Synthesis and Pharmacological Characterization of β_2-Adrenergic Agonist Enantiomers: Zilpaterol

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The β-adrenergic agonist **1** (zilpaterol) is used as production enhancer in cattle. Binding experiments of separated enantiomers on recombinant human β_2-adrenergic and μ-opioid receptors and functional studies showed that the (−)-**1** enantiomer accounts for essentially all the β_2-adrenergic agonist activity and that it exhibits less affinity toward the μ-opioid receptor than (+)-**1**, which is a μ-opioid receptor antagonist. X-ray crystallography revealed the absolute configuration of (−)-**1** to be 6*R*,7*R*.

Introduction

Activation of β_2-adrenergic receptors relax bronchial, vascular, intestinal, and uterine smooth muscles, increase ciliary beat frequency, and mediate mucociliary clearance.[1,2] Physiological responses to stimulation of the β-adrenergic receptors include increased lipolysis and reduced lipogenesis in adipose cells, increased glycogenolysis, increased protein synthesis, and reduced proteolysis in striated muscle fibers.[3,4] **1** (zilpaterol,[5] Figure 1) is a β-adrenergic agonist specifically used for increased rate of weight gain, improved feed efficiency, and increased carcass leanness in cattle fed in confinement for slaughter during the last 20−40 days on feed in an antibiotic-free and hormone-free manner and is not approved for any human use. (*rac*)-**1** as approved is a 1:1 mixture of (+)-**1** and (−)-**1** as hydrochlorides and does not contain any diastereoisomeric cis-form of **1**. The different binding, toxicity, and biological properties of various β_2-adrenergic agonist enantiomers have been reported.[6,7] (*R*)-**4** (levalbuterol[8]), the active enantiomer of (*rac*)-**4** (albuterol[9]), was introduced as a bronchodilator in the treatment of asthma, while the clinical value of switching from a 1:1 mixture to the pure eutomer is still up for discussion.[7] So far, the two enantiomers of (*rac*)-**1** had not been characterized individually with respect to their binding activity on β_2-adrenergic receptors or with respect to their biological functions. With the strategy of a chiral switch[10] in mind (to replace racemic (*rac*)-**1** by a putative eutomer) we prepared the individual enantiomers by chromatographic separation and conducted the binding experiments on recombinant expressed human β_2-adrenergic receptors and recombinant expressed human μ-opioid receptors. Additionally, we performed functional studies for agonist activity at β_2-adrenergic receptors in a quantitative ex vivo guinea pig trachea model and functional studies for agonist and antagonist activity at the μ-opioid receptors in a quantitative recombinant cellular assay. The absolute configuration of (−)-**1** was assigned by X-ray crystallography.

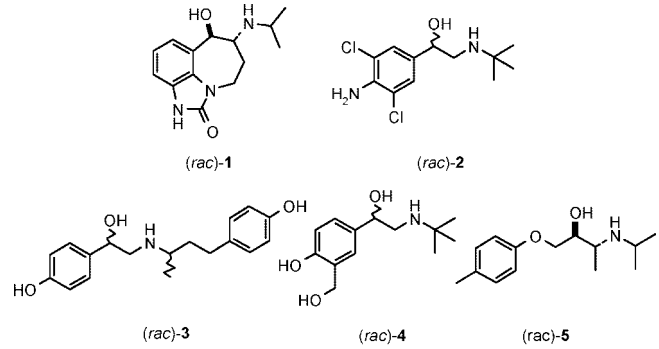

Figure 1. Comparison of the chemical structure of zilpaterol (*rac*)-**1** with several other β_2-adrenergic receptor agonists (**2**, **3**, **4**) and reference antagonist **5** (ICI 118551[11]).

