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A NEW CYTOKININ FROM *POPULUS* x *ROBUSTA*

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Key Word Index—*Populus* x *robusta*; Salicaceae; cytokinins; 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine; GC—MS; mass spectrometry.

Abstract—A new cytokinin was isolated from mature leaves of poplar. Its structure was determined by UV and MS and confirmed by synthesis as 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine. This cytokinin has medium activity in the soybean callus test but shows high activity in the radish leaf senescence test.

INTRODUCTION

It has been known for some time that mature leaves of poplar (*Populus* x *robusta* Schneid) contain high levels of cytokinin activity [1]. In a preliminary report we gave evidence for the identity of the major component of this activity [2]. This paper reports fully on the isolation, identification and biological activity of this new cytokinin.

Fractionation of the *n*-butanol-soluble basic material from mature poplar leaves on LH-20 Sephadex showed 4 discrete zones of cytokinin activity. The primary effort was directed to determining the nature of the cytokinin activity in the last eluting zone, as this had been shown to increase after red light treatment [3]. The nature of the cytokinins in the other zones from the LH-20 Sephadex column is at present under investigation.

RESULTS

Paper chromatography of the butanol-soluble basic material from mature poplar leaves showed a major zone of cytokinin activity at R_f 0·4–0·7. Chromatography of this zone on LH-20 Sephadex resulted in the distribution of cytokinin activity similar to that obtained by Hewett [3]. The relevant active fractions (40, 41 and 42) from 3 separate extractions were bulked, taken to dryness and triturated with EtOAc. All the cytokinin activity passed into the EtOAc. The EtOAc-soluble material was analysed by preparative GLC after silylation. The distribution of activity is shown superimposed on the GLC trace in Fig. 1. The nature of the cytokinin active peak was examined by GC–MS in two ways. Firstly a 10-sec/mass decade scan was taken from *m/e* 700 to *m/e* 20 on the apex of

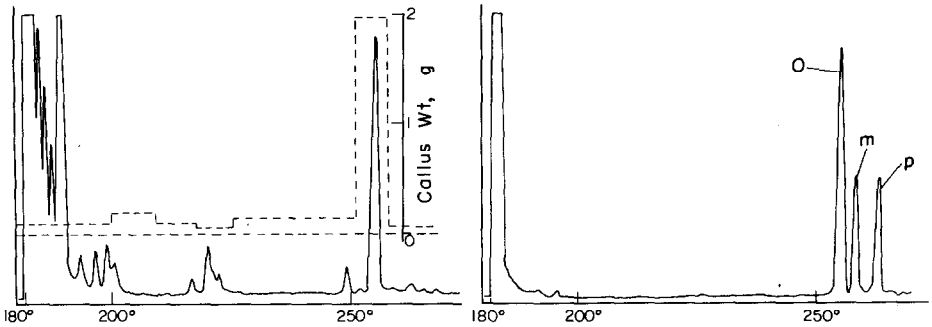

Fig. 1a. Biological activity obtained by preparative GLC of partially purified cytokinin from poplar leaves. 1b. GLC trace of mixture of equal amounts of *ortho*, *meta* and *para* isomers of 6-(hydroxybenzylamino)9-β-ribofuranosyl purine and cytokinin from poplar leaves.

