SUPPORTING INFORMATION

Exploring the Orthosteric Binding Site of the γ-Aminobutyric Acid Type A Receptors using 4-(Piperidine-4-yl)-1-hydroxypyrazoles 3- or 5-Imidazolyl substituted: Design, Synthesis, and Pharmacological Evaluation

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Content: Chemistry (Synthesis and NMR-spectral data, page S2), Elemental Analysis and analytical HPLC Data (page S9), Molecular Modeling (page S10), Pharmacology (page S12), and references (page S13).

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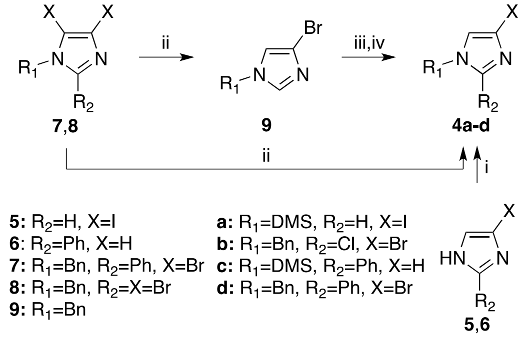
Experimental section.

Chemistry. *General procedures.* All reagents and solvents were obtained from commercial suppliers and used without further purification. Compound **10** and **11** was synthesized as described in the literature.[1] Air- and/or moisture-sensitive reactions were performed under a nitrogen atmosphere using syringe-septum cap techniques and with the use of flame-dried glassware. Anhydrous THF was obtained by distillation, and other anhydrous solvents were obtained by storage over 3 Å or 4 Å molecular sieves. Thin layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates, and compounds were visualized using UV (254 and 366 nm), $KMnO_4$ or Ninhydrin spray reagent. Dry column vacuum chromatography (DCVC)[2] was performed on Merck silica gel (0.015-0.040 mm). Melting points were recorded on a SRS OptiMelt apparatus in open capillary tubes and are uncorrected. ^{1}H- and ^{13}C-NMR was recorded on a 300 MHz Varian Mercury 300BB spectrometer equipped with a 5 mm ^{1}H{BB} probe, a 300 MHz Varian Gemini 2000BB spectrometer equipped with a 5 mm ^{31}P, ^{13}C{^{1}H, ^{19}F} probe, a Bruker Avance 400 MHz spectrometer equipped with a 5 mm PABBO BB{^{1}H, ^{19}F} Z-GRD probe or a Bruker Avance 600 MHz spectrometer equipped with a cryogenically cooled 5 mm CPDCH ^{13}C{^{1}H} Z-GRD probe, at 300 K. Data are tabulated in the following order: chemical shift (δ) [multiplicity (b, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constant(s) *J* (Hz), number of protons]. The solvent residual peak[3] or tetramethylsilane (δ 0.0 ppm) was used as internal reference. Elemental analyses were performed by Mr. J. Theiner, Department of Physical Chemistry, University of Vienna, Austria. Analytical HPLC (anal. HPLC) was performed on a Merck-Hitachi HPLC system consisting of an L-7100 pump, an L-7200 autosampler, and an L-7400 UV detector (254 nm), using an Chromolith SpeedROD RP-18 column (4.6 × 50 mm) eluted at a flow rate of 4.0 mL/min. A linear gradient elution was performed with eluent A (H_2O/TFA 100:0.1) containing 0% of solvent B (MeCN/H_2O/TFA, 90:10:0.01) rising to 100% of B during 5 min. Data were acquired and processed using the EZChrom Elite Software version 3.1.7 by Hitachi. The elemental analysis calculated values are within 0.4% of the found values and anal. HPLC purity is ≥ 95%, unless otherwise stated.

Synthesis of Imidazole building blocks. The imidazole building blocks was synthesized according to Scheme 1. Synthetic details are described below.

Scheme 1[a]

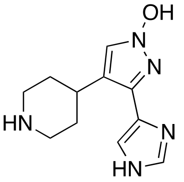


[a] *Reagents and conditions:* (i) Et_3N, dimethylsulfamoyl (DMS) chloride, CH_2Cl_2 or toluene, rt., (ii) EtMgBr, THF, 0 °C, (iii) H_2O, 0 °C to rt., (iv) ^{n}BuLi, THF, -78 °C, (v) C_2Cl_6, THF, -78 °C to rt.

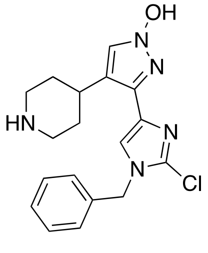
General procedures for Stille cross-coupling. Procedure A (12a,b,d, 13a,b,d). The 4-halogenated imidazole (**4a,b,d**, 1 equiv.) was dissolved in THF *or* CH_2Cl_2, under a nitrogen atmosphere, added ^{i}PrMgCl (1.2-2.3 equiv.), and stirred for 30 min. $SnBu_3Cl$ (1.1-1.4 equiv.) was added and the resulting mixture was stirred over-night before filtering and evaporation. The crude Stille reagent was re-dissolved in DMF and added **10** or **11** (1 equiv.), CuI (0.1-0.3 equiv.), CsF (2.0-2.1 equiv.), $Pd(PPh_3)_4$ (0.05-0.06 equiv.), and flushed with nitrogen. The reaction was stirred at 45-110 °C for 3-8 days before worked up by filtering over Celite and extraction using H_2O and Et_2O. The crude product was purified by DCVC.

Procedure B (12c, 13c). The halogenated imidazole (**4c**, 1 equiv.) was dissolved in THF, under a nitrogen atmosphere, and cooled to -78 °C. The mixture was added ^{n}BuLi (1.1-1.2 equiv.) and stirred for 1 h, then added Bu_3SnCl (1.2-1.3 equiv.). The resulting mixture was stirred over-night at rt. before filtering and evaporation. The crude Stille reagent was re-dissolved in DMF and added **10** *or* **11** (1 equiv.), CuI (0.2 equiv), CsF (2.1 equiv), $Pd(PPh_3)_4$ (0.05 equiv.), and flushed with nitrogen. The reaction was stirred at 90 °C for 6 days before worked up by filtering over Celite and extraction using H_2O and Et_2O. The crude product was purified by DCVC.

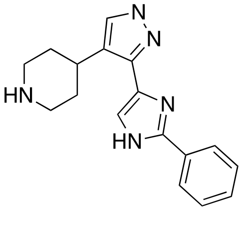
General procedure for acidic deprotection. Unless otherwise stated, a mixture of the protected compound in 48% aq. HBr was refluxed until the reaction was finished by TLC followed by evaporation and recrystallization from appropriate solvents.



