Synthesis of a Tritium-Labeled Indolidan Analogue and Its Use as a Radioligand for Phosphodiesterase-Inhibitor Cardiotonic Binding Sites

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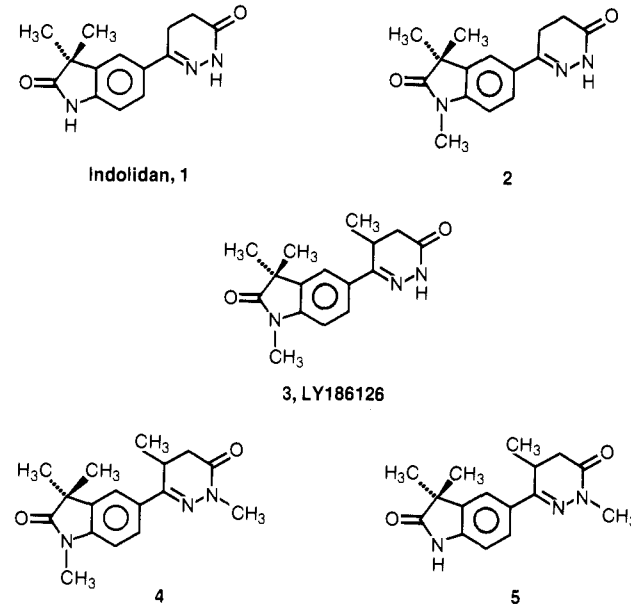
We have radiolabeled a structural analogue of indolidan, a potent phosphodiesterase-inhibitor cardiotonic, to permit biochemical studies regarding the interaction of this class of drugs with their pharmacological receptor. [^{3}H]-LY186126 (1,3-dihydro-3,3-dimethyl-1-[$^3H_3$]methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; [^{3}H]-**3**) was selected as a synthetic target because of its potency as a cardiotonic and the ability to readily incorporate three tritia via the indolone *N*-CH_3 substituent. Alkylation of a desmethyl precursor with tritium-labeled iodomethane resulted in [^{3}H]-**3** with a radiochemical purity of 98% and a specific activity of 79.2 Ci/mmol. This radioligand binds with high affinity to myocardial membrane vesicles. The binding was saturable, and K_d and B_{max} values of 4.1 nM and 383 fmol/mg protein were obtained. A series of indolidan congeners displaced [^{3}H]-**3** bound to myocardial vesicles, and K_i values for inhibition of binding were highly correlated with canine inotropic ED_{50} values, suggesting the specific binding of [^{3}H]-**3** to cardiac vesicles is pharmacologically relevant.

Several new classes of drugs have been introduced recently for treatment of congestive heart failure, a highly malignant and debilitating disease.[1-3] These include angiotensin converting enzyme inhibitors such as captopril[4] and enalapril[5] and cardiotonics such as amrinone.[6,7] Research stimulated by amrinone has spawned several interesting cardiotonics that have been studied clinically, including milrinone,[8-10] enoximone,[11,12] isomazole,[13-15] and indolidan.[16-20]

This class of cardiotonics displays inotropic and vasodilator activities both in vitro and in vivo, and both activities appear to contribute to the salutary hemodynamic effects these agents produce in congestive heart failure patients.[21-23] The inotropic and vasodilator effects of these drugs appear to result from their ability to inhibit phosphodiesterase.[20,24,25] The resulting increases in intracellular cyclic AMP (cAMP) concentrations stimulate contractility in the myocardium and in the vasculature produce a decrease in resistance. Phosphodiesterase exists in several isozymic forms, and in contrast to agents such as theophylline, which are relatively nonselective for the various isozymes, this class of cardiotonics selectively inhibits the low-K_m, cGMP-inhibitable form of the enzyme, often referred to as phosphodiesterase III (PDE III).[24,25] Currently recommended nomenclature for this isozymic form of PDE is Type IV PDE.[26] We discovered that indolidan (Chart I, compound 1), a cardiotonic currently

Chart I



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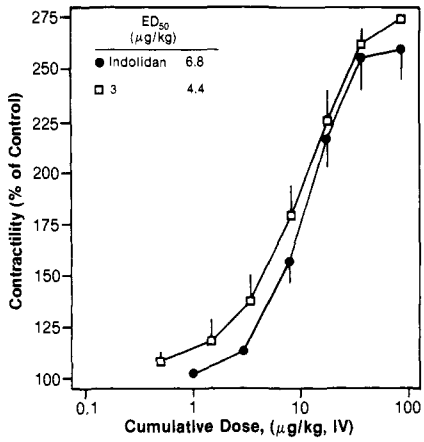


