SHORT REPORTS

IDENTIFICATION OF CYTOKININS ZEATIN AND ZEATIN RIBOSIDE IN *ABIES BALSAMEA*

C. H. ANTHONY LITTLE*, DONALD M. ANDREW*, PETER J. SILK† and GEORGE M. STRUNZ*

* Department of the Environment, Canadian Forestry Service, Maritimes Forest Research Centre,
P.O. Box 4000, Fredericton, N.B., E3B 5P7, Canada:
† New Brunswick Research and Productivity Council, P.O. Box 6000, Fredericton, N.B., E3B 5H1, Canada

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Key Word Index—*Abies balsamea*; Pinaceae; balsam fir; cambium; cytokinin; zeatin; zeatin riboside.

This paper reports the detection and identification of the cytokinins *trans*-zeatin and *trans*-zeatin riboside from the cambial region of the conifer balsam fir (*Abies balsamea* (L.) Mill.). This is the first positive identification of these growth hormones in the cambial region of a woody plant, and provides a firm basis for considering that cytokinins are involved with indoleacetic and abscisic acids in regulating cambial activity in trees [1]. Zeatin and/or zeatin riboside have previously been positively identified in only two other woody plant sources [2, 3], although they have been tentatively identified in many others [4–7].

Total ion current GC–MS of the derivatized presumptive *trans*-zeatin and *trans*-zeatin riboside fractions obtained after ion exchange, paper and TLC purification indicated the presence of complex mixtures. Limited mass scans for key ions characteristic of *trans*-TMSi$_2$-zeatin and *trans*-TMSi$_4$-zeatin riboside [3] gave peaks having the same retention times as those observed for authentic samples. Mass analysis at the appropriate retention times showed spectra identical to those of authentic samples [3]. The spectrum of *trans*-TMSi$_4$-zeatin riboside indicated a very high degree of purity, while that of *trans*-TMSi$_2$-zeatin showed some significant extraneous ions in the region below *m*/*e* 150. The spectrum characteristic of *trans*-TMSi$_3$-zeatin [3] was also identified, but no evidence for TMSi derivatives of dihydrozeatin [3] was found. On the basis of peak areas at *m*/*e* 260 (*trans*-TMSi$_2$-zeatin samples) and at *m*/*e* 536 (*trans*-TMSi$_4$-zeatin riboside samples), a concentration of *ca* 5 µg/kg fr. wt of cambial region was estimated for both cytokinins, not taking into account loss during the purification procedure.

EXPERIMENTAL

Bark was peeled from logs obtained from the base of the live crown of mature trees during the cambial growing season. Dead outer bark was removed and the resulting cambial region was diced and frozen in liquid N_2. Exposed xylem on the logs was wiped dry with Whatman No. 1 paper. The diced cambial region (1 kg fr. wt total) was homogenized in cold 80% MeOH and the extract (final concn 8 ml/g fr. wt) plus paper wipings were kept overnight on a shaker in the dark at 2°. The extract was filtered through cheesecloth and glass wool and the solids were re-extracted with fresh 80% MeOH. The MeOH was removed from the combined extracts by evapn under red. pres. at 40°. The aq. suspension was centrifuged at 2500 *g* for 45 min at 5°. The supernatant was acidified to pH 2.5 and extracted 3× with 0.3 vol. hexane or 4× with 0.6 vol. H_2O-saturated EtOAc. The aq. layer was centrifuged at 30000 *g* for 30 min at 2°, and the supernatant was passed through Rexyn 101 cation exchange resin (converted to the NH_4^+ form; 16–50 mesh; column 3.6 × 15 cm) at a flow rate of 60 ml/hr. The column was washed with 600 ml distilled H_2O, pH 2.5, followed by 600 ml 70% EtOH, then the cytokinins were eluted in 600 ml 2 N NH_4OH. NH_3 was evapd, the pH adjusted to 8.2 and the cytokinins were extracted 4× with 0.6 vol. H_2O-saturated *n*-BuOH. The combined *n*-BuOH layers were evapd, redissolved in 80% EtOH and chromatographed on Whatman No. 3 MM paper with *sec*-BuOH–25% NH_4OH (4:1). Zones corresponding to authentic *trans*-zeatin, *trans*-zeatin riboside and dihydrozeatin marker spots were eluted in 80% EtOH and rechromatographed on Si gel GF_{254}, first using *n*-BuOH–25% NH_4OH–H_2O (6:1:2; upper phase), then with $CHCl_3$–MeOH (9:1; multiple development, so as to separate completely the *cis* and *trans* isomers). TMSi derivatives were prepared by heating dried extracts and authentic samples in BSA for 1–2 hr at 80° in sealed tubes. GC–MS was performed with a Finnigan 3200 F-100 GC-quadrupole MS with 6100 Data System using 3% OV-1 on Chromosorb Q (80–100 mesh) in a 154 cm × 2 mm glass column at He flow rate of 40 ml/min for *trans*-zeatin and dihydrozeatin samples, and 80 ml/min for *trans*-zeatin riboside samples; injection temp. 250° and column temp. programmed from 190 to 250° at 6°/min for *trans*-zeatin and dihydrozeatin samples; injection and column temp. 275° for *trans*-zeatin riboside samples. MS were recorded at 70 eV.

