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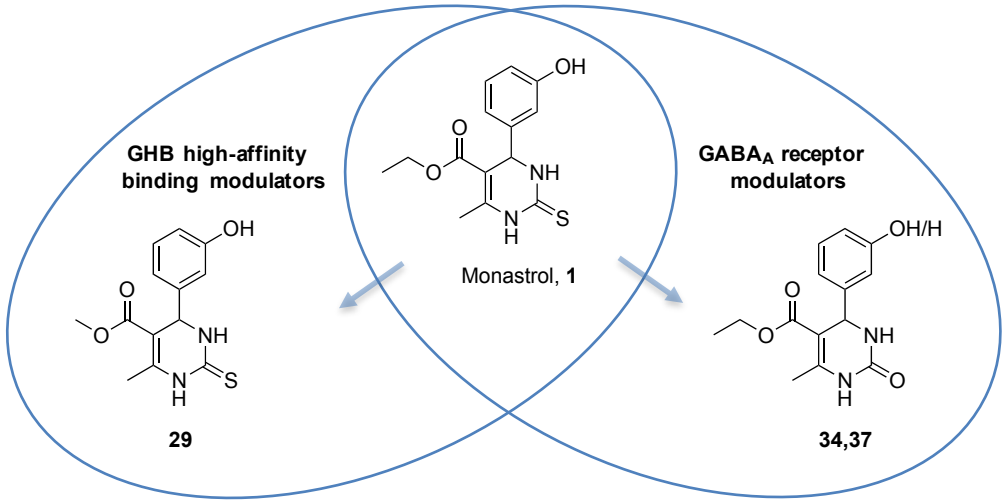
GHB high-affinity binding modulators
GABA$_A$ receptor modulators
OH
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Monastrol, **1**
OH
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O
NH
N
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OH/H
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34,37

Monastrol, a 3,4-dihydropyrimidin-2(1*H*)-thione, as structural scaffold for the development of modulators for GHB high-affinity binding sites and $\alpha_1\beta_2\delta$ $GABA_A$ receptors

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Abstract

The $\alpha_4\beta\delta$ subtype of the γ-aminobutyric acid (GABA) type A receptors ($GABA_ARs$) has been shown to be implicated in high-affinity binding of the neuromodulator γ-hydroxybutyric acid (GHB), but may not be the only GHB high-affinity binding sites. Monastrol has been identified as a modulator of GHB high-affinity binding and is furthermore reported as an allosteric modulator selective for the $\alpha_1\beta_2\delta$ $GABA_ARs$. Therefore, structural determinants for selectivity at the two targets were investigated. 39 structural diverse monastrol analogues were synthesized by employing the Biginelli cyclocondensation and examined for modulation of GHB high-affinity binding using the GHB-specific ligand [^{3}H]NCS-382 [(*E,RS*)-6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid] in rat brain homogenate. Only limited modifications were allowed on the monastrol scaffold in order to maintain modulation of GHB high-affinity binding. However, three analogues of monastrol (**11**, **12** and **24**) enhanced the maximal binding of [^{3}H]NCS-382 to a higher maximal level than seen for monastrol itself. Selected compounds were further characterized as modulators at $\alpha_1\beta_2\delta$, $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_1\beta_2$ $GABA_ARs$. Most of these modulators were shown to have δ-specific GABA-potentiating effects. The dual effect shown for monastrol to modulate the GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_AR$ activity was also shown for the compounds **11**, **18** and **24**. Compound **29** displayed minimal modulatory effect on $GABA_ARs$ and therefore appears to be a GHB high-affinity binding preferring modulator. However, compounds **34** and **37** were shown to be $\alpha_1\beta_2\delta$ $GABA_AR$ selective modulators, without modulatory effects on GHB high-affinity binding. Thus, our study shows that minor modifications in the structure of monastrol affects the selectivity profile for the two targets under study enabling separation of the dual activity.

Keywords: max 6

GHB high-affinity binding sites, $GABA_A$ receptor, allosteric modulators, monastrol analogues, Biginelli cyclocondensation

Highlights (3-5 bullet points (max 85 characters))

- 39 structural diverse monastrol analogues were synthesized
- Structural determinants for modulatory activity and selectivity were identified
- Modulators selective for $\alpha_1\beta_2\delta$ $GABA_AR$ and high-affinity GHB binding were identified

1. Introduction

γ-Hydroxybutyric acid (GHB) is a small endogenous compound proposed to be a neuromodulator in the mammalian brain.[1] GHB is both chemically and pharmacologically related to γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. GHB was first synthesized as a brain permeable GABA analogue in the 1960's[2] and simultaneously GHB was found to be a naturally occurring metabolite.[3] GHB has a complex pharmacology, comprising both high- and low-affinity binding sites.[1, 4] $GABA_B$ receptors are well-established low-affinity binding sites for GHB at which GHB acts as a weak partial agonist.[5, 6] Many of the effects observed with exogenously administered GHB have been ascribed to the $GABA_B$ receptors, e.g. hypothermia, dopamine release and sedation.[4, 7, 8, 9, 10] The $\alpha_4\beta\delta$ $GABA_A$ receptors ($GABA_A$Rs) have, however, been shown to be implicated in GHB high-affinity binding[11] and were shown to account for up to 40% of the GHB high-affinity binding, whereas the remaining 60% of GHB high-affinity binding sites remain elusive.[1]