Figure 1. Dose-dependent effects of indolidan and **3** on myocardial contractility in pentobarbital-anesthetized dogs. Increasing doses of drug were administered at 5-min intervals and peak responses recorded. Each point is the mean ± SEM of experimental values. Symbols without error bars indicate that errors fell within the area of the symbols. Control (base line) values were as follows: contractility, 50 g tension; heart rate, 127 ± 3 beats/min; mean arterial blood pressure, 99 ± 3 mmHg.

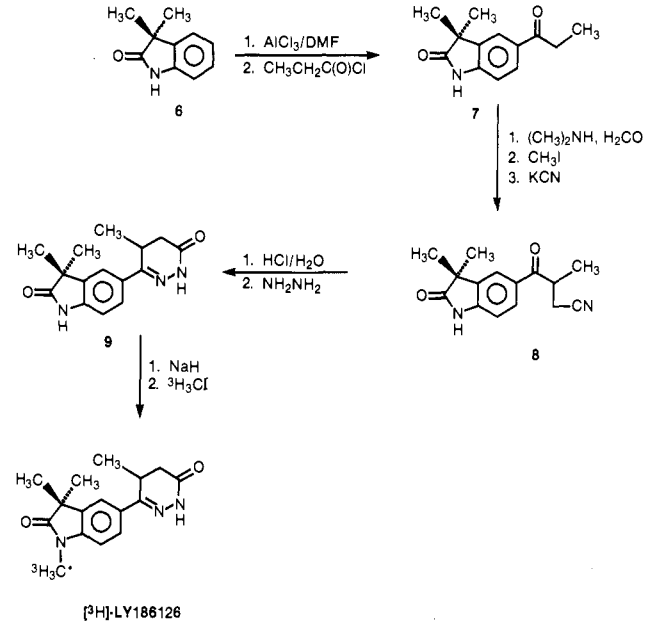
undergoing extensive clinical evaluation, was a potent and highly selective inhibitor of Type IV PDE. Using it as a biochemical tool, we determined that in canine cardiac muscle the subcellular origin of Type IV PDE appears to be the sarcoplasmic reticulum and thus referred to the enzyme as SR-PDE.[20] Other investigators have arrived independently at the same conclusion.[27] Using purified SR vesicles from canine ventricles, we found that indolidan is a linear competitive inhibitor of SR-PDE, with a K_i of 80 nM.[20]

A radioligand for the pharmacological receptor of the PDE-inhibitor cardiotonics would permit further studies regarding the molecular pharmacology of this class of drugs. Because of the potency and selectivity of indolidan as an inhibitor of Type IV PDE, we reasoned that it or a close congener would be a suitable radioligand candidate. In this paper we describe the synthesis of [^{3}H]-LY186126 (1,3-dihydro-3,3-dimethyl-1-[$^3H_3$]methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one; [^{3}H]-**3**) and biochemical studies demonstrating the pharmacological relevance of specific binding of this radioligand to canine cardiac vesicles.

Results and Discussion

Compound Selection. Indolidan is a potent cardiotonic, and canine studies revealed that plasma concentrations of approximately 22 ng/mL resulted in a 50% increase in contractility.[28] Therefore, we anticipated that biochemical studies would require radiolabeled material of high specific activity. Labeled iodomethane was selected as the reagent of choice since it can be obtained in high specific activity and can be used to introduce up to three tritia simultaneously. We anticipated that the indole NH of **1** could be regioselectively alkylated with iodomethane to form **2** (Chart I). However, alkylation at this site of the molecule leads to a substantial decrease in the compound's potency as an inotrope. For example, after iv administration to anesthetized dogs, inotropic ED_{50} values of indolidan and **2** were 6.8 and 52 μg/kg, respectively.[16] IC_{50} values of indolidan and **2** as inhibitors of SR-PDE were

Scheme I



150 and 760 nM, respectively, when determined with 1 μM cAMP.[20]