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D-HOMOCYSTEIC ACID FROM *PALMARIA PALMATA**

MAURICE V. LAYCOCK, A. GAVIN MCINNES and KEITH C. MORGAN
Atlantic Regional Laboratory, National Research Council of Canada, 1411 Oxford Street, Halifax, N.S., B3H 3Z1, Canada

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D-Homocysteic acid was isolated from the red seaweed *Palmaria palmata*. Although amino sulphonic acids are commonly found in aqueous extracts of red algae [1], D- or L-homocysteic acid has not previously been reported from any plant source. Amino acid analyses of hot 80% ethanolic extracts of *Palmaria palmata* showed three major free amino acids, glutamic acid, proline and a ninhydrin-positive compound that eluted from the long column of an automatic amino acid analyser well before aspartic acid. High-voltage paper electrophoresis showed this uncharacterized compound to be negatively charged at pH 6.5 with an electrophoretic mobility similar to that of aspartic acid. The analytical data for the isolated compound is consistent with homocysteic acid. The optical rotation, however, was negative at 589 nm indicating a mixture of 78% D-homocysteic acid and 22% of the L-isomer. It is uncertain if the observed proportions of the optical isomers occurred in the plant or if they resulted from racemization during the isolation procedures. However, when subjected to similar conditions, a pure sample of synthetic D-homocysteic acid showed some racemization resulting in 7% conversion to the L-isomer. Nevertheless, some L-isomer could occur in the plant since homocysteic acid is probably formed from L-methionine. A negative circular dichroism spectrum (peak at 215 nm) of the natural product compared to that of L-homocysteic acid confirmed that the compound isolated from *Palmaria palmata* was largely in the D-configuration. An IR spectrum (KBr pellet) of this material was identical to that of DL-homocysteic acid prepared by performic oxidation [2] of DL-homocystine. The 60 MHz ^{1}H NMR spectrum was identical to one previously reported for homocysteic acid [3], while the 20 MHz ^{13}C NMR spectrum contained the following characteristic resonances [4] and ^{13}C–H spin–spin couplings (4): C-1, δ_c 173.64; C-2, δ_c 54.25, J_{CH} = 147.2 Hz; C-3, δ_c 27.92, $J_{CH} \simeq$ 134 Hz; C-4, δ 49.31, J_{CH} = 136.2 Hz.

The concentration of homocysteic acid in the plant was found to be variable and dependent on the availability of nitrogen. Plants collected in early spring or taken from cultures supplied with nitrogen in excess contained 3.5 mg/g (dry wt) of homocysteic acid. In plants deprived of a source of nitrogen, the concentration of homocysteic acid fell to a minimum of 0.7 mg/g. Homocysteic acid was found in similar concentrations in tetrasporophytes and male plants collected at the same time of year from various locations in Nova Scotia.

EXPERIMENTAL

1 kg batch of *Palmaria palmata*, which was visually free of epiphytes, was soaked in 80% EtOH containing 1 M NH_4OH for 3 days at room temp. The extract was taken to dryness and the residue redissolved in 50 ml H_2O. After filtration through charcoal, the volume of the clear filtrate was reduced to *ca* 15 ml and left overnight in the refrigerator to allow inorganic salts to crystallize. Dowex 50 × 8, H^+ form (100 g) was then added to the mother liquors which were diluted with H_2O until all but the most acidic amino acids were adsorbed. This process was monitored by paper electrophoresis. Subsequently the soln (800 ml) containing the unknown amino acid was concd to *ca* 10 ml and applied to a column of QAE-Sephadex (2.5 × 45 cm) equilibrated with Py acetate buffer soln (0.5 M with respect to Py) at pH 5.0. Fractions were monitored by paper electrophoresis and those containing the unknown acidic amino acid, which was well separated from small amounts of glutamic and aspartic acids, were combined and concd by evapn. The pyridinium salt of the amino acid was transferred to a small column (1 × 2 cm) of Dowex 50 × 8, H^+ form to convert it to the free acid. The eluate was evapd to a clear syrup from which the acidic amino acid formed rosettes of needle shaped crystals (120 mg), which after recrystallizing twice from aq. EtOH had mp 270° (decomp.), $[\alpha]_D^{25}$ −10° (*c* 5 in N HCl). (Found: C, 26.09; H, 4.90; O, 43.65; N, 7.84; S, 17.51. Calc. for $C_4H_9O_5NS$: C, 26.22; H, 4.95; O, 43.67; N, 7.62; S, 17.50%).

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