Chemistry

The synthesis of the hydrochloride salts of (+)-**1** and (−)-**1** in optically pure form is outlined in Scheme 1. (*rac*)-**1**·HCl was treated with ammonia to give the corresponding free base which was subsequently reacted with benzyl chloroformate to furnish (*rac*)-**6** in 61% yield. The indirect enantioseparation approach utilizing intermediate (*rac*)-**6** was required, as (*rac*)-**1** is not sufficiently soluble in ethanol to allow multiple gram separation by the following HPLC step. Enantioselective chromatography was performed on 7.5 kg of amylose *tris*(3,5-dimethylphenylcarbamate) coated on a 20 μm silica support using ethanol as the mobile phase, and pure enantiomers (+)-**6** and (−)-**6** were recovered in 95% and 94% yields, respectively. The desired enantiomers of **1** were obtained after cleavage of the Cbz group using hydrogen and 10% palladium on carbon and final hydrochloride salt formation with 12 N HCl in 81% and 82% overall yields.

Absolute stereochemical assignment of (−)-**1** was performed on the basis of a crystal structure of analogue **7**, which was synthesized in one step and 51% yield from (−)-**1**. Figure 2 shows the X-ray structure of (6*R*,7*R*)-**7** in the crystal.

Binding Activity

Competitive binding assays on recombinant human β_2-adrenergic receptors in transfected Sf-9 cells were used to

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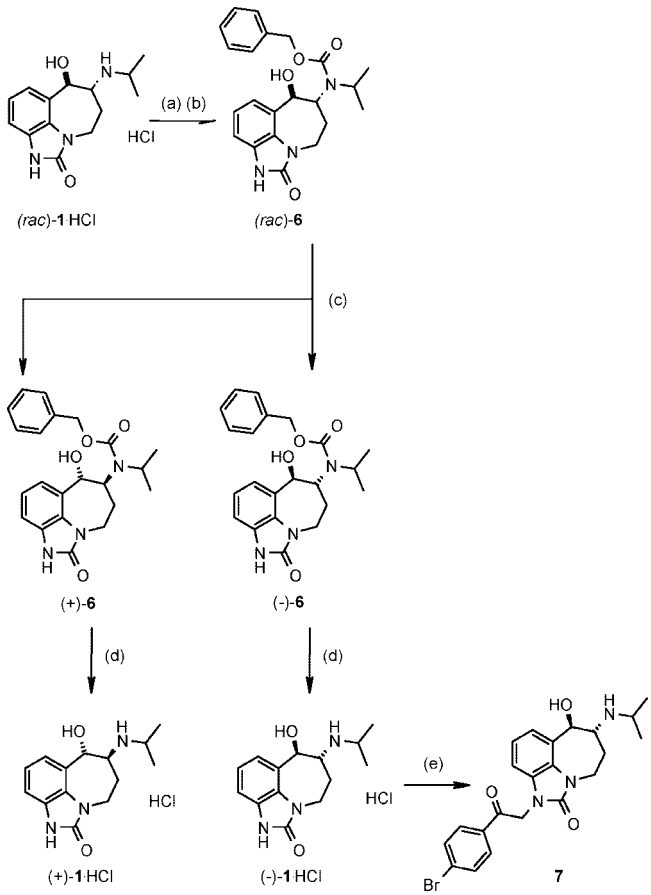
Scheme 1. Synthesis of Enantiomerically Pure (+)-**1** and (−)-**1**[a]



[a] Reagents and conditions: (a) ammonia (20% in water), room temp, 1 h, 96%; (b) benzyl chloroformate, $NaHCO_3$, acetone/toluene, room temp, 30 h, 61%; (c) amylose tris(3,5-dimethylphenylcarbamate) coated on a 20 μm silica support, EtOH, recovery of 95% for (+)-**6** and 94% for (−)-**6**; (d) Pd/C (10%), H_2 (4 bar), dioxane/CH_3COOH/H_2O, room temp, 5 h, then 12 N HCl, EtOH, 70 °C, 15 min, (+)-**1** 81% and (−)-**1** 82%; (e) Amberlite IRA-67 resin, then 2,4′-dibromoacetophenone, NaH, DMF, room temp, 18 h, 51%.