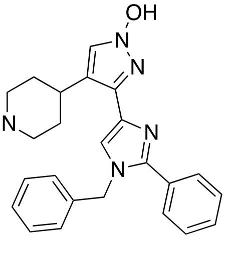
4-(1-hydroxy-3-(1*H*-imidazol-4-yl)-1*H*-pyrazol-4-yl)piperidinium bromide (2a). 12a (61 mg, 0.12 mmol) was dissolved in aqueous HBr (48%, 3 mL) and refluxed for 1 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 12 mL) and toluene (2 × 12 mL). Recrystallization from MeOH/Et_2O gave **2a** (27 mg, 54%) as brown solid: mp > 220 °C. ^{1}H-NMR (300 MHz, MeOD-d_4): δ 8.95 (d, J = 1.2 Hz, 1H), 7.89 (d, J = 1.3 Hz, 1H), 7.58 (s, 1H), 3.47 (b d, J = 12.8 Hz, 2H), 3.30-3.21 (m, 2H), 3.13 (tt, J = 12.1, 3.6 Hz, 1H), 2.14 (b d, J = 14.5 Hz, 2H), 1.89-1.75 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 135.5, 130.6, 128.1, 123.3, 122.7, 116.9, 45.5, 31.1, 31.0. Anal. ($C_{11}H_{15}N_5O{\cdot}2HBr{\cdot}1.5H_2O$) C, H, N (calc. 16.59, found 16.09).



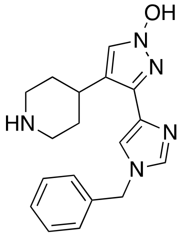
4-(3-(1-benzyl-2-chloro-1*H*-imidazol-4-yl)-1-hydroxy-1*H*-pyrazol-4-yl)piperidinium bromide (2b). **12b** (285 mg, 0.56 mmol) was dissolved in aqueous HBr (48%, 6 mL) and refluxed for 4 h. The reaction mixture was cooled to rt., evaporated and, re-evaporated with water (2 × 12 mL) and toluene (2 × 12 mL). Recrystallization from MeOH/MeCN resulted in **2b** (22 mg, 9%) as brown solid: mp > 230 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.60-7.57 (m, 1H), 7.51-7.48 (m, 1H), 7.43-7.24 (m, 5H), 5.23 (s, 2H), 3.33-3.18 (m, 3H), 3.04-2.91 (m, 2H), 2.06 (d, J = 13.3 Hz, 2H), 1.66-1.53 (m, 2H). ^{13}C-NMR (150 MHz, DMSO-d_6): δ 136.3, 134.9, 134.8, 130.1, 128.8, 127.9, 127.3, 120.7, 119.7, 118.6, 49.4, 44.0, 29.9, 29.8. Anal. ($C_{18}H_{20}ClN_5O{\cdot}1HBr{\cdot}0.5H_2O$) C, H, N. Purity by anal. HPLC (254 nm), 95.5%.



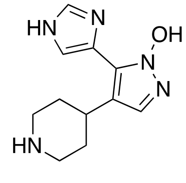
4-(1-hydroxy-3-(2-phenyl-1*H*-imidazol-4-yl)-1*H*-pyrazol-4-yl)piperidinium bromide (2c). 12c (271 mg, 0.58 mmol) was dissolved in AcOH (1 mL) and aqueous HBr (48%, 11 mL) and refluxed for 2 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 10 mL) and toluene (2 × 10 mL). Recrystallisation from MeOH/Et_2O gave **2c** (161 mg, 49%) as light brown crystals: mp > 230 °C. ^{1}H-NMR (300 MHz, MeOD-d_4): δ 8.01-7.95 (m, 3H), 7.72-7.65 (m, 3H), 7.62 (s, 1H), 3.48 (b d, J = 12.5 Hz, 2H), 3.29-3.13 (m, 3H), 2.19 (b d, J = 13.4 Hz, 2H), 1.85 (q d, J = 4.2 Hz, 12.2 Hz, 2H). ^{13}C-NMR (75 MHz, MeOD-d_4): δ 146.7, 134.0, 131.0, 130.7, 128.7, 128.6, 124.3, 123.4, 123.2, 118.1, 45.5, 31.2, 31.1. Anal. ($C_{17}H_{19}N_5O{\cdot}3HBr{\cdot}1H_2O$) C, H, N.



4-(3-(1-benzyl-2-phenyl-1*H*-imidazol-4-yl)-1-hydroxy-1*H*-pyrazol-4-yl)piperidinium bromide (2d). 12d (323 mg, 0.56 mmol) was dissolved in AcOH (1 mL) and aqueous HBr (48%, 11 mL) and refluxed for 2.5 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 10 mL) and toluene (2 × 10 mL). Recrystallization from MeOH/Et_2O gave **2d** (102 mg, 30%) as brown crystals: mp > 240 °C. ^{1}H-NMR (300 MHz, MeOD-*d₄*): δ 8.16 (s, 1H), 7.78-7.61 (m, 5H), 7.60 (s, 1H), 7.38-7.29 (m, 3H), 7.24-7.15 (m, 2H), 5.56 (s, 2H), 3.46 (b d, *J* = 12.3 Hz, 2H), 3.29-3.14 (m, 3H), 2.17 (b d, *J* = 14.2 Hz, 2H), 1.83 (q d, *J* = 4.1 Hz, 12.7 Hz, 2H). ^{13}C-NMR (75 MHz, MeOD-d_4): δ 147.0, 136.0, 134.0, 131.1, 130.8, 130.4, 130.4, 130.0, 128.7, 128.0, 123.8, 123.4, 123.1, 121.2, 53.2, 45.4, 31.1. Anal. ($C_{24}H_{25}N_5O{\cdot}2HBr{\cdot}1.5H_2O$) C, H (calc. 5.14, found 4.43), N. Purity by anal. HPLC (254 nm), 97.8%.

