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A NOVEL ABSCISIC ACID METABOLITE FROM SEEDS OF *ROBINIA PSEUDACACIA*

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Key Word Index—*Robinia pseudacacia*, Leguminosae; abscisic acid, conjugate of Metabolite C; conjugate of hydroxyabscisic acid; β-hydroxy-β-methylglutarylhydroxyabscisic acid.

Abstract—Abscisic acid and its novel metabolite, which was a conjugated form of hydroxyabscisic acid (Metabolite C), were isolated from seeds of *Robinia pseudacacia* L. The structure of the conjugate was shown to be (+)-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-methylglutarylhydroxyabscisic acid.

INTRODUCTION

Labelling studies in higher plants have suggested that exogenous abscisic acid (ABA) (**1**) is metabolized to phaseic acid (PA) (**2**) via hydroxyabscisic acid[†] (HOABA, Metabolite C) (**3**) and the resulting PA is converted to dihydrophaseic acid (DPA) (**4**) and its epimer (epi-DPA) (**4**) [1–5]. The conjugates have also been suggested to be formed from the free acids at each metabolic step [5, 6]. Among the free acids, PA and DPA have been identified as endogeneous metabolites of ABA in seeds of some plants [7, 8]. The only naturally occurring conjugate known so far is glucosyl ABA (**6**) which was isolated from immature seeds of yellow lupin [9].

In an investigation of natural metabolites of ABA in plants belonging to the Leguminosae, we have now found a novel conjugate of hydroxyabscisic acid along with ABA in the immature seeds of *Robinia pseudacacia*. This paper describes the isolation and structure of this compound.

RESULTS AND DISCUSSIONS

All steps of the isolation were monitored by a colour reaction on TLC [10] and by the inhibitory activity of the fractions on the growth of rice seedlings.

Fresh seeds (21 kg) of *R. pseudacacia* were extracted with MeOH, and an EtOAc-soluble part of the MeOH extract was chromatographed on Si gel to give two main fractions possessing the growth inhibitory activities. Further purification of the fractions by rechromatography afforded two growth inhibitors in yields of 2 and 36 mg respectively.

The minor inhibitor compound gave colourless needles, mp 163.5–164.2°, $[\alpha]_D^{20}$ +425° (EtOH; *c* 0.200), and was identified as (+)-ABA (**1**) by spectral comparison (PMR and ORD) with reference data [11].

The major inhibitor (**7**), $[\alpha]_D^{20}$ +560° (EtOH; *c* 0.013), showed a yellow-green fluorescent spot under UV at R_f 0.24 on Si gel TLC after spraying with 5% H_2SO_4 in EtOH followed by heating. This colouration was not distinguishable from that of ABA [10], but its R_f value (R_{ABA}[‡]: 0.28) showed that **7** was more polar than ABA.

The IR spectrum of **7** 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}). Compound **7** showed an UV absorption maximum at 258 nm (EtOH; ε 18 800) and a very intense positive Cotton effect with extrema at 286 ($[\alpha]^{20}$ +13 800°) and 245 nm ($[\alpha]^{20}$ −35 900°) (EtOH; *c* 0.0145) in the ORD curve, which are characteristic of ABA [12]. In the PMR spectrum of **7**, signals due to the protons of ABA lacking one of the 6′-*geminal* methyl groups were observed. In addition, the PMR 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 PMR data provided strong evidence that the inhibitor **7** was an ABA derivative modified at C-6′.

Treatment of **7** with diazomethane gave a dimethyl ester (**8**) whose PMR spectrum (90 MHz, $CDCl_3$) showed a singlet at δ 3.72 (6H) assignable to two carbomethoxyls. The parent ion peak at *m/e* 452 in the MS of **8** together with the data mentioned above indicated the molecular formula $C_{23}H_{32}O_9$ and consequently the molecular formula $C_{21}H_{28}O_9$ for **7**.

Alkaline hydrolysis of **7** followed by treatment with diazomethane gave two methyl esters. On GC–MS, one was identified as dimethyl β-hydroxy-β-methylglutarate (Me-HMG) by comparison with the MS of an authentic sample and the other was shown to be methyl phaseate (Me-PA), by MS comparison with a reference sample [13], which was derived from HOABA during the course of the esterification with diazomethane. Hence, conjugate **7** consisted of HOABA and HMG.

In the PMR spectrum of **7**, an AB-quartet (δ 4.15 and 4.19) indicated that the primary hydroxyl of HOABA

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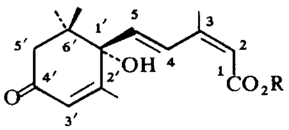
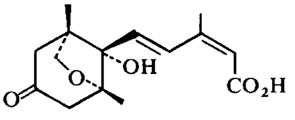
[*] All communications regarding this paper to K. Koshimizu at the above address.

