J Plant Growth Regul (1989) 8:181–197

Journal of
Plant
Growth
Regulation
© 1989 Springer-Verlag New York Inc.

Cytokinin Biochemistry in Relation to Leaf Senescence. III. The Senescence-Retarding Activity and Metabolism of 9-Substituted 6-Benzylaminopurines in Soybean Leaves*

Ren Zhang and David S. Letham

Plant Cell Biology Group, Research School of Biological Sciences, The Australian National University, Canberra City, ACT, 2601, Australia

Received August 12, 1988; accepted December 2, 1988

Abstract. A group of 14 9-substituted derivatives of 6-benzylaminopurine (BA), including the alanine conjugate, oxygen heterocyclic and alkyl derivatives, and compounds with a modified 9-ribose moiety, were assessed for their ability to retard soybean leaf senescence. The 9-alanine conjugate was very weakly active, and only two compounds, 9-(2-tetrahydropyranyl)-BA (9THP-BA) and 9-(2-tetrahydrofuranyl)-BA (9THF-BA), proved to be considerably more effective than BA. The metabolism of these three BA derivatives was determined to rationalize their differing activity. The alanine conjugate of BA was largely unmetabolized in leaf discs, but 9THP-BA and 9THF-BA released free BA and were also debenzylated to 9THP-adenine and 9THF-adenine, respectively. The three products of metabolism were identified by mass spectrometry. The enhanced activity of 9THP-BA and 9THF-BA, relative to that of BA, is attributed to their greater stability and their ability to gradually release free BA. This released BA was less susceptible to inactivation by alanine conjugate formation than was exogenous BA. The novel BA analogue 7-benzylaminooxazolo[5,4-d]pyrimidine, in which the 9-NH is replaced by oxygen, was inactive at 100 μM.

In the previous paper of this series (Zhang et al. 1987), the metabolism of 6-benzylaminopurine (BA) in soybean leaves was reported and the alanine conjugate was the dominant metabolite formed. Minimization of this conjugation is of both physiological and agronomic interest since such conjugation

* For part II, see Zhang et al. 1987

may result in inactivation of BA. 9-Substituted BA derivatives which slowly release free BA may possess enhanced senescence retarding activity in soybean because these compounds would not be directly subject to inactivation by alanine conjugation. Accordingly, we have now compared the activities of selected 9-substituted derivatives of BA and three BA analogues using soybean leaf bioassay systems in which cytokinins induce chlorophyll retention. The metabolism of two highly effective BA derivatives was also studied in an attempt to rationalize their enhanced activity.

Materials and Methods

Chromatographic Methods

Unless stated otherwise, all TLC was performed on layers spread with Merck silica gel 60 PF_{254}. For reversed phase TLC, layers spread with Merck silica gel 60 GF_{254} were impregnated with dimethylpolysiloxane-5X (Letham and Gollnow 1985) and are termed DMPS layers henceforth. The following solvents were used (proportions are by volume): A, 1-butanol-water-14 N ammonia (6:2:1, upper phase); B, methanol-water (1:1); C, chloroform-methanol (9:1); D, methanol-water (2:3); E, propyl acetate-acetic acid-water (20:9:5); F, 1-butanol saturated with water.

Chemicals

BA and 6-benzylamino-9-(2-tetrahydropyranyl)purine (9THP-BA) were purchased from Sigma, while [^{14}C]BA (12.8 mCi/mmol) and [^{3}H]BA (9 Ci/mmol) were obtained from Amersham and Shanghai Institute of Nuclear Research, China, respectively. [^{3}H]β-(6-benzylaminopurin-9-yl)alanine (9Ala-BA) was purified from soybean leaves which had been supplied with [^{3}H]BA (see Zhang et al. 1987).

The following compounds were synthesized by procedures detailed in the cited references: β-(6-benzylaminopurin-9-yl)propionitrile and ethyl 3-(6-benzylaminopurin-9-yl)propionate (Letham et al. 1983), 6-benzylamino-9-(3-hydroxypropyl)purine (Parker et al. 1986), 9Ala-BA (Letham et al. 1978), 6-benzylamino-9-(4-chlorobutyl)purine (Letham et al. 1978), 9-(2-tetrahydropyranyl)adenine (9THP-Ade) (Nagasawa et al. 1966), 6-benzoylaminopurine (Letham et al. 1972), and 6-benzylamino-2-chloropurine (Letham et al. 1978). 7-Benzylaminooxazolo[5,4-d]pyrimidine (BOP) was kindly provided by Dr C. H. Hocart of this laboratory and was synthesized as detailed previously (Hocart 1985). 10-(6-Benzylaminopurin-9-yl)decanal and 6-benzylamino-9-(10,11-dihydroxyundecyl)purine were synthesized by D. S. Letham (unpublished data). The other compounds were synthesized as detailed below.

6-Benzylamino-9-(2-tetrahydrofuranyl)purine (9THF-BA). BA (1.0 g), dry dioxane (5.0 ml), formic acid (0.5 ml), and 2,3-dihydrofuran (1.0 ml) were re-

fluxed under anhydrous conditions for 7 h. The mixture was evaporated to dryness and dissolved in ethyl acetate (40 ml). The resulting solution was shaken with 2% Na_2CO_3 (five 60-ml volumes), then with water (four 40-ml volumes), dried with anhydrous Na_2SO_4 and finally evaporated. The residue was crystallized from petroleum ether-ethyl acetate to yield 9THF-BA (0.65 g), m.p. 109–111°C. UV spectrum (in EtOH): λ_{max} (nm) 270.5 (ϵ = 17,500), shoulder 266.5 (ϵ = 17,360); λ_{min} 232.5 (ϵ = 2,200). Electron impact (EI) mass spectrum (m/z values and relative intensities in parentheses): 295 (M^+, 13), 267 (M − C_2H_4, 1), 252 (11), 225 (94), 224 (35), 209 (3), 198 (1.5), 197 (2.5), 148 (8), 121 (6), 120 (23), 119 (7), 106 (100). ^1H NMR spectrum (300 MHz, $CDCl_3$): δ 2.16 (2H, multiplet, N-9 CH_2), 2.54 (2H, multiplet, N-9 CH_2), 4.08 and 4.29 (both 1H, quartet and multiplet respectively, N-9 CH_2O), 4.90 (2H, broad singlet, benzylic CH_2), 6.15 (1H, broad signal, NH), 6.31 (1H, quartet, 4 lines of nearly equal intensity, N-9 CH), 7.3–7.5 (5H, multiplet, aromatic H), 7.85 (1H, singlet, purine H), 8.43 (1H, singlet, purine H).

