solvents, was recrystd from C_6H_6 and melted at 273–274°. Anal. $(C_{20}H_{10}N_2)$ C, H, N.

Acknowledgments.—We are indebted to Professor M. S. Newman, Ohio State University, for generous donation of samples of several rare hydrocarbons, including 1-, 3-, and 11-methylbenz[a]anthracene, and for detailed description of the synthesis of 1-bromo-3-methylnaphthalene. We are also grateful to Professor Charles B. Huggins, The University of Chicago, for permission to cite the biological test data obtained in his laboratory.

Specificity in Enzyme Inhibition. 1. Synthesis of 4-(4-Imidazolyl)-3-amino-2-butanone, 4-(4-Imidazolyl)-3-acetamido-2-butanone, and 4-(4-Imidazolylmethyl)-2,5-dimethyloxazole for Assay as Inhibitors of Histidine Decarboxylase

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Received December 10, 1970

A general approach to specific enzyme inhibition is discussed. The synthesis and results of assay of 4-(4-imidazolyl)-3-amino-2-butanone (2), 4-(4-imidazolyl)-3-acetamido-2-butanone (4), and 4-(4-imidazolylmethyl)-2,5-dimethyloxazole are described.

The receptor sites of many metabolic enzymes which utilize amino acids as substrates can be depicted as having 2 binding sites and 1 active site. The binding sites can be designated as specific and nonspecific. For example, if one views a model for the receptor site of a specific amino acid decarboxylase (Figure 1), the specific binding would be to the R group and would act to differentiate the amino acid to be used as a substrate. A nonspecific site would bind the amino group; the latter would act as the orienting function and would place the carboxyl group in juxtaposition to the site of chemical change, the active site.

On the basis of this model, an active-site-directed inhibitor of a decarboxylase enzyme should be capable of binding with the specific as well as the nonspecific sites of the enzyme but it should be incapable of undergoing the required chemical transformation, decarboxylation, at the active site. The same argument should apply to transaminases, aminopeptidases, aminesynthetases, certain oxidases, etc.

In order to investigate the applicability of this hypothesis, histidine was chosen as the substrate to be modified. L-Histidine decarboxylase is specific for the biosynthesis of histamine,² and a specific inhibitor of this enzyme should possess an imidazole ring which would approach and bind to the specific binding site, a basic N for binding to the nonspecific site, and a function incapable of decarboxylation to approach the active site. This phase of the investigation considered only reversible endo binding to the receptor.³ In the initial study of the requirements of histidine analogs for decarboxylase inhibitor activity 3 compds were prepared. The α -amino ketone **2** would be expected to have the specific and nonspecific binding



functions of histidine (1). The oxazole **3** would possess the basic nonspecific function and the specific imidazole ring but sterically might be less capable of endo binding to the receptor site. The *N*-acetyl- α -amino ketone **4** would have the specific binding function and the active-site-directed function which is incapable of decarboxylation, but it does not have the nonspecific binding group required for orientation of the Ac group to the active site.

It could be predicted that 2 would be an excellent and specific inhibitor, 3 would be specific but less active, and 4 would possess little or no activity as an inhibitor.

These compds were prepared from histidine \cdot HCl (1) which underwent decarboxylative acetylation in pyridine and Ac₂O soln to provide 4-(4-imidazolyl)-3-acetamido-2-butanone \cdot HCl (4) in 64% yield. Dakin and West⁴ were unable to characterize this material, since they failed to obtain a crystalline product. In this study a crystalline product was obtained and spectral data support the proposed structure. The maximum yield was secured with mild reaction conditions.

The hydrolysis of the acetamido ketone 4 to produce 4-(4-imidazolyl)-3-amino-2-butanone $\cdot 2HCl$ (2) was accomplished in 82% yield with 4 N HCl.

4-(4-Imidazolylmethyl)-2,5-dimethyloxazole HCl (3) was obtained in 40% yield by refluxing the acet-

⁽¹⁾ Taken in part from the dissertation presented by J. A. Weis, Sept 1968, to the Graduate School of the University of Kansas, Lawrence, Kan., in partial fulfillment of the requirements for the Doctor of Philosophy Degree.

⁽²⁾ G. Kahlson, E. Rosengren, and R. Thunberg, J. Physiol. (London), 169, 467 (1963).

