Biol. Chem. 237, 2481.
James, R. W. (1962), The Optical Principles of the Diffraction of X-rays, London, Bell, Chapter 9.
Kirkwood, J. G. (1949), *Rec. Trav. Chim. 68*, 649.
Kirkwood, J. G. (1954), *J. Polymer Sci. 12*, 1.
Luzzati, V. (1960), *Acta Cryst. 13*, 939.
Luzzati, V., Witz, J., and Nicolaieff, A. (1961), *J. Mol. Biol. 3*, 379.
Porod, G. (1951a), *Kolloid-Z. 124*, 83.
Porod, G. (1951b), *Kolloid-Z. 125*, 51.
Riseman, J., and Ullman, R. (1951), *J. Chem. Phys. 19*, 578.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Simha, R. (1940), *J. Phys. Chem. 44*, 25.
Tanford, C., Buzzell, J. G., Rands, D. G., and Swanson, S. A. (1955), *J. Am. Chem. Soc. 77*, 6421.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, Wiley, pp 445–6.
Weber, G. (1952), *Biochem. J. 51*, 155.
Weber, G., and Young, L. B. (1964a), *J. Biol. Chem. 239*, 1415.
Weber, G., and Young, L. B. (1964b), *J. Biol. Chem. 239*, 1425.
Williams, E. J., and Foster, J. F. (1960), *J. Am. Chem. Soc. 82*, 3741.

The Isolation and Characterization of 2-Hydroxyphenazine from *Pseudomonas aureofaciens**

Mark E. Levitch and Peter Rietz

ABSTRACT: 2-Hydroxyphenazine was isolated from culture supernatant liquid of *Pseudomonas aureofaciens* by solvent extraction, ion-exchange chromatography, and crystallization.

The compound was identified by comparison with chemically synthesized 2-hydroxyphenazine by paper chromatography and electrophoresis, infrared and ultraviolet spectrophotometry, and X-ray diffraction. Some preliminary studies on the biosynthesis of the compound are presented.

In the course of studies on the biosynthesis of phenazine-1-carboxylic acid (Levitch and Stadtman, 1964), a contaminant was observed during the early stages of isolation of the acid. Ultraviolet spectra of alkaline solutions showed maxima at 365 and 252 mμ, characteristic of the 1-carboxylic acid derivative, and an additional peak at 275 mμ. Partition of the mixture between chloroform and 1% aqueous sodium bicarbonate indicated that the contaminating compound was somewhat less acidic than phenazine-1-carboxylic acid, although a complete separation could not be effected by this procedure.

Haynes and his co-workers (1956) had described a phenolic fraction from *P. aureofaciens* which was insoluble in sodium bicarbonate solution, but gave no other chemical data. Kluyver (1956) described a red pigment which was isolated from chloroform extracts of *P. aureofaciens* cultures by column chromatography on alumina. Elemental analysis of this compound suggested to Kluyver an empirical formula of $C_{13}H_8N_2O_3$, for which he postulated a structure of (1) a hydroxyphenazine-1-carboxylic acid or (2) a phenazinecarboxylic acid *N*-oxide. Since the compound which exhibited an absorption maximum at 275 mμ in our alkaline extracts was also red and had weakly acidic properties, the possibility existed that this compound, the phenolic fraction of Haynes *et al.*, and the red pigment of Kluyver were the same compound. In this paper we report the isolation and characterization of this compound and some preliminary studies on its biosynthesis.

Experimental Procedure

Growth of the organism and cultural conditions for large-scale production (about 20 l. of medium) of phenazine compounds by *P. aureofaciens* are as described by Haynes *et al.* (1956). The chloroform extraction procedure was described in a previous publication (Levitch and Stadtman, 1964). The chloroform solution containing the phenazine compounds was extracted with a minimal amount of 0.1 N sodium hydroxide solution, which was then adjusted to pH 7.0 with 1 N HCl plus enough 1 M sodium phosphate to bring the phosphate concentration to 0.01 M. The substances in the

* From the Cellular Biochemistry Laboratory, Department of Dermatology, Medical School, and the Department of Industrial Health, The University of Michigan, Ann Arbor, Michigan. *Received August 3, 1965; revised November 1, 1965.* This work was supported by a grant from the U. S. Public Health Service (GM-09869).