The $GABA_A$Rs are pentameric ligand-gated chloride ion-channels. They can be assembled from 19 different $GABA_A$R subunits $\alpha_{1\text{-}6}$, $\beta_{1\text{-}3}$, $\gamma_{1\text{-}3}$, δ, ε, θ, π, and $\rho_{1\text{-}3}$[12] forming receptors showing distinct function and distribution depending of the subunit composition.[13] Synaptic $GABA_A$Rs are predominantly composed of α_1, α_2, and/or α_3 in combination with $\beta_{2/3}$ and γ_2 subunits, the $\alpha_1\beta_2\gamma_2$ representing the major subtype, whereas δ-containing $GABA_A$R have been shown to be located extrasynaptically and mediate tonic inhibition.[13-14] Specifically, α_1/δ-containing $GABA_A$R have been shown to be present in hippocampal interneurons, possibly mediating high sensitivity towards ethanol.[15] $\alpha_1\beta_2\delta$ receptors have been suggested to be silent receptors that upon modulation by endogenous neurosteroids could exert profound inhibition.[16]

Selective ligands targeting the orthosteric GHB high-affinity binding sites are highly useful pharmacological tools for studying these in isolation.[1] NCS-382 is representing a structural class of GHB analogues showing selectivity and high nanomolar affinity for the GHB high-affinity binding sites.[17] So far, the main focus has been on the development of GHB-like ligands for targeting the orthosteric GHB high-affinity binding sites and only recently allosteric modulators have been reported.[18]

Allosteric modulators have, on the other hand, been widely used to target and study the $GABA_A$Rs including benzodiazepines, barbiturates, steroids and naturally occurring compounds. Most

$GABA_AR$ modulators are non-selective towards the $GABA_AR$ subtypes[19] but a few compounds have been shown to have some selectivity towards the δ-containing receptors. DS1, DS2[20], AA29504[21], ethyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (monastrol, **1**) and the monastrol analogue JM-II-43A[22] are such compounds. More specifically, the synthetic compound monastrol is reported to selectively modulate the $\alpha_1\beta_2\delta$ $GABA_AR$ subtype.[22] Recently, monastrol and catechin were identified as the first GHB high-affinity allosteric modulators in a library screening of natural compounds and known $GABA_AR$ modulators.[18] The aim of this study was to dissect the structural determinants of monastrol for modulating GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_AR$ activity, respectively, assuming these effects are mediated via different molecular targets.

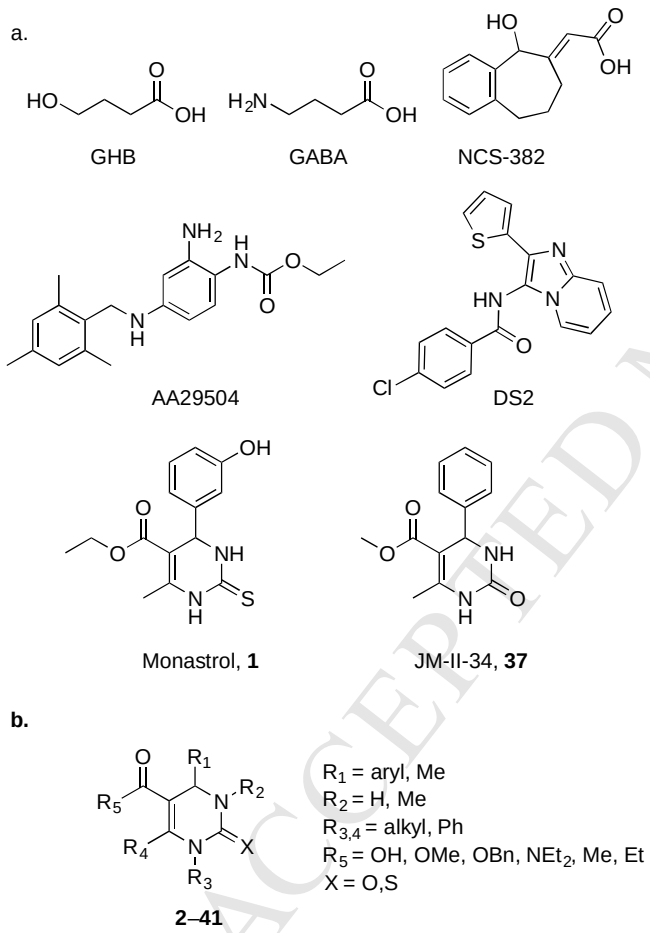


Figure 1. a Structures of GHB, GABA, NCS-382, and the δ selective $GABA_A$ receptors modulators DS2, AA29504, monastrol (**1)** and JM-II-34 (**37**). **B** General structure of the target monastrol analogues (**2**-**41**).