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Figure 1.

amido ketone 4 in Ac_2O . This material had been obtained previously only as the $AuCl_2$ complex.⁵

Compds 2, 3, and 4 were assayed as inhibitors against histidine decarboxylase and dopa decarboxylase. The results are shown in Table I.

	TABLE I	
	Conen, m M	% inhibition
	Histidine Decarboxylase ^a	
2	1.0	87
3	1.0	56
4	1.0	10
	Dopa Decarboxylase	
2	10.0	30
2	7.5	33
2	2.5	10
3	2.0	6
3	0.2	7
4	2.0	18

^a After the submission of this work in thesis form (ref 1) a report by S. R. Mardashev, N. A. Gonchar, and N. S. Dabagov, *Dokl. Acad. Nauk SSR*, **189** (4), 895 (1969), appeared.

As is evident from the results, 2 exhibits unique specificity as an inhibitor, being an excellent inhibitor of histidine decarboxylase and a poor inhibitor of dopa decarboxylase. Furthermore, the rationale for the preparation of 2, 3, and 4 is substantiated.

This approach has been utilized with other amino acids, and the results will be the subject of future reports.

Experimental Section⁶

4-(4-Imidazolyl)-3-acetamido-2-butanone \cdot HCl (4).—Ac₂O (141 g, 1.43 moles) and pyridine (94.5 g, 1.19 moles) were added to L-histidine \cdot HCl \cdot H₂O (1) (50 g, 0.24 mole). While stirring, the mixt was heated until gas evoln commenced (70°). External heating was stopped as the temp rose spontaneously to 100°. After 5 min, gentle heating was applied to maintain the reaction temp at 90° for 15 min. The mixt was allowed to cool over a 1-hr period. Excess volatile reactants were removed by distn *in vacuo*. Trace amts of volatile reactants were removed by steam distn *in vacuo*. The resulting aq soln was decolorized with activated charcoal, and H₂O was cryst from hot *i*-PrOH. The crystd mass was broken up, and addl *i*-PrOH was added to make a filterable slurry. The crystals were collected and re-

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crystd (*i*-PrOH) affording 35.4 g (64%): mp 165-168°; ir (Nujol) 1540 (amide II band), 1635 (amide C=O), and 1729 cm⁻¹ (C=O); nmr (D₂O) 8.82 (d, 1, J = 1.5 Hz, N=CHN), 7.46 (s, 1, NCH=C), 4.83 (m, 1, CHC=O), 3.33 (m, 2, CH₂), 2.38 (s, 3, COCH₃), and 2.10 ppm (s, 3, acetamide CH₃). Anal. (C₃H₁₂ClN₃O₂) C, H, N.

4-(4-Imidazolyl)-3-amino-2-butanone 2HCl (2).—Compd 4 (12 g, 52 mmoles) was dissolved in 120 ml of 4 N HCl. The soln was stirred while refluxing for 3 hr. H₂O was removed by distn *in vacuo*. Trace amts of H₂O were removed by codistn with *i*-PrOH *in vacuo*. The cryst residue was collected and washed with *i*-PrOH. Recrystn (MeOH-*i*-PrOH) provided yellow crystals weighing 9.69 g (82%): mp 212-215° (lit.4 205-206°); ir (Nujol) 1518 (+NH), 1585 (+NH), and 1725 cm⁻¹ (C=O); nmr (D₂O) 8.86 (d, 1, J = 1.5 Hz, N=CHN), 7.60 (s, 1, NCH=C), 4.72 (q, 1, CHCO), 3.37-3.73 (m, 2, CH₂), and 2.45 ppm (s, 3, COCH₃).

4-(4-Imidazolylmethyl)-2,5-dimethyloxazole HCl (3).— Compd 4 (12.3 g, 53 mmoles) was refluxed with stirring in Ac₂O (100 ml) for 50 min. When the mixt cooled, crystals formed. The product was collected and washed with Et₂O. The solid was dried *in vacuo* (60°) overnight. The dry material was dissolved in 95% EtOH, and the soln was decolorized with activated charcoal. The EtOH was removed by distn *in vacuo*, and the resulting thick gum spontaneously crystd. The solid mass was triturated with Me₂CO and collected and washed with Me₂CO. Recrystn from an abs EtOH-Me₂CO mixt furnished 4.5 g (40%) of product, mp 161-163°. An anal. sample of mp 163-165° was obtained by recrystn from CHCl₃-cyclohexane; ir (CHCl₃) 1585 (+NH), 1616 (+NH), and 1655 cm⁻¹ (oxazole C==N); nmr (CDCl₃) 14.41 (s, 2, +NH), 90.5 (d, 1, J = 1.5 Hz, N==CHN), 7.21 (s, 1, NCH==C), 3.97 (s, 2, CH₂), 2.36 (s, 3, N==CCH₃), and 2.28 ppm (s, 3, C==CCH₃). Anal. (C₉H₁₂-CIO) C, H, N.