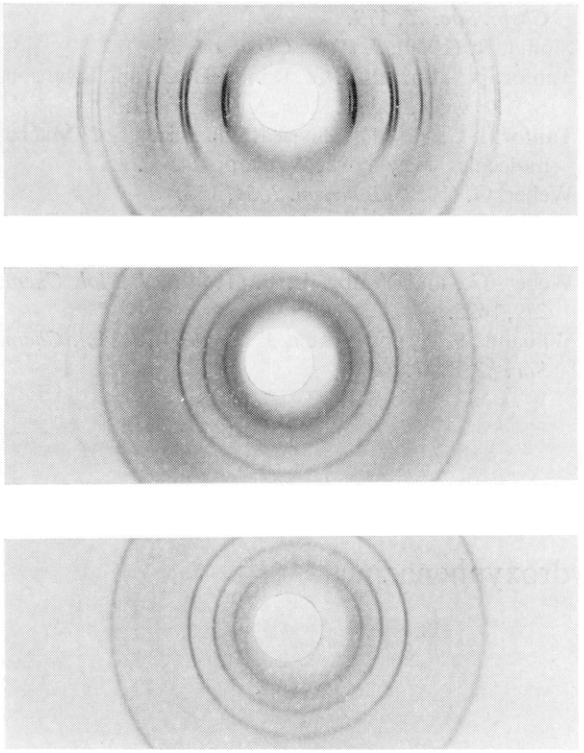

FIGURE 1: X-Ray Diffraction Patterns. A (top) 1-Hydroxyphenazine; B (middle) 2-hydroxyphenazine (synthetic); C (bottom) 2-hydroxyphenazine (natural product).

extract were then adsorbed on a column consisting of equal weights of Whatman cellulose powder (H. Reeve Angel Company, Clifton, N. J.) and triethylaminoethylcellulose (Calbiochem, Los Angeles, Calif.) which had been equilibrated with 0.005 M sodium phosphate (pH 7.0). Column dimensions were 4.3-cm diameter, 30-cm height. (A column of 1.2-cm diameter and 15-cm height can be used for preparations of less than 100 ml of culture supernatant.) The phenazine compounds were eluted with 0.05 M sodium phosphate (pH 7.0). The phenazine-1-carboxylic acid was eluted first in a very narrow band and was followed, after a considerable volume, by an orange band.

The fractions were made alkaline with 1 N NaOH and assayed spectrophotometrically at 275 and 252 mμ. The material from the second peak was crystallized twice from water to yield dark red crystals which, on drying in a vacuum desiccator at 50°, changed to an orange color. The compound did not give a definite melting point but melted over a broad range above 200° with decomposition.

The ultraviolet spectrum of this compound is identical with that of 2-hydroxyphenazine which is easily differentiated from that of the 1-hydroxy derivative (λ_{max}^{1NNaOH} 374 and 292 mμ; λ_{max}^{1NHCl} 384 and 275 mμ). Authentic samples of the 1- and 2-hydroxyphenazines were gifts of Dr. David Perlman of Squibb Institute for Medical Research. The compound exhibited the following ultraviolet absorption maxima (extinction coefficients are given in parenthesis): λ_{max}^{1NNaOH} 367 (7500), 275 (53,000), and 229 (27,000); λ_{max}^{1NHCl} 388 (18,000), 263 (62,000), and 217 (24,000).

Analysis of Natural Product

The elemental analyses were carried out by Huffman Laboratories, Inc., Wheatridge, Colo. The nitrogen analyses presented some difficulty, and a Kjeldahl method following reduction with iron and zinc in formic acid was found to give satisfactory analyses of the synthetic and natural products. Oxygen was determined directly using the Unterzaucher method. *Anal.* Calcd for $C_{12}H_8N_2O$: C, 73.45; H, 4.10; N, 14.27; O, 8.18. Found: C, 72.01; H, 4.97; N, 14.42; O, 8.28.

Paper Chromatography. The ascending technique was used on Whatman No. 1 paper with solvent systems 1 and 2 and on Schleicher and Schuell No. 589 Green Ribbon paper with solvent 3. The solvent systems employed were: (1) *sec*-butyl alcohol–concentrated ammonium hydroxide–water (100:14:36, v/v/v); (2) *sec*-butyl alcohol (water saturated)–glacial acetic acid (9:1 v/v); (3) 1 N acetic acid. R_F values were identical for 1- and 2-hydroxyphenazine and for the natural product, and are listed as follows for each solvent system: (1) 0.53; (2) 0.95; (3) 0.13.

