A Useful Synthesis of Nopaline, A Crown Gall Tumor Metabolite

Robert E. Jensen, Walter T. Zdybak,

Kyle Yasuda and William S. Chilton

Department of Chemistry, University of Washington
Seattle, WA 98195

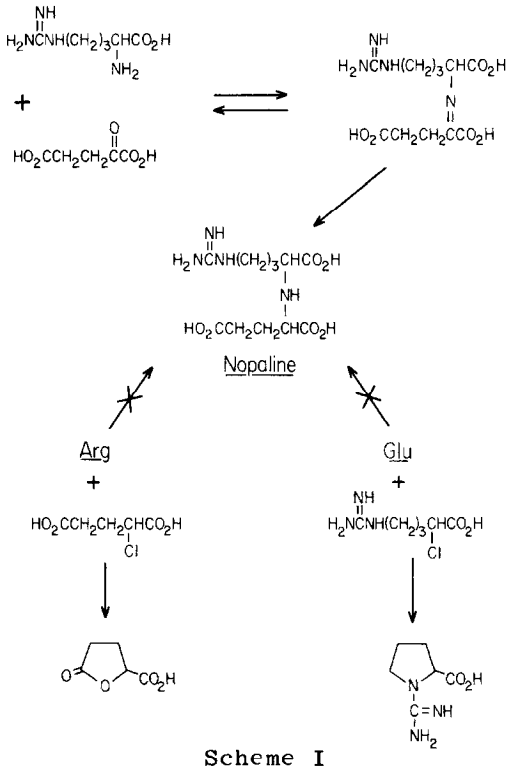
Received February 18,1977

Summary: Nopaline, a reductive conjugate of arginine and α-ketoglutaric acid found in plant tumors incited by Argobacterium tumefaciens, has been synthesized. Three routes were studied. Reduction of L-arginine and α-ketoglutaric acid with sodium cyanoborohydride gave an 80% yield of the diastereomers nopaline and isonopaline. This was by far the best method. The synthetic mixture of diastereomers is capable of replacing purified natural nopaline in biological experiments.

Crown gall tumors, incited in dicotyledonous plants by Agrobacterium tumefaciens, produce unusual reductive conjugates of several basic amino acids.[1-3] The best studied of these are octopine and nopaline (Scheme I), compounds not synthesized in normal plant tissue.[4-6] The bacterial strain which incites the tumor determines whether it will produce octopine or nopaline, independent of what host plant is used.[4] Large A. tumefaciens plasmids[7] are known to carry the genes which specify virulence and nopaline[8,9] or octopine[10,11] degradation by the bacterium, as well as nopaline or octopine production by the plant tumor.[10,12]

Interest in these metabolites stems from the possibility that they may be produced in bacteria-free tissue by transposed genetic information of bacterial origin.[4,10,12] Octopine and nopaline utilization are among the few genetic traits on A. tumefaciens virulence plasmids. Plasmid genetic manipulations have exploited octopine or nopaline utilization for direct genetic

ISSN 0006-291



Scheme I

selection of exconjugants or transformants.[11,12] Quantities of octopine and nopaline are needed for this purpose. Octopine, a formal reduction product of arginine and pyruvic acid has long been known in molluscs and is readily available synthetically. By contrast, nopaline is known only in crown gall and is not readily available.

Nopaline is formally the reductive conjugate of arginine and α-ketoglutaric acid. Attempts to duplicate the reductive synthesis in the laboratory have previously been unsuccessful.[13] We also found that reduction of arginine and α-ketoglutaric acid at pH 7 under three atmospheres of hydrogen with a platinum catalyst, analogous to a method for preparation of octopine,[14] gave only small amounts of nopaline because reduction of α-ketoglutaric acid to α-hydroxyglutaric acid competed with reversible Schiff base formation and imine reduction (Scheme I).

The yield of nopaline varied from 2-4%. Systematic variation in steps of 0.5 pH unit did not improve the yield significantly. Despite the low yield, this method was used to prepare and purify electrophoretically several hundred milligrams of nopaline. The crystalline product was identical chromatographically and electrophoretically to natural nopaline. The synthetic nopaline was utilized equally well as nopaline by arginine-requiring mutants of A. tumefaciens despite the possibility that it contains some of the glutamyl epimer iso-nopaline. The nopaline-arginine ratio in the hydrogenation product was improved only slightly by extraction of hydroxyglutaric acid and repeated reduction of the arginine in the presence of fresh portions of α-ketoglutaric acid. An 8.4% yield was obtained after five reduction cycles. No significant improvement in yield was obtained by using N^G-nitroarginine, nor by employing butyl esters rather than carboxylic acids.

Attempts to precondense arginine and α-ketoglutaric acid or their butyl esters by azeotropic methods led to only small amounts of nopaline accompanied by glutamic acid when the azeotroping solution contained alkoxide. The latter obviously arises from an undesired arginine-ketoglutaric acid transamination. In fact, a small amount of substance indistinguishable from nopaline was present in the alkaline azeotroped mixture before reduction. These indicators of disproportionation and transamination raise the probability of substantial racemization of the arginine which would significantly reduce the stereochemical homogeneity of nopaline prepared in this manner.