6-Benzylamino-9-(1-ethoxyethyl)purine. 6-Chloro-9-(1-ethoxyethyl)purine (0.29 g) synthesized according to McDonald et al. (1971), benzylamine (0.68 g) and *n*-propanol (1 ml) were refluxed for 3.5 h. After centrifugation, the reaction solution was evaporated *in vacuo* at 80°C thoroughly to remove all benzylamine. The product was stirred with water containing sufficient acetic acid to maintain a pH of about 5. An ethyl acetate solution (50 ml) of the insoluble fraction was decolourized with activated charcoal, extracted with 2% Na_2CO_3 (three 50-ml volumes) followed by water, and evaporated. The product was dried by addition and subseqeunt evaporation of a benzene-EtOH mixture and crystallized from benzene-petroleum ether (yield 0.25 g), m.p. 78–80°C. UV spectrum (in EtOH): λ_{max} (nm) 270.5 (ϵ = 18,400), shoulder 266 (ϵ = 18,150); λ_{min} 232.5 (ϵ = 2,200). EI mass spectrum: 297 (M^+, 12), 253 (5), 252 (M − OC_2H_5, 4), 238 (2), 225 (50), 224 (19), 209 (2), 198 (1), 197 (1.5), 148 (7), 121 (7), 120 (20), 119 (8), 106 (100), 91 (69), 73 (35). ^1H NMR spectrum (200 MHz, $CDCl_3$): δ 1.16 (3H, triplet, J = 7 Hz, $O \cdot CH_2 \cdot CH_3$), 1.75 (3H, doublet, J = 6 Hz, $CH \cdot CH_3$), 3.30–3.60 (2H, multiplet, nonequivalent methyleneoxy H), 4.92 (2H, broad doublet, benzylic CH_2), 5.94 (1H, quartet, J = 6 Hz, CH at N-9), 6.15 (1H, broad signal, NH), 7.30–7.45 (5H, multiplet, benzyl aromatic H), 7.94 (1H, singlet, purine H), 8.42 (1H, singlet, purine H).

[^3H]9THP-BA, [^{14}C]9THP-BA and [^3H]9THF-BA. Labelled BA (0.12 mg) was reacted with 2,3-dihydropyran or 2,3-dihydrofuran (10 μl) in the presence of dioxane and formic acid (60 and 6 μl, respectively) in a small reaction vial heated at 90°C for 8 h. After complete evaporation *in vacuo,* the synthesized compounds were separated by TLC and eluted with ethyl acetate. In these syntheses, [^{14}C]BA was used at the specific activity supplied, but [^3H]BA was diluted with carrier BA to give a specific activity of 42 mCi/mmol.

9-(2-Tetrahydrofuranyl)adenine (9THF-Ade). Adenine (Ade) (100 mg) was dried *in vacuo* at 90°C and suspended in dry dioxane (1.0 ml). Formic acid (0.1 ml) and dry 2,3-dihydrofuran (0.2 ml) were added and the mixture was refluxed with stirring under anhydrous conditions for 1 h. Dry purified dimethylsulphoxide (DMSO) (1.0 ml) was then added to completely solubilize the Ade.

After refluxing for a further 9 h, the mixture was evaporated *in vacuo* to yield a DMSO solution which was diluted with ethyl acetate (35 ml). The solution was then shaken with water (35 ml) containing sufficient Na_2CO_3 to yield a pH of 9, and then with water (18 ml). A portion of the ethyl acetate solution was subjected to TLC (solvent A) and the principal UV-absorbing zone was eluted and rechromatographed (solvent C) to yield compound A (R_f 0.65) and compound B (R_f 0.48). The EI mass spectrum of A (M^+, m/z 275) indicated that it was a di-(2-tetrahydrofuranyl)adenine. B was identified as 9THF-Ade. UV spectrum (in EtOH): λ_{max} (nm) 209, 260. EI mass spectrum: 205 (M^+, 18), 177 (3), 174 (2), 162 (67), 148 (3), 136 (82), 135 (100), 119 (4), 108 (40). Chemical ionization (CI) mass spectrum (CH_4), positive ion: 206 (MH^+, 41), 164 (19), 136 (100). CI mass spectrum (CH_4), negative ion: 204 (M-H, 100), 134 (42). ^1H NMR spectrum (300 MHz, $CDCl_3$): δ 2.16 (2H, multiplet, N-9 CH_2), 2.54 (2H, multiplet, N-9 CH_2), 4.08 and 4.30 (both 1H, quartet and multiplet respectively, N-9 CH_2O), 5.61 (2H, broad signal, NH_2), 6.31 (1H, quartet, 4 lines of nearly equal intensity, N-9 CH), 7.93 (1H, singlet, purine H), 8.37 (1H, singlet, purine H).

O-Succinyl BA 9-riboside. A solution of BA riboside (0.25 g) and succinic anhydride (0.25 g) in dry pyridine (9 ml) was kept at 23°C for 18 h when it was evaporated to dryness *in vacuo*. The residue was dissolved in dioxane and subjected to preparative TLC (solvent E). The layers were washed with MeOH prior to use. The principal UV absorbing zone (also the zone of highest R_f, 0.76) was eluted with 80% MeOH. The eluate was evaporated and the residue was dissolved in water maintained at pH 7–8 by addition of dilute ammonia. Dropwise addition of 30% acetic acid precipitated the product which was washed with water and then crystallized from ethyl acetate-MeOH; m.p. > 150°C, but indefinite due to decomposition (yield 0.15 g). Further product was recovered from the acetic acid mother liquids by extraction with ethyl acetate. Since the product did not react with periodate, the succinyl group was assigned to positions 2′ and/or 3′ of the ribose moiety. UV spectra (λ_{max}, nm): 75% EtOH with 0.15 N NH_4OH, 266–270 (plateau); 0.1 N HCl 265; EtOH, 266–271 (plateau). CI mass spectrum (CH_4): 486 (C_2H_5 adduct of M^+, 0.4), 458 (MH^+, 3), 358 (5), 254 (2), 226 (8), 101 (100); EI spectrum: 457 (M^+, 7), 357 (7), 268 (8), 254 (32), 225 (100), 224 (41), 148 (18), 106 (53), 100 (86).

6-Benzylaminopurine riboside 5′-aldehyde. BA riboside was first converted to the 2′:3′-isopropylidene derivative by the derivatization procedure developed for nucleosides by Hampton (1961). The product was crystallized from petroleum ether-diethyl ether, m.p. 90–91°C. UV spectrum (EtOH): λ_{max} (nm) 266–271 (plateau). EI mass spectrum: 397 (M^+, 55), 396 (52), 367 (17), 308 (28), 294 (11), 254 (31), 225 (100), 224 (38), 106 (43), 91 (22).