Paper Electrophoresis. Electrophoretograms were run on Whatman No. 1 paper on a Beckman Spinco Model R paper electrophoresis apparatus, using 0.05 M sodium carbonate, pH 11. The natural product and 2-hydroxyphenazine migrate at the same rate, 0.65 cm in 2 hr at 300 v. The 1-hydroxyphenazine derivative would be expected to migrate similarly. For comparison, phenazine-1-carboxylic acid migrates 1.7 cm under the same conditions.

pK Determinations. Since there is a shift of absorption maxima from 275 mμ in alkali to 263 mμ in acid, and both peaks are present at pH 7.0, it seemed likely that the shift is a result of the dissociation of the phenolic hydroxyl. The apparent pK_a of this group was determined spectrophotometrically as 7.5. Albert and Phillips (1956) found pK_a values of 7.5 and 8.5, respectively, for 2- and 1-hydroxyphenazine hydroxyls.

Synthesis of 2-Hydroxyphenazine. 1,2,4-Trihydroxybenzene (126 mg) was dissolved in 30 ml of diethyl ether and 500 mg of silver oxide was added. The suspension, in a 500-ml Erlenmeyer flask, was shaken for 1 hr at room temperature. The resulting solution of reddish brown quinone was filtered into a solution of 108 mg of *o*-phenylenediamine dissolved in 30 ml of diethyl ether. After standing for 2 hr at room temperature, the ether was evaporated on a rotary evaporator (without heating) and the residue dissolved in 100 ml of 0.05 N NaOH. This solution was made slightly acidic with 1 N HCl and extracted six times with 100-ml portions of benzene. The combined benzene extracts were then extracted with dilute aqueous alkali, the latter solution containing the crude 2-hydroxyphenazine. The yield, determined spectrophotometrically, was 15.6 mg. The compound was crystallized from distilled

water and its purity established by paper chromatography and electrophoresis. The synthesis is an adaptation of the method of Kehrmann and Cherpillod (1924). *Anal.* Calcd for $C_{12}H_8N_2O$: C, 73.45; H, 4.10; N, 14.27. Found: C, 73.00; H, 4.94; N, 14.33.

Infrared Spectra. Compounds were mounted in potassium bromide (infrared quality, Harshaw Chemical Co., Cleveland, Ohio) pellets and run in a Beckman IR-8 infrared spectrophotometer. The natural product and 2-hydroxyphenazine exhibited identical spectra. In the 700–900 wavenumber region of the spectra (characteristic of hydrogen out-of-plane deformation), the 2-hydroxyphenazine exhibits three strong maxima at 752, 825, and 854 wavenumbers, indicating 1, 2, and 4 adjacent hydrogens and characteristic of a 2-substituted phenazine, whereas the 1-hydroxyphenazine shows only two strong maxima at 736 and 760 wavenumbers, indicating 3 and 4 adjacent hydrogens, and characteristic of a 1-substituted phenazine, as shown by Corbett (1964).

X-Ray Diffraction. Powder patterns were run on a Norelco X-ray diffraction unit. The patterns for the natural product and the 2-hydroxyphenazine were identical and are shown in Figure 1 with that of 1-hydroxyphenazine.

Microbial Synthesis of 2-Hydroxyphenazine. Reaction mixtures were the standard incubation mixtures previously described (Levitch and Stadtman, 1964) for the study of pigment production by washed cell suspensions of *P. aureofaciens*. Duplicate flasks were used for each time interval. The phenazine compound was extracted and separated as previously described. After a lag of 6 hr, synthesis of 2-hydroxyphenazine was linear up to 12 hr. In this experiment 0.37 μmole of 2-hydroxyphenazine was synthesized in 12 hr, compared to a synthesis of 0.12 μmole of phenazine-1-carboxylic acid during the same period. Relative amounts of the two compounds synthesized vary with each batch of cells. Since previous studies (Levitch and Stadtman, 1964) had indicated the possibility that shikimic acid is a precursor of phenazine-1-carboxylic acid, incorporation of label from radioactive shikimic acid into 2-hydroxyphenazine was also studied. Incubation conditions and the results are shown in Table I.

