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Inhibitors of Polyamine Biosynthesis. 1. α-Methyl-(±)-ornithine, an Inhibitor of Ornithine Decarboxylase

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α-Methyl-(±)-ornithine (**5**) was obtained by two independent syntheses. In the first synthesis 1-phthalimidopentan-4-one was subjected to the Bucherer–Lieb reaction to provide 5-(3-phthalimidopropyl)-5-methylhydantoin. The latter was hydrolyzed to produce **5**. The second synthesis involved the reaction of 3-imino(4-nitrobenzyl)piperidin-2-one with phenyllithium to form a resonance-stabilized anion which on treatment with methyl iodide and acid hydrolysis provided **5** in good yields. α-Methyl-(±)-ornithine monohydrochloride was found *in vitro* to be a potent, reversible, competitive inhibitor of ornithine decarboxylase obtained from the prostate glands of rats. This inhibition was not abolished at high concentrations of pyridoxal phosphate.

The diamine putrescine and the polyamines spermidine and spermine are present in all animal and plant tissue tested and at least one of these is present in all microorganisms.[1] The exact physiological role of the polyamines is not known at present. Recent studies, however, afford evidence that these amines may control cell division and growth and may participate in many steps in the biosynthesis of protein and RNA.[2] Studies of both normal and neoplastic rapid-growth systems indicate that the synthesis and accumulation of polyamines are elevated shortly after a stimulus inducing proliferation. Furthermore, tissues which actively synthesize protein as prostate, bone marrow, and pancreas contain higher concentrations of polyamines than most other mammalian tissues.[3]

The biosynthesis of polyamines in mammalian tissue, plant tissue, and bacteria has been extensively studied. In mammalian tissue the decarboxylation of L-ornithine to produce putrescine is catalyzed by the enzyme ornithine decarboxylase (ORD). This enzyme requires pyridoxal phosphate (PLP) and has no activity toward L-lysine, L-arginine, or D-ornithine.[4] Spermidine is formed from putrescine by an enzyme system which catalyzes the decarboxylation of *S*-adenosylmethionine and the transfer of the propylamine moiety to putrescine. It appears that the same enzyme system catalyzes the formation of spermine from spermidine.[5]

In spite of numerous studies on the biosynthesis and accumulation of polyamines in proliferating tissue, it is not certain if the increase in polyamine levels in these tissues mediates the elevated rate of protein synthesis or if the elevated rate of protein synthesis produces the increase in polyamine levels. One way of elucidating the role of polyamines in proliferating tissue would be to block their biosynthesis and to determine if this causes inhibition of cellular proliferation. A likely candidate for this blockade is the enzyme ornithine decarboxylase since the decarboxylation of L-ornithine appears to be the rate-limiting step in polyamine synthesis,[6] and the activity of ORD is sharply increased in rapidly growing tissue.

There are very few reports in the literature of inhibitors of the enzyme ornithine decarboxylase. L-Canaline was found to inhibit pyridoxal-dependent enzymes including ornithine decarboxylase but this inhibition was reversed by excess pyridoxal phosphate.[7] A number of ornithine analogs were prepared in an attempt to obtain specific inhibition of ornithine decarboxylase. None of these compounds were found to be effective inhibitors.[8] Recently, α-hydrazino-L-ornithine was reported to be a potent inhibitor of ORD but a much less effective inhibitor of other pyridoxal-dependent enzymes. The inhibition of ORD by α-hydrazino-L-ornithine is completely abolished at high concentrations of PLP.[9] Also, inhibition by α-methylornithine of ORD obtained from regenerating rat liver was briefly reported in a recent symposium.[5]

