Supporting Information

Combination of a Beta Adrenoceptor Modulator and a Norepinephrine-Serotonin Uptake Inhibitor for the Treatment of Obesity

Cynthia D. Jesudason, James E. Baker, Robert D. Bryant, Jack W. Fisher, Libbey S. O'Farrell, Gregory A. Gaich, Minxia M. He, Steven D. Kahl, Aidas V. Kriauciunas, Mark L. Heiman, Mary A. Peters, Christopher J. Rito, Julie H. Satterwhite, Frank C. Tinsley, William G. Trankle and Anthony J. Shuker

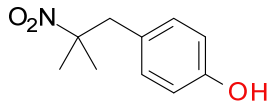
Table of Contents

A: Experimental Procedures for the synthesis of compound 4 and intermediates (7, 8, 9, and 10) and selected characterization

General Methods. All reagents and solvents for the synthesis of the compounds were analytical grade, purchased from Aldrich and used without further purification. ^{1}H NMR data were collected on a Varian System 500 spectrometer equipped with a 5 mm carbon cold probe and with the sample temperature maintained at 25 ^{o}C. Spectra were obtained employing a 45 degree pulse, 64 K datapoints, and a relaxation delay of 5.9 seconds. Spectral window was set from (-1) to 15 ppm. LCMS were obtained on an Agilent HP 1100 LCMS instrument equipped with a Waters Xterra C18 2.1 x 50mm 3.5µm column at a 50 ± 10 °C column temp using a 5-100% ACN/MEOH (50/50) w/0.2% NH_4Formate in 7.0 min then held at 100% for 1.0 min and a 1.0ml/min flow rate. High-resolution (positive ion) mass spectra (HRMS) were acquired using a Agilent LCMS with a TOF/QTOF mass spectrometer using both positive and negative ESI (electrospray) using 2uL inj vol of ~1.0mg/mL sample, 5-100% B in 15 min at 0.5uL/min flow, "A"= 0.1% formic acid in water, "B"=0.1% formic acid in acetonitrile, room temp, 190-700 nm UV/VIS on Zorbax SB-C8 3.0x150mm 3.5u column. Elemental analyses were determined by Schwarzkopf Microanalytical Laboratories and are ± 0.4% of the theoretical values unless otherwise indicated.

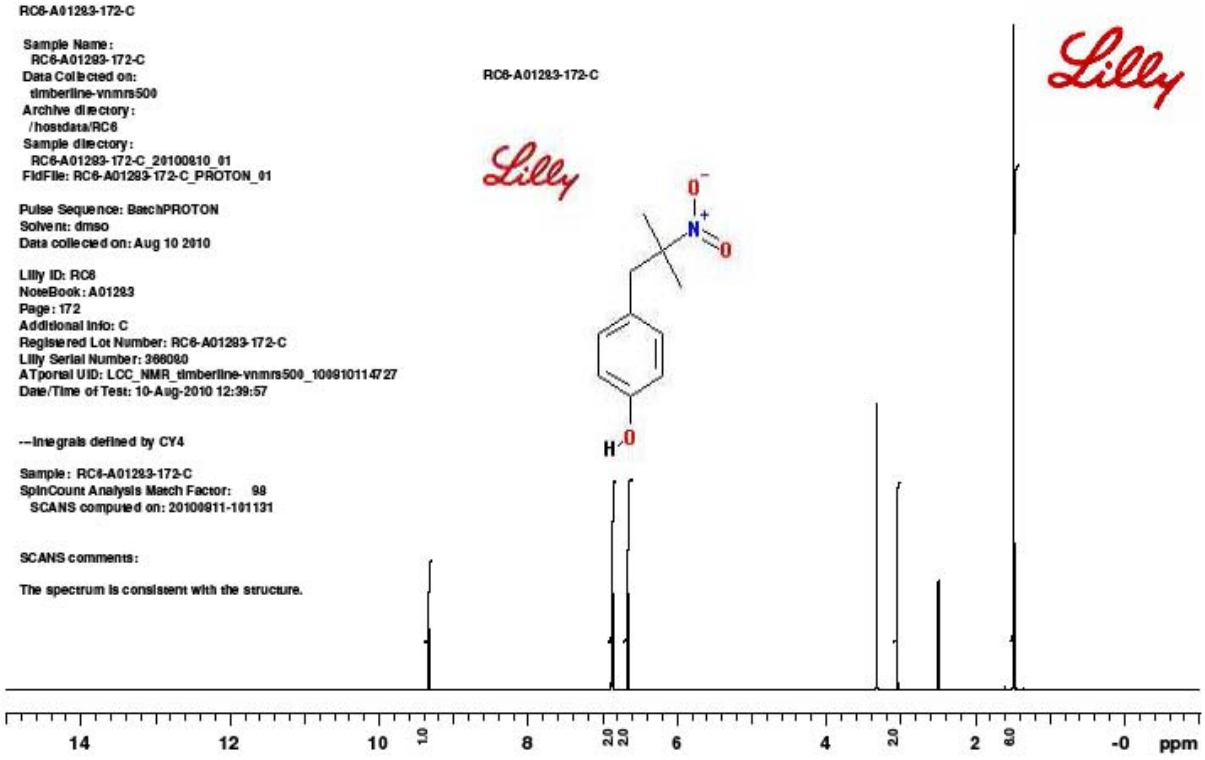
.