Fortunately, this methyl-induced diminution in potency could be counterpoised by placing a second methyl substituent at position 4 of the dihydropyridazinone ring (compound **3**, Chart I), resulting in a compound that mimics **1** biochemically and pharmacologically. The dose-dependent increases in contractility observed after iv administration of **3** were virtually identical with those elicited by **1** (Figure 1). Compound **3** appeared to be slightly more potent and efficacious than **1**, but these differences were not statistically significant. The ED_{50} values of these two drugs were 4.4 and 6.8 μg/kg, respectively. Since there is a significant correlation between the abilities of these cardiotonics to inhibit SR-PDE in vitro and their in vivo inotropic effects,[20] one would anticipate that **3** would be a potent inhibitor of SR-PDE. Its IC_{50} was 120 nM,[20] which would correspond to a K_i of approximately 40 nM. Thus, both in vitro and in vivo, **3** appears to be a suitable surrogate for **1**.

Synthesis of 3. A synthesis of **3** was devised that enabled introduction of the indolone methyl substituent in the final step (Scheme I). Friedel–Crafts reaction of 1,3-dihydro-3,3-dimethylindol-2-one (**6**) with propionyl chloride in the presence of an aluminum chloride/DMF melt produced the propiophenone **7** in 67% yield after recrystallization from THF. A Mannich reaction employing dimethylamine and formaldehyde and quaternization with iodomethane, followed by reaction with potassium cyanide produced the γ-keto nitrile **8**. Acid-catalyzed hydrolysis to the corresponding γ-keto acid, followed by hydrazine cyclization, completed construction of the dihydropyridazinone moiety of **9**.

Reaction of **9** with 1 equiv of sodium hydride, followed by 1 equiv of iodomethane led to formation of a major product. A difference nuclear Overhauser effect (NOE) experiment was conducted with this material to determine the precise regiochemistry of this alkylation. Irradiation of the *N*-methyl resonance at 3.25 ppm led to a NOE at the indolone H7 resonance at 6.86 ppm, thereby confirming the anticipated regiochemistry of the methylation reaction. Moreover, this material was identical with a sample of **3** synthesized from 1,3-dihydro-1,3,3-trimethylindol-2-one. A minor impurity was noted in the sample of **3** prepared by methylation of **9**; this corresponded to the dimethylated

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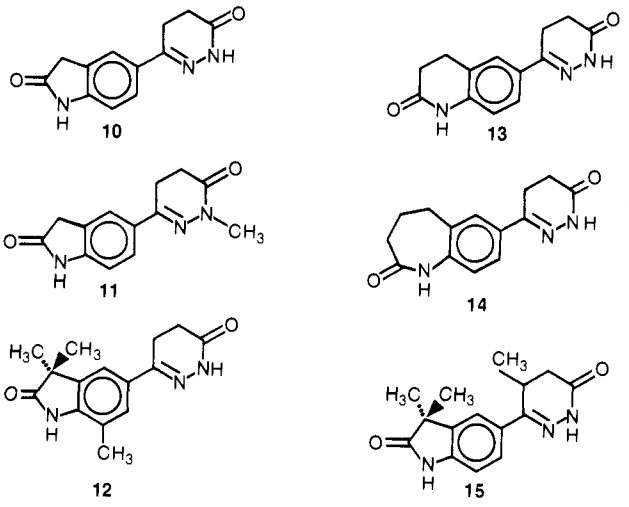
Chart II



byproduct **4** (Chart I), probably resulting from a slight excess of base in the reaction. None of the monomethyl regioisomer **5** could be detected by TLC, NMR, or HPLC. HPLC analysis revealed that the starting material **9**, product **3**, monomethyl isomer **5**, and dimethyl byproduct **4** were all well-separated; retention times were 3.12, 5.02, 5.53, and 10.47 min, respectively, when eluted from a C-18 reverse-phase column with acetonitrile/acetic acid/water (30/0.5/69.5). In a typical reaction, HPLC analysis of the unpurified reaction mixture revealed 92.5% of **3**, 1% of starting material **9**, and 6% of dimethylated byproduct **4**.

Syntheses of [^{3}H]-3. Alkylation of **9** with sodium hydride and tritium-labeled iodomethane (85 Ci/mmol) proceeded as expected to afford [^{3}H]-**3** with approximately 85% radiochemical purity. Approximately 15% of the radioactivity comigrated with dimethylated byproduct **4** upon TLC analysis. Preparative TLC followed by HPLC purification resulted in [^{3}H]-**3** with a radiochemical purity of 98%. The UV spectrum of [^{3}H]-**3** was indistinguishable from that of unlabeled material and the specific activity was calculated to be 79.2 Ci/mmol. The compound appears to be quite stable in radiolabeled form since storage for 4 months resulted in less than a 1% decrease in radiochemical purity.

