[CONTRIBUTION FROM CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

DL-5-Hydroxy-2-pyridinealanine, a Tyrosine Analog

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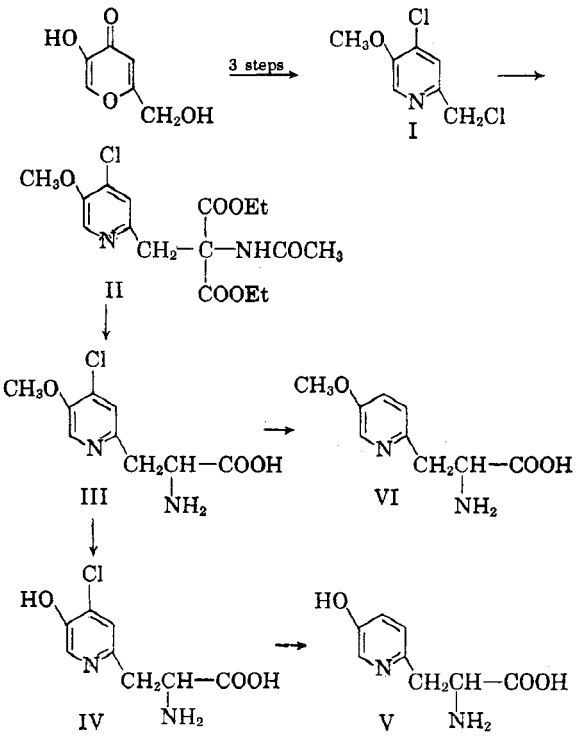
5-Hydroxy- and 5-methoxy-2-pyridine-DL-alanine and their 4-chloro derivatives were prepared from 2-hydroxymethyl-5-methoxy-4-pyridinol derived from kojic acid. The intermediate was converted to the dichloro derivative which was condensed with ethyl acetamidomalonate. Appropriate acid hydrolysis and hydrogenolysis steps afforded the desired products. 5-Hydroxy-2-pyridine-DL-alanine is a potent competitive antagonist of tyrosine in *Leuconostoc dextranicum* 8086 and is a moderately active growth inhibitor of *Escherichia coli* 9723. Tyrosine at high concentrations appears to be utilized by a pathway which is not inhibited by the analog in the latter organism.

Among previously reported amino acid antagonists, 3-fluorotyrosine appears to be the only analog which specifically inhibits tyrosine utilization. This compound is toxic for rats,[2a] and causes a growth inhibition of *Neurospora* which is competitively reversed by tyrosine.[2b] Two other potential tyrosine analogs, *p*-aminophenylalanine[3,4] and 3-nitrotyrosine,[4] inhibit the growth of *Escherichia coli*, but the growth inhibitions are reversed not only by tyrosine but also by phenylalanine and by tryptophan, respectively, in a manner which suggests that tyrosine is not specifically antagonized by these two compounds. In order to study tyrosine metabolism, a larger selection of competitive antagonists would be desirable, and the analog containing a pyridine ring in place of the benzene ring, 5-hydroxy-2-pyridinealanine, was considered since comparable modifications of *p*-aminobenzoic acid[5] and phenylalanine[6] had been successful in producing effective antimetabolites. This derivative was thus prepared and found to be a specific antagonist of tyrosine in certain microbial systems.

5-Hydroxy-2-pyridinealanine was synthesized through a sequence of reactions utilizing kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) as the starting material, and the mode of synthesis is indicated in the accompanying equations.



Initially, the dichlorination reaction to form 4-chloro-2-chloromethyl-5-methoxypyridine (I) was attempted by using a mixture of phosphorus pentachloride with phosphorus oxychloride; however, the reaction proceeded too vigorously and excessive decomposition products were obtained with only a small quantity of the desired product. In contrast, the reaction with phosphorus oxychloride alone proceeded smoothly and resulted in good yields of the desired compound when the temperature of the reaction mixture was not allowed to rise too rapidly. The hydrolysis of the acetamidomalonic ester derivative (II) was accomplished by heating under reflux for several hours with 48% hydrobromic acid. It was considered that cleavage of the 5-methoxy group might also occur; however, the product isolated from this hydrolytic mixture did not give a positive ferric chloride test indicating that the phenolic group was still methylated. Cleavage of the methoxy group was later achieved by heating 4-chloro-5-methoxy-2-pyridinealanine (III) in the presence of hydrobromic acid at temperatures of 165–170° for about four hours. The hydrogenolysis of the 4-chloro group in III and in 4-chloro-5-hydroxy-2-pyridinealanine (IV) was readily effected by catalytic

(1) Pre-doctoral Fellow (CF 10,779), National Cancer Institute, Public Health Service.