The above derivative (40 mg), 1,3-dicyclohexylcarbodiimide (80 mg), dry DMSO (0.73 ml), trifluoroacetic acid (8 μl) and pyridine (16 μl) were mixed and left under anhydrous conditions at 23°C for 18 h. Benzene (0.8 ml) was then added and the precipitate formed was removed by centrifugation. Chloroform (5 ml) was added to the supernatant and the resulting solution was extracted with water (3 times equal volume) and then evaporated. The residue was hydrolyzed by stirring with 10% (v/v) acetic acid at 100°C for 2 h to cleave the isopropylidene moiety. The aldehyde product was purified by TLC (solvent

F; positive reaction to 2,4-dinitrophenylhydrazine spray) and was then crystallized from methanol-water (yield 10 mg), m.p. 145°C (with decomp.). UV spectra (λ_{max}, nm): EtOH, 266–272 (plateau); 0.1 N HCl, 266. EI mass spectrum: 355 (M^+, 15), 337 (3), 327 (5), 268 (38), 254 (12), 225 (100), 224 (78), 149 (21), 148 (10), 120 (17), 106 (87), 91 (57).

Bioassay

Soybean (Glycine max [L.] Merr., cv. Anoka) plants were grown as described previously (Zhang et al. 1987). At late podfill, plants were taken out of potting mixture and transferred to water a few days before bioassay. Solutions of BA derivatives were prepared in water containing ethanol (0.5%) and Tween 80 (0.05%). Aliquots (5 μl) of the solutions were spread within 14 mm diameter lanolin rings on selected uniform leaflets showing slight yellowing. The plants were kept in a growth cabinet (28/22°C day/night temperature, 10 h day length). After an appropriate period (usually 5–7 days), the effect of different compounds on chlorophyll retention was evaluated; the treated areas of the leaves were punched out and the absorbance of 80% acetone extracts was determined at 665 nm as a measure of chlorophyll content.

Metabolism of BA Derivatives

Radioactively labelled BA derivatives (9Ala-BA, 9THP-BA and 9THF-BA) and BA itself were prepared as 7.5 μM aqueous solutions containing 0.2% DMSO and 0.05% Tween 80 which were supplied to excised leaf discs from soybeans at mid podfill stage. Methods of incubation and extraction were as described before (Zhang et al. 1987), but 70% methanol was used as solvent.

Purification of Metabolites for Identification by Mass Spectrometry

Aqueous solutions (100 μM) of 9THP-BA and 9THF-BA were supplied to excised soybean leaves (25 g) through the petiole in light (8 $\mu E\ m^{-2}\ s^{-1}$) at 23°C for 48 h. The leaves were extracted with 70% methanol. Supernatants after centrifugation were evaporated until the MeOH was removed, filtered through Whatman No. 1 filter paper and extracted with ethyl acetate (4 times with an equal volume). The ethyl acetate extracts were combined, evaporated and dissolved in 5% EtOH. The solutions were passed through paraffin-coated silica gel columns (Hall et al. 1987) which were washed with 5% EtOH and then eluted with 80% EtOH. The evaporated eluates were subseqeuntly purified by the following sequence of four TLC systems: (1) silica gel 60 PF_{254}, solvent A; (2) DMPS layer, solvent B; (3) silica gel 60 PF_{254}, solvent C; and (4) Merck silica gel HPTLC RP-2 F_{254} (prespread plate), solvent D. Between TLC systems 2 and 3, chromatography was carried out with Baker 10 SPE C_{18} columns (J. T. Baker Chemical Co., New Jersey, USA). To elute metabolites, 80% EtOH was used after TLC systems 1, 2 and 4, and also for Baker C_{18} columns, while EtOH-ethyl acetate (1:1) was used after TLC system 3. TLC

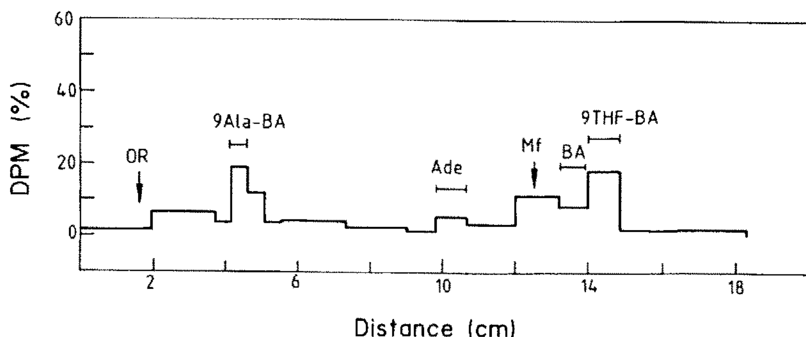

Fig. 1. Distribution of radioactivity over a thin-layer chromatogram (silica gel, solvent A) of extract from soybean leaf discs which had been supplied with [^{3}H]9THF-BA (7.5 μM) for 48 h. Mf denotes an unknown metabolite and OR indicates the origin. The positions of cochromatographed markers are indicated by the lines with bars.

system 1 separated BA from the new metabolite Mf derived from 9THF-BA (Fig. 1), but not from the new metabolite Mp derived from 9THP-BA. This resolution was achieved with system 2 (Fig. 2). The above four-system procedure yielded purified Mf and Mp and a metabolite likely to be BA for mass spectrometry. To distinguish different sources, the metabolites thought to be BA were termed BAf and BAp because they had been derived from 9THF-BA and 9THP-BA, respectively.

Methods for mass spectrometry has been described previously (Zhang et al. 1987).

Results

Comparative Activity of Some BA Derivatives

Fourteen 9-substituted BA derivatives have been compared with the parent compound (all at 100 μM) for ability to cause chlorophyll retention in leaves of intact soybean plants. The structures of these compounds are presented in Fig. 3. Compounds **3** and **7** were inactive, while **2, 4, 8, 9, 14** and **15** exhibited only very weak activity. The remaining compounds were much more active and their activities are compared with those of BA and compound **4** in Table 1 where chlorophyll retention data is presented. These results were obtained with cv. Anoka, but similar results were observed with cv. Bragg and Fiskeby V, and at 10 μM with Anoka (data not presented).

Of the 9-substituted derivatives tested, compounds **10** (9THF-BA) and **11** (9THP-BA) ranked equally as the most effective compounds, and both showed substantially higher activity than BA (compound **1**, Table 1). Both are cyclic ethers in which the ether oxygen is attached to the carbon joined to the purine N-9. Unexpectedly, the related acyclic compound **6**, synthesized for the first time in this study, was much less active, in fact slightly less effective than BA. BA riboside (compound **12**) was slightly more effective than BA, but modifica-

