Synthesis and Study of Pyridylalanine N-Oxides[1]

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Received May 9, 1968

The three pyridylalanine N-oxides, β-(2-, 3-, or 4-pyridyl 1-oxide)-DL-alanine, have been synthesized, and their biological activities in *Escherichia coli* 9723 and *Lactobacillus arabinosus* 17-5 have been determined. 4-Pyridylalanine N-oxide inhibits growth of *E. coli* at concentrations of 30 μg/ml and higher. The 2- and 3-pyridylalanine N-oxides are less effective inhibitors in this organism; the toxicities of all three N-oxides are reversed by supplements of phenylalanine or tyrosine. Evidence is given for the enzymatic reduction of the pyridylalanine N-oxides to the corresponding pyridylalanines in *E. coli* and for the probability that the toxicity of 4-pyridylalanine N-oxide in this organism is due to the formation of the more toxic 4-pyridylalanine. The pyridylalanine N-oxides are inhibitory to *L. arabinosus* only at very high concentrations; the organism does not reduce these compounds to the pyridylalanines.

Although the synthesis and biological study of structural analogs of the aromatic amino acids, phenylalanine and tyrosine, have been conducted in many laboratories for over two decades,[2] the study of new structural analogs remains interesting and often enlightening. The three pyridylalanines (the alanine side chain substituted in the 2, 3, and 4 positions of the pyridine ring) have been synthesized, and the 2- and 4-pyridylalanines[3-5] are well-documented antagonists of phenylalanine. Certain of these pyridylalanines have been employed in enzyme specificity studies and also serve as false feedback inhibitors.[6-8] Similarly, a tyrosine analog containing the pyridine ring, 5-hydroxy-2-pyridylalanine, has been a useful tool in the study of biological processes.[9,10] Further, 4,5-dihydroxy-2-pyridylalanine, a structural analog of 3,4-dihydroxyphenylalanine (DOPA), has been found to serve as a substrate for DOPA decarboxylase, while it inhibits the oxidation of DOPA by the enzyme, tyrosinase.[11] Certain substituted pyridine and quinoline N-oxides have been studied for fungistatic and bacteriostatic properties.[12] The N-oxides that were found to be active in the study were classified as "wide-spectrum" compounds.

The synthesis and the determination of the biological activity of the three pyridylalanine N-oxides, β-(2-, 3-, or 4-pyridyl 1-oxide)-DL-alanine, was interesting, therefore, in that these compounds could serve reasonably as either phenylalanine or tyrosine antagonists. The 4-pyridylalanine N-oxide, for example, might be expected to act as a tyrosine antagonist because of the presence of an oxygen atom *para* to the alanine substituent on the pyridine ring. The other two pyridylalanine N-oxides (alanine substitution in either the 2 or 3 position) would not be expected to be as effective tyrosine antagonists as the 4-substituted analog, because of the *ortho* and *meta* orientations of the oxygen atom; however, no selection of any of the three analogs as a most probable antagonist of phenylalanine can be made easily.

In the present study the three pyridylalanine N-oxides were synthesized,[13] and microbial growth inhibition studies were conducted. 4-Pyridylalanine N-oxide was found to be antagonistic to both phenylalanine and tyrosine in *Escherichia coli*. However, a strictly competitive reversal of the toxicity of 4-pyridylalanine N-oxide by either phenylalanine or tyrosine (or combinations of both) could not be demonstrated. The 2- and 3-pyridylalanine N-oxides are only weakly inhibitory to this organism, and the toxicities of the latter two compounds are reversed by low concentrations of either phenylalanine or tyrosine. Evidence is also given for the enzymatic reduction of the alanine-substituted pyridine N-oxides by *E. coli* to produce the corresponding pyridylalanines. Further evidence indicates that the rate of conversion of the pyridylalanine N-oxides to the corresponding pyridylalanines is an important factor in the inhibition of *E. coli*.

Experimental Section

General Procedures.—A Thomas-Hoover capillary melting point apparatus was employed for all melting point determinations, and the melting points reported are uncorrected. Paper chromatographic studies were conducted by the ascending technique using Whatman No. 1 chromatographic paper. Uv spectra were determined with a Beckman DBG recording spectrophotometer. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. The hydroxymethylpyridines and their N-oxides were obtained from Aldrich Chemical Co., Inc.