4-(2-methyl-2-nitropropyl) phenol

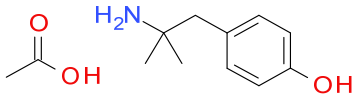


A mixture of 4-hydroxybenzyl alcohol (100.08 g, 806 mmol), 2-nitropropane (400 mL, 4.45 mol) and diglyme (800 mL) was heated to 38 °C. Potassium *t*-butoxide (45.29 g, 403.6 mmol) was added, and the mixture was heated to reflux at 132 °C with a Dean-Stark trap. Water began collecting in the trap, and continued at a high rate for approximately 1.5 h. When water collection slowed (around 2.5 h) then aliquots of solvent (30-40 mL each) were removed every thirty minutes. During the water collection and solvent removal the temperature rose from 132 °C to 149 °C. After 4 h, less than 1% of the 4-hydroxybenzyl alcohol remained by HPLC analysis. The heating mantle was removed, and the reaction mixture allowed to cool. When the temperature was 100^{o}C, water (200 mL was added, and the solution allowed to cool to room temperature. The solvent was removed on a rotary evaporator under vacuum until 593g of solution remained. Water (500 mL) and EtOAc (500 mL) were added and the layers were separated, and the aqueous layer was extracted with EtOAc (200 mL). The combined organic layers were extracted with 1*N* HCl (500 mL) and water (300 mL). The organic layer was distilled in vacuo to 261 g of oil to which EtOAc was added (160 mL). Heptane (3.4 L was added rapidly with vigorous stirring for 30 min, and the product crystallized to yield a beige solid (112.36 g, 71% yield > 98% purity by HPLC analysis). Another crop of crystals may be obtained from the filtrate by concentrating and filtering the solids, or by concentrating more fully to a solution and adding heptanes to crystallize.

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34 (s, 1H), 6.87 (d, *J* = 8.6 Hz, 2H, PhH), 6.66 (d, *J* = 8.6 Hz, 2H, PhH), 3.05 (s, 2H, CH_2), 1.48 (s, 6H, Me); Anal. Calcd for $C_{10}H_{13}NO_3$: C, 61.53; H, 6.71; N, 7.17. Found: C, 61.69; H, 6.86; N, 7.05.



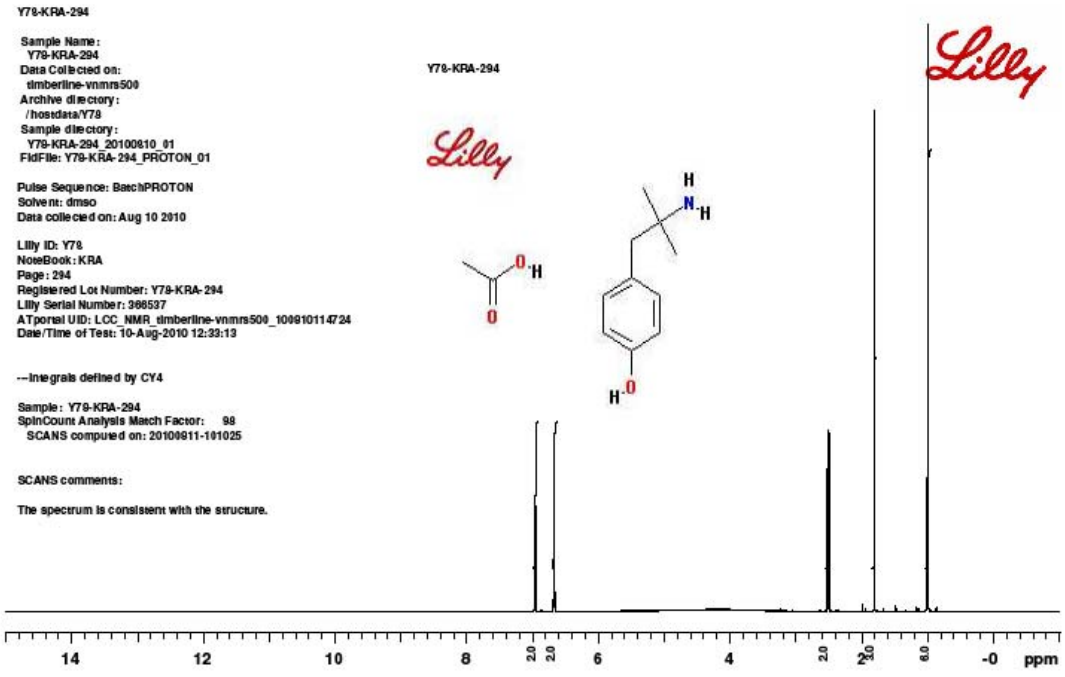
4-(2-amino-2-methylpropyl) phenol acetic acid salt



