Selective Inhibition of Xanthine Oxidase by 3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines

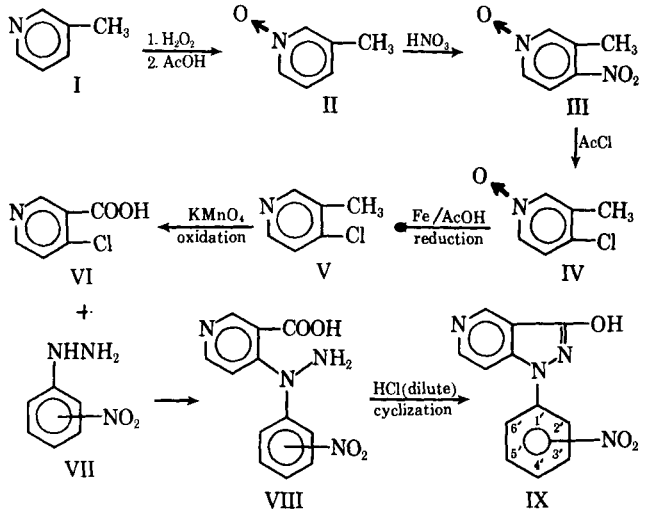
SURENDRA S. PARMAR▲, C. DWIVEDI, and BASHEER ALI

Abstract □ Some 3-hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]-pyridines were synthesized and characterized. The ability of the compounds to inhibit purine-catabolizing enzymes was evaluated. These pyrazolopyridines were found to exhibit selective inhibitory effects on xanthine oxidase, while adenosine deaminase, guanosine deaminase, and guanine deaminase were unaffected. 3-Hydroxy-1-(2′,4′-dinitrophenyl)-1*H*-pyrazolo[4,3-*c*]pyridine was the most potent inhibitor of xanthine oxidase.

Keyphrases □ 3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines—synthesis, inhibition of xanthine oxidase □ Xanthine oxidase—inhibition by 3-hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines

Administration of allopurinol (4-hydroxypyrazolo-[3,4-*d*]pyrimidine) concurrently with 6-mercaptopurine in therapy against adenocarcinoma 755 was shown to result in severalfold potentiation of 6-mercaptopurine, 6-methylthiopurine, 6-propylthiopurine, and 6-chloropurine (1). The selective action of 6-mercaptopurine on susceptible tumor cells was correlated with the absence or lowering of xanthine oxidase in these tumor cells (2). The usefulness of allopurinol in controlling hyperuricemia in patients with myeloproliferative diseases (3, 4) prompted synthesis of substituted allopurinol derivatives. Inhibitory effects of these compounds on bovine milk xanthine oxidase indicated the attachment of their phenyl moiety to a hydrophobic region present near the active site of the enzyme (5, 6). Kielley (7) showed that liver xanthine oxidase, a molybdoflavoprotein, possibly differs from milk xanthine oxidase due to low flavin adenine dinucleotide and iron content (8).

These observations led to synthesization of some purine analogs, 3-hydroxy-1-nitrophenyl-1*H*-pyrazolo-[4,3-*c*]pyridines, devoid of N-7 of the purine ring and possessing a hydrophobic phenyl ring at the 1-position. Attempts were made to evaluate their ability to inhibit liver xanthine oxidase and other purine-catabolizing deaminases, possibly through the participation of the active site of the enzyme, and also to ascertain if the requirement of a nitrogen atom at the 7-position of these purine analogs was essential for enzyme inhibition. These pyrazolo[4,3-*c*]pyridines were synthesized according to Scheme I.



Scheme I

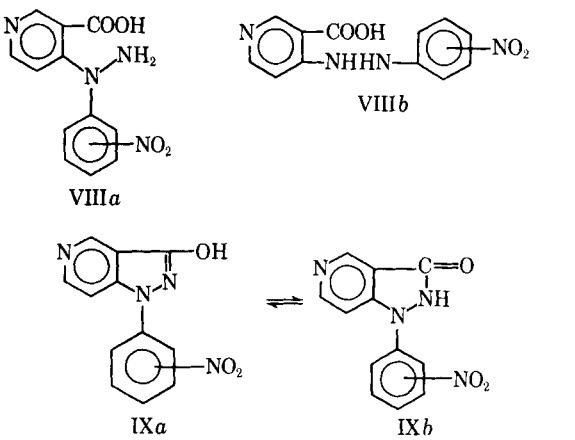
3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines (IX) were synthesized by cyclization of 4-(α-nitrophenylhydrazino)nicotinic acids (VIII). The reaction of 4-chloronicotinic acid (VI) with nitrophenylhydrazines (VII) was used for the preparation of 4-(α-nitrophenylhydrazino)nicotinic acids where diarylamino acids (VIII*a*) or hydrazo acids (VIII*b*) could be formed. The presence of two characteristic bands of a primary amino group in the IR spectrum and a positive Liebermann reaction (hydrozobenzene did not give this reaction) provided support in favor of formation of VIII*a* during the reaction. These 4-(α-nitrophenylhydrazino)nicotinic acids (VIII*a*) were cyclized with dilute HCl to yield the required pyrazolopyridines (IX).