For the microbiological assays employing *Escherichia coli* 9723 as test organism a previously reported inorganic salts–glucose medium[14] was employed; the experimental details have been reported.[15] For the assays employing *Lactobacillus arabino-*

(1) This work was supported in part by grants from The Robert A. Welch Foundation of Texas (Grant No. B-133) and from the U. S. Public Health Service (Grant No. AM07599-05).

(2) W. Shive and C. G. Skinner in "Metabolic Inhibitors. A Comprehensive Treatise," Vol. I, R. M. Hochster and J. H. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, pp 2–73.

(3) E. M. Lansford, Jr., and W. Shive, *Arch. Biochem. Biophys.*, **38**, 347 (1952).

(4) D. F. Elliot, A. T. Fuller, and C. R. Harrington, *J. Chem. Soc.*, 85 (1948).

(5) C. Niemann, R. N. Lewis, and J. T. Hays, *J. Am. Chem. Soc.*, **64**, 1678 (1942).

(6) T. W. Conway, E. M. Lansford, Jr., and W. Shive, *J. Biol. Chem.*, **237**, 2850 (1962).

(7) T. W. Conway, E. M. Lansford, Jr., and W. Shive, *Arch. Biochem. Biophys.*, **107**, 120 (1964).

(8) H. S. Moyed, *J. Biol. Chem.*, **236**, 2261 (1961).

(9) S. J. Norton, C. G. Skinner, and W. Shive, *J. Org. Chem.*, **26**, 1495 (1961).

(10) J. M. Ravel and W. Shive, *Biochem. Biophys. Res. Commun.*, **20**, 352 (1965).

(11) S. J. Norton and E. Sanders, *J. Med. Chem.*, **10**, 961 (1967).

(12) F. Leonard, F. A. Barkley, E. V. Brown, F. E. Anderson, and H. M. Green *Antibiot. Chemotherapy*, **6**, 261 (1956).

(13) 4-Pyridylalanine N-oxide has been synthesized by a different procedure than that reported herein: R. L. Bixler and C. Niemann, *J. Org. Chem.*, **23**, 575 (1958). The compound has not previously been studied biologically.

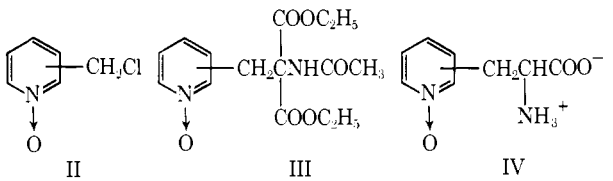
(14) E. H. Anderson, *Proc. Natl. Acad. Sci. U. S.*, **32**, 120 (1946).

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

sus 17-5 a previously described amino acid medium was used,[16] except phenylalanine and tyrosine were omitted from the basal medium. Phenylalanine and tyrosine were supplemented as required for limited growth of the organism. The amino acid analogs were dissolved in sterile H_2O and added to sterile assay tubes without being heated in all of the assays. The amount of growth was determined by optical density determinations (600 mμ) after 16–20 hr.

The chemical procedures for the organic synthesis for all three of the pyridylalanine N-oxides are the same. The experimental procedures below describe the synthesis of 4-pyridylalanine N-oxide (see Table I).

TABLE I



II III IV

a, 4 substitution; b, 3 substitution; c, 2 substitution

Compd	Yield, %	Mp, °C	Paper chromatography, R_f values: Solvent 1[a]	Solvent 2[b]	Formula[c]
IIa	50	136–138	...	...	$C_6H_6ClNO \cdot HCl$
IIb	70	91–93	...	...	$C_6H_6ClNO \cdot HCl$
IIc	70	109–111	...	...	$C_6H_6ClNO \cdot HCl$
IIIa	30	192–193	...	...	$C_{15}H_{20}N_2O_6$
IIIb	40	155–156	...	...	$C_{15}H_{20}N_2O_6$
IIIc	45	114–116	...	...	$C_{15}H_{20}N_2O_6$
IVa[d]	50	242–243 dec	0.16	0.11	$C_8H_{10}N_2O_3$
IVb	50	243–245 dec	0.18	0.10	$C_8H_{10}N_2O_3 \cdot HCl$
IVc	55	218–220 dec	0.24	0.14	$C_8H_{10}N_2O_3$

[a] The solvent was BuOH–AcOH–H_2O (4:1:1). [b] The solvent was EtOH–NH_4OH (19:1). [c] C, H, N analyses were made in every case. [d] Lit.[13] mp 238.2° dec.