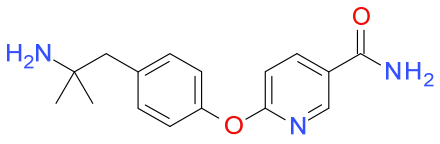
A one-gallon high-pressure reactor was charged with 4-(2-methyl-2-nitropropyl) phenol (120 g, 614 mmol), HOAc (35.2 mL, 614 mmol), 5% Palladium on carbon (24 g) wetted with EtOH (60 mL), and MeOH (1230 mL). the mixture was heated to 50 ^{o}C with agitation (600 rpm), and the reactor was purged with N_2 and pressurized to 50psi with H_2. After 15.5 h the reactor was purged with N_2, and the cooled mixture was filtered. The filter cake was washed with MeOH and the filtrate was concentrated to 514 g of slurry on a rotary evaporator. To this slurry was added EtOAc (2L) with vigorous agitation. After stirring for 1 h, the resulting crystals were filtered and washed with a small amount of EtOAc. The product was dried overnight in a 45 ^{o}C vacuum oven to yield 118.83 g (86%) of the product as small white needles

(mp 211-216 ^{o}C dec). This material was 99% pure by HPLC analysis, while another 9.0 g of material was obtained from the mother liquor, it was found to be only 88% pure.

^{1}H NMR (500 MHz, DMSO-d_6) δ 6.96 (d, *J* = 8.3 Hz, 2H, PhH), 6.67 (d, *J* = 8.3 Hz, 2H, PhH), 2.53 (s, 2H, $ArCH_2$), 1.82 (s, 3H, HOAc), 0.99 (s, 6H, CH_3); Anal. Calcd for $C_{12}H_{19}NO_3$: C, 63.98; H, 8.50; N, 6.23. Found: C, 64.08; H, 8.76; N, 6.33.



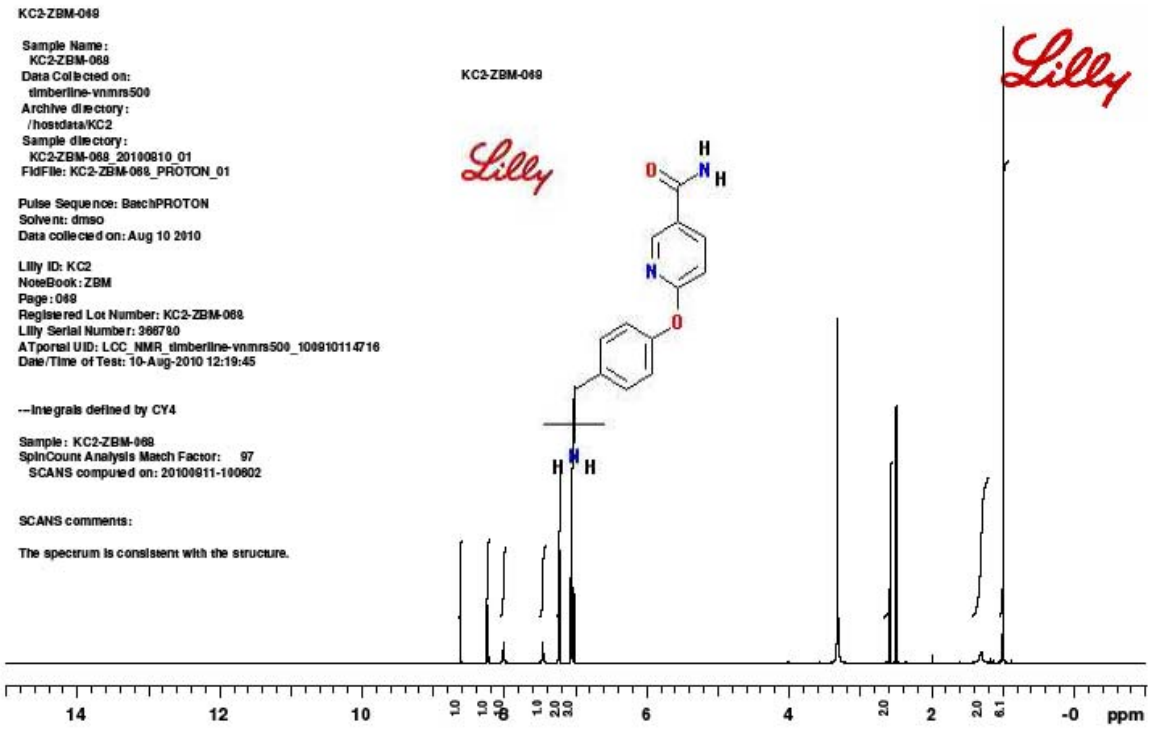
4-(2-amino-2-methylpropyl)phenoxy)-5-carboamidepyridine



