A New Opine Derived from Nopaline*

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Nopaline, \(\text{N}\-[4\text{-}[\text{aminomethylamino}]-\text{1S-carboxybutyl}]\)-2\(\text{R}\)-aminopentanediolic acid and isono-paline, \(\text{N}\-[4\text{-}[\text{aminomethylamino}]-\text{1S-carboxybutyl}]\)-2\(\text{S}\)-aminopentanediolic acid) have been synthesized and separated by crystallization. In addition, a derivative of each of these compounds that forms spontaneously from the parent compounds under the usual crystallization conditions was isolated and characterized. The chemical properties, elemental analysis, \(\text{H}^1\)NMR spectrum, and electrophoretic behavior of the derivative from nopaline are consistent with \(\text{N}\-[4\text{-}[\text{aminomethylamino}]-\text{1S-carboxybutyl}]\)-2-\(\text{R}\)-pyrrolidone-5\(\text{R}\)-carboxylic acid, also called pyronopaline. The presence of pyronopaline in crown gall tumor tissue and the catabolism of it by the bacterium \(\text{Agrobacterium tumefaciens}\) establish it as a new opine.

Crown gall tumors occur on almost all dicotyledonous plants as a result of wound infection by the bacterium, \(\text{Agrobacterium tumefaciens}\), and tumorigenicity in the bacterium is associated with the presence of a plasmid, the Ti plasmid, and DNA sequences complementary to part of the plasmid DNA, the T-DNA, are found stably incorporated in the tumor tissue and the catabolism of it by the bacterium \(\text{A. tumefaciens}\), strain C-58, and the crown gall tumor tissue (CG1CT-T37) were generously supplied by Dr. Milton P. Gordon, Department of Biochemistry, University of Washington, Seattle. Octopine dehydrogenase (EC 1.5.1.11) from \(\text{Pecten maximus}\) was from Sigma. All other chemicals were of analytical grade or better except where noted.

Methods—Infrared spectra were determined on a Perkin-Elmer Model 221 spectrophotometer and \(\text{H}^1\)-NMR spectra of samples dissolved in deuterium oxide were determined in the NMR Core Facility on a Bruker WH-400 spectrometer operating at 400 MHz. Chemical shifts are reported in ppm with respect to 3-(trimethylsilyl)-\(\text{tetradeutero}\) sodium propionate. Elemental analyses \((\text{C, H, and N})\) were done by Atlantic Microlab, Inc., Atlanta, Georgia. Oxygen was determined by difference.

HPLC was done on an LDC-HPLC with an absorbance monitor set at 196 nm connected to a digital integrator. All samples were 0.020 ml. System A consisted of a Regis column (SS ODS) of Sphereisorb, which was developed with H\(\text{PO}_4\)(0.015 M) at 1.0 ml/min. System B consisted of a Whatman column (10/25 SAX) of Partisil PXS, which was developed with sodium phosphate (0.0125 M, pH 3.50) at 3.0 ml/min. Electrophoresis was performed on paper strips (Whatman 1) in one of the following: sodium carbonate \((0.05 \text{ M}, \text{pH 10.0})\); potassium phosphate \((0.02 \text{ M}, \text{pH 7.0})\); formic acid-acetic acid-water \((5:15:80)\); or HCl \((0.01 \text{ M})\). The electric field was 100 V/cm. Guanidinium compounds were identified by the fluorescence produced with the phenanthrenequinone reagent described previously (18). The presence of secondary amines was determined by the nitroprusside reaction described by Feigl (19).

