and *Escherichia coli* were used as test organisms. A 0.2-mL portion of overnight culture growth was diluted with 100 mL of the molten agar medium to give final concentrations approximating 2×10^6 cfu/mL. The medium for the staphylococcal culture was supplemented with saponinlyzed horse blood at a final concentration of 2% and 2,3,5-triphenyltetrazolium chloride (TTC) was added, to give a final concentration of 0.02%, to the medium for *E. coli* as an indicator for growth of the culture. Aliquots (2 mL) of the molten agar containing the microorganisms were distributed to individual wells of a tissue culture dish. After the seeded agar medium had solidified, the test was performed by adding a few crystals of **9R** of **9S** to the center of a well with a wooden applicator. D-Cycloserine was used as reference compound for inhibition of growth, and several wells were left without compound as controls for culture growth. The test dishes were incubated at 35 °C for 24–30 h, at which time they were examined for inhibition of growth. The TTC indicator in the *E. coli* culture is reduced to a red color in the presence of growth. Inhibition, therefore, is indicated if the whole well or the portion surrounding the test compound remains pale yellow. Growth or inhibition of *S. aureus* was determined visually. Neither **9R** nor **9S** showed inhibitory activity.

Acknowledgment. We are grateful to Drs. H. Katzen and P. Vicario for enzyme inhibition assays and to M. Valiant and B. Weissberger for antibacterial assays.

Supplementary Material Available: A computer-generated drawing of **23S** derived from X-ray coordinates (Figure 1) and tables (Tables II–IV) containing the final fractional X-ray coordinates, temperature parameters, bond distances, and bond angles (4 pages). Ordering information is given on any current masthead page.

2,2-Difluoro-5-hexyne-1,4-diamine: A Potent Enzyme-Activated Inhibitor of Ornithine Decarboxylase

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Received March 22, 1988

2,2-Difluoro-5-hexyne-1,4-diamine was prepared in an eight-step sequence from ethyl 2,2-difluoro-4-pentenoate and tested as an inhibitor of mammalian ornithine decarboxylase. It produces a time-dependent inhibition of the enzyme in vitro which shows saturation kinetics, with $K_I = 10\ \mu M$ and $\tau_{1/2} = 2.4$ min. In rats, it produces a rapid, long-lasting, and dose-dependent decrease of ornithine decarboxylase activity in the ventral prostate, testis, and thymus. In contrast with the nonfluorinated analogue 5-hexyne-1,4-diamine (Danzin et al. *Biochem. Pharmacol.* **1983**, *32*, 941), 2,2-difluoro-5-hexyne-1,4-diamine is not a substrate of mitochondrial monoamine oxidase.

Ornithine decarboxylase (ODC, L-ornithine carboxylase, EC 4.1.1.17) is one of the rate-limiting enzymes in the biosynthesis of the polyamines spermidine and spermine.[1,2] Inhibitors of ODC show antitumoral activity[3] and are also useful in the treatment of human parasitic diseases.[3,4] Both substrate (ornithine) and product (putrescine) analogues can serve as inhibitors. In particular, α-ethynylputrescine is a potent irreversible inhibitor of ODC.[5] This compound, however, shows a mixed pharmacology owing to its in vivo oxidation to γ-ethynyl-GABA, which is an inhibitor of GABA metabolism.[6] While this oxidation, the first step of which is catalyzed by mitochondrial monoamine oxidase, can be avoided by the introduction of a methyl substituent at the δ-carbon, the consequent introduction of a second chiral center brings its own complications.[7] We sought to apply another solution to the problem of oxidation which circumvents the inconvenience of diastereomerism. Expecting that the two fluorine substituents would decrease the susceptibility of the primary amine to oxidation by monoamine oxidase,[8] we prepared 2,2-difluoro-5-hexyne-1,4-diamine (1). The synthesis of this compound and its behavior as an ODC inhibitor and an amine oxidase substrate are reported below.