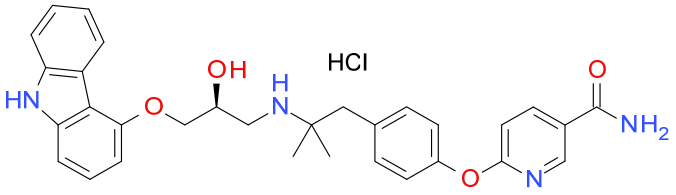
A mixture of 4-(2-amino-2-methylpropyl) phenol acetic acid salt (45.06 g, 200 mmol), powdered K_2CO_3 (691 g, 500 mmol), 6-chloronicotinamide (31.32 gm 200 mmol), DMAC (622 mL) and *iso*-octane (70 mL) was slowly heated to reflux at 140 ^{o}C. A water trap filled with *iso*-octane (70 mL) was used to collect water fromed in the reaction, and the reflux was maintained for 5.5 h. The mixture was allowed to

cool to room temperature, and the solids filtered and washed with EtOAc. The filtrate was concentrated in vacuo to 88.6 g of solid which was dissolved in EtOAc (500 mL). To this solution was added water (800 mL), 1*N* HCl (200 mL) and MeOH (50 mL). The pH of this mixture was adjusted to 7.2 with con. HCl, and the aqueous layer was separated and washed with methyl *t*-butyl either (500 mL). The product was crystallized by addition of 10 *N* NaOH (20 mL) which raised the pH to 11. This pH was maintained by addition of 10 *N* NaOH as needed during the course of the crystallization (90 min). The product was filtered, washed with water and dried in vacuo at 45 ^{o}C to 53.11 g (93%) of white solid which was > 99% pure by HPLC analyses.

^{1}H NMR (500 MHz, DMSO-d_6) δ 8.62 (d, *J* = 2.26, 1H), 8.24 (dd, *J* = 8.67, 2.26, 1H), 8.01 (br s, 1H), 7.46 (br s, 1H), 7.23 (d, *J* = 8.29 Hz, 2H, PhH), 7.05 (m, 3H, PhH), 2.61 (s, 2H, $ArCH_2$), 1.31 (br s, 2H), 0.99 (s, 6H, CH_3); MS (ESI): 286.6 $[M+H]^+$.



6-[4-[2-[[(2S)-3-(9H-carbazol-4-yloxy)-2-hydroxy-propyl]amino]-2-methyl-propyl]phenoxy]pyridine-3-carboxamide hydrochloride salt



A mixture of 4-(2-amino-2-methylpropyl)phenoxy)-5-carboamidepyridine (17.12 g, 60.0 mmol), (S)-4-(oxiranylmethoxy)-9H-carbazole (7.18 g, 30.0 mmol), HOAc (84.1 mg, 1.4 mmol), and water (10 mL) in MeOH (210 mL) was stirred at 60 ^{o}C for 22.5 h. The mixture was cooled and concentrated in vacuo to an oil. The residue was dissolved in EtOAc (150 mL) and water (60 mL) and the resulting layers were separated. The organic layer was extracted with a solution 1N HCl/water (28 mL/32 mL), and then with a solution of 1N HCl/water (2 x 2 mL/58 mL). The combined organic layers were washed with water (60 mL) and then were concentrated in vacuo to yield 16.01 g of a foam. Purification of the foam by falsh chloroform/methanol and later 25:4:0.1 chloroform/methanol/~28% ammonia as eluents yielded 10.0 g (63.53 %) of the free base.

A mixture of the free based prepared above (9.8 g, 18.68 mmol) in EtOAc (150 mL) and isopropanol (19 mL) was heated to 60 ^{o}C to obtain a solution. The stirred solution was made acidic by the dropwise addition of 56.5 mL (approx. 19.2 mmol HCl) of an approximately 0.34M HCl in EtOAc solution over 30 minutes. The resulting slurry was allowed to slowly cool for 1.75 h to ambient temperature. The mixture was filtered, and the filter cake washed with EtOAc (2 x 20 mL) and dried in vacuo at 50-60 ^{o}C to yield 10.04 g of the desired product as a white powder.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.32 (s, 1H), 9.06 (br s, 1H), 8.74 (br s, 1H), 8.62 (d, 1H), 8.26 (m, 2H), 8.04 (s, 1H), 7.51 (s, 1H), 7.4 (d, 1H), 7.28-7.32 (m, 4H), 7.07-7.10 (m, 5H), 6.72 (m, 1H), 6.06 (br s, 1H), 4.48 (m, 1H), 4.34 (m, 1H), 4.23 (m, 1H), 3.49 (m, 1H), 3.25 (m, 1H), 3.07 (m, 2H), 1.30 (s, 6H); HRMS m/e: calcd for $C_{31}H_{32}N_4O_4$ $[M]^+$ 525.2424, found 525.2414.