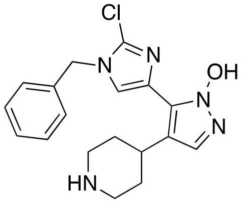


3-(1-benzyl-1*H*-imidazol-4-yl)-4-(piperidin-4-yl)-1*H*-pyrazol-1-ol (2e). 12b (200 mg, 0.40 mmol) was dissolved in CH_2Cl_2 (50 mL) and added 10% Pd/C (133 mg). The reaction mixture was hydrogenated at rt. for 4 h then filtered through Celite and eluted with MeOH. The combined organic phases were evaporated and purification by DCVC (Heptane/EtOAc, 0-100% EtOAc) provided Methyl 4-(3-(1-benzyl-1*H*-imidazol-4-yl)-1-hydroxy-1*H*-pyrazol-4-yl)piperidine-1-carboxylate (65 mg, 43%) as light oil. ^{1}H-NMR (300 MHz, MeOD-d_4): δ 9.02 (s, 1H), 7.77 (s, 1H), 7.48-7.36 (m, 6H), 5.47 (s, 2H), 4.11 (b d, *J* = 13.3 Hz, 2H), 3.68 (s, 3H), 2.95-2.74 (m, 3H), 1.84-1.75 (m, 2H), 1.49-1.35 (m, 2H). ^{13}C-NMR (100 MHz, MeOD-d_4): δ 157.8, 136.4, 135.8, 130.6, 130.4, 129.7, 129.2, 128.7, 124.4, 123.3, 119.3, 54.2, 53.4, 45.3, 33.9, 33.3. The resulting oil was was dissolved in aqueous HBr (48%, 4 mL) and refluxed for 4 h. The reaction mixture was cooled down to rt., evaporated, and re-evaporated with water (2 × 8 mL) and toluene (2 × 8 mL). Purification by ion-exchange (Amberlite IR-120 (H^+), sample was loaded with water, rinsed with water (2 × 20 mL), 1.4-dioxane (2 × 20 mL), and water (2 × 20 mL), eluted with aqueous NH_3 (2M, 3 mL) and evaporated) afforded **2e** as brown solid (21 mg, 16% over two steps): mp > 220 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.86 (s, 1H), 7.63 (s, 1H), 7.59 (s, 1H), 7.51-7.40 (m, 5H), 5.46 (s, 2H), 3.44 (b d, *J* = 12.6 Hz, 2H), 3.07-2.97 (m, 2H), 2.95-2.85 (m, 1H), 2.08-1.99 (m, 2H), 1.81-1.65 (m, 2H). Purity by anal. HPLC (254 nm), 96.8%.

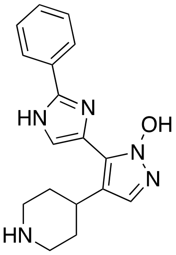


5-(1*H*-imidazol-4-yl)-4-(piperidin-4-yl)-1*H*-pyrazol-1-ol (3a). Under a nitrogen atmosphere, **4a** (605 mg, 2.0 mmol) was dissolved in anhydrous THF (50 mL) and added ^{i}PrMgCl (2.0 M, 1.3 mL, 2.60 mmol) drop wise and stirred at rt. for 30 min before Bu_3SnCl (0.7 mL, 2.60 mmol) was added. The resulting mixture was stirred over-night before filtering and evaporation. The formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (20 mL) and added **11** (371 mg, 0.82 mmol), $Pd(PPh_3)_4$ (64 mg, 0.06 mmol), CsF (250 mg, 1.65 mmol), and CuI (34 mg, 0.18 mmol). The resulting mixture was flushed with nitrogen and stirred at 45 °C for 4 days. Water (200 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 230 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. A crude purification by DCVC (Heptane/EtOAc, 0-50% EtOAc) provided **13a** (104 mg, 10% from **11**) as light oil. **13a** (104 mg, 0.21 mmol) was dissolved in aqueous HBr (48%, 6 mL) and refluxed for 1 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 15 mL) and toluene (2 × 15 mL). Purification by ion-exchange (Amberlite IR-

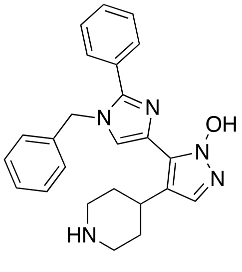
120 (H$^+$), sample was loaded with water, rinsed with water (2 × 20 mL), 1.4-dioxane (2 × 20 mL), and water (2 × 20 mL), eluted with aqueous NH_3 (2M, 3 mL) and evaporated) afforded **3a** as white solid (26 mg, 53% from **13a**): mp > 220 °C. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.82 (d, J = 1.1 Hz, 1H), 7.46-7.44 (m, 1H), 7.02 (s, 1H), 3.13-3.08 (m, 2H), 2.90-2.82 (m, 1H), 2.76-2.67 (m, 2H), 1.90-1.84 (m, 2H), 1.60-1.49 (m, 2H). Purity by anal. HPLC (254 nm), 96.8%.



4-(5-(1-benzyl-2-chloro-1*H*-imidazol-4-yl)-1-hydroxy-1*H*-pyrazol-4-yl)piperidinium bromide (3b). 13b (175 mg, 0.35 mmol) was dissolved in aqueous HBr (48%, 10 mL) and refluxed for 4 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 15 mL) and toluene (2 × 15 mL) to give **3b** (96 mg, 44%) as brown solid: mp > 230 °C. ^{1}H-NMR (400 MHz, MeOD-d_4): δ 7.75 (s, 1H), 7.43-7.29 (m, 5H), 7.18 (s, 1H), 5.32 (s, 2H), 3.50-3.38 (m, 3H), 3.18-3.08 (m, 2H), 2.21-2.14 (m, 2H), 1.89-1.75 (m, 2H). ^{13}C-NMR (100 MHz, MeOD-d_4): δ 137.0, 133.9, 130.3, 130.1, 129.9, 129.7, 128.9, 127.0, 132.2, 121.9, 51.8, 45.8, 31.4, 30.9. Anal. ($C_{18}H_{20}ClN_5O\cdot 3HBr\cdot 1.5H_2O$) C, H, N. Purity by anal. HPLC (254 nm), 97.6%.

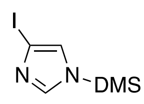


4-(1-hydroxy-5-(2-phenyl-1*H*-imidazol-4-yl)-1*H*-pyrazol-4-yl)piperidinium bromide (3c). Under a nitrogen atmosphere, **4c** (1.40 g, 5.6 mmol) was dissolved in anhydrous THF (50 mL) and cooled to -78 °C before ^{n}BuLi (1.6 M, 4.2 mL, 6.7 mmol) was added drop wise. The resulting solution was stirred for 1 h at -78 °C before Bu_3SnCl (1.8 mL, 6.7 mmol) was added. The mixture was allowed to reach rt. and stirring was continued for 23 h before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (25 mL) and added **11** (514 mg, 1.13 mmol), CsF (356 mg, 2.34 mmol), CuI (42 mg, 0.22 mmol), and $Pd(PPh_3)_4$ (67 mg, 0.06 mmol). The mixture was flushed with nitrogen and stirred at 90 °C for 6 days. Water (300 mL) and CH_2Cl_2 (300 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 300 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-50% EtOAc) provided **13c** (70 mg, 11% from **11**) as light yellow oil. **13c** (43 mg, 0.07 mmol) was dissolved in AcOH (0.5 mL) and aqueous HBr (48%, 2 mL) and refluxed for 1.5 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 10 mL) and toluene (2 × 10 mL). Recrystallization from MeOH/Et_2O gave **3c** (23 mg, 65% from **13c**) as light brown crystals: mp > 220 °C. ^{1}H-NMR (400 MHz, MeOD-d_4): δ 8.03-7.95 (m, 3H), 7.73-7.64 (m, 3H), 7.33 (s, 1H), 3.46 (b d, J = 12.5 Hz, 2H), 3.22-3.00 (m, 3H), 2.12 (b d, J = 13.6 Hz, 2H), 1.99-1.82 (m, 2H). ^{13}C-NMR (100 MHz, MeOD-d_4): δ 147.9, 134.1, 131.1, 130.7, 128.5, 125.1, 124.5, 122.9, 122.0, 121.2, 45.5, 31.2, 31.1. Anal. ($C_{17}H_{19}N_5O\cdot 2HBr\cdot 2H_2O$) C, H, N.

