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4-Hydroxy-1,2,3-triazole moiety as bioisostere of the carboxylic acid function: a novel scaffold to probe the orthosteric γ-aminobutyric acid receptor binding site

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KEYWORDS

hydroxy-1,2,3-triazole; bioisosterism, scaffold hopping, $GABA_A$ receptor.

ABSTRACT

The correct application of *bio(iso)steric replacement*, a potent tool for the design of optimized compounds, requires the continuous development of new isosters able to respond to specific target requirements. Among carboxylic acid isosters, as the *hydroxylated pentatomic heterocyclic* systems, the hydroxy-1,2,3-triazole represents one of the most versatile but less

investigated. With the purpose to enlarge its bioisosteric application, we report the results of a study devoted to obtain potential biomimetics of the γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS). A series of N_1- and N_2-functionalized 4-hydroxy-1,2,3-triazole analogues of the previous reported $GABA_AR$ ligands, including muscimol, 4-PIOL, and 4-PHP has been synthesized and characterized pharmacologically. Furthermore, this study led to development of straightforward chemical strategies directed to decorate the hydroxytriazole core scaffold, opening for further elaborative studies based on this system. The unsubstituted N_1- and N_2-piperidin-4-yl-4-hydroxy-1,2,3-triazole analogues (**3a**, **4a**) of 4-PIOL and 4-PHP showed weak affinity (high to medium micromolar range), whereas substituting the 5-position of the triazole core with a 2-naphthylmethyl or 3,3-diphenylpropyl led to binding affinities in the low micromolar range. Based on electrostatic analysis and docking studies using a $\alpha_1\beta_2\gamma_2$ $GABA_AR$ homology model we were able to rationalize the observed divergence in SAR for the series of N_1- and N_2-piperidin-4-yl-4-hydroxy-1,2,3-triazole analogues, offering more detailed insight into the orthosteric $GABA_AR$ binding site.

1. Introduction

A number of clear bioisosteric relationships[1] has been established for the carboxylic acid group, which successfully has been substituted by *hydroxylated pentatomic heterocyclic* systems such as thiadiazoles,[2, 3] 1,2,5-oxadiazoles,[4] pyrazoles,[5, 6] and isoxazoles.[7] Recently, the *4-hydroxy-1H-1,2,3-triazole* acidic system has been successfully used by the authors[8-11] and by others[12] as bioisostere of the carboxylic acid group. In fact, due to its acidic properties (pK_a ranged from 5 to 7, depending on the nature the substituents), this system is deprotonated to a large extent at physiological pH.[13, 14] These approaches successfully produced promising glutamate analogues,[8] novel Sortilin inhibitors,[12] new anti-cancer

compounds[9-11] and new immunosuppressive agents.[9] Compared to other above mentioned *hydroxylated pentatomic heterocyclic* systems, the hydroxy-1,2,3-triazole represents one of the most versatile but less investigated heterocycle. In particular, the three nitrogen atoms present in the triazole ring offer the possibility to regio-direct substituents in set directions with advantage to reach additional binding areas and improve properties as potency, as well as target selectivity. As an example of application of this concept, during the design of dihydroorotate dehydrogenase (*h*DHODH) inhibitors by mimicking the benzoic acid present in brequinar,[9, 15] the 1,2,3-triazole ring substitution allowed a fine tuning of the *chemical space* with the result of reaching optimized candidates.

With the purpose to widen the bioisosteric applications of the hydroxy-1,2,3-triazole system, in this paper we report the results of a work devoted to obtain potential biomimetics of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in CNS. In GABA neurotransmission, GABA activates the $GABA_A$ receptors ($GABA_ARs$), which belong to the family of ligand-gated ion channels. A high degree of structural heterogeneity of the $GABA_ARs$ has been revealed and is reflected in multiple receptor subtypes built up as pentameric assemblies comprised of 19 different $GABA_AR$ subunits: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, and ϱ_{1-3}.[16] A rich and complex pharmacology has been observed based on multiple subtypes, allosteric binding sites, and diverse subcellular and regional localization.[17] More detailed structural insight is emerging for the $GABA_ARs$ in terms of full-length crystal structures of related receptors and the more recent publication of the β_3 homopentameric $GABA_AR$.[18-21] Furthermore, extensive structure-activity relationship (SAR) studies have been performed over the years.[22, 23] Consequently, a large number of potent and selective ligands for the orthosteric $GABA_AR$ binding site have been reported. Especially, the conformational restriction of the structure of GABA by bioisosteric replacement of the carboxylic acid moiety with acidic heterocycles has been successful. Besides being carboxylic acid bioisosteres, these

heterocyclic rings allow for introduction of substituents of different shape, size, and electronic properties in well-defined positions useful for mapping the binding site.[24]

The broad range of ligands include muscimol, 5-(piperidin-4-yl)-3-isoxazolol (4-PIOL), 4-(piperidin-4-yl)-1-hydroxypyrazole (4-PHP), and 5-(piperidin-4-yl)-3-hydroxypyrazol (aza-4-PIOL) analogues (Figure 1), which all have supported the development of solid $GABA_AR$ homology models optimized for agonists or antagonist binding and identified specific cavities in the vicinity of the core part of the binding site for GABA.[25, 26]

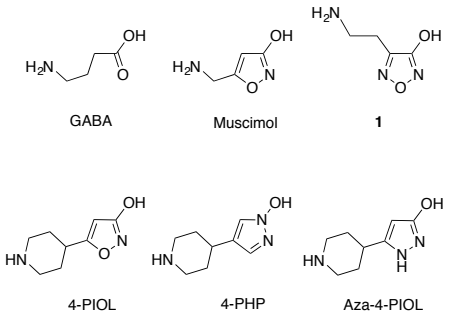

Figure 1. Reference compounds GABA, Muscimol, 4-PIOL, 4-PHP, Aza-4-PIOL and compound **1**.[4]

In the present study, we investigated the orthosteric $GABA_AR$ binding site by introducing the 4-hydroxy-1,2,3-triazole as a new bioisostere to the carboxyl group of GABA as described for the 3-hydroxyisoxazole, hydroxy-1,2,5-oxadiazole, and 1- and 3-hydroxypyrazole moieties of reported $GABA_AR$ ligands. In order to explore the potential of the aminoethyl substituted hydroxy-1,2,5-oxadiazole **1**[4, 27] (Figure 1), a low affinity $GABA_AR$ agonist, we designed the corresponding 4-hydroxy-1,2,3-triazole (**2a**) and hydroxythiadiazole analogues (**2b**) (Figure 2).

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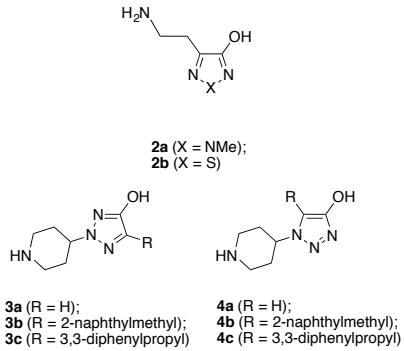


Figure 2. Compounds **2a–b**, **3a–c** and **4a–c**.

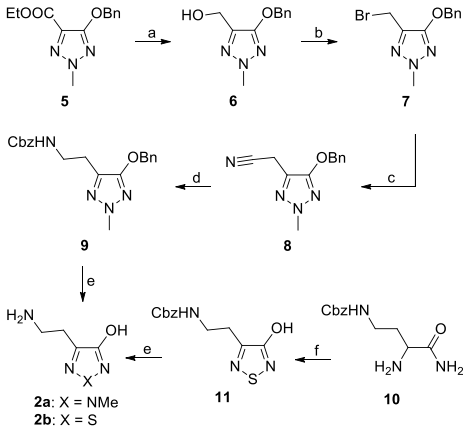
Subsequently, to challenge the above mentioned homology model and verify the structural similarity, binding modes, and bioisosteric potential of the 4-hydroxy-1,2,3-triazole, two regioisomeric series N_1- and N_2- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogues were synthesized (compounds **3a–c** and **4a–c**, respectively, Figure 2) corresponding to a selected subgroup of previously reported 4-PIOL, 4-PHP, and aza-4-PIOL analogues.[5, 6, 28] The syntheses and pharmacological properties at native $GABA_A$Rs in rat brain homogenate are reported and SARs are discussed using the above mentioned homology model.