4-Chloromethylpyridine 1-Oxide Hydrochloride (IIa).—4-Pyridylcarbinol 1-oxide (15.0 g, 0.12 mole), was added to $SOCl_2$ (~80 ml) with stirring over a period of about 10 min. After the initial exothermic reaction had subsided, the reaction mixture was heated for an additional 10 min. The clear solution was cooled in an ice bath and overlayed with an equal volume of ligroin. After vigorous scratching and stirring with a glass rod, the lower layer solidified to a light brown material. The solid was filtered off and washed several times (ligroin, Et_2O) to remove residual $SOCl_2$. After dissolving (EtOH) and decolorizing with Norit A, the product was crystallized (EtOH–EtOAc) (50:50), white needles, 8.6 g.

Ethyl 2-Acetamido-2-(4-pyridylmethyl 1-oxide)malonate (IIIa).—To a cool solution of 12.1 g (0.056 mole) of ethyl acetamidomalonate in 200 ml of dry EtOH containing 2.57 g (0.112 g-atom) of Na was added slowly with stirring 10 g (0.062 mole) of 4-chloromethylpyridine 1-oxide hydrochloride. After the addition was completed, the reaction mixture was heated under reflux for about 4 hr until the pH of an aliquot dissolved in H_2O had decreased to approximately pH 5. The precipitated NaCl was filtered off and the filtrate was taken to dryness *in vacuo*. The tan residue was dissolved (EtOH), decolorized with Norit A, and crystallized from EtOH–Et_2O. Recrystallization yielded 5.5 g of product.

β-(4-Pyridyl 1-oxide)-DL-alanine (IVa).—A solution of 4.6 g (0.014 mole) of IIIa in 50 ml of 6 *N* HCl was heated under reflux for 10 hr. The solution was concentrated *in vacuo* to approximately 10 ml, H_2O (50 ml) was added, and the solution was taken to dryness, *in vacuo*. The resulting solid was dissolved in a small volume of H_2O and neutralized (10% NaOH). The crude product was precipitated from the cold solution by the addition of 50:50 EtOH–Me_2CO. The amino acid was suspended in boiling EtOH, and H_2O was added dropwise until dissolution was complete. The hot solution was decolorized with Darco G-60 and the pure amino acid was crystallized from H_2O–EtOH–Me_2CO, yielding 1.0 g of white crystals, ninhydrin positive (red-brown color).

Results and Discussion

The synthesis of the pyridylalanine N-oxides was accomplished by employing the appropriate hydroxymethyl-substituted pyridine N-oxides as starting material. Conversion of the hydroxymethyl grouping to the chloromethyl grouping was accomplished by use of $SOCl_2$ without removal of the N-oxide function. The corresponding chloromethylpyridine N-oxides thus produced were then condensed with sodioacetamidomalonate diethyl ester. Acid hydrolysis of the resulting condensation products gave the desired pyridylalanine N-oxides. Certain physical data of the intermediates and final products are summarized in Table I.

All three of the synthesized pyridylalanine N-oxides were tested for biological activity in *E. coli* 9723. Both 3-pyridylalanine N-oxide (IVb) and 2-pyridylalanine N-oxide (IVc) were found to be only slightly inhibitory to this organism at concentrations up to 1000 μg/ml in the growth medium; the 2-substituted pyridine N-oxide was somewhat more inhibitory than the 3-substituted compound. The toxicity due to either the 2- or 3-pyridylalanine N-oxide could be reversed by supplements of either phenylalanine or tyrosine.