All pyrazolopyridines thus synthesized exhibited lactam–lactim tautomerism (IX*a* ⇌ IX*b*). The presence of a very strong carbonyl peak at 1630 cm.$^{-1}$ in the IR spectrum of these compounds suggested that these compounds exist predominantly in the lactam form (IX*b*).

EXPERIMENTAL

Analyses for carbon, hydrogen, and nitrogen were performed. Melting points were taken in open capillary tubes and were uncorrected. IR absorption spectra[1] and UV maxima[2] were obtained.

4-Nitro-3-picoline-1-oxide (III)—The method that Den Hertog and Combe (9) used for the preparation of 4-nitropyridine-1-oxide was followed for the synthesis of 4-nitro-3-picoline-1-oxide. To a



[1] Using a Perkin-Elmer spectrophotometer, model 137.
[2] Using a Hitachi-Perkin-Elmer UV-VIS spectrophotometer.

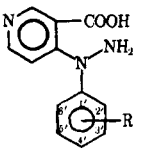


Table I—4-(α-Nitrophenylhydrazino)nicotinic Acid

R	Melting Point	Yield, %	Formula	Analysis % Calc.	Found
2′-NO_2	152° dec.	58	$C_{12}H_{10}N_4O_4$	C 52.55	52.19
				H 3.64	3.70
				N 20.43	20.14
4′-NO_2	270° dec.	64	$C_{12}H_{10}N_4O_4$	C 52.55	52.82
				H 3.66	3.85
				N 20.43	20.26
2′,4′-Di-NO_2	130° dec.	58	$C_{12}H_9N_5O_6$	C 45.14	45.60
				H 2.82	3.21
				N 21.94	21.52

mixture of hydrogen peroxide (30%, 75 ml.) and glacial acetic acid (75 ml.) was added 3-picoline (I) (19 g., 0.2 mole), and the mixture was heated at 50° for 48 hr. The reaction mixture was concentrated under reduced pressure. To the residue was added concentrated sulfuric acid (40 ml.) with cooling, and the resulting solution was added carefully to a mixture of fuming nitric acid (60 ml.) and concentrated sulfuric acid (40 ml.) in an ice bath. After complete addition, the reaction mixture was maintained at 90° for 2 hr. and then at 100° for an additional hour. The mixture was poured over crushed ice. Addition of 4 *N* sodium hydroxide with vigorous stirring until the solution was slightly acidic precipitated almost pure 4-nitro-3-picoline-1-oxide (III). The crude product on recrystallization from acetone gave 20.5 g. (87%) of 4-nitro-3-picoline-1-oxide, m.p. 136–137°.

4-Chloro-3-picoline-1-oxide (IV)—4-Chloro-3-picoline-1-oxide (IV) was prepared using the method of Ochiai (10) by the treatment of acetyl chloride on 4-nitro-3-picoline-1-oxide (III), m.p. 120–122°.

4-Chloro-3-picoline (V)—4-Chloro-3-picoline-1-oxide (IV) was reduced by the method that Essary and Schofield (11) used for the preparation of 4-amino-3,5-dimethylpyridine from its *N*-oxide. To the stirred solution of 4-chloro-3-picoline-1-oxide (IV) (5 g., 35 mmoles) in acetic acid (50 ml.) was added 3 g. of iron dust, and the mixture was heated on the water bath for 1.5 hr. After cooling, the reaction mixture was made alkaline (pH 10–11) with NaOH and filtered. The filtrate was extracted with ether which was dried over anhydrous K_2CO_3. On removing the excess solvent, 2.0 g. of 4-chloro-3-picoline was obtained as an oil, which was characterized by its picrate melting at 150–151° (11).

4-Chloronicotinic Acid (VI)—4-Chloro-3-picoline (V), on oxidation with aqueous $KMnO_4$ by the method of Herz and Murty (12), gave 4-chloronicotinic acid (VI), m.p. 162–163°.

