Supporting Information for

α_2-Adrenoreceptors Profile Modulation. 3.[1] (*R*)-(+)-meta-Nitrobiphenyline, a New Efficient and α_{2C}-Subtype Selective Agonist.

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Chemical Methodology

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian EM-390 instruments, respectively. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). IR spectra data, not shown because of the lack of unusual features, were obtained for all compounds reported and are consistent with the assigned structures. Optical activity was measured at 20 °C with a Perkin-Elmer 241 polarimeter. HPLC analyses were recorded on an Hewelett Packard 1090 I series chromatograph on a Hypersil 200 x 2.1 mm stainless steel column (packed with 5 μm particles, Hewelett Packard). The mobile phase was hexane/AcOEt (80/20) and the flow rate was set at 1.0 mL/min. The microanalyses were performed by the Microanalytical Laboratory of our department. The elemental composition of the compounds agreed to within ± 0.4% of the calculated value. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040-0.063 mm, Merck) by flash chromatography. The term “dried” refers to the use of anhydrous sodium sulfate. Compounds were named following the IUPAC rules proposed by Beilstein-Institut AutoNom (version 2.1), a software for systematic names in organic chemistry.

Biological Experiments

Culture of CHO clones expressing α_2-AR subtypes. The plasmids used to stably express the human α_{2A}-, α_{2B}- or α_{2C}-AR in CHO cells have been previously described.[11] Briefly, each vector contains an expression cassette comprising the CMV promoter/enhancer, the coding region of α_2C10-, α_2C2- or α_2C4 gene, the IRES derived from the encephalomyocarditis virus, the neomycin phosphotransferase gene, and a fragment from the rabbit β-globin gene containing an intron and a polyadenylation signal. Cells were transfected using the calcium-phosphate method and clones were selected in the presence of G418-sulfate (1 mg/mL). CHO-α_{2A}, CHO-α_{2B} and CHO-α_{2C} were routinely subcultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum. The clones used in this study respectively express 1.45 ± 0.3, 4.2 ± 0.8, and 2.7 ± 0.5 pmol of receptor /mg of membrane protein.

Binding experiments. Binding studies were performed on crude cell membrane preparations using the selective α_2-antagonist, [^{3}H]RX 821002, as radioligand. Briefly, membranes were incubated in a final volume of 400 μL of Tris-EDTA buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) containing the radioligand at a concentration corresponding to twice its *K*d value for the considered subtype (3 nM for CHO-α_{2A}, 10 nM for CHO-α_{2B} and 6 nM for CHO-α_{2C}) and increasing concentrations (10^{-11} to 10^{-4} M) of the competitor to be tested. After 30 minutes of incubation at room temperature, membrane bound radioactivity was collected by filtration through a fiberglass filter (Whatman GFC) using a Skatron Cell Harvester. Filters were then rapidly washed, and transferred to scintillation vials, and counted for radioactivity. Inhibition data were analysed using the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA), allowing non-linear regression analysis according to a one- or a two-site inhibition model.

Microphysiometry Instrument. Extracellular acidification was measured using a Cytosensor microphysiometer system (Molecular Devices, Menlo Park, CA, USA). CHO cells were seeded into 12 mm capsule cups (3 x 10^5 cells/cup) and placed at 37 °C under 5% CO_2 atmosphere. One day

post-seeding, the capsule cups were loaded into the cytosensor chambers and the cells perfused with a running medium (bicarbonate-free DMEM containing 0.584 g/L glutamine and 2.59 g/L NaCl) at a flow rate of 100 μL/min. For each 90 sec pump cycle, the pump was on for 60 sec and off for the following 30 sec and the pH value was recorded for 20 sec (from sec 68 to sec 88). The stimulation protocol was validated in preliminary experiments with three agonists (-)-Noradrenaline, Dexmedetomidine, and UK 14304. The rate of acidification in the chamber was calculated using the Cytosoft program (Molecular Devices). Changes in the rate of acidification were calculated as the difference between the maximum effect after agonist addition and the average of three measurements taken prior to agonist addition.

For antagonist studies, a control concentration-response curve to clonidine was conducted and the cells where then exposed to antagonist for at least 30 min prior to construction of a further clonidine concentration-effect curve in the presence of antagonist. Each chamber therefore acted as its own control. Antagonist data were analysed as the ability of the antagonist to shift the agonist concentration-effect curve and defined as K_b [antagonist]·M/concentration ratio^{-1}, where concentration ratio is the EC_{50} obtained in the presence of the antagonist divided by that obtained in the absence of the antagonist.[13] Data were expressed as pK_b [$-\log_{10}(K_b)$] and were means (±SEM) of three independent experiments.