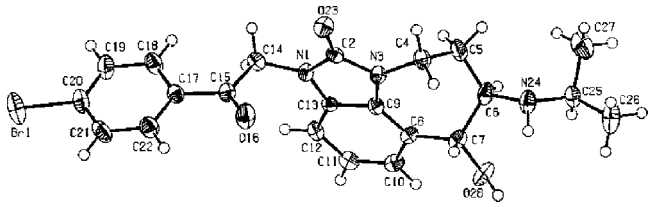


Figure 2. Molecular structure for (6*R*,7*R*)-**7** with displacement ellipsoids drawn at the 50% probability level.

Table 1. Binding Inhibition Values (IC_{50}) and Inhibitory Constants (K_i) of **1**, Standard β_2-Adrenergic Agonists, and a Selective β_2-Adrenergic Antagonist on Recombinant Human β_2-Adrenergic Receptors

compd	IC_{50} (μM)	K_i (μM)	nH
(−)-**1**	0.62	0.32	0.9
(*rac*)-**1**	1.1	0.58	0.9
2	1.3	0.57	0.9
3	0.33	0.18	0.9
4	0.98	0.51	0.8
5	0.003	0.001	1.5

determine the affinities of (*rac*)-**1** and its individual enantiomers, in comparison with standard β_2-adrenergic agonists **2** (clenbuterol[12]), **3** (ractopamin[13]), and **4** and the specific β_2-adrenergic antagonist **5** as reference (Table 1). We used well established

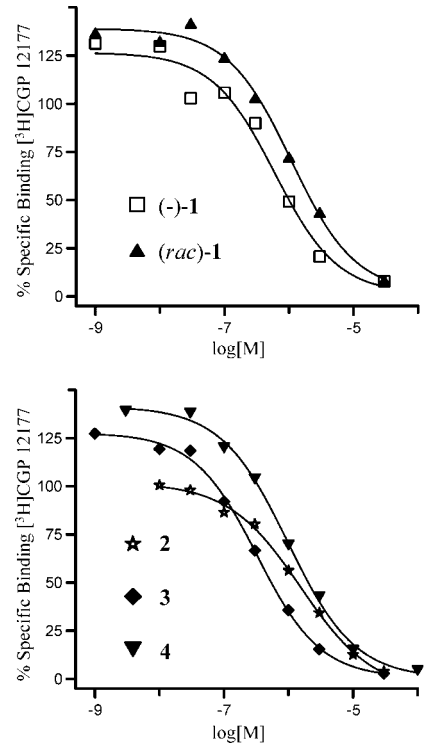


Figure 3. Binding dose−response curves for β_2-adrenergic agonists on the recombinant human β_2-adrenergic receptor in transfected Sf-9 cells.

human recombinant receptor assays as a surrogate for the bovine receptor assays, although we were aware that differences between species could occur. Sf-9 cells lack endogenous β-adrenergic receptors and therefore allow overexpression of single defined receptor subtypes for binding experiments. The tested compounds displaced the β_2-adrenergic receptor selective radioligand from a single binding site in a concentration-dependent manner as shown in Figure 3.

The observed K_i values for (*rac*)-**1** and (−)-**1** with respect to the recombinant human β_2-adrenergic receptor were 0.58 and 0.32 μM, respectively, whereas the binding affinity of the (+)-**1** was negligible: At 10 μM, (+)-**1** displaced only 3% of the radioligand at the β_2-adrenergic receptor, and the IC_{50} for (+)-**1** was not determined because of its weak binding affinity at 10 μM. Compound **3** had the strongest affinity of the agonists tested. The K_i was 1.8 times the K_i of (−)-**1**. The affinities of **2** and **4** were comparable to that of (*rac*)-**1** in this assay. The binding values obtained for the standard β_2-adrenergic agonists **3** and **4** and of the selective β_2-adrenergic antagonist **5** were in the same range as had been shown previously.[14−17] Our results demonstrate that the β_2-adrenergic receptor action of racemic (*rac*)-**1** essentially stems from the (−)-**1** enantiomer alone.