Y87-KNY-292

Sample Name:
Y87-KNY-292
Data Collected on:
timberline-vnmrs500
Archive directory:
/export/home/rboyer/vnmrsys/data/Y87
Sample directory:
Y87-KNY-292_20100818_01
FidFile: Y87-KNY-292_PROTON_01

Pulse Sequence: BaschPROTON
Solvent: dmso
Data collected on: Aug 16 2010

Lilly ID: Y87
NoteBook: KNY
Page: 292
Registered Lot Number: Y87-KNY-292
Lilly Serial Number: 377710
ATportal UID: LCC_NMR_timberline-vnmrs500_1008161427 52
Date/Time of Test: 16-Aug-2010 14:44:46

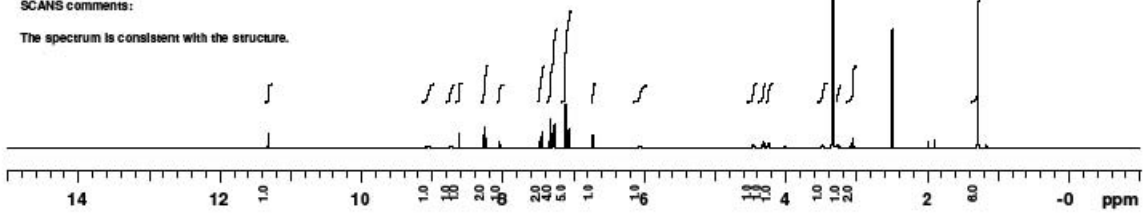
---Integrals defined by CY4

Sample: Y87-KNY-292
SpinCount Analysis Match Factor: 98
SCANS computed on: 20100818-095250

SCANS comments:

The specrum is consistent with the structure.

Y87-KNY-292

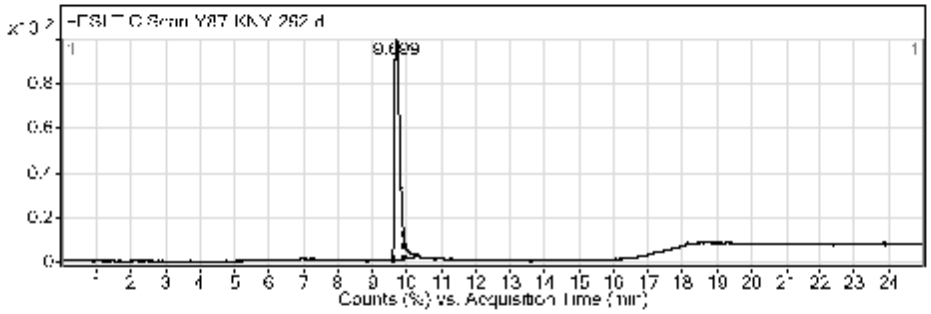


High Resolution Mass Spectrometry Analysis Report

Instrument Name	LCMS0018	**Data Filename**	Y87-KNY-292.d
Sample Name	Y87-KNY-292	**Acq Method**	C8_Long_XFRAG_Pos.m

User Chromatograms

Ionization Mode ESI



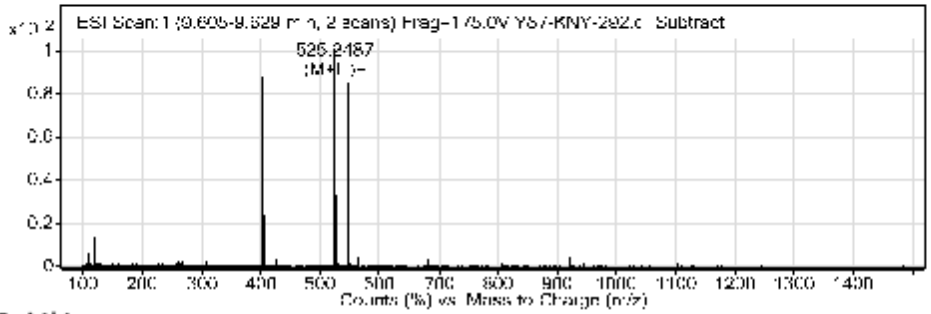
Integration Peak List

Peak	RT	Area	Area %
1	9.699	432994124	100

User Spectra

Spectrum Source
+ TIC Scan

Ionization Mode
Esi

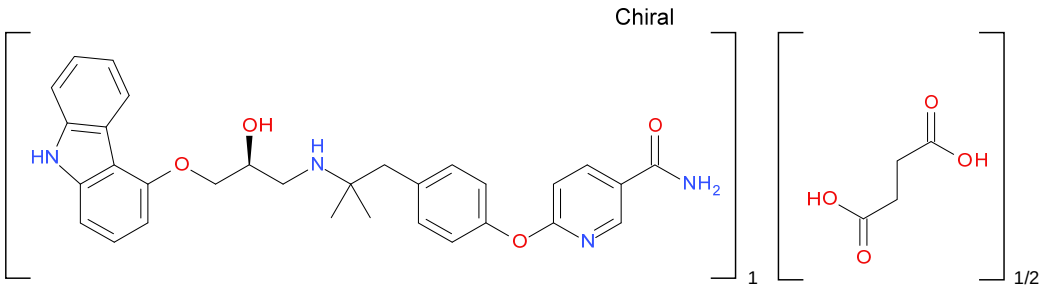


Peak List

m/z	Mass	Calc Mass	Formula (M)	Error (ppm)	Abundance
525.2487	524.2414	524.2424	C31 H32 N4 O4	1.75	25764.8

--- End Of Report ---

6-[4-[2-[[(2S)-3-(9H-carbazol-4-yloxy)-2-hydroxy-propyl]amino]-2-methyl-propyl]phenoxy]pyridine-3-carboxamide hemisuccinate salt