2. Result and discussion

2.1 Chemistry.

The target compounds **2a** and **2b** were synthesized as described in Scheme 1. The alcohol **6** was obtained from compound **5**[8] by reduction of the ethyl ester using $LiAlH_4$. Treatment of **6** with *N*-bromosuccinimide and triphenylphosphine afforded **7**, which, due to its instability, was converted into **8** using sodium cyanide immediately upon purification. Following a one-pot procedure, previously described by Petersen *et al*.,[29, 30] compound **8** was converted into **9** by reduction of the nitrile group followed by benzyloxycarbonyl (Cbz) protection of the

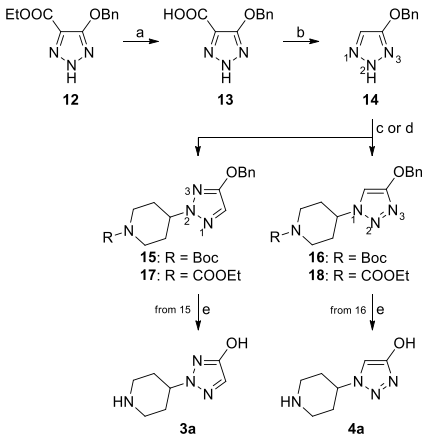
formed amine. This latter protection of the amino group was performed to optimize the purification procedure of **9**. Deprotection of **9** under acidic conditions afforded target compound **2a**. Compound **2b** was synthesized starting from **10** (Scheme 1), a compound previously described by Treder *et al*. in high yields.[31] Because, in our hands, the published synthetic scheme was not reproduced in satisfactory yields, we developed an alternative method starting from glutamic acid for the synthesis of **10** (please refer to Supplementary Information for synthetic details), which was obtained in an overall yield of 8% (four steps). Annulation of **10** and sulphur monochloride, a method previously described by Weinstock *et al*.,[32] and subsequent deprotection under acidic condition afforded target compound **2b**.



Scheme 1. Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C to rt, (b) PPh_3, NBS, CH_2Cl_2, –10 °C, (c) NaCN, $EtOH/H_2O$, rt, (d) BnOCOCl, $NaBH_4$, $NiCl_2$, MeOH, 0 °C to rt, (e) 2M HCl, reflux, (f) S_2Cl_2, DMF, rt.

Target compounds **3a**–**c** and **4a**–**c** were synthesized (Schemes 2 to 4) starting from **12**, which was prepared as previously reported.[8] Compound **14** (Scheme 2) was obtained from **12** in two steps starting by hydrolysis of the ethoxycarbonyl moiety followed by decarboxylation of the formed acid (**13**) at elevated temperatures. As for **12**, also compound **14** represent a valuable intermediate for the synthesis of regiosubstituted hydroxytriazoles. In analogy to

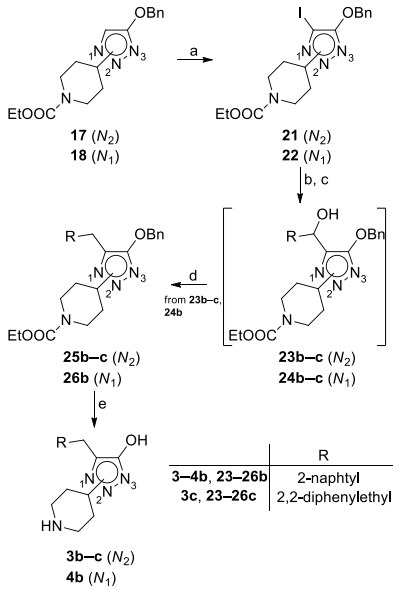
12[8] also **14** follows an alkylation scheme directed toward the N_2- and N_1- position of the triazole ring. Alkylation of **14** using tert-butyl 4-bromopiperidine-1-carboxylate (**19**) afforded a mixture of the N_2- (**15**) and N_1- (**16**) regioisomers, which were isolated using standard column chromatography in 60% and 10% yields, respectively. The substitution pattern between the N_2- and N_1- position was determined by 2D NMR analyses (please refer to Supplementary Information). Subsequent deprotection of compounds **15** and **16** under acidic conditions afforded compounds **3a** and **4a**, respectively.



Scheme 2. Reagents and conditions: (a) 6M NaOH, EtOH, 50 °C, (b) DMF, 130 °C, 6h, (c) tert-butyl 4-bromopiperidine-1-carboxylate (**19**), K_2CO_3, CH_3CN, reflux, (d) ethyl 4-bromopiperidine-1-carboxylate (**20**), Cs_2CO_3, 1,4-dioxane, reflux, (e) 6M HCl, reflux.

Target compounds **3b–c** and **4b–c** (Scheme 3 and 4) were obtained from intermediates **17** and **18**, which were synthesized as described for **15** and **16** (Scheme 2), using ethyl 4-bromopiperidine-1-carboxylate (**20**). Analogously to **15** and **16**, the N_2- (**17**) and N_1- (**18**) regioisomers were obtained in 63% and 17% yield, respectively. In order to obtain a higher yield of the N_1- isomer, different alkylation conditions were attempted. Interestingly, caesium carbonate in anhydrous 1,4-dioxane at reflux improved the ratio between the N_1- and N_2- regioisomers and a 1:1 mixture of **18** and **17** was obtained (isolated yields of 41% and 39%,

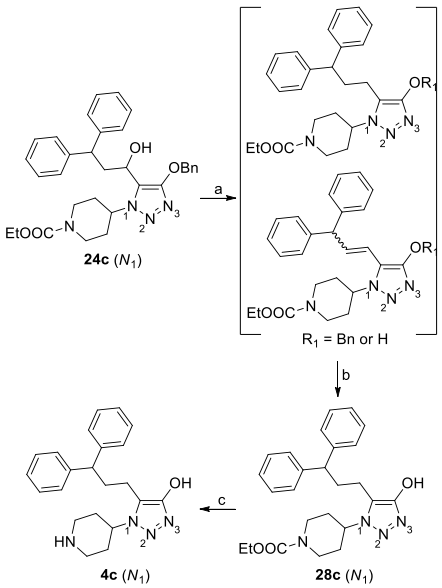
respectively). Iodination of **17** and **18** (Scheme 3) using iodine monochloride afforded compounds **21** and **22**, which were converted into the corresponding Grignard reagents using isopropylmagnesium chloride. Quenching of the Grignard reagents *in situ* with either 2-naphthaldehyde or 3,3-diphenylpropanal afforded the corresponding alcohol derivatives **23b–c** and **24b–c**. Ionic hydrogenation of the formed alcohol using triethylsilane and triflouoracetic acid[33] followed by deprotection under acidic conditions afforded target compounds **3b–c** and **4b**.



Scheme 3. Reagents and conditions: (a) ICl, AcOH, H_2O, 80 °C, (b) ^{i}PrMgCl, THF, –10 °C, (c) 2-naphthaldehyde or 3,3-diphenylpropanal (**27**), THF, 0 °C to rt, (d) Et_3SiH, TFA, CH_2Cl_2, 0 °C to rt, (e) 35 % HCl *v*/*v*, EtOH, reflux.

In contrast to compounds **23b–c** and **24b**, the ionic hydrogenation of **24c** (Scheme 4) afforded a mixture of saturated and unsaturated products (determined using LC-MS analysis), which could not be separated using conventional purification methods. However, the crude mixture

was hydrogenated using palladium on carbon, which afforded compound **28c**. Subsequent deprotection under acidic conditions afforded target compound **4c**.



Scheme 4. Reagents and conditions: (a) Et_3SiH, TFA, CH_2Cl_2, 50 °C, sealed tube, (b) H_2, Pd/C, MeOH, rt, (c) 35% HCl, EtOH, reflux.

2.2 Structure-activity relationship and electrostatic properties

The synthesized compounds **2a**–**b**, **3a**–**c**, and **4a**–**c** were characterized in receptor binding studies using rat brain membrane preparations, where the binding affinities of the compounds at native $GABA_AR$s were measured by displacement of [^{3}H]muscimol (Table 1). As previously reported for the corresponding 3-hydroxyisoxazole[34] the monocyclic analogues **2a** and **2b** showed no or low affinity for native $GABA_AR$s. Since these carboxylic acid isosteres show pK_a values in a range (pK_a 3.12–5.92) comparable to muscimol, a potent $GABA_AR$ agonist, the lack of affinity might reflect a suboptimal conformation of the pharmacophoric elements of the compounds.

Also, the N_2- and N_1- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogues of 4-PIOL (**3a** and **4a**, respectively) displayed low $GABA_AR$ affinities in the high to medium micromolar range comparable to aza-4-PIOL and more than 5-fold lower than 4-PIOL and 4-PHP. Introduction of 2-naphthylmethyl in the 5-position of the N_1- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogue (**4b**) led to a 20-fold increase in affinity compared to the non-substituted analogue. Similar receptor affinity was observed by introduction of the 2-naphthylmetyl substituent in the 5-position of the N_2- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogue **3b**. Replacing the naphthylmethyl to a more flexible 3,3-biphenylpropyl moiety did not change the receptor affinity for the 5-substituted N_1- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogue **4c**. In contrast, the corresponding structural change for the N_2- piperidin-4-yl-4-hydroxy-1,2,3-triazole analogue **3c** was detrimental for affinity and led to a compound with complete loss of $GABA_AR$ affinity.

Considering the overall structural similarity of the core scaffolds of 4-PIOL, 4-PHP, **3a** and **4a**, and because the substituted analogues of **3a** and **4a** to an extent showed affinity, a high desolvation energy of the non-substituted analogues **3a** and **4a** could be the reason for the lack of receptor affinity observed in the binding study. A similar case was previously reported for the corresponding 3-hydroxypyrazol analogue of 4-PIOL (aza-4-PIOL).[6] Using the program Jaguar,[35] the free energies of solvation for the zwitterionic forms of 4-PHP (–77.9 kcal/mol), aza-4-PIOL (–101.2 kcal/mol), **3a** (–88.5 kcal/mol), and **4a** (–97.2 kcal/mol) were calculated, indicating a significantly higher desolvation energy penalty for compounds **3a** and **4a** than for 4-PHP.