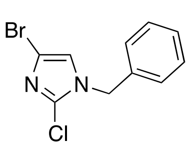


4-(5-(1-benzyl-2-phenyl-1*H*-imidazol-4-yl)-1-hydroxy-1*H*-pyrazol-4-yl)piperidinium bromide (3d). 13d (156 mg, 0.28 mmol) was dissolved in AcOH (1 mL) and aqueous HBr (48%, 9 mL) and refluxed for 2 h. The reaction mixture was cooled to rt., evaporated, and re-evaporated with water (2 × 11 mL) and toluene (2 × 10 mL) to give **3d** (135 mg, 73%) as brown solid: mp > 240 °C. ^{1}H-NMR (300 MHz, MeOD-d_4): δ 8.09 (s, 1H), 7.79-7.64 (m, 5H), 7.41-7.34 (m, 3H), 7.31 (s, 1H), 7.26-7.20 (m, 2H), 5.57 (s, 2H), 3.50-3.42 (m, 2H), 3.22-3.13 (m, 2H), 3.12-3.04 (m, 1H), 2.11 (b d,

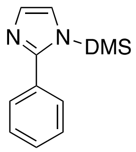
J = 14.5 Hz, 2H), 1.96-1.82 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 148.1, 135.6, 134.1, 131.3, 130.9, 130.9, 130.5, 130.2, 129.2, 125.1, 125.0, 123.6, 122.0, 120.6, 53.5, 46.6, 45.5, 43.6, 31.1, 31.1. Anal. ($C_{24}H_{25}N_5O\cdot3HBr\cdot1.5H_2O$) C, H, N. Purity by anal. HPLC (254 nm), 95.2%.



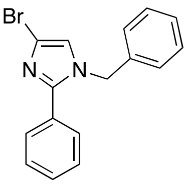
4-iodo-*N,N*-dimethyl-1*H*-imidazole-1-solfonamide (4a). Under a nitrogen atmosphere, 4(5)-iodo-1*H*-imidazole (**5**, 1.38 g, 7.1 mmol) was dissolved in anhydrous CH_2Cl_2 (70 mL) and added Et_3N (3.00 mL, 21.4 mmol) and dimethylsulfamoyl (DMS) chloride (0.94 mL, 8.8 mmol). The resulting mixture was stirred for 26 h at rt. before the organic phase was washed with water (150 mL) and aqueous $Na_2S_2O_3$ (10%, 150 mL). The organic phases was dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-40% EtOAc) provided **4a** (1.25 g, 58%) as a white solid: mp 130-133 °C (lit.[4] 131.5 °C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.76 (d, J = 1.5 Hz, 1H), 7.32 (d, J = 1.5 Hz, 1H), 2.88 (s, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 137.9, 122.6, 84.4, 38.2.



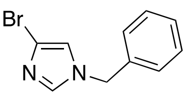
1-benzyl-4-bromo-2-chloro-1*H*-imidazole (4b). Under a nitrogen atmosphere, **9** (2.34 g, 9.9 mmol) was dissolved in anhydrous THF (80 mL) and cooled to -78 °C. ^{n}BuLi (2.5 M, 4.50 mL, 11.3 mmol) was added drop wise and the resulting mixture was stirred at -78°C for 30 min before hexachloroethane (5.22 g, 22.0 mmol) in anhydrous THF (10 mL) was added. The solution was allowed to reach rt. and stirred for 19 h. Water (100 mL) and saturated aqueous $NaHCO_3$ (80 mL) was added and the mixture was extracted with Et_2O (3 × 200 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-15% EtOAc) provided **4b** (1.56 g, 58%) as a yellow solid: mp 41-43 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.42-7.29 (m, 3H), 7.22-7.13 (m, 2H), 6.83 (s, 1H), 5.05 (s, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 175.6, 134.6, 129.3, 128.8, 127.6, 120.5, 113.8, 50.9.



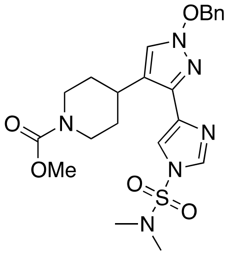
***N,N*-dimethyl-2-phenyl-1*H*-imidazole-1-sulfonamide (4c).** Under a nitrogen atmosphere, 2-phenyl-1*H*-imidazole (**6**, 8.08 g, 56.3 mmol) was dissolved in anhydrous toluene (80 ml) and added Et_3N (10.6 mL, 76.5 mmol) and dimethylsulfamoyl (DMS) chloride (6.1 mL, 57.18 mmol). The resulting mixture was stirred overnight at rt. before filtering. The filter was washed with toluene (40 mL) and the organic phase was evaporated. Purification by DCVC (Heptane/EtOAc, 0-75% EtOAc) provided **4c** (5.21 g, 37%) as white solid: mp 109-110 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.69-7.65 (m, 2H), 7.46-7.43 (m, 3H), 7.42 (d, J = 2.2 Hz, 1H), 7.09 (d, J = 1.6 Hz, 1H), 2.48 (s, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 147.6, 130.5, 130.13, 129.97, 128.0, 127.8, 122.2, 37.8.