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(2) (b) H. K. Mitchell and C. Niemann, *J. Am. Chem. Soc.*, **69**, 1232 (1947).

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(6) E. M. Lansford, Jr., and W. Shive, *Arch. Biochem. Biophys.*, **38**, 347 (1952) and references cited therein.

hydrogenation using palladium black as catalyst. When the hydrogenation reaction mixture was not neutralized, the dihydrochloride salt of 5-hydroxy-2-pyridinealanine (V) precipitated, but when the hydrogenated solution was neutralized with concentrated ammonium hydroxide following hydrogenolysis, the free amino acid was isolated.

Concurrent with the above synthetic work, an alternate route of synthesis of 5-hydroxy-2-pyridinealanine was considered which involved the preparation and subsequent hydrolysis of an appropriately substituted hydantoin using procedures patterned after those reported for the synthesis of tyrosine.[7,8] In order to determine if the heterocyclic group would interfere in the condensation, 2-pyridinecarboxaldehyde was condensed with hydantoin and the unsaturated intermediate was hydrogenated and subsequently hydrolyzed to yield 2-pyridinealanine. Since at this stage the former procedure had yielded the desired amino acid antagonist, further work on this procedure was discontinued.

All four of the β-substituted alanines which were prepared in this study were examined for their microbial inhibitory activity; the derivatives studied included 5-methoxy-2-pyridine-(VI), 4-chloro-5-methoxy-2-pyridine (III), 4-chloro-5-hydroxy-2-pyridine-(IV), and 5-hydroxy-2-pyridine-alanine (V). Using *Leuconostoc dextranicum* 8086 and *Escherichia coli* 9723 as the test organisms, III and IV were inactive up to a concentration level of 200 γ/ml. Testing at higher concentration levels was not feasible due to their relatively poor solubility in the assay media. VI was inhibitory to the growth of *E. coli* at its limit of solubility, 600 γ/ml., but was relatively nontoxic toward *L. dextranicum*. In contrast to the relatively poor inhibitory properties of III, IV, and VI, 5-hydroxy-2-pyridinealanine (V) was inhibitory to growth of *L. dextranicum* and *E. coli* at levels of 2 and 6 γ/ml., respectively, and was more extensively studied with respect to its biological properties than the other three derivatives.

Using *L. dextranicum*, growth inhibition by V was competitively reversed over a 100-fold range of increasing DL-tyrosine concentrations with an inhibition index (ratio of inhibitor to substrate necessary for complete inhibition of growth) of about 10 as indicated in Table I. Under the testing conditions used in these assays, not only was phenylalanine inactive in reversing this toxicity, but at high concentrations it slightly augmented the toxicity of the analog.

For *E. coli* 9723, the toxicity of 5-hydroxy-2-pyridinealanine is prevented by tyrosine in a noncompetitive manner (Table II). Levels of tyrosine at 2, 6, and 20 γ per ml. results in readings of approximately 20, 40, and 70, respectively, over a range of ten-fold increases in inhibitor concentrations. These results suggest that appropriately high concentrations of tyrosine can by-pass the inhibitory effect of the analog; however, this does not occur in the presence of a supplement of phenylalanine (100 γ per ml.) as indicated in Table II, and under these conditions a competitive reversal by tyrosine over more than a 100-fold range of concentrations of inhibitor are observed with an inhibition index of approximately 100. These data suggest that there exists two pathways for the utilization of tyrosine, one a major pathway initially inhibited by the 5-hydroxy-2-pyridinealanine, and another less effective pathway in common with phenylalanine which can also effectively inhibit the utilization of tyrosine by this route. Thus, only in the presence of exogenous phenylalanine can a

TABLE I

REVERSAL OF 5-HYDROXY-2-PYRIDINEALANINE TOXICITY IN *Leuconostoc dextranicum* 8086 BY DL-TYROSINE[a]

5-Hydroxy-2-pyridine-DL-alanine, γ/ml.	Supplement, DL-Tyrosine, γ/ml.				
	None	0.2	2.0	6.0	20.0
	Galvanometer Readings[b]				
0	61	63	63	63	64
0.6	43	56			
2	5	43	60		
6	1	1	46	60	64
20			4	53	62
60				5	54
200					13

[a] Incubated at 30° for about 18 hr. No phenylalanine, tyrosine media as described in the Experimental section. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100.

