ARTICLES

Derivatization of Abscisic Acid as the *p*-Nitrobenzyl Ester

James Velasco,* G. Ram Chandra, and Nagabhushanam Mandava

A quantitative procedure was developed for derivatizing nanogram quantities of abscisic acid (ABA) as the *p*-nitrobenzyl ester (PNB-ABA). As the ester, an increase in UV absorbance of 63.9% was obtained compared to ABA. Thus, a more sensitive measurement of ABA is possible by methods based on UV absorption. The lower limits of detection of the PNB ester of cis and trans isomers of ABA by high-performance liquid chromatography (LC) was found to be about 5 ng using a fixed wavelength detector at 254 nm. With a variable wavelength detector at 268 nm, a level of detection of about 4 ng is possible. Examination of thin-layer chromatography (TLC) plates under UV light enables detection of 1–2 μg of PNB-ABA ester. Identity of the PNB-ABA ester was confirmed by infrared (IR), nuclear magnetic resonance (NMR), and mass spectra (MS).

Several procedures have been used to quantitatively measure abscisic acid (ABA), a plant growth inhibitor. Milborrow (1967) measured 0.2 μg of underivatized ABA by optical rotary dispersion. Using gas chromatography (GC) with a flame ionization detector, Davis et al. (1968) detected 25 ng of ABA as the trimethylsilyl derivatives, and Lenton et al. (1971) detected near microgram levels as the methyl ester derivative. Seeley and Powell (1970) used an electron-capture detector in their GC procedure and were able to measure as little as 100 pg of ABA as the methyl ester. Sweetser and Vatvars (1976) measured 14 ng of ABA by high-performance liquid chromatography (LC) using a 254-nm detector.

The possibility of enhancing the detection and measurement of ABA by spectrophotometry was suggested by the findings of Knapp and Krueger (1975). They reported that the UV absorption of *p*-nitrobenzyl esters of fatty acids, C_{12}–C_{18}, was greater than that of the free acids.

We report the derivatization and quantitative measurement of ABA as the *p*-nitrobenzyl ester (PNB-ABA).

EXPERIMENTAL SECTION

Chemicals. The following chemicals were used: (±)-2-*cis*-4-*trans*-ABA from Sigma Chemical Co., St. Louis, Mo.; *O*-*p*-nitrobenzyl-*N*,*N*-diisopropylisourea (PNBDIU) from Regis Chemical Co., Morton Grove, Ill.; (±)-2-*cis*-4-*trans*-[2-^{14}C]ABA, sp act. 44.3 μCi/mg, purity 98% by TLC, from Amersham/Searle, Arlington Heights, Ill.; *p*-nitrobenzyl alcohol, ACS grade, glass distilled solvents from Burdick and Jackson Laboratories, Muskegon, Mich.

Esterification at Microgram Levels. One milligram (3.6 μmol) of PNBDIU in 100 μL of $CHCl_3$–MeOH (9:1) was added to a 2-mL reaction vial, and the solvent was evaporated under a stream of nitrogen. To the vial was added 1 μmol (265 μg) of 2-*cis*-ABA in 265 μL of $CHCl_3$–MeOH (9:1). The vial was sealed tightly with a Teflon-lined screw cap, placed in a heating block at 80 °C, removed after 2 h, and then uncapped. The solvent was allowed to evaporate to about 75 μL while still warm.

Purification by Thin-Layer Chromatography (TLC). Plates 20 × 20 cm were layered 0.35-mm thick with silica gel (Machrey-Nagel, Brinkmann Instruments, Westbury, N.Y.), air-dried, activated at 110 °C for 1 h, and stored. Just before use the plates were reactivated at 110 °C for 10 min and scored in half to permit two developments per plate. The entire esterified solution was spotted along the 20 cm side in six or seven spots. The vial was rinsed two times with 50-μL portions of $CHCl_3$, and the rinse solution was added to the spots. The chromatogram was developed in diethyl ether–hexane (7:3) for 1 h, removed, and viewed in a cabinet under 360-nm UV light. The PNB-ABA spots were marked, scraped off the plate, transferred to a small fritted column containing a thin layer of anhydrous sodium sulfate, and eluted with 10 mL of MeOH. The eluate was caught in a volumetric flask and adjusted to 10 mL with MeOH. This solution contained an equivalent of 26.5 μg of ABA/mL.