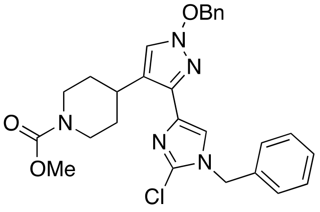
1-benzyl-4-bromo-2-phenyl-1*H*-imidazole (4d). Under a nitrogen atmosphere, **7**[5] (2.00 g, 5.1 mmol) was dissolved in anhydrous THF (50 mL) and cooled to 0 °C. EtMgBr (3 M, 1.90 mL, 5.7 mmol) was added drop wise and the resulting mixture was stirred for 2 h before quenched with saturated aqueous $NaHCO_3$ (25 mL) and water (40 mL). The aqueous phase was extracted with Et_2O (4 × 50 mL) and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated to provide **4d** (1.57 g, 98%) as a white solid: mp 130-131 °C. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.55-7.50 (m, 2H), 7.42-7.30 (m, 6H), 7.12-7.07 (m, 2H), 6.90 (s, 1H), 5.20 (s, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 148.0, 135.8, 129.2, 129.0, 128.9, 128.6, 128.5, 128.0, 126.5, 119.9, 115.2, 50.6.



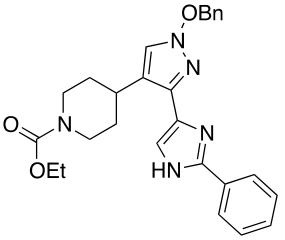
1-benzyl-4-bromo-1*H*-imidazole (9). Under a nitrogen atmosphere, **8**[6] (4.02 g, 10.2 mmol) was dissolved in anhydrous THF (90 mL) and cooled to 0 °C. EtMgBr (3 M, 10.8 mL, 32.4 mmol) was added drop wise and the resulting mixture was stirred for 2 h. Water (50 mL) was added and the phases separated. The aqueous phase was extracted with Et_2O (3 × 100 mL) and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-50% EtOAc) provided **9** (1.71 g, 71%) as white solid: mp 87-88 °C (lit.[7] 88-90 °C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.44-7.33 (m, 4H), 7.22-7.15 (m, 2H), 6.87 (d, J = 1.6 Hz, 1H), 5.09 (s, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 137.3, 129.5, 129.0, 127.9, 118.9, 116.1, 99.6, 52.0.



Methyl 4-(1-(benzyloxy)-3-(1-(*N,N*-dimethylsulfamoyl)-1*H*-imidazole-4-yl)-1*H*-pyrazol-4-yl) piperidine-1-carboxylate (12a). Under a nitrogen atmosphere, **4a** (329 mg, 1.09 mmol) was dissolved in anhydrous THF (30 mL) and added ^{i}PrMgCl (2.0 M, 0.7 mL, 1.44 mmol) drop wise. The mixture was stirred at rt. for 30 min before addition of Bu_3SnCl (0.4 mL, 1.44 mmol). Stirring was continued over-night before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (20 mL) and added **10** (259 mg, 0.59 mmol), $Pd(PPh_3)_4$ (35 mg, 0.03 mmol), CsF (182 mg, 1.20 mmol) and CuI (25 mg, 0.13 mmol). The resulting mixture was flushed with nitrogen and stirred at 45 °C for 3 days. Water (200 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 200 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-60% EtOAc) provided **12a** (61 mg, 21% from **10**) as light oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.91 (d, J = 1.41 Hz, 1H), 7.59 (d, J = 1.51 Hz, 1H), 7.42-7.28 (m, 5H), 6.74 (s, 1H), 5.27 (s, 2H), 4.16 (b s, 2H), 3.69 (s, 3H), 3.27 (tt, J = 12.1 Hz, 3.5 Hz, 1H), 2.92 (s, 6H), 2.90-2.81 (m, 2H), 1.88 (b d, J = 12.7 Hz, 2H), 1.36-1.22 (m, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.9, 137.9, 136.3, 135.8, 133.7, 129.7, 129.3, 128.6, 122.3, 121.2, 113.7, 80.7, 52.4, 44.3, 38.2, 33.0, 31.7.

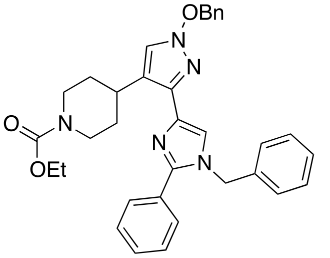


Methyl 4-(3-(1-benzyl-2-chloro-1*H*-imidazol-4-yl)-1-(benzyloxy)-1*H*-pyrazol-4-yl)piperidine-1-carbozylate (12b). Under a nitrogen atmosphere, **4b** (778 mg, 2.9 mmol) was dissolved in anhydrous CH_2Cl_2 (50 mL) and added ^{i}PrMgCl (2 M, 1.7 mL, 3.4 mmol) drop wise. The mixture was stirred at rt. for 30 min before addition of Bu_3SnCl (1.0 mL, 3.5 mmol). Stirring was continued over-night before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (25 mL) and added **10** (469 mg, 1.06 mmol), $Pd(PPh_3)_4$ (61 mg, 0.05 mmol), CsF (324 mg, 2.13 mmol), and CuI (40 mg, 0.21 mmol). The resulting mixture was flushed with nitrogen and stirred at 110 °C for 3 days. Water (250 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 200 mL) and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-50% EtOAc) provided **12b** (296 mg, 55% from **10**) as clear oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.43-7.24 (m, 10H), 7.18-7.14 (m, 1H), 6.71 (s, 1H), 5.25 (s, 2H), 5.13 (s, 2H), 4.18 (b s, 2H), 3.71 (s, 3H), 3.27 (tt, J = 11.6 Hz, 3.7 Hz, 1H), 2.87 (t, J = 13.2 Hz, 2H), 1.93-1.86 (m, 2H), 1.72-1.64 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.9, 136.7, 135.4, 135.1, 133.8, 131.6, 129.7, 129.2, 129.0, 128.6, 128.4, 127.6, 121.5, 121.0, 117.7, 80.6, 52.4, 50.4, 44.3, 32.8, 31.6.

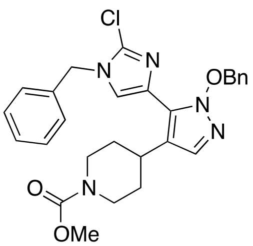


Ethyl 4-(1-(benzyloxy)-3-(2-phenyl-1*H*-imidazol-4-yl)-1*H*-pyrazol-4-yl)piperidine-1-carboxylate (12c). Under a nitrogen atmosphere, **4c** (352 mg, 1.4 mmol) was dissolved in anhydrous THF (13 mL) and cooled to -78 °C before ^{n}BuLi (1.6 M, 1.0 mL, 1.6 mmol) was added drop wise. The resulting solution was stirred for 1 h at -78°C before

Bu_3SnCl (0.5 mL, 1.8 mmol) was added. The mixture was allowed to reach rt. and stirring was continued for 23 h before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (25 mL) and added **10** (621 g, 1.4 mmol), CsF (443 mg, 2.9 mmol), CuI (52 mg, 0.3 mmol) and $Pd(PPh_3)_4$ (88 mg, 0.1 mmol). The mixture was flushed with nitrogen stirred at 90 °C for 6 days. Water (200 mL) and CH_2Cl_2 were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 300 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-75% EtOAc) provided **12c** (271 mg, 41% from to **10**) as yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.92-7.86 (m, 2H), 7.50-7.25 (m, 9H), 6.74 (s, 1H), 5.28 (s, 2H), 4.21 (b s, 2H), 4.14 (q, *J* = 7.1 Hz, 2H), 3.02-2.79 (m, 3H), 1.92 (b d, *J* = 13.1 Hz, 2H), 1.41-1.23 (m, 5H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.4, 146.3, 133.4, 130.2, 129.7, 129.6, 129.2, 128.7, 128.5, 127.8, 127.5, 125.1, 121.2, 120.8, 80.8, 61.3, 44.2, 37.5, 32.6, 32.4, 14.7.

