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SHORT REPORTS

N^2-(1,3-DICARBOXYPROPYL) ORNITHINE IN CROWN GALL TUMOURS

JOHN L. FIRMIN* and ROGER G. FENWICK†

*John Innes Institute, Colney Lane, Norwich NR4 7UH; †ARC Food Research Institute, Colney Lane, Norwich NR4 7UA, U.K.

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; crown gall tumours; novel amino acids; structural elucidation; N^2-(1,3-dicarboxypropyl) ornithine.

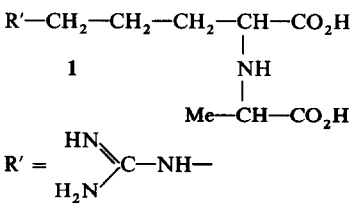
Abstract—A novel acidic amino acid present in crown gall tumours induced on *Nicotiana tabacum* cv White Burley by *Agrobacterium tumefaciens* has been identified as N^2-(1,3-dicarboxypropyl) ornithine.

INTRODUCTION

Crown gall tumours may be initiated on a variety of dicotyledonous plants by inoculation with an oncogenic strain of *Agrobacterium tumefaciens*. An interesting feature of the tumour tissue is the presence of the unusual amino acids octopine, N^2-(D-1-carboxyethyl)-L-arginine [1] (**1a**) or nopaline, N^2-(1,3-dicarboxypropyl) arginine [2] (**2a**), depending on the particular strain of bacterium used to initiate the tumour.

The observation that tumours containing octopine or nopaline are specifically induced by *A. tumefaciens* strains capable of metabolizing octopine or nopaline respectively [3–5] has led to the theory that the genes controlling the metabolism of these compounds in the bacterium are incorporated into the infected plant cells [6]. Recent work [7] has shown that the TI plasmid, found only in oncogenic strains of *A. tumefaciens*, determines the strain specificity with regard to octopine or nopaline utilization, and that the synthesis of these compounds in crown gall cells is also determined by genes located on this plasmid.

Analogues of octopine, octopinic acid, N^2-(D-1-carboxyethyl)-L-ornithine [8] (**1b**), and lysopine, N^2-(D-1-carboxyethyl)-L-lysine [9] (**1c**) usually occur in association with octopine, but as far as we are aware no analogues of nopaline have been reported.



Octopine, **1a**

R′ = H_2N—

Octopinic acid, **1b**

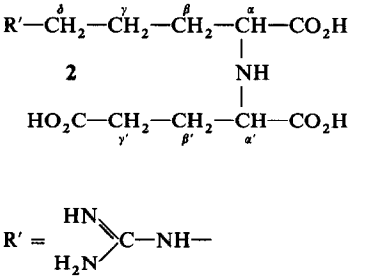
R′ = H_2N—CH_2—

Lysopine, **1c**

RESULTS AND DISCUSSION

Chromatographic studies on the free amino acids of crown gall tumours induced on *N. tabacum* by nopaline-metabolizing strains of *A. tumefaciens* (38-9, T-37) revealed an unidentified ninhydrin positive, Sakaguchi negative compound not present in tumours induced by an octopine-metabolizing strain of *A. tumefaciens* (B6) or in *N. tabacum* leaf tissue. The new compound was homogeneous on chromatography and electrophoresis, and was completely separated from nopaline (**2a**) by only two solvent systems. GC–MS of the heptafluorobutyric (HFB) *n*-propyl ester derivative [10] indicated a molecular formula $C_{20}H_{27}N_2O_6F_7$ for the derivative, corresponding to an atomic composition of $C_{10}H_{18}N_2O_6$ for the parent compound. Aqueous $KMnO_4$ oxidation of the new compound yielded mainly ornithine and glutamic acid, identified by chromatographic comparison with the authentic compounds; under identical conditions nopaline yielded mainly arginine and glutamic acid [11].

These data suggested that the unidentified compound was an analogue of nopaline, N^2-(1,3-dicarboxypropyl) ornithine, which we have named 'nopalinic acid' (**2b**) by analogy with octopine (**1a**) and octopinic acid (**1b**). The proposed structure was readily synthesized since molecules containing arginine residues yield the cor-



Nopaline, **2a**

R′ = H_2N—

Nopalinic acid, **2b**

responding analogues of ornithine on hydrazinolysis [12]. Thus, treatment of nopaline with aqueous hydrazine gave, after purification, nopalinic acid (**2b**), which was inseparable from the natural material by PC, high voltage paper electrophoresis and GLC (HFB *n*-propyl ester). Low resolution MS of the HFB *n*-propyl ester derivatives of both natural and synthetic nopalinic acid were identical.

Nopalinic acid constitutes an additional member to the group of unusual free amino acids found in crown gall tumours [1, 2, 8, 9] and its association with nopaline in the two tumour lines investigated appears to be analogous to the occurrence of octopinic acid [8] with octopine [1] in other tumour lines. A further analogy is suggested by the observation that those strains of *A. tumefaciens* which induced nopaline and nopalinic acid synthesis in the tumours, could also specifically metabolise these compounds.

The optical configuration of the assymetric centres in nopalinic acid could not be established in this study, since the configurations in nopaline (**2a**) are not yet known.