Binding Studies. In preliminary communications, we described a high-affinity binding site for [^{3}H]-**3** in highly purified sarcoplasmic reticulum vesicles from canine ventricles.[29-31] Binding was saturable, proportional to vesicle protein, inactivated by protease treatment, and required divalent cations. Data were consistent with the presence of a single binding site, and K_d and B_{max} values of 3.9 nM and 890 fmol/mg protein, respectively, were obtained.[31] For the present studies, we explored the use of a less purified membrane preparation containing a mixture of both sarcolemmal and sarcoplasmic reticulum vesicles.[32] With these myocardial vesicles (MV), a single binding site with a K_d of 4.1 nM was observed for [^{3}H]-**3**, a value indistinguishable from that observed with highly purified SR vesicles. The only discernible difference was an approximately 2-fold lower B_{max} (383 fmol/mg protein); consequently, this more readily obtained myocardial vesicle preparation was utilized for all binding studies reported in this paper.

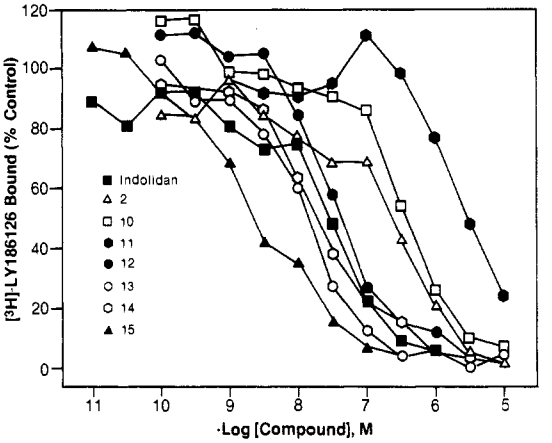


Figure 2. Displacement of [^{3}H]-LY186126 binding to crude myocardial vesicles by indolidan and some analogues. Myocardial vesicle protein was incubated with 10 mM of [^{3}H]-LY186126 either in the presence or absence of various concentrations of indolidan and its analogues. After 1 h the percent radioligand bound was determined. For additional details, consult the Experimental Section. Each data point represents the mean of triplicate experiments.

The abilities of **1** and several of its analogues (Chart II) to compete for [^{3}H]-**3** binding were studied. For these experiments myocardial vesicles were incubated with 10 nM of the radiolabel, either in the presence and absence of various concentrations of the unlabeled compound; the percent of [^{3}H]-**3** bound was determined and data are displayed in Figure 2. Compound **1** and all of the congeners specifically displaced the radioligand from myocardial vesicles in a concentration-dependent fashion, and the K_i of **1** was 5.4 nM. The descending portion of the sigmoidal concentration–response curves of the most potent compounds (**1**, **12–15**) occurred between 1 and 100 × 10^{-9} M. Concentrations of 10^{-6} M led to complete displacement of [^{3}H]-**3** binding.

The order of potency of these compounds as inhibitors of radioligand binding appeared to correlate in a qualitative sense with their potencies as positive inotropes in anesthetized dogs. For example, compound **11** has no proton on the pyridazinone nitrogen, and we have reported previously that this produced a substantial decrease in potency as a positive inotrope after iv administration to anesthetized dogs (ED_{50} = 271 μg/kg).[16] Data in Figure 2 reveal that this agent is also impotent as an antagonist of [^{3}H]-**3** binding (K_i = 61 400 nM). Alternatively, the quinolin-2-one analogue, **13**, was quite potent as a positive inotrope (iv ED_{50} = 3.3 μg/kg)[16] and as an antagonist of [^{3}H]-**3** binding (K_i = 2.5 nM). Compound **15** was the most potent antagonist of radioligand binding (K_i = 0.54 nM), and it was also the most potent inotrope in this series (iv ED_{50} = 3.2 μg/kg).

To study this correlation more quantitatively, the K_i values of these analogues as inhibitors of the radioligand binding were plotted vs their ED_{50} values as positive inotropes (Figure 3). The two phenomena appear to be highly correlated, with a highly significant ($p < 0.001$) correlation coefficient of 0.9525.

