when examined by the rice seedling test (Figure II-6).

The effect of ABA, PA, HMG-HOABA and MeHMG-HOABA on stomatal aperture was examined using the isolated epidermis of *Commelina communis* L. As shown in Figure II-7, ABA caused almost complete stomatal closure at a concentration of $10^{-6}$ M, while PA and HMG-HOABA required a concentration of $10^{-3}$ M to exhibit this activity.

Since the activities of HMG-HOABA in both bioassays were comparable to that of PA, it was suggested that PA might be released

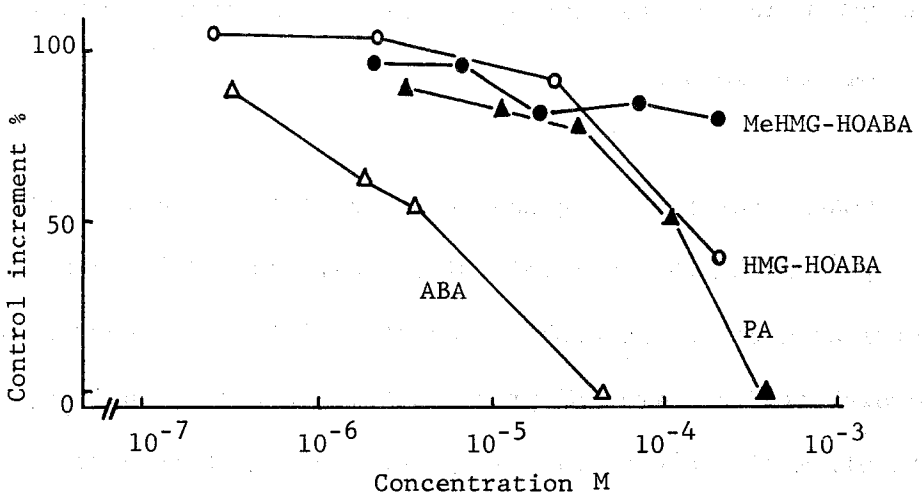


Figure II-6 Inhibitory activity on second leaf sheath elongation of rice seedlings.

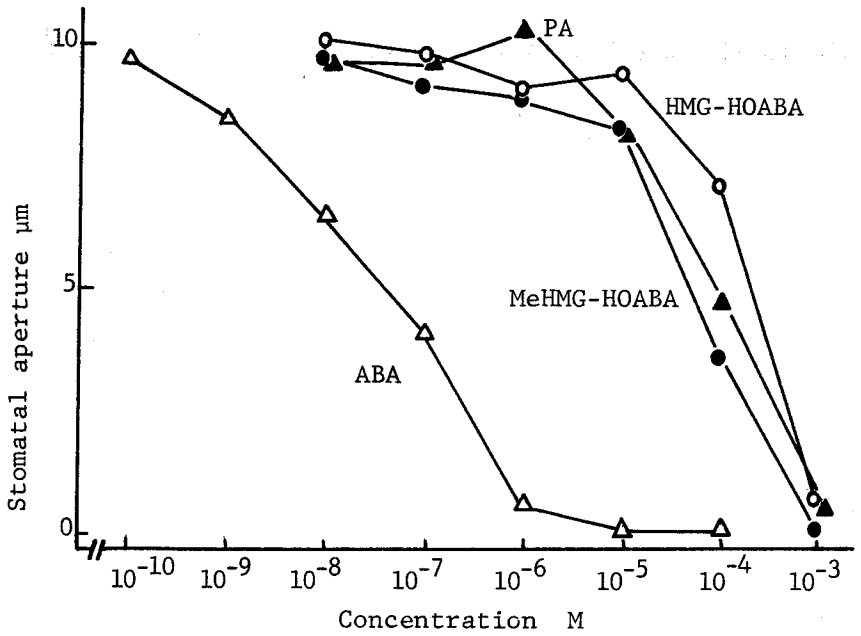


Figure II-7 Stomatal closure activity in isolated epidermis of *Commelina communis*.

from HMG-HOABA *in vivo* during bioassays and cause growth inhibition or stomatal closure.

HOABA, the first degradative metabolite of ABA, is rearranged easily to PA by the intramolecular vinylogous addition of the hydroxyl at C-6' to the double bond at C-2' [25]. Covering of the hydroxyl at C-6' with HMG prevents this rearrangement. Further metabolism of the conjugate in plants to PA, if it occurs, will require the cleavage of the ester linkage. The content of HMG-HOABA in the immature fruit of *R. pseudacacia* was higher than that of ABA and PA. This fact suggests that HMG-HOABA plays an important role in the metabolism of ABA during fruit development.

## EXPERIMENTAL

Melting points were measured on a hot stage and are uncorrected. $^1$HNMR spectra were obtained on a Hitachi Model R-22 spectrometer (90 MHz). Chemical shifts ($\delta$) are expressed from TMS as internal standard, and coupling constants (J) in Hz. Hereafter, singlet, doublet, double doublet, triplet, quartet and multiplet will be abbreviated to s, d, dd, t, q and m, respectively. MS, IR and UV spectra were recorded on a JEOL JMS-01SG-2 mass spectrometer, a Hitachi EPI-G3 infrared spectrometer and a Shimadzu UV-200, respectively. Optical rotations and optical rotatory dispersion curves were measured with a Jasco Model J-5 ORD recorder. In GC-MS, a JEOL JGC-20K gas chromatograph was connected to the mass spectrometer. The following chromatographic materials were used: silica gel H (Type 60, Merck) and granular charcoal (activated charcoal for chromatography, Wako Pure Chemical Industries, Tokyo). Silica gel $60F_{254}$ precoated plates (Merck, layer thickness 0.25 mm) were used for TLC and preparative TLC. Celite 545 was washed successively with distilled water, methanol and acetone, and then dried at 130° for 5 hr before use.

*Rice seedling test* [15]

The rice (Tanginbozu) seeds were soaked in ethanol for 5 min and sterilized with 1% antiformin ($NaClO_4$) for 1 hr, and washed

with running tap water for 3 hr. The sterilized seeds were allowed to a germinate in water for two days at 30°. The seedlings were then placed in a glass tube containing 2 ml of the sample solution, and the seedlings were allowed to grow in a tube sealed with a sheet of polyethylene film under continuous illumination at 30°. The length of the second leaf sheath was measured after seven days.

*Extraction and preliminary separation*

Fresh fruit of *R. pseudacacia*, harvested in July 1977, were soaked in methanol and left at 5° for 2 months, and then filtered. The methanol extract was concentrated at 30° *in vacuo*. The aqueous solution was adjusted to pH 3 with 1N HCl and extracted with ethyl acetate. The ethyl acetate extract (78 g) was chromatographed on granular charcoal (80 g) eluted with 30, 50, 60, 65, 70, 80 and 100% acetone in water. Inhibitory activity was found in the 65 and 70% eluates. The residue (3.2 g) from the 65 and 70% eluates was placed on the top of a column of Celite 545 (600 g) impregnated with 360 ml of 1M phosphate buffer (pH 5.4). The column was eluted with benzene containing 0, 5, 10, 20, 30, 40 and 50% *n*-butanol in a volume of 2 liters per fraction. Inhibitory activity and the ABA-like colourlation on TLC were found in the 5, 10, 30 and 40% *n*-butanol eluates.

*Isolation of ABA and MeHMG-HOABA*

The fraction eluted with 5% *n*-butanol was concentrated to give a gum (325 mg), which was then chromatographed on silica gel (14 g) with benzene containing acetic acid (1%) and ethyl acetate. The ethyl acetate content was increased 10% from 10% to 100%, every tenth 15 ml fraction. Fractions No. 19-22 were combined and concentrated to give a gum, which was recrystallized from *n*-hexane and benzene to yield (+)-ABA (5.2 mg) as colourless needles, which showed a very intense positive Cotton effect with extrema at 290 ($[\alpha]^{21}$: +33000°) and 249 nm ($[\alpha]^{21}$: -87000°) (EtOH, *c* 0.010) in the ORD curve. $^1$HNMR (90 MHz, $CDCl_3$): δ 1.01 (3H, s), 1.12 (3H, s), 1.92 (3H, s), 2.03 (3H, s), 2.27 (1H, d, *J*=18 Hz), 2.50 (1H, d, *J*=18 Hz), 5.77 (1H, br.s), 5.94 (1H, br.s), 6.15 (1H, d, *J*=16 Hz), 7.83 (1H, d, *J*=16 Hz).

Fractions No. 34-36 were combined and concentrated to give MeHMG-HOABA (100 mg). $[\alpha]_D^{15}$ +243° (EtOH; *c* 0.15); ORD, $[\alpha]_{290}^{15}$ +16000°, $[\alpha]_{268}^{15}$ ± 0°, $[\alpha]_{247}^{15}$ -40000° (EtOH; *c* 0.0015); $^1$HNMR (90 MHz, acetone-$d_6$): δ 1.13 (3H, s), 1.37 (3H, s), 1.97 (3H, s), 2.08 (3H, s), 2.52 (2H, s), 2.68 (4H, s), 3.67 (3H, s), 4.20 (1H, d, *J*=11 Hz), 4.30 (1H, d, *J*=11 Hz), 5.80 (1H, br.s), 5.95 (1H, br.s), 6.40 (1H, d, *J*=17 Hz), 7.95 (1H, d, *J*=17 Hz). MeHMG-HOABA was methylated with diazomethane in the same manner as HMG-HOABA. MS of methyl ester of MeHMG-HOABA, *m/z* (rel. int.): 452 ($M^+$, 4), 420 (13), 403 (16), 361 (27), 294 (6), 276 (44), 263 (10), 244 (26),

221 (93), 190 (100), 125 (62).

*Isolation of PA*

The fraction (170 mg) eluted with 10% *n*-butanol from the Celite column was applied to a silica gel (14 g) column and eluted with benzene containing acetic acid (1%) and ethyl acetate. The amount of ethyl acetate added was increased 10% from 20% to 100%, every tenth 15 ml fraction. Fractions No. 19-28 were combined and further purified by preparative TLC (solvent, benzene-ethyl acetate-acetic acid, 12:7:1) to give PA (9.6 mg). PA, $^{1}$HNMR (90 MHz, acetone-$d_6$): $\delta$ 1.04 (3H, s), 1.22 (3H, s), 2.13 (3H, s), 2.33 (1H, dd, $J$=18 and 2.4 Hz), 2.40 (1H, dd, $J$=18 and 2.4 Hz), 2.77 (1H, dd, $J$=18 and 3.0 Hz), 2.86 (1H, d, $J$=18 Hz), 3.65 (1H, d, $J$=7.6 Hz), 3.95 (1H, dd, $J$=7.6 and 3.0 Hz), 5.80 (2H, br.s), 6.65 (1H, d, $J$=16 Hz), 8.23 (1H, d, $J$=16 Hz).

*Methylation of PA*

PA (5.5 mg) was treated with ethereal diazomethane and recrystallized from benzene and ethyl acetate to yield MePA (2.8 mg) as colourless needles. MePA. UV $\lambda_{max}^{EtOH}$ nm ($\varepsilon$): 265 (21400); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3430, 2980, 2950, 2880, 1720, 1690, 1640, 1600, 1240, 1160; $^{1}$HNMR (90 MHz, $CDCl_3$): $\delta$ 1.04 (3H, s), 1.25 (3H, s), 2.02 (3H, br.s), 2.50 (2H, br.s), 2.60 (2H, s), 3.72 (3H, s), 3.77 (1H, d, $J$=8 Hz), 3.96 (1H, d, $J$=8 Hz), 5.78 (1H, br.s), 6.23 (1H, d,

$J$=16 Hz), 8.16 (1H, d, $J$=16 Hz); MS $m/z$ (rel. int.): 294 ($M^+$, 60), 276 (22), 262 (17), 217 (16), 205 (8), 195 (6), 177 (25), 168 (40), 163 (35), 154 (38), 140 (15), 139 (55), 135 (34), 125 (100), 122 (73), 121 (50), 99 (24).