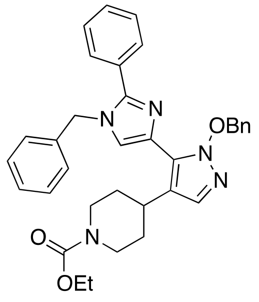


Ethyl 4-(3-(1-benzyl-2-phenyl-1*H*-imidazole-4-yl)-1-(benzyloxy)-1*H*-pyrazol-4-yl)piperidine-1-carboxylate (12d). Under a nitrogen atmosphere, **4d** (497 mg, 1.6 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL) and added ^{i}PrMgCl (2 M, 1.8 mL, 3.6 mmol) drop wise. The mixture was stirred at rt. for 2 h before addition of Bu_3SnCl (0.5 mL, 1.8 mmol). Stirring was continued over-night before filtering and evaporation. The formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (30 mL) and added **10** (730 mg, 1.6 mmol), CsF (519 mg, 3.4 mmol), CuI (51 mg, 0.3 mmol), and $Pd(PPh_3)_4$ (108 mg, 0.1 mmol). The mixture was stirred at 90 °C for 8 days. Water (500 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (5 × 300 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-40% EtOAc) provided **12d** (366 mg, 41% from **10**) as yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.61-7.55 (m, 2H), 7.42-7.23 (m, 12H), 7.17-7.12 (m, 2H), 6.71 (s, 1H), 5.24 (s, 4H), 4.16 (b s, 2H), 4.10 (q, *J* = 7.1 Hz, 2H), 3.36 (tt, *J* = 11.9 Hz, 3.4 Hz, 1H), 2.84 (t, *J* = 12.8 Hz, 2H), 1.96 (b d, *J* = 12.6 Hz, 2H), 1.38-1.28 (m, 2H), 1.24 (t, *J* = 7.0 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.3, 147.4, 137.4, 136.5, 135.8, 133.8, 130.3, 129.5, 128.9, 128.8, 128.5, 128.5, 128.3, 127.8, 126.6, 121.3, 120.6, 118.2, 80.5, 61.0, 60.3, 50.5, 44.3, 32.0, 14.2.



Methyl 4-(5-(1-benzyl-2-chloro-1*H*-imidazol-4-yl)-1-(benzyloxy)-1*H*-pyrazol-4-yl)piperidine-1-carboxylate (13b). Under a nitrogen atmosphere, **4b** (2.04 g, 7.5 mmol) was dissolved in anhydrous CH_2Cl_2 (100 mL) and added ^{i}PrMgCl (2 M, 6.3 mL, 12.6 mmol) drop wise and stirred at rt. for 30 min before addition of Bu_3SnCl (2.8 mL, 9.9 mmol). Stirring was continued over-night before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was re-dissolved in anhydrous DMF (40 mL) and added **11** (829 mg, 1.9 mmol), $Pd(PPh_3)_4$ (108 mg, 0.09 mmol), CsF (575 mg, 3.8 mmol), and CuI (35 mg, 0.18 mmol). The resulting mixture was flushed with nitrogen and stirred at 110 °C for 3 days. Water (400 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 400 mL) and the combined organic phases were dried over $MgSO_4$, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-60% EtOAc) provided **13b** (175 mg, 18% from **11**) as white solid: mp 119-121 °C. ^{1}H-NMR (400

MHz, $CDCl_3$): δ 7.45-7.12 (m, 12H), 5.18 (s, 2H), 5.09 (s, 2H), 4.23 (b s, 2H), 3.73 (s, 3H), 3.28 (tt, *J* = 12.1 Hz, 3.6 Hz, 1H), 2.90 (t, *J* = 13.3 Hz, 2H), 1.91 (d, *J* = 13.6 Hz, 2H), 1.61-1.48 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.9, 135.0, 133.2, 132.2, 130.2, 130.1, 129.7, 129.1, 129.0, 128.5, 128.4, 127.4, 125.6, 121.9, 119.9, 80.1, 52.4, 50.4, 44.4, 32.5, 31.8.



Ethyl 4-(5-(1-benzyl-2-phenyl-1*H*-imidazol-4-yl)-1-(benzyloxy)-1*H*-pyrazol-4-yl)piperidine-1-carboxylate (13d). Under a nitrogen atmosphere, **4d** (593 mg, 1.9 mmol) was dissolved in anhydrous CH_2Cl_2 (20 mL) and added 2.0 M ^{i}PrMgCl (2.0 M, 1.94 mL, 3.9 mmol) drop wise. The mixture was stirred at rt. for 3 h before addition of Bu_3SnCl (0.7 mL, 2.6 mmol). Stirring was continued for 23 h before filtering and evaporation. Formation of the Stille reagent was verified by ^{1}H-NMR. The crude Stille reagent was dissolved in anhydrous DMF (25 mL) and added **11** (538 mg, 1.2 mmol), CsF (358 mg, 2.4 mmol), CuI (51 mg, 0.3 mmol) and $Pd(PPh_3)_4$ (69 mg, 0.06 mmol). The mixture was flushed with nitrogen and stirred at 90 °C for 6 days. Water (200 mL) and CH_2Cl_2 (200 mL) were added and the mixture was filtered through Celite. The aqueous phase was extracted with Et_2O (3 × 200 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and evaporated. Purification by DCVC (Heptane/EtOAc, 0-50% EtOAc) provided **13d** (369 mg, 55% from **11**) as yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.54-6.98 (m, 17H), 5.13 (s, 2H), 5.11 (s, 2H), 4.15 (b s, 2H), 4.05 (q, *J* = 6.9 Hz, 2H), 3.36 (tt, *J* = 11.9 Hz, 3.5 Hz, 1H), 2.80 (t, *J* = 12.5 Hz, 2H), 1.98-1.87 (m, 2H), 1.59-1.42 (m, 2H), 1.21-1.15 (m, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.6, 147.9, 136.6, 133.4, 130.6, 130.1, 129.7, 129.0, 129.0, 128.9, 128.6, 128.6, 128.4, 128.1, 126.6, 126.5, 121.7, 120.5, 80.1, 61.0, 53.4, 50.5, 44.4, 32.5, 14.7.