In contrast to 2- or 3-pyridylalanine N-oxide, 4-pyridylalanine N-oxide is a fairly good inhibitor in *E. coli* 9723 (see Table II); complete inhibition of growth usually resulted at concentrations of 30 μg/ml and higher in the growth medium. Although either phenylalanine or tyrosine reverse the inhibition of the analog, as shown in Table II, the reversal is not strictly competitive. It can be seen from this experiment that increasing concentrations of either phenylalanine or tyrosine tend to overcome the effect of the inhibitor in a noncompetitive fashion. The results of inhibition experiments in *E. coli* were quite varied, and a consistent pattern of reversal of toxicity by either phenylalanine or tyrosine (or combinations of both) could not be obtained.

TABLE II

REVERSAL OF 4-PYRIDYLALANINE N-OXIDE TOXICITY IN *E. coli* 9723 BY PHENYLALANINE AND TYROSINE[a]

4-Pyridylalanine N-oxide, μg/ml	Optical density at 600 mμ; Supplement; Phenylalanine, μg/ml[b]: None	2	6	20	60	Tyrosine, μg/ml: 6	20	60
0	0.72	0.72	0.72	0.74	0.74	0.72	0.71	0.71
3	0.22	0.18	0.54			0.29	0.72	
10	0.07	0.15	0.40	0.72		0.13	0.38	
30	0.03	0.13	0.31	0.60		0.08	0.16	0.73
100	0.02	0.06	0.24	0.53	0.70	0.03	0.04	0.58
300	0.00	0.05	0.04	0.31	0.37	0.01	0.01	0.46
1000				0.30	0.35		0.01	0.40

[a] Incubated 16 hr at 37°. [b] The basal inorganic salts–glucose medium used and the experimental details are described in ref 13 and 14.

Biological activities of the three analogs were also determined in *L. arabinosus* 17-5 employing an amino acid medium[16] in which phenylalanine and tyrosine had been omitted from the basal solution. The assay medium prepared therefrom was supplemented with 10 μg/ml each of DL-phenylalanine and DL-tyrosine to pro-

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

mote growth of the organism. All three of the pyridylalanine N-oxides were only very slightly inhibitory at concentrations up to 1000 μg/ml of the assay medium. 3-Pyridylalanine N-oxide was slightly more inhibitory in this organism than either 2- or 4-pyridylalanine N-oxide.

Because of the inconsistency of both phenylalanine and tyrosine to reverse competitively 4-pyridylalanine N-oxide toxicity in *E. coli* 9723, the question arose as to whether any enzymatic chemical modification of the pyridylalanine N-oxide was occurring. Such a process affecting the chemical nature of the inhibitor might explain the inconsistencies in the bioassays, since the rate of inhibitor modification might be affected by a variety of factors (*e.g.*, slight variations in incubation temperature, size and age of the inoculum, etc.).

Paper chromatographic analyses were conducted on the *E. coli* assay media (after bacterial growth) which had been supplemented with phenylalanine to prevent inhibition. In all cases it was found that the pyridylalanine N-oxides tested were almost completely converted to a ninhydrin-positive substance which exhibited a higher R_f value in acidic, basic, and neutral chromatographic solvents. Except with very high concentrations of either 2- or 3-pyridylalanine N-oxide, no phenylalanine supplementation was necessary because of the low toxicity of these compounds; however, conversion of these compounds to ones of unknown structure resulted in all cases.

It was reasonable to assume that enzymatic reduction of the N-oxide function of the pyridine ring might occur. If such were the case, the enzyme(s) responsible might be expected to act on any pyridine N-oxide regardless of the substitution on the pyridine ring and to function as a pyridine N-oxide reductase. As shown in Figure 1 (a paper chromatograph tracing), certain substituted pyridine N-oxides were tested in growing cultures of *E. coli* 9723 to determine whether they could be reduced to the corresponding substituted pyridines. It can be seen that 2-hydroxymethylpyridine N-oxide is apparently converted to the 2-hydroxymethylpyridine. Also tested, but not shown in the tracing, were the 3- and 4-hydroxymethylpyridine N-oxides. Both compounds were also apparently converted to the corresponding hydroxymethylpyridines by growing cultures of the organism as determined by paper chromatography.

In all solvent systems tested the R_f values for the three pyridylalanine N-oxides were found to be similar; the same is true for the enzymatic products obtained from these N-oxides. The R_f values for a given pyridylalanine N-oxide and its enzymatic product, however, are significantly different. The general correspondence between R_f values for the pyridylalanine N-oxides and the like correspondence of R_f values for the enzymatic products might be taken to indicate that a similar enzymatic conversion is occurring in each case.

