Phytochemistry, Vol. 27, No. 3, pp. 673 676, 1988.
Printed in Great Britain.

0031 9422/88 \$3.00+0.00

β-D-GLUCOPYRANOSYL PHASEIC ACID FROM SHOOTS OF *LYCOPERSICON ESCULENTUM*

N. J. CARRINGTON, G. VAUGHAN and B. V. MILBORROW

University of New South Wales, P.O. Box 1, Kensington 2033, Australia

(*Revised received* 4 *August* 1987)

Key Word Index—*Lycopersicon esculentum*; *Solanaceae*; phaseic acid, *O*-β-D-glucopyranosyl phaseic acid, HPLC, GCMS, NMR.

Abstract—The glucose ester of phaseic acid has been isolated and characterized from tomato shoots (*Lycopersicon esculentum* cv. Grosse Lisse) fed with *RS*-[2-^{14}C]ABA and has been shown to occur naturally. The metabolite was isolated by reverse and normal phase HPLC as the tetra-acetyl derivative and identified by a combination of ^{1}H NMR (500 MHz), CIMS and TLC. Evidence for the natural occurrence of the glucose esters of 4′-dihydrophaseic and 4′-*epi*-dihydrophaseic acid was also obtained.

INTRODUCTION

Inactivation of the phytohormone abscisic acid (ABA) occurs via two main pathways, by oxidation and by conjugation [1]. Oxidation of the 8′-methyl group of ABA produces the unstable 8′-OH ABA which rearranges to phaseic acid (PA) and is reduced to the two epimeric dihydrophaseic acids, of which the 4′-*S* predominates. The importance of conjugation in the inactivation of plant hormones is underlined by the presence of conjugates, particularly glucosyl derivatives, of the metabolites of ABA. For example, the 4′-*O*-β-D-glucoside of dihydrophaseic acid (DPAGS) is the most abundant metabolite of ABA [2] in tomato plants.

Until now the glucose ester of phaseic acid has not been identified and characterized chemically, its presence in aqueous residues of plant extracts has been inferred from the release of free phaseic acid by basic hydrolysis: the free acid could, however, have been released from other conjugates, from the 1′-*O*-β-glucoside of phaseic acid or even HMG-HOABA which could release 8′-OH ABA thereby allowing phaseic acid to be formed. For convenience we have used the numbering system for PA that was proposed for ABA [3]. We now report the characterization of β-D-glucosyl phaseic acid.

RESULTS

Derivatisation of TLC of [2-^{14}C] *PAGE*

Samples of a ^{14}C-labelled material (R_f 0.67) from *RS*-[2^{14}C] ABA (R_f 0.92) and its reduction products were chromatographed on HPTLC plates after hydrolysis (R_f 0.84), acetylation (R_f 0.94) and methylation (R_f 0.66) and detected by autoradiography. A large reduction in polarity upon acetylation suggested the presence of a sugar residue and as treatment with diazomethane has no effect this suggested the presence of an esterified carboxyl group.

After microgram amounts of the metabolite were isolated its structure was confirmed by NMR and MS to be β-D-glucopyranosyl phaseate (PAGE). It was isolated as the acetyl derivative after HPLC in three systems. Reverse-phase HPLC was used to chromatograph the metabolite in the underivatised form and then as the acetyl derivative. Chromatography on a normal-phase silica column gave the acetylated metabolite (AcPAGE) with no impurities detectable by NMR. Diode array detection enabled a UV spectrum of the purified metabolite, in the eluting solvent, to be obtained. Approximately 200 μg of AcPAGE were obtained after 30 mg ABA had been fed to 74 g fresh weight of tomato shoots.

The UV spectrum of ABAGE exhibits a shoulder at 240 nm and λ_{max} to 273 nm is higher than that of the Me ester (265 nm). PAGE by comparison has a λ_{max} at 273 nm, again, with no shoulder at 240 nm, and so contrasts with the λ_{max} of dihydrophaseic acid 4′-*O*-β-D-glucopyranoside methyl ester (MeDPAGS), 265 nm. Comparison of the UV spectra of PA and PAGE and its derivatives with the glucose esters and glucosides of the ABA-diols [5] shows PAGE resembles the glucose esters rather than the glucosides, the shift in λ_{max} of PA (from 261 to 273 nm) when the glucose ester is formed is very similar to the corresponding changes observed with the diols and ABA.