Summary and Conclusions

In this paper we have described an efficient preparation of **3** in high specific activity, tritium-labeled form. The radiolabeled form of this dihydropyridazinone cardiotonic is readily purified and appears to be surprisingly stable.

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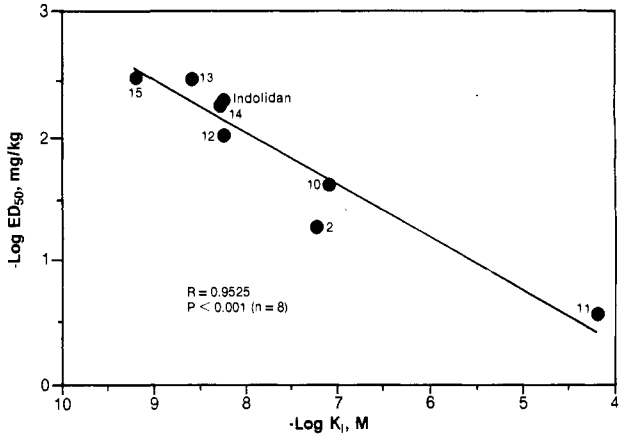

Figure 3. Correlation of ED_{50} values of indolidan and its analogues as positive inotropes (iv, anesthetized dogs) with their K_i values as antagonists of [^{3}H]-LY186126 binding to myocardial vesicles. K_i values for inhibition of radioligand binding were determined as described in the Experimental Section. Positive inotropic ED_{50} values were taken from ref 16.

Binding of [^{3}H]-**3** to myocardial vesicles was saturable and could be displaced by a series of indolidan analogues. Since the potencies of these compounds as inhibitors of [^{3}H]-**3** binding correlated well with their potencies as positive inotropes in dogs, this binding appears to be pharmacologically relevant. [^{3}H]-**3** is readily prepared in radiolabeled form and, on the basis of the data reported herein, appears to be a useful radioligand for a site of action of the PDE-inhibitor cardiotonics. As such it may be a useful biochemical tool to probe the molecular pharmacology of this class of drugs. Moreover, this binding could be performed with relatively crude myocardial vesicles, indicating that displacement of [^{3}H]-**3** binding may provide a useful means to screen for phosphodiesterase-inhibitor cardiotonics.

Experimental Section

Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are not corrected. Proton magnetic resonance (^{1}H NMR) spectra were obtained by use of a Bruker WM-270 spectrometer. Difference NOE (nuclear Overhauser effect) spectra were obtained by substracting free induction decays accumulated with the decoupler off-resonance from similar accumulations with particular resonances irradiated, followed by Fourier transformation of the difference signals. The procedure was not optimized for maximum NOE measurement; the usual irradiation period was 2.0 s, followed by a preaccumulation delay of 0.03 s. Mass spectra were recorded from a Varian MAT CH-5 spectrometer. HPLC studies were performed on a Waters 6000A chromatograph; the detector was a Waters operated at 315 nM. For the HPLC specific activity determinations, equal fractions from the column were collected in vials containing PCS scintillation fluid (Amersham), and the radioactivity was measured in a Packard Model 3375 liquid scintillation spectrometer. Microanalytical data were provided by the Physical Chemistry Department of the Lilly Research Laboratories; only symbols of elements analyzed are given, and they were within 0.4% of theoretical values unless indicated otherwise.

Except where noted, a standard procedure was used for product isolation. This involved quenching by addition to water, filtration or exhaustive extraction with a solvent (washing of extract with aqueous solutions, on occasion), drying over an anhydrous salt, and evaporation of solvent under reduced pressure. Particular solvents, aqueous washes (if needed), the drying agents are mentioned in parentheses after the phrase "product isolation". The preparations of compounds **1**, **2**, and **10–15** have been described previously.[16]

1,3-Dihydro-3,3-dimethyl-5-(1-oxopropyl)-2*H*-indol-2-one (7). Dimethylformamide (19.3 mL, 245 mmol) was added dropwise to aluminum chloride (117 g, 877 mmol). During this addition, the temperature rose to 90 °C, and then the resulting melt was allowed to cool to 50 °C. A mixture of 1,3-dihydro-3,3-dimethyl-2*H*-indol-2-one[16] (14.1 g, 87.6 mmol) and propionyl chloride (7.61 mL, 87.6 mmol) was added in portions. The reaction was stirred at 70 °C for 3 h and then the mixture was carefully poured onto ice. Concentrated hydrochloric acid (100 mL) was slowly added, and the mixture was cooled to 0 °C overnight. The precipitate was filtered and recrystallized from THF to provide 12.7 g (67%) of product as a white solid with a mp of 207–210 °C; mass spectrum (70 eV), *m/e* (relative intensity) 217 (28, M^+), 188 (100). Anal. ($C_{13}H_{15}NO_2$) C, H, N.