To confirm that the pyridylalanine N-oxides were being converted by the bacterial enzyme activity to the corresponding pyridylalanines, several studies were initiated. 4-Pyridylalanine was synthesized according to the procedure of Bixler and Niemann[13] and compared (see Figure 1) with the enzymatic product of 4-pyridylalanine N-oxide. It became evident after comparing R_f values in several chromatographic solvents that enzymatic reduction of 4-pyridylalanine N-oxide was occurring, resulting in the formation of 4-pyridylalanine. Further substantiation of this conversion is that the compound enzymatically produced from 4-pyridylalanine N-oxide is converted photochemically by uv light into a yellow product (as yet unidentified) which has an absorption spectrum identical with that of uv-irradiated 4-pyridylalanine, λ_{max} 440 mμ. Uv irradiation of 4-pyridylalanine N-oxide has no observable effect on its absorption spectrum.

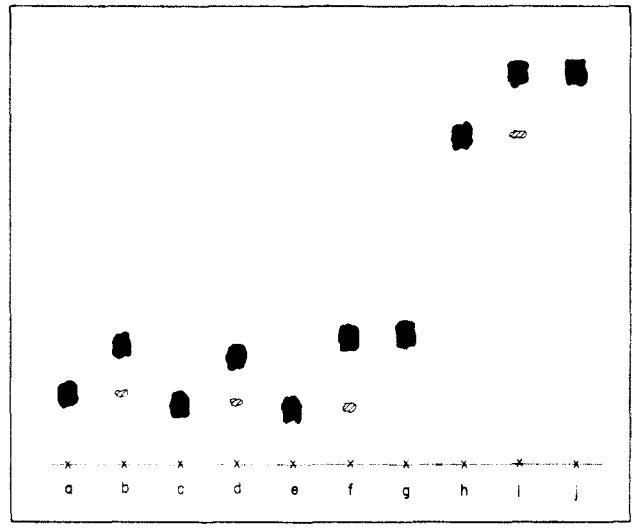


Figure 1.—Ascending paper chromatogram tracing indicating the reduction of various substituted pyridine N-oxides to the corresponding substituted pyridines by growing cells of *E. coli* 9723: a, 2-pyridylalanine N-oxide; b, 2-pyridylalanine N-oxide added to growth medium; c, 3-pyridylalanine N-oxide; d, 3-pyridylalanine N-oxide added to growth medium; e, 4-pyridylalanine N-oxide; f, 4-pyridylalanine N-oxide added to growth medium; g, 4-pyridylalanine; h, 2-hydroxymethylpyridine N-oxide; i, 2-hydroxymethylpyridine N-oxide added to growth medium; j, 2-hydroxymethylpyridine. Those compounds added to the growth medium were incubated (1 mg/ml) with growing cells of *E. coli* 9723. The inorganic salts–glucose medium, to which the pyridylalanine N-oxides were added, was supplemented with 100 μg of phenylalanine/ml to prevent growth inhibition. After heavy growth (~16 hr), cells were removed by centrifugation, and aliquots of the supernatants were spotted on paper. All spots were detected after chromatography in EtOH–NH_4OH (19:1) by ninhydrin and/or uv light.

Information concerning the nature of the enzymatic products from the pyridylalanine N-oxides was also obtained by chemical reduction studies. The 2- and 3-pyridylalanine N-oxides were reduced (Fe–AcOH) under conditions in which only the N-oxide function is affected.[17] The resulting 2- and 3-pyridylalanines were compared by paper chromatography with the corresponding enzymatic products and were found to have identical R_f values.

A color reaction,[18] specific for the pyridine ring, was employed to compare the visible absorption spectra of the chemically reduced 2- and 3-pyridylalanine N-oxides (and the synthetic 4-pyridylalanine) with the absorption spectra obtained from the enzymatic products of the 2-, 3-, and 4-pyridylalanine N-oxides. Table III summarizes the findings of this study. These data leave little doubt that the reduction of pyridylalanine

(17) H. J. den Hertog and W. P. Combé, *Rec. Trav. Chim.*, **70**, 581 (1951).
(18) E. Asmus and H. Garschagen, *Z. Anal. Chem.*, **139**, 81 (1953); *Chem. Abstr.*, **47**, 10410*i* (1953).