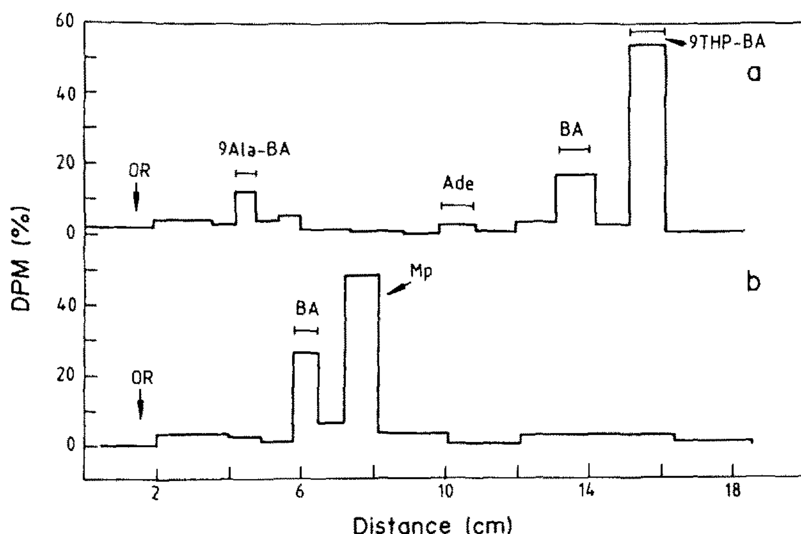

Fig. 2. Distribution of radioactivity over thin-layer chromatograms of extract from soybean leaf discs which had been supplied with [^{14}C]9THP-BA (7.5 μM) for 48 h. **a**, original extract separated by TLC on silica gel (solvent A); **b**, rechromatography of the eluate of the BA zone from **a** (DMPS layer with solvent B). Mp denotes an unknown metabolite and OR the origin.

tion of the ribose moiety by oxidation (compound **13**) or by conjugation (compounds **14** and **15**) depressed activity to varying degrees. The remaining compounds tested (**2**, **3**, **4**, **5**, **7**, **8** and **9**) were BA derivatives with a substituted alkyl group at N-9. All were either inactive or much less active than BA with one exception; compound **5**, with a 4-chlorobutyl group at N-9, was slightly more effective than the parent purine. It is noteworthy that the weakly active alkyl derivatives include compound **4** (9Ala-BA), the alanine conjugate to which BA is actively metabolized in soybean leaves (Zhang et al. 1987).

In addition to the 9-substituted BA derivatives discussed above, three BA analogues (**16**, **17**, **18**; Fig. 1) were also compared with BA for senescence-retarding activity. Compound **18** equalled the activity of BA, while **16** and **17** were inactive.

Metabolic Comparison of Selected BA Derivatives

The two most effective compounds 9THF-BA and 9THP-BA, and the main metabolite of BA in soybean leaves, 9Ala-BA, were labelled isotopically and their metabolism in soybean leaf discs was compared with that of labelled BA. Percentages of radioactivity in different metabolite zones after TLC (solvent A) of leaf disc extracts are presented in Table 2.

BA was converted largely to a metabolite which cochromatographed with 9Ala-BA as expected. 9Ala-BA exhibited great stability and no appreciable radioactivity due to BA was detected after two-dimensional TLC, although some ^{3}H did cochromatograph with BA marker in one-dimensional TLC (Table

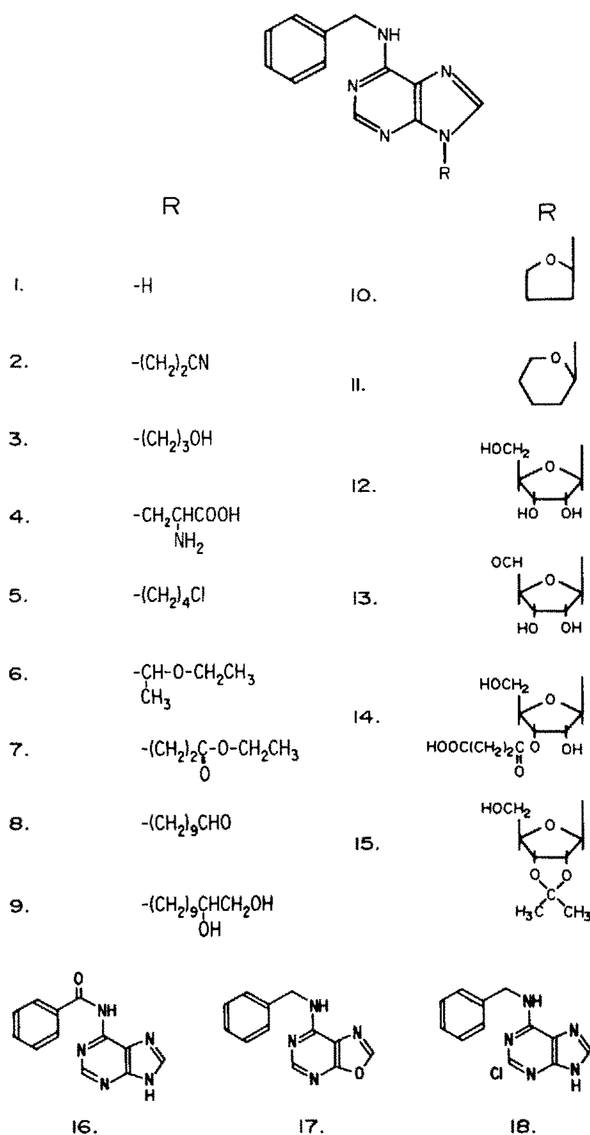

Fig. 3. Structures of BA derivatives tested in soybean leaf senescence assay. **1**, BA; **2**, β-(6-benzylaminopurin-9-yl)propionitrile; **3**, β-6-benzylamino-9-(3-hydroxypropyl)purine; **4**, 9Ala-BA; **5**, 6-benzylamino-9-(4-chlorobutyl)purine; **6**, 6-benzylamino-9-(1-ethoxyethyl)purine; **7**, ethyl 3-(6-benzylaminopurin-9-yl)propionate; **8**, 10-(6-benzylaminopurin-9-yl)decanal; **9**, 6-benzylamino-9-(10,11-dihydroxyundecyl)purine; **10**, 6-benzylamino-9-(2-tetrahydrofuranyl)purine (9THF-BA); **11**, 6-benzylamino-9-(2-tetrahydropyranyl)-purine (9THP-BA); **12**, BA riboside; **13**, 6-benzylaminopurine riboside 5′ aldehyde; **14**, O-succinyl 6-benzylaminopurine riboside; **15**, 2′,3′-isopropylidene 6-benzylaminopurine riboside; **16**, 6-benzoylaminopurine; **17**, 7-benzylamino-oxazolo[5,4-d]pyrimidine (BOP); **18**, 6-benzylamino-2-chloropurine (2-chloro-BA).

Table 1. Leaf chlorophyll retention induced by BA derivatives. Solutions (100 μM) of the derivatives were applied to the surface of soybean leaves within lanolin rings. The identity of the numbered compounds is given in Fig. 3.

Compound	A (665 nm)	Compound	A (665 nm)
Control[a]	0.07	**10**	0.43
1 (BA)	0.29	**11**	0.41
4	0.09	**12**	0.35
5	0.33	**13**	0.27
6	0.22		

[a] A 0.2% aqueous solution of DMSO containing Tween 80 (0.05%).