Instrumental Analysis. These analyses were made with 0.038 μM methanolic solutions of PNB-ABA, 2-*cis*-ABA, *p*-nitrobenzyl alcohol (PNB-OH), and the methyl ester of ABA (Me-ABA). The absorbance maxima of these solutions were measured with a Beckman Model DB spectrophotometer, and molar absorptivities were calculated. Infrared (IR) spectra were measured on a Nicolet 7199-IR spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian A-60 A instrument with $CDCl_3$ as solvent. Mass spectra (MS) were measured at 70 eV ionization on an LKB 9000 GC–MS spectrometer (LKB-Produkter, Bromma, Sweden). The sample was introduced into the ion chamber through a GC column (temperature, 230 °C) which was 0.75% SE-30.

Esterification at Nanogram Levels. Efficiency of esterification at nanogram levels was determined by use of radioactive ABA. 2-*cis*-4-*trans*-[2-^{14}C]ABA in 50-ng portions, about the level of interest, was reacted with PNBDIU in the weight ratios (reagent:acid) of 10:1, 100:1,

Seed Research Laboratory (J.V., G.R.C.) and Plant Hormone and Growth Regulators Laboratory (N.M.), Science and Education Administration, U.S. Department of Agriculture, Agricultural Research Center, Beltsville, Maryland 20705.

Table I. Spectrophotometric Measurements of PNB-ABA and Related Compounds

compd	MW	mM	μg/mL	λ max, nm	A	molar absorptivity	λ ratio 254/max
ABA	264	0.038	10.0	252	0.86	22 700	1.0
PNB-OH	153	0.038	5.8	272	0.30	9 200	0.70
PNB-ABA	399	0.038	15.2	268	1.40	37 200	0.78
Me-ABA	278	0.038	10.6	265	0.92	24 100	0.94

500:1, and 1000:1. The volume of solvent used, $CHCl_3$–MeOH (9:1), was 200 μL; and the reaction conditions were the same as described above. TLC of the esterified mixture was also as described above, but PNB-OH was spotted at each end (10 μg) and in the middle (5 μg) of the plate to indicate the uniformity of the chromatographic development. The entire developed area on the plate was scraped off in 1-cm strips parallel to the solvent front and transferred to scintillation vials. To the vials was added 10 mL of toluene containing 40 mg of PPO (2,5-diphenyloxazole) and 5 mg of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene). Radioactivity was counted by liquid scintillation procedures, and the counting efficiency for ^{14}C was 60–61%. Efficiency of esterification was determined from the total counts recovered and the counts at the origin.

Recovery of Nanogram Levels of ABA. To each of 10, 20, 40, 60, and 80 ng of [^{14}C]ABA was added 1000 times as much PNBDIU by weight. The volume of each reaction mixture was adjusted to 200 μL with $CHCl_3$–MeOH (9:1). Esterification, TLC, and measurement of radioactivity were the same as described above.

Isomerization. A 1:1 equilibrium mixture of 2-cis and 2-trans isomers of ABA was prepared by irradiation of 2-*cis*-ABA with UV light. A solution of 1 μmol of ABA (265 μg) in 265 μL of MeOH was placed in a 2-mL reaction vial and diluted to about 1 mL with $CHCl_3$. The vial was tightly capped and placed about 2–3 cm in front of a UV lamp (366 nm, rated at 7000 $\mu W/cm^2$ at 38 cm). After 4 h of irradation, the vial was uncapped and the solvent was evaporated. The irradiated ABA was reacted with 1 mg (3.6 μmol) PNBDIU in 300 μL of $CHCl_3$–MeOH (9:1) at 80 °C for 2 h. TLC development, removal of the zones containing the PNB-ABA, and elution of the ester were the same as outlined in the section for esterification of ABA at microgram levels.