*Isolation of HMG-HOABA*

The 30 and 40% $n$-butanol eluates, on removal of the solvent left a gum (220 mg) which was rechromatographed on silica gel (14 g) with benzene containing acetic acid (1%) and ethyl acetate. Ethyl acetate content was increased from 60% by 20% step, every tenth fraction, at a volume of 15 ml per fraction. Fractions No. 10-30 were combined and concentrated to give HMG-HOABA (95 mg) as an intractable gum. HMG-HOABA. $^1$HNMR (90 MHz, $CD_3OD$): $\delta$ 1.10 (3H, s, 6'-Me), 1.37 (3H, s), 1.95 (3H, s, 2'-Me), 2.05 (3H, s, 3-Me), 2.48 (2H, s, 5'-$H_2$), 2.67 (4H, br.s), 4.15 (1H, d, $J$=11 Hz), 4.29 (1H, d, $J$=11 Hz), 5.76 (1H, br.s, 2-H), 5.98 (1H, br.s, 3'-H), 6.23 (1H, d, $J$=17 Hz, 5-H), 7.79 (1H, d, $J$=17 Hz, 4-H).

*Methylation of HMG-HOABA*

Excess ethereal diazomethane was added to a solution of HMG-HOABA (10 mg) in methanol. After a few minutes the solvent was evaporated to give a gum of the dimethyl ester of HMG-HOABA. When a mixture of HMG-HOABA and diazomethane in ether and methanol was allowed to stand overnight, HMG-HOABA was decomposed into MePA and

MeHMG *via* the dimethyl ester of HMG-HOABA. MS of dimethyl ester of HMG-HOABA, *m/z* (rel. int.): 452 ($M^+$, 2), 420 ($M^+$-32, 2), 403 (20), 361 (4), 294 ($M^+$-158, 40), 276 (27), 263 (17), 244 (19), 221 (21), 190 (21), 177 (28), 167 (40), 154 (38), 139 (42), 135 (41), 125 (100), 122 (69).

*Alkaline treatment of HMG-HOABA*

HMG-HOABA (1.0 mg) was dissolved in 1 N methanolic NaOH (1 ml). The solution was allowed to stand overnight. After adjustment of pH 3 with 2 N HCl, it was then extracted with ethyl acetate. The ethyl acetate extracts were washed with water and concentrated to give a gum, which was treated with ethereal diazomethane for GC-MS analysis.

*GC-MS of the methyl esters of HMG-HOABA hydrolyzate*

GC was performed using a 2 m x 2 mm i.d. glass column containing 3% OV-1 on Chromosorb W, helium flow-rate 18 ml/min, temperature programmed from 80 to 180° at 10° per min. MS of MeHMG, *m/z* (rel. int.): 175 ($M^+$-15, 2), 143 (20), 117 (53), 101 (12), 85 (31), 74 (6), 48 (26), 31 (100). MS of MePA: *m/z* (rel. int.) 294 ($M^+$, 22), 276 (13), 262 (8), 217 (6), 205 (4), 195 (7), 177 (17), 163 (19), 154 (25), 140 (5), 135 (33), 125 (79), 122 (51), 121 (43), 48 (100).

*Synthesis of MeHMG by Reformatsky reaction* [46]

Zinc dust (150 g) was purified by washing it rapidly with dilute NaOH solution, water, dilute acetic acid, water, ethanol, acetone and ether in this order. It was then dried in a vacuum oven at 100°. In a clean, dry, 500 ml-three-necked flask, fitted with a mechanical stirrer, a 250 ml separatory funnel, and a reflux condenser, the upper end of which was protected by a calcium chloride drying tube, was placed 20 g of powdered zinc. A solution of 50 g of methyl bromoacetate and 40 g of methyl acetoacetate in 100 ml of dry benzene was placed in the separatory funnel. About 10 ml of this solution was added to the zinc, and the flask was warmed until the reaction started. The mixture was then stirred and the rest of the solution added at such a rate that the reaction mixture refluxed, care being taken that the reaction did not become too vigorous. The addition took about 1 hr. The reaction mixture was refluxed for 2 hr on a water bath after the addition of the solution was complete. The flask was then cooled in an ice bath and the reaction mixture hydrolyzed by the addition of 200 ml of cold 10% sulfuric acid with vigorous stirring during the addition. The acid layer was drawn off and the benzene solution extracted twice with 50 ml of 10% sodium carbonate solution, then with 25 ml of 5% sulfuric acid, and finally with two 25 ml portions of water. The benzene solution was dried with 5 g of magnesium sulfate. The solution was filtered, the solvent removed by distillation at

atmospheric pressure from an oil bath and the residue fractionated under reduced pressure. The ester was collected at 60-70°/5 mm Hg. The total yield was 5 g (8%). $^{1}$HNMR (90 MHz, neat): δ 1.33 (3H, s), 2.64 (4H, s), 3.63 (6H, s ); MS *m/z* (rel. int.): 175 ($M^{+}$-15), 143 (20), 117 (53), 101 (12), 85 (31), 74 (6), 48 (26), 31 (100).

*Isolation of MePA*

HMG-HOABA (10 mg) was hydrolyzed and esterified in the same way described above. Mixture of the methyl esters gave MePA (6 mg) on removal of MeHMG *in vacuo* (5 mm Hg) at 70°.

*Preparation of nitrosoethylurea and diazoethane* [47]

In a 200 ml-flask was placed 20 g of a 24% aqueous ethylamine solution, and concentrated HCl was added until the solution was acid to methyl red. Water was added to bring the total weight to 50 g, 30 g of urea was added, and the solution was boiled gently under reflux for 2.6 hr. The solution was cooled to room temperature, 11 g of 95% sodium nitrite was dissolved in it, and the whole was cooled to 0°. A mixture of 60 g ice and 10 g of concentrated sulfuric acid in a 500 ml-beaker was surrounded by an efficient freezing mixture, and cold ethylene-nitrite solution was run in slowly with mechanical stirring at such a rate that the temperature did not rise above 0°. Nitrosoethylurea rose to the surface as a crystalline foamy precipitate which was filtered at

once with suction, and pressed well on the filter. The crystals were stirred to a paste with about 10 ml of cold water, sucked as dry as possible, and dried in a vacuum desicator to constant weight. The yield was 3.1 g (22%).

In a 500 ml round-bottomed flask were placed 5 ml of 50% aqueous KOH solution and 200 ml of ether, and 0.5 g of nitroso-ethylurea was added with shaking. The flask was fitted with a condenser set for distillation. The lower end of the condenser carried an adapter passing through a two-holded rubber stopper and dipping below the surface of 20 ml of ether in a 100 ml flask and cooled in ice. The reaction flask was placed in a water bath at 50° and brought to the boiling point of ether with occasional shaking. The ether solution containing diazoethane was reserved in a freezer until use.

*Ethylation of MeHMG-HOABA and PA*

Excess ethereal diazoethane was added to a solution of MeHMG-HOABA (5.8 mg) and left at room temperature overnight. The solvent was evaporated to give a gum of the ethyl ester of MeHMG-HOABA. Ethyl ester of PA was also obtained by treatment of PA (2.0 mg) with diazoethane in the same manner described above for MS comparison with ethyl ester of MeHMG-HOABA. Ethyl ester of MeHMG-HOABA, $^{1}$HNMR (90 MHz, $CDCl_3$): $\delta$ 1.05 (3H, s), 1.28 (3H t, $J$=8 Hz), 1.36 (3H, s), 1.91 (3H, br.s), 2.01 (3H, t, $J$=8 Hz), 2.48 (1H,

br.s), 2.51 (1H, br.s), 2.60 (2H, s), 2.65 (2H, br.s), 3.72 (3H, s), 4.04 (1H, d, $J$=12 Hz), 4.17 (2H, q, $J$=8 Hz), 4.42 (1H, d, $J$=12 Hz), 5.76 (1H, br.s), 5.96 (1H, br.s), 6.04 (1H, d, $J$=16 Hz), 7.86 (1H, d, $J$=16 Hz); MS $m/z$ (rel. int.): 466 ($M^+$, 4), 448 ($M^+$-18, 2), 433 (10), 420 (10), 375 (22), 308 (5), 290 (40), 272 (9), 262 (26), 244 (25), 234 (80), 207 (26), 203 (28), 190 (100), 17 (21), 161 (34), 141 (49), 139 (73), 117 (41), 111 (25). Ethyl ester of PA. $^1$HNMR (90 MHz, $CDCl_3$): $\delta$ 1.03 (3H, s), 1.28 (3H, t, $J$=8 Hz), 2.00 (3H, br.s), 2.50 (2H, br.s), 2.63 (2H, br.s), 3.74 (1H, d, $J$=8 Hz), 3.95 (1H, d, $J$=8 Hz), 4.17 (2H, q, $J$=8 Hz), 5.78 (1H, d, $J$=8 Hz), 6.20 (1H, d, $J$=16 Hz), 8.18 (1H, d, $J$=16 Hz); MS $m/z$ (rel. int.): 308 ($M^+$, 66), 290 ($M^+$-18, 17), 263 (30), 244 (13), 221 (19), 177 (28), 167 (54), 163 (42), 149 (22), 139 (93), 135 (37), 125 (61), 122 (100), 109 (28).

*Stomatal closure test*

This was conducted according to the method of Tucker and Mansfield except the $CO_2$-free air was not used [48,49]. *Commelina communis* L. grown at Kyoto University was used. The youngest, fully expanded leaves were cut in the morning and immediately floated on water and kept in darkness for 7 hr prior to use, to ensure that the stomata were closed at the beginning of incubation. Strips of epidermis about 5 mm square were detached from the abaxial leaf surfaces under dim light.

To 130 µg of ABA, PA or HOABA in 66 µl of methanol, 5 ml of a $10^{-2}$ M PIPES buffer (pH 6.8) and $10^{-1}$ M $NaNO_3$ were added to make the total solution 5 ml. Thus solution was serially diluted with buffer to make concentrations ranging from $10^{-4}$ to $10^{-10}$ M. Methanol up to 15% (v/v) did not affect stomatal opening under illumination.

Five replicate strips were floated on 5 ml of the test solution in 10 ml vials and incubated at 24°. Illumination was provided by a tungsten lamp (300 W) and the light intensity on the incubation vials was approximately 12 klx. After 3 hours, three replicate strips were transferred to glass slides in a drop of test solution and the width of the aperture of 10 stomata on each strip were measured within 5 minutes at a magnificaiton of 15 x 40 fold with the aid of an eye-piece micrometer. The mean stomatal aperture of the control was 10.0 µm.

CHAPTER III

# CHIRALITY OF THE ACYL GROUP OF β-HYDROXY-β-METHYLGLUTARYLHYDROXYABSCISIC ACID

Hydroxyabscisic acid (HOABA), a highly unstable metabolite of ABA, has been shown to occur in the fruit of *Robinia pseudacacia* as a stable conjugate, β-hydroxy-β-methylglutarylhydroxyabscisic acid (HMG-HOABA) [50]. However, the chirality at the β-carbon of the HMG group has not been established. If one of the carboxyl groups of HMG is stereospecifically esterified with HOABA *in vivo*, C-3 of the HMG residue must possess either *R*- or *S*-configuration. This chapter discusses the absolute configuration at C-3 of HMG determined by the selective reduction of the HMG moiety to give mevalonolactone. A convenient method for the optical resolution of (*R*,*S*)-mevalonolactone is also described.