2,3-Dihydro-γ,2-dioxo-β,3,3-trimethyl-1*H*-indole-5-butanoic Acid (16). A mixture of formaldehyde (5.6 mL of a 37% aqueous solution, 69.1 mmol) and dimethylamine hydrochloride (7.0 g, 86.4 mmol) was stirred at room temperature for 30 min. Acetic anhydride (34.8 mL, 369 mmol) was added, the reaction was heated to 45 °C, an exothermic reaction ensued, and the temperature reached 110 °C. After the reaction cooled to 90 °C, 1,3-dihydro-3,3-dimethyl-5-(1-oxopropyl)-2*H*-indol-2-one (12.5 g, 57.6 mmol) was added in one portion. The reaction was heated to 120 °C for 3 h and cooled. Acetone (200 mL) was added and the mixture was refluxed for 20 min. Solvents were removed in vacuo, water was added, and the solution was extracted thrice with methylene chloride (discarded). Product isolation (6 N sodium hydroxide, methylene chloride, brine, Na_2SO_4) provided 5.4 g of the Mannich base as an oil.

Iodomethane (26.1 mL, 419 mmol) was added to a solution of the unpurified Mannich base (5.4 g, 19.7 mmol) in 50 mL of acetone. The reaction was stirred at room temperature for 72 h and then refluxed for 1 h. Solvent was removed to provide 6.8 g of quaternarized product as an oil.

A solution of potassium cyanide (2.6 g, 39.2 mmol) in 50 mL of water was added to a solution of the quaternarized Mannich base (6.8 g, 16.3 mmol) in 50 mL of methanol, and the reaction was stirred overnight. Product isolation (water, ethyl acetate, water, brine, Na_2SO_4) and flash chromatography (0–2% methanol in methylene chloride gradient, silica gel) provided 2.7 g of homogeneous nitrile **8** as a foam: ^{1}H NMR ($CDCl_3$) δ 1.42 (d, 3, $CHCH_3$), 1.46 (s, 6, $C(CH_3)_2$), 2.70 (m, 2, CH_2), 3.80 (m, 1 $CHCH_3$), 7.02 (d, 1, indolone H7), 7.85 (s, 1, indolone H4), 7.86 (d, 1, indolone H6); mass spectrum (70 eV) *m/e* (relative intensity) 256 (24, M^+), 188 (100).

A mixture of this nitrile (2.7 g, 10.5 mmol) and 33 mL of 6 N hydrochloric acid were refluxed for 3 h. The mixture was cooled, diluted with water, and stored at 0 °C overnight. The precipitate was recrystallized from DMF/water to provide 2.08 g (72%) of the titled carboxylic acid: mp 232–234 °C; ^{1}H NMR (DMSO-d_7) δ 1.10 (d, 3, $CHCH_3$), 1.30 (s, 6, $C(CH_3)_2$), 2.39 and 2.73 (dd and dd, each 1, CH_2), 3.88 (m, 1, $CHCH_3$), 6.97 (d, 1, indolone H7), 7.93 (d, 1, indolone H6), 7.94 (s, 1, indolone H4); mass spectrum (70 eV) *m/e* (relative intensity) 275 (20, M^+), 188 (100). Anal. ($C_{15}H_{17}NO_4$) C, H, N.

1,3-Dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one (9). A mixture of 2,3-dihydro-γ,2-dioxo-β,3,3-trimethyl-1*H*-indole-5-butanoic acid (1.0 g, 3.64 mmol) and hydrazine hydrate (471 μL of an 85% aqueous solution, 8.0 mmol) in 10 mL of ethanol was refluxed for 4 h and then slowly cooled to 0 °C. The precipitate was filtered to afford 790 mg (80%) of product as white crystals with mp 267–269 °C; mass spectrum (70 eV) *m/e* (relative intensity) 271 (100, M^+), 256 (76). Anal. ($C_{15}H_{17}N_3O_2$) C, H, N.