TABLE III

ABSORBANCE MAXIMA FOR SUBSTANCES TESTED EMPLOYING A PYRIDINE RING SPECIFIC COLOR REACTION[a]

Substance tested[b]	Absorbance max, mμ
Enzymatic product of 2-pyridylalanine N-oxide	485
Chemical reduction product of 2-pyridylalanine N-oxide	485
Enzymatic product of 3-pyridylalanine N-oxide	485
Chemical reduction product of 3-pyridylalanine N-oxide	485
Enzymatic product of 4-pyridylalanine N-oxide	604
4-Pyridylalanine	606
Enzymatic product of 4-hydroxymethylpyridine N-oxide	614
4-Hydroxymethylpyridine	614

[a] The color reaction for the pyridine ring is a reported procedure[18] employing HCl, chloramine, CN^-, and barbituric acid as reagents. The wavelengths of maximum absorbance were determined by scanning on a recording spectrophotometer. Pyridine N-oxides do not give the color reaction. [b] All the enzymatic products tested were from the growth media of *E. coli* 9723 which had been supplemented with a substituted pyridine N-oxide. Chemical reductions of the 2- and 3-pyridylalanine N-oxides were conducted with Fe–AcOH.[17] The 4-pyridylalanine was synthesized by the procedure of Bixler and Niemann.[13]

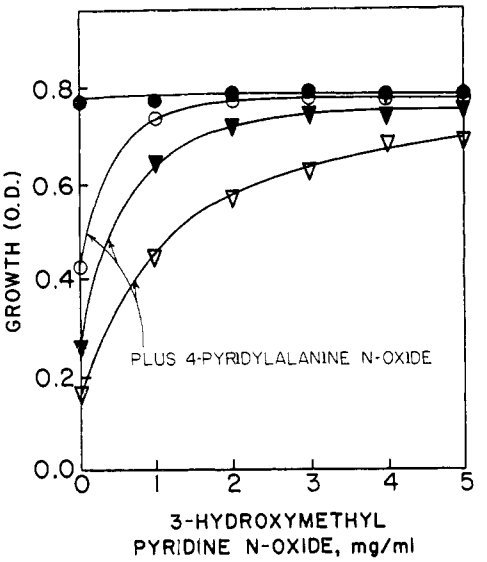


Figure 2.—The effect of 3-hydroxymethylpyridine N-oxide on the inhibition of *E. coli* 9723 by 4-pyridylalanine N-oxide. 4-Pyridylalanine N-oxide concentrations: ●—●, none; ○—○, 10 μg/ml; ▼—▼, 30 μg/ml; ▽—▽, 100 μg/ml. Duplicate tubes at each concentration level of 3-hydroxymethylpyridine N-oxide were run, and the optical density readings above are averages of the individual readings at each level. See the Experimental Section and ref 13 and 14 for the basal inorganic salts–glucose medium and the experimental details.

N-oxides (and other pyridine N-oxides) to the corresponding pyridylalanines (or other substituted pyridines) does occur in *E. coli* 9723.

That the reduction of the N-oxide is enzyme catalyzed is shown by the fact that when sufficiently high concentrations of the analogs are employed to effect complete inhibition of bacterial growth, there is very little or no chromatographic evidence of pyridine N-oxide reduction.

Another question of interest was whether the toxicity of the 4-pyridylalanine N-oxide in *E. coli* is due to the unaltered compound or to its reduction product. This question might be resolved in the studies with *E. coli* if the conversion of 4-pyridylalanine N-oxide to 4-pyridylalanine could be prevented. In other experiments it was found that the various hydroxymethylpyridine N-oxides (employed as starting compounds in the synthesis of the pyridylalanine N-oxides) were nontoxic to *E. coli* even at very high concentrations. As has been shown earlier (see Figure 1) the hydroxymethyl pyridine N-oxides are reduced in growing cultures of *E. coli* to the corresponding hydroxymethylpyridines. It was decided to test whether the hydroxymethylpyridine N-oxides at high concentrations could exert a sparing effect on the reduction of 4-pyridylalanine N-oxide. In other words, by greatly increasing the total concentration of pyridine N-oxides available for enzymatic reduction, the rate of reduction of the 4-pyridylalanine N-oxide might be diminished significantly. Further, if it is 4-pyridylalanine and not its N-oxide that is the more potent inhibitor, a diminished rate of reduction of 4-pyridylalanine N-oxide should be reflected in a decreased growth inhibition in the microorganism.