HMG-HOABA was reduced with borane so that C-3 of the HMG moiety retained its configuration after reduction and hydrolysis of the ester [51], as shown in Figure III-1. On TLC (silica gel, solvent system 1), the reduction product was observed as a quenched spot ($R_f$ 0.50) under UV radiation (HMG-HOABA, $R_f$ 0.31). It decomposed to two compounds during purification by preparative TLC. One was identified as mevalonolactone and the other as PA by comparison with authentic samples ($^1$HNMR and MS). This showed that the free carboxyl group of HMG was reduced and that of the HOABA moeity was not. The instability of the reduction product

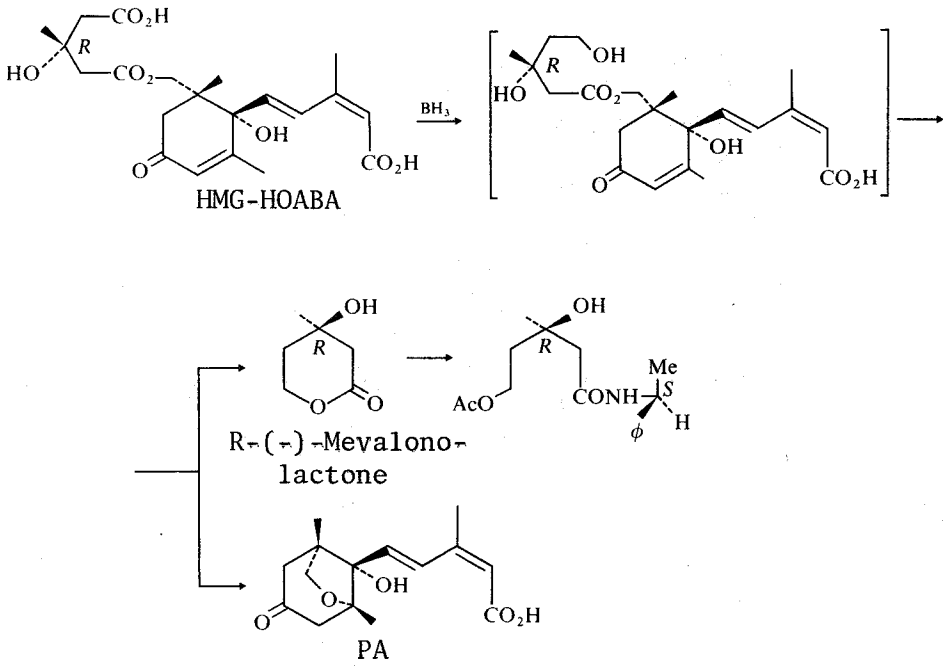


Figure III-1 Reduction and derivatization of HMG-HOABA.

can be ascribed to attack of the ester carbonyl by the primary hydroxyl group formed from the free carboxyl groups of the HMG moiety.

The enantiomeric composition of the mevalonolactone was determined by HPLC analysis. Treatment of (*R*,*S*)-mevalonolactone with (*S*)-(-)-1-phenyl-ethylamine in tetrahydrofuran gave two diastereomeric amides, the monoacetates of which were separated by HPLC (column, 30 cm x 3.9 mm i.d. μ-Porasil; flow rate, 1.5 ml/min; solvent system 2; pressure, 33 kg/cm$^2$; detection, $UV_{254\ nm}$; $R_t$s of diastereomers 10.7 and 11.7 min), as shown in Figure III-2 a). These were then hydrolyzed to give optically active mevalonolactone.

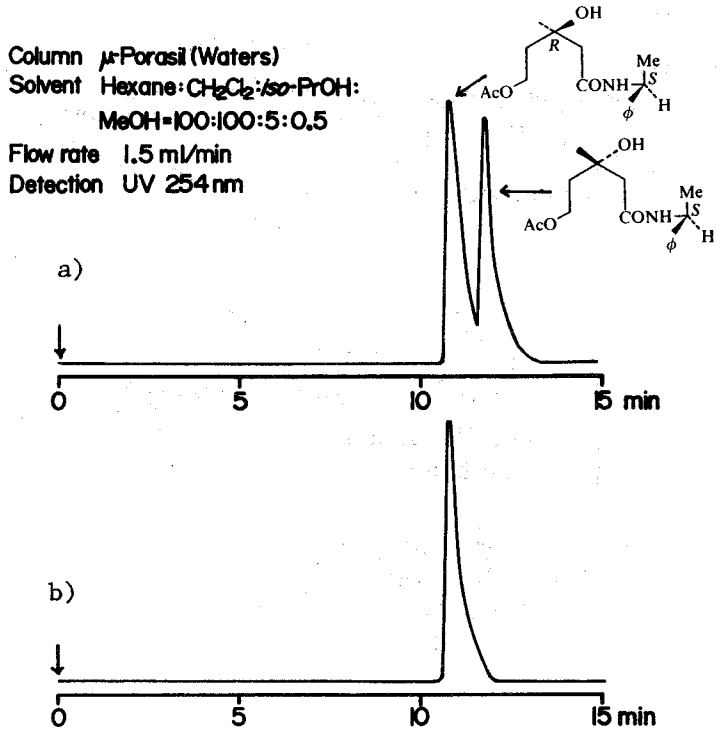


Figure III-2 HPLC separation of acetate-amides derived from mevalonolactone.
a) (*R*,*S*)-Mevalonolactone.
b) Mevalonolactone obtained from HMG-HOABA.

By measruing the optical rotation of the mevalonolactones, the first eluted diastereomer was shown to be (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]-mevalonamide and the second one was shown to be (3*S*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]-mevalonamide [52]. The monoacetyl-(*S*)-phenylethylamide of the mevalonolactone derived from HMG-HOABA (identified by MS comparison with the diastereomers obtained above) was similarly analysed by HPLC to give a peak which was co-chromatographed with the first diastereomer, as shown in Figure III-2 b).

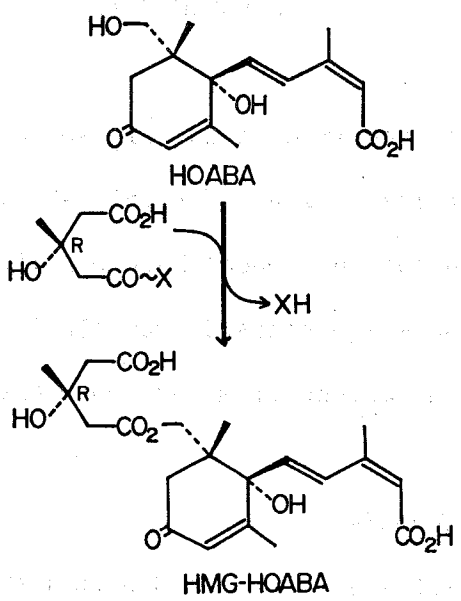


Figure III-3 Putative acylation mechanism of HOABA.

The mevalonolactone derived from HMG-HOABA was, thus, proved to have the *R*-configuration. This result indicates that the absolute configuration at the acyl group of HMG-HOABA is *R*.

If HMG-HOABA were derived *in vivo* by the reaction of HOABA with HMG-CoA, the C-3 of the acyl group must have *S*-configuration. Interestingly, however, the absolute configuration at C-3 is *R*, suggesting that HOABA is not acylated by the usual mechanism through HMG-CoA. It is possible that another acylation mechanism is involved in the acylation of HOABA, as shown in Figure III-3.

## EXPERIMENTAL

For general experimental details see Chapter II. GC-mass spectra were obtained using a Hitachi M-70 mass spectrometer. HPLC was carried out on a model M-6000 A liquid chromatograph (Waters). Borane was purchased from Aldrich Chemical Co., Inc., and (*R,S*)-mevalonolactone from Wako Pure Chemical Industries.

*Reduction of HMG-HOABA*

HMG-HOABA (50 mg) was reduced with 1 M borane (1.0 ml) in dry tetrahydrofuran at -18° for 30 min under a stream of dry nitrogen and left at room temperature overnight. Water (0.6 ml) was added to the reaction mixture at 0°. Tetrahydrofuran was removed and the products were partitioned into ethyl acetate at pH 3.

*Purification and derivatization of mevalonolactone and PA derived from the reduction products of HMG-HOABA*

The ethyl acetate extract (43 mg) was applied to Merck precoated silica gel plates (0.25 mm) which were then developed to 34 mm in benzene-ethyl acetate-acetic acid (11:8:1) (system 1). The zone of $R_f$ 0.24-0.30 was scraped off and eluted with ethyl acetate. The ethyl acetate eluate (4 mg) was further purified by silica gel TLC in benzene-ethyl acetate-acetic acid (7:12:1) (system 3). The ethyl acetate eluates of the zones $R_f$ 0-0.33 and 0.58-

0.78 gave mevalonolactone (0.6 mg) and PA (1.0 mg), respectively. Mevalonolactone was treated with (*S*)-(-)-1-phenylethylamine (10 mg), followed by acetylation with acetic anhydride-pyridine (1:2), to afford the monoacetate of (*S*)-1-phenylethylamide (2.0 mg). An aliquot of PA was methylated by ethereal diazomethane for GC-MS. The GLC conditions were: column, 1.8 m x 2 mm i.d. glass column packed with 2% OV-17 on Gaschrom Q (100-120 mesh); flow rate, 20 ml helium/min; column temperature, 135° for mevalonolactone and 180° for MePA.

Mevalonolactone, $^1$HNMR (90 MHz, $CDCl_3$): δ 1.35 (3H, s), 1.86 (1H, d, *J*=5 Hz), 1.95 (1H, d, *J*=5 Hz), 2.50 (1H, d, *J*=17 Hz), 2.59 (1H, d, *J*=17 Hz), 4.43 (2H, m); GC-MS *m/z* (rel. int.): 112 ($M^+$-18), 60), 88 (4), 81 (100), 72 (2), 69 (9), 68 (6), 66 (3), 60 (6), 54 (15), 53 (38), 52 (17), 50 (9).

PA. $^1$HNMR (90 MHz, acetone-$d_6$): δ 1.04 (3H, s), 1.22 (3H, s), 2.13 (3H, s), 2.33 (1H, dd, *J*=18 and 2.4 Hz), 2.40 (1H, dd, *J*=18 and 2.4 Hz), 2.77 (1H, dd, *J*=18 and 3.0 Hz), 2.86 (1H, d, *J*=18 Hz), 3.65 (1H, d, *J*=7.6 Hz), 3.95 (1H, dd, *J*=7.6 and 3.0 Hz), 5.80 (2H, br.s), 6.65 (1H, d, *J*=16 Hz), 8.23 (1H, d, *J*=16 Hz).

MePA, GC-MS *m/z* (rel. int.): 294 ($M^+$, 60), 276 (22), 262 (17), 217 (16), 205 (8), 195 (6), 177 (25), 167 (40), 163 (35), 154 (38), 140 (15), 139 (55), 135 (34), 125 (100), 122 (73), 121 (50), 99 (24).