Table 1. Pharmacological data and ionization constants for reference compounds GABA, 4-PIOL, 4-PHP, Aza-4-PIOL, and compounds **1**, **2a–b**, **3a–c** and **4a–c**.

	[^{3}H]muscimol binding K_i (μM)[a] [pK_i±SEM]	pK_{a1}[b]
GABA	0.049[c]	4.04 ±0.02[c]
1	13[c]	3.12 ±0.02[c]
2a	>100	5.92 ±0.02
2b	75 [4.13±0.04]	4.54 ±0.03
4-PIOL	9[d]	5.3[d]
4-PHP	10[d]	5.4[d]
Aza-4-PIOL	>100[d]	6.7[d]
3a	>100	6.36 ±0.01
4a	55 [4.26±0.05]	6.51 ±0.03
3b	3.3 [5.49±0.04]	-
4b	2.4 [5.62±0.04]	-
3c	>100	-
4c	1.6 [5.80±0.03]	-

[a]$GABA_A$ receptor binding affinities at rat synaptic membranes: IC_{50} values were calculated from inhibition curves and converted to K_i values. Data is given as the mean [mean pK_i ± SEM] of three to five independent experiments. [b]The ionization constants of compounds **2a–b**, **3a**, and **4a** were determined by potentiometric titration using a GLpK_a apparatus (Sirius Analytical Instruments Ltd., Forest Row,East Sussex, UK). [c]Data from Lolli *et al*.[4] [d]Data from Krall *et al*.[22]

The heterocyclic carboxylic acid bioisosteres interacting with the $GABA_AR$ in general resembles the electrostatic properties of the carboxylic acid in GABA.[23] As shown for 4-PHP and aza-4-PIOL (Figure 3A,B), the electronegative charge is centred in the area around the hydroxy group and the neighbouring nitrogen allowing the ligands to interact in a bidentate manner with the conserved α_1-Arg66 in the GABA binding site. In contrast, the electrostatic profile shows a slightly different charge distribution for compounds **3a** and **4a** (Figure 3C,D) which could indicate that this bidentate interaction could be compromised leading to reduced

binding affinity. The higher pK_a values observed for the hydroxytriazoles (pK_a 6.36–6.51) compared to that of 4-PHP (pK_a 5.4), and 4-PIOL (pK_a 5.3) (Table 1) might also add to lower binding. The N_1- and N_2- piperidin-4-yl hydroxytriazoles, as the 3-hydroxypyrazole aza-4-PIOL, are thus less acidic than 4-PHP and protonated to a greater extent under physiological pH, which in turn might lead to a weaker interaction in the orthosteric $GABA_AR$ binding site.

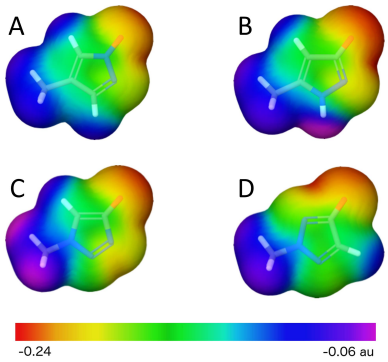


Figure 3. Electrostatic potential mapped on the surface of the molecular density for the anionic form of (A) 4-methyl-1-hydroxypyrazole, (B) 5-methyl-3-hydroxypyrazole, (C) 1-methyl-4-hydroxy-1,2,3-triazole, and (D) 2-methyl-4-hydroxy-1,2,3-triazole ring systems. Increasing negative potential coloured from purple/blue over green to red. Calculations were carried out with Jaguar[35] using the cc-PVDZS basis set and the B3LYP hybrid potential. Au, atomic units.

2.3. Molecular modelling.

To further assess the obtained pharmacological data, the binding modes of the synthesized compounds were evaluated using the reported homology model of the $\alpha_1\beta_2\gamma_2$ $GABA_AR$ in the antagonist bound state.[26] The obtained docking poses for the ligands match the binding mode previously reported,[26] with the amine moiety forming hydrogen bonds with β_2-Glu155 and

the backbone carbonyl of β_2-Tyr157 and the hydroxytriazole moiety forming a bidentate interaction to α_1-Arg66 mimicking the binding interactions of GABA. As reported for 4-PIOL and 4-PHP, two different orientations of the triazole-piperidine core scaffold of **4a** are possible while still maintaining the bidentate interactions described above (Figure 4B,C). The naphthylmethyl substituted analogue **4b** is able to bind in either of the two orientations (Figure 4B,C), whereas the diphenylpropyl substituted analogue **4c** adopts a binding pose where the triazole moiety is found in the aforementioned alternative orientation (180° flip), thus the substituent is accommodated in the more spacious cavity below the core scaffold (Figure 4C). The binding site optimized for **3a** shows a marked difference in the conformation of α_1-Arg66, with the side chain moving to a position further towards the membrane, thus allowing it to form a bidentate interaction with **3a** and **3b**, and with the hydrophobic substituent reaching out into the previously reported cavity above the core scaffold (Figure 4A). The more bulky diphenylpropyl substituted analogue, **3c**, is not able to interact with α_1-Arg66 in this conformation, likely due to limited space in the aforementioned cavity. Unlike **4c**, the suggested 180° flip as described for **4a** and analogues is not optimal for this series of compounds (**3a–c**).

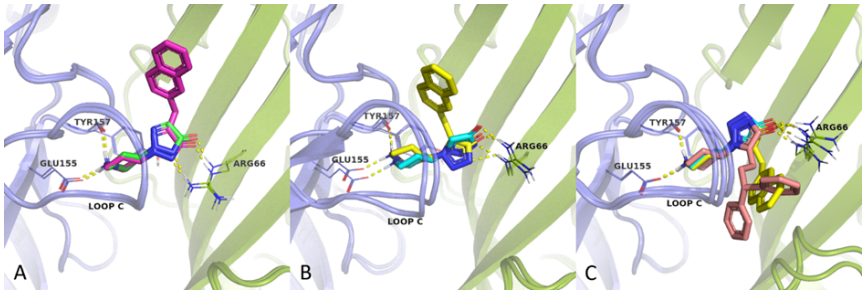


Figure 4. Compounds **3a** (A, green), **3b** (A, pink), **4a** (B and C, cyan), **4b** (B and C, yellow) and **4c** (C, salmon) docked into the $\alpha_1\beta_2\gamma_2$ $GABA_AR$ homology model. Residues surrounding

the ligand binding site from the principal side (light-teal carbons) and complementary side (olive-green carbons) are shown. Hydrogen bonds are depicted with yellow dashes.

3. Conclusions

In this study we show that the 4-hydroxy-1,2,3-triazole ring system is a valid bioisostere for previous identified five membered heterocyclic carboxylic acid bioisosteres as ligands for the $GABA_A$Rs. A series of 4-hydroxy-1,2,3-triazole analogues were synthesized and characterized pharmacologically at native rat $GABA_A$Rs. In general, the synthesized N_1- and N_2- piperidin-4-yl analogues displayed affinities in the medium to low micromolar range (K_i values of 1.6–55 μM). Despite previous identified cavities in the vicinity of the core of the orthosteric binding site, the two structural closely related series of substituted analogues (**3b–c** and **4b–c**) displayed slightly different SAR indicating different binding modes. These results were rationalized by using a homology model for the orthosteric binding site of the $\alpha_1\beta_2\gamma_2$ $GABA_A$R implying a 180° flip of the core scaffold of the N_1- piperidin-4-yl analogues **4b** and **4c** enabling accommodation of the larger substituent of **4c** in the more spacious cavity below the core scaffold. This binding mode is not optimal for the corresponding N_2- piperidin-4-yl analogue **3c**.

The synthesis strategy applied in this study included directed alkylation of the triazole ring system useful for future application of this heterocyclic moiety, which, in the present study, has offered a more detailed insight into the architecture and flexibility of the orthosteric binding site in the $GABA_A$R.