TABLE I: Incorporation of Label into 2-Hydroxyphenazine.

Radioactive Substrate	2-Hydroxyphenazine		% Incorporation
	μmoles	cpm[d]	
Glycerol-2-^{14}C (1.1×10^7 cpm)[a]	1.33	7.5×10^4	0.68
Shikimic acid-G.L.[e]-^{14}C (2×10^6 cpm)[b]	1.33	4.3×10^4	2.15

[a] Incubation mixture contained 500 μmoles of glycerol, 50 μmoles of DL-lysine, 50 μmoles of sodium 3,3-dimethylglutarate buffer (pH 7.4), 200 mg (wet weight) of cells,[c] in a final volume of 2.1 ml in a 50-ml Erlenmeyer flask incubated 12 hr on a rotary shaker at 30°. [b] Incubation mixture contained 1 mmole of glycerol, 100 μmoles of DL-lysine, 50 μmoles of sodium 3,3-dimethylglutarate buffer (pH 7.4), 200 mg (wet weight) of cells,[c] in a final volume of 4 ml in a 50-ml Erlenmeyer flask. Incubation conditions as in *a*. [c] *P. aureofaciens* cells were preincubated with 0.05 M sodium 3,3-dimethylglutarate buffer for 4 hr, centrifuged in a Servall SS-3 centrifuge for 10 min at 36,000 × *g*. They were washed once with fresh buffer and suspended in buffer at a concentration of 200 mg (wet weight)/ml. Such cells gave consistently higher yields of phenazine compounds than cells which were not preincubated. [d] Radioactivity measurements were determined on a Nuclear-Chicago Model D-47 gas flow counter. Samples were plated on ridged metal planchets at infinite thinness. [e] Generally labeled.

Discussion

The chemical data presented indicate that the compound isolated from culture filtrates of *P. aureofaciens* is a monohydroxyphenazine. Paper chromatography and electrophoresis support this hypothesis. The ultraviolet and infrared spectra and the X-ray diffraction patterns confirm the structure of the natural product as 2-hydroxyphenazine. It should be noted that the analytical values for carbon and hydrogen do not agree with calculated values. It is possible that the compound is not completely pure. However, the physical data leave no question as to the identity of this compound. This is the first report of a β-substituted hydroxyphenazine isolated from natural material. α-Substituted hydroxyphenazines are quite common in nature. They include pyocyanine (Wrede and Strack, 1929) (1-hydroxy-5-methylphenazine); iodinin (Clemo and McIlwain, 1938) (1,6-dihydroxyphenazine 5,10-dioxide), 1,6-dihydroxyphenazine (Akabori and Nakamura, 1959), and 1,6-dihydroxyphenazine 5-oxide (Gerber and Lechevalier, 1964). If one assumes that hydroxylation occurs after formation of the phenazine ring system, it is not difficult to rationalize the occurrence of both α- and β-hydroxyphenazines. However, this is unlikely since free phenazine has not been shown to occur in nature, but, if the ring is synthesized with the hydroxyl group preformed, it is unlikely that the two different hydroxylated rings are derived from a common precursor. Such considerations lead us to test the suitability of *P. aureofaciens* as a biological system for the study of the biosynthesis of 2-hydroxyphenazine. Preliminary labeling data, as shown in Table I, indicate that the system is adequate for such a study. The incorporation of label from shikimic acid-G.L.-^{14}C gives rise to a more highly labeled product than that from glycerol-1,3-^{14}C, and suggests that shikimate may serve as a precursor of 2-hydroxyphenazine, as in other

phenazine compounds (Levitch and Stadtman, 1964; Millican, 1962; MacDonald, 1963).

Acknowledgment

We are grateful to Mr. Benjamin Weiss for performing and interpreting the X-ray diffraction patterns, and to Dr. Samuel Krimm for interpreting the same patterns.

References

Akabori, H., and Nakamura, M. (1959), *J. Antibiotics (Tokyo) A12*, 17.

Albert, A., and Phillips, J. N. (1956), *J. Chem. Soc.*, 1294.

Clemo, G. R., and McIlwain, H. (1938), *J. Chem. Soc.*, 479.