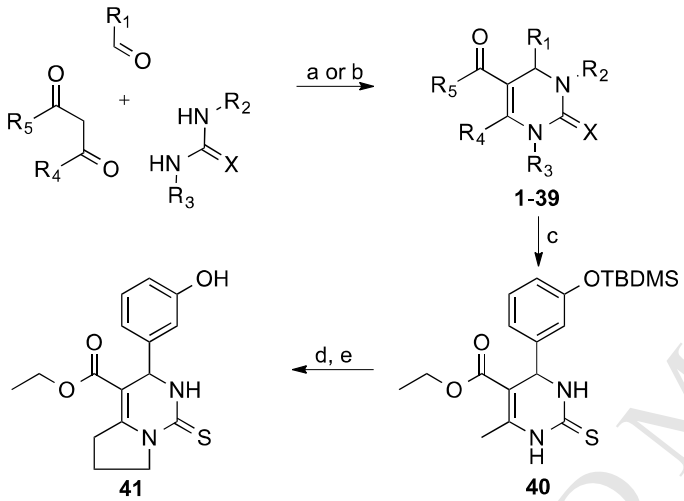
2. Results

2.1. Chemistry

A series of monastrol analogues were synthesized to elucidate the structure-activity relationship (SAR) for the modulatory effect of monastrol on GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_AR$ activity. The structural design of analogues was based on systematic investigation of different parts of the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold constituting the core scaffold of monastrol (Figure 1): the aromatic ring system and substituents (R_1), the urea moiety (X, R_2, R_3), the methyl substituent (R_4) and the acyl substituent (R_5).

The synthetic procedures for the synthesis of compounds **1**-**41** are depicted in Scheme 1. Compounds **1**-**11**, **13**, **14**, **17**, **19**-**21**, **25**, **28**-**30**, **32**-**34** and **36**-**37** were synthesized according to modified procedures previously described in the literature (please refer to Experimental Section for further information). The Biginelli three component reaction involving cyclocondensation of a 1,3-dicarbonyl, an aromatic aldehyde and urea or thiourea[23, 24] was used to synthesize the target compounds as racemic mixtures. Analogues (**1**-**17**, **24**-**39**) differing in the R_1, R_4, R_5 and X regions were synthesized using a microwave-mediated Biginelli reaction employing ytterbium(III) trifluoromethanesulfonate ($Yb(OTf)_3$) to catalyze the reaction.[25] The yields varied greatly depending on the substituents. This procedure showed not to be applicable for *N*-substituted thioureas. Instead, analogues **18**-**23** were synthesized by a modified Biginelli reaction using TMSCl to catalyze the reaction of 3-hydroxybenzaldehyde, ethyl acetoacetate and *N*-substituted thioureas.[26] The bicyclic product **41** was synthesized by TBDMS-protection of the phenol group of monastrol to give **40**, thereafter cyclization by the use of *n*-BuLi and 1,2-dibromoethane to obtain the cyclized product, subsequent deprotection with MeONa gave the desired compound **41** as previously described for similar compounds.[27] The two enantiomers of monastrol were separated on a Chiralpak IF semi-prep column (10x250 mm, 5μm), using a flow of 6 ml/min, injection size of app. 5 mg and heptane/2-propanol (90:10) as the mobile phase. Identification of the enantiomers was confirmed by optical rotation, comparable to literature values.[28]

Scheme 1. Synthesis of monastrol analogues **1-41**[a]



[a]Reagents and conditions: (a) $Yb(OTf)_3$ cat, THF or MeCN, 120 °C (microwave), 1-97%, (b) TMSCl, DMF, rt, 5-80%, (c) TBDMSCl, imidazole, DMF, 80 %, (d) 1. N-BuLi, THF, -10°C, 2. $BrCH_2CH_2Br$, THF, -10°C, 8 %, (e) MeONa, MeOH, 28 %.

2.2. Pharmacology

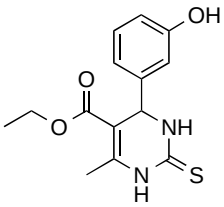
2.2.1. Modulation of GHB high-affinity binding

The synthesized monastrol analogues were initially pharmacologically characterized using the well-known [^{3}H]NCS-382 binding assay on rat cortical membranes[29] to investigate their modulatory effect on the GHB high-affinity binding, similar to previously reported for monastrol.[18] Table 1 gives an overview of the synthesized compounds and their ability to enhance [^{3}H]NCS-382 binding.

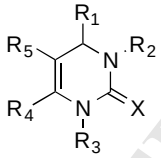
In the majority of cases, structural modifications of the lead compound, monastrol (**1**), led to loss of modulatory effect on the high-affinity GHB binding, i.e. no enhancement of [^{3}H]NCS-382 binding was observed. However, of the 39 synthesized analogues, six compounds (**11**, **12**, **18**, **24**, **25**, **29**) did show modulatory effects to the same or higher level than monastrol (**1**) itself (Table 1).

Interestingly, in terms of the modulatory capacity, no apparently stereoselectivity was seen for monastrol as both stereoisomers increased the cooperativity factor (α value) to a similar extent as the racemate.

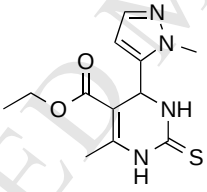
Table 1. Modulatory effects of monastrol (**1**) and the synthesized analogues (**2**–**41**) on [^{3}H]NCS-382 binding (rat cortical membranes). The cooperativity factor (α) and the dissociation constant of the modulator (K_b) were estimated by curve fitting (see Experimental section). Average data are based on triplicate measurements from at least three independent experiments, except for the stereoisomers of monastrol, which were tested only twice.