High-Performance Liquid Chromatography. Aliquots of PNB-ABA solutions were diluted with MeOH so that the concentration was equivalent to 1 μg of ABA/mL (1.5 μg of ester/mL). The instrument was a Perkin Elmer Model 1220, equipped with a fixed wavelength (254 nm) UV absorption detector having a cell volume of 8 μL. A polar bonded phase column, 4.6 mm × 25 cm Whatman Partisil PAC, was used with the sensitivity of the UV detector set at 0.2 optical density units and attenuation at 1. Flow rate of the hexane–$CHCl_3$–MeOH (75:20:5) solvent system was 1 mL/min.

Gas Chromatography. A Hewlett-Packard gas chromatograph, Model 5830A, was used with a 4 ft × 0.25 in. glass column packed with 2% OV-17 and 1% OV-210. Temperatures were set at 250 °C for injection port, 200 °C for column, and 300 °C for electron-capture detector. The radioelement of the detector was a ^{63}Ni source. Flow rate of carrier gas, argon with 5% methane, was 55 mL/min and the sensitivity of the detector was 2^8 attenuation.

RESULTS AND DISCUSSION

Figure 1 is the thin-layer chromatogram, as viewed under UV light, of a reaction mixture after 1 μmol of ABA has been derivatized as the *p*-nitrobenzyl ester. The PNB-ABA spot is the second from the top and could be visualized under UV light because of the *p*-nitrobenzyl group. Nearly 1 μg of PNB-ABA on the TLC plates could be detected. The top spot was identified as *p*-nitrobenzyl alcohol, a breakdown product of the PNBDIU reagent during the reaction. The reagent as well as any unesterified ABA remain at the plate origin.

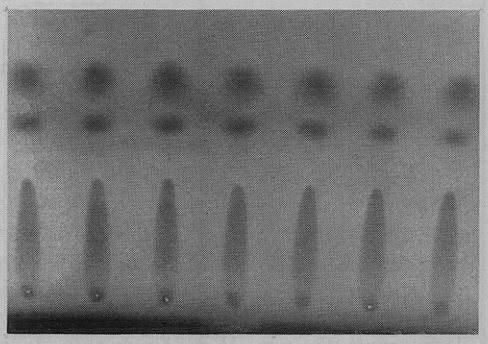

Figure 1. TLC development of ester reaction mixture viewed under UV light. Spots in ascending order from origin are: PNBDIU reagent plus unreacted ABA, PNB-ABA, *p*-nitrobenzyl alcohol (breakdown product of PNBDIU).

Table I shows that the molar absorptivity of PNB-ABA (37 200) was greater than the sum of the molar absorptivities of its acid and alcohol components. The absorbance of PNB-ABA was 63.9% higher than that of ABA.

To confirm the identity of the PNB-ABA ester, we examined the IR, NMR, and MS spectra of a purified sample. The IR spectrum exhibited bands for tertiary hydroxyl at 3440 cm^{-1}, conjugated ketone carbonyl at 1664 cm^{-1}, ester carbonyl at 1715 cm^{-1}, and ester C–O–C linkage at 1230 and 1145 cm^{-1}. The resonance signals in NMR appeared at: 1.03 and 1.13 ppm (2 s, 3 H each) for C-1 *gem*-methyl protons, 1.9 ppm (s, 3 H) for C-5 methyl, 2.07 ppm (s, 3 H) for C-9 methyl, 2.37 and 2.41 ppm (m, 2 H) for C-2 methylene, 5.3 ppm (s, 2 H) for benzylic protons, 5.6–6.2 ppm (m, 3 H) for C-4, C-7, and C-8 olefinic hydrogens, and 7.2 ppm (unresolved, 1 H) for C-10 proton. The aromatic protons in the *p*-nitrobenzyl moiety showed at 7.55 and 8.15 ppm, which corresponded to protons of ortho and meta substitution to a benzylic methylene group. Thus in the PNB-ABA ester, all the protons except the OH proton were fully accounted for. MS data (Figure 2) indicated the following fragments: *m/e* 399 (M^+), 381 (M – 18), 365, 343, 263 (M – 136), 246 (M – 153), 207, 190 (base peak), 162, 137, 134, 106, 91, and 78. The fragmentation pattern suggests that PNB-ABA besides exhibiting a molecular ion at *m/e* 399, loses water, the *p*-nitrobenzyl group, and *p*-nitrobenzyl alcohol from the molecular ion, thereby showing the fragments at *m/e* 381, 263, and 246. As was observed for ABA and its methyl ester by Most et al. (1970) and Cornforth et al. (1966), PNB-ABA also gave a base peak at *m/e* 190 ($C_{11}H_{10}O_3$) and other intense peaks at *m/e* 162 ($C_{10}H_{10}O_2$) and 134 ($C_9H_{10}O$). The fragment at *m/e* 190 would be formed as result of a sequence of reactions: cleavage of the 2,3-carbon–carbon bond in PNB-ABA, loss of isobutene, and cyclization after elimination of *p*-nitrobenzyl alcohol (*m/e* 343 → 190). Gray et al. (1974) suggested that the fragment at *m/e* 190 further breaks down to *m/e* 162 (loss of CO), which in turn