From a screening of **1** against a panel of various receptors, the μ-opioid receptor unexpectedly showed different binding properties for the enantiomers. We tested the binding affinities of (*rac*)-**1** and its individual enantiomers at the μ-opioid receptor agonist site in transfected HEK-293 cells. Results were identical with the free bases or the HCl salts (data not shown). The tested compounds displaced the μ-opioid receptor selective radioligand from a single binding site, explicitly the agonist site, in a concentration-dependent manner (Figure 4).

The observed binding data of (*rac*)-**1**, (+)-**1**, and (−)-**1** are provided in Table 2. (−)-**1** bound weakly to the μ-opioid receptor, whereas (*rac*)-**1** and (+)-**1** exhibited greater binding affinity. More specifically, the K_i (and IC_{50}) of (−)-**1** was 10

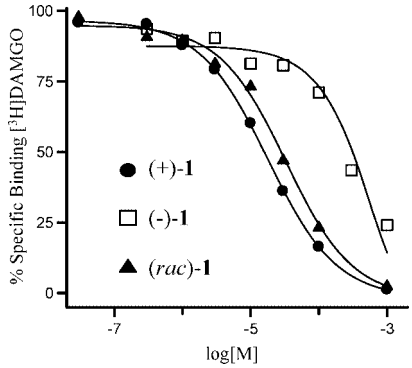


Figure 4. Binding dose−response curves for (*rac*)-**1** and its individual enantiomers on cell membranes of HEK-293 cells full of the recombinant expressed human μ-opioid-receptor.

Table 2. Binding Inhibition Values IC_{50} and Inhibitory Constants (K_i) of (*rac*)-**1**·HCl and Its Enantiomers (+)-**1**·HCl and (−)-**1**·HCl on Recombinant Expressed Human μ-Opioid Receptors

compd	IC_{50} (μM)	K_i (μM)	nH
(+)-**1**·HCl	17	7	0.9
(−)-**1**·HCl	320	130	0.9
(*rac*)-**1**·HCl	31	13	0.9

Table 3. EC_{50} (nM) and E_{max} Values (%) for β_2-Adrenergic Agonist Compounds in the Functional Ex Vivo Guinea Pig Trachea Bioassay

compd	EC_{50} (nM)	E_{max} (%)
(+)-**1**	6.9×10^3	105
(−)-**1**	8.7	102
(*rac*)-**1**	13	100
2	6.2	105
3	9.1	100
4	19	103

times the K_i (and IC_{50}) of (*rac*)-**1** and more than 18 times the K_i (and IC_{50}) of (+)-**1**.

Biological Activity

The purpose of the quantitative ex vivo guinea pig trachea study[18] was to investigate the biological functions of single enantiomers of **1**on the β_2-adrenergic receptor in comparison to **2**−**4**. We used the well established guinea pig trachea assay to demonstrate functionality, irrespective of species. We confirmed that the bronchodilator effects of (*rac*)-**1** and (−)-**1** were due to activation of the β_2-adrenergic receptor because these effects were antagonized by the specific β_2-adrenergic receptor antagonist **5**. In sum, all compounds induced a concentration-dependent and **5**-sensitive relaxation, and the maximum response (E_{max}) to each was similar to that evoked by reference **4** (Table 3).

The dose−response curves (Figure 5) elucidate the individual agonist potency of each compound tested. (−)-**1** has about 1.5 fold potency over the racemate (*rac*)-**1**, which is less than the theoretical value of 2, but clearly demonstrates the higher potency of (−)-**1**. (−)-**1** has about 3 orders of magnitude higher potency than (+)-**1**. (−)-**1** appears to account for essentially all the β_2-adrenergic agonist activity of (*rac*)-**1**. The maximum responses (E_{max}) of (−)-**1** and (*rac*)-**1** were similar to that induced by the reference **4**, indicating that (−)-**1** and (*rac*)-**1** behave as full agonists at the β_2-adrenergic receptors in this tissue.