[†] The 6′-hydroxymethyl derivative of ABA which has been called 'Metabolite C' is named 'hydroxyabscisic acid' (HOABA) in this paper.

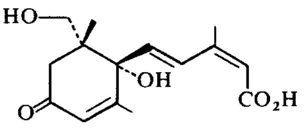
[‡] R_{ABA} = R_f value of 7 ÷ R_f value of ABA



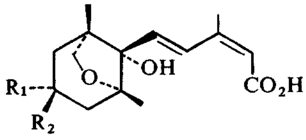
(1 R = H
(6) R = glucosyl



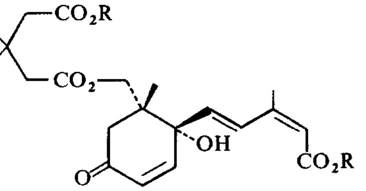
(2)



(3)



(4) R_1 = H, R_2 = OH
(5) R_1 = OH, R_2 = H



(7) R = H
(8) R = Me

was acylated with HMG. This was supported by the parent ion peak at m/e 452 (M^+) and the prominent peak at m/e 294 (M^+ − 158) in the MS of **8**. The Me-PA obtained above showed a negative plain ORD curve between 250 and 350 nm which was in good agreement with the data reported by Milborrow [2, 14]. The stereochemistry of the 6′-oxymethylene of **7** was, therefore, the *R* configuration as shown in the structure for **7**. Consequently **7** was shown to be (+)-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-methylgutarylhydroxyabscisic acid (HMG-HOABA).

The inhibitory activity of HMG-HOABA in the growth of rice (Tan-ginbozu) seedlings was about 1/100 of (+)-ABA.

HMG-HOABA was stable in MeOH, while the dimethyl ester was gradually decomposed into Me-PA and Me-HMG by solvolysis in MeOH at room temperature.

HOABA, the first degradative metabolite of ABA, is rearranged easily to PA by the intramolecular vinylogous addition of the 6′-hydroxymethyl group to the double bond at C-2′ [11]. The rearrangement was prevented by the acylation of the 6′-hydroxymethyl group with HMG. Further metabolism of the conjugate in plants to phaseic acid, if it occurs, will require the cleavage of the ester linkage. HMG-HOABA is expected to occur widely in the plant kingdom and we are continuing to investigate the distribution.

EXPERIMENTAL

Bioassay. The rice (Tan-ginbozu) seedling assay followed the method reported in a previous paper [15].

TLC. Samples were spotted on a precoated Si gel plate (Merck, layer thickness 0.25 mm), developed with C_6H_6–EtOAc–AcOH (7:12:1), and detected as fluorescent spots under UV-light (254 nm) after spraying with 5% H_2SO_4 in EtOH followed by heating.

Extraction and preliminary separation. Fresh seeds of *R. pseudacacia* L., harvested in July 1976, were soaked in MeOH, left at 5° for 2 months, and then filtered. The MeOH extract was conc. at 30° *in vacuo*, and the aq. soln extracted with EtOAc. The EtOAc extract (150 g) was chromatographed on Si gel (4 kg) and eluted with C_6H_6 containing 5, 10, 20, 35, 50, 65, 80 and 100% EtOAc, and EtOAc containing 5, 10, 20 and 100% MeOH.

Isolation of ABA. The fraction eluted with 65% EtOAc was conc. to give a gum (2.5 g), which was chromatographed on Si gel (65 g) and eluted with C_6H_6–EtOAc with increasing EtOAc content. The fraction eluted with C_6H_6–EtOAc (1:1) was evapd to leave a residue (774 mg), which was rechromatographed on charcoal (10 g) eluted with H_2O containing increasing Me_2CO (5% steps). The 70 and 75% Me_2CO eluates were conc. to give a gum (19 mg) and recrystallized from C_6H_6–hexane to yield (+)-ABA (**1**) (2 mg) as colourless needles, which showed a very intense Cotton effect with extrema at 290 ($[\alpha]^{21}$ + 33000°) and 249 nm ($[\alpha]^{21}$ − 87000°) (EtOH; *c* 0.010) in the ORD curve. PMR (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) and 7.83 (1H, *d*, *J* = 16 Hz).