*Preparation and separation of diastereomers derived from (R,S)-mevalonolactone*

(*S*)-(-)-1-Phenylethylamine (80 mg) was added to (*R,S*)-mevalonolactone (60 mg) in 3 ml of tetrahydrofuran and the mixture left at room temperature. After evaporation of tetrahydrofuran, ethyl acetate (50 ml) was added to the residues and washed with 0.1 N HCl, followed by water. Ethyl acetate was removed *in vacuo* to yield an oily residue, which was then acetylated with acetic anhydride and pyridine (1:2) at room temperature overnight. The diastereomers (120 mg) were separated by preparative HPLC: column, 30 cm x 7.8 mm i.d. μ-Porasil; flow rate, 7 ml/min; pressure, 33 $kg/cm^2$, solvent, *n*-hexane-dichloromethane-isopropanol-methanol, 100:100:0.5:0.5 (system 2); detection, $UV_{254\ nm}$. GC-MS was carried out using a 1 m x 3 mm glass column containing 3% OV-1 coated on Chromosorb W (80-100 mesh) with a helium flow rate 13 ml/min and a column temperature of 192°.

(3*R*)-5-*O*-Acetyl-1-[(*S*)-phenylethyl]-mevalonamide, $[\alpha]_D^{24}$ -58.8° (EtOH; *c* 1.30); UV $\lambda_{max}^{EtOH}$ nm (ε): 258 (190): IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3670, 3430, 2970, 2930, 1735, 1655, 1605; $^1$HNMR (90 MHz, $CDCl_3$): δ 1.22 (3H, s), 1.48 (3H, d, *J*=7 Hz), 1.83 (2H, t, *J*=7 Hz), 2.02 (3H, s), 2.28 (1H, d, *J*=15 Hz), 2.36 (1H, d, *J*=15 Hz), 4.18 (2H, t, *J*=7 Hz), 5.08 (1H, q, *J*=7 Hz), 6.65 (NH, d, *J*=7 Hz), 7.25 (5H s); GC-MS *m/z* (rel. int.): 293 ($M^+$, 2), 278 (2), 275 (2), 260 (2), 246 (2), 233 (3), 218 (2), 216 (2), 215 (5), 206 (6), 200 (4), 188 (2), 174 (4),

163 (2), 148 (10), 132 (5), 120 (100), 106 (77), 105 (62).

(3*S*)-5-*O*-Acetyl-1-[(*S*)-phenylethyl]-mevalonamide, $[\alpha]_D^{24}$ -67.5° (EtOH; *c* 0.83); UV $\lambda_{max}^{EtOH}$ nm (ε): 258 (190): IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3670, 3430, 2970, 2930, 1735, 1655, 1605; $^1$HNMR (90 MHz, $CDCl_3$): δ 1.24 (3H, s), 1.48 (3H, d, *J*=7 Hz), 1.80 (2H, t, *J*=7 Hz), 2.00 (3H, s), 2.29 (1H, d, *J*=15 Hz), 2.34 (1H, d, *J*=15 Hz), 4.16 (2H, t, *J*=7 Hz), 5.07 (1H, q, *J*=7 Hz), 6.78 (NH, d, *J*=7 Hz), 7.27 (5H, s); GC-MS *m/z* (rel. int.): 293 ($M^+$, 2), 278 (2), 275 (3), 260 (2), 246 (2), 233 (4), 218 (2), 216 (2), 215 (6), 206 (7), 200 (5), 188 (2), 174 (4), 163 (22), 148 (10), 132 (6), 120 (100), 106 (77), 105 (63).

*Hydrolysis of diastereomers*

The faster eluting diastereomer (3*R*) (29 mg) was dissolved in 6 N NaOH (3 ml), refluxed for 4 hr and partitioned with ethyl acetate. The aqueous layer was acidified with 6 N HCl and then extracted with ethyl acetate. The ethyl acetate layer was washed with a small amount of water and concentrated to give (*R*)-(-)-mevalonolactone (9.6 mg), $[\alpha]_D^{26}$ -15° (EtOH; *c* 0.48), IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3650, 3580, 3430, 2960, 2920, 1735, 1600. The slower eluting diastereomer (43 mg) was hydrolyzed in the same manner to give (*S*)-(+)-mevalonolactone (16.7 mg), $[\alpha]_D^{26}$ +19° (EtOH; *c* 0.84), IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3650, 3580, 3430, 2960, 1735, 1600.

CHAPTER IV

# SCREENING OF CONJUGATES OF ABA METABOLITES IN FRUIT

Some researchers have reported that ABA is released from extracts of plant tissues by base-catalyzed hydrolysis [4,53,54]. In the peel of oranges matured on the tree, the amount of ABA released by hydrolysis exceeds the content of free acid ten fold [55]. Milborrow showed that ABA and PA were released by saponification of the aqueous residue from the tomato plants supplied with $^{14}C,^{3}H$-ABA [33]. At present only ABAGE has been identified as a conjugated form which can release ABA by hydrolysis [37]. However it may be erroneous to conclude that all of the base-labile ABA-conjugates are glucose ester; small amounts of other adducts could be overlooked. Conjugated forms of PA, DPA and epi-DPA also require further attention. This chapter describes the examination of the screening method of base-hydrolyzable conjugates in fruit.

GC is usually used for detecting and quantifying the free metabolites of ABA. The GC procedure requires preliminary derivatization such as methylation or trimethylsilylation. Convenient detection and quantification of ABA metabolites by HPLC were searched.

Of the two HPLC columns examined, μ-Bondapak $C_{18}$ was found suitable for quantification of the ABA metabolites. The five ABA metabolite-standards, ABA, *t*-ABA, PA, DPA and epi-DPA, can be separated by a μ-Bondapak $C_{18}$ column using 35% (v/v) methanol in 0.1%

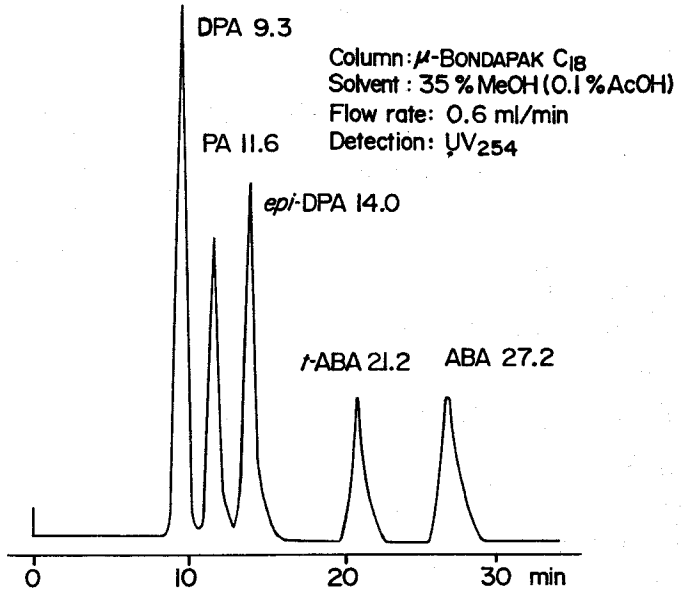


Figure IV-1 HPLC separation of ABA metabolites.

(v/v) acetic acid at a flow rate of 0.6 ml/min, as shown in Figure IV-1. The lower limit of detection of five ABA metabolites was 50 ng/injection, and quantification was linear up to 10 g using a $UV_{254\ nm}$ detector. The μ-Porasil column separated ABA, *t*-ABA and PA when a solution of 15% (v/v) acetonitrile in chloroform acidified with 0.1% acetic acid was passed through at a flow rate of 3.0 ml/min. However DPA and epi-DPA were not eluted within 30 min because they had higher polarity.

Next the conditions for hydrolysis and the methods of extraction were examined. Avocado fruit was chosen as the test material because ABA has been shown to be released by the alkaline hydrolysis of the extract of avocado fruit [6]. The extraction procedure

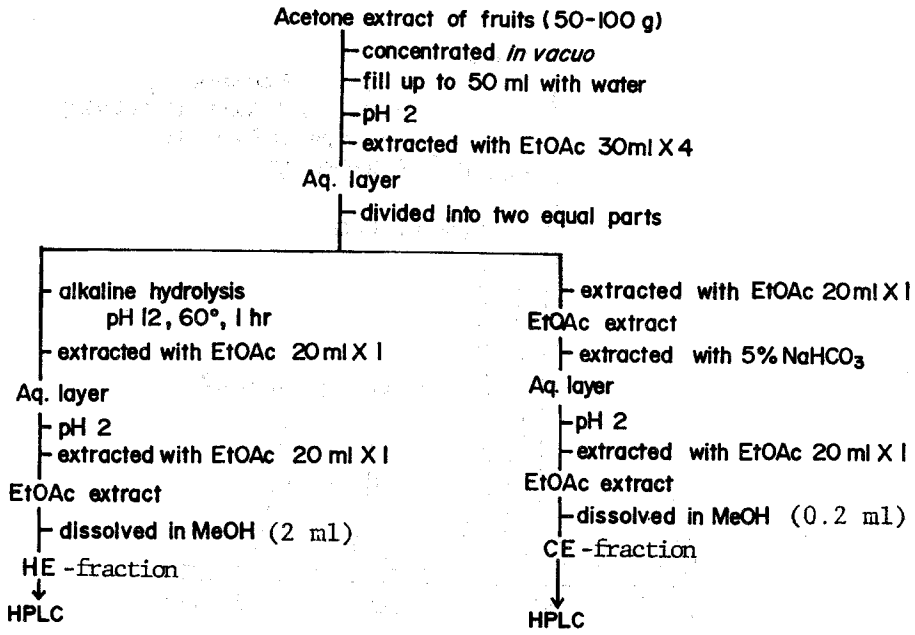


Figure IV-2 Hydrolysis and extraction method for screening.

for HPLC analysis is summarized in Figure IV-2. One avocado fruit (275 g) was extracted with acetone. Methanol is not suitable for extraction since ABAGE undergoes methanolysis to give glucose and MeABA during extraction. Water was added to the acetone extract to make the total volume 50 ml, and the pH of the solution was adjusted to pH 2 with 1 N HCl. This acidic aqueous solution was washed with ethyl acetate four times in order to remove free metabolites. The aqueous layer of this solution was then separated into two equal parts. One part was adjusted to pH 12 with 3 N NaOH and hydrolyzed at 60° for 1 hr. This solution was washed with 20 ml of ethyl acetate and adjusted to pH 2 with 3 N HCl and extracted with 20 ml of ethyl acetate. This ethyl acetate extract

was dried over sodium sulfate and concentrated *in vacuo* at 30°. The resultant residue was dissolved in 2 ml of methanol (HE-fraction). Since free metabolites of ABA, especially DPA and epi-DPA, can not be completely removed from the aqueous solution by ethyl acetate, it is possible to mistake the residual free metabolites for metabolites released by hydrolysis. To avoid such a mistake, the other part of the aqueous solution was extracted with 20 ml of ethyl acetate once more without hydrolysis. The acidic ethyl acetate-soluble part was dissolved in 0.2 ml of methanol (CE-fraction) and the CE-fraction was regarded as the control for the HE-fraction.

The free ABA metabolites in the HE- and CE-fraction were quantified using the HPLC procedure described above. Although ABA was not detected in either fraction, the HE-fraction was shown to contain 320 μg/100 g fruit weight of DPA, while the DPA content of the CE-fraction was 72 μg/100 g fruit weight (see Figure IV-3). This suggests that the content of ABA conjugates depends on the physiological state and/or the variety of avocado. The fraction corresponding to DPA was collected by HPLC and it was converted to methyl ester, which was gas-chromatographed on an OV-1 column to give a peak corresponding to MeDPA. These results indicate that DPA in the HE-fraction was released by alkaline hydrolysis and that conjugate(s) of DPA occurr in the avocado fruit.