The freebase (102. 14 g, 194. 69 mmol) in 511 mL of EtOAc and 1150 mL of EtOH was heated at 50-52°C until a clear amber solution was obtained. A solution of succinic acid (11. 50 g, 97. 34 mmol, 0. 50 mole equivalents) in 200 mL of EtOH at 50°C was added to the solution. An additional 150 mL of EtOH was added. The resulting solution was allowed to cool. At 46°C the mixture was seeded. Product began to crystallize at 43°C. The mixture was stirred overnight at ambient temperature. The resulting slurry was cooled to 10°C and filtered under vacuum. The filter cake was rinsed with 2x50 mL of 3:1 EtOH: EtOAc. The collected product was air dried under vacuum for 15 min. This gave a wet cake with a mass of 122 g which was further dried under vacuum.

^{1}H NMR (500 MHz, DMSO-d_6): δ 11.29 (s, 1H), 8.64 (d, 1H), 8.26 (m, 2H), 8.07 (s, 1H), 7.57 (s, 1H), 7.45 (d, 1H), 7.25-7.34 (m, 4H), 7.02-7.12 (m, 5H), 6.71 (d, 1H), 4.18-4.22 (m, 3H), 2.98-3.13 (m, 2H), (m, 1H), 2.77 (s, 2H), 2.31 (s, 2H, ½ mole equivalent of succinic acid); 1.09 (s, 6H).

Functional beta 1, 2 & 3 Assays

The genes encoding the human β_1-adrenergic receptor (Frielle *et al.*, *Proc. Natl. Acad. Sci.*, 84:7920-7924, 1987), the human β_2-adrenergic receptor (Kobika *et al.*, *Proc. Natl. Acad. Sci.*, 84:46-50, 1987, Emorine *et al.*, *Proc. Natl. Acad. Sci.*, 84:6995-6999, 1987) and the human β3 adrenergic receptor (Granneman *et al.*, *Molecular Pharmacology*, 44(2):264-70, 1993) are individually subcloned into a phd expression vector (Grinnell *et al.*, *Bio/Technology*, 5:1189-1192, 1987) and transfected into the DXB-11 Chinese hamster ovary (CHO) cell line by calcium phosphate precipitation methodology. The stably transfected cells are grown to 95% confluency in 95% Dulbecco's modified Eagles Medium (DMEM), 5% fetal bovine serum and 0.01% proline. Media is removed and the cells are washed with phosphate buffered (pH 7.4) saline (without magnesium and calcium). Cells are then lifted using an enzyme free cell dissociation solution (Specialty Media, Lavallette, New Jersey) and pelleted by centrifugation.

Cells from each of the above cell lines are resuspended and added (20,000/well) to a 96-well plate. Cells are incubated at 37°C with representative compounds of the invention for 20 minutes in buffer (Hank's balanced salt solution, 10 mM HEPES, 0.1% BSA, 1 mM L-ascorbic acid, 0.2% dimethyl sulfoxide, 1 mM 3-isobutyl-1-methylxanthine, pH 7.4). After halting the incubation with quench buffer (50 mM Na Acetate, 0.25% Triton X-100, pH 5.8), the c-AMP level is quantified by scintillation proximity assay (SPA) using a modification of the commercially available c-AMP kit (Amersham, Arlington Heights, IL) with rabbit anti-cAMP antibody (ICN Biomedicals, Aurora, Ohio) for the kit.

Sigmoidal dose response curves, from the whole cell receptor coupled c-AMP assay are fit to a four parameter logistic equation using non linear regression: $y=(a-d)/(1+(Dose/c)^b)+d$ where a and d are responses at zero and maximal dose, b is the slope factor and c is the EC_{50} as previously described (DeLean et al., *Am. J. Physiol*., 235, E97-E102, 1978). EC_{50} is assessed as the concentration producing 50% of the maximum response to each agonist.

Isoproterenol is accepted in the art as a non-selective β agonist and is widely used as a comparator in evaluating the activity of compounds. See *Trends in Pharm. Sci.*, 15:3, 1994. The % intrinsic activity (E_{max}) of representative compounds of the invention is assessed relative to isoproterenol by the compound's maximal response divided by the isoproterenol maximal response times 100. The β agonist data for LY377604 is shown in Figures 1-3.

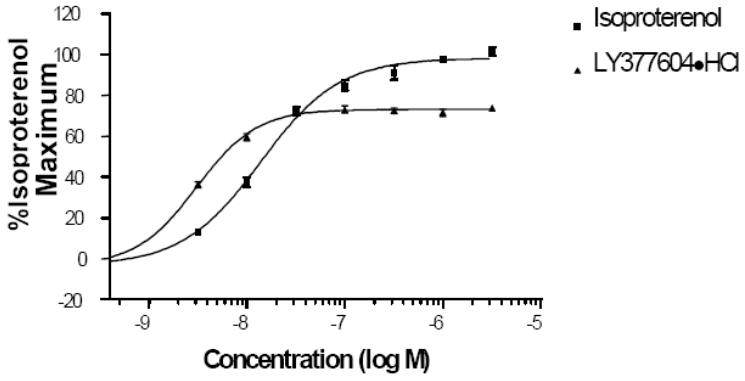


Figure 1. Stimulation of cAMP accumulation in CHO cells transfected with the human β_3 adrenergic receptor. Shown are concentration-effect curves for isoproterenol (n=6) and LY377604▪HCl (n= 14).