The aglycone of AcPAGE was confirmed as PA after basic hydrolysis which released one major, UV absorbing product. The HPLC R_t of the compound (21.2 min) and the λ_{max} (265 nm) in acidic ethanol were identical with those of an authentic standard. The positive ion GC/CIMS of the methylated product also had the same characteristic fragment ions as those of methyl phaseate (MePA) previously determined [8, 9] by EIMS. Prominent ions were $[MH]^+$ at *m/z* 295, $[MH-H_2O]^+$ at *m/z* 277, $[MH-MeOH]^+$ at *m/z* 263, and other ions at *m/z* 245, 233, 125, 89 and 69. The differences in relative intensities between these and the earlier spectra probably reflect the different modes of ionisation.

Acid hydrolysis was used to isolate and identify the glycone released from AcPAGE. The acid hydrolysis product was trimethylsilylated and compared with the TMS derivative of D-glucose. Positive ion GC/MS subsequently proved both products to have very similar retention times (for α- and β-anomers) and mass spectra.

These results confirm that PAGE is composed of a phaseic acid molecule esterified with glucose.

Mass spectrometry

Evidence for PAGE as a glucose ester of phaseic acid was provided by both positive and negative ion CIMS of the acetyl derivative.

No parent ion was detected in the positive ion spectrum ($MH^+ = 611$) but ions at *m/z* 281 and 263 were found which are attributed to $[MH - \text{tetraacetylglucose}]^+$ and $[MH - \text{tetraacetylglucose} - H_2O]^+$ although the latter could also be a tetraacetylglucose ion. Characteristic fragment ions of the tetraacetylglucosyl residue which selectively lose acetic acid or ketene[10] were also observed. These were $[\text{tetraacetylglucose} + H]^+$ at *m/z* 331, $[\text{tetraacetylglucose} + H - HOAc]^+$ at *m/z* 263 and $[\text{tetraacetylglucose} + H - CH_2CO]^+$ at *m/z* 169.

The negative ion spectrum revealed a greater array of fragment ions but included the expected parent ion region. Two major fragment ions $[M-H]^-$ at *m/z* 609 $[M-H]^-$ and $[M - \text{tetraacetylglucose} - H_2O]^-$ at *m/z* 262. Other characteristic ions were at *m/z* 592, 490, 430, 370, 279, 168 and 125.

Loss of the tertiary hydroxyl group from the molecular ion is a common feature in both CI and EI mass spectra of ABA[11] and its metabolites [2, 9, 12, 13]. The presence of an ion $[M - H_2O]^-$ at *m/z* 592 indicates that the 1′-OH group of the phaseic acid residue is unsubstituted in PAGE.

NMR

The chemical shifts and coupling constants of the aglycone were consistent with those of methyl phaseate (MePA) as reported by Milborrow [14] (Table 1).

Further proof that the 1′-OH group of the PA is unsubstituted in AcPAGE is provided by the signal at $\delta 2.08$, attributed to the hydrogen of the 1′-OH group.

The signals in AcPAGE arising from the sugar moiety identified it as D-glucose: the chemical shifts are almost identical with those of pentaacetylglucose recorded at 300 MHz, in the same solvent, by Vaughan [5]. Minor differences in the positions of the C-2 and C-4 hydrogen atom signals were observed which is AcPAGE are centred at $\delta 5.20$ and 5.14 while in pentaacetylglucose that at C-2 is at $\delta 5.14$ and C-4 at 5.15. This probably reflects the interaction of the glucose with the aglycone in AcPAGE.

The coupling constant of the anomeric doublet of the glucosyl residue in AcPAGE is 8.0 Hz. This is in the range of 8–10 Hz indicating a β-linkage and not an α-linkage [15] (2–5 Hz). Furthermore, the integrals of the glucose hydrogens and the presence of just one signal of an anomeric hydrogen atom establish that only one glucose moiety is present.

PAGE, therefore, has been identified as the β-D-glucopyranosyl ester of phaseic acid.