4. Experimental section

4.1 Chemistry

4.1.1 General methods

Compounds **10**, **19**, **20** and **27** were synthesized as reported in Supplementary Information, while compounds **5** and **12** were prepared as described in the literature.[8] All chemical reagents and solvents (analytical grade) were obtained from commercial sources (Sigma Aldrich, Alfa Aesar, or TCI) and used without further purification. Air- and/or moisture sensitive reactions were performed under a nitrogen atmosphere using syringe-septum techniques and dried glassware. Anhydrous solvents were dried over 4 Å molecular sieves or by distillation (THF) prior to use from Na and benzophenone under nitrogen atmosphere. ^{i}PrMgCl (in THF) was titrated prior to use as described elsewhere.[36] Thin layer chromatography (TLC) on silica gel was carried out using 5 × 20 cm plates with a silica layer of 0.25 mm in thickness. Purification of synthesized compounds were performed using flash column chromatography on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM) or by the use of a CombiFlash Rf 200 apparatus (Teledyne Isco) with 5–200 mL/min, 200 psi (with automatic injection valve) using RediSep Rf Silica columns (Teledyne Isco). Melting points (mp) were measured on a Büchi 540 apparatus in open capillary tubes and are uncorrected. Analytical high performance liquid chromatography (HPLC) analyses were performed on a Perkin Elmer Flexar UHPLC system equipped with an UHPLC Acquity BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters) and a 20 μL loop. Elution of analysed samples were performed using mixtures of eluent A (H_2O/TFA, 100/0.1) and eluent B (CH_3CN/TFA, 100/0.1) at a flow rate of 0.5 mL/min. For HPLC control, data collection, and data handling Chromera Software ver. 4.1.0 was used. Alternatively, analytical HPLC analyses were performed on an Ultimate 3000 HPLC system (Thermo Scientific) with an LPG-3400A pump, a WPS-3000SL autosampler, and a DAD-3000D detector using a Gemini® NX-C18 column (3 μm, 110 Å, 4.6 × 250 mm) and eluents A (H_2O/TFA, 100/0.1) and B (CH_3CN/H_2O/TFA, 90/10/0.1) at a flow rate of 1 mL/min. For HPLC control, data collection, and data handling, Chromeleon Software

ver. 6.80 was used. The purity of the analysed compounds is ≥95%, unless otherwise stated. Preparative reversed phase HPLC was carried out on an Ultimate 3000 HPLC system (Thermo Scientific) with a LPG-3200BX pump, a Rheodyne 7125i injector, a 10 mL loop, and a MWD-3000SD detector (200, 210, 225, and 254 nm) using a preparative Phenomenex Gemini NX-C18 column (5 μm, 21.2 × 250 mm) and eluents A (H_2O/TFA, 100/0.1) and B (CH_3CN/H_2O/TFA, 90/10/0.1) at a flow rate of 20 mL/min. For HPLC control, data collection, and data handling, Chromeleon Software ver. 6.80 was used. ^{1}H and ^{13}C NMR were recorded either on a Bruker Avance 300 MHz, a Jeol JNM-ECZR 600 MHz, or a Bruker Avance 600 MHz spectrometer equipped with a cryogenically cooled 5 mm CPDCH 13C[1H] 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 or TMS were used as internal reference.[37] Elementary analyses were performed by Mr. J. Theiner, Department of Physical Chemistry, University of Vienna, Austria. HPLC-HRMS analyses were performed on a system comprised of an Agilent 1200 HPLC system comprising of a quaternary pump with a built-in degasser, a thermostated column compartment, an autosampler, and a photodiode array detector, coupled with a Bruker microOTOF-QII mass spectrometer equipped with an electrospray ionization (ESI) source and operated via a 1:99 flow splitter. Mass spectra were acquired in positive ionization mode, using drying temperature of 200 °C, a capillary voltage of –4100V, nebulizer pressure of 2.0 bar, and drying gas flow of 7 L/min. A solution of sodium formate clusters was injected in the beginning of each run to enable internal mass calibration. Chromatographic separation was acquired on a Phenomenex Luna C18(2) column (150 mm × 4.6 mm, 3 µm, 100 Å) maintained at 40 °C, using a flow rate of 0.8 mL/min and a linear gradient of the binary solvent system water-acetonitrile-formic acid (eluent A: 95/5/0.1, and eluent B: 5/95/0.1) rising from 0% to 100% of eluent B over 20 minutes. Data was acquired using Compass HyStar Ver.

3.2 (Bruker Daltonic GmbH, Germany) and processed using Compass DataAnalysis Ver. 4.0 (Bruker Daltonic GmbH, Germany).

*4.1.2. (5-(Benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)methanol (**6**).* $LiAlH_4$ (0.36 g, 9.6 mmol) was added to a cooled (0 °C) solution of compound **5**[8] (2.50 g, 9.6 mmol) in anhydrous THF (125 mL). The reaction mixture was stirred for 2 h at 0 °C before it was quenched by adding in sequence water (0.37 mL), 15% *w/w* NaOH (0.37 mL) and then water (0.37 mL). The volatiles were evaporated *in vacuo* and the residue was taken up in water. The resulting mixture was extracted with Et_2O (3 × 100 mL) and the combined organic phase was washed with brine (150 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to afford compound **6** as colourless oil (1.82 g, 87%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.50–7.28 (m, 5H), 5.22 (s, 2H), 5.05 (t, J = 5.5 Hz, 1H), 4.36 (d, J = 5.5 Hz, 2H), 3.95 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 157.4, 136.5, 131.8, 128.3, 128.0, 127.8, 71.4, 52.2, 41.3. HRMS (ESI-TOF): *m/z* calculated for $C_{11}H_{12}N_3O$ $[M\div H_2O+H]^+$, 202.0975. Found, 202.0974 (ΔM=0.3 ppm).

*4.1.3. 4-(Benzyloxy)-5-(bromomethyl)-2-methyl-2H-1,2,3-triazole (**7**).* PPh_3 (1.58 g, 6.0 mmol) was added to a stirred solution of **6** (1.10 g, 5.0 mmol) in anhydrous CH_2Cl_2 (30 mL) at –10 °C. To the resulting mixture, NBS (1.07 g, 6.03 mmol) was added in small portions over 30 min. The reaction mixture was stirred for 1 h at –10 °C before the solvent was evaporated *in vacuo*. Purification of the resulting residue by flash chromatography (CH_2Cl_2) afforded compound **7** as colourless oil (1.13 g, 81%), which was used immediately upon purification in the synthesis of compound **8** due to stability issues of **7**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.43–7.22 (m, 5H), 5.18 (s, 2H), 4.38 (s, 2H), 3.93 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 158.0, 136.2, 128.7, 128.6, 128.4, 128.0, 72.2, 42.0, 20.4.

*4.1.4. 2-(5-(Benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)acetonitrile (**8**).* A solution of **7** (1.13 g, 4.0 mmol) in EtOH (20 mL) was added dropwise to a solution of NaCN (0.39 g, 8.0 mmol) in EtOH/water (9:1 *v*/*v*, 25 mL). The reaction mixture was stirred for 48 h at rt before the volatiles were evaporated *in vacuo*. The resulting residue was taken up in water and extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with water (1 × 100 mL), brine (1 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–20% EtOAc) afforded **8** as colourless oil (0.63 g, 69%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.50–7.30 (m, 5H), 5.24 (s, 2H), 4.05–3.90 (m, 5H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 156.8, 136.1, 128.3, 128.1, 127.8, 121.8, 117.0, 71.7, 41.7, 12.3. HRMS (ESI-TOF): *m*/*z* calculated for $C_{12}H_{13}N_4O$ $[M+H]^+$, 229.1084. Found, 229.1085 (ΔM=0.5 ppm).

*4.1.5. Benzyl (2-(5-(benzyloxy)-2-methyl-2H-1,2,3-triazol-4-yl)ethyl)carbamate (**9**).* Benzyl chloroformate (0.65 mL, 4.6 mmol) and $NiCl{\cdot}6H_2O$ (54 mg, 0.23 mmol) were added to a stirred solution of **8** (0.52 g, 2.3 mmol) in MeOH (20 mL) at 0 °C. $NaBH_4$ (0.69 g, 18 mmol) was then added in small portions over 1 h while keeping the temperature at 0 °C, whereupon the reaction mixture was allowed to reach rt and stirred for 24 h before water was added (300 mL). The resulting mixture was extracted with CH_2Cl_2 (6 × 100 mL) and the combined organic phase was washed with brine (200 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by column chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–70% EtOAc) afforded **9** (0.28 g, 35%) as white solid: mp 44–46 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.51–7.23 (m, 10H), 5.19 (s, 2H), 4.99 (s, 2H), 3.92 (s, 3H), 3.21 (q, J = 6.9 Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 157.4, 155.9, 137.1, 136.5, 128.9, 128.3, 128.2, 127.9, 127.7, 127.65, 127.6, 71.4, 65.1, 41.2, 39.2, 23.8. HRMS (ESI-TOF): *m*/*z* calculated for $C_{20}H_{23}N_4O_3$ $[M+H]^+$, 367.1765. Found, 367.1760 (ΔM=1.3 ppm).

*4.1.6. 5-(2-Aminoethyl)-2-methyl-2H-1,2,3-triazol-4-ol hydrochloride (**2a**).* A solution of **9** (0.18 g, 0.50 mmol) in MeOH/2M HCl (1:4 *v/v*, 25 mL) was refluxed for 72 hours. The resulting solution was washed with EtOAc (3 × 15 mL) and evaporated *in vacuo*. Recrystallization from ^{i}PrOH/Et_2O afforded **2a** (40 mg, 45%) as white solid: mp 191–192 °C. ^{1}H NMR (300 MHz, D_2O): δ 3.94 (s, 3H), 3.29 (t, J = 7.0 Hz, 2H), 2.96 (t, J = 7.0 Hz, 2H). ^{13}C NMR (75 MHz, D_2O, int. std. MeOH): δ 156.1, 128.2, 41.5, 38.8, 21.7. HRMS (ESI-TOF): *m/z* calculated for $C_5H_{11}N_4O$ $[M+H]^+$, 143.0927. Found, 143.0927 (ΔM=0.5 ppm).