In a second study, we determined the relative agonist and antagonist potency and efficacy of (*rac*)-**1** and its single enantiomers on μ-opioid receptors. This assay for functionality

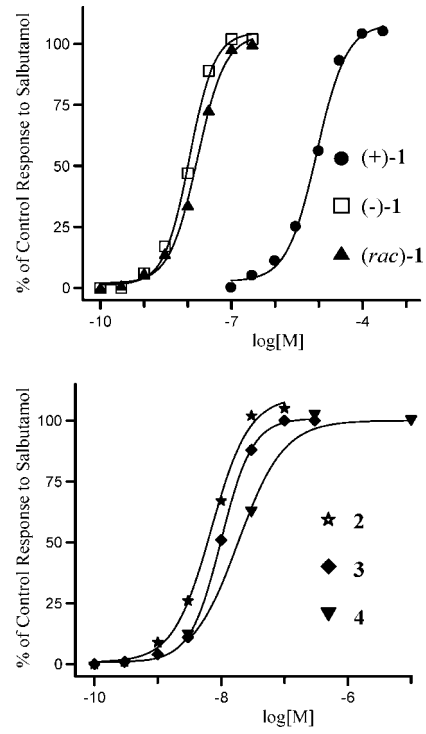


Figure 5. Agonist dose−response curves for β_2-adrenergic agonists in a quantitative and functional ex vivo guinea pig trachea bioassay.

Table 4. Antagonistic Activities of (*rac*)-**1** and Its Single Enantiomers on Recombinant Expressed Human μ-Opioid Receptors

compd	IC_{50} [a] (μM)	E_{max} (%)
(+)-**1**·HCl	1.4×10^2	96
(−)-**1**·HCl	$>10^3$	nc[b]
(*rac*)-**1**·HCl	3.0×10^2	93

[a] At highest reasonable concentrations, activities did not reach a plateau. [b] Not calculable.

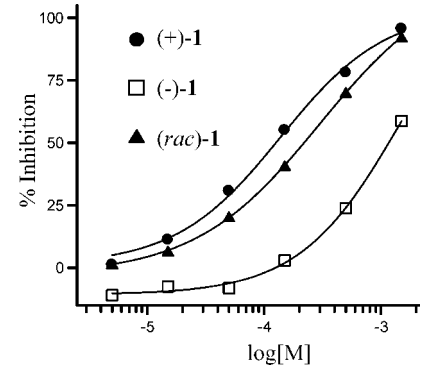


Figure 6. Antagonist dose−response curves for (*rac*)-**1** and its enantiomers (as hydrochlorides) on an AequoScreen cell line recombinantly expressing human μ-opioid receptors.

on the human recombinant μ-opioid receptor is based on aequorin-derived luminescence triggered by receptor-mediated changes in intracellular calcium levels.[19,20] In this assay, none of the compounds tested showed agonistic activity on human μ-opioid-receptors. (+)-**1** accounts essentially for all the antagonist activity of (*rac*)-**1** on μ-opioid-receptors (Table 4).

In a concentration-dependent manner, both enantiomers and the racemate of **1** antagonized μ-agonist-triggered luminescence by binding on the agonist site of the μ-opioid receptor (Figure

6); however, (+)-**1** appears to account for essentially all the μ-opioid antagonist activity of (*rac*)-**1**.

Conclusion

We have demonstrated that (−)-**1** accounts for essentially all of the β_2-adrenergic agonist activity of (*rac*)-**1** and have assigned its absolute configuration to be 6*R*,7*R*.[21] The approximate 2-fold difference in the affinity values of (*rac*)-**1** and (−)-**1** correlated well with the 1:1 composition of the racemate. Our binding and cellular assay studies demonstrated the hitherto unknown activity of the β_2-adrenergic receptor distomer (+)-**1** and racemate (*rac*)-**1** on the μ-opioid receptor and reduced activity of the β_2-adrenergic receptor eutomer (−)-**1** on the μ-opioid receptor.