2). When 9THF-BA and 9THP-BA were supplied, 18% and 43% of extracted radioactivity, respectively, was due to unmetabolized cytokinin after uptake for 48 h. Hence 9THP-BA was more stable than 9THF-BA. Substantial proportions (>7%) of radioactivity were detected in the BA zones on TLC chromatograms (solvent A) of extracts from discs supplied with either labelled 9THF-BA (Fig. 1) or 9THP-BA (Fig. 2). Essentially all of this radioactivity derived from 9THF-BA cochromatographed with authentic BA when eluted with 80% EtOH and rechromatographed and this metabolite was termed BAf. However, much of the ^3H in the BA zone derived from 9THP-BA was due to an unknown metabolite termed Mp (see Fig. 2); most of the remainder cochromatographed with BA and the metabolite involved was designated BAp. A peak of radioactivity (8–11%) derived from 9THF-BA was also due to an unknown metabolite (termed Mf, see Fig. 1). It behaved as a single peak when rechromatographed on a DMPS layer (solvent B) and exhibited an R_f (0.27) almost identical to that of Mp in this system. Some radioactivity derived from both 9THF-BA and 9THP-BA cochromatographed with Ade (Table 2). Rechromatography confirmed that Ade was in fact a minor metabolite. No radioactivity peak was detected in the adenosine zone. 9Ala-BA appeared to account for 12.5–19% of the radioactivity in the extracts of discs supplied with 9THF-BA and 9THP-BA. This was only about one third of the percentage derived from BA.

Identification of BAf and BAp

BAf and BAp cochromatographed with authentic BA during normal phase TLC (solvents A and C) and during reversed phase TLC on DMPS and Merck RP-2 layers. They exhibited a mass spectrum (Fig. 4) identical to that of authentic BA.

Identification of Mf and Mp

Because of the close structural similarity between 9THP-BA and 9THF-BA and the similar R_f values exhibited by Mf and Mp, these metabolites could be analogous. To facilitate identification, [^{3}H]Mf (0.05 μM) purified by TLC was

Table 2. Radioactivity distribution on TLC[a] chromatograms of soybean leaf disc extracts. The leaf discs had been supplied with labeled 9-substituted BA derivatives (7.5 μM).

Compound and Period Supplied		Extracted Radioactivity (% of supplied)	% of Radioactivity in TLC Zones					
			9Ala-BA	Ade	Mf or Mp[b]	BA	9THF-BA	9THP-BA
[^{14}C]BA (Control)	24 h	48.0	45.7	11.3		11.6		
	48 h	47.3	51.7	4.7		8.4		
[^{3}H]9Ala-BA	48 h	45.1	62.7	0.4		4.3[c]		
[^{3}H]9THF-BA	24 h	53.3	13.6	6.4	8.5	7.4	28.5	
	48 h	52.9	18.9	5.5	11.2	8.7	18.5	
[^{14}C]9THP-BA	48 h	66.9	12.5	2.5	<u>11.0</u>	<u>6.6</u>		43.1

[a] Silica gel 60 PF_{254}, solvent A; the data underlined were obtained after rechromatography of the BA zone eluate (DMPS layer, solvent B).
[b] Mf and Mp were unknown metabolites derived from 9THF-BA and 9THP-BA respectively; they were subsequently identified as THF-Ade and THP-Ade, respectively (see text).
[c] After rechromatography (as in a), no ^{3}H peak occurred in the BA zone.

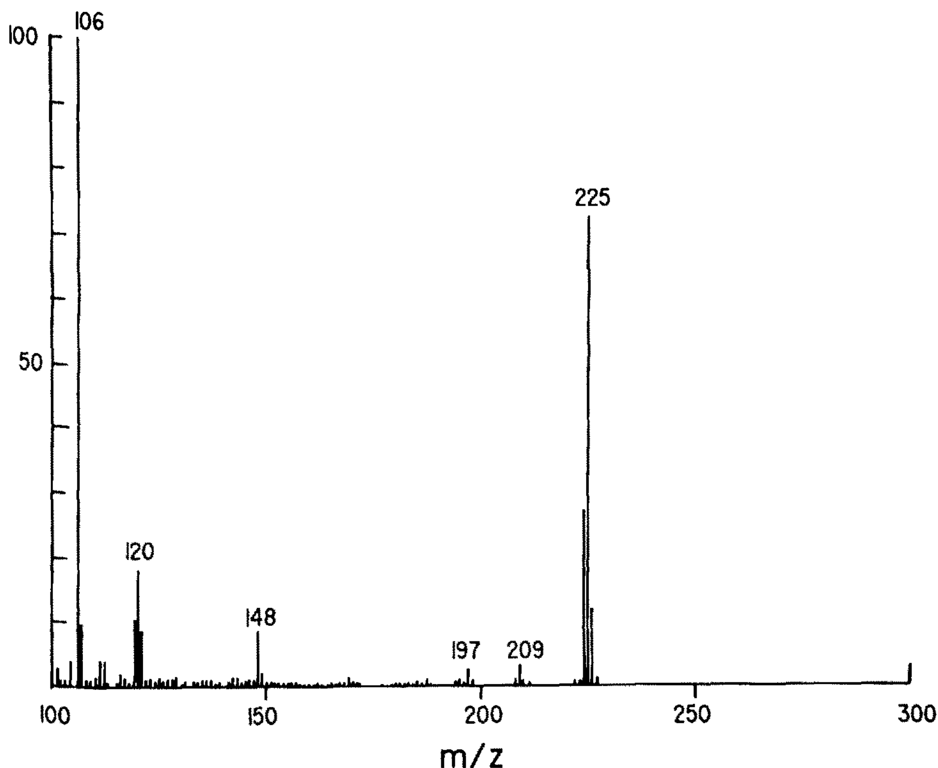

Fig. 4. EI mass spectrum of BAp. The spectrum of BAf was identical.

supplied to soybean leaf discs for 32 h. The radioactivity distribution of the extract on a thin-layer chromatogram (solvent A) revealed basically two peaks; one occurred at the R_f value of Mf and the other at the position of Ade. This suggested that Mf contained an intact Ade moiety and was possibly an N^6 cleavage product. Furthermore, acid hydrolysis of [^{3}H]Mf and [^{14}C]Mp, under conditions (0.5 N HCl at 23°C for 20 h) that would cleave 9-THP and 9-THF groups, yielded hydrolysates each containing a labelled compound which cochromatographed with Ade (see Fig. 5). Hence Mf and Mp were probably derived from 9THF-BA and 9THP-BA, respectively, by cleavage of the N^6-benzyl group.

To establish structures unequivocally, Mp and Mf were purified and mass spectra determined. The CI mass spectrum (CH_4) of Mp exhibited, as positive ions, a clear MH^+ ion at m/z 220 and an intense peak at m/z 136 attributable to a protonated Ade fragment (Fig. 6). The peak at m/z 164 corresponds to a C_2H_5 adduct ion of Ade (i.e. 135 + 29). The negative ion spectrum (not shown) exhibited the base peak at m/z 218, an $(M\text{-}H)^-$ ion. The EI mass spectrum (Fig. 7) showed a molecular ion at m/z 219, intense peaks at m/z 135 and 136 (base peak) and an ion at m/z 108 which is derived from the purine ring system (see Shannon and Letham, 1966).

