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A DIRECT METHOD FOR ASSIGNMENT OF ABSOLUTE CONFIGURATIONS IN CROWN GALL METABOLITES: THE STRUCTURE OF NOPALINE

HANS AARON BATES,* JOSEPH J. MAGRATH, and ALKA KAUSHAL

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

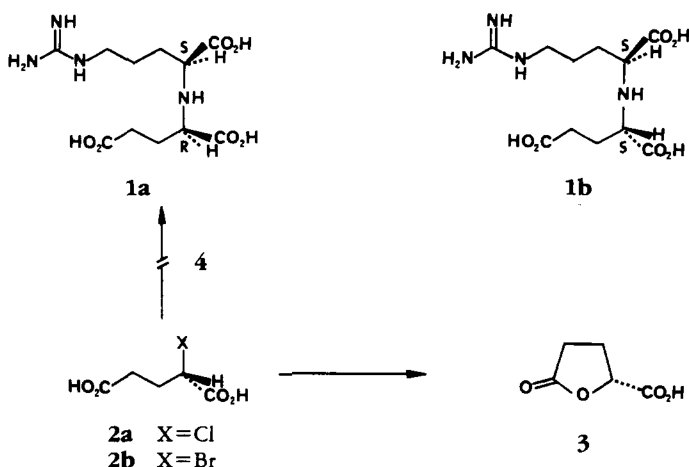
ABSTRACT.—The absolute configurations of the crown gall tumor metabolite nopaline (**1a**) have been determined by a direct degradative method. Oxidation of nopaline with $KMnO_4$ afforded (*S*)-arginine and (*R*)-glutamic acid. Separation of these two amino acids was accomplished easily and simultaneously with determination of chirality by chromatography on a reverse-phase column eluted with chiral eluent. The present technique can easily determine absolute configurations on submilligram quantities. In contrast, an attempted determination of absolute configuration by synthesis from (*S*)-arginine and chiral 2-chloropentanedioic acid was unsuccessful.

Crown gall tumors, incited by the bacterium *Agrobacterium tumefaciens*, produce a variety of unusual metabolites (1), including opines and opalines, which cannot be metabolized by the dicotyledonous host plant but are utilized as nutrients by the parasitic bacterium. Structurally, opines and opalines may be envisioned as conjugates of two amino acids joined at a common nitrogen. Biosynthetically, they are derived through reductive alkylation of an amino acid with a 2-oxocarboxylic acid (1, 2). Thus, the opines are derived from 2-oxopropanoic acid (pyruvic acid), while the opalines are derived from 2-oxopentanedioic acid (2-ketoglutaric acid). A segment of bacterial Ti plasmid DNA that codes for the enzymes that synthesize these abnormal metabolites is transferred to the plant cells. Therefore, the identity of the opine or opaline synthesized by the host plant is determined by the strain of *A. tumefaciens* that incites the tumor rather than by the host plant. Plant cells containing the transferred plasmid DNA proliferate as an uncontrolled cancerous growth. The parastic bacterium is able to catabolize the same opine or opaline that its Ti plasid DNA forces the host plant to synthesize. Accordingly, these abnormal metabolites may be viewed as chemical mediators of parasitism by *A. tumefaciens.*

Nopaline (**1a**) is an opaline originally isolated from crown gall tissue cutures derived from *Opuntia vulgaris* (nopal or sabra cactus) infected by *A. tumefaciens* (3). Subsequently, nopaline was isolated from crown gall tumors of other plants including sunflower, potato, carrot, tobacco, and the succulent *Kalanchoe diagremontiana* infected by various strains of *A. tumefaciens* (4). Nopaline was known to be derived from arginine, and the gross structure, undefined with respect to absolute configuration, was elucidated by elemental analysis, ms studies, and degradation (3). The gross structure was confirmed by synthesis of a diastereomeric mixture of nopaline and isonopaline by reductive alkylation of arginine with 2-oxopentanedioic (5, 6). At the outset of our investigation, the absolute configuration of nopaline was unknown. In this paper, we describe the absolute configuration of nopaline (**1a**) and a new direct method for assignment of absolute configurations to crown gall metabolites.

RESULTS AND DISCUSSION

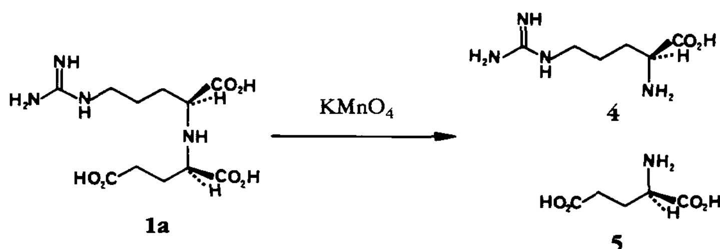
Our initial strategy for determining the absolute configuration of nopaline (**1a**) involved preparing all possible diastereomers of nopaline defined with respect to absolute configuration at both chiral centers, a classical approach employed by this group and many other investigators (2, 7-11). In this respect we were slightly encouraged by a report that arginine (**4**) reacts with 2-chloropentanedioic acid (**2a**) to produce nopaline,



albeit in very low yield (6). The reaction of NH_3 with 2-chloropentanedioic acid provided a more encouraging precedent (12). In our hands, however, reaction of arginine with 2-chloropentanedioic acid produced no detectable amount of nopaline but only lactone **3**. We anticipated that a reaction of arginine with 2-bromopentanedioic acid (**2b**) might be more successful; however, an attempt to prepare optically actice **2b** from (*S*)-glutamic acid afforded only lactone **3**.