Table I. Effects of Preincubation with Different Effectors on the Time of Half-Inactivation of ODC by 1

additions to incubation media	time of half-inactivation of ODC, min
5 μM 1	9 ± 1
5 μM 1 + 1 mM L-ornithine	85 ± 5
5 μM 1 + 1 mM L-2-methylornithine	>200

Chemistry

The synthesis of 2,2-difluoro-5-hexyne-1,4-diamine (1) is outlined in Scheme I. The starting material chosen was ethyl 2,2-difluoro-4-pentenoate (**2**), readily available in one step from allyl 2-hydroperfluoroethyl ether[9] by a modification of the literature procedure.[10,11] Ester **2** was reduced in quantitative yield ($NaBH_4$) to give alcohol **3** as a colorless, mobile oil. Reaction with triflic anhydride afforded triflate **4**, an oil which colors on standing, which was transformed into crystalline phthalimide **5** in good yield. Ozonolysis of the double bond gave aldehyde **6**, allowing the introduction of the ethynyl unit with ethynyl-

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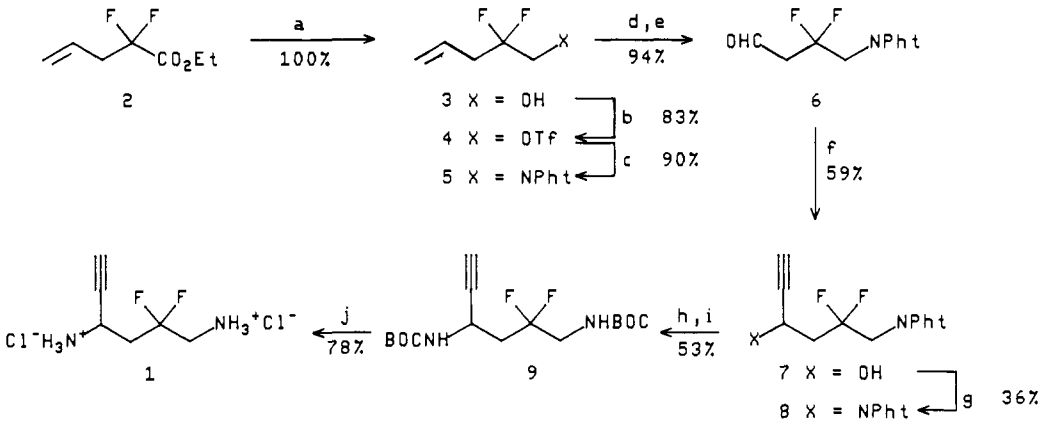
Scheme I[a]



[a] (a) $NaBH_4$, EtOH; (b) Tf_2O, py, CH_2Cl_2; (c) PhtNK, DMF; (d) O_3, CH_2Cl_2; (e) Me_2S; (f) HCCMgBr, THF; (g) PhtNH, DEAD, Ph_3P, THF; (h) $MeNHNH_2$, MeOH, THF; (i) $(BOC)_2O$, K_2CO_3, H_2O, THF; (j) HCl, Et_2O.

magnesium bromide. The resulting alcohol **7** could be isolated as a white solid in moderate to good yield after column chromatography. Under the reaction conditions chosen, the potential elimination of fluoride did not pose any serious problems. The transformation of alcohol **7** into bis-phthalimide **8** was possible with the Mitsunobu procedure,[12] although the yield was only moderate. Hydrazinolysis gave the free diamine which was purified as its bis-*tert*-butylcarbamate **9**. Finally, treatment with anhydrous HCl/Et_2O gave the target compound **1** as its dihydrochloride.

Enzyme Inhibitory Activity

Incubation of ODC preparation with 2,2-difluoro-5-hexyne-1,4-diamine (**1**) resulted in a time-dependent loss of enzyme activity which followed pseudo-first-order kinetics for approximately 2 half-lives (Figure 1). Loss of activity was related to the concentration of inhibitor. By plotting the time of half-inactivation ($t_{1/2}$) as a function of the reciprocal of the inhibitor concentration ($1/I$) according to Kitz and Wilson,[13] a straight line was obtained (Figure 1). This line did not pass through the origin but intercepted the positive y axis, demonstrating saturation effects and hence the involvement of the enzyme's active site in the inhibitory process. Kinetic constants for the time-dependent inhibition of ODC can be extrapolated from Figure 1. The apparent dissociation constant (K_I) is 10 μM, and the time of half-inactivation extrapolated at infinite concentration of inhibitor ($\tau_{1/2}$) is 2.4 min. Further studies on ODC inactivation by 2,2-difluoro-5-hexyne-1,4-diamine (**1**) showed the protective effects of the natural substrate, L-ornithine, and of the competitive inhibitor L-2-methylornithine[14] (Table I). These results confirm that the inactivation is active site directed. Furthermore, the presence of dithiothreitol (5 mM) in the preincubation medium and the absence of lag time before the onset of inhibition rule out the possibility of inactivation via affinity labeling by a diffusible alkylating species.[15] Incubation with **1** at 10 μM concentration resulted in 95% inactivation of ODC after 30 min. Prolonged (24 h) dialysis of this inactivated ODC against a buffer solution containing sodium phosphate (30 mM, pH 7.1), pyridoxal phosphate (0.1 mM), and dithiothreitol (5 mM) (conditions where the native enzyme is stable) did not lead to any significant regeneration of the enzyme activity (93% inactivation was still observed after the dialysis), thus demonstrating the irreversibility of the process.