Identification of PAGE reduction products

The 4′-*O*-β-D-glucopyranoside of DPA is often the most abundant metabolite formed from ABA so evidence

Table 1. ^{1}H NMR spectra (500 MHz) of *O*-acetyl PAGE and β-D-glucose pentaacetate in $[^2H]CHCl_3$

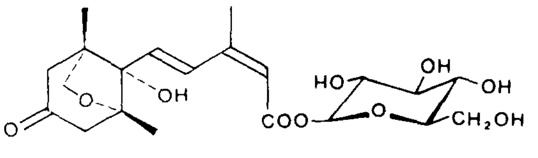
	AcPAGE (PA residue)*				MePA†	
H		*	*J* (Hz)		*	*J* (Hz)
OMe-1		—		*s*	3.74	
2	*s*	5.76		*m*	5, 82	
Me-3	*d*	2.00	2.4	*d*	2.02	2
4	*d*	8.03	15.5	*d*	8.19	16
5	*d*	6.27	15.5	*d*	6.25	16
OH-1′	*s*	2.08		*s*	2.09	
3′	*q*	2.63	18.8	*s*	A2.54 B2.52	<2
5′	*q*	2.49	18.2, 27.7	*m*		
Me-7′	*s*	1.22		*s*	1.25	—
H_S-8′	*d*	3.77	7.95	*q*	3.98	8, 2
H_R-8′	*q*	3.98	1.6, 8.05		3.78	8
Me-9′	*s*	1.02		*s*	1.02	—
PAGE (Sugar residue)*					β-D-Glucose pentaacetate‡	
1	*d*	5.76	$J_{1,2} = 8$	*d*	5.73	8
2	*dd*	5.20	$J_{2,3} = 10$	*t*	5.14	10
3	*dd*	5.27	$J_{3,4} = 9$	*t*	5.26	9
4	*dd*	5.14	$J_{4,5} = 11$	*t*	5.15	10
5	*m*	3.86–3.83	$J_{5,6}A = 2$	*ddd*	3.85	2, 5, 10
6(A)	*dd*	9.29	$J_{6A,6B} = 13$	*dd*	4.29	5, 12
6(B)	*m*	4.09–4.12		*dd*	4.12	2, 12

*500 MHz.

†100 MHz [14].

‡300 MHz [5].

§Chemical shifts measured from $CHCl_3$ at $\delta 7.24$ (internal standard).



for the simultaneous occurrence of the glucose esters of DPA (DPAGS) and its 4′-epimer (*epi*-DPAGS) was sought. Reduction of PAGE with borohydride at pH 6.0 (to prevent hydrolysis) produced DPAGS and *epi*-DPAGS whose positive ion CIMS as acetyl derivatives were compared to the EIMS of DPA and *epi*-DPA as reported [9, 12, 13]. Characteristic ions at *m/z* 265, 247, 239, 125 and 109 were common to spectra of all four compounds while those of AcDPAGE and Ac*epi*-DPAGE also showed ions at *m/z* 331, 263 and 169, attributable to the glucosyl residue [10]. The glucose esters of DPA and *epi*-DPA had to be hydrolysed and subjected to negative-ion GC-MS as pentafluorobenzyl (PFB) esters.

DISCUSSION

The glucose ester of phaseic acid has now been characterized and shown to be formed from *RS*-ABA although earlier results [4] have shown that only the natural *S*-ABA is converted into PA. PAGE was shown to occur naturally in tomato plants but in much smaller amounts than DPAGS. The glucose esters of DPA and *epi*-DPA were also identified but they occur naturally in very small concentrations. The release of DPA and *epi*-DPA by alkaline hydrolysis of bean extracts has been reported previously [13] so the glucose esters may have been the source of these compounds in bean shoots.

Although PAGE was present in tomato shoots that had not been supplied with ABA in much smaller quantities than DPAGS, it was detected by negative ion CIMS. The glucose esters of DPA and *epi*-DPA had to be isolated by HPLC, hydrolysed and subjected to negative ion GC/MS as pentafluorobenzyl (PFB) derivatives before they could be confirmed. The characteristic ions $[M-PFB]^-$ at *m/z* 323, $[M-PFB-H_2O]^-$ at *m/z* 305, $[M-PFB-CH_2CO]^-$ at *m/z* 281, $[M-PFB-MeCOOH]^-$ at *m/z* 263 and $[M-PFB-MeCOOH-H_2O]^-$ at *m/z* 245 from both samples and the identical chromatographic properties of synthetic and natural samples of DPAGE and *epi*-DPAGE established that these metabolites occur naturally in tomato shoots in small quantities.