*4.1.7. Benzyl (2-(4-hydroxy-1,2,5-thiadiazol-3-yl)ethyl)carbamate (**11**).* A solution of S_2Cl_2 (0.45 mL, 5.7 mmol) in anhydrous DMF (20 mL) was added dropwise to a solution of **10** (0.47 g, 1.9 mmol) in anhydrous DMF (10 mL). The reaction mixture was stirred for 12 hours at rt before poured into 200 mL of iced water. The mixture was filtered, the filtrate was acidified to pH 1 and extracted with Et_2O (4 × 100 mL). The combined organic phase was washed with brine (1 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2/MeOH 95:5 *v/v*) afforded **11** (0.090 g, 17%) as white solid: mp 86–87 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.59 (br s, 1H), 7.42–7.26 (m, 5H), 4.99 (s, 2H), 3.37 (t, J = 7.1 Hz, 2H), 2.83 (t, J = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 162.5, 155.9, 149.9, 137.1, 128.2, 127.6, 127.5, 65.0, 38.1, 29.0. HRMS (ESI-TOF): *m/z* calculated for $C_{12}H_{14}N_3O_3S$ $[M+H]^+$, 280.0750. Found, 280.0744 (ΔM=2.4 ppm).

*4.1.8. 4-(2-Aminoethyl)-1,2,5-thiadiazol-3-ol hydrochloride (**2b**).* A solution of **11** (81 mg, 0.29 mmol) in MeOH/2M HCl (1:3 *v/v*, 16 mL) was refluxed for 72 h. The resulting solution was washed with EtOAc (3 × 10 mL) and evaporated *in vacuo*. Trituration of the resulting residue with $^{i}Pr_2O$ afforded **2b** (34 mg, 64%) as white solid: mp 215–217 °C. ^{1}H NMR (600 MHz, D_2O): δ 3.49 (t, J = 6.8 Hz, 2H), 3.18 (t, J = 6.8 Hz, 2H). ^{13}C NMR (150 MHz, D_2O): δ

162.0, 148.1, 37.1, 26.1. HRMS (ESI-TOF): *m/z* calculated for $C_4H_8N_3OS$ $[M+H]^+$, 146.0383. Found, 146.0385 (ΔM=1.8 ppm).

*4.1.9. 5-(Benzyloxy)-2H-1,2,3-triazole-4-carboxyic acid (**13**).* 6M NaOH (14.2 mL, 85.0 mmol) was added to a solution of **12**[8] (3.5 g, 14.2 mmol) in EtOH (100 mL). The reaction mixture was heated at 50 °C for 24 h. Upon cooling to rt, the reaction mixture was neutralized with 6M HCl and the solvents were evaporated. The residue was taken up in water and 1M HCl was added until pH 1. The resulting suspension was filtered and the solid was washed with hexane to give **13** (3.1 g, quant.) as white solid: mp 172 °C (dec.). ^{1}H NMR (600 MHz, DMSO-d_6): δ 14.05 (br s, 1H), 7.54–7.27 (m, 5H), 5.32 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.2, 159.8, 136.5, 128.4, 128.1, 128.0, 121.6, 71.5. HRMS (ESI-TOF): *m/z* calculated for $C_{10}H_{10}N_3O_3$ $[M+H]^+$, 220.0717. Found, 220.0712 (ΔM=2.3 ppm).

*4.1.10. 4-(Benzyloxy)-2H-1,2,3-triazole (**14**).* **13** (3.5 g, 16.0 mmol) was dissolved in anhydrous DMF (50 mL) and the resulting solution heated at 130 °C for 6 h. Upon cooling to rt, water (500 mL) was added and the mixture was extracted with Et_2O (5 × 100 mL). The combined organic phase was washed with water (2 × 100 mL), brine (2 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2/EtOAc, 95:5 *v/v*) afforded **14** (2.25 g, 70%) as white solid: mp 100–102 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 14.1 (br s, 1H), 7.29–7.49 (m, 6H), 5.19 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.5, 136.6, 128.4, 128.1, 128.0, 118.7, 71.4. HRMS (ESI-TOF): *m/z* calculated for $C_9H_{10}N_3O$ $[M+H]^+$, 176.0818. Found, 176.0812 (ΔM=3.6 ppm).

*4.1.11. tert-Butyl 4-(4-(benzyloxy)-2H-1,2,3-triazol-2-yl)piperidine-1-carboxylate (**15**) and tert-butyl 4-(4-(benzyloxy)-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**16**).* K_2CO_3 (1.7 g, 12.6 mmol) was added to a solution of **14** (1.1 g, 6.3 mmol) in CH_3CN (35 mL). The reaction

mixture was heated at reflux and tert-butyl 4-bromopiperidine-1-carboxylate (**19**, 2.2 g, 8.2 mmol) was added in portions over 48 h. The reaction mixture was cooled at rt and the solvent was evaporated *in vacuo*. The resulting residue was taken up in water (200 mL) and extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 10%–40% EtOAc) afforded **15** (first eluting, N_2-isomer) and **16** (second eluting, N_1- isomer) as white solids. **15** (1.36 g, 60%): mp 87–88 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.48–7.30 (m, 6H), 5.17 (s, 2H), 4.52 (tt, J = 10.8, 4.0 Hz, 1H), 3.94 (d, J = 13.3 Hz, 2H), 3.08–2.86 (m, 2H), 2.09–1.96 (m, 2H), 1.78 (qd, J = 4.3, 11.6 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 153.9, 136.3, 128.4, 128.2, 128.1, 118.6, 78.9, 71.5, 60.6, 41.8, 31.0, 28.0. HRMS (ESI-TOF): m/z calculated for $C_{19}H_{26}N_4O_3Na$ $[M+Na]^+$, 381.1897. Found, 381.1895 (ΔM=0.6 ppm). **16** (0.21 g, 10%): mp 106–107 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.85 (s, 1H), 7.49–7.29 (m, 5H), 5.15 (s, 2H), 4.59 (tt, J = 11.3, 3.8 Hz, 1H), 4.03 (d, J = 12.9 Hz, 2H), 3.05–2.80 (m, 2H), 2.08–1.96 (m, 2H), 1.79 (qd, J = 12.2, 4.3 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 153.7, 136.5, 128.4, 128.1, 128.0, 105.6, 78.9, 71.5, 57.7, 42.1, 31.7, 28.1. HRMS (ESI-TOF): m/z calculated for $C_{19}H_{27}N_4O_3$ $[M+H]^+$, 359.2078. Found, 359.2068 (ΔM=2.7 ppm).

*4.1.12. 2-(Piperidin-4-yl)-2H-1,2,3-triazol-4-ol hydrochloride (**3a**).* **15** (0.25 g, 0.70 mmol) was suspended in 6M HCl (10 mL) and the suspension was heated at reflux for 48 h. Upon cooling to rt, the reaction mixture was washed with EtOAc (2 × 10 mL) and the aqueous phase evaporated *in vacuo*. Recrystallization from EtOH/Et_2O afforded **3a** (90 mg, 63%) as white crystals: mp 259–263 °C. ^{1}H NMR (300 MHz, D_2O): δ 7.17 (s, 1H), 4.65 (tt, J = 10.3, 4.3 Hz, 1H), 3.52 (dt, J = 13.3, 3.9 Hz, 2H), 3.28–3.13 (m, 2H), 2.42–2.14 (m, 4H). ^{13}C NMR (75 MHz, D_2O): δ 158.4, 120.1, 58.1, 42.7, 27.9. HRMS (ESI-TOF): m/z calculated for $C_7H_{13}N_4O$ $[M+H]^+$, 169.1084. Found, 169.1083 (ΔM=0.6 ppm).

*4.1.13. 1-(Piperidin-4-yl)-1H-1,2,3-triazol-4-ol hydrochloride (**4a**).* **16** (0.17 g, 0.47 mmol) was suspended in 6M HCl (10 mL) and the suspension heated at reflux for 48 h. Upon cooling to rt, the reaction mixture was washed with EtOAc (2 × 10 mL) and the aqueous phase evaporated *in vacuo*. Recrystallization from EtOH/Et_2O afforded **4a** (20 mg, 21%) as white crystals: mp 243 °C (dec.). ^{1}H NMR (300 MHz, D_2O): δ 7.36 (s, 1H), 4.73 (m, 1H), 3.57 (dt, J = 6.9, 3.2 Hz, 2H), 3.22 (td, J = 13.2, 3.2 Hz, 2H), 2.49–2.36 (m, 2H), 2.34–2.16 (m, 2H). ^{13}C NMR (75 MHz, D_2O): δ 157.5, 107.8, 56.3, 42.9, 28.3. HRMS (ESI-TOF): *m/z* calculated for $C_7H_{13}N_4O$ $[M+H]^+$, 169.1084. Found, 169.1085 (ΔM=0.9 ppm).