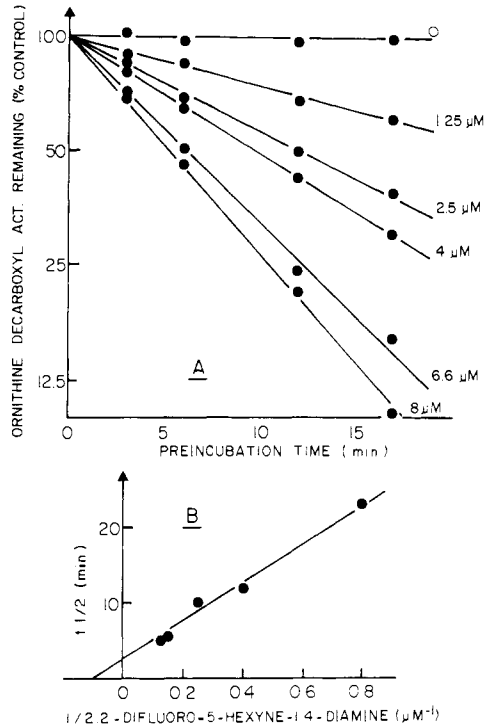


Figure 1. Time- and concentration-dependent inhibition of rat liver ODC in vitro. (A) ODC was incubated at 37 °C with 30 mM phosphate buffer (pH 7.1), 5 mM dithiothreitol, 0.1 mM pyridoxal phosphate, and the indicated concentrations of **1**. At given time intervals, 25-μL aliquots were assayed for remaining enzyme activity according to a published procedure.[5] (B) The times of half-inactivation ($t_{1/2}$) are plotted against the reciprocal of the inhibitor concentration, according to Kitz and Wilson.[13]

The selectivity of 2,2-difluoro-5-hexyne-1,4-diamine (**1**) was explored in vitro. The compound, similarly to other diamines,[16] activates *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50), but the activation is weak (K_a = 1 mM) compared to that of putrescine (K_a = 16 μM). We found that **1** is a potent competitive inhibitor of hog kidney diamine oxidase (K_i = 2.7 μM) and of rat aorta semicarbazide-sensitive amine oxidase (K_i = 0.1 μM), two Cu^{2+}-dependent amine oxidases (EC 1.4.3.6). As expected,

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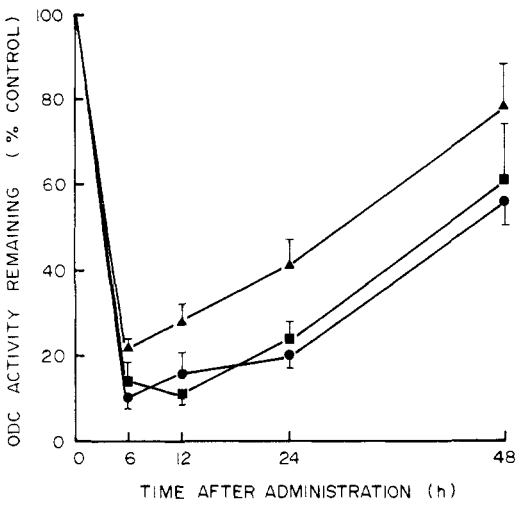

Figure 2. Effect of a single administration of 1 on ODC activity in ventral prostate, testis, and thymus. Rats were given 1 (50 mg/kg body weight) by gavage at time zero. At given time intervals animals were sacrificed, and ODC activity was immediately measured in ventral prostate (●), testis (■), and thymus (▲) as described in the Experimental Section. ODC activities are expressed as percentage of control value, each value being the mean ± SEM of five animals. In control animals, ODC activities were 310 ± 60, 35 ± 3, and 60 ± 6 nmol of CO_2 h^{-1} (g of wet tissue)$^{-1}$ in ventral prostate, testis, and thymus, respectively.