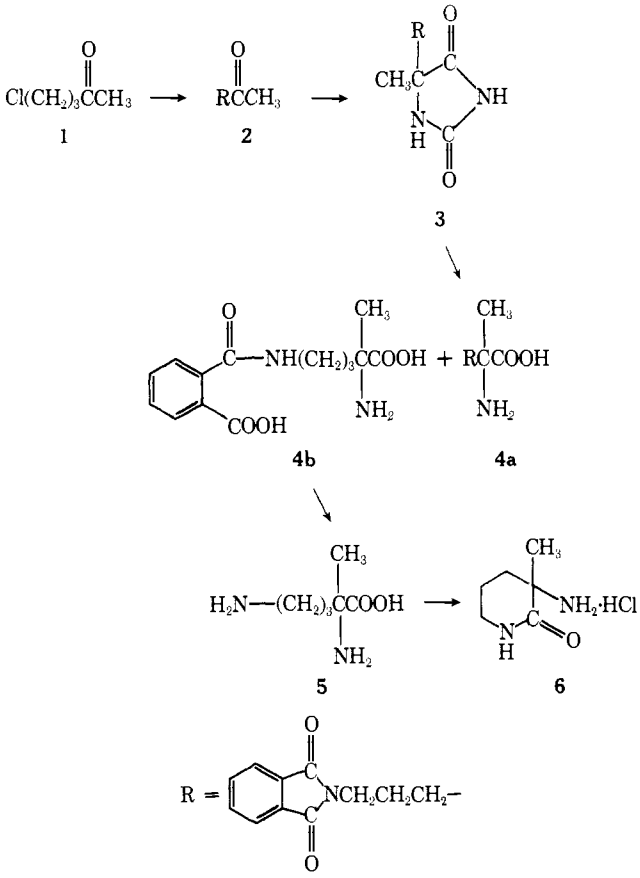
The present communication describes the synthesis of α-methylornithine and the evaluation of its inhibitory effect on ornithine decarboxylase obtained from rat prostate gland.

Results and Discussion

The target compound, α-methylornithine (**5**), was obtained *via* two independent syntheses. In the first synthe-

sis (Scheme I) 5-chloro-2-pentanone (1) was allowed to react with potassium phthalimide to provide 2 in moderate yields. The latter was subjected to the Bucherer-Lieb synthesis to provide the hydantoin 3[10] which was treated with a barium hydroxide solution, under pressure, to provide 4a. Tlc examination of the product indicated the presence of two ninhydrin-positive components. This was attributed to the partial hydrolysis of the phthalimido group in 4a to provide 4b since hydrolysis of the crude product (4a and 4b) with hydrochloric acid provided 5·2HCl. The latter was purified using an Amberlite IR-120 (H^+) ion exchange resin. The α-methylornithine was treated with 1 equiv of hydrochloric acid to provide 5·HCl. Crystallization of 5·HCl from aqueous acetone provided the monohydrate (5·HCl·H_2O). The latter produced the anhydrous 5·HCl by heating at 150°. When 5·HCl was heated at 250° it melted and resolidified to provide the lactam 6.

Scheme I



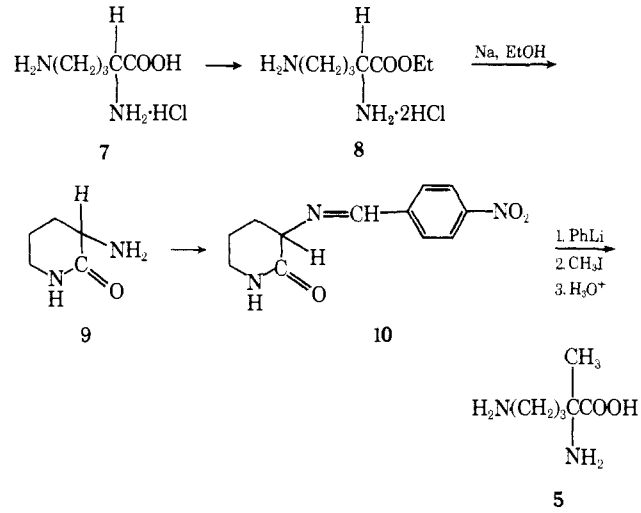
The second synthesis of 5 is represented in Scheme II. In this synthesis the α-methyl group was added to the ornithine molecule using a synthetic scheme similar to that used to obtain 6-α-methylpenicillin G sodium.[11] 3-Amino-2-piperidone (9) was prepared using a previously reported method by treatment of 8 with sodium in dry ethanol.[12] Treatment of 9 with *p*-nitrobenzaldehyde with concomitant removal of water, by azeotropic distillation with benzene, provided the Schiff base 10. Treatment of 10 with 2.0 molar equiv of phenyllithium in tetrahydrofuran at −76° resulted in the formation of a brilliant blue solution, presumably due to the formation of the resonance stabilized anion of 10. This solution was stirred for 4 hr at −78° and was treated with 10 molar equiv of methyl iodide in dimethylformamide. The product after work-up provided crude 5 in 67% yield. Purification by crystallization of the monohydrochloride provided the monohydrate of 5·HCl identical with that obtained from the first synthesis. The optimization of yields, identification of side products, and the general applications of this reaction to the synthesis of α-substituted amino acids is presently under investigation.