Monastrol, **1**



2-**39**



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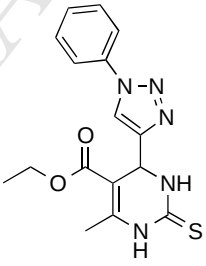

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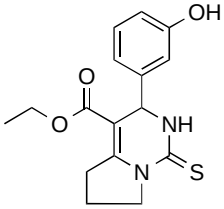

41

Compound	Chemical modification	α ($\log\alpha$ ± SEM	K_b (μM) (pK_b ± SEM)	Maximal binding ± SEM (% of control)
Monastrol, **1**[a]		2.00 (0.31 ± 0.05)	110 (3.96 ± 0.16)	185 ± 7.1
(*S*)-monastrol[b]		2.35 (0.37 ± 0.02)	111 (3.95 ± 0.07)	208 ± 12
(*R*)-monastrol[b]		1.87 (0.27 ± 0.02)	329 (3.48 ± 0.11)	171 ± 5.3
R_1 modification				
2	R_1 = Ph		NA	NA
3	R_1 = 3-Me-C_6H_4		NA	NA
4	R_1 = 4-Me-C_6H_4		NA	NA
5[c]	R_1 = 2-Me-C_6H_4		-	-

6[c]	R_1 = 3-Br-C_6H_4		-	-
7	R_1 = 3-F-C_6H_4		NA	NA
8	R_1 = 3-Cl-C_6H_4		NA	NA
9	R_1 = 3-NO_2-C_6H_4		NA	NA
10	R_1 = 4-OH-C_6H_4		NA	NA
11[e]	R_1 = 3,4-OH-C_6H_3	2.62	210	246 ± 21
		(0.42 ± 0.03)[*]	(3.68 ± 0.02)	
12[e]	R_1 = 3,5-OH-C_6H_3	3.06	134	247 ± 3.0
		(0.49 ± 0.01)[**]	(3.83 ± 0.06)	
13	R_1 = 3-pyridine		NA	NA
14	R_1 = 3-OMe-C_6H_4		NA	NA
15	R_1 = Het.cycle		NA	NA
16	R_1 = Het.cycle		NA	NA
17	R_1 = Me		NA	NA

R_2 and R_3 modifications

18	R_3 = Me	1.97	87	177 ± 1.3
		(0.29 ± 0.01)	(4.06 ± 0.01)	
19	R_3 = Et		WA	WA
20	R_3 = Bu		NA	NA
21	R_3 = Ph		NA	NA
22	R_3 = Bn		NA	NA
23	R_2 = R_3 = Me		NA	NA

R_4 modification

24	R_4 = Et	2.66	109	245 ± 13
		(0.42 ± 0.04)	(3.98 ± 0.08)	
25[d]	R_4 = Pr	-	-	198 ± 17
26	R_4 = ^{i}Pr		WA	WA
27	R_4 = ^{t}Bu		WA	WA
28	R_4 = Ph		NA	NA

R_5 modification

29[e]	R_5 = CO_2Me	2.37	347	194 ± 3.7
		(0.38 ± 0.01)[*]	(3.46 ± 0.06)	
30	R_5 = CO_2H		NA	NA

31	$R_5 = CO_2Bn$	NA	NA
32	$R_5 = CONEt_2$	NA	NA
33	$R_5 = COCH_3$	WA	WA

Mixed profile

34	X = O	NA	NA
35	R_3 = Me, $R_5 = CO_2Me$	NA	NA
36	X = O, R_1 = Ph	NA	NA
37	X = O, R_1 = Ph, $R_5 = CO_2Me$	NA	NA
38	R_4 = Et, $R_5 = COCH_2CH_3$	WA	WA
39	$R_4 = CH_2OCH_3$, $R_5 = CO_2Me$	WA	WA
41	$R_3, R_4 = (CH_2)_{3-}$	NA	NA

NA: no activity (defined as compounds that did not significantly increase the binding compared to control; student's t-test; data not shown); *WA*: weak activity (defined as compounds that only enhanced the binding to max. 130% at 100 µM and consequently not tested further).

[a] Data from ref[18]

[b] In one of the experiment for (R)-monastrol, the plateau of the concentration-response curve was not fully reached. Due to shortage of compounds, the pooled data were therefore used in a similar manner to fit both (*S*) and (*R*)-stereoisomers of monastrol. Hence, no statistical analysis was conducted on these values. Using this method did not change the values for (*S*)-monastrol.

[c] Compound insolubility precluded testing.

[d] Due to solubility problems, the plateau of the concentration-response curve was not reached. Consequently the data could not be analyzed by nonlinear regression. Therefore, no value for α or K_b was obtained for this compound.

[e] $\log\alpha$ means were compared and analyzed statistically by one-way ANOVA followed by Dunnett's multiple comparison test with significance levels indicated; * $p<0.05$ and ** $p<0.01$.

The 3-hydroxyphenol part of monastrol was found to be crucial for modulation of [^{3}H]NCS-382 binding. Any variation of the aromatic substitution pattern of the 4-aryl moiety (**2**–**10**, **13**–**17**) was shown to be detrimental for the enhancement of [^{3}H]NCS-382 binding. However, the 3,4-, 3,5-dihydroxy and C6-ethyl analogues, **11**, **12** and **24** enhanced the maximal binding of [^{3}H]NCS-382 to a higher maximal level than seen for monastrol (**1**) itself (Figure 2 and Table 1). The effect seen for the 3-hydroxy containing compounds could not be achieved by substituting the 3-hydroxy group

for a pyrazol or triazole moiety, **15** and **16**, set up for accepting a hydrogen bond in the corresponding position.