Corbett, J. F. (1964), *Spectrochim. Acta 20*, 1665.

Gerber, N. N., and Lechevalier, M. P. (1964), *Biochemistry 3*, 598.

Haynes, W. C., Stodola, F. H., Locke, J. M., Pridham, T. G., Conway, H. F., Sohns, V. E., and Jackson, R. W. (1956), *J. Bacteriol. 72*, 412.

Kehrmann, F., and Cherpillod, F. (1924), *Helv. Chim. Acta 7*, 973.

Kluyver, A. J. (1956), *J. Bacteriol. 72*, 406.

Levitch, M. E., and Stadtman, E. R. (1964), *Arch. Biochem. Biophys. 106*, 194.

MacDonald, J. C. (1963), *Can. J. Microbiol. 9*, 809.

Millican, R. C. (1962), *Biochim. Biophys. Acta 57*, 407.

Wrede, F., and Strack, E. (1929), *Z. Physiol. Chem. 181*, 58.

(*S*)- and (*R*)-1-Cyano-2-hydroxy-3-butene from Myrosinase Hydrolysis of *epi*-Progoitrin and Progoitrin*

M. E. Daxenbichler, C. H. VanEtten, and I. A. Wolff

ABSTRACT: Enzymatic hydrolysis of *epi*-progoitrin, the major thioglucoside from crambe seed, at pH 3.0 produced the previously characterized (*R*)-goitrin and an unknown nitrile. The nitrile was isolated and shown to be (*S*)-1-cyano-2-hydroxy-3-butene. Measurements were obtained for the yield of (*R*)-goitrin and (*S*)-1-cyano-2-hydroxy-3-butene as a function of the pH during the hydrolysis of *epi*-progoitrin. Hydrolysis of progoitrin from rutabaga seed formed the enantiomeric (*S*)-goitrin and (*R*)-1-cyano-2-hydroxy-3-butene. In the Cahn–Ingold–Prelog system *epi*-progoitrin has the (*S*) configuration at the asymmetric carbon atom of the aglycon and may, therefore, also be designated as a salt of 2-(*S*)-hydroxy-3-butenylglucosinolate.

The major thioglucoside in the seed of *Crambe abyssinica* Hochst ex R. E. Fries is *epi*-progoitrin[1] (Ia) (Daxenbichler *et al.*, 1965). Its structure was established in part through characterization of (*R*)-5-vinyloxazolidine-2-thione [(*R*)-goitrin] (IIIa) as the product obtained by mustard myrosinase hydrolysis of the thioglucoside at near neutral pH. In the course of our work, we observed that the yield of (*R*)-goitrin from *epi*-progoitrin by myrosinase hydrolysis at pH 3 was poor although other data showed complete hydrolysis of the thioglucoside. From early literature, as well as from recent reports by Virtanen and Saarivirta (1962) and Schwimmer (1960), it was presumed that a nitrile might be formed in addition to (*R*)-goitrin when the enzymatic hydrolysis of *epi*-progoitrin was carried out at an acidic pH. We have now characterized the nitrile as (*S*)-1-cyano-2-hydroxy-3-butene (IIa), and report here the amounts of IIa formed under different conditions of myrosinase hydrolysis of *epi*-progoitrin. The enantiomeric (*R*)-1-cyano-2-hydroxy-3-butene (IIb) from progoitrin (Ib) of rutabaga seed was also isolated and characterized.

Stereochemical interrelationships among the thioglucosides (I) and their derived nitriles (II) and oxazolidinethiones (III) are shown below. The reasonable assumption is made that no inversion of configuration occurs in these reactions at the asymmetric center of the aglycon. Greer (1956) and Astwood *et al.* (1949) showed the derivation of goitrin from progoitrin obtained from seeds of the *Brassica* genus. This goitrin was later assigned the absolute configura-

* From the Northern Regional Research Laboratory, Peoria, Ill. *Received June 28, 1965; revised November 23, 1965.* Presented in part at the Division of Agricultural and Food Chemistry, 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug 30–Sept 4, 1964. The Northern Regional Research Laboratory is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[1] Authors regret earlier erroneous use of (*R*) in referring to the *epi*-progoitrin molecule (Daxenbichler *et al.*, 1965).