Experimental Section

β_2-Adrenergic Receptor Binding Assay. In vitro pharmacology binding assays on human β_2-adrenergic receptor in cells were conducted based on assays described elsewhere;[22,23] for details see Supporting Information.

μ-Opioid Receptor Binding Assay. In vitro pharmacology binding assays on the μ-opioid receptor agonist site were conducted based on assays described elsewhere;[24] for details see Supporting Information.

β_2-Adrenergic Receptor Agonist Activity Assay. The determination of function as β_2-adrenergic receptor agonist using a quantitative ex vivo guinea pig trachea bioassay was conducted based on assays described elsewhere;[18] for details see Supporting Information.

Functional Human Recombinant μ-Opioid Receptor Activity Assay. We determined the agonist and antagonist activity on the human recombinat μ-opioid receptor. A functional assay, based on aequorin-derived luminescence triggered by receptor-mediated changes in intracellular calcium levels, used to examine relative potency and efficacy and the inhibitory properties of test items was conducted based on assays described elsewhere;[19,20] for details see Supporting Information.

Chemistry. General Methods. (*rac*)-**1**·HCl was kindly provided by Intervet International B.V., Boxmeer, The Netherlands.[25] **2** and **3** were purchased from ChemPacific. **4** was from Sigma Aldrich and **5** from Tocris Bioscience. All chemicals and analytical grade solvents were used as supplied. Amberlite IRA-67 was purchased from Fluka and Chiralpak AD from Daicel. ^{1}H and ^{13}C spectra were recorded at 300/400 MHz and 75/100 MHz using a Bruker DPX300 or DRX400 spectrometer. Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. HPLC−MS data were collected using an Agilent HPLC/MSD 1100 system including a mass detector G1946D SL with ESI-source and an evaporating light detector Sedex 75. Melting points were recorded using a HWS Mainz Laboratoriumstechnik SG 2000 melting point apparatus and are uncorrected. Rotary power determinations were recorded using a Perkin-Elmer 343 polarimeter with a path length of 0.1 dm, while the concentrations (*c*) are given in g/100 mL.

Preparation of (6*R*,7*R*)-*rel*-4,5,6,7-Tetrahydro-7-hydroxy-6-[(1-methylethyl)amino]imidazo[4,5,1-*jk*][1]benzazepin-2(1*H*)-one ((*rac*)-1). A 4 L reaction vessel equipped with mechanical stirring was charged with (*rac*)-**1**·HCl (304.6 g, 1.023 mol) and water (1.5 L). An aqueous solution of ammonia (20%, 150 mL) was added in portions while stirring. Stirring was then continued at room temperature for 1 h. When the mixture was stirred in an ice bath for another hour, a precipitate formed, which was subsequently collected, washed with water (1 L), washed with petroleum ether (500 mL), coevaporated with toluene, and dried under reduced pressure to afford 256.6 g (96%) of (*rac*)-**1** as a colorless solid which was used in the next step as obtained. Mp 177 °C (dec); *m*/*z* 262.0 $(M + H)^+$; HRMS, *m*/*z* 262.1548 $(M + H)^+$.