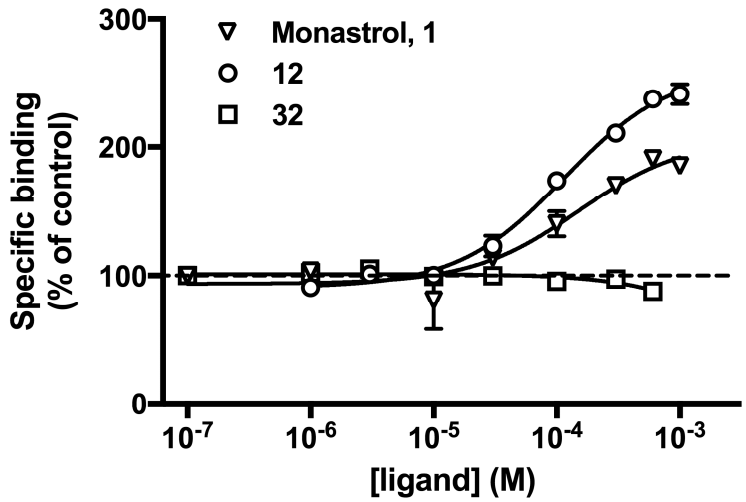

Figure 2. Concentration-dependent modulation of [^{3}H]NCS-382 (16 nM) binding to rat cortical membranes by monastrol (**1**) and selected analogues (**12** and **32**). Each point is mean ± S.D. of triplicate measurements of a single representative experiment with at least two additional experiments giving similar results. Average α and K_b values are summarized in Table 1.

Introduction of a methyl group on the N_1 nitrogen in the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold was allowed (**18**) whereas larger substituents such as an ethyl (**19**), a butyl (**20**) and a phenyl (**21**) group abolished the modulatory effect. Neither introduction of an additional methyl group in the N_3 nitrogen (**23**), nor cyclisation of the allowed side chains in the 6- and N_1-position (**41**) retained modulatory effect. Substitution of the methyl group at the 6-position of the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold by ethyl (**24**) led to a compound that increased the maximal binding of the radioligand to a higher level than monastrol (**1**). A propyl in this position (**25**) also retained modulatory effect, but larger alkyl groups (**26**–**27**) or a phenyl group (**28**) abolished activity.

Furthermore, only small modifications in the ester group was accepted, where the methyl ester (**29**) did show modulatory effect, but could not be replaced by the corresponding benzylic ester (**31**), carboxylic acid (**30**) or diethylamide (**32**). Interestingly, exchanging the thiocarbonyl group at the 2-position in monastrol for an oxocarbonyl (**34**) was detrimental for activity.

Overall, these results show that only minor modifications are allowed to retain the modulatory effect at high-affinity GHB binding. A summary of the allowed modifications in the core scaffold of monastrol is shown in Figure 3.

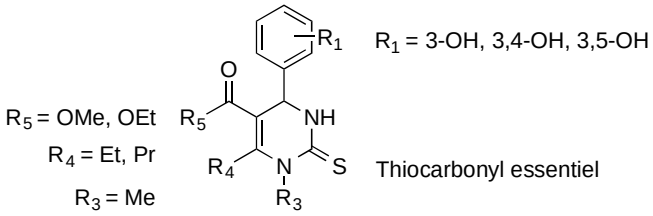

Figure 3. Representative structure of monastrol and the allowed modifications that retain the modulatory properties on GHB high-affinity binding.

2.2.2. GABA-potentiating effects of selected compounds

Monastrol (**1**) and compound **37 (**JM-II-43A) have previously been shown to selectively modulate the $\alpha_1\beta_2\delta$ $GABA_AR$.[22] Therefore, monastrol (**1**), **37** and five other analogues (**11**, **18**, **24**, **29** and **34**) were further characterized on different $GABA_AR$ subtypes representing the synaptic ($\alpha_1\beta_2\gamma_2$s) and extrasynaptic ($\alpha_1\beta_2\delta$) $GABA_ARs$ and on $\alpha_1\beta_2$ $GABA_ARs$ for comparison, expressed in *Xenopus laevis* oocytes, by use of the two-electrode voltage clamp technique (TEVC). Compounds **11**, **18**, **24** and **29** were chosen based on the observed modulatory effect on the GHB high-affinity binding, whereas **34**, the oxycarbonyl analog of monastrol, was selected based on previous report on modulatory effect of **37** on $\alpha_1\beta_2\delta$ $GABA_A$ receptors.[22]