The above observations indicated that Mp was 9-(2-tetrahydro-

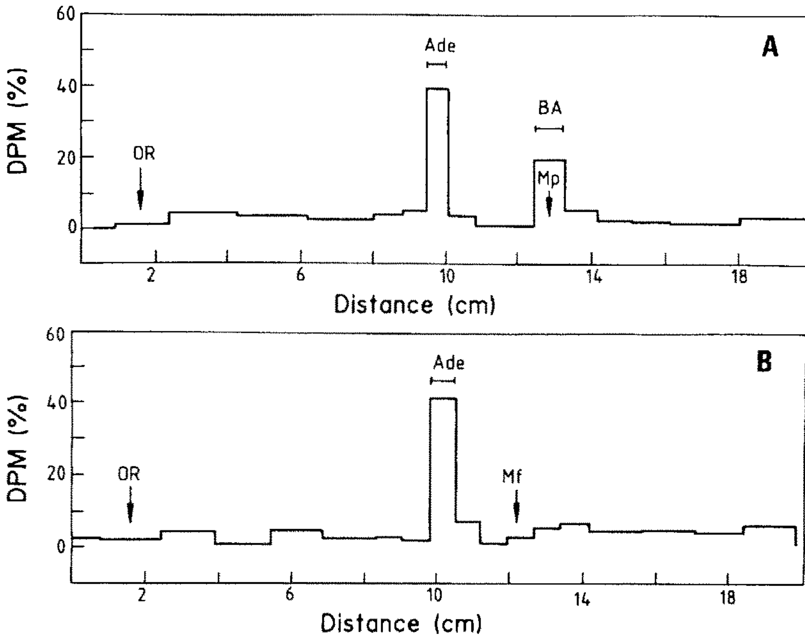

Fig. 5. Distribution of radioactivity over silica gel thin-layer chromatograms (solvent A) of hydrolyzed eluates from the BA zone shown in Fig. 2a (A) and from the Mf zone shown in Fig. 1 (B). Both eluates had been subjected to acid hydrolysis (0.5 N HCl at 23°C for 20 h). The calculated positions of Mf and Mp are denoted by arrows.

pyranyl)adenine (9THP-Ade) and this compound was then synthesized chemcially. Mp and 9THP-Ade exhibited identical mass spectra and cochromatographed in both normal phase and reverse phase TLC systems. Hence it was concluded that Mp and 9THP-Ade were identical.

Unfortunately, Mf did not give a completely satisfactory mass spectrum due to the presence of impurities; however, the EI spectrum did show peaks at m/z 205, 162, 136 and 135 which are characteristic of synthetic 9THF-Ade (see Materials and Methods). Also, synthetic 9THF-Ade and Mf cochromatographed in four TLC systems (two normal phase, two reversed phase). Hence these two compounds were probably identical.

Discussion

The Relative Activity of BA Derivatives

The two most effective retardants of soybean leaf senescence found in this study were the related 9-substituted derivatives of BA, 9THP-BA and 9THF-BA, both of which were more effective than BA (Table 1). 9THF-BA was synthesized for the first time in the present study, while 9THP-BA has been reported previously to be more active than BA in evoking several growth responses (see review by Letham 1978). In promoting lettuce seed germination, 9THP-BA and BA were equally active (Pietraface and Blaydes 1981), but

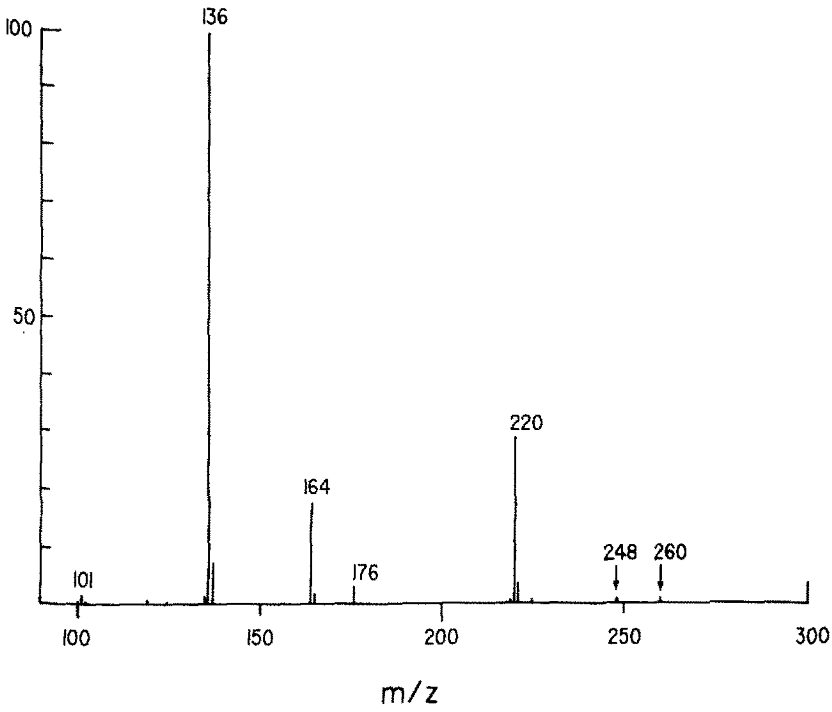

Fig. 6. CI mass spectrum of Mp. The reagent gas was CH_4.

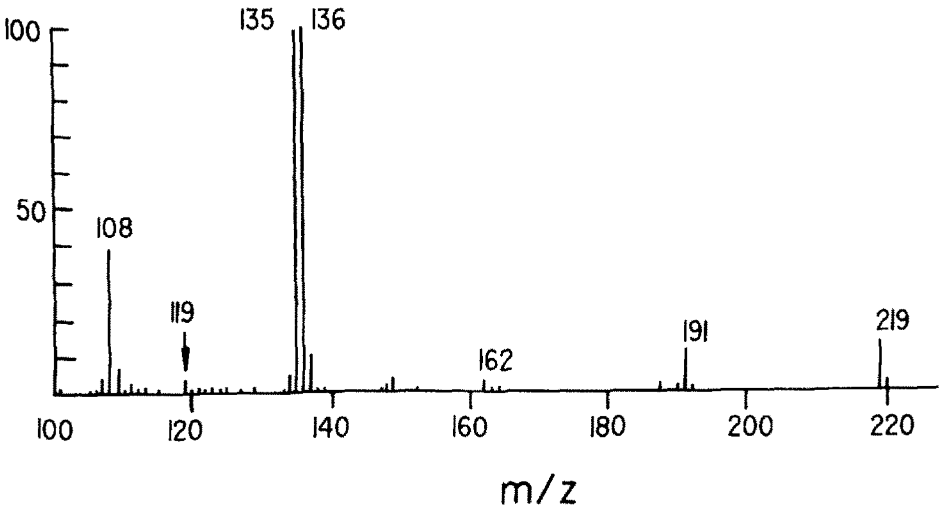

Fig. 7. EI mass spectrum of Mp.