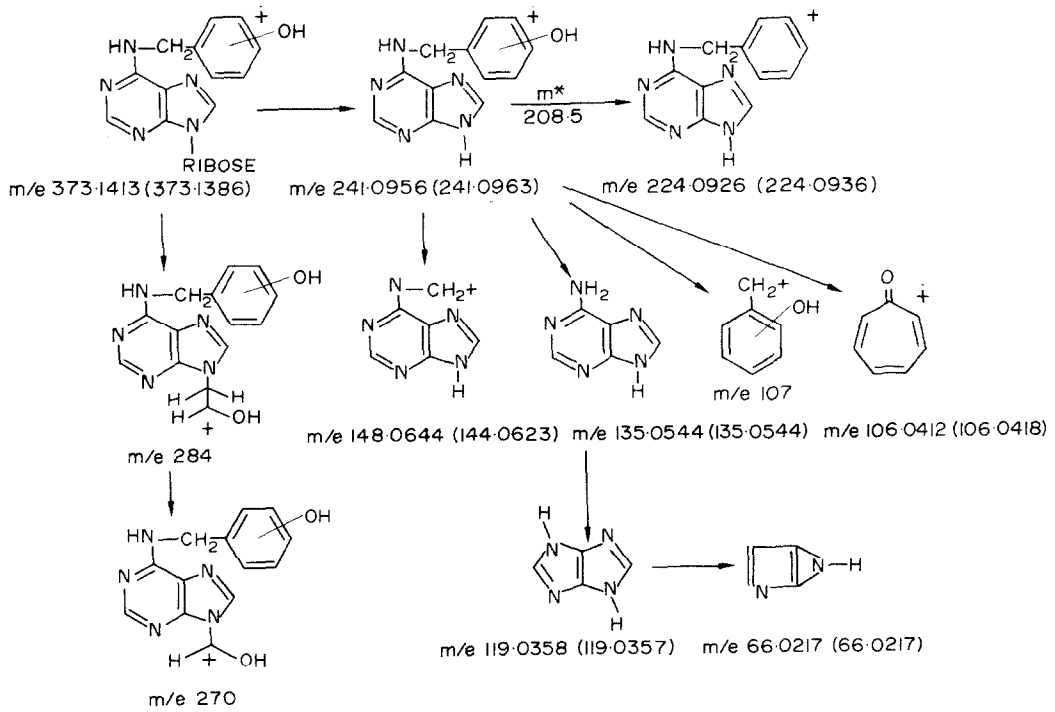

Fig. 2. Fragmentation scheme for cytokinin from poplar leaves.

the peak. The MS thus obtained showed the following major ions: *m/e* 661 (8·5% M^+), 646 (4·8% $M\text{-}15^+$), 428 (3·7%), 386 (4·0%), 342 (43%), 313 (49%), 312 (34%), 259 (8·5%), 245 (17%), 243 (11·5%), 230 (24%), 217 (23%), 179 (11·5%), 147 (14·5%), 103 (29%) and 73 (100%). Examination of the isotope peaks of the parent ion suggested the presence of 4 TMS groups in the molecule. The ions at *m/e* 259, 245, 243, 230, 217, 179 and 147 were considered to be indicative of a ribose moiety [4]. Little structural information could be gained from the remainder of the spectrum. Secondly, 1 sec/mass decade scans were taken from *m/e* 700 to *m/e* 100 at 5 successive points on the leading and trailing edges of the peak. These spectra were identical, and hence it was concluded that the peak was homogeneous.

The total extracted material (50 μg estimated by GLC) was purified by preparative GLC. After removal of the TMS groups with 0·01 M AcOH it gave the following UV spectra: λ_{max}^{EtOH} 266, $\lambda_{max}^{EtOH-HOAc}$ 260, $\lambda_{max}^{EtOH-NH4OH}$ 265 nm. A low resolution MS of the purified material showed the following major ions: *m/e* 373 (1% M^+), 284 (0·8%), 270 (1·3%), 241 (20%), 224 (4·3%), 178 (10%), 164 (23%), 148 (6·7%), 136 (40%), 135 (100%), 119 (10%), 108 (83%), 107 (??%), 106 (37%) and 66 (20%).

The presence of major ions at *m/e* 135, 119, 108 and 66 indicated that the molecule contained an adenine fragment [5]. The ions at *m/e* 284 $(M-C_3H_5O_3)^+$ and 270 $(M-C_4H_7O_3)^+$ together with those at *m/e* 178 (adenosine $-C_3H_5O_3)^+$ and 164 (adenosine $-C_4H_7O_3)^+$ were taken as strong evidence that the molecule was a ribosyladenine [6]. The UV data indicated that it was a 9-riboside. Letham [7] has suggested that the appearance of an ion at *m/e* 148 is a characteristic feature of the MS of N^6-substituted adenines having a $-CH_2-$ group adjacent to the N^6. Thus it was concluded that the remaining unit of the side chain had a mass of 93 a.m.u. and hence was certainly aromatic. The loss of 17 a.m.u. from the *m/e* 241 ion indicated the presence of an –OH group on the side chain as did the fact that the fully silylated molecule must have contained 4 TMS groups. From this it was concluded that the unknown compound was a 6-(hydroxybenzylamino)-9-ribofuranosylpurine. The proposed MS fragmentation

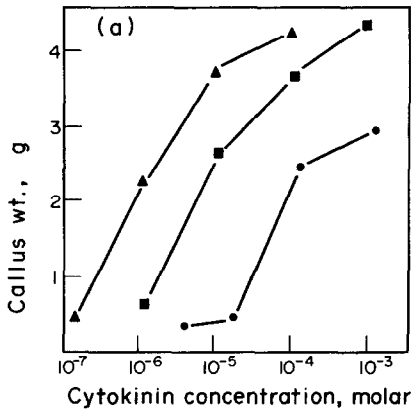

Fig. 3a. Effects of ▲—zeatin riboside, ■—benzyladenine riboside and ●—*o*-hydroxybenzyladenosine on the growth of soybean callus tissue.

scheme for this molecule is shown in Fig. 2. The composition of the ions shown was determined by high resolution MS.

The position of the –OH group was determined by comparison of the low resolution MS of the 3 synthetic isomers with that of the natural product. The natural product was clearly not the *m*-OH isomer as this isomer, as might be expected, showed only a very weak *m/e* 224 ion. The spectra of the *o*-OH and *p*-OH isomers showed MS which were very similar. Although the *o*-OH isomer gave an MS closer to the natural product it was felt that additional information was needed before an unambiguous identification could be made. The MS of the TMS derivatives of the 3 isomers were identical with each other and with the TMS derivative of the natural product. However, on the GLC system used for the isolation, these derivatives could be separated completely, and so it was shown by coinjection (Fig. 1b) that the natural product was 6-(*o*-hydroxybenzylamino)-9-*β*-D-ribofuranosyl-purine.