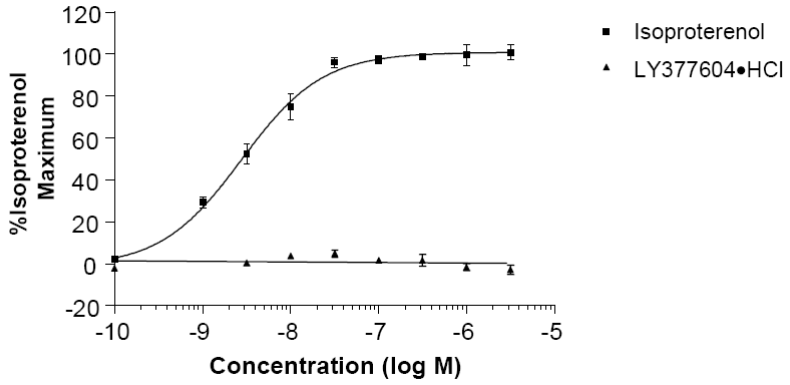


Figure 2. Inability of LY377604▪HCl to stimulate cAMP accumulation in CHO cells transfected with the human β_1 adrenergic receptor. Shown are concentration-effect curves for isoproterenol (n=4) and LY377604▪HCl (n=4).

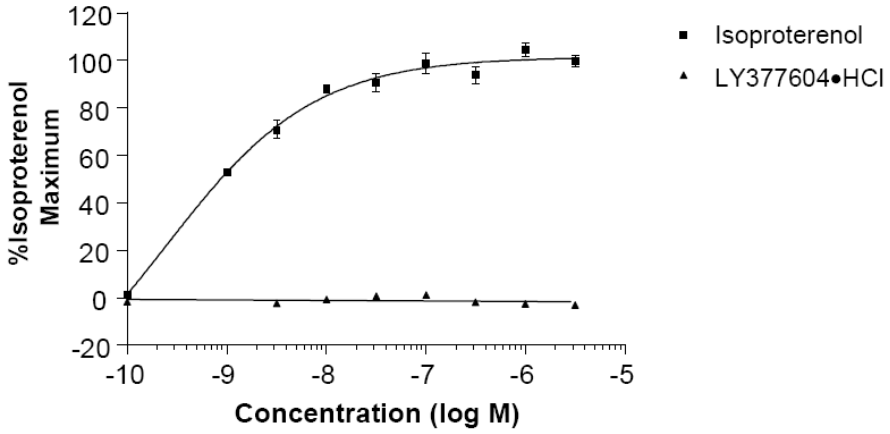


Figure 3. Inability of LY 377604▪HCl to stimulate cAMP accumulation in CHO cells transfected with the human β_2 adrenergic receptor. Shown are concentration-effect curves for isoproterenol (n=4) and LY 377604▪HCl (n=4).

Beta 1 & 2 Binding Assays

Receptor Membranes:

Membranes prepared from Sf9 cells expressing either the human β_1- or β_2-adrenergic receptor (AR) were obtained from NEN Life Sciences (Boston MA) and stored at –80^{o}C until use. Frozen membranes were diluted into assay buffer (50 mM Tris-HCl, 10 mM magnessium chloride, pH 7.4) and homogenized using a tight-fitting teflon/glass homogenizer (10 strokes at setting 4 on an overhead motorized stirrer). Diluted membranes were kept on ice until assayed.

[^{125}I]Iodocyanopindolol Binding to β_1- and β_2-AR using SPA:

(-)-3-[^{125}I]Iodocyanopindolol ([^{125}I]ICP, 2000 Ci/mmol, Amersham Pharmacia Biotech, Piscataway NJ) was diluted in assay buffer (50 mM Tris-HCl, 10 mM magnesium chloride, pH 7.4) and incubated with β_1- or β_2-AR membranes in clear bottom, opaque 96 well microplates (CoStar #3632, Corning NY) in a total final volume of 0.2 ml. Order of addition for the reagents was as follows: 20 μl of compound or buffer control, 50 μl of [^{125}I]ICP (0.05 nM final concentration), 80 μl of membranes (0.4 μg for β_1-AR, 0.25 υg for β_2-AR), and 50 μl of wheat germ agglutinin SPA beads (Cat #RPNQ0003, PEI Type A, Amersham Pharmacia Biotech, 0.125 mg). Following a 10-hour incubation at room temperature to allow for receptor-ligand equilibrium and stable bead settling conditions, the 96-well plates were counted in a Wallac Microbeta 1450 microplate scintillation counter at 1 minute per well. Nonspecific binding was determined in the presence of 1 μM unlabeled propranolol. LY377604 hemisuccinate and (S)-(-)-propranolol (Cat #P-8688, Sigma-Aldrich Co, St. Louis MO) were serially diluted in assay buffer containing 10% β-hydroxycyclodextrin (Cat #33,260-7, Sigma-Aldrich Co, St. Louis MO), with final β-hydroxycyclodextrin concentrations of 1% in the assay. Total binding was determined in the presence of assay buffer only containing identical concentrations of β-hydroxycyclodextrin.