Nopaline and isonopaline were synthesized from \(\text{L-arginine}\) and 2-oxoglutaric acid and were isolated as described by Jensen et al. (16) (see Scheme 1). The ammonium salts of the mixture of isomers obtained from synthesis and subsequent ion exchange chromatography were dissolved in water \((0.1 \text{ g/ml})\) and the pH of the solution was adjusted to pH 3.2 with \(\text{HCl}\) \((1 \text{ M})\). After the solution was left at 4 °C overnight, crystals enriched in nopaline were obtained. Recrystal-lization was accomplished by suspension of the crystals at room temperature in \(\text{H}_2\text{O}\) \((0.1 \text{ g/ml})\). The \(\text{pH}\) was adjusted to 4.5-5.0 with aqueous ammonia \((2 \text{ M})\) to dissolve the crystals, and then adjusted to 3.2 with \(\text{HCl}\) \((2 \text{ M})\). The solution was chilled as before, and the crystals were collected by filtration. They were washed with a small quantity of cold \(\text{H}_2\text{O}\) and dried at room temperature in vacuo. The product contained less than 1% of isonopaline, \(\text{L-arginine}\), or 2-oxoglutarate by HPLC or paper electrophoresis. Additional crystallization produced no significant change in the physical properties recorded in Table I. Isonopaline was crystallized by the addition of 3 volumes of ethanol \((85\%)\) to the mother liquor and chilling overnight at 4 °C. After an additional crystallization from ethanol/water, its physical properties became constant. Naturally occurring nopaline was isolated from crown gall tumor tissue, which was grown in Murishige and Skoog minimal medium without phytohormones at

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1 The abbreviation used is: HPLC, high performance liquid chromatography.
23 °C (about 3 weeks) and harvested by filtration through cheesecloth. The tissue was immediately placed in a plastic bag and frozen between two blocks of dry ice. It was shipped (overnight) in an insulated container of dry ice. The frozen tissue was dried in vacuo, and the dried material was extracted by suspension in water (60 ml/g, 4 °C) in a Waring blender (2 min). The suspension was centrifuged (10,000 × g, 50 min), and the supernate was applied to a column (0.079 times the volume of the extract, 1.56 diameter to height) of Dowex 50W-X8 (OH− form). The column was washed with two volumes of water, and the nopaline (determined by HPLC, system A or B) was eluted with aqueous NH₄Cl (0.1 M). The eluate that contained nopaline was applied to a column (0.36 times the volume of sample) of Dowex 50W-X8 (H+ form) at 4 °C. The column was washed with one column volume of water, and the nopaline was eluted with aqueous ammonia (1 M). The eluate that contained nopaline was taken to dryness and left in vacuo overnight. The residue was dissolved in water (10 ml/g) and the pH was adjusted to 3.2 with HCl (1 M). After 15 h at 4 °C, a crystalline material was filtered that corresponded to nopaline (at least 98%) by HPLC.

The minimal medium for bacterial growth was the mineral salt mixture described by Petit and Tempé (13) to which glucose was added to make the final concentration 0.005 g/ml. The control medium was the mineral salt mixture from which the ammonium sulfate was omitted. In order to test whether they would support bacterial growth, either nopaline, isonopaline or the pyrrolidone-5R-carboxylic acid derivative of nopaline (to be described) was added to the control medium to make the final concentration 0.0045 g/ml.

A single colony from a plate on minimal medium was adapted to liquid medium that contained nopaline. When visibly turbid, the nopaline liquid culture was inoculated (1% inoculum) into minimal liquid medium and into nopaline liquid medium. When turbid, these two cultures were used as inoculum to test the nopaline, isonopaline, and pyrrolidone-5S-carboxylic acid derivative of nopaline (to be described) was added to the control medium to make the final concentration 0.0045 g/ml.

Properties and Structure of Nopaline Derivative—During the analysis by HPLC, it was discovered that a new chemical species appeared in fractions of nopaline and isonopaline (pH 3.2), particularly when the solutions were warmed (60–100 °C). A sufficient amount of the new compounds was synthesized from nopaline and isonopaline (Scheme 1) to permit characterization and structure determination. The properties of these derivatives, particularly the former, are consistent with those of the parent compounds in which the glutamic acid portion has cyclized to the pyrrolidonecarboxylic acid. Accordingly, it will be shown below that the guanidino group of nopaline remains, whereas the secondary amine and a carboxyl group disappear. In addition, the differences in the 1H-NMR spectra between the parent compound and the derivative are very similar to those in the spectra of glutamic acid and its pyrrolidonecarboxylic acid derivative. Furthermore, the results of elemental analysis correspond very well with the changes predicted for the two compounds. Therefore, the derivative formed from nopaline is designated N-[4-[(aminoiminomethyl)amino]-1S-carboxybuty]-2-pyrrolidone-5R-carboxylic acid or pyronopaline.