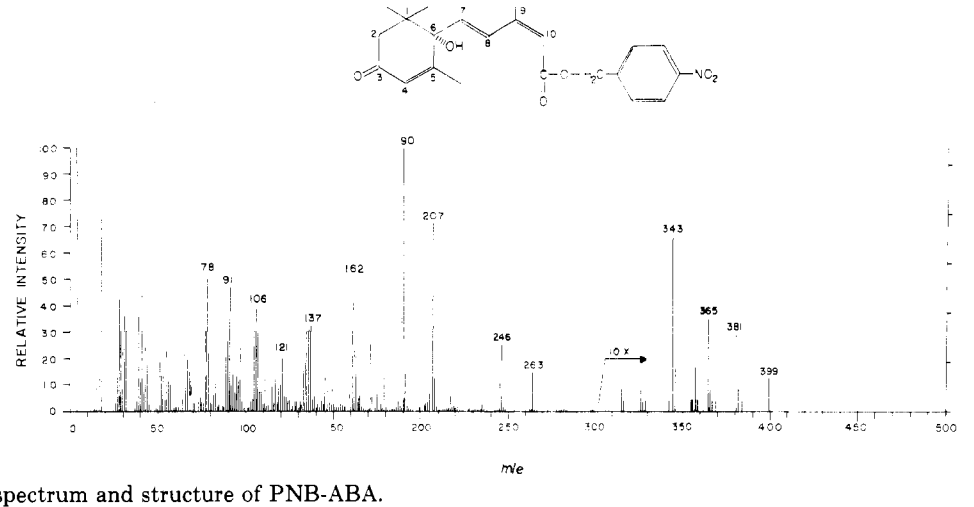

Figure 2. Mass spectrum and structure of PNB-ABA.

Table II. Effect of PNBDIU Quantity on the Percent Esterification of 50 ng of ABA

PNBDIU, μg	PNB-ABA, %[a]
0.5	0.01
5.0	11.03
25.0	84.63
50.0	99.91

[a] Determined by radioactive count of PNB-ABA separated by TLC.

Table III. Formation and Recovery of [^{14}C]ABA as PNB-ABA after TLC[a]

[^{14}C]ABA		recovered	
ng	CPM	CPM	%
10	56	51	91.1
20	112	97	86.6
40	224	218	97.3
60	336	317	94.3
80	448	435	97.1

[a] Ratio [^{14}C]ABA:PNBDIU (1:1000), counting efficiency 60%.

yields m/e 134 (loss of CO_2); this then gives the fragments at m/e 106 (loss of CO) and 91 (loss of CO_2). Hence, the mass spectral data clearly establishes structure I shown in Figure 2 for PNB-ABA.

Microgram amounts of ABA were esterified at a 3.6:1 mole ratio of reagent to acid. The optimum weight ratio for esterifying nanogram levels was determined by use of radioactive ABA. The results of the test with 50-ng portions of ABA (Table II) showed that 99.1% was esterified when the weight of PNBDIU was 1000 times that of the ABA, or 50 μg.