Table I. Effect of Pyridoxal Phosphate Concentration on the Rate of Decarboxylation of L-Ornithine by Dialyzed and Nondialyzed Ornithine Decarboxylase from Rat Prostate Glands

	Concn of pyridoxal phosphate, *M*	Vel[a] (nmol of CO_2/mg of wet wt tissue per hr)
Nondialyzed enzyme	2.0×10^{-4}	0.217
	2.0×10^{-5}	0.186
	2.0×10^{-6}	0.123
Dialyzed[b] enzyme	2.0×10^{-4}	0.187
	2.0×10^{-5}	0.146
	2.0×10^{-6}	0.119

[a] The reaction was carried out in the presence of 1.9×10^{-4} *M* L-ornithine. [b] The 100,000g supernatant from the rate prostate gland was dialyzed at 4° for 16 hr against 1 l. of 1.0×10^{-2} *M* phosphate buffer (pH 7.5) containing 5×10^{-3} *M* dithiothreitol.

Scheme II



The inhibition by 5·HCl of the enzymatic decarboxylation of L-ornithine was measured *in vitro*. A soluble cell-free extract from the prostate gland of rats was used as the source of the enzyme ornithine decarboxylase (ORD). ORD activity was measured by determining the amount of $^{14}CO_2$ released from ^{14}C-carboxy-labeled (±)-ornithine in the presence of pyridoxal phosphate (PLP). Contrary to the findings of Pegg, *et al.*,[13] we observed no enhancement of ORD activity on dialyzing the enzyme preparation. Furthermore, the ORD activity was diminished on dialysis (Table I), probably due to aging since a similar decrease in activity was observed on storage of other enzyme preparations. The effect of PLP concentration on the activity of ORD was also studied (Table I). The activity of ORD at a constant concentration of the substrate was found to be highly dependent on PLP concentration. In subsequent experiments the nondialyzed enzyme was used and all measurements were carried out in the presence of either 2.0×10^{-4} or 2.0×10^{-6} *M* concentrations of PLP.

In the presence of 2.0×10^{-6} *M* PLP, the ORD activity was approximately 65% of that in the presence of 2 ×

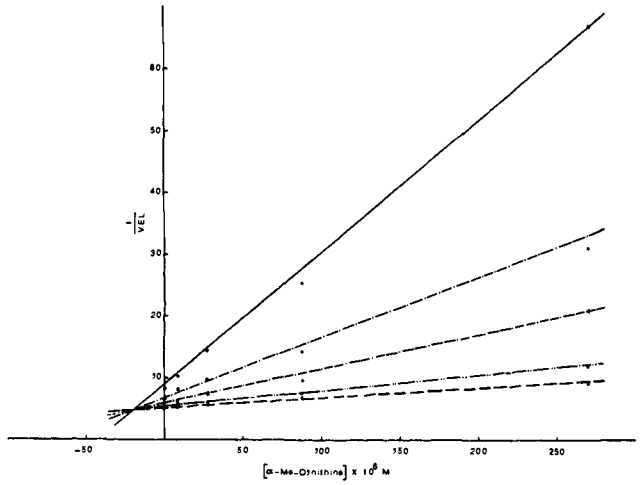


Figure 1. Dixon plots of the effect of α-methyl-(±)-ornithine on the rate of L-ornithine decarboxylation by ornithine decarboxylase from the prostate glands of rats in the presence of 2×10^{-6} *M* pyridoxal phosphate. The concentrations of L-ornithine used were: 8.64×10^{-4} *M* (----), 4.03×10^{-4} *M* (-·-·-·-), 1.72×10^{-4} *M* (--------), 9.6×10^{-5} *M* (-·····), and 4.8×10^{-5} *M* (——). Each point is the average of two values. Vel = nmol of CO_2/mg of wet weight tissue per hr.

10^{-4} *M* PLP. We also observed an apparent substrate inhibition at concentrations of L-ornithine of 5×10^{-4} *M* or higher. Under these conditions, ORD from rat prostate had an apparent K_m for L-ornithine of 4.7×10^{-5} *M* and a V_{max} of 0.22 nmol of CO_2/mg of wet weight tissue per hr. The addition of 5·HCl in concentrations varying from 8.7×10^{-6} to 2.7×10^{-4} *M* resulted in the supression of the production of $^{14}CO_2$. It appears from the Dixon plots[14] of these data (Figure 1) that 5·HCl is a competitive inhibitor of decarboxylation of L-ornithine by ORD. The K_i for 5·HCl was calculated to be 2.0×10^{-5} *M*.