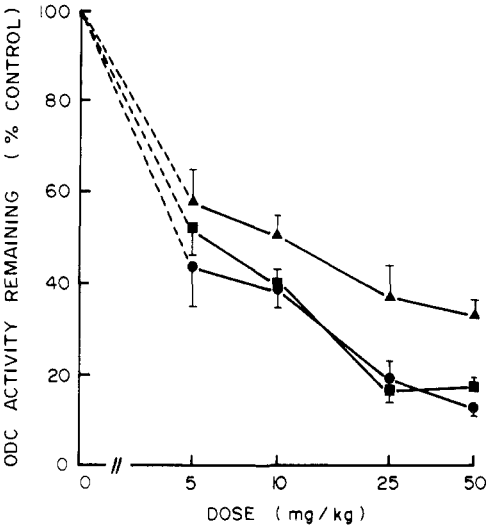

Figure 3. Dose–effect relationship between a single oral administration of 1 and ODC activity in ventral prostate (●), testis (■), and thymus (▲). ODC activity was measured 6 h after administration of the compound to rats and is expressed as percentage of control values (see legend of Figure 2). Each value is the mean ± SEM of five animals.

it is not a substrate of rat liver mitochondrial monoamine oxidase (EC 1.4.3.4). When used at 0.1 and 1 mM, the formation of H_2O_2 was less than 2% of that detected with 0.1 mM benzylamine; moreover, no inhibition of benzylamine oxidation occurred at these concentrations.

Since 2,2-difluoro-5-hexyne-1,4-diamine (1) is almost as potent in vitro as the two best inhibitors of ODC reported so far, i.e. (2*R*,5*R*)-6-heptyne-2,5-diamine (K_i = 3 μM, $\tau_{1/2}$ = 1.7 min)[7] and (*E*)-2-(fluoromethyl)dehydroornithine (K_i = 2.7 μM, $\tau_{1/2}$ = 2.6 min),[17] we have tested the compound in vivo. As expected from the in vitro data, 2,2-difluoro-5-hexyne-1,4-diamine (1) produces a strong inhibition of ODC in various rat tissues (Figures 2 and 3). The inhibition is rapid, long-lasting, and dose-dependent. However, as distinct from the enzyme-activated irreversible inhibitors of ODC studied so far,[7,18-20] 2,2-difluoro-5-hexyne-1,4-diamine (1) causes an ODC inhibition as pronounced in the testis as in the ventral prostate. Furthermore, the inhibition in these two organs is more prolonged than that observed for most of the other inhibitors except the esters of (*E*)-2-(fluoromethyl)dehydroornithine,[20] and in the thymus it is more prolonged than that observed even for these esters.

Conclusion

2,2-Difluoro-5-hexyne-1,4-diamine (1) is a potent time-dependent inhibitor of mammalian ODC, and our data are consistent with this compound being an enzyme-activated irreversible inhibitor. The presence of the fluorine substituents prevents oxidation by monoamine oxidase, as was predicted. The capability of 2,2-difluoro-5-hexyne-1,4-diamine (1) to inhibit ODC in tissues as different as ventral prostate, thymus, and testis with an almost equal efficiency and its particularly long duration of action were, however, somewhat unexpected. It has been established that the various ODC inhibitors so far available have different biological profiles.[3] The interesting properties of 2,2-difluoro-5-hexyne-1,4-diamine (1) warrant an examination of its pharmacological properties.

Experimental Section

Melting points were obtained with a Büchi melting point apparatus. ^{1}H NMR data were recorded on a Varian Associates EM-390 spectrometer (90 MHz) with Me_4Si as internal standard. ^{19}F NMR data were obtained with the same instrument (84.7 MHz) with C_6F_6 as internal standard. Chemical shifts are reported in ppm relative to $CFCl_3$ = 0 ($\delta(C_6F_6)$ = 163 ppm).