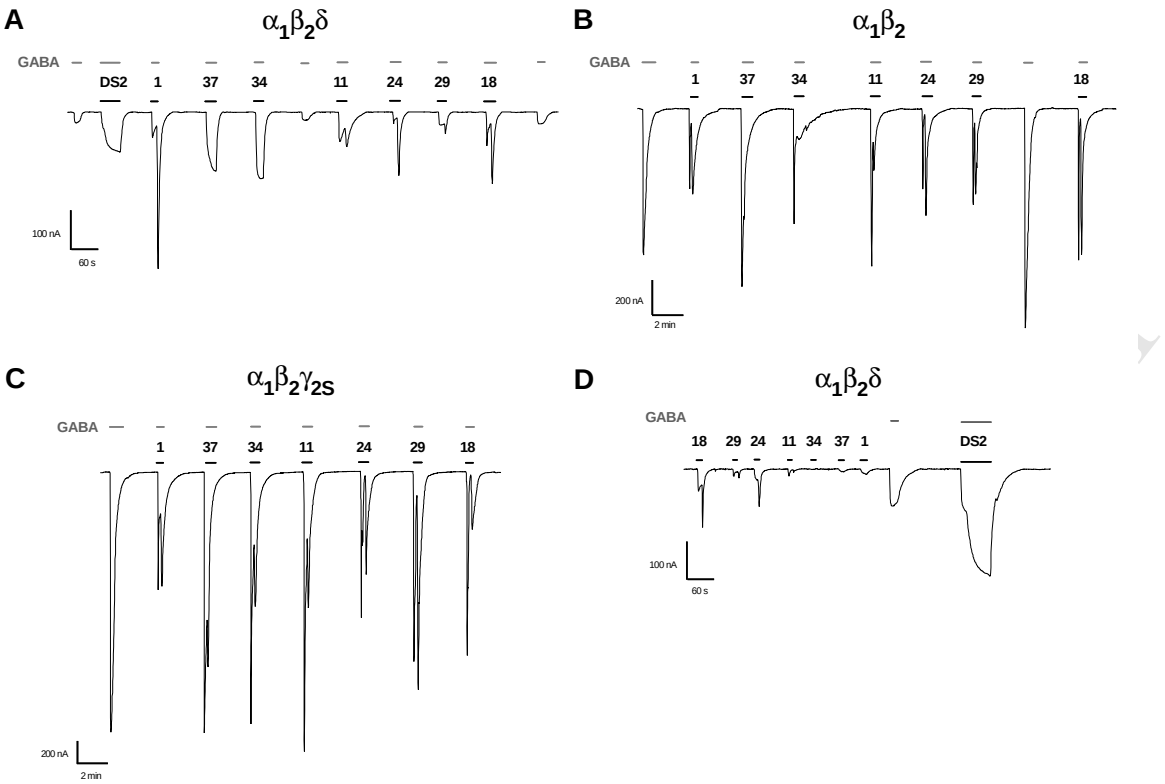

Figure 4. Pharmacological characterization of monastrol (**1**) and analogues (**11**, **18**, **24**, **29**, **34**, and **37**) at human α1β2δ (5:1:5), α1β2 (1:1) and α1β2γ2s (5:1:5) $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. Representative traces showing the effect of 1 mM of monastrol and analogues on currents evoked by 1 mM GABA at α1β2δ (**A**), α1β2 (**B**) and α1β2γ2s (**C**) $GABA_A$ receptors. Monastrol and the analogues showed no or only minimal agonistic effects in a concentration of 1 mM at α1β2δ receptors (**D**). DS2 is a positive allosteric modulator of the δ subunit-containing $GABA_A$ receptors and was applied to confirm the expression of the δ subunit. Data are from 4 to 15 oocytes from at least two different oocyte batches.

Compounds **1**, **11**, **18**, **24**, **29**, **34**, and **37** were tested at a concentration of 1 mM for their effect on the currents evoked by 1 mM GABA at the human $\alpha_1\beta_2\delta$, $\alpha_1\beta_2\gamma_2$s and $\alpha_1\beta_2$ $GABA_AR$. This concentration was previously used for monastrol (**1**) as a modulator of $\alpha_1\beta_2\delta$ $GABA_ARs$.[22] At the $\alpha_1\beta_2\delta$ receptors, all compounds seemed to increase the amplitude of the GABA-mediated current. The thiocarbonyl-containing compounds (**1**, **11**, **18**, **24**, and **29**) showed pronounced rapid rebound currents upon termination of compound application (Figure 4A), the amplitudes of which were, however, not considered feasible to permit quantification. The amplitudes of the initial responses

are given in Table 2 together with the amplitudes for compounds **34** and **37**, lacking the thiocarbonyl group and showing no tail responses.

The GABA-potentiating effects were δ-specific, as the compounds did not potentiate the GABA currents at the $\alpha_1\beta_2$ (Figure 4B) as well as at the $\alpha_1\beta_2\gamma_2$s (Figure 4C) $GABA_A$Rs. In fact, tendencies for reduced GABA responses appeared especially for monastrol (**1**). Furthermore, all compounds had no or only minimal agonistic effects on their own (Figure 4D).

Table 2. Effects of 1 mM monastrol (**1**) and 6 analogues (**11**, **18**, **24**, **29**, **34**, and **37**) on the response of 1 mM GABA at human $\alpha_1\beta_2\delta$ (5:1:5) $GABA_A$ receptors expressed in *X. laevis* oocytes. Compounds **11**, **18**, **24** and **29** gave rebound effects upon termination of compound application. The initial response for all compounds is given as fold current increase compared to the response of 1 mM GABA (mean ± S.E.M., n=6).