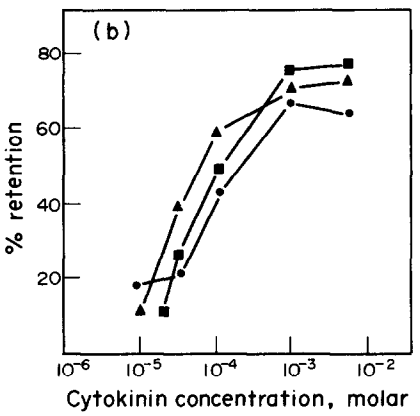

Fig. 3b. Effects of ▲—zeatin riboside, ■—benzyladenine riboside and ●—hydroxybenzyadenosine on the retention of chlorophyll in radish leaf discs.

DISCUSSION

Synthetic 6-(benzylamino)-purines have long been known to be active as cytokinins and Kuraishi [8] has shown that synthetic 6-(*o*-hydroxybenzylamino)-purine is an active cytokinin in the radish leaf expansion test. However, the isolation of 6-(*o*-hydroxybenzylamino)-9-*β*-D-ribofuranosyl-purine from mature poplar leaves deserves comment on two counts. Firstly, it represents the first isolation of naturally occurring purinyl cytokinin having an aromatic side chain and thus demonstrates that mevalonic acid is not the only source of the side chains of naturally occurring cytokinins. Secondly, the cytokinin is present in high levels (*ca* 0·1 μg/g fr. wt) in fully expanded leaves and this raises the question as to its physiological role. Since the leaves in which it was found had ceased cell division for some time it is unlikely to be acting as a regulator of cell division in this system and, in fact, its potency as a cell division factor is somewhat low. Hewett has demonstrated that the levels of populin increase transiently after short periods of exposure to red light and that the populin levels can be correlated both the lighting conditions and degree of leaf expansion [3]. It seems therefore that the role of this compound may be more in terms of controlling leaf expansion, and possibly senescence since its activity as a senescence retarding factor is high.

EXPERIMENTAL

General. Mps, determined on a hot stage microscope, are uncorrected. Cytokinin activity was monitored throughout the purification procedure by the soybean callus bioassay [9]. The radish leaf senescence test was carried out as described by Kefford *et al.* [10].

Extraction of leaf material. The first 4 fully expanded leaves from greenhouse-grown plants were harvested, extracted and a butanol-soluble basic fraction prepared as previously described. Extracts were chromatographed on Whatman 3MM paper in *i*-BuOH–25% NH_4OH. Sephadex chromatography was carried out on a 90 × 2·5 cm column of LH-20 Sephadex using 35%

EtOH as eluant at a flow rate of 15 ml/hr. 30 ml fractions were collected. Material from 3 extracts of 180 g fr. wt were bulked for preparative GC.

Gas–liquid chromatography. TMS derivatives were prepared by reacting the dried extract with bis-(trimethylsilyl)-trifluoroacetamide in acetonitrile (1:4) at 90° for 30 min in a sealed vial. GLC was carried out on a PYE 104 instrument with dual flame ionization detectors. Standard conditions for cytokinin analysis were: 1% SE-52 (on 100–120 mesh Gas Chrom Q), columns (2·8 m × 4 mm i.d), temp. programmed 180–300° at 4°/min, N_2 flow 40 ml/min. Preparative GLC was carried out on a PYE 104 instrument (modified by R. Horgan) using the same conditions as above. Split ratio was 8:1 and samples were collected in 10 × 0·2 cm pieces of glass tubing.

Mass spectrometry. GC–MS spectra were obtained on an AEI MS-30 single beam instrument operating at 24 eV. GLC conditions as above with a helium flow of 40 ml/min. GC–MS interface was a silicon rubber membrane separator operating at 230°. Spectra were recorded using the automatic sensitivity control to compensate for changes in total ion current during scans. Low resolution MS as probe samples were obtained using the above instrument. High resolution MS as probe samples were obtained on an AEI MS-9 instrument at a static resolution of 10000. Mass measurements and determination of elemental composition of ions were carried out by an AEI DS 30 data system.

Synthesis of 6-(hydroxybenzylamino)-9-β-D-ribofuranosylpurines. The three isomers were prepared by the reaction of 6-chloropurine-9-β-D-riboside with the relevant hydroxybenzylamine in refluxing *n*-BuOH. The compounds were recrystallized to constant m.p. from EtOH. Mps *o*-isomer 188–190°, *p*-isomer 207–208°, and *m*-isomer 171–174°.

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