Isolation of HMG-HOABA. The fractions eluted with 5 and 10% MeOH from the above Si gel column were combined and conc. to give a gum (12.5 g), which was chromatographed on charcoal (150 g) eluted with H_2O containing increasing (10%) amounts of Me_2CO. The fractions eluted with 60 and 70% Me_2CO, on removal of the solvents, left a gum (660 mg), which was chromatographed on Celite (200 g) impregnated with 120 ml of 1 M phosphate buffer (pH 5.4). The column was eluted with C_6H_6 containing an increasing conc. of *n*-BuOH (10% steps). The 30 and 40% *n*-BuOH eluates (94 mg) were rechromatographed on charcoal (1.5 g)–Celite (3.0 g) eluted with H_2O containing increasing (10%) amounts of Me_2CO. The 60, 70 and 80% Me_2CO eluates (52 mg) were purified by PLC on Si gel with C_6H_6–Me_2CO–AcOH (13:6:1) to give HMG-HOABA (**7**) (36 mg) as an intractable gum. PMR (90 MHz, CD_3OD): δ 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) and 7.79 (1H, *d*, *J* = 17 Hz, 4-H).

Methylation of HMG-HOABA. Excess ethereal CH_2N_2 was added to a solution of HMG-HOABA (10 mg) in MeOH. After a few min the solvent was evap. to give a gum of the dimethyl ester of HMG-HOABA (**8**). When a mixture of HMG-HOABA and CH_2N_2 in Et_2O–MeOH was allowed to stand overnight, HMG-HOABA was decomposed into Me-PA and Me-HMG via the dimethyl ester of HMG-HOABA. The MS of the dimethyl ester of HMG-HOABA showed ions at m/e (rel. int.) 452 (M^+, 2), 420 (M^+ − 32, 2), 403 (2), 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) and 122 (69).

Alkaline treatment of HMG-HOABA. HMG-HOABA (1.0

mg) was dissolved in 1 N NaOH (1 ml). The soln was allowed to stand overnight and, after adjustment to pH 3 with 2 N HCl, extracted with EtOAc. The EtOAc extracts were washed with H_2O and conc. to give a gum, which was treated with ethereal CH_2N_2 for GC–MS analysis.

GC–MS of the methyl esters of HMG-HOABA hydrolysate. This was performed using a 2 m × 2 mm 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 dimethyl β-hydroxy-β-methylglutarate: *m/e* (rel. int.) 175 (M^+ −15, 2), 143 (20), 117 (53), 101 (12), 85 (31), 74 (6), 48 (26) and 31 (100). MS of methyl phaseate: *m/e* (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) and 48 (100).

Isolation of Me-PA. HMG-HOABA (10 mg) was hydrolyzed and esterified in the same way described above. A mixture of the methyl esters gave Me-PA (6 mg) on removal of Me-HMG *in vacuo* (5 mm Hg) at 70°. $IR_{max}^{CHCl_3}$ cm^{-1}: 1720 and 1610: PMR (90 MHz, $CDCl_3$): δ 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) and 8.16 (1H, *d*, $J = 16$ Hz).

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REFERENCES

1. Milborrow, B. V. (1970) *J. Exp. Botany* **21**, 17.
2. Milborrow, B. V. (1969) *Chem. Commun.* 966.
3. Walton, D. C. and Sondheimer, E. (1972) *Plant Physiol.* **49**, 285.
4. Tinelli, E. T., Sondheimer, E., Walton, D. C., Gaskin, P. and MacMillan, J. (1973) *Tetrahedron Lett.* 139.
5. Milborrow, B. V. (1975) *Phytochemistry* **14**, 123
6. Zeevaart, J. A. D. and Milborrow, B. V. (1976) *Phytochemistry* **15**, 493.
7. MacMillan, J. and Pryce, R. J. (1968) *Chem. Commun.* 124
8. Martin, G. C., Dennis, F. G., Jr., Gaskin, P. and MacMillan, J. (1977) *Phytochemistry* **16**, 605.
9. Koshimizu, K., Inui, M., Fukui, H. and Mitsui, T. (1968) *Agric. Biol. Chem.* **32**, 789.
10. Antoszewski, R. and Rudnicki, R. (1969) *Anal. Biochem.* **32**, 233.
11. Milborrow, B. V. (1971) in *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W. ed.) p. 137. Academic Press, London
12. Milborrow, B. V. (1974) *Ann. Rev. Plant Physiol.* **25**, 259.
13. MacMillan, J. and Pryce, R. J. (1969) *Tetrahedron* **25**, 5903.
14. Milborrow, B. V. (1975) *Phytochemistry* **14**, 1045.
15. Koshimizu, K., Fukui, H., Mitsui, T. and Ogawa, T. (1966) *Agric. Biol. Chem.* **30**, 941.