Octopine is more conveniently prepared by alkylation of arginine with chloropropionic acid than by the reductive method.[15] The alkylating agent for preparation of nopaline by this method,

chloroglutaric acid, has been used to alkylate ammonia in preparation of glutamic acid. Competing internal displacement produces some α-hydroxyglutarolactone (Scheme I). Extension of this method to preparation of nopaline gave α-hydroxyglutarolactone and only traces of nopaline because the concentration of arginine cannot be raised sufficiently to compete with internal displacement. The complimentary alkylation of glutamic acid with 2-chloro-5-guanidopentanoic acid gave only N-amidinoproline by internal displacement (Scheme I). Masking of the guanido group as the N-nitro derivative similarly gave N-amidinoproline.

Among all the methods investigated the only practical one is reduction of arginine and α-ketoglutaric acid with the selective reducing agent sodium cyanoborohydride at controlled pH. Yields approaching 80% of crystalline nopaline were obtained by this method. The ^{13}C-NMR spectrum of synthetic nopaline, measured at pH 13, is consistent with the structure previously assigned: arginyl β-, γ- and glutamyl β-carbons at 29.5, 30.9 and 31.5 ppm downfield from TMS; glutamyl γ-C, 35.2 ppm; arginyl δ-C, 41.5 ppm; two unresolved α-C, 63.4 ppm; guanido-C, 157 ppm; three carboxyl-C, 182.6, 182.9 and 183.2 ppm.

Natural nopaline at self pH is weakly levorotatory and has a weak negative rotatory dispersion consistent with D-configuration at one α-carbon and L at the other. The weakly dextrorotatory synthetic nopaline prepared from L-arginine is presumed to be a diastereomeric mixture of the weakly levorotatory nopaline and of the strongly dextrorotatory isonopaline having L-configuration at both centers. The isonopaline content of the synthetic product is under investigation. Synthetic nopaline was capable of serving as sole nitrogen source for ten strains of A. tumefaciens known to be nopaline-utilizing strains, and was incapable

of serving as nitrogen source for eleven strains known to be unable to utilize nopaline.

Materials and Methods

A solution of 6.96 g (.04 mole) arginine and 29.22 g (.2 mole) α-ketoglutaric acid in 60 ml water was adjusted to pH 7.0, and 9.43 g (.12 mole) sodium cyanoborohydride (Aldrich Chem. Co.) was added. After 48 hr of stirring the mixture was treated with Bio Rad AG 50W-x8 cation resin (H^+) until evolution of HCN ceased. The solution and suspended resin was poured over a 150 ml column of the same resin. Nopaline was eluted from the resin with 4 column volumes of 10% ammonia. One quarter of the eluate was lyophilized giving an 85% yield of nopaline electrophoretically free of arginine and pyronopaline. Three quarters of the eluate was concentrated in vacuo and crystallized from water giving 75% yield of crystalline nopaline mp 116-120°, dec. The overall yield of nopaline was 8 g.

Acknowledgements

We thank the NSF Undergraduate Research Participation Program for support (R.E.J. and K.Y.) and Alice Montoya and Mary Dell Chilton for testing synthetic nopaline on A. tumefaciens strains. This work supported in part by NIH Grant CA 13015.

References

1. Goldmann, A., Tempé, J., and Morel, G. (1968) C.R. Acad. Sci., 162, 630-631.
2. Goldmann, A., Thomas, D. W., and Morel, G. (1969) C. R. Hebd. Sceances Acad. Sci. Ser. D, 268, 852-854.
3. Kemp, J.D. (1977) Biochem. Biophys. Res. Commun., in press.
4. Petit, A., Delhaye, S., Tempé, J., and Morel, G. (1970) Physiol. Vég., 8, 205-213.
5. Holderbach, E., and Beiderbeck, R. (1976) Phytochem., 15, 955-956.
6. Kemp, J.D. (1976) Biochem. Biophys. Res. Commun., 69, 816-822.
7. Zaenen, I., Van Larebeke, N., Teuchy, H., Van Montagu, M., and Schell, J. (1974) J. Mol. Biol., 86, 109-127.
8. Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R.A., and Schell, J. (1974) Nature, 252, 169-170.
9. Watson, B., Gurrier, T.C., Gordon, M.P., Chilton, M.-D., and Nester, E.W. (1975) J. Bacteriol., 123, 255-264.
10. Bomhoff, G., Klapwijk, P.M., Kester, H.C.M., Schilperoort, R.A., Hernalsteens, J.P., and Schell, J. (1976) Mol. Gen. Genet., 145, 177-181.
11. Chilton, M.-D., Farrand, S.K., Levin, R.L., and Nester, E.W. (1976) Genetics, 83, 609-618.
12. Montoya, A.L., Chilton, M.-D., Gordon, M.P., Sciaky, D., and Nester, E.W. (1977) J. Bacteriol., in press.
13. Thoai, N.V., and Robin, Y. (1961) Biochem. Biophys. Acta, 52, 221-232.
14. Herbst, R.M., and Swart, E.A. (1946) J. Org. Chem., 11, 368-377.
15. Izumiya, N., Wade, R., Winitz, M., Otey, M.C., Birnbaum, S.M., Koegel, R.J., and Greenstein, J.P. (1957) J. Am. Chem. Soc., 79, 652-658.