EXPERIMENTAL

Feeding, extraction and chromatography. Tomato shoots (*Lycopersicon esculentum* L. cv. Grosse Lisse) (10 g) were fed *RS*-[2-^{14}C]ABA (6.4 µCi; 25.6 µCi/mol) or unlabelled ABA (30 mg to 70 g plants) and extracted after 7 days as described previously [2]. The combined Me_2CO–0.2% HOAc (4:1) extracts were filtered and evapd, 95% EtOH and HOAc were then added to give a 3:2 mixture which was filtered and passed through a column of 4 Sep-Pak C_{18} cartridges (Millipore-Waters, Milford, MA, U.S.A.) connected in series and washed with 20 ml 0.2% aq HOAc. The ABA and metabolites retained by the column were then eluted with 20 ml 95% EtOH–0.2% aq HOAc (7:13).

The HPLC apparatus was as described in [4] except that the eluate from ^{14}C-labelled samples was passed through a Precision Radioactivity Monitor (Reeve Analytical, Glasgow, Scotland). The monitor was connected via an HP-IB interface bus (IEEE-488) to an HP-85B computer (Hewlett-Packard, Corvallis, OR, U.S.A.) to record elution profiles of radioactivity. Unlabelled material was detected by replacing the radioactivity monitor with an HP104A Diode Array Detector (Hewlett-Packard, Waldbronn, F.R.G.)

Reverse-phase HPLC was carried out on a Techsil 10 C_{18} semi-prep. column, 250 × 8 mm i.d. (HPLC Technology, West Macclesfield, Cheshire, England) and all normal-phase HPLC on a Techsil 10 silica semi-prep. column, 250 × 8 mm i.d..

The metabolites eluted in EtOH–0.2% aq HOAc were dried and re-dissolved in 300 µl aq EtOH–H_2O (2:1) 150 µl sub-samples were chromatographed by reverse-phase HPLC at 2 ml/min, in EtOH–0.2% aq HOAc (3:17) isocratically for 15 min followed by a linear gradient, to 90% EtOH–0.2% HOAc (3:7) over 30 min. After a further 30 min another linear gradient over 18 min to 95% EtOH–0.2% aq HOAc (3:2) was applied, followed immediately by a purge to 95% EtOH for 15 min.

The fraction collected between 12.0 to 22.0 min contained both [2-^{14}C]DPAGS and a smaller, unidentified peak of unknown identity, subsequently characterised as phaseic acid glucose ester [1]. The whole fraction was collected and re-chromatographed on the same column in 95% EtOH–0.2% aq HOAc (2:23) which gave clear separation of the two peaks. [2-^{14}C]PAGE (16.5–20.5 min) was acetylated and chromatographed on a C_{18} column in 95% EtOH–0.2% aq HOAc (7:13) at 2 ml/min. The AcPAGE fraction (17.0–21.0 min) was dried and re-chromatographed by normal-phase HPLC, in isopropanol–hexane (3:21) at 2 ml/min. The AcPAGE fraction was collected from 20.0 to 24.0 min.

Derivatives. Derivatised and underivatised [2-^{14}C]PAGE samples were chromatographed on 100 × 100 mm silica gel HPTLC plates (60 F_{254}) (E. Merck, Darmstadt) with marker ABA in $CHCl_3$–MeOH–H_2O (75:22:3). Radioactive bands were detected by autoradiography.

PAGE (*ca* 50 µg) was subjected to basic hydrolysis (aq. NH_3 0.880, 30 min, 27°) and the dried hydrolysate chromatographed by reverse-phase HPLC in 95% EtOH–0.2% aq HOAc (15.85) at 4 ml/min. A single UV absorbing peak (PA) was collected (19.0–23.0 min), methylated and analysed by GCMS.