	Fold current increase ($I_A/I_{control}$ ± S.E.M.)
	Initial response
Monastrol, 1	2.9 ± 0.27
11	2.7 ± 0.28
18	3.1 ± 0.54
24	1.4 ± 0.22
29	1.3 ± 0.22
34	6.2 ± 0.43
37	5.9 ± 0.41

To further investigate the rebound current observed for some of the compounds, the concentration-response relationship of monastrol (**1**), which showed the most pronounced rebound trace, was examined (Figure 5).

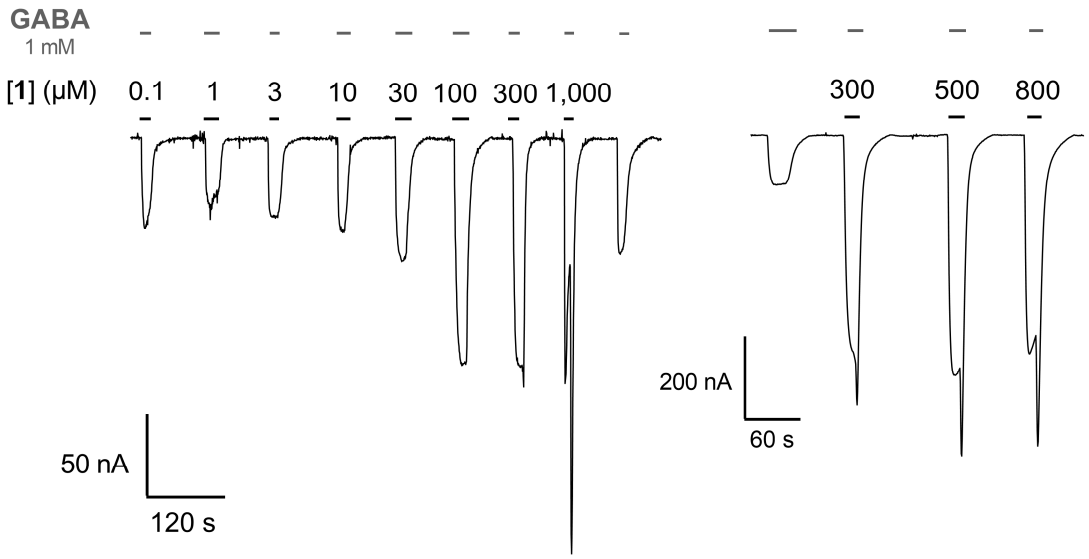

Figure 5. Representative traces showing the effects of varying concentrations of monastrol (**1**) on currents evoked by 1 mM GABA at human $\alpha_1\beta_2\delta$ (5:1:5) $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. Data are from 6 to 9 oocytes from two different oocyte batches.

At a concentration of 100 µM and below, no rebound current for monastrol (**1**) was observed, while the rebound currents were present and increased in amplitude with higher concentrations, suggesting distinct concentration-dependent receptor effects.

3. Discussion

The dihydropyrimidin-2(1*H*)-thione monastrol is a well-studied molecule, originally described as a kinesin inhibitor arresting mammalian cell mitosis.[30] Later, Lewis et al. showed that monastrol potentiated GABA currents in recombinant $\alpha_1\beta_2\delta$ $GABA_AR$ expressed in HEK293T cells to a similar extent as phenobarbital.[22] Furthermore, monastrol was found to significantly reduce [^{3}H]NCS-382 dissociation rates and induce conformational changes in the high-affinity GHB binding site, demonstrating a positive allosteric modulation of radioligand binding.[18]

Considering the dual activity of monastrol as a δ-selective $GABA_AR$ allosteric modulator[22] and a modulator of GHB high-affinity binding[18], monastrol comprises an interesting lead compound in an effort to separate the structural determinants for these modulatory effects. Furthermore, the single step Biginelli three-component reaction[23] enables efficient synthesis of a considerable number of compounds covering a systematic SAR study on the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold of monastrol.

Based on the obtained SAR for the monastrol analogues of the present study in modulating GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_A$ receptor activity, the structural determinants for effect differ for the two targets. 41 compounds were characterized for modulation of GHB high-affinity binding at rat cortical membranes in a [^{3}H]NCS-382 binding assay. For retaining modulatory effect of GHB high-affinity binding only very limited modifications were allowed on the structural scaffold of monastrol. Variation of the aromatic substitution pattern of the 4-aryl moiety in 4-aryl-3,4-dihydropyrimidin-2(1*H*)-thione was not tolerated. However, a few compounds did enhance the level of modulation of [^{3}H]NCS-382 binding. The 3,4- and 3,5-dihydroxy analogues, **11** and **12**, and compound **24** with an ethyl C6 side chain enhanced the maximal binding of [^{3}H]NCS-382 to a higher maximal level than seen for monastrol (**1**) itself. Also, the thiourea substituent was crucial for the ability to enhance [^{3}H]NCS-382 maximal binding.