4-[α-(2′-Nitrophenyl)hydrazino]nicotinic Acid (VIIIa)—2-Nitrophenylhydrazine (VII) (1 g., 7 mmoles) was added to a suspension of 4-chloronicotinic acid (VI) (1 g., 7 mmoles) in 15 ml. of absolute ethanol, and the mixture was refluxed for 8 hr. The reaction mixture was concentrated under reduced pressure and cooled. The solid mass which separated out was collected by filtration and purified by dissolving it in dilute NH_4OH (approximately 50%) and reprecipitating by dropwise addition of dilute HCl. On recrystallization from ethanol, the crude product gave 1.0 g. (58%) 4-[α-(2′-nitrophenyl)hydrazino]nicotinic acid (VIII*a*) melting at 152° dec.; IR (KBr) 3220 and 3360 $cm.^{-1}$ (—NH_2) (Table I).

3-Hydroxy-1-(2′-nitrophenyl)-1*H*-pyrazolo[4,3-*c*]pyridine (IX)—4-[α-(2′-Nitrophenyl)hydrazino]nicotinic acid (VIII*a*) (0.5 g., 2 mmoles), distilled water (15 ml.), and concentrated HCl (1.5 ml.) were refluxed for 5 hr. The reaction mixture was filtered and concentrated to one-third of its original volume and cooled. The crude product which separated out was filtered and recrystallized from concentrated hydrochloric acid–water (1:2), m.p. 202° dec.; yield 0.25 g. (47%); IR (KBr) 1650 $cm.^{-1}$ (C=O) (Table II).

BIOCHEMICAL STUDIES

Normal healthy albino rats (100–150 g.), kept on an *ad libitum* diet, were used throughout the studies. The rats were decapitated, and the livers were immediately homogenized in 0.25 *M* ice-cold sucrose (1:9 ratio) in a homogenizer[3]. The homogenate was centrifuged at 100,000×*g* for 30 min., and the clear supernatant (nonparticulate or soluble fraction) thus obtained was used for the assay of purine-catabolizing enzymes (13–16).

Adenosine Deaminase (Adenosine Aminohydrolase, E.C. 3.5.4.4)—The enzyme activity was determined by estimation of the disappearance of adenosine at 265 nm. (17). The reaction mixture in final concentration consisted of 0.05 *M* phosphate buffer (Na_2HPO_4; KH_2PO_4) in a total volume of 1 ml. (pH 7.0) 0.0001 *M* adenosine and 0.1 ml. 5% supernatant equivalent to 500 mcg. protein. Prior to the addition of the substrate, the tubes containing enzyme preparations, with or without compounds, were preincubated for 10 min. at 37°. The substrate was added, and the reaction was allowed to run for exactly 15 min. after which 0.5 ml. 10% cold perchloric acid was added. After centrifugation, the supernatants were read for adenosine at 265 nm. The enzyme activity, found to be proportional to the decrease in optical density (O.D.), was expressed in terms of change in O.D./mg. protein/15 min.

Guanosine Deaminase—The enzyme activity was determined by estimation of disappearance of guanosine at 245 nm. using assay conditions similar to those used for adenosine deaminase.

Guanine Deaminase (Guanine Aminohydrolase, E.C. 3.5.4.3)—The enzyme activity was determined by estimation of the disappearance of guanine at 245 nm. (18). The assay system of 1 ml. consisted of 0.05 *M* tromethamine–HCl buffer (pH 8.0), 0.0001 *M* guanine, 0.1 ml. 5% supernatant equivalent to 500 mcg. protein as an enzyme source, and glass-distilled water. Before the reaction was started, the tubes containing enzyme were preincubated at 37° for 10 min. The reaction was started by addition of guanine and allowed to run for 15 min. at 37°. To this mixture was added 0.5 ml. of 10% cold perchloric acid to stop the reaction. After centrifuging, the supernatants were read for guanine at 245 nm. The assay conditions were the same as those reported for adenosine deaminase.

Xanthine Oxidase (Xanthine–O_2 Oxidoreductase, E.C. 1.2.3.2)—Estimation of uric acid formed from the oxidation of hypoxanthine at 290 nm. was used (19) for determining xanthine oxidase activity. The reaction mixture in a total volume of 1 ml. consisted of 0.05 *M* tromethamine–HCl buffer (pH 8.0), 0.00015 *M* hypoxanthine, 0.2 ml. of 10% supernatant equivalent to 2 mg. protein, and glass-distilled water. The use of different concentrations of hypoxanthine indicated that 0.00015 *M* was the optimum concentration for maximum activity. After centrifuging out the proteins, the supernatants were read for uric acid at 290 nm. The assay conditions used were the same as those reported for adenosine deaminase. The enzyme activity was expressed as change in O.D./mg. protein/30 min.