A sample of AcPAGE (15 µg) was hydrolysed in 1 M HCl (50 µl, 100°, 6 hr). The hydrolysate was then extracted (× 3) with Et_2O. The phase was neutralized, dried and trimethylsilylated with 'Sil-Prep' (Applied Sciences, Deerfield, ILL, U.S.A.). The product was analysed by GCMS and compared to the TMSi derivative of standard D-glucose.

Reduction of PAGE to DPAGE and epi-*DPAGE.* PAGE was reduced as described in ref. [5]. The sample (80 µg) in EtOH–K-Pi 0.1 M buffer (pH 6.0; 2:1, 1 ml, 0°), was treated with $NaBH_4$ (3 mg) at 0, 5, 10 and 30 min immediately followed by dropwise additon of a satd NaH_2PO_4 soln to return the pH to 6.0 and stirred continuously. Aq HOAc (2 ml 0.2%) was added, the EtOH removed under vacuum, loaded onto two C_{18} Sep-Paks in series and washed with 20 ml 0.2% aq HOAc. The products were eluted with 10 ml EtOH–0.2% aq HOAc (7:13:2) and dried. Aliquots (50 µl) were chromatographed by reverse-phase HPLC in 95% EtOH–0.2% HOAc (2:23) at 4 ml/min. Two major peaks, DPAGE and *epi*-DPAGE, were collected from 9.0–12.0 min and 19.0–24.0 min respectively, they were dried, acetylated and rechromatographed in 95% EtOH–0.2% aq HOAc (9:11) or 95% EtOH–0.2% HOAc (7:13) respectively at 2 ml/min. The AcDPAGE (16.0–22.0 min) and Ac*epi*-DPAGE (28.0–32.0 min) were further purified by normal-phase HPLC in isopropanol–hexane (3:7). AcDPAGE (6.0–9.0 min) and Ac*epi*-DPAGE (16.0–19.0 min) were analysed by negative ion CIMS with direct probe insertion.

The natural occurrence of DPAGE and *epi*-DPAGE was established by hydrolysing the appropriate HPLC fractions from the reverse-phase 95% EtOH–0.2% HOAc column. The AcPFB esters were then synthesized by the method of ref. [6] and analysed by GCMS on a BP-1 capillary column with negative ion monitoring.

Mass spectra were obtained under the conditions of ref. [7]. MePA formed after hydrolysis of DPAGE was injected on to a 3% OV-225 GC column (1.5 m × 2 mm id.) held at 150° for 1 min then programmed to rise 10°/min to 300°. The PFB-AcDPA, PFB-Ac*epi*-DPA and the TMS derivatives were run on a bonded fused silica BP-1 capillary GC column (12 min × 0.33 mm) (Scientific Glass Engineering, Melbourne, Australia) on the same temperature programme. The PFB-derivatives were analysed by NICI MS, the TMS-derivatives by PICI MS. AcPage, AcDPAGE and Ac*epi*-DPAGE were analysed by direct probe insertion and volatilized by increasing the ion source temperature.

AcPAGE PIC MS (methane) *m/z* (rel. int.): 331 (18), 309 (3), 281 (30), 271 (3), 263 (33), 243 (6), 235 (12), 225 (3), 205 (100), 177 (15), 169 (24), 133 (11), 105 (12), 77 (20), 59 (2).

AcPAGE NICI MS (methane) *m/z* (rel. int.): 611 $[M+H]$ (26), 609 $[M-H]$ (100), 592 (2), 562 (5), 529 (4), 353 (1), 322 (1), 304 (3), 287 (4), 279 (23), 262 (97), 210 (4), 185 (8), 168 (35), 162 (1), 143 (2), 126 (17), 125 (14), 119 (9), 108 (8), 99 (2), 82 (1), 81 (1).

MePA from hydrolysis of PAGE, PICI MS (methane) *m/z* (rel. int.): $[M+1]^-$ 295 (13), 277 $[M+1-H_2O]^-$ (100), 263 (33), 245 (9), 233 (5), 125 (5), 111 (4), 89 (20), 69 (16), 61 (25).