Using this screening method, the conjugates in 11 kinds of fruits were examined. The chromatograms of the HE- and CE-fraction

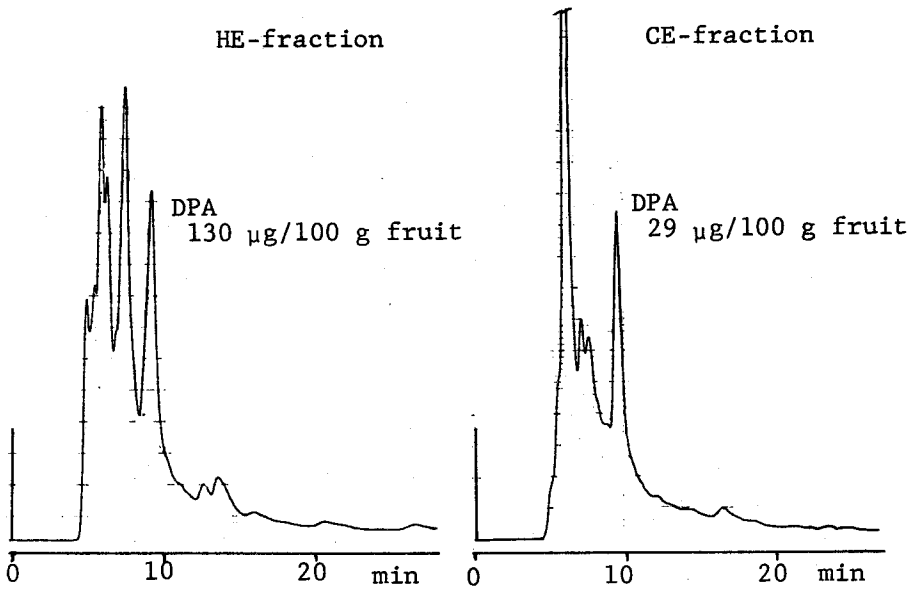


Figure IV-3 HPLC trace of HE- and CE-fraction of avocado fruit. HPLC condition, see Figure IV-1; injection volume, HE-fraction: 1 µl, CE-fraction: 3 µl.

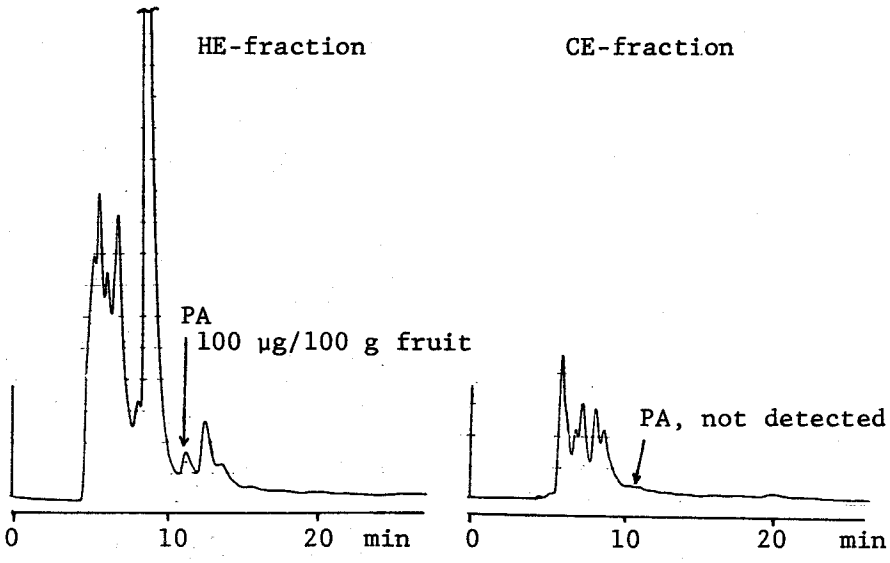


Figure IV-4 HPLC trace of HE- and CE-fraction of cherry fruit. HPLC condition, see Figure IV-1; injection volume, HE-fraction: 2 µl, CE-fraction: 2 µl.

Table IV-1 ABA metabolites released by alkaline hydrolysis.

(μg/100 g fruit weight)

| | Avocado | | Tomato | | Peach | | Prince melon | | Orange | | Cherry | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CE | CE | CE | HE | CE | HE | CE | HE | CE | HE | CE | HE |
| ABA* | ND | ND | ND | ND | 5 | 31 | ND | ND | ND | 77 | ND | ND |
| PA | ND | ND | ND | ND | 4 | 8 | ND | 8 | ND | ND | ND | ND |
| DPA | 29 | 130 | ND | ND | 10 | 73 | ND | 16 | ND | ND | ND | ND |
| epi-DPA | ND | ND | 3 | 45 | ND | ND | ND | 14 | ND | ND | ND | ND |

ND (not detected) : Apple, Kiwi, Papaya, Citron, Longan and Papaw.

* : ABA + *t*-ABA.

of cherry fruit, also shown in Figure IV-4, indicate that base-labile PA conjugate(s) occurr in their fruits. The results of the quantification, summarized in Table IV-1, show that tomato, peach prince melon and orange fruits contain base-hydrolyzable conjugates of ABA metabolites as well as avocado and cherry fruits. The extracts of apple, kiwi, papaya, citron, longan, and papaw did not release detectable amounts of ABA metabolites by hydrolysis. However, these results do not indicate that there are no conjugates of ABA metabolites in these fruits: Other conjugated forms which can not be hydrolyzed by alkali might occur in these fruits.

## EXPERIMENTAL

For general experimental details see Chapter II.

*Fruit materials*

One avocado (*Persea amerocana* Mills., 275 g), one tomato (*Lycopersion esculentum* Mill., 268 g), one peach (*Prunus persica* Batsch., Hakuto, 264 g), one prince melon (*Cucumis melo* L., 468 g), one orange (*Citrus sinensis* Osbeck, 184 g), 15 cherries (*Prunus avium* L., Napoleon, 168 g), one apple (*Malus pumila* Mill. var. domestica C. K. Schn., Kokko, 126 g), two kiwis (*Actinidia chinensis* Planch., 196 g), one papaya (*Carica papaya* L., 357 g), two citrons (*Citrus junos* Tanaka, Uzu, 84 g), and two longans (*Euphoria longana* Lam., 42 g) fruits were obtained from a local supplier. Ten papaws (*Asimina triloba* Dun., 270 g) were collected from the papaw-tree grown at Kyoto University.

*Preparation of standards of ABA, t-ABA, PA, DPA and epi-DPA*

(±)-ABA was purchased from Tokyo Chemical Industry Co., Ltd. and recrystallized from *n*-hexane and toluene, mp 188.3-189.5°. (±)-ABA (10 mg) in methanol was allowed to stand under a luminescent lamp for a week, and (±)-*t*-ABA isomerized from (±)-ABA was isolated from the fruit of *Robinia pseudacacia* as described in Chapter II. DPA and epi-DPA were prepared by reduction of MePA

[44]. Crystalline MePA (8.3 mg), obtained by methylation of PA with diazomethane, was dissolved in methanol (0.8 ml) at 0° and successively water (1.0 ml) and small amounts of sodium borohydride were added. After 1 hr water was added to make the solution 5 ml which was then extracted with ethyl acetate. The crude epimeric MeDPAs (9 mg) were separated by multiple development on silica gel TLC plates in *n*-hexane-ethyl acetate (1:1) to give the more polar compound, MeDPA (2 mg) and the less polar compound, Me-epi-DPA (2 mg). MeDPA was developed on silica gel TLC at $R_f$ 0.16, Me-epi-DPA at $R_f$ 0.22 (solvent, *n*-hexane-ethyl acetate, 2:3).

MeDPA, mp 138-139°; UV $\lambda_{max}^{EtOH}$ nm (ε): 268 (21000); $^1$HNMR (90 MHz, $CDCl_3$): δ 0.97 (3H, s, 6'-Me), 1.19 (3H, s, 2'-Me), 1.21-2.34 (4H, m, 3',5'-$H_2$), 2.05 (3H, s, 3-Me), 3.73 (3H, s, OMe), 3.77 (2H, br.s, 6'-$CH_2$), 3.87-4.48 (1H, m, 4'-H), 5.74 (1H, br.s, 2-H), 6.40 (1H, d, *J*=16 Hz, 5-H), 8.03 (1H, d, *J*=16 Hz, 4-H).

Me-epi-DPA, UV $\lambda_{max}^{EtOH}$ nm (ε): 268 (19000); $^1$HNMR (90 MHz, $CDCl_3$): δ 0.94 (3H, s, 6'-Me), 1.22 (3H, s, 2'-Me), 1.20-2.14 (4H, m, 3',5'-$H_2$), 2.03 (3H, br.s, 3-Me), 3.72 (3H, s, OMe), 3.91 (1H, d, *J*=8 Hz, 6'-CH), 3.98 (1H, m, 4'-H), 4.10 (1H, d, *J*=8 Hz, 6'-CH), 5.74 (1H, br.s, 2-H), 6.15 (1H, d, *J*=16 Hz, 5-H), 8.01 (1H, d, *J*=16 Hz, 4-H).

Each methyl ester was hydrolyzed with 1 N NaOH to yield DPA (1.5 mg) and epi-DPA (1.5 mg).

*HPLC analysis*

A model M-6000A liquid chromatograph (Waters Assoc., Milford, Ma, USA) with a model 440 UV absorption monitor and a model U6K universal injector was used. The chromatography was carried out on μ-Bondapak $C_{18}$ reversed-phase or μ-Porasil adsorption column (30 cm x 3.9 mm i.d., Waters Assoc.), to which a precolumn (5 cm x 2 mm) packed with same materials were fitted. All solvents were used after sonication. Retention times for authentic samples were; for μ-Bondapak $C_{18}$ (solvent, methanol-water-acetic acid, 35:65:0.1; flow rate, 0.6 ml/min; pressure, 66 kg/cm$^2$): ABA, 27.2 min; *t*-ABA, 21.2 min; PA, 11.6 min; DPA, 9.3 min; epi-DPA, 14.0 min; for μ-Porasil (solvent, acetonitrile-chloroform-acetic acid, 15:85:0.1; flow rate, 3.0 ml/min; pressure, 66 kg/cm$^2$): ABA, 4.7 min; *t*-ABA, 2.7 min; PA, 11.4 min).

*Identification of DPA released from an avocado fruit extract by GC*

The fraction corresponding to DPA was collected and treated with diazomethane. This was then analysed by GC (Shimadzu GC-7A). The chromatography was carried out on a 2 m x 3 mm i.d. glass column packed with 2% SE-30 on Chromosorb W [nitrogen flow rate 50 ml/min, temperature 200° and detector FID]. Retention times for authentic samples were; MeABA, 5.2 min; Me-*t*-ABA, 7.4 min; MePA, 6.2 min; MeDPA, 7.3 min; Me-epi-DPA, 6.9 min.

CHAPTER V

# A NEW CONJUGATE OF DIHYDROPHASEIC ACID FROM AVOCADO FRUIT

The results of the screening described in Chapter IV suggest that the conjugate(s) of DPA in avocado fruit occur in higher levels than in other fruit. Isolation of a DPA conjugate was attempted using alkaline hydrolysis as a monitoring method.