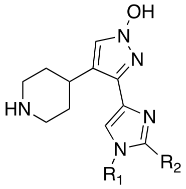
Elemental Analysis and analytical HPLC Data.

Compound		Anal. HPLC (Wavelength)	Elemental analysis calculated (found)		
			C	H	N
2a	$C_{11}H_{15}N_5O\cdot2HBr\cdot1.5H_2O$	-	31.30 (31.42)	4.78 (4.37)	16.59 (16.09)
2b	$C_{18}H_{20}ClN_5O\cdot1HBr\cdot0.5H_2O$	95.5% (254 nm)	48.28 48.17	4.95 (4.58)	15.64 (15.35)
2c	$C_{17}H_{19}N_5O\cdot3HBr\cdot1H_2O$	-	35.81 (35.45)	4.24 (4.62)	12.28 (11.86)
2d	$C_{24}H_{25}N_5O\cdot2HBr\cdot1.5H_2O$	97.8% (254 nm)	49.00 (48.97)	5.14 (4.43)	11.90 (11.49)
2e	-	96.8% (254 nm)	-	-	-
3a	-	96.8% (254 nm)	-	-	-
3b	$C_{18}H_{20}ClN_5O\cdot3HBr\cdot1.5H_2O$	97.6% (254 nm)	34.45 (34.71)	4.18 (3.82)	11.16 (10.84)
3c	$C_{17}H_{19}N_5O\cdot2HBr\cdot2H_2O$	-	40.26 (40.63)	4.97 (4.65)	13.81 (13.68)
3d	$C_{24}H_{25}N_5O\cdot3HBr\cdot1.5H_2O$	95.2% (254 nm)	43.07 (43.09)	4.67 (4.80)	10.46 (10.92)

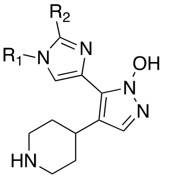
Molecular Modeling.

A model of the extracellular domain of GABA$_A$R constructed using an iterative approach with the orthosteric binding site optimized using an induced fit docking protocol, has previously been reported[8], and was used here. The binding site optimized around 3-(naphthalen-2-ylmethyl)-5-phenyl-4-(piperidin-4-yl)-1-hydroxypyrazol (compound **2p** in reference[1]) was characterized using the program GRID,[9, 10] with a grid spacing of 0.33 Å and otherwise default settings. The following probes were used: C3, COO- and N2+, contoured at -2, -10 and -10 kCal/mol, respectively. Subsequently, ligands **2a-e** and **3a-d**, respectively, were docked into the binding site as described previously,[8] in their zwitterion form, with a negatively charged hydroxyl moiety and a positively charged amine moiety. 200 poses per ligand were included in the post-docking minimization step. The attained docking poses were subsequently refined using the "None (refine only)" ligand sampling option in the Glide 5.8 docking program.[11-14]

Table 1. Pharmacological data and Glide XP docking scores for 4-PHP, **1**, **2a-e**, and **3a-d**: GABA$_A$R binding affinities at rat synaptic membranes and functional characterization at the human $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$R transiently expressed in tsA201 cells in the FMP blue Assay.



2a-e



3a-d

	R$_1$	**R$_2$**	[^{3}H]Muscimol Binding K_i (μM)^a [pK_i ± SEM]	$\alpha_1\beta_2\gamma_{2S}$ tsA201 cell line IC$_{50}$ (μM)^b [pIC$_{50}$ ± SEM]	Glide XP Docking score (kcal/mol)
Gabazine			0.074^f	0.24^f	-9.193
4-PHP			10^c	>500^c	-7.341
1	-	-	0.030^c	0.21^c	Orientation left: -10.636 Orientation right: -9.821
2a	H	H	5.1 [5.31 ± 0.08]	~30 [~4.5]	-8.532
2b	Bn	Cl	0.74 [6.14 ± 0.02]	n.d.	-10.282
2c	H	Ph	2.5 [5.61 ± 0.05]	9.7 [5.01 ± 0.06]	-9.562
2d	Bn	Ph	0.28 [6.57 ± 0.05]	0.21 [6.67 ± 0.05]	-9.109/10.929^d
2e	Bn	H	0.75 [6.13 ± 0.04]	n.d.	-10.221
3a	H	H	7.5 [5.14 ± 0.08]	n.d.	-9.373
3b	Bn	Cl	0.83 [6.09 ± 0.04]	n.d.	-9.683
3c	H	Ph	0.014 [7.86 ± 0.04]	0.69 [6.16 ± 0.04]	-10.229
3d	Bn	Ph	0.23 [6.64 ± 0.04]	0.78 [6.10 ± 0.08]	-11.284^e

[a] IC$_{50}$ values were calculated from inhibition curves and converted to K_i values. Data is given as the mean [mean pK_i ± S.E.M] of three to four independent experiments. [b] For the characterization of the antagonists, assay concentrations of GABA of 8 μM (EC$_{85}$-EC$_{95}$) were used. [c] From reference[1]. [d] First score is for the pose shown in Figure 2B, second score is for a similar pose, only with the benzyl and phenyl substituents having switched places, via a 180° twist around the

1-hydroxypyrazole-imidazolyl bond. [e] Second score is for a docking pose that was flipped 180° around the bond connecting the piperidine and 1-hydroxypyrazole moieties. [f] From reference.[15] n.d.: not determined.

For binding mode of Gabazine, please refer to reference.[8] The suggested binding mode of compounds **2c** and **3d** is shown in Figure 1 and Figure 2, respectively.

Figure 1. 4-PHP analogue **2c** docked into the homology model of the $\alpha_1\beta_2\gamma_2$ GABA$_A$R. Contours of favorable van der Waals interactions calculated with the program GRID are depicted with grey mesh (C3 probe, isolevel = -2 kcal/mol). Contours of favorable hydrogen bond acceptor sites are depicted with red surfaces (COO- probe, isolevel = -10 kcal/mol). Contours of favorable hydrogen bond donor sites are depicted with blue surfaces (N2+ probe, isolevel = -10 kcal/mol).