α-Hydrazino-L-ornithine is also reported to be a potent competitive inhibitor of ORD. This inhibition, however, was abolished in the presence of concentrations of PLP of 1.0×10^{-4} *M* or higher.[9] It was therefore of interest to examine the effect of increasing the concentration of PLP on the inhibition of ORD by 5·HCl. At a concentration of 2×10^{-4} *M* PLP the apparent K_m for L-ornithine was 9.7×10^{-5} *M* and the V_{max} was 0.59 nmol of CO_2/mg of wet weight tissue per hr. The inhibition of ORD by 5·HCl was only slightly affected by the 100-fold increase in PLP concentration and the apparent K_i for 5·HCl was 3.0×10^{-5} *M* (Figure 2). Comparison of this value (3.0×10^{-5} *M*) with the K_m for L-ornithine (9.7×10^{-5} *M*) indicates that 5·HCl was a potent inhibitor of the rat prostate ORD.

The decarboxylation of a concentration of L-ornithine by varying concentrations of ORD was studied in presence of 2.0×10^{-4} *M* PLP with or without 5·HCl. The release of $^{14}CO_2$ was linear with enzyme concentration and passing through the origin (Figure 3) indicating the complete reversibility of the inhibition of ORD by 5·HCl.

Conclusion

α-Methyl-(±)-ornithine hydrochloride (5·HCl) is a potent, reversible, competitive inhibitor of ornithine decarboxylase obtained from the prostate glands of rats. The inhibition by 5·HCl may be greater than that reported here as racemic 5·HCl was used in the enzyme testing and its resolution may indicate that the inhibitory activity resides mainly in one of the antipodes. Unlike the inhibition of ORD by α-hydrazino-L-ornithine,[9] the inhibition of ORD by 5·HCl was not abolished at high concentrations of PLP. It is not clear, however, if this differential

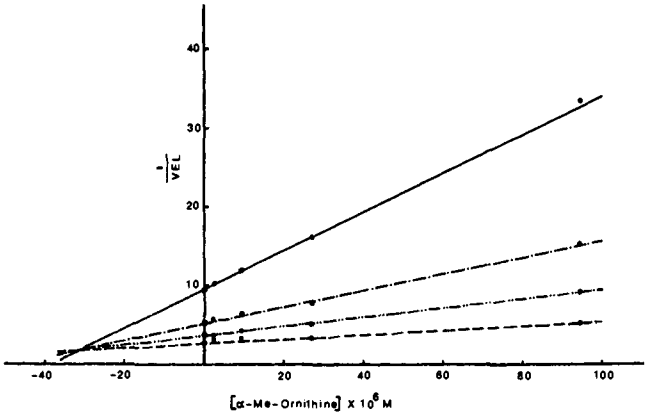


Figure 2. Dixon plots of the effect of α-methyl-(±)-ornithine on the rate of L-ornithine decarboxylation by ornithine decarboxylase from the prostate glands of rats in the presence of 2×10^{-4} *M* pyridoxal phosphate. The concentrations of L-ornithine used were: 1.9×10^{-4} *M* (----), 9.3×10^{-5} *M* (-·-·-·-), 4.8×10^{-5} *M* (-·····), and 2.0×10^{-5} *M* (——). Each point is the average of two values. Vel = nmol of CO_2/mg of wet weight tissue per hr.

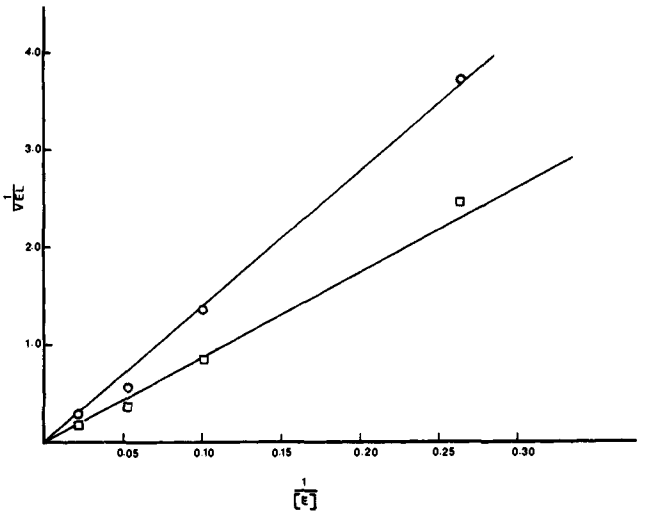


Figure 3. An Ackerman–Potter plot of the reciprocal of the velocity *vs.* the reciprocal of the enzyme concentration, with or without 2.7×10^{-5} *M* α-methyl-(±)-ornithine. [E] = mg of wet weight tissue; Vel = nmol of CO_2/hr.