Measurement of MAPK activation. CHO cells were seeded at a density of 8 x 10^5 cells/cm^2 in 60-mm culture dishes. One day post-seeding, cells were placed for 24 h in FCS-free DMEM. Cell layers were then exposed for 10 min to the compound to be tested, rapidly rinsed with ice-cold PBS and harvested in 0.3 mL of RIPA buffer (10 mM Tris-HCl pH 7.5, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM Na-orthovanadate, 1 mM PMSF, and 0.5 mM aprotinin). Proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Bucks, UK). The active forms of MAPK were revealed by chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences) using anti-phosphorylated Erk antibody (SantaCruz Biotechnologies, Santa Cruz, CA, USA). Equal

protein loading was assessed by reprobing the blots with anti-Erk2 antibody (SantaCruz Biotechnologies) that do not distinguish phosphorylated and unphosphorylated forms of MAPKs.

Measurement of cAMP. CHO cells were detached in PBS containing 1 mM EDTA, collected by gentle centrifugation (400 g, 5 min) and suspended in Hepes-buffered DMEM (pH 7.4). Aliquots of the cell suspension were incubated in a 200 μL final volume of Hepes-buffered DMEM containing 10 μM forskolin, alone or in combination with 10 μM of the compound to be tested. After 15 minutes at 37 °C, the reaction was stopped by adding 1.8 mL of methanol/formic acid (95/5, v/v). The alcoholic extract was centrifuged (3000 g, 10 min, 4 °C) and an aliquot of supernatant was evaporated. The dry samples were taken up in acetate buffer containing 0.1% NaN_3, and their cAMP content was determined by RIA using an Immunotech kit (Immunotech, Marseille, France).

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Crystallographic Data Collection and Refinement Procedures

The colourless crystals of the hydrogen dibenzoyl-L-tartrate salt of (*S*)-(-)-**5**, in suitable form for X-ray diffraction measurements, were grown by slow evaporation of a methanol : butanol (1 : 1, *vol*.) mixture. Diffraction data were collected at 200(2) K on an Oxford Diffraction PX Ultra CCD diffractometer, equipped with Oxford Diffraction Enhance Ultra optics and an Oxford Cryosystems apparatus, using $Cu_{K\alpha}$ radiation (λ = 1.5418 Å). Lattice constants were determined from the setting angles of 10931 reflections at θ 8.8-58.9°. Intensity data were corrected for absorption effects by the ABSPACK multiscan procedure, using Oxford Diffraction software.[14] Details of crystal data and refinement parameters are summarized below.

Formula $C_{35}H_{35}N_3O_{13}$, M_w = 705.65; Crystal system, monoclinic; Space group I 2; a = 19.627(3) Å, b = 7.479(1) Å, c = 23.206(4) Å, α = 90°, β = 93.23(1)°, γ = 90°, V = 3401.0(9) Å^3, Z = 4, $F(000)$ = 1480, D_c = 1.378 g cm^{-3}, μ = 0.900 mm^{-1}. Crystal size 0.15 x 0.20 x 0.60 mm, Transmission factors range for absorption correction: 0.902 - 1.057.

Data collection θ range, 3.8 - 72.4°; measured reflections 23740, unique reflections 5559 (R_{int} = 0.023), observed reflections 5347 ($F_o \geq 4.0\sigma(F_o)$ threshold). Number of parameters 502, restraints 11. Final R values: R_1 (observed refl.) = 0.0292, R_1 (all refl.) = 0.0307, wR_2 (observed refl.) = 0.0778, wR_2 (all refl.) = 0.0828; GoF = 1.068. $\Delta\delta_{max/min}$ in final ΔF Fourier, 0.169/-0.228 e Å^{-3}.