*4.1.14. Ethyl 4-(4-(benzyloxy)-2H-1,2,3-triazol-2-yl)piperidine-1-carboxylate (**17**) and ethyl 4-(4-(benzyloxy)-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**18**).* Cs_2CO_3 (17.3 g, 53 mmol) was added to a solution of **14** (4.6 g, 26.5 mmol) in anhydrous 1,4-dioxane (100 mL). The reaction mixture was heated at reflux and ethyl 4-bromopiperidine-1-carboxylate (**20**, 18.8 g, 80 mmol) was added in small portions over 72 h. The reaction mixture was cooled to rt, neutralized by adding 1M HCl, and the volatiles were removed *in vacuo*. The resulting residue was taken up in water (100 mL) and extracted with EtOAc (3 × 100 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 15%–40% EtOAc) afforded **17** (first eluting, N_2- isomer) and **18** (second eluting, N_1- isomer) as colourless oil and white solid, respectively. **17** (3.45 g, 39%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.50–7.30 (m, 6H), 5.17 (s, 2H), 4.54 (tt, J = 10.7, 4.0 Hz, 1H), 4.05 (q, J = 7.1 Hz, 2H), 4.03–3.89 (m, 2H), 3.18–2.90 (m, 2H), 2.05 (dd, J = 12.8, 3.0 Hz, 2H), 1.79 (ddd, J = 15.9, 12.1, 4.3 Hz, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.1, 154.6, 136.3, 128.4, 128.2, 128.1, 118.6, 71.5, 60.8, 60.4, 41.9, 31.0, 14.6. HRMS (ESI-TOF): *m/z* calculated for $C_{17}H_{23}N_4O_3$ $[M+H]^+$, 331.1765. Found, 331.1760 (ΔM=1.5 ppm). **18** (3.6 g, 41%): mp 98–100

°C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.85 (s, 1H), 7.48–7.31 (m, 5H), 5.15 (s, 2H), 4.62 (tt, J = 11.3, 4.0 Hz, 1H), 4.14–4.01 (m, 4H), 3.09–2.89 (m, 2H), 2.06–1.99 (m, 2H), 1.82 (qd, J = 12.3, 4.4 Hz, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 160.1, 154.5, 136.5, 128.4, 128.1, 128.0, 105.6, 71.5, 60.9, 57.6, 42.2, 31.6, 14.6. HRMS (ESI-TOF): m/z calculated for $C_{17}H_{23}N_4O_3$ $[M+H]^+$, 331.1765. Found, 331.1761 (ΔM=1.0 ppm).

*4.1.15. Ethyl 4-(4-(benzyloxy)-5-iodo-2H-1,2,3-triazol-2-yl)piperidine-1-carboxylate (**21**).* A solution of ICl (0.12 g, 0.73 mmol) in AcOH (2 mL) were added to a solution of **17** (0.20 g, 0.61 mmol) in AcOH (3 mL). Water (7 mL) was added and the resulting mixture was heated at 80 °C for 24 h. A solution of sodium thiosulfate 15-20% *w/w* was added and the reaction mixture was concentrated *in vacuo*. Water (50 mL) was added and the mixture was extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–25% EtOAc) afforded **21** as colourless oil (0.21 g, 76%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.54–7.28 (m, 5H), 5.23 (s, 2H), 4.67–4.50 (m, 1H), 4.05 (q, J = 7.1 Hz, 2H), 4.02–3.88 (m, 2H), 3.14–2.90 (m, 2H), 2.12–1.97 (m, 2H), 1.78 (qd, J = 12.2, 4.1 Hz, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.3, 154.6, 136.0, 128.5, 128.3, 128.2, 77.2, 72.0, 61.3, 60.8, 41.8, 30.9, 14.6. HRMS (ESI-TOF): m/z calculated for $C_{17}H_{22}N_4O_3I$ $[M+H]^+$, 457.0731. Found, 457.0732 (ΔM=0.3 ppm).

*4.1.16. Ethyl 4-(4-(benzyloxy)-5-iodo-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**22**).* A solution of ICl (0.14 g, 0.88 mmol) in AcOH (4 mL) were added to a solution of **18** (0.22 g, 0.68 mmol) in AcOH (6 mL). Water (14 mL) was added and the resulting mixture was heated at 80 °C for 24 h. A solution of sodium thiosulfate 15–20% *w/w* was added and the reaction mixture was concentrated *in vacuo*. Water (50 mL) was added and the mixture was extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried

over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–30% EtOAc) afforded **22** as white solid (0.19 g, 60%): mp 103–107 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.48–7.31 (m, 5H), 5.31 (s, 2H), 4.54 (tt, J = 11.4, 4.1 Hz, 1H), 4.14–4.07 (m, 2H), 4.06 (q, J = 7.1 Hz, 2H), 3.15–2.94 (m, 2H), 2.04–1.96 (m, 2H), 1.89 (qd, J = 12.1, 4.4 Hz, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 161.9, 154.6, 136.6, 128.5, 128.2, 128.1, 71.4, 65.0, 60.9, 57.9, 42.2, 31.1, 14.6. HRMS (ESI-TOF): m/z calculated for $C_{17}H_{21}N_4O_3INa$ $[M+Na]^+$, 479.0551. Found, 479.0556 (ΔM=1.2 ppm).

ha formattato: Inglese (Regno Unito)

*4.1.17. Ethyl 4-(4-(benzyloxy)-5-(1-hydroxy-3,3-diphenylpropyl)-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**24c**).* A 1.7M solution of ^{i}PrMgCl in THF (0.67 mL, 1.1 mmol) was added dropwise to a cooled (–10 °C) solution of **22** (0.48 g, 1.0 mmol) in anhydrous THF (7 mL). The resulting mixture was stirred at the same temperature for 2 h. A solution of 3,3-diphenylpropanal (**27**, 0.24 g, 1.1 mmol) in anhydrous THF (3 mL) was added and the mixture was allowed to reach rt. After 48 h, saturated aqueous NH_4Cl (7 mL) was added and the mixture stirred for 30 min before it was evaporated *in vacuo*. The residue was taken up in water (50 mL) and extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2/EtOAc, 85:15 *v/v*) afforded **24c** (0.26 g, 46%) as white solid: mp 62–66 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.38–7.31 (m, 5H), 7.29–7.21 (m, 8H), 7.20–7.14 (m, 2H), 5.62 (d, J = 5.3 Hz, 1H), 5.27 (s, 2H), 4.47 (dt, J = 8.2, 5.9 Hz, 1H), 4.38 (tt, J = 10.6, 4.6 Hz, 1H), 4.05 (q, J = 7.1 Hz, 2H), 4.01 (t, J = 8.0 Hz, 1H), 4.01–3.95 (m, 2H), 2.96–2.71 (m, 2H), 2.64–2.53 (m, 2H), 1.96–1.87 (m, 2H), 1.80–1.67 (m, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.8, 154.5, 144.5, 144.1, 136.9, 128.5, 128.4, 128.2, 127.8, 127.7, 127.65, 127.6, 126.2, 126.1, 120.8, 71.1, 60.8, 60.3, 55.7, 47.0, 42.4, 42.3, 31.9,

31.3, 14.6. HRMS (ESI-TOF): *m/z* calculated for $C_{32}H_{37}N_4O_4$ $[M+H]^+$, 541.2809. Found, 541.2804 (ΔM=1.0 ppm).

*4.1.18. Ethyl 4-(5-(3,3-diphenylpropyl)-4-hydroxy-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**28c**).* Et_3SiH (0.3 mL, 1.8 mmol) and TFA (0.67 mL, 8.7 mmol) were added to a solution of **24c** (0.17 g, 0.31 mmol) in CH_2Cl_2 (6 mL). The reaction mixture was heated at 50 °C in a sealed tube for 48 h. After cooling, CH_2Cl_2 was added up to 50 mL and the resulting mixture washed with 2M NaOH (50 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic phases were washed with brine (50 mL), dried over anhydrous $MgSO_4$, and evaporated. The crude product was dissolved in MeOH (20 mL) and added Pd/C (15 mg). The reaction mixture was put under a hydrogen atmosphere and stirred for 16 h. The reaction mixture was filtered through a PVDF filter (0.45 μm) and the volatiles were evaporated *in vacuo*. Purification by preparative HPLC (gradient 50%–70% solvent B over 10 min) afforded **28c** (0.11 g, 81%) as colourless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.37–7.25 (m, 8H), 7.21–7.15 (m, 2H), 4.05 (q, J = 7.0 Hz, 2H), 4.05–3.98 (m, 2H), 3.95 (t, J = 7.8 Hz, 1H), 2.90–2.72 (m, 2H), 2.49–2.44 (m, 2H), 2.30–2.23 (m, 2H), 1.84–1.74 (m, 4H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.4, 154.5, 144.5, 128.5, 127.6, 126.2, 117.0, 60.8, 54.6, 50.0, 42.4, 33.1, 31.5, 19.7, 14.6. HRMS (ESI-TOF): *m/z* calculated for $C_{25}H_{31}N_4O_3$ $[M+H]^+$, 435.2391. Found, 435.2395 (ΔM=1.0 ppm).