dependence on PLP of the two inhibitors is due to a difference in the mechanism of inhibition of ORD or a difference in the interaction with PLP. It has been reported that analogs of ornithine substituted on either the α- or ε-amino group or on the β position are inactive as inhibitors of ORD[8] indicating that the unsubstituted aminopropyl moiety of the ornithine molecule is required for binding to the enzyme. On the other hand, substitution on the α carbon or replacement of the α-amino group with other groups capable of reacting with PLP results in a potent inhibitor, indicating the less rigid structural requirement for binding of the α carbon to the enzyme.

Experimental Section

The melting points were determined in open capillary tubes with a Thomas-Hoover apparatus and are uncorrected. Elemental analysis was performed by M-H-W Laboratories, Garden City, Mich. The ir spectra were obtained with a Perkin-Elmer 237B or Beckman IR-9 spectrophotometer. The nmr spectra were taken on a Varian A-60D in $CDCl_3$ or D_2O with TMS or DSS as internal standards, respectively. Mass spectra analyses were performed on AEI MS-30 at 70 eV and 200° chamber temperature. Optical rotation measurements were obtained on a Perkin-Elmer 141 polarimeter. All spectral data were consistent with the proposed structures. Radioactivity was measured using a Beckman LS-150 liq-

uid scintillation counter. Calculations of the kinetic parameters for the enzyme studies were performed on CDC 6400 computer using the method of Wilkinson.[15]

1-Phthalimidopentan-4-one (2). Freshly distilled 5-chloro-2-pentanone (48.3 g, 0.40 mol) was dissolved in DMF (400 ml). Potassium phthalimide (89.0 g, 0.48 mol) was added and the suspension was stirred at 80° for 4 hr. The cold mixture was treated with H_2O (700 ml) and extracted with $CHCl_3$ (3 × 250 ml). The $CHCl_3$ was extracted with 1 *N* NaOH solution (2 × 300 ml), dried (anhydrous Na_2SO_4), and concentrated to a yellow liquid. The residue was poured into ice H_2O and stirred and the white solid was filtered off. The solid after recrystallization (EtOH-H_2O, 1:2) provided 39.4 g (42.5%) of the title compound, mp 74.5–75.5°. *Anal.* ($C_{13}H_{13}NO_3$) C, H, N.

5-(3-Phthalimidopropyl)-5-methylhydantoin (3). A solution of **2** (0.925 g, 0.004 mol) in EtOH (25 ml) was treated dropwise over 2 hr with a solution of $NaHSO_3$ (0.4168 g, 0.004 mol) in H_2O (10 ml). The reaction mixture was treated with a solution of KCN (0.5265 g, 0.008 mol) and $(NH_4)_2CO_3$ (1.8219 g, 0.016 mol) in H_2O (15 ml). The reaction mixture was held at 55° for 18 hr and then boiled under reflux for 1 hr to decompose the excess $(NH_4)_2CO_3$. The mixture was evaporated to dryness, and the residue was dissolved in H_2O (50 ml) and extracted with $CHCl_3$ (2 × 50 ml). The H_2O phase was evaporated to dryness; the residue was treated with anhydrous MeOH and filtered. The filtrate was evaporated to dryness to provide the title compound with some inorganic salts. This product, after three recrystallizations from water, had mp 215–220° and was homogenous on tlc.