Synthesis of (6*R*,7*R*)-*rel*-(1-Methylethyl)-(4,5,6,7-tetrahydro-7-hydroxy-2-oxoimidazo[4,5,1-*jk*][1]benzazepin-6-yl)carbamic Acid Phenylmethyl Ester ((*rac*)-6). A 6 L reaction vessel equipped with mechanical stirring under N_2 was charged with (*rac*)-**1** (125 g, 0.48 mol) and acetone (3.5 L). Solid $NaHCO_3$ (160 g, 1.91 mol) was then added. The resulting suspension was stirred and cooled to 10 °C, while benzyl chloroformate (0.59 mol) diluted in toluene (to a total volume of 200 mL) was rapidly added dropwise. After the mixture was stirred at room temperature for 15 h, an additional solution of benzyl chloroformate (55 mL, 50% solution in toluene) was added. The resulting mixture was again stirred at room temperature for 15 h and then filtered. The mother liquor was reserved. Two additional batches (125 and 83 g scale) were then synthesized using the same procedure. The resulting solids were combined and suspended in water (4 L). This mixture was stirred for 30 min and filtered. The resulting solid was again suspended in water (3 L), and the mixture was stirred for 15 min. After filtration, the solid was collected and washed with water (2 × 1 L), petroleum ether (500 mL), and diethyl ether (500 mL) and dried under reduced pressure: 267 g of (*rac*)-**6** as a colorless solid. The reserved mother liquor was concentrated under reduced pressure, and the resulting residue was triturated in an aqueous solution at a pH of ≤4 (HCl) to form a precipitate, which in turn was rinsed with water and dried under reduced pressure to afford an additional 42 g of (*rac*)-**6** (61% overall). Mp 169 °C; *m*/*z* 413.4 $(M + NH_4)^+$, 396.4 $(M + H)^+$, 378.4 $(M + H - H_2O)^+$; HRMS, *m*/*z* 396.1917 $(M + H)^+$. Anal. ($C_{22}H_{25}N_3O_4$) C, H, N.

(6*S*,7*S*)-(1-Methylethyl)-(4,5,6,7-tetrahydro-7-hydroxy-2-oxoimidazo[4,5,1-*jk*][1]benzazepin-6-yl)carbamic Acid Phenylmethyl Ester ((+)-6) and (6*R*,7*R*)-(1-Methylethyl)-(4,5,6,7-tetrahydro-7-hydroxy-2-oxoimidazo[4,5,1-*jk*][1]benzazepin-6-yl)carbamic Acid Phenylmethyl Ester ((−)-6). The enantiomers were prepared in optically pure form by preparative enantioselective HPLC. Preparative separation of (*rac*)-**6** was performed on a Prochrom LC200 DAC HPLC column (20 cm × 60 cm) filled with Chiralpak AD (particle size 20 μm, 7.5 kg), with ethanol as the mobile phase at a flow rate of 18 L/h. Thus, 197 g of (*rac*)-**6**, split into eight batches of 20−26 g and each dissolved in 1 L of methanol at 50 °C, were injected onto the column. Analytical HPLC: Chiralpak AD-H (4.6 mm × 250 mm), *n*-hexane/ethanol 60:40 (volume), 0.8 mL/min, $T = 25$ °C. UV: 220 nm. For (+)-**6**: 93.8 g (237 mmol, recovery 95%), >99% ee, $t_R = 8.99$ min; $[\alpha]_D^{20}$ +28° (*c* 3.27, MeOH). For (−)-**6**: 92.1 g (234 mmol, recovery 94%), >99% ee, $t_R = 13.56$ min; $[\alpha]_D^{20}$ −27° (*c* 0.63, MeOH). ^{1}H NMR spectra of (+)-**6** and (−)-**6** were identical to the ones reported above for (*rac*)-**6**.

Synthesis of (6*S*,7*S*)-4,5,6,7-Tetrahydro-7-hydroxy-6-[(1-methylethyl)amino]imidazo[4,5,1-*jk*][1]benzazepin-2(1*H*)-one Hydrochloride ((+)-1·HCl). (+)-**6** (47 g, 119 mmol), 1,4-dioxane (750 mL), acetic acid (450 mL), water (300 mL), and Pd/C (10%, 9.4 g) were charged into a 2 L bomb reactor fitted with magnetic stirring. Following a purge with N_2, the mixture was stirred at room temperature for 5 h under H_2 atmosphere (4 bar, adjusted upon consumption of H_2). The resulting mixture was filtered through Celite, and the resulting solid was washed with 1,4-dioxane. Volatiles were then removed under reduced pressure. A second batch (46 g, 116 mmol) was then synthesized using the same procedure. The crude product mixtures were combined and taken up in a minimum amount of water. The resulting solution was adjusted to pH 9−10 using aqueous ammonia (30%). This aqueous solution was then successively extracted with 500 mL portions of ethyl acetate. The organic layers were combined and then dried over $MgSO_4$. After filtration and concentration under reduced pressure, 53 g (203 mmol) of (+)-**1** was obtained. This pale-yellow solid and ethanol (600 mL) were charged under an inert atmosphere of N_2 to a 2 L three-necked round-bottomed flask equipped with a magnetic stirrer, condenser, and thermometer. The mixture was stirred at 70 °C, while 20.8 mL (250 mmol) of concentrated aqueous HCl (12 N) was added dropwise. The resulting mixture was stirred at 70 °C for an additional 15 min and then allowed to attain room temperature. The mixture was then cooled with an ice bath, resulting