TABLE II

REVERSAL OF 5-HYDROXY-2-PYRIDINEALANINE TOXICITY IN *Escherichia coli* 9723 BY DL-TYROSINE[a]

5-Hydroxy-2-pyridine-DL-alanine, γ-Ml.	DL-Tyrosine, γ/ml.					
	None	0.2	0.6	2.0	6.0	20.0
	Galvanometer Readings[b]					
0	78	76	81	78	75	79
2	55	69	69	73		
6	3	40	47	58	67	
20		5	7	26	53	
60				22	45	
200				17	46	73
600					40	69
Media[c] supplemented with 100 γ/ml. DL-phenylalanine						
0	73	72	72	74	73	73
2	40	60	68			
6	2	10	50	56		
20		3	7	46	66	
60			5	12	55	69
200				3	14	65
600					5	32

[a] Incubated at 37° for about 12 hr. [b] A measure of culture turbidity; distilled water reads 0, an opaque object 100. [c] Salts-glucose media described in Ref. 11.

(7) H. L. Wheeler and C. Hoffman, *Am. Chem. J.*, 45, 368 (1911).

(8) W. J. Boyd and W. R. Robson, *Biochem. J.*, 29, 542 (1935).

competitive relationship of tyrosine and the pyridine analog be observed.

From these results, it is apparent that 5-hydroxy-2-pyridinealanine is a specific and effective antagonist of tyrosine in both *L. dextranicum* and *E. coli*.

EXPERIMENTAL[9]

Microbiological assays. For the assays using *Leuconostoc dextranicum* 8086, a previously described amino acid medium[10] was employed, except that the phenylalanine and tyrosine were omitted from the basal media and the concentration of aspartic acid was reduced to 4 γ/ml. of assay medium. Tyrosine was supplemented as indicated in the Tables. The medium was further modified by adding 0.02 γ/ml. of pantethine, by increasing the Salts A concentration fourfold, and by adding 0.2 γ/ml. of calcium pantothenate. A previously reported inorganic salts-glucose medium[11] was used for *Escherichia coli* 9723, and the experimental detail has been reported elsewhere.[12]

The amino acid analogs were dissolved in sterile water and added to sterile assay tubes without being heated in all of the assays. The amount of growth was determined turbidimetrically in terms of galvanometer readings so adjusted that distilled water read 0 and an opaque object 100.

Organic intermediates. 2-Hydroxymethyl-5-methoxy-4H-pyran-4-one was prepared from kojic acid by interaction with dimethyl sulfate, m.p. 157–158°.[13] The ring oxygen of the latter derivative was replaced by nitrogen using concentrated ammonium hydroxide to form 2-hydroxymethyl-5-methoxy-4-pyridinol, m.p. 171–173°.[14]

4-Chloro-2-chloromethyl-5-methoxypyridine. To 165 ml. of cooled phosphorus oxychloride was added slowly with stirring 54.0 g. of 2-hydroxymethyl-5-methoxypyridinol. The exothermic reaction was controlled by cooling, and, after the addition was completed, the flask was fitted with a condenser and the reaction mixture was heated under reflux for about 2 hr. The mixture changed from a bluish color to maroon during this period. The volatile inorganic chlorides were distilled *in vacuo*, the residue was chilled in an ice bath, and small chips of ice were added to decompose the residual phosphorus oxychloride. The resulting residue was then taken up in 300 ml. of water, and decolorized with charcoal. The aqueous solution was finally neutralized with 10% sodium hydroxide, and a white crystalline product separated. This material was recrystallized from ethanol-water to yield 40.4 g. of long white needles, m.p. 72–73°.

Anal. Calcd. for $C_7H_7Cl_2NO$: C, 43.78; H, 3.67; N, 7.29. Found: C, 43.97; H, 3.92; N, 7.09.

Ethyl 2-acetamido-2-(4-chloro-5-methoxy-2-pyridinemethyl)-malonate. To a cool solution of 36.0 g. of ethyl acetamidomalonate dissolved in 300 ml. of ethanol containing 3.7 g. of sodium was added slowly with stirring 32.0 g. of 4-chloro-2-chloromethyl-5-methoxypyridine. After addition was completed the reaction mixture was heated under reflux for about 24 hr. The precipitated sodium chloride was removed by filtration, and the filtrate was then poured over ice water. The resulting crystalline material was finally recrystallized from ethanol-water to yield 38.4 g. of product, m.p. 150–151°.

Anal. Calcd. for $C_{15}H_{21}ClN_2O_6$: C, 51.55; H, 5.68; N, 7.52. Found: C, 51.64; H, 5.65; N, 7.29.