The overall scheme of isolation is shown in Figure V-1. Mature avocado fruit (*Persea americana*, 19 kg) was extracted with

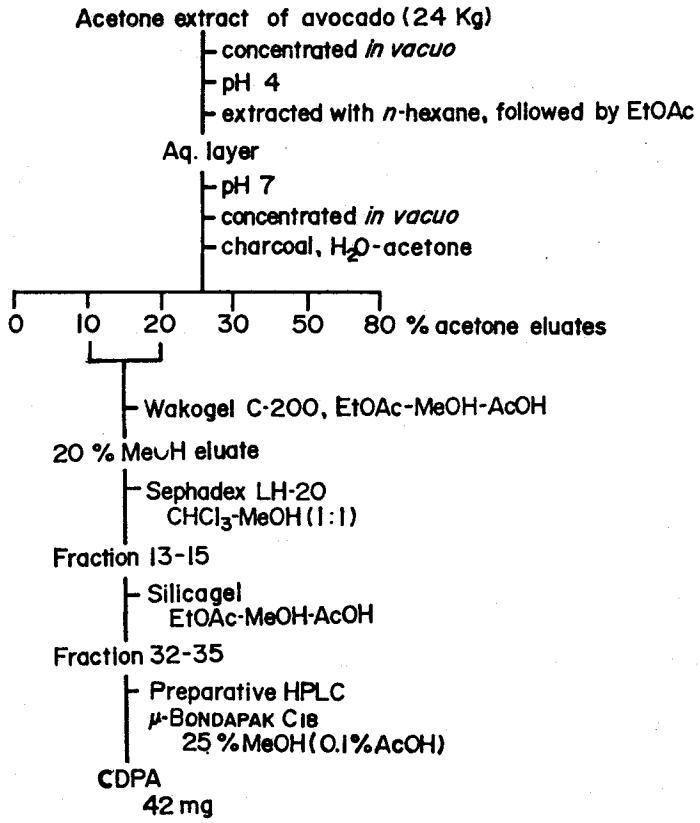


Figure V-1 Purification procedure of DPA conjugate from avocado fruit.

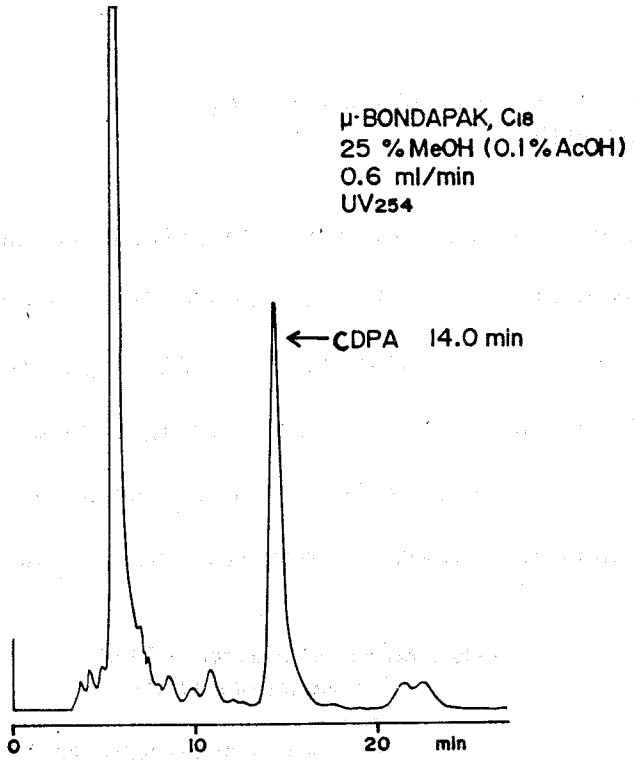


Figure V-2 HPLC trace of 10 and 20% acetone eluates.

acetone and the extract was concentrated *in vacuo* to give an aqueous concentrate, which was adjusted to pH 4 and washed successively with *n*-hexane and ethyl acetate. After neutralization, the aqueous solution was chromatographed on charcoal to give 0, 10, 20, 30, 40, 50 and 80% acetone eluates. An aliquot of each eluate was hydrolyzed with 0.1 N NaOH at 60° for 1 hr. HPLC analysis of the hydrolyzate of each eluate showed that DPA was released from the 10 and 20% acetone eluates by hydrolysis. These two eluates were combined and analyzed by HPLC using μ-Bondapak

$C_{18}$ (Figure V-2). The fractions containing UV-absorbing compounds were collected and each fraction was evaporated and hydrolyzed. HPLC analysis of the resultant residue showed that DPA was released from the fraction containing a compound with the peak at $R_t$ 14.0 min, suggesting that the fraction includes a conjugate of DPA. The combined fractions (10 and 20% acetone eluates) were rechromatographed successively on Wako gel C-200, Sephadex LH-20 and silica gel. The final purification by preparative HPLC afforded a DPA conjugate (CDPA) in a yield of 42 mg.

CDPA, $[\alpha]_D^{27}$ -20° (EtOH; $c$ 0.209), exhibited a UV absorption maximum at 257 nm ($\varepsilon$ 15600) and IR absorption bands at 3400, 2920, 2880, 1690, 1630 and 1600 $cm^{-1}$. As shown in Figure V-3, the $^1$HNMR

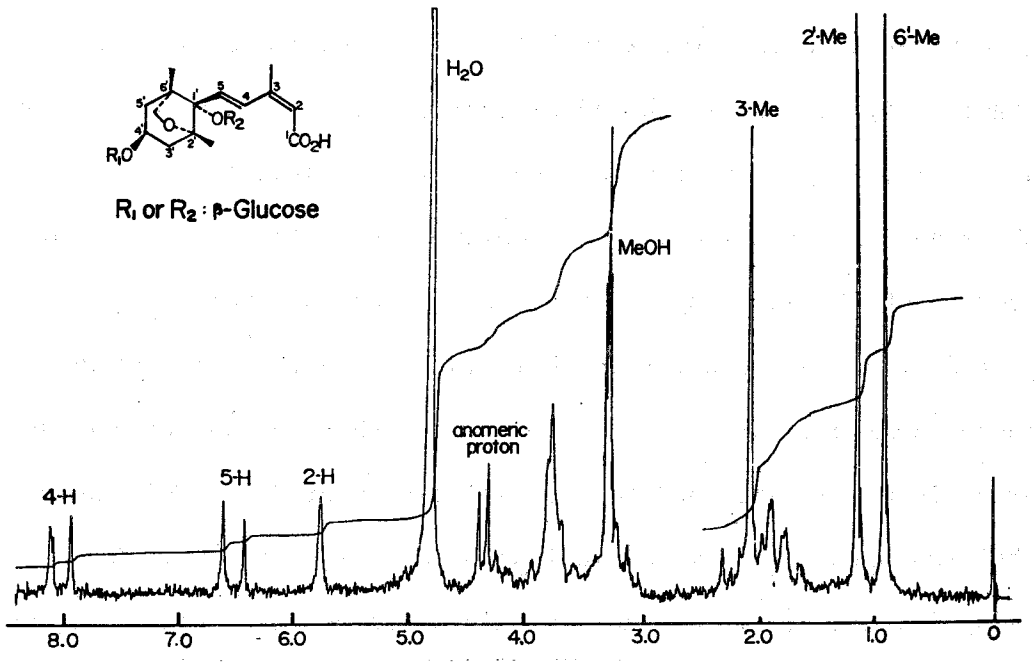


Figure V-3 $^1$HNMR spectrum of CDPA ($CD_3OD$).

spectrum of CDPA showed that it consisted of one mole of DPA and of β-glucose. CDPA was more easily hydrolyzed with acid than with alkali, and released glucose which was detected by GC after derivatization to hexaacetyl sorbitol. On acid hydrolysis of CDPA, however, none of the DPA released was detected by HPLC analysis because DPA decomposes with strong acid. CDPA was also hydrolyzed by β-glucosidase to yield DPA and glucose. Treatment of CDPA with diazomethane did not cleave the bond between glucose and DPA, and gave monomethyl ester (CDPA-Me) whose $^1$HNMR spectrum showed a singlet at δ 3.70 (3H) assignable to a carbomethoxyl. These chemical properties indicate that CDPA is not a β-glucosyl ester but a β-glucoside of DPA.

Acetylation of CDPA-Me with pyridine and acetic anhydride gave a tetra acetate (CDPA-Me-Ac) whose $^1$HNMR spectrum shown in Figure V-4, indicates a broad singlet (12 H) at δ 2.07 due to four acetoxyls, suggesting that a free tertiary hydroxyl at C-1' of CDPA-Me-Ac has remained. The doublet ascribable to the β-anomeric proton, which was observed at δ 4.35 in the spectrum of CDPA, appeared at δ 4.59 with little shift in the spectrum of CDPA-Me-Ac. These $^1$HNMR data provide strong evidence that the anomeric carbon of β-glucose is attached to the C-4' oxygen of DPA. This was further supported by chemical ionization mass spectrometry (CIMS) using isobutane, ammonia and ammonia-$d_3$ as reactant gases (Figure V-5). Isobutane CI mass spectrum of CDPA-Me-Ac showed a quasi-

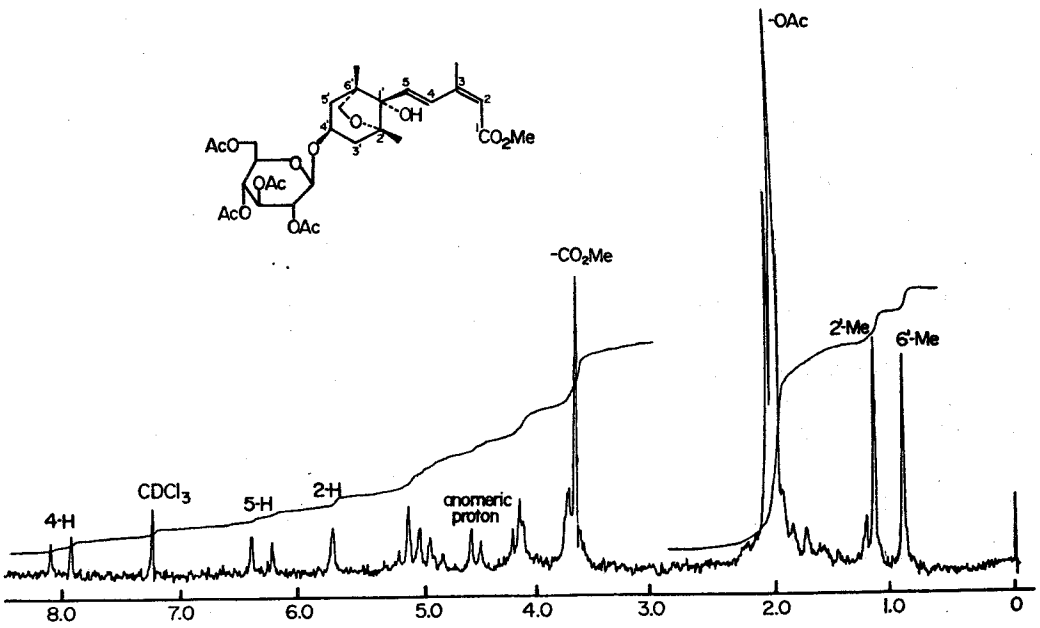


Figure V-4 $^{1}$HNMR spectrum of CDPA-Me-Ac ($CD_3OD$).