EXPERIMENTAL

Spectroscopic methods. MS of the heptafluorobutyric *n*-propyl ester derivative [10] of nopalinic acid were obtained at 70 eV. PMR spectra (90 MHz) were determined in D_2O–C_5D_5N using DSS as internal standard. IR spectra were recorded as *KBr discs.*

Chromatographic methods. Descending chromatograms were run on Whatman 3 MM paper, prewashed in 5% HOAc if used for preparative purposes. The solvent systems were *A*, PhOH–EtOH–H_2O–0.88 NH_4OH (120:40:40:1); *B*, PhOH–H_2O (3:1); *C*, Py–*iso*-AmOH–H_2O (8:4:7); *D*, *n*-PrOH–0.88 NH_4OH (3:2); *E*, MeOH–Py–H_2O (20:5:1); *F*, *n*-BuOH–Py–H_2O (1:1:1); *G*, *sec*-BuOH–HCO_2H–H_2O (5:1:1). Electrophoretograms were run on Whatman 3MM paper at voltage gradients of 30–60 V/cm for 2 hr in pH 3.6, 0.05 M HOAc or pH 9.5, 0.05 M $Na_2B_4O_7$. Amino acids were located by dipping the paper through 0.2% (w/v) ninhydrin in Me_2CO and heating at 100°. Guanidine containing compounds were revealed by the Sakaguchi reaction: papers were dipped through 0.1% (w/v) 8-hydroxyquinoline in Me_2CO and then sprayed with 0.2% (v/v) Br_2 in 0.5 M aq. NaOH.

Bacterial strains. A. tumefaciens strains B6 and T-37 were obtained from Dr D. Butcher, ARC Unit of Developmental Botany, Cambridge; he also kindly supplied a sample of crown gall teratoma induced on *N. tabacum* by strain T-37. Strain 38-9 was obtained from Professor R. H. Hamilton, Pennsylvania State University, U.S.A.

Initiation and culture of crown gall tumours. Tumours were induced on bacteria-free, 8 week-old *N. tabacum* cv White Burley plants by inoculation with either *A. tumefaciens* strain 38-9 or strain B6. Tumours 0.5–1 cm diameter were transferred to a solid medium [13] containing 200 μg/ml each of vancomycin (Lilly, Basingstoke) and carbenicillin (Glaxo, Greenford), and were subcultured at monthly intervals on to a solid medium without added antibiotics. Samples of tumour cultured as described, and plated on to a complete medium showed no visible bacterial growth.

Isolation of nopalinic acid from crown gall tumours. Tumour tissue was frozen in liquid N_2 and lyophilized for 48 hr. Dry tissue was ground with acid washed sand, and the resulting powder stirred with H_2O (4°), 40 ml/g dry wt of tissue, for 1 hr. The slurry was cleared by centrifugation and after re-extraction of the pellet with a second aliquot of H_2O, the combined supernatants were passed through a column of Zerolit 225, 4% D.V.B., 52–100 mesh, H^+ form, 5 ml resin/g dry wt of tumour extracted. After washing with 10 bed vols of H_2O, cationic compounds were eluted with 2 M NH_4OH (10 ml/ml resin). NH_4OH was removed by evaporation and nopalinic acid was isolated by preparative PC in solvent D (R_f 0.18 ± 0.02).

Synthesis of nopalinic acid. Synthetic nopaline [11] (300 mg) in aq. hydrazine (20 ml, 50% v/v) was heated for 2 hr at 85°. After removal of hydrazine by repeated evaporation with H_2O, the crude product, (240 mg dissolved in 100 ml H_2O) was passed through a column of Dowex 1 × 8 (30 ml bed vol.) 20–50 mesh, ^-OH form. The column was washed with H_2O (600 ml) before elution with 2 M HOAc (300 ml), which was subsequently removed by evaporation. Crystallization from aq. EtOH afforded the product (130 mg, 42%) as colourless microcrystals.

Synthetic nopalinic acid was ninhydrin positive and Sakaguchi negative and melted above 300° (uncorr.) with decomposition; high resolution MS of the HFB *n*-propyl ester derivative showed a M^+ at 524.17581, corresponding to an atomic composition $C_{20}H_{27}N_2O_6F_7$ (524.17567). Field desorption MS on the underivatized amino acid (**2b**) indicated a M^+ at *m/e* 263 $(M+H)^+$ and showed two successive losses of H_2O to yield *m/e* 245 and 227. Elemental analysis was consistent with the expected composition. (Found: C, 45.8; H, 6.9; N, 10.4. $C_{10}H_{18}N_2O_6$ requires C, 45.9; H, 6.8; N, 10.7%). The PMR spectrum showed characteristic signals at δ 2.4 (6H, *m*), 2.8 (2H, *t*, *J* = 6 Hz, C − γ′) 3.42 (2H, *t*, *J* = 7 Hz, C − δ), 4.03 (2H, *m*, C − α + α′). The IR spectrum exhibited both carboxylate and ammonium bands: γ max 3410, 3120, 2640 (w), 2080 (w), 1720, 1640, 1400 cm^{-1}.

Oxidative degradation of natural and synthetic nopalinic acid. Aq. samples were treated with a molar excess (× 1.5) of $KMnO_4$ for 8 hr at 20°. After centrifugation, the supernatant was analysed by PC (solvent D) and electrophoresis at pH 9.5).

Bacterial degradation of nopalinic acid. A. tumefaciens strains B6, T-37 and 38-9 cultured in a complete liquid medium [14] were inoculated (*ca* 10^7 cells/ml) into a minimal medium [15] containing synthetic nopalinic acid (80 μg/ml) as the sole N source, and GlC (4 mg/ml). Mean generation time, measured by increase in A_{630nm} at 30° was 5 hr ± 0.5 (T-37 and 38-9); strain B6 (octopine-metabolising) did not grow under these conditions.

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