1,3-Dihydro-1,3,3-trimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one (3; LY186126). Sodium hydride (19 mg of a 60% dispersion in oil, 0.476 mmol) was added in one portion to a solution of 1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl-2*H*-indol-2-one (129 mg, 0.476 mmol) in 5 mL of dry DMF, and the reaction was stirred for 15 min at room temperature. Iodomethane (740 μL of a 0.64 M solution in DMF, 0.476 mmol) was added in one portion and the reaction was allowed to proceed for 2 h. Water was added and the mixture was extracted thrice with ethyl acetate. The organic layer was washed twice with water and then brine and dried (Na_2SO_4). Removal of solvent under reduced pressure provided 140 mg of essentially homogeneous product. HPLC analysis using a Waters C-18 4.5 mm × 25 cm column eluted with acetonitrile/acetic acid/water (30/0.5/69.5) at 2300 psi and at

a flow rate of 2 mL/min revealed this sample was composed of LY186126 (92.5%), starting material **9** (1%), and dimethylated byproduct 4 (6%); retention times were 5.02, 3.12, and 10.47 min, respectively. Recrystallization from THF/hexane provided 88 mg of LY186126 as white crystals with mp 217–219 °C; mass spectrum (70 eV) *m/e* 285 (100, M^+). Anal. ($C_{16}H_{19}N_3O_2$) C, H, N.

1,3-Dihydro-1,3,3-trimethyl-5-(1,4,5,6-tetrahydro-1,4-dimethyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one (4; Dimethyl Byproduct). Sodium hydride (88 mg of a 60% dispersion in oil, 2.2 mmol) was added to a solution of **3** (1,3-dihydro-1,3,3-trimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one) (568 mg, 2.0 mmol) in 25 mL of DMF, and the reaction was stirred for 20 min at room temperature. Iodomethane (136 μL, 2.2 mmol) was added and the reaction was stirred overnight. Product isolation (water, ethyl acetate, water, brine, Na_2SO_4) provided 550 mg (92%) of essentially homogeneous product as a white solid. An analytical sample was prepared by recrystallization from ethyl acetate to afford **4** as white crystals with mp 178–179 °C; mass spectrum (70 eV) *m/e* 299 (100, M^+). Anal. ($C_{17}H_{21}N_3O_2$) C, H, N.

1,3-Dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-1,4-dimethyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one (5; Monomethyl Byproduct). Methylhydrazine (108 μL, 2 mmol) was added to a suspension of 2,3-dihydro-γ,2-dioxo-β,3,3-trimethyl-1*H*-indole-5-butanoic acid (254 mg, 0.92 mmol) in 2.5 mL of ethanol, and the reaction was refluxed for 7 h. After cooling, solvent was removed and the product was recrystallized from THF to afford 165 mg of **5** as a white solid with mp 229–232 °C; mass spectrum (70 eV) *m/e* (relative intensity) 285 (100, M^+). Anal. ($C_{16}H_{19}N_3O_2$) C, H, N.

1,3-Dihydro-3,3-dimethyl-1-[$^3H_3$]methyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one ([^{3}H]-3). Sodium hydride (6.6 mg of an 80% dispersion in oil, 0.22 mmol) was added to a solution of 1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-indol-2-one (59.5 mg, 0.22 mmol) in DMF at room temperature. After 15 min a solution of [^{3}H]iodomethane (25 Ci at 85 Ci/mmol; 0.29 mmol) in 4 mL of THF was added, and the reaction was stirred for 2 h at room temperature. Volatile materials were removed in vacuo. Product isolation (ethyl acetate, water, brine, Na_2SO_4) provided 8.9 Ci of material that was approximately 85% radiochemically pure [^{3}H]-**3** as judged by TLC analysis.

A 400 mCi portion of this material was applied to two 1000-μm silica GF TLC plates, and they were developed with chloroform/methanol (40/1); the major UV band was removed and was eluted with ethanol to provide approximately 200 mCi of radioactivity. This material was further purified by HPLC on a Techsphere ODS (5 μm, 25 cm × 4.6 mm) column eluted isocratically with acetonitrile/THF/water/acetic acid (100/25/375/0.5) to provide 190 mCi of [^{3}H]-**3** with a radiochemical purity of 98%.