The structure was determined by direct methods, with SIR97,[15] and was completed by difference Fourier procedures and refined on F^2 by full-matrix least-squares, with SHELXL.[16] In view of the ΔF features, molecules of solvent present in the lattice were considered to be water molecules introduced by the commercial solvents used for the crystallization procedures. One of these molecules lay in a special position, on a twofold axis; the others were assigned fractional occupancies, on consideration of their contact distances. Fixed 0.50 population parameters (pp) were assigned to these molecules on the basis of refinement cycle results in which their pp values were allowed to refine. In the final refinement cycles, all non-hydrogen atoms were refined anisotropically. Hydrogens attached to the carbon atoms were introduced in calculated positions, each riding in the course of the refinement on the respective carrier atom, whereas the positions of the hydrogens bound to the nitrogen and oxygen atoms, located from ΔF maps, were allowed to refine. The hydrogen temperature factors were given by the formula $U_H = nU_E^{eq}$, where U_E^{eq} is the isotropic equivalent temperature factor of the carrier E atom and n was assigned the value 1.2 for

E = C_{CH2}, C_{CH} or N, and the value 1.5 for E = C_{CH3} or O. The positions of hydrogen atoms of the water molecules were refined with geometrical restraints; the hydrogens of one of the water fractions were not located and were not introduced in the calculations. With a -0.07(11) value of the Flack parameter[17] [0.96(12) value for the enantiomeric model], based on measurement of 1942 Friedel pairs, the absolute structure could be safely assigned.

For graphics the ORTEP program was used.[18] Details on the crystal structure investigation have been deposited at the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK, and may be obtained free of charge from deposit@ccdc.cam.uk by quoting the code CCDC-630732.

Description of the Structure

The structure consists of (*S*)-(-)-**5** cations, hydrogen dibenzoyl-L-tartrate anions and interspersed solvate water molecules, the latter being in a partially disordered arrangement. All of these structure components participate in a system of hydrogen bond linkages. The geometry of the imidazolinium cycle is regular (Figure 5 and Table 2), due to protonation of both nitrogen sites, and is in agreement with the geometry found for the same cycle in recent structural determinations.[19, 20] The ring is planar, all deviations of non-hydrogen atoms from the least-squares plane through them being less than 0.058(2) Å. The two rings of the diphenyl moiety in the cation form a dihedral angle of 48.93(2)°, and the plane of the NO_2 substituent is at an angle of 2.30(6)° with that of the phenyl group to which it is attached. In the anion (Figure 6), values of the carboxyl C-O distances (Table 2) clearly reveal that only one of these groups is dissociated; the other one, whose hydrogen atom was located, is involved in a chain of hydrogen-bonded anions along the crystallographic *b* direction (Figure 7). A view of the packing in proximity to the *a*-*c* plane is shown in Figure 8.

Table 2. Values of selected bond distances in the structure of (-)-**5** hydrogen dibenzoyl-L-tartrate salt.

C1-N1	1.298(2)	C7-C12	1.484(2)
C1-N2	1.301(2)	C19-O4	1.307(2)
N1-C2	1.460(2)	C19-O5	1.210(2)
N2-C3	1.467(3)	C28-O8	1.251(2)
C2-C3	1.518(3)	C28-O9	1.243(2)

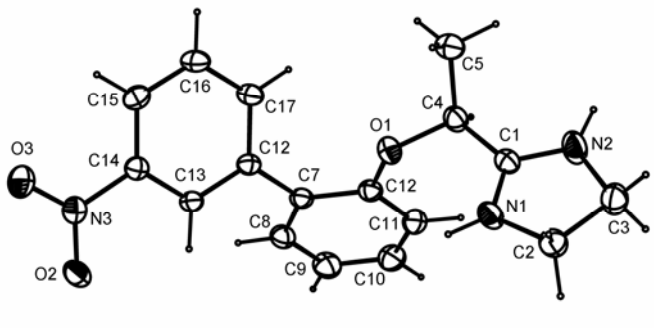

Figure 5. A view of the (-)-**5** cation showing atomic labels and 30% probability ellipsoids.

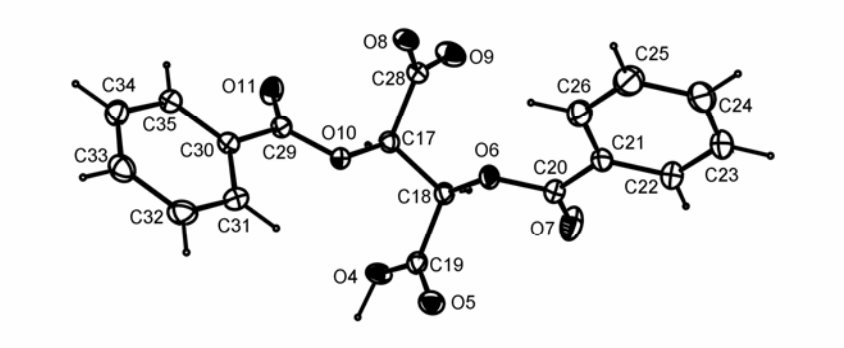

Figure 6. A view of the hydrogen dibenzoyl-L-tartrate anion in the structure of the (-)-**5** salt.