Purification of Enzymes.—Histidine decarboxylase (EC 4.1.1.22) was prepd by a modification of methods described by Hakanson⁷ and Levine and Watts.⁸ Whole rat fetuses (19–20 days gestation) obtd from Sprague–Dawley rats (Carworth Farms) were homogenized in 2 vol of 0.1 M NaOAc, pH 5.5. After centrifugation for 45 min at 90,000g, the supernatant was fractionated using (NH₄)₂SO₄. The protein pptng between 25 and 45% sath was dissolved in 0.1 M K₃PO₄, pH 7.0, and dialyzed overnight at 5° against H₂O. The dialyzed fraction was dild with 0.05 K₃PO₄, pH 7.0, to a concen of 40 mg of protein per ml, and could be stored at -15° for several months with no loss of activity.

Aromatic L-amino acid decarboxylase (EC 4.1.1.26) was prepd from guinea pig kidneys by the method of Clark, *et al.*⁹ The kidneys were homogenized in 4 vol of H₂O and were centrifuged at 20,000g for 30 min. The protein fraction pptd from the supernatant by (NH₄)₂SO₄ between 37 and 55% satn was dissolved in 0.05 *M* K₃PO₄, pH 7.0, and dialyzed overnight against H₂O at 5°. The vol after dialysis was adjusted to a concn of 65 mg of protein per ml and the enzyme was stored at -15° .

Assay of Histidine Decarboxylase.—Histidine decarboxylase activity was determined by measuring the $CO_{2^{-14}C}$ produced from L-histidine-carboxyl-¹⁴C as previously described.¹⁰ The standard reaction mixt contd 100 µmoles of K₃PO₄ buffer (pH 6.8), 0.25 µmole of L-histidine contg 0.25 µCi of L-histidinecarboxyl-¹⁴C, 0.01 µmole of pyridoxal 5-phosphate, and 2 mg 0.05 mole) of the histidine decarboxylase prepn. Inhibitors were added at various conces in 0.1 ml of H₂O. The final vol was made to 1.0 ml with H₂O. The mixt was incubated for 90 min at 37°. Controls were included to correct for nonenzymatic decarboxylation.

Assay of Aromatic L-Amino Acid Decarboxylase.—Aromatic L-amino acid decarboxylase activity was determined using DL-dopa-carboxyl-¹⁴C as the substrate and measuring the $CO_2^{-14}C$ in the same manner as with histidine decarboxylase.¹⁰ The std reaction mixt contd 50 µmoles of K₃PO₄ buffer (pH 6.8), 0.5 µmole of DL-dopa contg 0.11 µCi of DL-dopa-carboxyl-¹⁴C, 0.033 µmole of pyridoxal 5-phosphate, and 0.13 mg of the decarboxylase prep. Inhibitors were added in 0.1 ml of H₂O, and the final vol

⁽⁶⁾ Melting points were obtd on a calibrated Thomas-Hoover Uni-Melt and are cor. Ir data were recorded on IR-8 and IR-10 spectrophotometers and nmr data on Varian Associates Model A-60 and A-60-A spectrometers, using MedSi or Na 3-(trimethylsilyl)-1-propanesulfonate as internal standards and are reported as δ (ppm). Microanalyses were performed on an F and M CHN analyzer Model 185 in this department and by Midwest Microlab, Inc., Indianapolis, Ill. Where anal, are indicated only by symbols of the elements, anal, results obtained for those elements were within $\pm 0.4\%$ of the theor values.

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was made to 0.5 ml with H_2O . The mixt was incubated for 5 min at 37°. Controls were included to correct for nonenzymatic decarboxylation.