The unsuitability of this classical approach stimulated us to consider a fundamentally different strategy toward assignment of absolute configuration in nopaline. To this end, we envisioned a degradation that would cleave nopaline (**1a**) into arginine (**4**) and glutamic acid (**5**) with preservation of chirality. Aqueous permanganate has been utilized to oxidize secondary amines to imines, which hydrolyze to primary amines and ketones (13). Indeed this reaction has been applied to a number of opines (11, 14) and opalines. Nopaline is known to afford a mixture of arginine and glutamic acid as well as 4-guanidinobutyric acid, which presumably arises from further oxidation of 5-guanidino-2-oxopentanoic acid (3, 5, 14, 15).

We have confirmed that $KMnO_4$ oxidizes nopaline (**1a**) to arginine (**4**) and glutamic acid (**5**); however, the yield was low (25% total) when the customary excess permanganate was employed. The stoichiometry of this oxidation requires only $2/3$ mole of permanagante/1 mole of nopaline. Under these conditions, arginine and glutamic acid were formed in 60% and 30% yield, respectively. The remaining tasks, separation of arginine and glutamic acid and determination of their chirality, were performed easily and simultaneously via liquid chromatography on a reverse-phase column eluted with a chiral eluent consisting of (*S*)-proline and cupric acetate (16). Post-column derivatization with *o*-phthalaldehyde allowed selective detection of the two amino acids by fluorescence on a microgram scale. Thus, oxidation of nopaline produced (*S*)-arginine and (*R*)-glutamic acid while the synthetic diastereomer, isonopaline, afforded (*S*)-arginine and (*S*)-glutamic acid. Because none of the enantiomeric amino acids were



formed, no racemization occurs during oxidation. Accordingly, nopaline and isonopaline have structures **1a** and **1b**, respectively. Thus, nopaline, like all other opalines and opines previously investigated, possesses the *S*-configuration at one amino acid and the *R*-configuration at the other amino acid. In nopaline the arginine portion possesses the *S*-configuration, as does the basic amino acid in all opines and opalines derived from basic amino acids.

The correct absolute configurations of nopaline had previously been proposed based upon the ability of nopaline to serve as a substrate for octopine dehydrogenase (17). While this investigation was in progress, the absolute configuration of the glutamic acid fragment of nopaline was established as *R* because it did not react with (*S*)-glutamate decarboxylase (15). The present method for determining the absolute configuration of crown gall tumor metabolites is general and can be performed easily on submilligram quantities. Our method is an attractive alternative to the previously utilized enzymatic methods, which require separation of opine degradation products, tend to utilize *lack* of reactivity as evidence, and may be precluded by unavailability of suitable enzymes. Assignment of absolute configurations by the present method is more general and much less tedious than by the classical method involving the synthesis of each authentic diastereomer. We anticipate that our method will find wide application to structure determination of other crown gall tumor metabolites.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^1H and ^{13}C nmr were observed with a Varian CFT-80 at 80 and 20 MHz, respectively. TMS or DSS (δ 0.0) and dioxane (δ 67.39), respectively, were utilized as internal standards. Analytical tlc was performed on silica gel eluted with *t*-BuOH-H_2O-pyridine (50:47:3) and paper electrophoresis was performed with 0.1 M pH 3 citrate buffer at 500 V for 3.5 h. Sakaguchi reagent was utilized for visualization. Other methods have previously been described (7).

(*S*)-2-CHLOROPENTANEDIOIC ACID.—Compound **2a** was prepared from (*S*)-glutamic acid (18) and was recrystallized from C_6H_6. ^1H nmr (Me_2CO-d_6) δ 2.3 (2 H, m), 2.5 (2 H, m) 4.37 (1 H, dd).

ATTEMPTED PREPARATION OF (*S*)-2-BROMOPENTANEDIOIC ACID (**2b**).—In an attempt to prepare (*S*)-2-bromopentanedioic acid, an aqueous solution of (*S*)-glutamic acid was treated with 6 *M* HBr and $NaNO_2$ at 0° for 24 h (7). The solution was extracted with Et_2O and the Et_2O was evaporated to afford a 23% yield of 2,3-dihydro-5(4*H*)-oxofuran-1-carboxylic acid (**3**). Physical and spectral properties were in agreement with literature values (19).