This hypothesis was tested employing 3-hydroxymethylpyridine N-oxide to retard the reduction of 4-pyridylalanine N-oxide; the results are summarized in Figure 2. Increasing concentrations of 3-hydroxymethylpyridine N-oxide have virtually no effect on the growth of *E. coli* 9723 in the absence of inhibitor; however, the toxicity of the inhibitor is greatly decreased as the concentration of the hydroxymethylpyridine N-oxide increases. In another experiment the effects of increasing concentrations of 3-hydroxymethylpyridine N-oxide on the toxicity of 4-pyridylalanine to *E. coli* were tested. It was found that supplements of the nontoxic N-oxide were completely without effect on the toxicity of 4-pyridylalanine. Inhibition studies have shown that both phenylalanine and tyrosine prevent the toxic effect of 4-pyridylalanine in *E. coli* 9723.

Our interpretation of these findings is that the toxicity of 4-pyridylalanine N-oxide to *E. coli* 9723 is largely due to the conversion of this compound to the toxic 4-pyridylalanine. It is to be remembered that none of the three pyridylalanine N-oxides that were studied are toxic to *L. arabinosus* at concentrations below 1000 μg/ml. Paper chromatographic studies of the growth medium employed for *L. arabinosus* have indicated that this organism does not catalyze any detectable reduction of substituted pyridine N-oxides during growth to the corresponding substituted pyridines. The failure of this reduction process to occur may explain the poor inhibition properties of the pyridylalanine N-oxides in this organism. We have found that the reduction product, 4-pyridylalanine, is fairly toxic to *L. arabinosus* (over 90% inhibition at 200 μg/ml).

It is tempting to speculate on the possible use of the pyridylalanine N-oxides for the selective inhibition of those microorganisms which have a pyridine N-oxide reductase. It may well be that 4-pyridylalanine N-oxide could be employed as a reagent to determine the presence of such an enzyme activity in a given microorganism (based on the ability of the compound to inhibit growth of the organism). We have recently found, however, that a simple colorimetric procedure[18] is more

suitable for determining pyridine N-oxide reductase activity in whole cell suspensions of a given microorganism. 4-Hydroxymethylpyridine N-oxide has been employed as substrate; the enzymatically produced 4-hydroxymethylpyridine may then be determined spectrophotometrically. We are presently engaged in the isolation and study of the pyridine N-oxide reductase activity from *E. coli* 9723.

Carcinogenicity of Lactones. III.[1] The Reactions of Unsaturated γ-Lactones with L-Cysteine

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Received April 8, 1968

The reactions of a series of unsaturated γ-lactones and related compounds, ranging in carcinogenicity from inactive to potent, with cysteine have been studied, and it has been shown that such reactions will usually not provide meaningful indications of carcinogenicity or of other biological activities. In fact such a criterion can often prove misleading. However, a chemical basis has emerged which may have predictive value: carcinogenic lactones (for example, 4-hydroxypent-2-enoic acid lactone and 4-hydroxy-2,4-hexadienoic acid lactone) undergo Michael addition of the nucleophilic thiol group to the conjugated unsaturation(s) giving rise to S-alkylated adducts. No normal *in vivo* processes are known which can reverse such alkylations and any cellular modification of this type would probably be permanent. The inactive lactones (in which the double bonds are not conjugated with the carbonyl group) are also subject to rapid attack by the cysteine thiol group; however, addition occurs at the lactone carbonyl giving rise to S-acylated intermediates which then rearrange rapidly in neutral solution to yield N-acylated cysteines. In contrast to the effectively irreversible *in vivo* alkylation reactions cellular damage resulting from such acylation processes can readily be repaired by various proteolytic enzymes.