DL-*4-Chloro-5-methoxy-2-pyridinealanine.* A solution of 30.0 g. of ethyl 2-acetamido-2-(4-chloro-5-methoxy-2-pyridinemethyl)malonate in 100 ml. of 48% hydrobromic acid was heated under reflux for about 7 hr. The resulting solution was then reduced to about 50 ml. *in vacuo*, and adjusted to *p*H 6 with concentrated amonium hydroxide. After cooling in the refrigerator for several hours, some material precipitated which was ninhydrin positive. This product was recrystallized from water, and only one ninhydrin-positive hydrolysis product was indicated by paper chromatography. There was recovered 19.5 g. of material, m.p. 216–218°.

Anal. Calcd. for $C_9H_{11}ClN_2O_3$: N, 12.15. Found: N, 12.14.

DL-*5-Methoxy-2-pyridinealanine.* To a solution of 1.4 g. of 4-chloro-5-methoxy-2-pyridinealanine in 50 ml. of water was added 200 mg. of palladium black, and the mixture was treated with hydrogen under 3 atm. for about 3 hr. The catalyst was recovered and the filtrate was neutralized with concentrated ammonium hydroxide. The resulting solution was then cooled for several hours, and 0.75 g. of white crystalline material separated, m.p. 224–227° dec.

Anal. Calcd. for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.16; N, 14.28. Found: C, 55.10; H, 5.99; N, 14.22.

DL-*4-Chloro-5-hydroxy-2-pyridinealanine.* A solution of 6.0 g. of 4-chloro-5-methoxy-2-pyridinealanine and 10 ml. of 48% hydrobromic acid was placed in a stainless steel bomb and heated at 165–170° for about 4 hr. The cooled solution was then diluted with 300 ml. of water and neutralized with concentrated ammonium hydroxide to yield a dark red mixture. After cooling this latter solution, about 0.5 g. of brick red material separated leaving a light yellow colored supernatant which was reduced in volume to about 150 ml. and finally placed in the refrigerator for several hours. A white crystalline product separated which was found to give a positive ferric chloride test. Recrystallization of this material from water gave 3.5 g. of product, m.p. 180–182° dec.

Anal. Calcd. for $C_8H_9ClN_2O_3 \cdot 1^1/_2H_2O$: C, 39.44; H, 4.96; N, 11.50. Found: C, 39.70; H, 4.36; N, 11.53.

DL-*5-Hydroxy-2-pyridinealanine dihydrochloride.* To a solution of 0.5 g. of 4-chloro-5-hydroxy-2-pyridinealanine in 50 ml. of water was added 200 mg. of palladium black, and the resulting mixture was treated with hydrogen gas under about 3 atm. for 4 hr. The catalyst was removed, and the filtrate was concentrated *in vacuo* to approximately 20 ml. Upon cooling a crop of white crystals formed which were recrystallized from water-ethanol to yield 0.3 g. of product, m.p. 151–153° dec.

Anal. Calcd. for $C_8H_{10}N_2O_2 \cdot 2HCl$: C, 37.67; H, 4.74; N, 10.98. Found: C, 37.84; H, 5.06; N, 10.80.

Ultraviolet absorption spectrum at *p*H 3: λ_{max} 288–289 mμ; λ_{min} 248 mμ. Spectrum at *p*H 12: λ_{max} 303 mμ; λ_{min} 268 mμ.

5-(2-Pyridinemethylidine)hydantoin. Following the general procedure for the preparation of 5-(*p*-hydroxybenzal)-hydantoin,[10] a mixture of 16 ml. of 2-pyridinecarboxaldehyde, 13.5 g. of hydantoin, 6 ml. of diethylamine, and 15 ml. of pyridine were placed in a glass-lined steel bomb and heated at 100° for about 20 hr. The solvents were removed and the semisolid residue was taken up in water, decolorized

(9) All melting points are uncorrected. The ultraviolet spectra were determined on a Beckman DK-2 Recording Spectrophotometer using a 10 γ/ml. aqueous solution of the compound. The paper chromatograms were determined by the ascending technique, and the colors were developed with ninhydrin reagent. The authors are indebted to Dr. J. M. Ravel and Mrs. Jean Humphreys for assistance with the biological assays, to Mr. Charles Hedgcoth for the elemental analyses, and to Mr. Allen Lane for technical assistance with some of the intermediates.

(10) J. M. Ravel, L. Woods, B. Felsing, and W. Shive, *J. Biol. Chem.*, **206**, 391 (1954).