ATTEMPTED ALKYLATION OF (*S*)-ARGININE WITH (*S*)-2-CHLOROPENTANEDIOIC ACID (**2a**).—An equimolar aqueous mixture of (*S*)-arginine, (*S*)-2-chloropentanedioic acid, and 205 mol% $Ba(OH)_2$ was heated to 55° for 48 h, conditions, which previously affected a similar alkylation (7). No nopaline was detected by ^1H nmr or electrophoresis. Only **3** was detected. Use of 310 mol% **2a** and 600-1100 mol% $Ba(OH)_2$ also afforded no nopaline.

DIASTEREOMERIC MIXTURE OF NOPALINE (**1a**) AND ISONOPALINE (**1b**).—Method A. $NaBH_3CN$ (470 mg, 7.54 mmol, 450 mol) was added to a solution of (*S*)-arginine HCl (350 mg, 1.68 mol) and 2-oxopentanedioic acid (1.47 g, 10.0 mmol, 600 mol%) in H_2O (3.0 ml). After 48 h at 20°, HCl (2.75 ml, 12 M) was added, the solution was stirred another hour, concentrated, and chromatographed on AG 50W-X8 (1.6 × 8 cm, 100 to 200 mesh, equilibrated with 2 M HCl). The column was washed with H_2O (90 ml), and nopaline was eluted with NH_4OH (0.6 M, 200 ml). Evaporation of the solvent afforded at 60:40 mixture of nopaline (**1a**) and isonopaline (**1b**) as a glassy solid (534 mg, 90% yield).

Method B. A solution of (*S*)-arginine (**4**) and 2-oxopentanedioic acid in formamide was reduced with $NaBH_4$ (5) to afford a 30:70 mixture of nopaline and isonopaline as a white solid (70% yield).

NOPALINE (**1a**).—Nopaline was obtained by fractional recrystallization of the diastereomeric mixture produced by method B from H_2O in 3% yield (5). ^1H nmr (D_2O) δ 1.75 (4 H, m), 2.08 (2 H, q), 2.50 (2 H, t), 3.15 (2 H, t), 3.65 (1 H, t), 3.75 (1 H, t). ^{13}C nmr (D_2O δ 24.90, 25.14, 27.03, 31.5 (broad), 41.22, 61.83, 63.22, 156.39, 173.64, 178.12. Other physical and spectral properties as noted (5).

ISONOPALINE (**1b**).—The filtrate that remained after precipitation of nopaline was evaporated to dryness and recrystallized from 95% EtOH to afford isonopaline in 7% yield (5). ^1H nmr (D_2O) δ 1.75 (4 H, m), 2.08 (2 H, q), 2.50 (2 H, t), 3.15 (2 H, t), 3.54 (2 H, t). ^{13}C nmr (D_2O) δ 24.70, 25.19, 26.30, 31.3 (broad), 41.25, 61.16, 61.59, 156.39, 173.61, 178.12.

DEGRADATION OF NOPALINE AND ISONOPALINE.—Nopaline or isonopaline (1.2 mg, 4 μmol) dissolved in H_2O (0.85 ml) was oxidized with $KMnO_4$ (0.43 mg, 2.7 μmol, 69 mol%) for 2 h at 20°, at which point decolorization indicated consumption of the $KMnO_4$. The solution was filtered and analyzed by hplc. Centrifugation was less effective, and filtration through a C_{18}-Sep-Pak retained arginine.

LIQUID CHROMATOGRAPHY.—Liquid chromatography was performed with two Waters micro C-18 columns (30 × 0.39 cm) connected in series and two Waters model M-45 pumps (one for eluent and one for derivatization reagent) controlled by a model 660 solvent programmer. The column was eluted at 0.56 ml/min with a chiral eluent consisting of aqueous proline (0.017 M) and cupric acetate (0.008 M) (16). Post-column derivatization was accomplished with buffered *o*-phthalaldehyde-thioethanol reagent (20), which was pumped into a zero dead-volume reactor tee fitting at 0.84 ml/min at an angle 180° from the column eluent. The mixture of eluent and derivatization reagent emerged at 90° from each inlet and flowed through stainless steel tubing (50 × 0.009 cm) into a Waters model 420 fluorescence detector with excitation filter=340 nm and emission filter ≥ 440 nm. In our hands, cupric precipitate (soluble in aqueous EDTA) tended to form in the tubing after the tee-fitting. This was flushed from the system daily with H_2O.

Calibration of the column with standard solutions of (*R*)- and (*S*)-glutamic acid and arginine afforded the following mean retention volumes: (*R*)-glu, 2.70; (*S*)-glu, 2.80; (*R*)-arg, 3.18; (*S*)-arg, 3.42 ml (all±0.05 ml). The detector showed half-scale deflection at gain=8 in response to approx 1.5 μg (10 nmoles) of amino acid. The two columns in series possessed a total of 3000 theoretical plates. Analysis of the filtrate (5 μl) obtained from oxidation of nopaline indicated the presence of (*R*)-glu (2.66 ml) and (*S*)-arg (3.50 ml) in 60% and 30% yields, respectively. The identity of the amino acids was confirmed by coinjection.

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