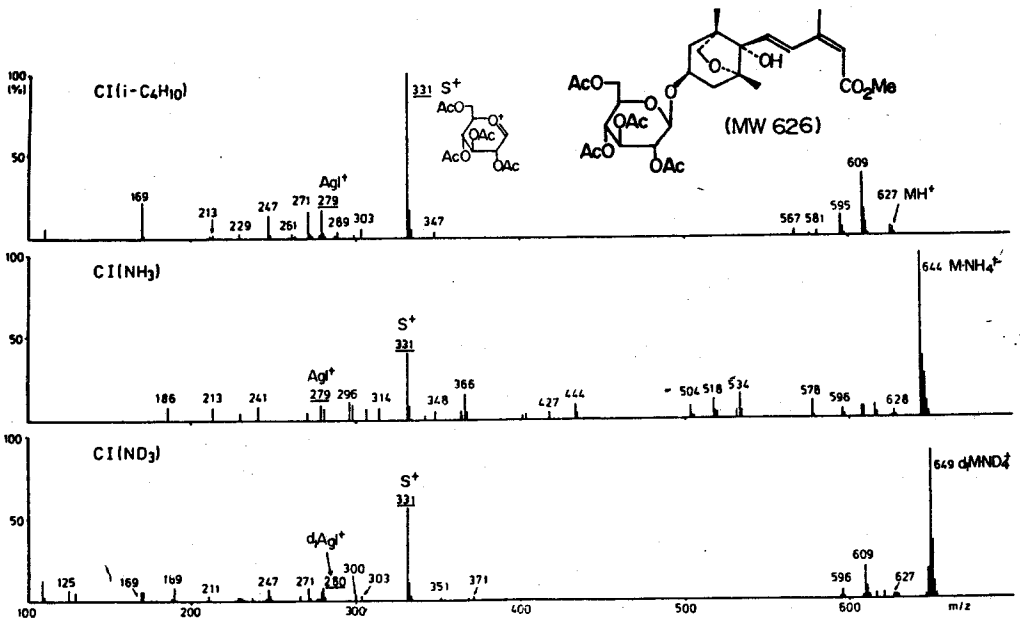


Figure V-5 Chemical ionization mass spectra of CDPA-Me-Ac.

molecular ion ($QM^+$) peak at *m/z* 627 corresponding to the mass weight of protonated tetraacetyl-β-glucoside of MeDPA ($MH^+$). Oxonium-type sugar (tetraacetylglucose) ion and aglycone (MeDPA) ion, produced by the cleavage of the glucosidic bond, were observed at *m/z* 331 and 279, respectively. The presence of a free hydroxyl of DPA moiety was demonstrated by the shifting technique using ammonia ($NH_3$) and ammonia-$d_3$ ($ND_3$) as reactant gases. As was expected, the peaks at *m/z* 644 ($d_1NMD_4^+$) and 279 ($Agl^+$) in the ammonia CI mass spectrum shifted to *m/z* 649 ($d_1MND_4^+$) and 280 ($d_1Agl^+$), respectively, in the ammonia-$d_3$ CI mass spectrum, while sugar ion at *m/z* 331 was not shifted. These data indicate that CDPA is dihydrophaseic acid-4'-*O*-β-glucoside (DPA-4'-β-GS). CDPA was identified by comparison of the spectral data (IR, $^1$HNMR and CIMS) of CDPA-Me-Ac with those of methyl dihydrophaseate-4'-*O*-β-tetraacetylglucoside (MeDPA-4'-β-AcGS) synthesized from MeDPA and α-acetobromo glucose under the presence of $AgNO_3$ by the method of Koenig-Knorr [56]. The DPA used for the synthesis was also isolated from the ethyl acetate extract of avocado fruit.

DPA-4'-β-GS is pretty stable to alkali, however a part of the compound contributes to the release of DPA from the extract of avocado fruit by alkaline hydrolysis. The possibility that unstable conjugates of DPA such as glucosyl ester might occur in avocado fruit besides DPA-4'-β-GS can not be excluded.

DPA-4'-β-GS as well as DPA did not inhibit the growth of rice

seedlings at a concentration of 100 ppm.

DPA-4'-β-GS was also suggested to be present in the fruit of grape (*Vitis vinifera*) by HPLC and ECD-GC analyses. DPA-4'-β-GS is expected to occur widely in plants.

## EXPERIMENTAL

For general experimental details see Chapter II. Column chromatography were carried out on Wako gel C-200 (Wako Pure Chemical Industries) and Sephadex LH-20 (Pharmacia Fine Chemicals). CI mass spectra were obtained using a Shimadzu LKB-9000 A mass spectrometer equipped with a chemical ionization source.

### *Extraction and fractionation*

Mature fruit of avocado imported from U. S. A. were purchased from a local supplier, soaked in 30 liters of acetone containing BHT (5 μg/ml) for 3 weeks, and then filtered. The acetone extract was concentrated at 30° *in vacuo*, and the aqueous solution was adjusted to pH 4 with 3 N HCl, and washed with 2 liters of *n*-hexane 3 times followed with 2 liters of ethyl acetate 3 times.

### *Isolation of DPA-4'-β-GS*

The aqueous solution neutralized with 3N $NH_3$ was chromatographed on charcoal (1.2 kg) and eluted with water containing 0, 10, 20, 30, 40, 50 and 80% acetone. An aliquot (1/1000) of each fraction was added to 0.5 ml of 0.1 N NaOH and hydrolyzed at 60° for 1 hr. The ethyl acetate soluble acidic part was dissolved in 1.0 ml of methanol and analyzed by HPLC (column, μ-Bondapak $C_{18}$ 30 cm x 3.9 mm i.d.; solvent, methanol-water-acetic acid, 35:65:0.1;

flow rate, 0.6 ml/min; pressure, 66 kg/cm$^2$; detection $UV_{254\ nm}$; $R_t$ of DPA, 9.3 min). DPA was found in the 10 and 20% acetone eluates. These two fractions were combined and an aliquot was injected to μ-Bondapak $C_{18}$ column (solvent, methanol-water-acetic acid, 25:75:0.1; flow rate, 0.6 ml/min; pressure, 66 kg/cm$^2$; detection, $UV_{254\ nm}$). The main peaks were observed at $R_t$ 5.8, 8.3, 10.8, 14.0 and 22.1 min in the HPLC. The fractions including UV-absorbing compounds were collected by HPLC and each fraction was hydrolyzed in the same manner as described above. HPLC analysis of the hydrolyzates showed that DPA was released from the fraction corresponding to a compound with a peak at $R_t$ 14.0 min. Subsequent purification of CDPA was monitored by detection using HPLC. The combined fractions (10 and 20% acetone eluates, 8.2 g) was applied to a Wako gel C-200 (300 g) column and eluted with ethyl acetate containing an increasing content of methanol and acetic acid (5%) in a volume of 1 liter per fraction. The fraction eluted with 20% methanol, on removal of the solvents, left a gum (580 g), which was chromatographed on Sephadex LH-20 (70 g) eluted with 50% chloroform in methanol in a volume of 10 ml per fraction. The fractions No. 13-15 were combined and evaporated to leave a residue (289 mg), which was rechromatographed on silica gel (20 g) eluted with ethyl acetate containing an increasing amount of methanol and acetic acid (2 %). The fraction eluted with 20% methanol was concentrated to give a gum (110 mg), which was purified by preparative

HPLC (column, μ-Bondapak $C_{18}$ 30 cm x 7.8 mm i.d.; flow rate, 1.3 ml/min; solvent, methanol-water-acetic acid, 25:75:0.1; pressure, 70 kg/cm$^2$; detection, $UV_{254\ nm}$) to give DPA-4'-β-GS (42 mg) as an intractable gum. $^1$HNMR (90 MHz, $CD_3OD$): δ 0.90 (3H, s, 6'-Me), 1.13 (3H, s, 2'-Me), 1.55-2.35 (4H, m, 3',5'-$H_2$), 2.04 (3H, s, 3-Me), 3.00-4.00 (9H, m, 4'-H, 6'-$CH_2$ and glucose protons), 4.35 (1H, d, *J*=7 Hz, anomeric proton), 5.77 (1H, br.s, 2-H), 6.53 (1H, d, *J*= 15 Hz, 5-H), 7.98 (1H, d, *J*=15 Hz, 4-H).

*Isolation of DPA*

The ethyl acetate-soluble acidic part was extracted with sodium bicarbonate solution. The aqueous extract was acidified to pH 2 with 2 N NCl and re-extracted with ethyl acetate. The ethyl acetate extract (2 g) was chromatographed on charcoal (200 g) eluted with acetone-water with increasing content of acetone. The fractions eluted with 50 and 65% acetone were concentrated to give a gum (320 mg), which was rechromatographed on Wako gel C-200 (10 g) eluted with toluene-ethyl acetate containing 2% acetic acid. The fractions eluted with 60 and 80% ethyl acetate, on removal of the solvents, gave crude DPA (61 mg), which was treated with diazomethane and subsequently recrystallized from toluene-*n*-hexane to yield MeDPA (25 mg) as colourless needles, mp 138-139°. UV $\lambda_{max}^{EtOH}$ nm (ε): 268 (21000); IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3600, 3450, (br), 2940, 2890, 1700, 1630, 1600, 1440, 1370, 1154, 990; $^1$HNMR (90 MHz, $CDCl_3$): δ

0.96 (3H, s), 1.18 (3H, s), 1.20-2.30 (4H, m), 2.05 (3H, br.s), 3.73 (3H, s), 3.78 (2H, br.s), 3.87-4.68 (4H, m), 5.78 (1H, br.s), 6.44 (1H, d, $J$=16 Hz), 8.07 (1H, d, $J$=16 Hz).

*Methylation and acetylation of DPA-4'-β-GS*

Excess ethereal diazomethane was added to a methanolic solution of DPA-4'-β-GS (8 mg). After 3 hours, the solvent was evaporated to give a gum of the monomethyl ester of DPA-4'-β-GS (MeDPA-4'-β-GS), which was then acetylated overnight with acetic anhydride-pyridine (1:1) at room temperature. The solvent was removed *in vacuo* to yield tetraacetate of MeDPA-4'-β-GS (MeDPA-4'-β-AcGS) (9.4 mg). $[\alpha]_D^{25}$ -41° (EtOH; $c$ 0.146); UV $\lambda_{max}^{EtOH}$ nm (ε): 267 (17700); IR $\nu_{max}^{CHCl_3}$ cm$^{-1}$: 3660, 3590, 3470, 2930, 2880, 1755, 1710, 1630, 1600; $^1$HNMR (90 MHz, $CDCl_3$): δ 0.97 (3H, s), 1.20 (3H, s), 2.07 (12H, br.s, OAc x 4), 2.11 (3H, s), 1.55-2.35 (4H, m), 4.59 (1H, d, $J$=7 Hz), 4.88-5.35 (3H, m), 5.83 (1H, br.s), 6.42 (1H, d, $J$=15 Hz), 8.12 (1H, d, $J$=15 Hz).

*Acid hydrolysis of DPA-4'-β-GS*

DPA-4'-β-GS (3 mg) was hydrolyzed with 1 N $H_2SO_4$ at 120° for 2 hr in a sealed tube. The solution was washed with ethyl acetate. The aqueous residue was neutralized with barium hydroxide solution and centrifuged. The supernatant was evaporated and dissolved in water. A small amount of sodium borohydride was added to the

aqueous solution. After addition of acetone, the aqueous solution was neutralized with acetic acid and evaporated to dryness. The reduction product was acetylated with acetic anhydride in pyridine at room temperature overnight, and analyzed by GC. GC was performed using a 2 m x 3 mm i.d. glass column containing 2% OV-1 on Chromosorb W [nitrogen flow rate 30 ml/min, temperature 210°, detector FID]. The retention time (7.6 min) of the acetate was identical with that of an authentic sample of hexaacetyl sorbitol.