AcDPAGE, PICI MS (methane) *m/z* (rel. int.): $[M+1-H_2O]^+$ 637 (55), 603 (1), 595 (45), 577 (12), 551 (3), 537 (1), 523 (3), 411 (3), 369 (1), 331 (80), 207 (10), 271 (18), 265 (3), 247 (8), 229 (2), 211 (9), 169 (100), 139 (7), 127 (8), 109 (51), 97 (5), 83 (6), 69 (2), 61 (59).

Ac*epi*-DPAGE, PICI MS (methane) *m/z* (rel. int.): $[M+1-H_2O]^-$ 636 (4), 623 (2), 594 (21), 577 (6), 553 (22), 537 (1), 521 (2), 411 (3), 369 (1), 331 (100), 307 (1), 289 (8), 271 (23), (265) 23, 257 (7), 247 (13), 229 (7), 211 (10), 207 (12), 169 (80), 165 (12), 139 (8), 127 (7), 109 (40), 97 (2), 83 (4), 61 (23).

ACPFB DPA, NICI MS (methane) *m/z* (rel. int.): 323 $[M-PFB]^-$ (100), 317 [impurity?]$^-$ (12), 305 $[M-PFB-H_2O]$ (14), 263 $[M-PFB-HOAc]$ (4).

AcPFB *epi*-DPA, NICI MS (methane) *m/z* (rel. int.): 323 $[M-PFB]^-$ (100), 305 $[M-PFB-H_2O]^-$ (14), 281 (4), 263 $[M-PFB-HOAc]^-$ (31).

^{1}*H NMR* spectra of AcPAGE (100 μg) were recorded in $[^2H]CHCl_3$ at 500 MHz on a Bruker AM-500 spectrometer in pulsed Fourier transform mode with quadrature detection. Spectral accumulation parameters were: sweep width 5000 Hz, 8192 addresses, radiofrequency pulse of approximately 5.8 μsec duration, 2 sec recycle time, approximately 100 accumulations and 30° probe temperature with $CHCl_3$ as an int. standard at δ7.24 ppm. 1D and 2D (COSY) ^{1}H spectra were obtained.

Acknowledgements We thank Dr K. Cross for the ^{1}H NMR and Dr A. M. Duffield for the mass spectra. The work was supported by the Australian Research Grants Scheme.

REFERENCES

1. Loveys, B. R. and Milborrow, B. V. (1983) *The Metabolism of Abscisic Acid: S. E. B. Symposia.* (Crozier, A. and Hillman J. R., eds), p. 71. Cambridge University Press, Cambridge.
2. Milborrow, B. V. and Vaughan, G. T. (1982) *Aust. J. Plant Physiol* **9**, 361.
3. Boyer, G. L., Milborrow, B. V., Wareing, P. F. and Zeevaart, J. A. D. (1986). In *Plant Growth Substances* 1985 (Bopp, M., ed.), p. 99. Springer Berlin.
4. Vaughan, G. T. and Milborrow, B. V. (1984) *J. Exp. Botany* **35**, 110.
5. Vaughan, G. T. (1985) Ph.D. Thesis. University of NSW.
6. Netting, A. G. and Duffield, A. M. (1985) *Biomed. Mass Spectrom.* **12**, 668.
7. Nonhebel, H. M. and Milborrow, B. V. (1986) *J. Exp. Botany* **37**, 1533.
8. MacMillan, J. and Pryce, R. T. (1969) *Tetrahedron* **25**, 5903.
9. Tietz, D., Dörffling, D., Worhle, D., Enxleben, I. and Liemann, F. (1979) *Planta* **147**, 168.
10. Hogg, A. M. and Nagabhushan, T. L. (1972) *Tetrahedron Letters* **47**, 4827.
11. Gray, R. T., Mallaby, R., Ryback, G. and Williams, V. P. (1974) *J. Chem. Soc. Perkin II*, 919.
12. Takeda, N., Harada, K.-I., Suzuki, M., Tatematsu, A., Hirai, N. and Koshimizu, K. (1984) *Agric. Biol. Chem.* **48**, 685.
13. Zeevaart, J. A. D. and Milborrow, B. V. (1976) *Phytochemistry* **15**, 493.
14. Milborrow, B. V. (1975) *Phytochemistry* **14**, 1045.
15. Coxon, B. (1972) *Methods Carbohydr. Chem.* **6**, 513.