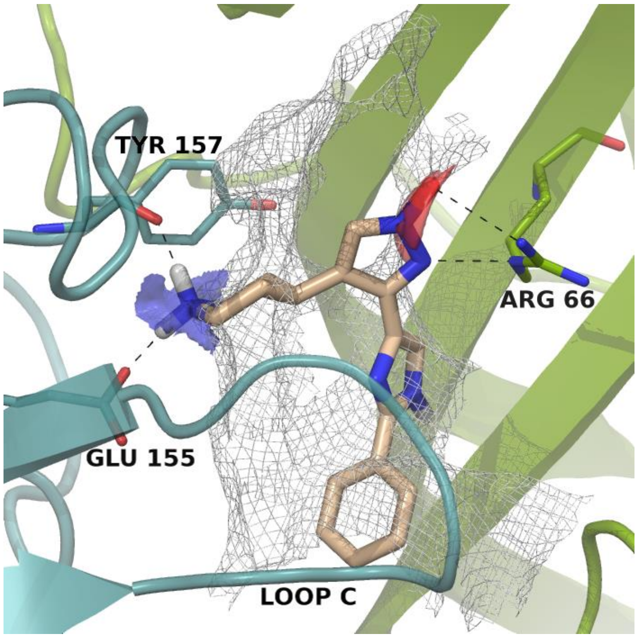
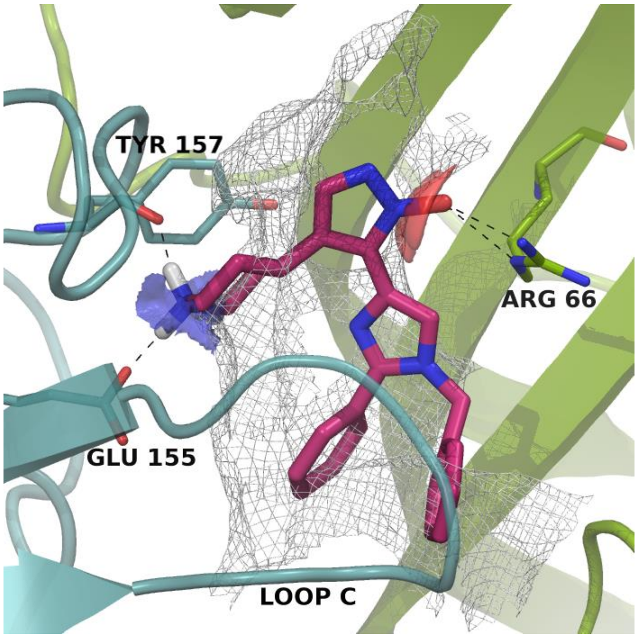


Figure 2. 4-PHP analogue **3d** docked into the homology model of the $\alpha_1\beta_2\gamma_2$ GABA$_A$R. Contours of favorable van der Waals interactions calculated with the program GRID are depicted with grey mesh (C3 probe, isolevel = -2 kcal/mol). Contours of favorable hydrogen bond acceptor sites are depicted with red surfaces (COO- probe, isolevel = -10 kcal/mol). Contours of favorable hydrogen bond donor sites are depicted with blue surfaces (N2+ probe, isolevel = -10 kcal/mol).



Pharmacology.

*Characterization of **2a-e** and **3a-d** in Muscimol Binding assay.*

Affinity for GABA$_A$Rs in rat cortical synaptosomes was determined using 5 nM [3H]Muscimol as previously described.[16] Rat membrane preparations used in the receptor binding experiment was prepared according to a method previously described.[17] The binding data were analyzed by a non-linear regression curve-fitting procedure using PRISM Graphpad Prism 4.0b (GraphPad Software Inc., San Diego, CA, USA). K_i values were calculated from IC$_{50}$ values by means of the Cheng-Prusoff equation.[18]

*Functional characterization of **2a**,**c**,**d** and **3c**,**d** in the FMP assay.*

Materials. Culture medium, serum, and antibiotics were purchased from Invitrogen (Groningen, The Netherlands). GABA was purchased from Sigma-Aldrich (St. Louis, MO), and the FLIPR Membrane Potential™ Blue (FMP) dye was purchased from Molecular Devices (Crawley, UK). The cDNAs encoding for the human □$_1$, □$_2$ and □$_{2s}$ cDNAs were kind gifts from Dr. P.J. Whiting and Merck, Sharp & Dohme (Harlow, Essex, UK) and the human □$_1$-pCDNA3 plasmid was a kind gift from Dr. D.S. Weiss. The subcloning of the □$_1$, □$_2$ and □$_{2s}$ cDNAs from their original vectors to pCDNA3.1 has been described previously[19].

Cell culture and transfections. The tsA201 cells were maintained in Dulbecco's Modified Eagle Medium + Glutamax™-I, supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO$_2$ atmosphere. The cells were split into 10 cm (2 × 10^6 cells) tissue culture plates and transfected the following day with a total of 8 µg cDNA using Polyfect® Transfection Reagent according to the protocol of the manufacturer (Qiagen, Hilden, Germany). To facilitate the expression of ternary receptor complexes in cells expressing the □$_1$□$_2$□$_{2s}$ combination, a cDNA ratio of 1:1:3 (□$_1$:□$_2$:□$_{2s}$) was used for the transfections of the cells. The ability of diazepam to potentiate EC_{20} GABA-evoked responses in the FMP Blue assay confirmed the incorporation of the □$_{2s}$ subunit in receptors assembled in these cells (data not shown).

FLIPR™ Membrane Potential (FMP) Blue Assay. The FMP Blue assay was performed in poly-D-lysine-coated, black 96-well plates (Becton Dickinson, Bedford, MA). Transfected tsA201 cells were seeded into these plates 16 to 24 h before the experiment. On the day of the experiment the medium was aspirated, and the cells were washed with 100 µl Krebs buffer (140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-glucose, pH 7.4). 50 µl Krebs buffer was added to each well (in the antagonist experiments, the buffer was supplemented with various concentrations of test compound) together with 50 µl Krebs buffer supplemented with FMP dye (1.0 mg/ml), after which the plate was incubated at 37 °C in humidified 5% CO_2 for 30 min. Then the plate was then assayed in a FlexStation3 Benchtop Multi-Mode Microplate Reader (Molecular Devices) measuring emission at 565 nm [in fluorescence units (FU)] caused by excitation at 525 nm a total of 90 sec before and after addition of 33 µl agonist solution in Krebs buffer. Experiments were performed in duplicate at least three times for each receptor. The concentration-response curves for GABA and the concentration-inhibition curves for antagonists in the assay were constructed based on the differences in the fluorescence units (△FU) between the maximal fluorescence levels recorded before and after addition of eight different concentrations of the various ligands.

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