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BIOSYNTHESIS OF PHENAZINES - THE ROLE OF PHENAZINE-1,6-DICARBOXYLIC ACID

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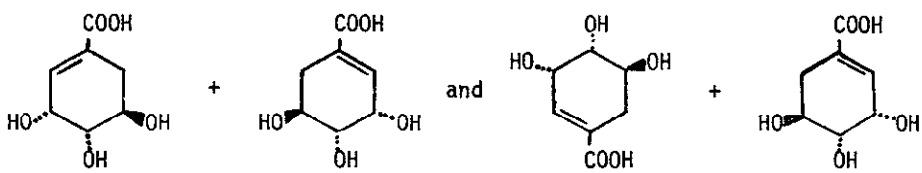
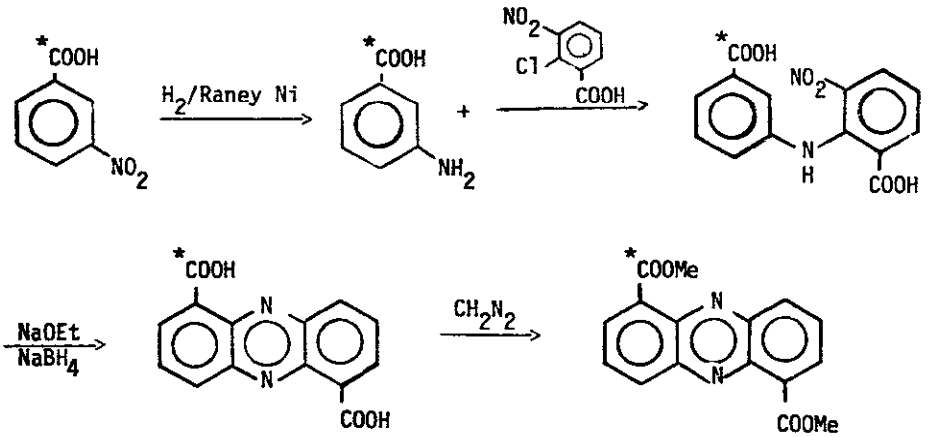
The phenazine skeleton occurs in some 30 presently known microbial antibiotics. The role of shikimic acid as precursor has been established for iodinin (1,6-dihydroxyphenazine-5,10-dioxide),[1,2] pyocyanine (the zwitterion of 1-hydroxy-5(N)-methylphenazine)[3] and phenazine-1-carboxylic acid.[3] The biosynthetic pathway to pyocyanine branches off from the shikimate pathway at chorismic acid.[4] Furthermore, phenazine-1-carboxylic acid has been shown to be a precursor of pyocyanine.[5] Since shikimic acid appears to be a general precursor for phenazines, several investigators have studied the incorporation of variously ^{14}C-labeled shikimic acids. The results have led to the conclusion that for iodinin and phenazine-1-carboxylic acid the possible pairing schemes of two shikimic acid molecules (or chorismic acid molecules generated therefrom) can be narrowed down to two:[1,2]



A B

Detection of phenazine-1,6-dicarboxylic acid from a bacterial source[6] and the incorporation of two molecules shikimic acid into iodinin and phenazine-1-carboxylic acid[9] suggest that this metabolite lies on the biosynthetic pathway after shikimic (or chorismic) acid. However, ^{2}H-labeled phenazine-1,6-dicarboxylic acid, fed to Pseudomonas iodinum, was not incorporated into iodinin.[7] Recently it was shown that scheme B must operate in the biosynthesis of iodinin.[9]

We have fed ^{14}C-monocarboxyl-labeled dimethylphenazine-1,6-dicarboxylate to Pseudomonas aureofaciens and found a 7.4% incorporation of the label into phenazine-1-carboxylic acid, labeled exclusively in the carboxyl carbon. m-Nitrobenzoic-carboxyl-^{14}C acid, 4.86 mci/mmole,[8] was diluted to a specific activity of 27.85 µci/mmole. It was catalytically reduced to m-aminobenzoic-carboxyl-^{14}C acid. Coupling with inactive 2-chloro-3-nitrobenzoic acid led to 6-nitrodiphenylamine-2,3'-^{14}C-dicarboxylic acid which was reductively cyclized[5] to phenazine-1-^{14}C,6-dicarboxylic acid mp > 365°, (lit[5] > 290°), TLC silicagel, MeOH:$CHCl_3$ 1:1, R_f = 0.25. The diacid was methylated with diazomethane.