in the formation of a precipitate. The precipitate was collected, washed with diethyl ether, and dried under reduced pressure to provide 57 g (191.5 mmol, 81% over two steps and >99% ee) of (+)-**1**·HCl as a colorless solid. $[\alpha]_D^{20}$ +33 (*c* 1.01, water); mp 195 °C (dec); *m*/*z*, 262.0 $(M + H)^+$; HRMS, *m*/*z*, 262.1548 $(M + H)^+$. Anal. ($C_{14}H_{20}N_3O_2Cl$) C, H, N.

(6*R*,7*R*)-4,5,6,7-Tetrahydro-7-hydroxy-6-[(1-methylethyl)amino]imidazo[4,5,1-*jk*][1]benzazepin-2(1*H*)-one Hydrochloride ((−)-1·HCl). Using the procedure described for (+)-**1**·HCl, an amount of 90 g (227 mmol) of (−)-**6** was converted into 55.4 g (186 mmol) of (−)-**1**·HCl in 82% yield (over two steps), its spectroscopic data being identical to the ones obtained for (+)-**1**·HCl. >99% ee; $[\alpha]_D^{20}$ −33 (*c* 1.03, water); HRMS, *m*/*z*, 262.1548 $(M + H)^+$. Anal. ($C_{14}H_{20}N_3O_2Cl$) C, H, N.

Synthesis of (6*R*,7*R*)-1-[2-(4-Bromophenyl)-2-oxoethyl]-4,5,6,7-tetrahydro-7-hydroxy-6-[(1-methylethyl)amino]imidazo[4,5,1-*jk*][1] benzazepin-2(1*H*)-one (7). General method for the preparation of the free bases from the corresponding hydrochloride salts of **1** on small scale is as follows: A 0.1 M solution of **1**·HCl in water was treated with Amberlite IRA-67 resin for 20 min at room temperature and then filtered. The filtrate was freeze-dried, and the free bases of **1** were obtained as white solids. Following the general procedure for the preparation of the free bases, (−)-**1** (750 mg, 2.87 mmol) was dissolved in dry DMF (15 mL) and NaH (172 mg, 4.3 mmol, 60% dispersion in mineral oil) was added. After 10 min, 2,4′-dibromoacetophenone (956 mg, 3.44 mmol) was added and the resulting reaction mixture was stirred for 20 h at room temperature. Water was then added, and the resulting solution was extracted with diethyl ether (2×), and the combined organic layers were washed with 5% $NaHCO_3$ (3×). After drying over $MgSO_4$, the organic phase was evaporated to dryness, and the residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 9:1 v/v). **7** was obtained as a bright-yellow solid (0.66 g, 1.14 mmol, 50%). Mp 169−170 °C (dec); $[\alpha]_D^{20}$ −27° (*c* 0.45, EtOH); *m*/*z*, 460.1 $(M)^+$, 457.9; HRMS, *m*/*z*, 458.1068 $(M + H)^+$.

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Supporting Information Available: Details of β_2-adrenergic receptor binding assay, μ-opioid receptor binding assay, β_2-adrenergic receptor agonist activity assay, and functional human recombinant μ-opioid receptor activity assay; ^1H and ^{13}C spectra and HRMS, elemental analysis, HPLC results of selected compounds; and crystallographic data for **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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