2,2-Difluoro-4-penten-1-ol (3). A solution of ethyl 2,2-difluoro-4-pentenoate (2) (147 g, 0.9 mol) in absolute EtOH (350 mL) was added dropwise to stirred slurry of $NaBH_4$ (34 g, 0.9 mol) in absolute EtOH (550 mL). The temperature of the mixture was allowed to rise to 34 °C, whereupon cooling (ice/salt bath) was applied and the temperature was maintained at 16 °C by controlling the rate of addition of the ester. Upon completion of the addition (2 h), the cooling bath was removed and the mixture was stirred at room temperature for 15 h. The EtOH was evaporated and the residue was taken up in CH_2Cl_2 (500 mL). H_2SO_4 (350 mL, 4 N) was added, the phases were separated, and the aqueous phase was extracted twice with CH_2Cl_2. The organic phases were combined, washed twice with H_2SO_4 (2 N), dried over Na_2SO_4, and evaporated. Distillation of the residue gave the alcohol 3: yield 111 g (100%); bp 44–50 °C (12 Torr); ^{1}H NMR ($CDCl_3$) δ 6.1–5.7 (1 H, m), 5.3 (2 H, m), 3.70 (2 H, t, *J* = 12 Hz), 3.2 (1 H, br s), 2.70 (2 H, dt, *J* = 6, 15 Hz); ^{19}F NMR ($CDCl_3$) δ 109 (tt, *J* = 12, 15 Hz).

Anal. ($C_5H_8F_2O$) calcd: C, 49.18; H, 6.60. Found: C, 50.72; H, 7.33.

2,2-Difluoro-4-pentenyl Trifluoromethanesulfonate (4). To a solution of alcohol 3 (78 g, 0.64 mol) and pyridine (55.7 mL, 0.7 mol) in CH_2Cl_2 (500 mL), cooled under N_2 to 0 °C, was added dropwise a solution of $(CF_3SO_2)_2O$ (204 g, 0.7 mol) in CH_2Cl_2 (250 mL). Upon completion of the addition (45 min) the mixture was allowed to warm to room temperature and stirred for 30 min. It was then cooled to 0 °C and H_2O (350 mL) was added. The phases were separated, and the aqueous phase was extracted twice with CH_2Cl_2. The organic phases were combined, washed twice with H_2O, dried over $MgSO_4$, and evaporated. Distillation of the residue gave triflate 4: yield 134 g (83%); bp 50–52 °C (12 Torr); ^{1}H NMR ($CDCl_3$) δ 6.0–5.5 (1 H, m), 5.3 (2 H, m), 4.50 (2 H, t,

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J = 11 Hz), 2.75 (2 H, dt, J = 6, 15 Hz); ^{19}F NMR ($CDCl_3$) δ 75 (3 F, s), 106 (2 F, tt, J = 11, 15 Hz).

Anal. ($C_6H_7F_5O_3S$) H; C: calcd, 28.35; found, 29.08.

2,2-Difluoro-1-phthalimido-4-pentene (5). A mixture of triflate 4 (130 g, 0.51 mol) and potassium phthalimide (123 g, 0.67 mol) in DMF (1.2 L) was heated under N_2 at 120–130 °C (oil bath temperature) for 21 h. The mixture was cooled to 10 °C, and H_2O (2 L) was added. The mixture was extracted with Et_2O (4 × 1.5 L). The combined extracts were washed with NaOH (3 × 150 mL, 2 N) and H_2O (3 × 500 mL), dried over $MgSO_4$, and evaporated to give phthalimide **5**: yield 115 g (90%); mp 74–77 °C; ^{1}H NMR ($CDCl_3$) δ 7.9 (4 H, m), 5.9 (1 H, m), 5.3 (2 H, m), 4.10 (2 H, t, J = 14 Hz), 2.75 (2 H, dt, J = 7, 16 Hz); ^{19}F NMR ($CDCl_3$) δ 102.5 (tt, J = 16, 14 Hz).

A sample was crystallized from Et_2O/pentane, mp 78–80 °C. Anal. ($C_{13}H_{11}F_2NO_2$) C, H, N.