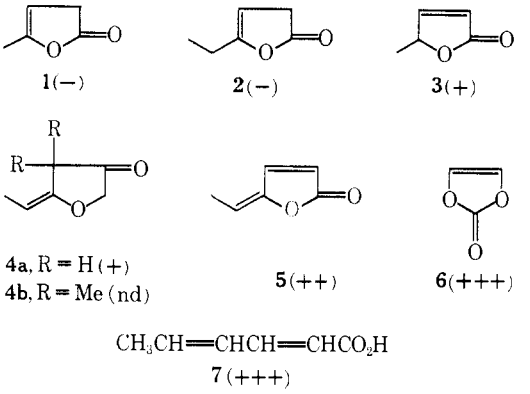
It is well known that compounds containing the lactone function exhibit a broad range of physiological properties[3] including carcinogenic[4] and antitumor[5] activities. Certain properties, for example, antibacterial activity, of many lactones (and of related compounds) are inhibited by the addition of cysteine and other sulfhydryl-containing compounds, and several attempts have been made to correlate physiological potency of lactones with their cysteine reactivity.[4,6,7]

One of the most recent studies of this kind was made by Dickens and Cooke[7] who measured the rates of thiol disappearance and hydrolysis for a number of cysteine–carcinogenic lactone reactions. However, the very approximate correlation between the rate of thiol disappearance and carcinogenicity which emerged was rather unsatisfactory. In retrospect, this is not surprising since cysteine is a trifunctional compound and attack by the thiol group need not be the initial nor the rate-determining step. Accordingly, a systematic examination of the products formed, and of the reaction pathways involved, was begun in the hope that some of the anomalies[7] might be clarified.[8]

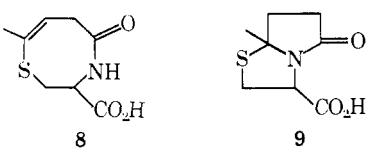
Of the compounds examined by Dickens and Cooke those selected for detailed study are shown in Chart I with their relative carcinogenicities.[4,7,9]

Chart I

Relative Carcinogenicity (in parentheses) of Selected γ-Lactones and Related Compounds (from the Data of Dickens, *et al.*[4,7,9])



The reaction of 4-hydroxypent-3-enoic acid lactone (**1**) with cysteine was first investigated by Cavallito and Haskell.[6] However, since the assignment of structure **8**



to the product isolated was made prior to the advent of modern spectroscopic analytical methods it was considered desirable to reexamine the reaction. A product identical with that described by Cavallito and Haskell

(1) Part II: J. B. Jones, C. H. Koo, I. P. Mellor, S. C. Nyburg, and J. M. Young, *Can. J. Chem.*, **46**, 813 (1968).

(2) Research Fellow of the National Cancer Institute of Canada, 1966–1968.

(3) Leading references to the extensive literature on this subject are given by (a) L. J. Haynes, *Quart. Rev.* (London), **2**, 46 (1948); (b) H. W. Buston and S. K. Roy, *Arch. Biochem.*, **22**, 1 (1949); (c) D. G. Wenzel and C. M. Smith, *J. Am. Pharm. Assoc.*, **47**, 792 (1958); (d) R. Rondanelli, *Arch. Intern. Pharmacodyn.*, **135**, 289 (1962).

(4) F. Dickens and H. E. H. Jones, *Brit. J. Cancer*, **15**, 85 (1961); **17**, 100, 691 (1963); **19**, 392 (1965).

(5) S. M. Kupchan, R. J. Hemingway, and J. C. Hemingway, *Tetrahedron Letters*, 149 (1968), and earlier papers; S. M. Kupchan, R. J. Hemingway, and R. W. Doskotch, *J. Med. Chem.*, **7**, 803 (1964), and later papers.

(6) C. J. Cavallito and T. H. Haskell, *J. Am. Chem. Soc.*, **67**, 1991 (1945), and references therein.

(7) F. Dickens and J. Cooke, *Brit. J. Cancer*, **19**, 404 (1965).

(8) In the final paragraph of their paper, Dickens and Cooke[7] also conclude that much chemical work is required in this area since the chemical literature contains surprisingly few relevant data.

(9) F. Dickens, H. E. H. Jones, and H. B. Waynforth, *Brit. J. Cancer*, **20**, 134 (1966).