Protein Estimation—Protein determinations were done by the method of Lowry *et al.* (20), using bovine serum albumin as the standard. Readings were taken at 750 nm.

RESULTS AND DISCUSSION

As is evident from Table III, 3-hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines selectively inhibit liver xanthine oxidase alone,

[3] Potter Elvehjem.

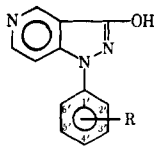


Table II—3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines[a]

R	Melting Point	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found	UV $\lambda_{max.}$, nm.[b] 1 *N* NaOH	UV $\lambda_{max.}$, nm.[b] H_2O	UV $\lambda_{max.}$, nm.[b] 1 *N* HCl
2′-NO_2	202° dec.	47	$C_{12}H_8N_4O_3 \cdot HCl$	C 49.23 H 3.07 N 19.14	48.92 3.36 18.82	244	256	234
4′-NO_2	>300°	56	$C_{12}H_8N_4O_3 \cdot HCl$	C 49.23 H 3.07 N 19.14	49.18 3.23 19.74	245	255	234
2′,4′-Di-NO_2	192° dec.	47	$C_{12}H_7N_5O_5 \cdot HCl$	C 42.66 H 2.37 N 20.74	43.01 2.72 20.32	246	260	250

[a] Hydrochloride salts. [b] UV absorption maxima were taken at concentrations of 3.3×10^{-5} *M*.

whereas no inhibition was observed with other purine deaminases such as adenosine deaminase, guanosine deaminase, and guanine deaminase under similar experimental conditions.

Results shown in Table IV clearly indicate the greater effectiveness of 3-hydroxy-1-(2′,4′-dinitrophenyl)-1*H*-pyrazolo[4,3-*c*]pyridine compared to 3-hydroxy-1-(2′-nitrophenyl)-1*H*-pyrazolo[4,3-*c*]-pyridine and 3-hydroxy-1-(4′-nitrophenyl)-1*H*-pyrazolo[4,3-*c*]pyridine. All these compounds produced complete inhibition of liver xanthine oxidase when used at a final concentration of 1×10^{-3} *M*. This result was also reflected by the I_{50} values of these compounds: 3.2×10^{-4} *M*, 4.7×10^{-4} *M*, and 5.2×10^{-5} *M*, respectively. The ratio of the inhibitory effectiveness of these compounds, as determined by calculating the ratio of the concentration of the inhibitor to the substrate giving 50% inhibition (Table IV), was found to be 2.133, 3.133, and 0.347, respectively. Thus, changes observed in the inhibitory effects of pyrazolopyridine by the introduction of different substituents in their phenyl group at the 1-position could presumably account for increased hydrophobic binding of the inhibitor to the crude preparation of liver xanthine oxidase as compared to the substrate hypoxanthine. Results from these laboratories are comparable with those of Baker and Wood (6) where 9-phenylguanine was found to show significant inhibition, possibly by interaction of the phenyl group with the hydrophobic binding region of bovine milk xanthine oxidase.

The greater binding ability of 6-phenylallopurinol achieved by the introduction of the nitro substituent and, consequently, the increased inhibition were reflected by severalfold inhibition of milk xanthine oxidase (5). These results provided evidence that nitro substitution in the phenyl ring at the 1-position of pyrazolopyridine plays an important role in producing enzyme inhibition and that both the 2′- and 4′-positions presumably contribute independently toward such inactivation, since maximum inhibition was observed with the 2′,4′-dinitro-substituted compound. Recently, Massey *et al.* (21) showed that inhibition of milk xanthine oxidase is due to

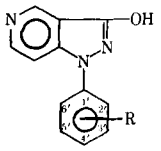


Table III—Effect of 3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines on Rat Liver Adenosine Deaminase, Guanosine Deaminase, Guanine Deaminase, and Xanthine Oxidase[a]

R	Adenosine Deaminase	Guanosine Deaminase	Guanine Deaminase	Xanthine Oxidase
None (control)	0.260 ± 0.015	0.222 ± 0.014	0.290 ± 0.012	0.126 ± 0.008
2′-NO_2	0.255 ± 0.012	0.228 ± 0.020	0.288 ± 0.013	Nil[b]
4′-NO_2	0.263 ± 0.013	0.218 ± 0.018	0.295 ± 0.022	Nil[b]
2′,4′-Di-NO_2	0.258 ± 0.009	0.215 ± 0.008	0.292 ± 0.010	Nil[b]

[a] Details of assay procedure and units of activities (change in extinction ΔE/mg. protein) given here are described in the text. All values are the means with *SE* of four duplicate experiments. Final concentrations of the compounds used were 2 m*M*. [b] No change in extinction (ΔE) denotes 100% enzyme inhibition.