Data Analysis:

Sigmoidal dose-response curves from the SPA binding assay were fit to a four parameter logistic equation using nonlinear regression analysis:

y=(a-d)/(1+(Dose/c)b)+d

where a and d are responses at zero and maximal dose, b is the slope factor and c is the IC_{50}. IC_{50} was assessed by the concentration at which the response was 50% maximum. The affinity constants (K_i) were calculated from the equation shown below, assuming simple competitive interaction:

$$K_i = \frac{IC_{50}}{1+[L]/K_d}$$

where [L] is the concentration of radioligand and the K_d is the equilibrium dissociation constant of the radioligand and the receptor. The binding data for LY377604 is shown in Figures 4 and 5.

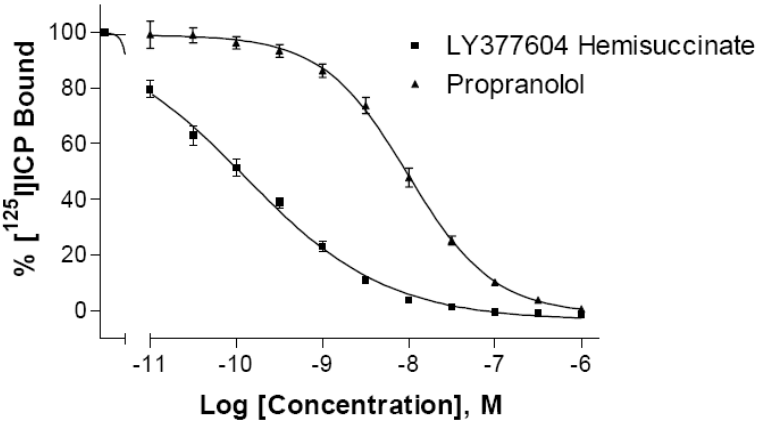


Figure 4. Inhibition of [^{125}I]iodocyanopindolol receptor binding to membranes prepared from Sf9 cells expressing the human β_1 adrenergic receptor. Shown are concentration response curves for propanolol (n=4) and LY377604▪hemisuccinate (n=4).

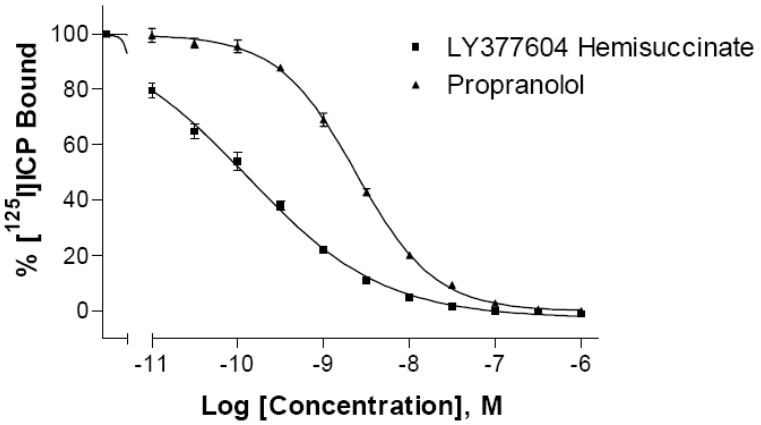


Figure 5. Inhibition of [^{125}I]iodocyanopindolol receptor binding to membranes prepared from Sf9 cells expressing the human β_2 adrenergic receptor. Shown are concentration response curves for propanolol (n=4) and LY377604▪hemisuccinate (n=4).

Indirect calorimetry

Indirect calorimetry experiments were performed following a published procedure (Chen, Y.; Heiman, M. L. Chronic leptin administration promotes lipid utilization until fat mass is greatly reduced and preserves lean mass of normal female rats. Regul Pept **2000** 92:113–119). Specifically, 24-h caloric expenditure and respiratory quotient (RQ) were measured by using an open circuit system (Oxymax; Columbus Instruments, Columbus, OH). RQ was measured as the ratio of volume of CO_2 produced to the volume of O_2 consumed. Caloric expenditure was calculated as the product of calorific value of oxygen and volume of O_2 consumed per kilogram of body weight, where calorific value of

oxygen = 3.815 + 1.232 (RQ). Total calories expended were calculated to determine daily fuel use. To calculate the proportion of protein, fat and carbohydrate that used during the 24-h period, we used Flatt's proposal and formula as other derived constants (24). Locomotor activity was measured by counting number of new beam breaks (ambulatory) and same beam breaks (fine movement) during each period of 24 h in the calorimeter. Comparisons between groups of data (mean $\pm$ sem) were analyzed using SigmaStat (SPSS Inc., Chicago, IL) ANOVA using Student-Newman-Keuls (Student-Newman-Keuls) test for multiple comparisons.