α-Methylornithine Monohydrochloride (5·HCl). The compound **3** (1.0 g, 0.0033 mol) was added to a suspension of $Ba(OH)_2 \cdot 8H_2O$ (2.08 g, 0.0066 mol) in H_2O (25 ml). The mixture was heated in a pressure bottle at 160° for 3 hr. The mixture was cooled, filtered, treated with $(NH_4)_2CO_3$ until no further precipitation occurred, heated to boiling, and filtered. The filtrate was evaporated to dryness to provide an oil which showed two ninhydrin-positive spots on tlc (silica gel with 60:20:20 *n*-BuOH–HOAc–H_2O as a solvent). The residue was treated with 6 *N* HCl (5.0 ml); the mixture was boiled under reflux for 20 hr and cooled. The precipitate formed was filtered off and identified as o-phthalic acid (mp 205–208°). The filtrate was evaporated to an oil and the residue was treated with acetone; the mixture was stirred and cooled. The white solid which separated was filtered and washed with cold acetone. The precipitate was dissolved in water and the solution was adjusted to pH 5–6 by the addition of NH_4OH. The solution was applied to a column of Amberlite IR-120 ion exchange resin, the column was washed with water until washings were neutral, and the amino acid was eluted with 1 *M* NH_4OH. The eluate was evaporated to dryness and the residue was titrated with 0.1 *N* HCl to produce the monohydrochloride. The solution was treated with decolorizing charcoal, boiled, and filtered through a Celite filter bed. The filtrate was evaporated in dryness and the residue was crystallized from water–acetone to provide **5**·HCl·H_2O (yield 44%). *Anal.* ($C_6H_{17}N_2O_3Cl$) C, H, N. The sample when heated at 150° provided the anhydrous salt. *Anal.* ($C_6H_{15}N_2O_2Cl$) C, H; N: calcd, 15.34; found, 15.84. The sample was heated at 250° to produce **6**·HCl as shown by its ir spectrum.

L-Ornithine Ethyl Ester Dihydrochloride (8·2HCl). The title compound was prepared using the method of Yamada, *et al.*,[16] in 97% yield, mp 176–177°.

3-Aminopiperidone (9). This compound was obtained by the method described by Golankiewicz, *et al.*,[12] in 86% yield. The product was used without distillation.

3-Imino(4-nitrobenzyl)piperidin-2-one (10). A suspension of *p*-nitrobenzaldehyde (7.55 g, 0.05 mol) in benzene (100 ml) was added to a solution of **9** (4.92 g, 0.043 mol) in benzene (100 ml) in a flask connected to a Dean-Stark distillation receiver. The reaction mixture was heated under reflux for 2 hr, filtered while hot, and allowed to cool. The precipitate obtained was recrystallized from benzene to provide 6.91 g (64%) of the title compound, mp 155–157°. *Anal.* ($C_{12}H_{13}N_3O_3$) C, H, N.

α-Methylornithine Monohydrochloride (5·HCl). A suspension of Li wire (0.56 g, 0.08 mol) in dry THF was treated with bromobenzene (6.91 g, 0.044 mol). The phenyllithium solution was cooled to −78° and treated dropwise with a solution of **10** (4.94 g, 0.02 mol) in dry THF (400 ml) under N_2. The mixture was kept at −78° during the addition and a brilliant blue solution was obtained. The solution was stirred at −78° for 4 hr and then treated dropwise, over a period of 2 hr, with a solution of CH_3I (56.8 g, 0.4 mol) in DMF (200 ml). The red reaction mixture was allowed to warm to room temperature and then filtered through a sintered glass filter. The filtrate was concentrated to 100 ml and diluted with 200 ml of H_2O. The solution was extracted with $CHCl_3$ (3 × 200 ml) and the $CHCl_3$ was dried (anhydrous Na_2SO_4), and the solvent was evaporated under vacuum. The residue was dissolved in THF (10 ml) and treated with 2 *N* HCl (125 ml) and the mixture was boiled under reflux for 20 hr. The cooled mixture was filtered to remove *p*-nitrobenzaldehyde and the filtrate was extracted with $CHCl_3$ (2 × 250 ml). The aqueous phase was concentrated to about 50 ml, adjusted to pH 4.0-5.0, and applied on a column of Amberlite IR-120 (H^+) ion exchange resin. The column was washed with H_2O until the washings were neutral and the amino acid was eluted with 1 *M* NH_4OH. The eluate was evaporated to dryness to provide 1.94 g of **5** (67%). This product was titrated with 0.1 *N* HCl to provide **5**·HCl which after decolorization with charcoal, evaporation of the solvent, and recrystallization from acetone–water provided **5**·HCl·H_2O. This product was identical with **5**·HCl·H_2O obtained from the previous synthetic route, $[\alpha]^{23}D$ 0° (*c* 2).

Animals. Adult male Sprague–Dawley rats (220–250 g) were used. The animals were fed on a diet of Purina Chow and water *ad libitum*.