(11) E. H. Anderson, *Proc. Natl. Acad. Sci., U. S.*, **32**, 120 (1946).

(12) F. W. Dunn, J. M. Ravel, and W. Shive, *J. Biol. Chem.*, **219**, 809 (1956).

(13) K. N. Campbell, F. J. Ackerman, and B. K. Campbell, *J. Org. Chem.*, **15**, 221 (1950) report a m.p. of 157–158°.

(14) J. W. Armit and T. J. Nolan, *J. Chem. Soc.*, 3023 (1931) report a m.p. of 173–175°.

with Darco G-60, and finally recrystallized from water to yield 15.6 g. of product, m.p. 228–229°.

Anal. Calcd. for $C_9H_7N_3O_2$: C, 57.14; H, 3.73. Found: C, 57.01; H, 3.57.

5-(2-Pyridinemethyl)hydantoin. To a solution of 5 g. of 5-(2-pyridinemethylidine)hydantoin in 150 ml. of 95% alcohol was added 2 g. of palladium on charcoal, and the mixture was treated with hydrogen gas under about 3 atm. with continuous shaking for about 12 hr. The catalyst was recovered, and the filtrate was reduced to a small volume *in vacuo*. Upon cooling, crystals separated which were finally recrystallized from methanol-water to yield 3.9 g. of product, m.p. 171–172°.

Anal. Calcd. for $C_9H_9N_3O_2$: N, 22.00. Found: N, 21.91.

DL-*2-Pyridinealanine.* A mixture of 3.5 g. of 5-(2-pyridinemethyl)hydantoin, 10 g. of barium hydroxide, and 15 ml. of water was placed in a stainless steel beaker fitted with a reflux condenser, and heated over a steam cone until the evolution of ammonia ceased. The reaction mixture was filtered, the filter was washed several times with boiling water, and the combined filtrates were then cooled and treated with carbon dioxide. After filtration, the filtrate was finally adjusted to *p*H 4.5 with concentrated sulfuric acid to remove the remaining barium present, and the resulting solution was treated with Darco G-60, filtered, and the filtrate was reduced to a small volume *in vacuo*. Upon chilling, a precipitate formed which was recrystallized from alcohol-water to yield 1.3 g. of material, m.p. 209–210°. This product was chromatographically identical with material prepared by a previously reported procedure.[15]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF BUFFALO]

The Synthesis of Phthaloyltauryl Peptides[1]

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A number of phthaloyl peptide esters have been prepared by treatment of β-phthalimidoethanesulfonyl chloride with amino acid esters or dipeptide esters. These esters were converted to carboxylic acids by alkaline hydrolysis.

Although the occurrence of taurine in the body and its formation from cysteine has been recognized for some time, recent investigation had indicated that taurine may also be formed by unknown, pyridoxal independent, routes.[4] High concentrations of taurine have been reported in the supernatant fluid of Ehrlich Ascites tumors.[5] In an attempt to isolate a naturally occurring tumor inhibitor from Ehrlich Ascites tumor fluid, taurine was isolated.[6] Although taurine was void of inhibitory properties in mouse tumors and mammalian cell cultures, it was suggested that taurine may be a degradation product of the original inhibitor.[6]

In order to investigate a possible biological significance of the taurine moiety, some taurine derivatives were prepared. The present paper reports the synthesis of phthaloyltauryl peptides which may be considered as analogs of the corresponding carboxamido peptides. These are the first of a number of model compounds synthesized as potential antineoplastic agents. Preliminary results indicate some tumor inhibitory activity in Krebs 2 carcinoma for I and IV.[7,8]

The phthaloyltauryl peptides were prepared by treatment of amino acid esters or dipeptide esters with β-phthalimidoethanesulfonyl chloride (I) in the presence of triethylamine. The resulting products were then saponified with 1*N* sodium hydroxide at room temperature but attempted removal of the phthaloyl group by treatment with hydrazine and then hydrochloric acid under the conditions employed by Sheehan and Frank did not give tauryl peptides.[9] It was shown by the use of paper partition chromatography that the products of the reaction were taurine, phthalhydrazide and the constituent amino acids.

In order to substantiate the structures of the compounds prepared infrared spectra were obtained and neutralization equivalents determined when applicable. Absorptions typical of the phthaloyl group were found in all spectra and neutralization equivalents indicated that the acids were monocarboxylic.

I was first prepared by Miller *et al.*, by a rather circuitous route.[10] Phthalic anhydride was treated with ethanolamine, phosphorus pentachloride and then thiourea. Oxidation of the resulting inter-

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