*4.1.19. Ethyl 4-(4-(benzyloxy)-5-(naphthalen-2-ylmethyl)-1H-1,2,3-triazol-1-yl)piperidine-1-carboxylate (**26b**).* A solution of ^{i}PrMgCl in THF (1.7M, 0.69 mL, 1.2 mmol) was added dropwise to a cooled solution (–10 °C) of **22** (0.50 g, 1.1 mmol) in anhydrous THF (7 mL). The mixture was stirred 1 h before a solution of 2-naphthaldehyde (0.19 g, 1.2 mmol) in anhydrous THF (3 mL) was added. The resulting mixture was allowed to reach rt. After 48 h, saturated aqueous NH_4Cl (5 mL) was added and the mixture stirred for 30 min before it was

evaporated *in vacuo*. The residue was taken up in water (50 mL) and extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–40% EtOAc) afforded the alcohol intermediate **24b** (0.42 g, 80%). HRMS (ESI-TOF): *m/z* calculated for $C_{28}H_{31}N_4O_4$ $[M+H]^+$, 487.2340. Found, 487.2338 (ΔM=0.4 ppm). **24b** (0.40 g, 0.83 mmol) was dissolved in CH_2Cl_2 (30 mL) and Et_3SiH (0.21 mL, 1.3 mmol) was added. The solution was cooled at 0 °C, TFA (1.8 mL, 23 mmol) was added and the resulting mixture was allowed to reach rt and stirred for 20 h. CH_2Cl_2 was added up to 50 mL and the resulting mixture was washed with 2M NaOH (50 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 10%–35% EtOAc) afforded **26b** (0.30 g, 77%) as colourless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.90–7.78 (m, 3H), 7.66 (s, 1H), 7.52–7.45 (m, 2H), 7.37–7.25 (m, 6H), 5.31 (s, 2H), 4.58 (tt, J = 11.5, 4.0 Hz, 1H), 4.20 (s, 2H), 4.02 (q, J = 7.1 Hz, 2H), 4.00–3.89 (m, 2H), 2.97–2.77 (m, 2H), 1.81 (qd, J = 12.3, 4.5 Hz, 2H), 1.73–1.62 (m, 2H), 1.15 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 156.8, 154.5, 137.0, 135.1, 133.0, 131.8, 128.3, 127.9, 127.85, 127.6, 127.4, 126.7, 126.4, 126.2, 125.8, 118.3, 71.3, 60.8, 55.0, 42.3, 31.5, 26.7, 14.5. HRMS (ESI-TOF): *m/z* calculated for $C_{28}H_{31}N_4O_3$ $[M+H]^+$, 471.2391. Found, 471.2387 (ΔM=0.8 ppm).

*4.1.20 Ethyl 4-(4-(benzyloxy)-5-(naphthalen-2-ylmethyl)-2H-1,2,3-triazol-2-yl)piperidine-1-carboxylate (**25b**)*. A solution of ^{i}PrMgCl in THF (1.7M, 1.4 mL, 2.4 mmol) was added dropwise to a cooled solution (–10 °C) of **21** (1.0 g, 2.2 mmol) in anhydrous THF (15 mL). The mixture was stirred 1 h before a solution of 2-naphthaldehyde (0.38 g, 2.4 mmol) in anhydrous THF (5 mL) was added. The resulting mixture was allowed to reach rt. After 48 h, saturated aqueous NH_4Cl (10 mL) was added and the mixture stirred for 30 min before it was

evaporated *in vacuo*. The residue was taken up in water (50 mL) and extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–35% EtOAc) afforded the alcohol intermediate **23b** (0.64 g, 60%) as colourless oil. HRMS (ESI-TOF): *m/z* calculated for $C_{28}H_{30}N_4O_4Na$ $[M+Na]^+$, 509.2159. Found, 509.2167 (ΔM=1.5 ppm). **23b** (0.62 g, 1.3 mmol) was dissolved in CH_2Cl_2 (45 mL) and Et_3SiH (0.33 mL, 2.0 mmol) was added. The solution was cooled at 0 °C, TFA (2.7 mL, 36 mmol) was added and the resulting mixture was allowed to reach rt and stirred for 20 h. CH_2Cl_2 was added up to 50 mL and the resulting mixture was washed with 2M NaOH (50 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–20% EtOAc) afforded **25b** (0.30 g, 50%) as colourless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.85 (d, J = 7.7 Hz, 1H), 7.83–7.78 (m, 2H), 7.69 (s, 1H), 7.51–7.43 (m, 2H), 7.37– 7.26 (m, 6H), 5.19 (s, 2H), 4.49 (tt, J = 10.9, 4.1 Hz, 1H), 4.07–4.00 (m, 4H), 4.00–3.92 (m, 2H), 3.09–2.93 (m, 2H), 2.07–2.00 (m, 2H), 1.79 (qd, J = 11.6, 4.4 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.0, 154.6, 136.5, 136.3, 133.0, 131.7, 130.5, 128.3, 128.0, 127.9, 127.8, 127.5, 127.4, 127.2, 126.4, 126.1, 125.5, 71.4, 60.8, 60.2, 41.9, 31.0, 29.5, 14.6. HRMS (ESI-TOF): *m/z* calculated for $C_{28}H_{31}N_4O_3$ $[M+H]^+$, 471.2391. Found, 471.2384 (ΔM=1.3 ppm).

*4.1.21 Ethyl 4-(4-(benzyloxy)-5-(3,3-diphenylpropyl)-2H-1,2,3-triazol-2-yl)piperidine-1-carboxylate (**25c**)*. A solution of ^{i}PrMgCl in THF (1.7M, 1.4 mL, 2.5 mmol) was added dropwise to a cooled solution (–10 °C) of **21** (1.0 g, 2.2 mmol) in anhydrous THF (15 mL). The mixture was stirred 1 h before a solution of 3,3-diphenylpropanal (**27**, 0.52 g, 2.5 mmol) in anhydrous THF (5 mL) was added. The resulting mixture was allowed to reach rt. After 48

h, saturated aqueous NH_4Cl (5 mL) was added and the mixture stirred for 30 min before it was evaporated *in vacuo*. The residue was taken up in water (50 mL) and extracted with Et_2O (3 × 50 mL). The combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, gradient 0%–35% EtOAc) afforded the alcohol intermediate **23c** (0.36 g, 30%) as colourless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.41–7.30 (m, 5H), 7.30–7.20 (m, 8H), 7.18–7.11 (m, 2H), 5.24 (d, J = 5.2 Hz, 1H), 5.17 (s, 2H), 4.48 (tt, J = 10.7, 4.0 Hz, 1H), 4.32 (dt, J = 8.2, 5.6 Hz, 1H), 4.05 (q, J = 7.1 Hz, 2H), 4.03–4.00 (m, 1H), 3.99–3.92 (m, 2H), 3.11–2.95 (m, 2H), 2.56–2.43 (m, 2H), 2.06–2.00 (m, 2H), 1.83–1.74 (m, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 156.6, 154.6, 145.1, 144.3, 136.6, 134.2, 128.4, 128.36, 128.3, 128.2, 128.0, 127.9, 127.8, 127.6, 126.1, 126.0, 71.3, 61.7, 60.8, 60.2, 46.9, 41.9, 40.9, 30.9, 14.6. HRMS (ESI-TOF): m/z calculated for $C_{32}H_{36}N_4O_4Na$ $[M+Na]^+$, 563.2629. Found, 563.2618 (ΔM=1.8 ppm). **23c** (0.34 g, 0.62 mmol) was dissolved in CH_2Cl_2 (30 mL) and Et_3SiH (0.60 mL, 3.7 mmol) was added. The solution was cooled at 0 °C and TFA (1.3 mL, 17 mmol) was added and the resulting mixture was allowed to reach rt and stirred for 72 h. CH_2Cl_2 was added up to 50 mL and the resulting mixture was washed with 2M NaOH (50 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic phase was washed with brine (50 mL), dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Purification by flash chromatography (petroleum ether 40–60 °C/EtOAc, 85:15 *v/v*) afforded **25c** (0.25 g, 78%) as colourless oil. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.41–7.31 (m, 5H), 7.28–7.22 (m, 8H), 7.17–7.12 (m, 2H), 5.16 (s, 2H), 4.46 (tt, J = 10.8, 4.1 Hz, 1H), 4.05 (q, J = 7.1 Hz, 2H), 4.00–3.92 (m, 2H), 3.92 (t, J = 7.7 Hz, 1H), 3.10–2.94 (m, 2H), 2.41–2.37 (m, 2H), 2.32–2.27 (m, 2H), 2.04–1.99 (m, 2H), 1.77 (qd, J = 12.3, 4.3 Hz, 2H), 1.19 (t, J = 7.1 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 156.9, 154.6, 144.7, 136.5, 131.1, 128.4, 128.37, 128.1, 128.0, 127.6, 126.1, 71.4, 60.8, 60.0, 49.9, 41.9, 33.4, 30.9, 21.7, 14.6. HRMS (ESI-TOF): m/z calculated for $C_{32}H_{37}N_4O_3$ $[M+H]^+$, 525.2860. Found, 525.2856 (ΔM=0.7 ppm).