Materials and Solutions. DL-[1-^{14}C]Ornithine (12.8 mCi/mmol) and $NaH^{14}CO_3$ (8.4 mCi/mmol) were obtained from New England Nuclear, Inc.; pyridoxal phosphate and dithiothreitol from Sigma Chemical Co.; and ethanolamine and Permablend III from Packard Instruments. The homogenization solution was prepared to contain sucrose, 250 m*M*; Na_2HPO_4, 8.4 × 10^{-3} *M*, and KH_2PO_4, 1.6 × 10^{-3} *M* (pH 7.5); dithiothreitol, 5 × 10^{-3} *M*; and pyridoxal phosphate, 2.0 × 10^{-4} or 2.0 × 10^{-6} *M*. The incubation solution contained Na_2HPO_4, 15.4 m*M*, and KH_2PO_4, 9.6 m*M* (pH 7.0); dithiothreitol, 5 × 10^{-3} *M*; and pyridoxal phosphate, 2 × 10^{-4} or 2 × 10^{-6} *M*. The counting cocktail contained 5.5 g of Permablend III dissolved in 1 l. of 1 part toluene and 2 parts methanol.

Tissue Extracts. Rats were decapitated. Their prostate glands were removed and placed in ice-cold homogenization solution. The glands were blotted, weighed, and homogenized in 3 vol of homogenization solution with a hand homogenizer. The homogenates were centrifuged at 10,000g for 10 min at 2° and the supernatants were then centrifuged at 100,000g for 90 min at 2°. This high-speed supernatant was used in all determinations of enzymatic activity.

Assay of Ornithine Decarboxylase Activity. A mixture of 900 μl of the incubation solution and 100 μl of the tissue extracts was placed in a 25-ml conical flask. The reaction was initiated by the addition of 0.6 μCi of DL-[1-^{14}C]ornithine monohydrochloride diluted to 10 μl with an appropriate amount of nonradioactive L-ornithine and the **5**·HCl solution if needed. The flask was sealed with a rubber stopper holding a polypropylene center well (Kontes) containing 100 μl of ethanolamine. The mixtures were incubated at 37 ± 0.1° for the desired amount of time and the reaction was terminated by the injection of 0.5 ml of 2 *N* HCl through the rubber stopper. The flasks were allowed to stand at 37° for 40 min to ensure complete absorption of $^{14}CO_2$. The polypropylene cups with their contents were carefully placed in a counting vial and mixed with 10 ml of the counting cocktail. The nonenzymatic production of $^{14}CO_2$ was determined by replacing the 100 μl of tissue extract with 100 μl of the homogenization solution. The extent of and time required for absorption of $^{14}CO_2$ by ethanolamine was determined using $NaH^{14}CO_3$. 97% of the $^{14}CO_2$ was absorbed after 40 min. The counting efficiency was determined to be 79.5% using [^{14}C]toluene as an internal standard. The enzyme reaction was linear with respect to time and enzyme concentration over the ranges used. Enzymatic activity is reported as nanomoles of CO_2 released per milligram of wet weight of tissue per hour.

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Notes

Nitrofurfuryl Heterocycles. 12.† 4-Amino-6-(5-nitro-2-furyl)isoxazolo[5,4-*d*]pyrimidines and 4-Amino-2-(5-nitro-2-furyl)pyrimido[4,5-*d*]pyrimidines

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In two previous papers in this series it was shown that the attachment of a condensed pyrimidine ring system at the 2 position of the nitrofuran ring would give compounds possessing exceptional antibacterial activity. Those papers described the antibacterial activity of numerous 4-amino-2-(5-nitro-2-furyl)quinazoline[2] and 4-amino-6-(5-nitro-2-furyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine[3] analogs. The earlier concept[2] is further exemplified by the present study on isoxazolo[5,4-*d*]pyrimidine and pyrimido[4,5-*d*]pyrimidine analogs.

The 4-amino-3-alkyl-6-(5-nitro-2-furyl)isoxazolo[5,4-*d*]pyrimidine analogs were prepared by the same sequence of reactions used to prepare the 4-amino-6-(5-nitro-2-furyl)-1*H*-pyrazolo[3,4-*d*]pyrimidines[3] except that 5-amino-4-cyanoisoxazoles were substituted for 5-amino-4-cyanopyrazoles. This synthesis is similar to the isoxazolo[5,4-*d*]pyrimidine synthesis reported by Desimoni, *et al.*[4]

The synthesis of Taylor, *et al.*,[5] for the preparation of pyrimido[4,5-*d*]pyrimidines was modified to allow for the introduction of the nitrofuryl group and is summarized in Scheme I.

Pertinent physical data for compounds **7**, **8**, and **13–19** are summarized in Table I. The antibacterial testing data, obtained by standard procedures, for these compounds are summarized in Table II. Although there are too few examples reported here to allow comment on structure–activity relationships within each class, we believe these data give further support to our previously published concept[2] for antibacterial activity.