The fact that both derivatives react with the phenanthrenequinone reagent to give a fluorescent compound indicates that the guanidino group remains intact. However, the nitroprusside test for secondary amines was negative with both derivatives, whereas the test was positive with both nopaline and isonopaline (although only weakly so in the case of the latter compound). In addition, comparison of the titration curves of the parent compounds with those of their derivatives showed that the appearance of the latter was associated with the disappearance of a group with a pKₐ of 9.2 (presumably the amine). The loss of the amine was further determined by HPLC.

### Scheme 1

**Properties and structure of nopaline derivative**

**Table 1**

| Compound | Nopaline | Isonopaline | Pyronopaline |
|----------|----------|-------------|--------------|
| **Empirical formula** | C₇H₉N₂O₅.H₂O | C₇H₉N₂O₅.H₂O | C₇H₉N₂O₅ |
| **Properties** | | | |
| **Optical rotation** | [a]° = 19 ± 1 | [a]° = 27 ± 1 | [a]° = 40 ± 1 |
| **Solubility in HzO (mg/ml, 22 °C)** | 6.3 | 21.3 | 34.5 |

*The numbers in parentheses are the calculated values based on the empirical formula.*
demonstrated by the results of electrophoresis where the nopaline derivative moved more slowly toward the anode than nopaline at pH 2, whereas they move at equal rates toward the cathode at neutral pH. The loss of a carboxyl group is indicated by the fact that the nopaline derivative also moved more slowly than nopaline toward the cathode upon paper electrophoresis at pH 10.0.

Fig. 1 shows the $^{1}H$-NMR spectra of nopaline (A), its derivative (B), and of isonopaline (C). Because of their chemical shifts, their relative sizes, and their splitting, the two peaks at 3.65 and 3.75 ppm in the nopaline spectrum are attributed to the hydrogens on the $\alpha$-carbons of the glutamate and arginine portions of nopaline. These peaks become overlapping in isonopaline, and in the compound derived from nopaline they shift to 4.15 ppm and overlap even more. The fact that the chemical shift of the analogous hydrogen in reference spectra of glutamate moves from 3.7 to 4.16 upon the formation of the corresponding pyrrolidonecarboxylic acid (20) strongly supports the contention that the nopaline derivative is a similar compound.

The peak at 3.2 ppm in all three spectra is attributed to the hydrogens on the $\delta$-carbon of the arginine portion, and the peaks between 1.6 and 2.0 ppm are attributed to the other methylene carbons of arginine by comparison with spectra of arginine and octopine. The peak at 2.5 ppm in the nopaline spectrum is attributed to the hydrogens on the $\gamma$-carbon of the glutamate portion, and as expected, it becomes much more complex in the derivative. The peak at 2.1 ppm is attributed to the hydrogens on the $\beta$-carbon of the glutamate portion, and the additional peak at 1.6 ppm in the spectrum of the derivative must be due to one of the same two hydrogens, which become nonequivalent in the pyrrolidonecarboxylic acid ring. The peak at 0.1 ppm is due to an impurity consistently seen in the deuterium oxide.

In addition, further spectroscopic evidence for the proposed structure comes from the fact that the nopaline derivative has a shoulder in the UV spectrum at 205 nm, attributed to the lactam, in addition to the peak at 196 nm that is characteristic of the guanidino groups of nopaline, isonopaline, and arginine.

The structure of the nopaline derivative is finally confirmed by the results of elemental analysis (Table I), which correspond to the empirical formula of a compound derived from nopaline by the loss of water. Since the pyrrolidonecarboxylic acid apparently crystallizes without the mole of water of crystallization that is characteristic of nopaline and isonopaline (15, 17), there is a difference of 2 mol of water in their respective empirical formulae, one of crystallization and one of formation of the internal amide.