Determination of Specific Activity. Compound **3** has an ultraviolet absorbance pattern with a maximum at 306 nm (ϵ = 23 875). The UV spectrum of [^{3}H]-**3** was indistinguishable from that of unlabeled compound. The concentration of a sample of [^{3}H]-**3** in ethanol was measured with the absorbance at 306 nM, and the radioactivity present was then determined. These measurements were repeated on samples containing various concentrations of labeled compound. The specific activity was calculated to be 79.2 Ci/mmol.

Pharmacological Methods. Experiments in Anesthetized Dogs. Mongrel dogs of either sex (7–14 kg) were anesthetized with sodium pentobarbital (35 mg/kg, iv). A positive-pressure pump was used to ventilate dogs through an endotracheal tube (18 strokes/min, 20 mL/kg per stroke) and a heating pad maintained body temperature at 37–38 °C. Femoral arterial blood pressure was measured through a polyethylene catheter filled with heparin solution (16 units/mL) and connected to a Statham pressure transducer. The femoral vein was cannulated for iv drug administration. Heart rate was derived by means of a cardiotachometer that was triggered by the arterial pressure pulse. A Walton-Brodie strain-gauge arch sutured to the right ventricle of the heart measured cardiac contractility. Tension on the gauge was adjusted to 50 g, which corresponded to 10 mm of recorder pen deflection. Rapid iv injection of 50 mL of 5% dextran and mechanical compression of the aorta demonstrated that contractility measurements were independent of changes in preload and afterload. Subcutaneous pin electrodes provided a lead II ECG. Increasing doses of test compounds were administered iv in volumes of 0.25–4.0 mL at 5-min intervals; no responses occurred with appropriate vehicle injections. ED_{50} values were determined by linear regression analysis.

Preparation of Myocardial Vesicles. Myocardial vesicles (MV) were prepared from ventricles of pentobarbital-anesthetized dogs as described by Jones and co-workers.[32] Aliquots of the vesicles were stored at −80 °C until used. Under these conditions no loss of PDE activity was detected after 6 months of storage. Vesicle protein was determined by the method of Lowry.[33]

Binding of [^{3}H]-3 to Myocardial Vesicles. Displacement binding studies were carried out at 25 °C using canine myocardial vesicles. Approximately 100 μg of vesicle protein was incubated for 60 min in 1.0 mL of 50 mM Tris·Cl, pH 7.4, 5 mM $MgCl_2$, 0.01% bovine serum albumin (BSA, fraction V), and 10 nM [^{3}H]-**3** (79.2 Ci/mmol), either in the presence or absence of displacing ligands. Upon completion of incubation, the membrane suspension was filtered with a vacuum apparatus (Brandel cell harvestor) onto Whatman GF/C filters pretreated with 50 mM Tris·Cl, pH 7.4, 0.05% BSA to minimize nonspecific binding. Filters were washed four times with cold (0–4 °C) 50 mM sodium phosphate, pH 7.4. Total radioactivity retained by the filter was determined by scintillation techniques. Specific binding was defined as the portion of total bound radioactivity displaced by 100 μM indolidan. Solutions of displacing ligands were prepared in DMSO; this solvent had no detectable effect on binding of [^{3}H]-**3**. The concentration of displacing ligands was varied over a range of 0.003–100 μM in half log increments, and binding determinations were performed in triplicate. K_i values were obtained from displacement curves by nonlinear least squares analysis using LIGAND[34] and LUNDON software (LUNDON Software, Inc.; Cleveland). Protein concentrations were determined by BCA protein assay[35] using BSA (fraction V) as protein standard.

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Registry No. **1**, 100643-96-7; **3**, 100644-00-6; [$^3H_3$]-**3**, 120476-88-2; **4**, 120476-83-7; **5**, 120476-84-8; **6**, 19155-24-9; **7**, 120476-85-9; **7** Mannich base derivative, 120496-33-5; **7** quaternized Mannich base derivative, 120476-87-1; **8**, 120476-86-0; **9**, 100644-04-0; **10**, 70386-01-5; **11**, 101345-97-5; **12**, 100644-01-7; **13**, 101345-95-3; **14**, 103240-37-5; **15**, 100644-04-0; **16**, 100644-10-8; phosphodiesterase, 9025-82-5.

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