*Enzymatic hydrolysis of DPA-4'-β-GS*

DPA-4'-β-GS (6 mg) in 0.1 M acetate buffer (pH, 5, 0.5 ml) was mixed with 0.5 ml of the same buffer containing β-glucosidase (10 mg) and allowed to stand at 28° for 24 hr. The solution was adjusted to pH 2 with 1 N HCl and extracted with 1.5 ml of ethyl acetate 3 times. DPA in the ethyl acetate extract was detected by the same HPLC procedure described in *isolation of DPA-4'-β-GS*. The aqueous layer was neutralized with 1 N $NH_3$ and evaporated to dryness. The resulting residue was extracted with methanol. The methanol extract was treated in the same manner described in *acid hydrolysis of DPA-4'-β-GS* to give hexaacetyl sorbitol.

*CIMS of MeDPA, MeDPA-4'-β-GS and MeDPA-4'-β-AcGS*

Operating conditions were as follows: ion source temperature, 230°; electron energy, 500 eV; accelerating voltage, 3.5 kV; reac-

tant gas pressure, approximately 0. Torr (isobutane, ammonia and ammonia-$d_3$); emmission current, 250 A. The samples were introduced into the ion source by means of a direct probe and volatilized at probe temperature of approximately 230-240°.

CIMS (isobutane) of MeDPA, *m/z* (rel. int.): 297 (27, $MH^+$), 279 (100, $MH^+$-$H_2O$), 265 (6, $MH^+$-MeOH), 261 (4, $MH^+$-$2H_2O$), 247 (9, $MH^+$-$H_2O$-MeOH), 223 (6), 183 (4), 171 (4), 155 (5), 127 (3), 125 (4).

CIMS (isobutane) of MeDPA-4'-β-GS, *m/z* (rel. int.): 459 (2, $MH^+$), 441 (7, $MH^+$-$H_2O$), 297 (21, $AOH_2^+$), 293 (5), 279 (100, $A^+$), 265 (5), 261 (5), 247 (12), 163 (11 $S^+$), 147 (7), 145 (10, $S^+$-$H_2O$), 127 (6, $S^+$-$2H_2O$).

CIMS (isobutane) of MeDPA-4'-β-AcGS, *m/z* (rel. int.): 627 (5, $MH^+$), 626 (6), 609 (38, $MH^+$-$H_2O$), 595 (13), 567 (4), 447 (6), 347 (4), 331 (100, $S^+$), 303 (5), 297 (1, $AOH_2^+$), 279 (17, $A^+$), 271 (16), 247 (14), 169 (22), 110 (6).

CIMS (ammonia) of MeDPA-4'-β-AcGS, *m/z* (rel. int.): 644 (100, $MNH_4^+$), 628 (5), 616 (8), 609 (8), 608 (8), 596 (7), 544 (11), 534 (15), 518 (12), 504 (8), 444 (10), 427 (6), 404 (6), 402 (5), 366 (15, $SOH \cdot NH_4^+$), 348 (5, $SNH_3^+$), 331 (40, $S^+$), 314 (7, $AOH \cdot NH_4^+$), 306 (10), 298 (11), 296 (11, $ANH_3^+$), 281 (8), 279 (9, $A^+$), 271 (5), 246 (9), 230 (6), 213 (8), 186 (8).

CIMS (ammonia-$d_3$) of MeDPA-4'-β-AcGS, *m/z* (rel. int.): 649 (100, $d_1MND_4^+$), 648 (40), 609 (2), 522 (2), 371 (25, $SOD \cdot ND_4^+$),

347 (7), 331 (12, $S^+$), 320 (5, $d_1AOD \cdot ND_4^+$), 300 (14), 280 (5, $d_1A^+$), 250 (9), 190 (5).

*Synthesis of MeDPA-4'-β-AcGS*

Crystalline MeDPA (15 mg) was dissolved in 1 ml of dry ether. α-Acetobromoglucose (30 mg) and $Ag_2CO_3$ (15 mg) was added to the ether solution and left at room temperature for 25 hr. Ethyl acetate (10 ml) was added to the reaction mixture and washed 3 times with 1 ml of water. Ethyl acetate was removed to give a gum (30 mg), which was chromatographed on charcoal (1.8 g) eluted with methanol-acetone mixtures. The fractions eluted with 10, 20 and 40% methanol in acetone were combined (26 mg) and purified by preparative TLC on silica gel with *n*-hexane-ethyl acetate (2:3) to give MeDPA-4'-β-AcGS. The total yeild was 23 mg (73 %). $[\alpha]_D^{10}$ -27° (EtOH; *c* 0.230); UV $\lambda_{max}^{EtOH}$ nm (ε): 267 (17200); IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3490, 2930, 2880, 1755, 1710, 1630, 1600; $^1$HNMR (90 MHz, $CDCl_3$): δ 0.97 (3H, s), 1.21 (3H, s), 2.07 (12H, br.s), 2.12 (3H, s), 1.55-2.35 (4H, m), 3.75 (3H, s), 3.65-3.95 (3H, m), 4.15-4.35 (2H, m), 4.59 (1H, d, *J*=7 Hz), 4.88-5.35 (3H, m), 5.82 (1H, br.s), 6.41 (1H, d, *J*=15 Hz), 8.11 (1H, d, *J*=15 Hz). CIMS (isobutane), *m/z* (rel. int.): 627 (5, $MH^+$), 262 (7), 609 (43, $MH^+-H_2O$), 595 (15), 567 (5), 347 (5), 331 (100, $S^+$), 303 (7), 297 (3, $AOH_2^+$), 279 (22, $A^+$), 271 (21), 247 (20), 169 (22), 110 (8).

*Identification of DPA-4'-β-GS from fruit of grape*

Fruit (190 g) of grape was soaked in 300 ml of acetone for one week. The acetone extract was concentrated at 30° *in vacuo*, and washed with ethyl acetate. An aliquot (500 mg) of the aqueous residue was chromatographed on charcoal (2 g) eluted with water-acetone mixtures. The fraction eluted with 50% acetone in water was analyzed by the same HPLC procedure used in *isolation of DPA-4'-β-GS*. The HPLC trace showed the UV-absorbing peak which was co-chromatographed with DPA-4'-β-GS. The fraction corresponding to DPA-4'-β-GS was collected and methylated with diazomethane followed by acetylation with acetic anhydride in pyridine for ECD-GC analysis.

GC was performed using a 2 m x 3 mm i.d. glass column containing 2% WE-30 on Chromosorb W, nitrogen flow rate 60 ml/min, temperature 280°. An electron capture detector (Shimadzu ECD-7) was used at 300° with a detection current of 1.0 nA. On ECD-GC, the derivatized sample obtained above gave a peak ($R_t$ 11.3 min) which was cochromatographed with an authentic sample of MeDPA-4'-β-AcGS.

*Rice seedling test*

The rice seedling test was performed in the same method described in Chapter II.

# CHAPTER VI

# CONCLUDING REMARKS

Studies on ABA were begun separately to identify the causal substance of such differing phenomena as bud dormancy and fruit abscission. After its isolation and structure elucidation, an extensive amount of knowledge has accumulated, and ABA is now recognized as one of the central endogenous regulators of higher plants with important hormonal functions.

The author has investigated endogenous metabolites of ABA and found two new conjugated metabolites. One was isolated from fruit of *Robinia pseudacacia*, and chemical and spectral evidence showed that it was a conjugate of hydroxyabscisic acid, β-hydroxy-β-methylglutarylhydroxyabscisic acid (HMG-HOABA). The C-3 of the HMG group was shown to possess *R*-configuration by HPLC analysis of the reduced product. HMG-HOABA had biological activities comparable to those of PA in rice seedling and stomatal closure tests. The other, a conjugate of DPA, was found in avocado (*Persea americana*) fruit by the systematic screening of conjugated forms in 12 kinds of fruit. The conjugate was identified as dihydrophaseic acid-4'-*O*-β-glucoside (DPA 4-β-GS) from chemical and spectrometric analyses. The occurrence of DPA-4'-β-GS in grape was also confirmed by HPLC and ECD-GC analyses.

During the investigation of HMG-HOABA and DPA-4'-β-GS, four other conjugates have been identified by other research workers.

Milborrow observed that $^{14}$C-ABA fed to apple seeds was metabolized to ABA-1'-*O*-α and β-glucoside (ABA-1'-α and β-GS) and PA-1'-*O*-β-glucoside (PA-1'-β-GS) [57]. ABA maltosyl ester (ABA-1'-α-Me) was isolated from birch branches by Semdner and co-workers [58].

Metabolism of ABA is now supposed as shown in Figure VI-1, including seven conjugated forms. ABA is metabolized to PA *via* HOABA and the resulting PA is converted to DPA and epi-DPA. The free acids form conjugates as an acylated form or glucosides or glucosyl esters. At present HMG-HOABA is the only conjugate that does not possess sugar moiety. Though a conjugate of epi-DPA has

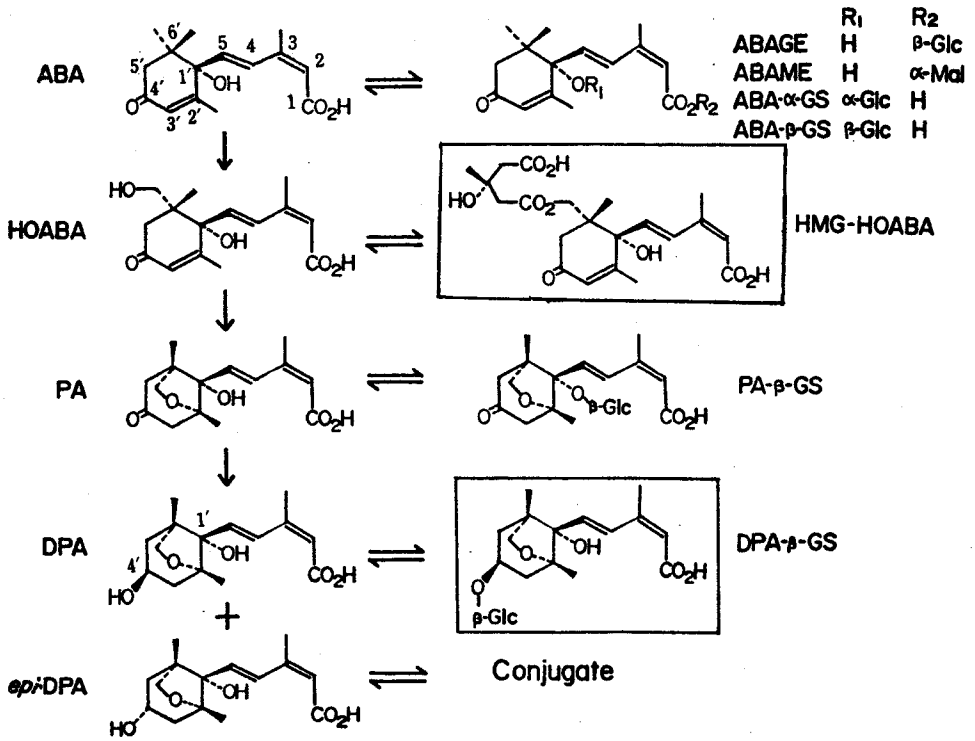


Figure VI-1 Metabolism of ABA.

not yet been found, epi-DPA also seems to form glucoside as well as DPA.

Most of the conjugates have been isolated from various plant species. Considering that the metabolic rate of ABA depends on plant species and physiological state, free acids accumulated in plant tissues at a high level may be converted to conjugated forms. This is supported by the experimental evidence that ABA supplied to tomato shoots is rapidly converted to ABAGE [29]. The conjugates may be a storage form and/or a transportation form, and contribute to the regulation of free acids contents in plant tissues.

Hereafter it is expected that the relation between physiological processes and changes in ABA level is more correctly comprehended.

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