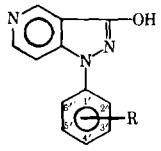


Table IV—Effect of 3-Hydroxy-1-nitrophenyl-1*H*-pyrazolo[4,3-*c*]pyridines on Rat Liver Xanthine Oxidase

R	Percent Inhibition[a] 1×10^{-3} *M*	5×10^{-4} *M*	1×10^{-4} *M*	5×10^{-5} *M*	1×10^{-5} *M*	I_{50} Value[b]	[(I)/(S)]$_{0.5}$[c]
2′-NO_2	>100	67.0 ± 2.32	26.6 ± 1.88	10.5 ± 1.14	None	3.2×10^{-4}	2.133
4′-NO_2	>100	53.5 ± 1.48	16.0 ± 1.43	2.3 ± 0.08	None	4.7×10^{-4}	3.133
2′,4-Di-NO_2	>100	>100	73.3 ± 2.81	48.0 ± 2.35	10.7 ± 0.95	5.2×10^{-5}	0.347

[a] All values given represent the percent inhibition with *SE* of four duplicate experiments. Molar concentrations given are final concentrations of compounds used in the reaction mixture. [b] Represents the molar concentration of compound required to produce 50% inhibition of the enzyme under the experimental conditions described in the text. [c] Represents the ratio of concentration of inhibitor to substrate giving 50% inhibition.

the formation of an inactive complex of reduced enzyme having lower valency state molybdenum with alloxanthine, a metabolic product of allopurinol. At present, it is difficult to determine the exact nature of enzyme inhibition. Synthesis of new compounds and kinetic studies to evaluate their inhibitory activity using purified enzyme preparations could possibly elucidate the mechanism of action of such selective inhibition of liver xanthine oxidase.

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▲ To whom inquiries should be directed.

Electrokinetic Studies of Bacteria I: Effect of Nature, Ionic Strength, and pH of Buffer Solutions on Electrophoretic Mobility of *Streptococcus faecalis* and *Escherichia coli*

HANS SCHOTT▲ and C. Y. YOUNG

Abstract □ The characteristics of the exterior surface of bacteria are important because it is the first region of the cell with which dissolved nutrients and drugs come into contact. The electrical properties of the outer layer of the cell wall were studied in detail for the Gram-positive *Streptococcus faecalis* by microelectrophoresis. A few experiments were made with the Gram-negative *Escherichia coli*. Incubation time had only a minor effect on the electrophoretic mobility, even when comparing bacteria during the exponential growth phase and during the stationary phase. Increasing concentrations of buffer electrolytes reduced the mobility. When the mobility of *S. faecalis* was measured as a function of pH between 2.5 and 10.8 at constant ionic strength with phosphate, acetate, and biphthalate buffer systems, all points fell on a single curve, indicating the absence of specific ion effects. The curve had a plateau between the pH values of 4 and 7. It dropped sharply at lower pH and rose slowly at higher pH. The extrapolated isoelectric point was at pH 2.3. *S. faecalis* was negatively charged over the entire pH range studied. According to the apparent ionization constant, the group responsible for that charge was the carboxyl group. The charge density at the outer portion of the cell wall was one carboxyl group per (48 Å)^2. For *E. coli*, the value was one carboxyl group per (31 Å)^2.

Keyphrases □ Bacteria—electrokinetic studies □ Electrokinetics—bacteria □ *Streptococcus faecalis*, electrophoretic mobility—effect of nature, ionic strength, and pH of buffer solutions □ *Escherichia coli*, electrophoretic mobility—effect of nature, ionic strength, and pH of buffer solutions □ Electrophoresis—bacteria

From the colloid-chemical viewpoint, bacteria, like all small particles, can be characterized by size, shape, hydration, and charge. The latter two properties are determined by the characteristics of the bacterial surface, specifically, of the outer layer of the cell wall. The nature of the exterior of bacteria is of interest because it is the first region of the cell with which nutrients and antibacterial agents come into contact. Their interac-