The Stability and Rate of Formation of Pyronopaline—The conversion of both nopaline and its diastereoisomer to the respective pyrrolidonecarboxylic acid derivatives goes to completion in 30 min at pH 3.2 and 120 °C (autoclave), whereas the cyclization of neither nopaline nor isonopaline is demonstrable above pH 5.5. No significant reverse reaction of the nopaline derivative is seen at 100 °C in NaOH, Na$_2$CO$_3$ (both at 0.1 M), or HCl (3 M), a condition known to promote the conversion of the pyrrolidonecarboxylic acid derivative of glutamate to glutamic acid (21). Since nopaline cyclizes rapidly under the latter conditions, the lack of significant reverse reaction is due to the equilibrium and not to the rate of the reaction. Both nopaline and isonopaline react at pH 3.2 and 60 °C to form their respective pyrrolidonecarboxylic acid derivatives in a first order reaction with rate constants of $4.6 \times 10^{-7}$ s$^{-1}$ and $0.35 \times 10^{-7}$ s$^{-1}$, respectively. The faster reaction of the natural isomer, nopaline, is also apparent at 22 and 100 °C.

Occurrence and Metabolism of Pyronopaline—Analysis of extracts of crown gall tumors by HPLC (both systems) demonstrated that the concentration of the pyrrolidonecarboxylic acid derivative of nopaline, identified by retention time and analysis of samples mixed with authentic compounds, in the tumor is 52 μg/g. In addition, the fact that the concentration of both nopaline and its derivative in bacteria-free tumor extract (pH 5.3) did not change for 10 days at room temperature indicates that the derivative was indeed present in the tumor and is not an artifact of extraction or isolation.

Table II contains the results of experiments to test the ability of A. tumefaciens (strain C-58) to utilize the isomers and derivatives of nopaline as a source of carbon and nitrogen. Both nopaline and its pyrrolidonecarboxylic acid derivative are metabolized, whereas isonopaline is completely ineffective. The results are the same for both preinduced and uninduced bacteria. As expected, the doubling time in minimal medium is greater than that in either nopaline or pyronopaline, since metabolism by the organism is more complex in the former medium. However, the fact that the doubling time in pyronopaline is longer than that in nopaline suggests that one or more additional processes are required for growth on the derivative as well.

**DISCUSSION**

Goldman et al. (8) first reported that nopaline was unstable upon exposure to acid (chromatography on a column of Dowex-50, H$^+$ form). We have now identified the reason for this instability and the principal product to which nopaline is converted. Since the isolation of nopaline commonly includes crystallization of the zwitterionic form (pH in water solution, about 3.2) from mixtures of water and ethanol (15, 17), the final product might suffer from reduced yield and contamination with the pyrrolidonecarboxylic acid derivative. The contamination would account for the discrepancies in the literature (15, 17) with regard to the melting point of nopaline and for the fact that the optical rotation reported here is higher than that reported by the previous workers. Crystallized...
zation of the products from synthetic mixtures may result in a preparation composed predominantly of the unnatural isomer, isonopaline, since it converts more slowly than nopaline to the more soluble pyrrolidonecarboxylic acid derivative. In fact, one commercial source of nopaline was found by HPLC to be composed predominantly of the unnatural isomer.

The fact that isonopaline did not support the growth of A. tumefaciens confirms the results of Hatanaka et al. (17). However, since octopine will be metabolized by this strain of the organism only if it is previously induced by nopaline (13), it was necessary to investigate whether isonopaline would be metabolized by preinduced cells. That the latter compound is not metabolized by either cells shows that the R configuration at the C-2 of the glutamate portion of nopaline is required for transport, metabolism, or both.

The growth of both forms of bacteria on pyronopaline as a carbon and nitrogen source shows that the compound with the cyclic form of the glutamate portion of nopaline is active in induction, transport, and metabolism. The additional processes, indicated by the doubling time, that are required for growth in pyronopaline probably include the opening of the pyrrolidone ring, and investigations of this reaction are underway.

Although the presence of pyronopaline in the crown gall tumor tissue and its metabolism by A. tumefaciens qualify the compound as an opine, whether the pyronopaline present in the crown gall tumor tissue is a result of a spontaneous process or an enzymatic process depends upon the pH inside the tumor. The pH of our aqueous extract of the tumor indicates that it may be the result of an enzymatic process. Work is currently in progress to identify such enzymatic activity.

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