3,3-Difluoro-4-phthalimidobutanal (6). A solution of olefin **5** (115 g, 0.45 mol) in CH_2Cl_2 (2.2 L) was divided into batches of 50 mmol (250 mL), and ozonized (ca. 1.3 mmol of O_3/min) at −78 °C until 10 min after the appearance of a blue coloration. Me_2S (15 mL/batch) was added, and the mixture was stirred at room temperature overnight. The solution was washed twice with saturated $NaHCO_3$ and twice with H_2O, dried over $MgSO_4$, and evaporated to give the aldehyde **6** as a yellow paste: yield 108 g (94%); ^{1}H NMR ($CDCl_3$) δ 9.9 (1 H, m), 7.9 (4 H, m), 4.22 (2 H, t, J = 14 Hz), 3.07 (2 H, dt, J = 2, 17 Hz); ^{19}F NMR ($CDCl_3$) δ 99 (ddd, J = 17, 14, 2 Hz).

Trituration with cyclohexane gave a white solid, mp 76–78 °C.

2,2-Difluoro-1-phthalimido-5-hexyn-4-ol (7). A stream of dry acetylene[22] was passed through THF (40 mL) cooled to 10 °C for 15 min, the cooling both was removed, and the solution was allowed to warm to 20 °C. EtMgBr (1.47 M in THF, 85 mL, 127 mmol) was added dropwise over 20 min, and the mixture was stirred for 45 min, while the acetylene stream was maintained. The stream was then cut, and the mixture was cooled to 10 °C under N_2. A solution of aldehyde **6** (32.2 g, 12 mmol) in THF (450 mL) was added dropwise over 25 min, and the solution was stirred for 1.5 h, brought to room temperature, recooled to 10 °C, and quenched with aqueous NH_4Cl. The phases were separated, and the organic phase was washed twice with aqueous NH_4Cl and then evaporated. The aqueous phases were combined and extracted with EtOAc. The organic extract was combined with the residue from the THF phase, washed with brine, dried over $MgSO_4$, and evaporated. The residue was triturated with Et_2O to give alcohol **7** as a yellow solid: yield 20.7 g (59%); mp 147–157 °C; ^{1}H NMR (CD_3OD/$(CD_3)_2SO$) δ 7.9 (4 H, m), 4.67 (td, J = 6, 2 Hz), 4.5–3.7 (2 H, m), 3.03 (1 H, d, J = 2 Hz), 2.40 (2 H, ddd, J = 18, 15, 6 Hz); ^{19}F NMR (CD_3OD/$(CD_3)_2SO$) δ 99 (m).

A sample was recrystallized from EtOAc/hexane, mp 176–178 °C. Anal. ($C_{14}H_{11}F_2NO_3$) C, H, N.

2,2-Difluoro-1,4-diphthalimido-5-hexyne (8). A solution of alcohol **7** (8.88 g, 31.8 mmol), Ph_3P (16.7 g, 63.7 mmol), and phthalimide (5.12 g, 34.8 mmol) in THF (500 mL) was cooled in ice under N_2. Diethyl azodicarboxylate (7.5 mL, 47.4 mmol) was added dropwise over 10 min, and the mixture was stirred at 0 °C for 2 h and at room temperature for 65 h. The THF was evaporated, and the residue was purified by flash chromatography[23] (800 g of SiO_2, eluant EtOAc + hexane 1:1, then neat EtOAc). The fractions containing the product **8** (R_f 0.34, EtOAc + hexane 1:1) and Ph_3P were further purified by trituration with Et_2O. The fractions containing **8** and $EtO_2CNH{\cdot}NHCO_2Et$ were purified by trituration with MeOH. The two fractions were combined: yield 4.85 g, 36%; ^{1}H NMR ($CDCl_3$) δ 7.8 (8 H, m), 5.60 (1 H, ddd, J = 9, 4, 2 Hz), 4.08 (2 H, t, J = 14 Hz), 3.5–2.5 (2 H, m), 2.43 (1 H, d, J = Hz); ^{19}F NMR ($CDCl_3$) δ 104 (m).

A sample was triturated once more with MeOH: mp 201–203 °C. Anal. ($C_{22}H_{14}F_2N_2O_4$) H, N; C: calcd, 64.71; found, 63.86.