Thirty mg of the radioactive diacid was fed to two 1000-ml portions of a production medium of Ps. aureofaciens which had been grown on a rotary shaker for 6 hrs as described earlier.[3] After growth for another 22 hrs the pigments were isolated and placed on a 40 x 600 mm Florisil column (100/200 mesh) in chloroform. Elution with chloroform, followed by chloroform-methanol mixtures with gradually increasing methanol content up to 5% separated phenazine-1-carboxylic acid as a green-yellow band sharply from phenazine-1,6-dicarboxylic acid which remained as a dark yellow band in the upper 30 mm of the column. The isolated and purified[3] phenazine-1-carboxylic acid was measured by liquid scintillation[3] and showed an activity indistinguishable from the background. However, relatively high activity was detected in the material remaining in the upper part of the column. Thus, phenazine-1,6-dicarboxylic acid was either not incorporated or did not pass through the cell walls of the bacteria.

Furthermore, the chromatographic technique employed had clearly separated it from phenazine-1-carboxylic acid.

Phenazine-1-^{14}C,6-dicarboxylic acid, 25 mg, was suspended in 5 ml methanol and treated with an excess of etheral diazomethane at 0°. The resulting dimethylester, 23 mg, was fed, as before, to Ps. aureofaciens. After extraction of the pigments the acidic material was separated from possibly remaining dimethylester and chromatographed on Florisil, as before. Isolated and purified phenazine-1-carboxylic acid showed radioactivity which remained constant upon further recrystallization. An incorporation of 7.4% ($\frac{\text{total activity isolated}}{\text{total activity fed}}$ x 200) was calculated. The active phenazine-1-carboxylic acid was decarboxylated in a heated tube under a stream of CO_2-free nitrogen at 290° with copper chromite as described earlier.[3] Phenazine was collected from the colder part of the tube and counted. Carbon dioxide was trapped as barium carbonate. In a closed apparatus carbon dioxide was liberated, and taken up in β-phenylethylamine and counted as described.[3] Table 1 shows that all activity resided in the carboxyl carbon as would be expected for the incorporation of the intact diacid (or possibly its dimethylester) into the mono acid.

Table 1

Specific activities[a)]

Phenazine-1-carboxylic acid	phenazine	CO_2
100%	0%	96.7%

a) averages of two runs

Thus, phenazine-1,6-dicarboxylic acid, possibly in its dimethyl ester form, is a direct precursor of phenazine-1-carboxylic acid and the shikimic (chorismic) acid pairing scheme A may be discarded. That the diacid itself was not incorporated as such, but only its dimethyl ester is possibly a question of polarity and concomitant lack of cell wall transport. A similar problem may have been encountered in the feeding of the ^{2}H-labeled diacid to Ps. iodinum.[7]

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References

1. U. Hollstein and D. A. McCamey, J. Org. Chem., 38, 3415 (1973).

2. R. B. Herbert, F. G. Holliman and J. B. Sheridan, Tetrahedron Letters, 4201 (1974).

3. U. Hollstein and L. G. Marshall, J. Org. Chem., 37, 3510 (1972).

4. R. P. Longley, J. E. Halliwell, J. J. R. Campbell and W. M. Ingledew, Can. J. Microbiol., 18, 1357 (1972).

5. M. E. Flood, R. B. Herbert and F. G. Holliman, Chem. Commun., 1514 (1970).

6. N. N. Gerber, J. Heterocycl. Chem., 6, 297 (1968).

7. M. E. Flood, R. B. Herbert and F. G. Holliman, J. Chem. Soc., Perkin Trans I, 1, 622 (1972).

8. California Bionuclear Corporation.

9. R. B. Herbert, F. G. Holliman and J. B. Sheridan, Tetrahedron Letters, 639 (1976).