*4.1.22 5-(Naphthalen-2-ylmethyl)-2-(piperidin-4-yl)-2H-1,2,3-triazol-4-ol hydrochloride (**3b**).* A solution of **25b** (0.23 g, 0.49 mmol) in EtOH/35% HCl (1:2 *v*/*v*, 15 mL) was heated at reflux for 24 h. Upon cooling to rt, the solvents were evaporated *in vacuo*. Purification by preparative HPLC (gradient 20%–50% solvent B over 15 min) followed by conversion of the obtained product into the hydrochloric salt using 2M HCl afforded **3b** (32 mg, 20%) as pale yellow solid: mp 200–203 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.50 (s, 1H), 9.03 (br s, 2H), 7.87–7.79 (m, 3H), 7.68 (s, 1H), 7.50–7.42 (m, 2H), 7.39 (d, J = 8.4 Hz, 1H), 4.52 (tt, J = 9.8, 4.3 Hz, 1H), 4.01 (s, 2H), 3.33–3.24 (m, 2H), 3.09–2.99 (m, 2H), 2.21–2.06 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 156.1, 136.9, 133.0, 131.6, 130.5, 127.9, 127.5, 127.4, 127.2, 126.3, 126.1, 125.5, 57.3, 41.7, 29.3, 27.9. HRMS (ESI-TOF): *m*/*z* calculated for $C_{18}H_{21}N_4O$ $[M+H]^+$, 309.1710. Found, 309.1711 (ΔM=0.3 ppm).

*4.1.23 5-(3,3-Diphenylpropyl)-2-(piperidin-4-yl)-2H-1,2,3-triazol-4-ol hydrochloride (**3c**).* A solution of **25c** (0.21 g, 0.38 mmol) in EtOH/35% HCl (1:2 *v*/*v*, 15 mL) was heated at reflux for 24 h. Upon cooling to rt, the solvents were evaporated *in vacuo*. Recrystallization from MeOH/Et_2O afforded **3c** (82 mg, 52%) as white solid: mp 246–249 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.27 (s, 1H), 9.21 (s, 2H), 7.38–7.21 (m, 8H), 7.21–7.09 (m, 2H), 4.49 (tt, J = 10.0, 4.8 Hz, 1H), 3.96 (t, J = 7.5 Hz, 1H), 3.32–3.22 (m, 2H), 3.12–2.98 (m, 2H), 2.42–2.25 (m, 4H), 2.21–2.06 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.8, 144.8, 131.1, 128.4, 127.6, 126.0, 57.0, 50.1, 41.6, 33.5, 27.8, 21.8. HRMS (ESI-TOF): *m*/*z* calculated for $C_{22}H_{27}N_4O$ $[M+H]^+$, 363.2179. Found, 363.2182 (ΔM=0.8 ppm).

*4.1.24 5-(Naphthalen-2-ylmethyl)-1-(piperidin-4-yl)-1H-1,2,3-triazol-4-ol hydrochloride (**4b**).* A solution of **26b** (0.21 g, 0.46 mmol) in EtOH/35% HCl (1:2 *v*/*v*, 15 mL) was heated at reflux for 24 h. Upon cooling to rt, the solvents were evaporated *in vacuo*. Purification by

preparative HPLC (gradient 20%–40% solvent B over 10 min) followed by conversion of the obtained product into the hydrochloric salt using 2M HCl afforded **4b** (73 mg, 46%) as pale yellow solid: mp 258–261 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.34 (br s, 1H), 8.99 (br s, 1H), 7.90–7.81 (m, 3H), 7.71 (s, 1H), 7.51–7.44 (m, 2H), 7.37 (dd, J = 8.4, 1.7 Hz, 1H), 4.67 (tt, J = 10.9, 3.9 Hz, 1H), 4.19 (s, 2H), 3.31–3.25 (m, 2H), 3.03–2.94 (m, 2H), 2.21–2.11 (m, 2H), 1.85–1.77 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.8, 135.7, 133.0, 131.8, 128.3, 127.6, 127.4, 126.8, 126.3, 126.0, 125.7, 116.8, 52.3, 42.0, 28.4, 26.6. HRMS (ESI-TOF): m/z calculated for $C_{18}H_{21}N_4O$ $[M+H]^+$, 309.1710. Found, 309.1708 (ΔM=0.5 ppm).

*4.1.25 5-(3,3-Diphenylpropyl)-1-(piperidin-4-yl)-1H-1,2,3-triazol-4-ol hydrochloride (**4c**).* A solution of **28c** (93 mg, 0.21 mmol) in EtOH/35% HCl (1:2 *v/v*, 15 mL) was heated at reflux for 24 h. Upon cooling to rt, the solvents were evaporated *in vacuo*. Recrystallization from MeOH/Et_2O afforded **4c** (43 mg, 51%) as white solid: mp 232–234 °C. ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.82 (s, 1H), 9.16 (br s, 1H), 8.88 (br s, 1H), 7.37–7.24 (m, 8H), 7.23–7.13 (m, 2H), 4.28 (tt, J = 11.0, 4.0 Hz, 1H), 3.97 (t, J = 7.8 Hz, 1H), 3.40–3.37 (q, J = 7.0 Hz, 0.6H, $(CH_3CH_2)_2O$), 3.37–3.33 (m, 2H), 3.00–2.91 (m, 2H), 2.48–2.45 (m, 2H), 2.30–2.24 (m, 2H), 2.22–2.13 (m, 2H), 2.02–1.95 (m, 2H), 1.09 (t, J = 7.0 Hz, 0.9 H, $(CH_3CH_2)_2O$). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.4, 144.5, 128.5, 127.6, 126.2, 117.2, 64.9, 51.9, 50.1, 42.1, 33.0, 28.4, 19.7, 15.1. HRMS (ESI-TOF): m/z calculated for $C_{22}H_{27}N_4O$ $[M+H]^+$, 363.2179. Found, 363.2179 (ΔM=0.2 ppm). Anal. calcd ($C_{22}H_{26}N_4O\cdot1.25HCl\cdot0.1Et_2O$): C, 64.76; H, 6.85; N, 13.49. Found: C, 65.14; H, 6.45; N 13.18.

4.2. Determination of ionization constants.

The ionization constants of compounds **2a–b**, **3a–c** and **4a–c** were determined by potentiometric titration with the GLpK_a apparatus (Sirius Analytical Instruments Ltd, Forest Row, East Sussex, UK). The pK_a values were obtained as mean of four titrations: aqueous

solutions (ionic strength adjusted to 0.15M with KCl) of the compound (20 mL, about 1 mM) were initially acidified to pH 1.8 with 0.5 N HCl and then titrated with standardized 0.5N KOH to pH 12.2 at constant temperature of 25(±0.1) °C under argon atmosphere.

4.3 Molecular modelling

4.3.1 Docking of selected compounds. A model of the extracellular domain of $GABA_AR$ constructed using an iterative approach with the orthosteric binding site optimized using an induced fit docking protocol,[38-41] has previously been reported,[26] and is used here with the compounds **3a** and **4a**. Subsequently ligands **3b–c**, and **4b–c**, were docked into the binding site as described previously,[26] except 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 7.7 docking program.[42-45] Finally the obtained models were minimized using the MacroModel 11.8 program.[46]

*4.3.2 Calculation of solvation energies of 4-PHP and **2a***. Solvation energies were calculated in Jaguar[35, 47] version 9.8 on B3LYP/6-31+G** optimized geometries using the Poisson Boltzmann Finite[48-50] element method as implemented in Jaguar. Gas phase optimized geometries (B3LYP/6-31+G**) were used as reference. Default settings were used except for the SCF convergence threshold, which was set to "ultrafine". Calculations were performed on the anionic forms the triazole moiety of the compounds.

4.4 Pharmacology

Characterization of compounds **2a–b**, **3a–c**, and **4a–c** in muscimol binding: the binding assay was performed using rat brain synaptic membranes of cortex and the central hemispheres from male SPRD rats with tissue preparation as described in the literature.[51] On the day of the experiment, the membrane preparation was quickly thawed, homogenized in 50 volumes of ice-cold buffer (50 mM Tris–HCl buffer, pH 7.4), and centrifuged at 48,000g for 10 min at 4

°C. This washing step was repeated four times and the final pellet was re-suspended in buffer. The assay was carried out in 96-wells plates, by incubation of membranes (70–80 μg protein) in 200 μL buffer, 25 μL [^{3}H]muscimol (5 nM final concentration), and 25 μL test substance in various concentrations, for 60 min at 0 °C. The reaction was terminated by rapid filtration through GF/C filters (Perkin Elmer Life Sciences), using a 96 well Packard FilterMate cellharvester, followed by washing with 3 × 250 μL of ice-cold buffer. The dried filters were added Microscint scintillation fluid (PerkinElmer Life Sciences), and the amount of filterbound radioactivity was quantified in a Packard TopCount microplate scintillator counter. The experiments were performed in triplicate at least three times for each compound. Non-specific binding was determined using 1.0 mM GABA. The binding data was analysed by a non-linear regression curve-fitting procedure using GraphPad Prism v. 6.00 (GraphPad Software, CA, USA). IC_{50} values were calculated from inhibition curves and converted to K_i values using the modified Cheng–Prusoff equation.[52]

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Notes. The authors declare no competing financial interest.

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ABBREVIATIONS USED

γ-Aminobutyric acid (GABA), GABA type A receptor ($GABA_AR$), 5-(piperidin-4-yl)-3-isoxazolol (4-PIOL), 4-(piperidin-4-yl)-1-hydroxypyrazole (4-PHP), and 5-(piperidin-4-yl)-3-hydroxypyrazol (aza-4-PIOL).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at: XXXXXX

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