Acknowledgment.—The authors gratefully acknowledge the support of this project by the National Institutes of Health Grants GM-09254 and GM-01341. We wish to thank Dr. R. J. Taylor, Jr., and F. J. Leinweber, McNeil Laboratories, Fort Washington, Pa., for the histidine decarboxylase and dopa decarboxylase assays.

5-Homopyridoxals, 5-Thiopyridoxal, and Related Compounds. Synthesis, Tautomerism, and Biological Properties¹

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Received January 19, 1971

Homopyridoxals with 1 or 2 additional CH₂ groups in the 5 position have been obtained by controlled oxidn of the corresponding homopyridoxols with MnO₂. More vigorous oxidn yielded the corresponding 4-homopyridoxic acids. 4-Deoxyhomopyridoxols have also been prepd from homopyridoxols by hydrogenolysis with hydrazine. Reaction of hydrazine- d_4 with pyridoxol gave 4-deoxypyridoxol in which the α^2 and α^4 Me groups and the 6 position were deuterated. These deuterations as well as the formation of 4-deoxypyridoxol have been rationalized assuming the formation of quinone methide intermediates. 5-Thiopyridoxal was prepd and was found to be a hemiacetal in the narrow pH range in which it was stable. Likewise, the two homopyridoxals exist in a cyclic (hemiacetal) form in acid and neutral soln, whereas in an alkaline medium a marked tendency to revert to the aldehyde form, particularly in the 2 C homolog, has been observed. Derivatives of both the aldehyde and hemiacetal forms of these pyridoxal analogs have been obtained. Pyridoxal was found to undergo a Cannizzaro reaction when treated with alkali. The oximes of homopyridoxals and the 4-deoxyhomopyridoxals are inhibitors of pyridoxal phosphokinase. The effect of some of these compds on Saccharomyces carlsbergensis, tissue culture cells, and certain enzymes *in vivo* has also been determined.

In efforts to develop more selective antagonists of vitamin B₆ that might be active as anticancer agents² we previously synthesized a series of homologs of pyridoxol (I, R = CH₂OH) by extension and branching of the 4- and 5-hydroxymethyl side chains.^{3,4} Compds obtained by extension of the 5 position (I, R = CH₂-OH; n = 2-4) were found to be inhibitors of Saccharomyces carlsbergensis⁴ but were ineffective in inhibiting mammalian systems.⁵



It was hoped that by modifying the 4-hydroxymethyl to a formyl (I, R = CHO; n = 2,3) or Me (I, R = CH₃; n = 2,3), improved inhibitors could be obtained, since they would more closely resemble the biologically more active form of vitamin B₆ or the well-known antimetabolite 4-deoxypyridoxol (I, R = CH₃; n = 1), resp. (It has also been found that when 5-deoxypyridoxol was converted to the corresponding 4-aldehyde, toxicity was increased markedly.⁶)

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In addn to compds of the types already mentioned, we have synthesized 5-thiopyridoxal (XVIII) and two 5-homopyridoxic acids (VIII and XIII). Chem properties of these compds, particularly ring-chain tautomerism, have been studied, and have been compared with those of pyridoxal. Some of these compds have been evaluated for their biol and enzymatic activity in several systems.

Chemistry. Synthesis.—Scheme I depicts the synthesis of the homopyridoxals (III and X), homopyridoxic acid (VIII and XIII), their derivs, and 4-deoxyhomopyridoxols (VI and XII); and Scheme II that of 5-thiopyridoxal (XVIII) and its ethyl acetal deriv.

Oxidn of the 4-CH₂OH group to the CHO and COOH groups has been carried out with MnO_2 as shown in Scheme I. Conditions for this oxidn had to be varied for each compd. Probably because of the ring-chain tautomerism of these compds (see below), the length of the side chain had a profound effect on the oxidizability of the 4-CH₂OH group. Thus conditions⁷ that had been worked out earlier for the oxidn of pyridoxol to pyridoxal and 4-pyridoxic acid could not be applied.

In the synthesis of 5-thiopyridoxal (XVIII, Scheme II), it was necessary to block the SH group of 5-thiopyridoxol by benzoylation, as in XVI. This was accomplished in a more direct manner and in better yield (from the blocked chloro derivative XIV) than has been reported previously.⁸ The most crucial step in this synthesis was the deblocking step to yield 5-thiopyridoxal from XVII. Both acid and alkaline hydrolysis of the thiobenzoate XVII gave a mixt of products, but a base-catalyzed transesterification with

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