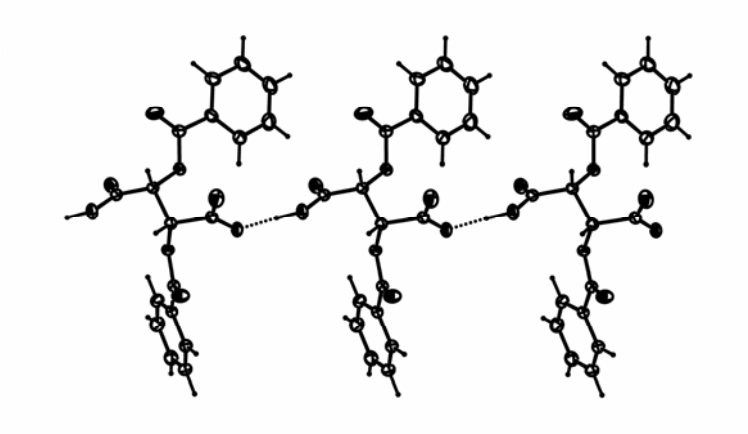

Figure 7. Part of one of the chains of hydrogen-bonded anions in the structure of the (-)-**5** salt. The spacing along the chain corresponds to the length of the crystallographic *b* axis.

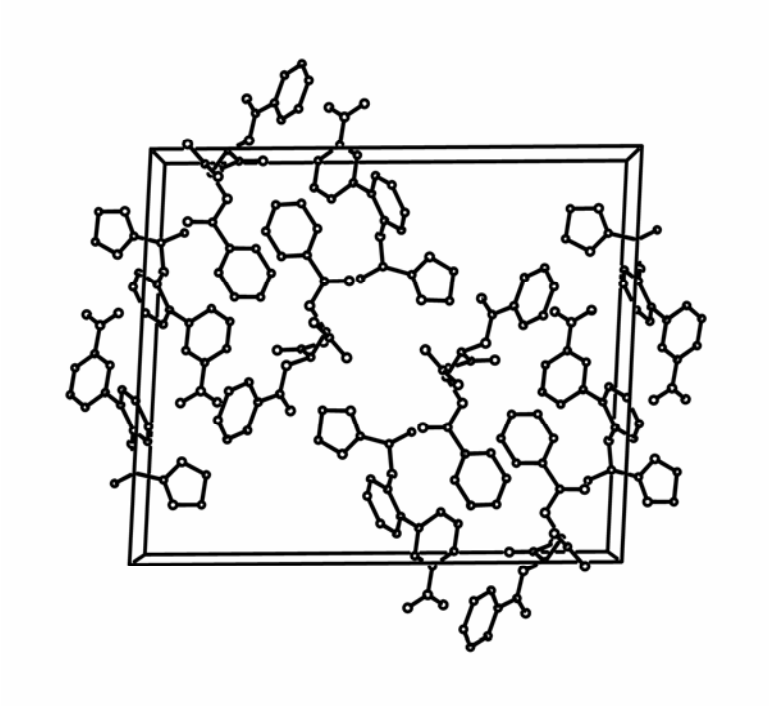

Figure 8. View of the packing in the structure of the (-)-**5** salt, in proximity of the *a*-*c* face of the crystallographic unit cell. Hydrogen atoms and solvent molecules have been omitted for clarity.

References for the Crystal Structure Determination

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Table 3. Elemental Analyses

Compd	Formula	Calculated			Found		
		C%	H%	N%	C%	H%	N%
(*R*)-(+)-5 oxalate salt	$C_{17}H_{17}N_3O_3 \cdot H_2C_2O_4$	56.86	4.77	10.47	56.96	5.03	10.21
(*S*)-(-)-5 oxalate salt	$C_{17}H_{17}N_3O_3 \cdot H_2C_2O_4$	56.86	4.77	10.47	56.51	4.49	10.38
7a	$C_{26}H_{26}N_4O_4$	68.11	5.72	12.22	67.82	5.99	12.01
7b	$C_{26}H_{26}N_4O_4$	68.11	5.72	12.22	67.74	5.84	11.87
(*R*)-(-)-8	$C_{17}H_{17}N_3O_3$	62.93	4.93	9.79	62.59	5.13	10.04