9THP-BA was slightly less active than BA in the *Amaranthus* betacyanin bioassay and in the cucumber cotyledon chlorophyll synthesis bioassay (Gasque 1982). With tobacco leaf disc chlorophyll retention bioassay (Young and Letham 1969), 9-(2-tetrahydropyranyl)isopentenyladenine markedly exceeded its parent cytokinin isopentenyladenine in activity. Hence a 9-(2-tetrahydropyranyl) moiety appears to be a structural feature which can enhance cytokinin activity in some plant systems. Cytokinin free bases are probably the "active" forms of cytokinins (Letham and Palni 1983). Hence the activity of 9THP-BA and 9THF-BA is probably a consequence of slow cleavage of the pyranyl and furanyl moieties, respectively, as discussed further below.

Several of the 9-substituted alkyl derivatives of BA tested in the soybean senescence assay (compounds **2–9**, Fig. 3) have been assessed for activity previously in other bioassays. Thus, compound **3** was much less active than BA in the soybean callus assay (Fox and Chen 1968); it was inactive in the soybean leaf senescence assay reported herein (Table 1). Compound **5**, a 9-chloroalkyl derivative of BA, exhibited activity which approached that of BA in the radish cotyledon expansion assay (Letham et al. 1978, 1982); it was slightly more effective than BA in retarding soybean leaf senescence. Compound **7** was inactive in the radish cotyledon assay (Letham et al. 1983) and also in the soybean senescence test (Table 1). The alanine conjugate 9Ala-BA showed only very weak activity in several classical cytokinin bioassays (Letham et al. 1983) and also in the soybean leaf assay. The remaining three alkyl-type derivatives, including two with long chains (compounds **8** and **9**), have not been tested previously for cytokinin activity, but all were very weakly active in the soybean senescence assay.

The 9-riboside of BA (compound **12**, Fig. 3) exhibited senescence-retarding activity which exceeded that of BA, but in some commonly used cytokinin bioassays, it was less active than BA itself (Letham 1978). Because of the activity of compound **12**, the effect of modification (oxidation and substitution) of the ribose moiety was studied (compounds **13–15**). However this resulted in diminished activity. Compound **14**, with a succinyl group, might be expected to release BA riboside as a result of esterase action.

The basis of the relationship between structure and cytokinin activity is still obscure as far as 9-substituted cytokinins are concerned. Thus it is not possible to explain why the related compounds **3** and **5** differ so greatly in activity, one being inactive and the other highly active. While there may be a connection between chemical lability of the N-9 substituent and cytokinin activity in some sets of compounds (Young and Letham 1969), susceptibility to enzymic dealkylation is probably the critical factor that largely determines activity of 9-alkyl-type cytokinins. Thus, compound **5** is highly active probably because it is readily dealkylated to yield free BA, while **3** is not susceptible to dealkylation and is inactive because of its stability. Until the structural requirements for enzymic dealkylation of purines in plant tissues are elucidated, the structure-activity relations of N-9 alkyl cytokinins will probably remain an enigma.

Although amides of Ade exhibit moderate cytokinin activity, including activity in a Chinese cabbage leaf senescence bioassay (Letham et al. 1972), compound **16** was inactive in the soybean senescence bioassay. A 2-chloro substituent has little effect on cytokinin activity in the tobacco pith callus

bioassay (Hecht et al. 1970, Dammann et al. 1974); similarly, in the soybean senescence assay (Table 1), 2-chloro-BA (compound **18**) exhibited activity similar to that of BA. A newly synthesized compound tested in the present study was compound **17**, namely, 7-benzylaminooxazolo[5,4-d]pyrimidine (BOP). Although this compound is closely related to BA, it was inactive. This is the first assessment of the possible cytokinin activity of this compound which would be resistant to glucosylation and conjugation to alanine at N-9. The inactivity of BOP emphasizes for the first time the importance of the nitrogen at position 9 of the purine ring in cytokinin structure-activity relationships.

The Metabolism of 9-Substituted Derivatives of BA

The observed high activity of 9THP-BA and 9THF-BA relative to BA itself could be a consequence of one or more of the following factors: (1) enhanced uptake by the leaves caused by the THP or THF moiety; (2) the resistance of 9THP-BA and 9THF-BA to metabolism which inactivates cytokinins, especialy alanine conjugation; (3) the ability of 9THP-BA and 9THF-BA to slowly release free BA and provide desirable concentrations of the latter over a prolonged period; (4) 9THP-BA and 9THF-BA per se are more active than BA.

The extracted radioactivity data in Table 2 do not support the first proposition. However, the proportions of radioactivity in various metabolites (Table 2), and the mass spectrometric identification of BA as a metabolite of 9THP-BA and 9THF-BA give clear support to the suggestions (2) and (3). While proposal (4) cannot be excluded, 9-substitution with an alkyl-type group generally markedly reduces activity in bioassays (Letham 1978). Hence the direct activity of 9THP-BA and 9THF-BA per se is probably less than that of BA.

The mechanism for the cleavage of the 9THP and 9THF groups is obscure. These groups can be cleaved by acid since 9THP-BA and 9THF-BA are analogous to acetals. Compounds could be ranked as follows with respect to stability to acid hydrolysis: 9THP-BA > compound **6** > 9THF-BA (data not shown). However, pH values below 3 are required for appreciable hydrolysis. Hence the cleavage observed in soybean leaves is probably due to a dealkylating enzyme. The cleavage of substituents other than sugar moieties at position 9 of BA have been considered before. Thus, Fox et al. (1971) studied the metabolism of 9-methyl-BA labelled with ^{14}C in the methyl group using tobacco callus tissue cultures. The methyl derivative was found to be rapidly metabolized but no metabolites were identified definitively, although it was claimed that conversion to free BA occurred. The metabolism of 9-methyl-BA in germinatng lettuce seed was studied by Pietraface and Blaydes (1981). Metabolism was appreciable only during the initial period of uptake; similarly in the present study of metabolism of 9THF-BA, degradation was rapid only during the first 24 h (see Table 2). Chromatographic studies of the lettuce seed extracts suggested that BA nucleotide and BA riboside were metabolites, but free BA was not detected. Again, in a study of the metabolism of 9-(4-chlorobutyl)BA in radish cotyledons (Letham et al. 1978), no free BA was identified definitively, but conversion to the 7- and 9-glucosides of BA was established unequivocally by mass spectrometry and other methods. Formation of these

glucosides presumably requires prior conversion to BA. However, in the present study of metabolism of 9THP-BA and 9THF-BA, free BA was identified unambigously as a metabolite. Hence this is the first definite demonstration of conversion of a 9-substituted cytokinin, other than a 9-glycoside, to the free cytokinin base.