Experimental Section

All melting points were determined in open capillaries using a Mel-Temp melting point apparatus and are corrected. Ir spectra were determined as Nujol mulls on a Perkin-Elmer Model 135 Infracord. The nmr spectrum was obtained on a Varian A-60A instrument using Me_4Si as an internal standard.

***N*-(4-Cyano-3-methyl-5-isoxazolyl)-2-furamide** (1). To a stirred solution of 940 g (7.65 mol) of 5-amino-4-cyano-3-methylisoxazole[6] in 1 l. of pyridine was added slowly 995 g (7.65 mol) of 2-furoyl chloride. After completing the addition, the solution was heated on a steam bath for 2 hr and poured into 4 l. of ice–H_2O, and the solids were removed by filtration. The solids were suspended in 2 l. of cold H_2O and the mixture was acidified with concentrated HCl. The crude product was filtered and dried to give 300 g (54.2%): mp 227.5–228° (aqueous MeOH). *Anal.* ($C_{11}H_9N_3O_3$) C, H, N.

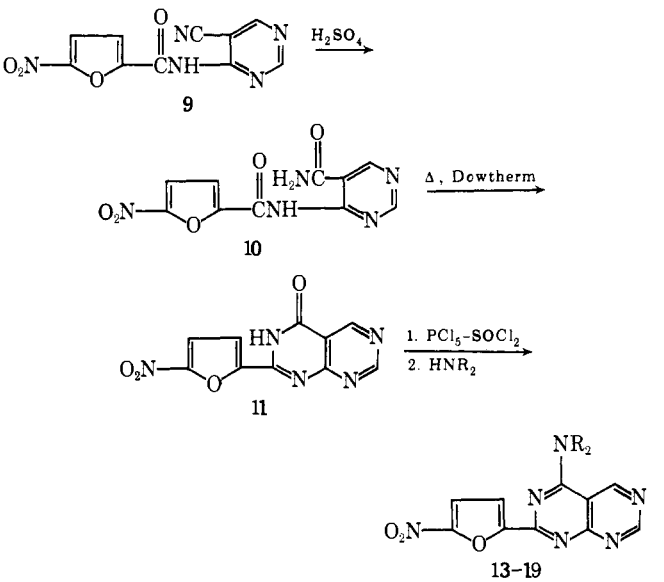
***N*-(4-Cyano-3-ethyl-5-isoxazolyl)-2-furamide** (2) was prepared similarly in 83.7% yield from 2-furoyl chloride and 5-amino-4-cyano-3-ethylisoxazole:[6] mp 171–172.5° (aqueous MeOH). *Anal.* ($C_{11}H_9N_3O_3$) C, H, N.

6-(2-Furyl)-3-methylisoxazolo[5,4-*d*]pyrimidin-4(5*H*)-one (3). Compound 1 (217 g, 1.0 mol) was added in small portions during about 1 hr to a stirred, warm (50–60°) solution of 510 g of NaOH pellets and 850 ml of 30% H_2O_2 in 2 l. of H_2O. Considerable effervescence occurred which was controlled by the periodic addition of a few milliliters of EtOAc. After the exothermic reaction had ceased (0.5–1 hr), the mixture was heated on a steam bath for 2–3 hr. The clear solution was chilled, acidified slowly with glacial AcOH, and filtered. The crude product was washed thoroughly with H_2O and dried to give 188 g (86.6%): mp 306–308° ($MeNO_2$). *Anal.* ($C_{10}H_7N_3O_3$) C, H, N.

3-Ethyl-6-(2-furyl)isoxazolo[5,4-*d*]pyrimidin-4(5*H*)-one (4) was prepared similarly in 76% yield from **2**: mp 269–269.5° ($MeNO_2$). *Anal.* ($C_{11}H_9N_3O_3$) C, H, N.

3-Methyl-6-(5-nitro-2-furyl)isoxazolo[5,4-*d*]pyrimidin-4(5*H*)-one (5). To 200 ml of concentrated H_2SO_4 was added in portions with stirring at 25–30° (external cooling needed) 72.5 g (0.33 mol) of **3**. A solution of 33 ml of concentrated HNO_3 in 66 ml of concentrated H_2SO_4 was added dropwise at 25–30° with cooling during 15 min. The temperature was kept at 25° for 10 min following the addition and then lowered to <10° for 1 hr. After pouring the mixture cautiously into 2 l. of ice–H_2O, the

Scheme I



†For the preceding paper in this series, see ref 1.