In a reciprocal test to determine the recoverability of nanogram quantitities of ABA, PNBDIU was reacted with the acid in the weight ratio of 1000:1. The average recovery of 10–80 ng of ABA, as its ester, from the TLC plates was 93.3% (Table III).

Figure 3 shows UV absorption traces for PNB-ABA during separation by LC. A single peak (top curve) was obtained for 15 ng of the ester of 2-*cis*-ABA in 10 μL of solution (10 ng of *cis*-ABA). The double peaks were obtained for an equivalent amount of PNB ester prepared from a sample of ABA that had been irradiated with UV light; they represent the esters of the cis and trans isomers of ABA. Each peak represents about 7.5 mg of the ester, or an equivalent of 5 ng of ABA. The peak areas could be increased 22% by use of a variable wavelength detector

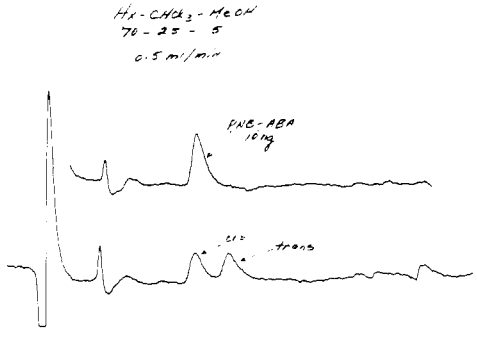

Figure 3. LC of PNB ester of ABA. Top superimposed curve is for the PNB ester of 2-*cis*-ABA. Lower curve is for the ester formed from UV-irradiated ABA.

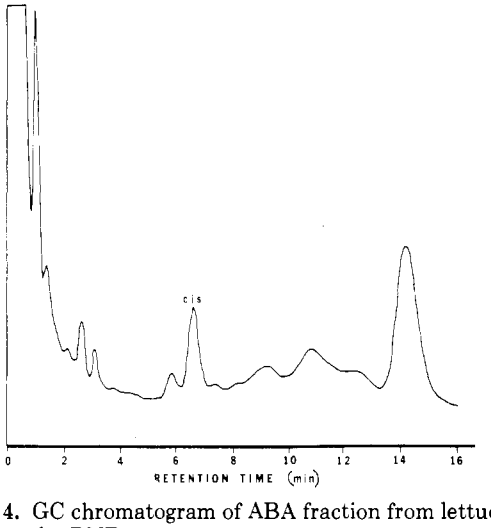

Figure 4. GC chromatogram of ABA fraction from lettuce seed extract as the PNB ester.

set at 268 nm; then the lower limit of detection of the ester would be near the 4-ng level.

ABA was purified from lettuce seed extract and its PNB ester was analyzed by both LC (this report) and GC procedure (Seeley and Powell, 1970). The LC profile of the seed PNB-ABA was similar to separation of the cis form as shown in Figure 3 while the GC profile is shown in Figure 4. The GC profile for ABA is in the cis form with a retention time of 6.8 min. The peak area represents an equivalent of about 2 ng of ABA.

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Comparative Metabolic Fate of 2,4-Dichlorophenoxyacetic Acid in Plants and Plant Tissue Culture

C. S. Feung, S. L. Loerch, Robert H. Hamilton, and Ralph O. Mumma*

The metabolic fate of 2,4-dichlorophenoxyacetic acid (2,4-D) in soybean cotyledon callus tissue culture was compared with the metabolism in soybean and corn plants to determine the usefulness of plant tissue culture as a research tool for pesticide analysis. Both plants and callus tissue were able to metabolize 2,4-D by common metabolic pathways, by way of ring hydroxylation as well as conjugation of the carboxyl with amino acids and with sugars. Qualitatively the metabolites of 2,4-D were the same in callus tissue and in plants but they varied quantitatively. Soybean callus tissue formed amino acid conjugates and hydroxylated derivatives more extensively than soybean or corn plants. The plants contained a higher relative proportion of hydroxylated derivatives than amino acid conjugates. Therefore, 2,4-D metabolism in callus tissue is a good indication of the operating metabolic pathways.

The metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) has been studied extensively in plants (Butts and Fang, 1956; Andreae and Good, 1957; Klämbt, 1961; Thomas et al., 1964; Faulkner and Woodcock, 1964; Audus, 1964; Hilton, 1966; Ojima and Gamborg, 1968; Fleeker and Steen, 1971; Hagin et al., 1970; Hamilton et al., 1971; Chkanikov et al, 1976) and in plant tissue cultures (Bristol et al., 1977; Feung et al., 1971, 1972, 1973, 1975, 1976). Soybean cotyledon callus tissue cultures rapidly convert externally applied 2,4-D-*1*-^{14}C into at least seven biologically active amino acid conjugates, at least two biologically inactive ring-hydroxylated metabolites, and, to a smaller extent, into glucose or sugar esters (Feung et al., 1973, 1974).

Experimentally it is easier and quicker to identify metabolites from plant callus tissue than from whole plants because of the absence of interfering plant pigments and starches (Mumma and Hamilton, 1976). Until experiments are conducted comparing pesticide metabolism in both callus tissue and in the whole plant, the potential advantages of using plant callus tissue as a research tool will remain in doubt. Therefore, we now report the comparative metabolism of 2,4-D by soybean and corn plants and by soybean callus tissue. Results of 2,4-D metabolism by corn endosperm tissue cultures under somewhat different conditions were previously reported (Feung et al., 1975).

MATERIALS AND METHODS

Soybean (*Glycine max* (L.) Merrill var. *Acme*) and corn (*Zea mays* (L.) [Su-1]) were grown in soil in the greenhouse for 2 weeks prior to treatment with 2,4-D-*1*-^{14}C (sp act. 52 mCi/mmol or 236 μCi/mg). With the aid of a syringe, 5 μCi 2,4-D-*1*-^{14}C (21 μg), dissolved in 10 μL of ethanol–acetone solution (1:1), was directly injected into the stem of each plant. Soybean and corn plants were separately harvested 14 days following treatment by cutting at the soil surface, and the tissue was stored in plastic bags at −18 °C for 24 h.

Callus tissue, derived from cotyledon of soybean (*Glycine max* (L.) Merrill var. *Acme*), was grown on autoclaved agar nutrient medium (Miller, 1963) plus α-naphthaleneacetic acid (NAA) in a 125-mL flask for 4 weeks. Then, 5 μCi 2,4-D-*1*-^{14}C (21 μg) in 10 μL of ethanol–acetone (1:1) was aseptically injected into three callus clumps (8–10 g total) which were then allowed to grow on the agar in the growth chamber for 7 days. The callus clumps were harvested and stored in plastic bags at −18 °C for 24 h prior to extraction.

The procedures used for extraction and fractionation of 2,4-D-*1*-^{14}C metabolites of callus tissues and plants were the same as those previously described (Feung et al., 1976). The frozen 2,4-D-*1*-^{14}C treated soybean and corn plants were separately cut into pieces 1 cm in length and subsequently homogenized in a Waring Blendor with 95% ethanol. The frozen callus tissue was also directly ground in a Waring Blendor with 95% ethanol. The homogenates were filtered with suction. The residue was boiled in 80% ethanol for 5 min, filtered with suction, and washed six times with 80% ethanol. The ethanol filtrate was combined, concentrated, adjusted to pH 2 with 3 N H_3PO_4, and then extracted four times with diethyl ether. The aqueous phase was subsequently extracted twice with 1-butanol and the 1-butanol layer was evaporated to dryness. This residue was dissolved in 15 mL of distilled water and adjusted to a pH of 4.5 with 1% $NaHCO_3$, Emulsin was added (Nutritional Biochemical Corporation), and the solution was incubated at room temperature for 72 h. The aglycons were obtained by acidification and extraction with diethyl ether. This aglycon solution and the original ether extract were evaporated to dryness, 90% ethanol (1 mL) was added to these residues, and the resulting solutions were stored at −18 °C for 12 h. The cold alcoholic solution of the original ether extract was then centrifuged at 300*g* for 5 min and the precipitate was discarded (precipitate

Pesticide Research Laboratory and Graduate Study Center and The Departments of Biology and Entomology, The Pennsylvania State University, University Park, Pennsylvania 16802.

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