While 9THP-BA and 9THF-BA both yielded free BA in soybean leaves, 9Ala-BA did not release free BA in detectable amounts, and this could account for its very low activity. The metabolism of the zeatin-alanine conjugate, lupinic acid, has been studied in two plant tissues. Like 9Ala-BA, it exhibited high stability relative to the corresponding base or riboside (Parker et al. 1978, Palni et al. 1984). Lupinic acid is also weakly active in cytokinin bioassays (Letham et al. 1983, Palni et al. 1984). Interestingly, the ratios, radioactivity due to BA/radioactivity due to 9Ala-BA (Table 2), indicate that free BA released from 9THP-BA and 9THF-BA is less susceptible to alanine conjugation than is exogenous BA. This would be expected to contribute to the effectiveness of 9THP-BA and 9THF-BA.

The present study has established that 9THP-BA and 9THF-BA are subject to both N^6 debenzylation and to N-9 substituent cleavage in soybean leaves. Debenzylation was more prominent in the metabolism of these BA derivatives than in that of BA itself. This N^6 sidechain cleavage probably involves an imino intermediate formed enzymatically by elimination of a hydrogen atom from both the NH group at position 6 and the benzylic methylene. It is possible to suggest structural alternatives to 9THP-BA and 9THF-BA which could exhibit enhanced senescence-retarding activity because they would not be susceptible to this oxidative metabolism. Compounds of this type with a 9-tetrahydropyranyl group are: 6-(2-phenylethyl)-9-(2-tetrahydropyranyl)purine (I), 6-(*trans*-styryl)-9-(2-tetrahydropyranyl)purine (II), 6-benzyloxy-9-(2-tetrahydropyranyl)purine (III), 6-(N-benzylmethylamino)-9-(2-tetrahydropyranyl)-purine (IV), and 6-phenylaminopurine (V). The analogues of compounds I, II and III without a THP group and BA are all approximately equally active in the tobacco pith cytokinin bioassay (Henderson et al. 1975), whereas the analogues of IV and V are less effective than BA. Hence compounds I, II and III particularly merit synthesis and testing as senescence retardants for soybean. These compounds all lack an exocyclic N atom at position 6 of the purine ring.

References

Dammann LG, Leonard NJ, Schmitz RY, Skoog F (1974) Cytokinins: synthesis of 2-, 8-, and 2,8-substituted 6-(3-methyl-2-butenylamino)purines and their relative activities in promoting cell growth. Phytochemistry 13:329–336

Fox JE, Chen C-M (1968) Cytokinin incorporation into RNA and its possible role in plant growth. In: Wightman F, Setterfield G (eds) Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, pp 777–789

Fox JE, Sood CK, Buckwalter B, McChesney JD (1971) The metabolism and biological activity of a 9-substituted cytokinin. Plant Physiol 47:275–281

Gasque CE (1982) Comparison of cytokinin activities of 9-substituted N^6-benzyladenines in the *Cucumis* and *Amaranthus* bioassays. Phytochemistry 21:1501–1507

Hall PJ, Badenoch-Jones J, Parker CW, Letham DS, Barlow BA (1987) Identification and quantifi-

cation of cytokinins in the xylem sap of mistletoes and their hosts in relation to leaf mimicry. Aust J Plant Physiol 14:429–438
Hampton A (1961) A new procedure for the conversion of ribonucleosides to 2′,3′-*O*-isopropylidene derivatives. J Amer Chem Soc 83:3640–3645
Hecht SM, Leonard NJ, Schmitz RY, Skoog F (1970) Cytokinins: synthesis and growth-promoting activity of 2-substituted compounds in the N^6-isopentenyladenine and zeatin series. Phytochemistry 9:1173–1180
Henderson TR, Frihart CR, Leonard NJ, Schmitz RY, Skoog F (1975) Cytokinins with different connecting links between purine and isopentenyl or benzyl groups. Phytochemistry 14:1687–1690
Hocart CH (1985) PhD Thesis, Aust Natl Univ, Canberra, pp 210–222
Letham DS (1978) Cytokinins. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds: A comprehensive treatise. Vol. 1. Elsevier/North Holland, Amsterdam, pp 205–263
Letham DS, Gollnow BI (1985) Regulators of cell division in plant tissues. XXX. Cytokinin metabolism in relation to radish cotyledon expansion and senescence. J Plant Growth Regul 4:129–145
Letham DS, Palni LMS (1983) The biosynthesis and metabolism of cytokinins. Ann Rev Plant Physiol 34:163–197
Letham DS, Palni LMS, Tao G-Q, Gollnow BI, Bates CM (1983) Regulators of cell division in plant tissues. XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. J Plant Growth Regul 2:103–115
Letham DS, Parker CW, Gordon ME (1972) The cytokinin activities and metabolism of 6-acylaminopurines. Physiol Plant 27:285–290
Letham DS, Summons RE, Entsch B, Gollnow BI, Parker CW, MacLeod JK (1978) Glucosylation of cytokinin analogues. Phytochemistry 17:2053–2057
Letham DS, Tao G-Q, Parker CW (1982) An overview of cytokinin metabolism. In: Wareing PF (ed) Plant Growth Substances 1982. Academic Press, London, pp 143–153
McDonald JJ, Leonard NJ, Schmitz RY, Skoog F (1971) Cytokinins: synthesis and biological activity of ureidopurines. Phytochemistry 10:1429–1439
Nagasawa N, Kumashiro I, Takenishi T (1966) Tetrahydro-2-pyranyl derivatives of purines. J Org Chem 31:2685–2688
Palni LMS, Palmer MV, Letham DS (1984) The stability and biological activity of cytokinin metabolites in soybean callus tissue. Planta 160:242–249
Parker CW, Entsch B, Letham DS (1986) Inhibitors of two enzymes which metabolize cytokinins. Phytochemistry 25:303–310
Parker CW, Letham DS, Gollnow BI, Summons RE, Duke CC, MacLeod JK (1978) Metabolism of zeatin by lupin seedlings. Planta 142:239–251
Pietraface WJ, Blaydes DF (1981) Activity and metabolism of 9-substituted cytokinins during lettuce seed germination. Physiol Plant 53:249–254
Shannon JS, Letham DS (1966). The mass spectra of cytokinins and other 6-aminopurines. New Zealand J Sci 9:833–842
Young H, Letham DS (1969) 6-(Substituted amino)purines: synthesis by a new method and cytokinin activity. Phytochemistry 8:1199–1203
Zhang R, Letham DS, Wong OC, Nooden LD, Parker CW (1987) Cytokinin biochemistry in relation to leaf senescence. II. The metabolism of 6-benzylaminopurine in soybean leaves and the inhibition of its conjugation. Plant Physiol 83:334–340