1,4-Bis(*tert*-butylcarbamoyl)-2,2-difluoro-5-hexyne (9). A solution of bis-phthalimide **8** (4.85 g, 11.9 mmol) and $MeNHNH_2$ (2.46 mL, 46 mmol) in MeOH/THF (50 mL + 50 mL) was heated at gentle reflux under N_2 for 22 h. The mixture was cooled to room temperature, and the solvents were evaporated, finally with EtOH azeotrope. The residue was suspended in MeOH (200 mL), water (10 mL) and concentrated HCl (20 mL) were added, and the mixture was stirred for 30 min. The solids were removed by filtration and washed with H_2O. The filtrate and washings were evaporated, and the residue was taken up in H_2O/THF (25 mL + 25 mL). Na_2CO_3 (5 g, 47 mmol) and $(BOC)_2O$ (10 g, 46 mmol) were added, and the mixture was stirred at room temperature overnight and then extracted with Et_2O (3 × 50 mL). The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography[23] (200 g of SiO_2, eluant pentane + Et_2O, 2:1) to give the bis-carbamate **9**: yield 2.2 g (53%); R_f (pentane + Et_2O 2:1) 0.31; ^{1}H NMR ($CDCl_3$) δ 4.8 (3 H, m), 3.57 (2 H, dt, J = 6, 14 Hz), 2.3 (3 H, m), 1.47 (18 H, s); ^{19}F NMR ($CDCl_3$) δ 104 (m).

A sample was recrystallized from pentane: mp 108–115 °C. Anal. ($C_{16}H_{26}F_2N_2O_4$) C, H, N.

2,2-Difluoro-5-hexyne-1,4-diamine Dihydrochloride (1). To a solution of bis-carbamate **9** (2.2 g, 6.3 mmol) in Et_2O (10 mL) was added Et_2O/HCl (20 mL). The mixture was stirred at room temperature for 4 days. The precipitate was collected, washed with Et_2O, and recrystallized from MeOH/iPr_2O to give diamine dihydrochloride **1**: yield 1.09 g (78%); mp >200 °C dec; ^{1}H NMR (CD_3OD) δ 4.4 (1 H, m partially obscured by OH signal), 3.70 (2 H, t, J = 15 Hz), 3.43 (1 H, d, J = 2 Hz), 2.80 (2 H, dt, J = 7, 15 Hz); ^{19}F NMR (CD_3OD) δ 103 (q, J = 15 Hz).

Anal. ($C_6H_{10}F_2N_2{\cdot}2HCl$) C, H, N.

Enzyme Preparations and Inhibition Studies. ODC preparation was obtained from the liver of rats which had been injected with thioacetamide (150 mg/kg of body weight) 18 h before sacrifice and was purified about 10-fold by acid treatment at pH 4.6 as described by Ono et al.[24] The specific activity of this preparation was 0.2 mmol of CO_2 min^{-1} (mg of protein)$^{-1}$. The kinetic constants of the time-dependent inhibition were determined essentially as described previously.[5] *S*-Adenosyl-L-methionine decarboxylase was prepared and assayed for determination of activation constant as already described.[16] Preparation of mitochondrial monoamine oxidase, assay of its activity, and assay of hog kidney diamine oxidase (Sigma, St. Louis, MO) were likewise performed according to ref 16. Rat aorta semicarbazide-sensitive amine oxidase was prepared and assayed according to ref 25.

Measurement of Ornithine Decarboxylase Activity (ex Vivo). Male rats of the Sprague–Dawley strain (200–220 g body weight) were purchased from Charles River. Animals had access to standard diet and water ad libitum and were kept under a constant 12 h light/12 h dark lighting schedule. They were killed by decapitation at about the same time of day to minimize effects due to diurnal fluctuations. Drugs dissolved in water were given by gavage. Rats given water served as controls. Immediately after sacrifice the ventral prostate, testis, and thymus were excised and homogenized, and the corresponding ODC activities were measured according to a published procedure.[16]

Data Processing. Kinetic constants were calculated from Kitz and Wilson plots[13] (time-dependent inhibition) and from Dixon plots[26] (competitive inhibitions), using a least-squares fit of the data points with a Hewlett-Packard 9820 calculator. ODC activities, measured ex vivo, were the mean ± SEM of five animals.

Acknowledgment. We wish to thank Dr. Peter Haslam for technical assistance, the staff of the Analytical Chemistry Section for the microanalyses, and Nicole Kuentz for typing the manuscript.

Registry No. 1, 110483-06-2; 1·2HCl, 110483-05-1; **2**, 110482-96-7; **3**, 110482-97-8; **4**, 110482-98-9; **5**, 110482-99-0; **6**, 110483-00-6; **7**, 110483-02-8; **8**, 110483-03-9; **9**, 110483-04-0; ODC, 9024-60-6.

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