A limited number of compounds (**1**, **11**, **18**, **24**, **29**, **34**, **37**) were characterized in different $GABA_AR$ subtypes expressed in *Xenopus laevis* oocytes, representing synaptic and extrasynaptic $\alpha_1\beta_2$-containing $GABA_ARs$. The tested compounds were shown to modulate the $\alpha_1\beta_2\delta$ subtype selectively over $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_{2s}$ $GABA_ARs$. In contrast to what has been reported for monastrol at $\alpha_4\beta_3\delta$ receptors expressed in *Xenopus laevis* oocytes[18], no direct agonist activity on $\alpha_1\beta_2\delta$ receptors in the absence GABA was observed for any of the tested compounds. At the $\alpha_1\beta_2\delta$ $GABA_AR$, GABA showed a low efficacy compared to that at $\alpha_1\beta_2$ and the $\alpha_1\beta_2\gamma_2$s receptors (as exemplified in Figure 4), which is in agreement with a previous TEVC study.[16]
The $\alpha_1\beta_2\delta$ selective $GABA_A$ modulation displayed by monastrol (**1**) and **37** is consistent with previous reports,[22] however, **37** seems to be a more potent modulator in our hands.

In addition to the increase in the overall current amplitude, a significant rebound current was seen for monastrol (**1**) and the thiocarbonyl containing analogues **11**, **18**, **24** and **29** at the end of the application period, which correlates with previous findings for monastrol.[22] It is difficult to explain the exact mechanism for these effects other than to suggest that the compound could bind with different affinities stabilizing distinct conducting states of the receptor. Rebound currents at $GABA_ARs$ have previously been reported for the barbiturate, pentobarbital[31], which blocks the receptors in millimolar concentrations and potentiates the GABA response at lower concentrations. Hence, the large tail observed upon termination of pentobarbital application has been suggested to reflect recovery from receptor block when the compound is washed away, which is translated into a repopulation of the conducting state of the channel.[31] Assuming a similar mechanism for monastrol

(**1**) and its analogues (**11**, **18**, **24**, **29**), the amplitude of the tail response, which reflects recovery from block, could then be used as a measure of the modulatory effect of the compounds. However, there are at least two caveats for quantifying the magnitude of these currents: 1) Since the conventional TEVC technique utilized in this study does not allow for the accurate recording of the very rapid currents exemplified in Figure 4, the amplitudes of the rebound traces shown in the figure could be underestimated. Therefore, a fast application and recording system would have been necessary. 2) Because the rebound effect is seen immediately after switching from compound application to washing buffer, the exact concentration of the compound giving rise to the modulatory effect is not known. Therefore, a direct comparison between the different compounds would not be possible. In general, the oxocarbonyl containing compounds, **34** and **37**, were shown to be more effective than the thiourea analogues, monastrol (**1**), **11** and **18**, as modulators of $\alpha_1\beta_2\delta$ $GABA_AR$ activity.

In the present study, selective modulators for each of the studied targets as well as dual active modulators were identified. The thiocarbonyl containing analogue **29** displaying a low degree of $GABA_AR$ modulation was shown to be a GHB high-affinity binding preferring modulator. Furthermore, compound **11** proved to modulate GHB high-affinity binding to a higher extent than monastrol (**1**) and was shown to be a less potent modulator at $\alpha_1\beta_2\delta$ $GABA_ARs$ than monastrol (**1**). This indicates that GHB high-affinity binding sites and at $\alpha_1\beta_2\delta$ $GABA_ARs$ are separate molecular targets. Leaving room for further development of GHB selective modulators based on the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold. A pronounced preference for the oxocarbonyl containing analogues for modulation of $\alpha_1\beta_2\delta$ $GABA_A$ receptor activity was observed, making compounds **34** and **37** selective for this specific target. Furthermore, apart from monastrol (**1**), three dual active compounds were identified, **11**, **18**, and **24** being modulators of both GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_AR$ activity.

In general, dihydropyrimidin-2(1*H*)-ones or -thiones represents a class of heterocylic compounds with significant pharmacological efficiency. They exhibit a diverse pharmacological profile like calcium channel modulation, α_{1a}-adrenoreceptor antagonism, antibacterial, antifungal and other related properties.[32] In this study, the synthesized monastrol analogues were only examined for modulation of high-affinity GHB binding and activity of specific $GABA_AR$ subtypes. However, the structural determinants reported for activity for the abovementioned targets, especially the absolute stereochemistry at the C4 stereocenter and the hetero substituent at the 2-position, seem to differ

from the pattern found for the targets of the present study. For instance, the (*S*)-monastrol has been established as the effective enantiomer of monastrol regarding kinesin inhibition.[33] As demonstrated here, both enantiomers of monastrol seem to be modulators of GHB high-affinity binding. This leaves some room for selectivity for the (*R*)-enantiomer in this context. In addition, substituting oxygen for sulfur at position 2 results in the loss of kinesin inhibition, which differ from the structural preference for $\alpha_1\beta_2\delta$ $GABA_AR$ activity.[33]

In conclusion, a series of structural diverse analogues of monastrol has been synthesized by employing the Biginelli cyclocondensation. These analogues have been pharmacologically evaluated as modulators at two distinct targets: GHB high-affinity binding sites and $\alpha_1\beta_2\delta$ $GABA_ARs$. This SAR study highlighted some of the functional groups contributing to the modulatory effect and selectivity for each target. For instance, the nature of the heteroatom in 2-position was determinant for selectivity for either of the targets as shown for compounds **34**, **37** and **24**. The dual effect shown for monastrol (**1**) to positively modulate both GHB high-affinity binding and $\alpha_1\beta_2\delta$ $GABA_AR$ activity was also shown for the compounds **11** and **18** in the present study. Thus, our study provides structural information for further development of the 4-aryl-3,4-dihydropyrimidin-2(1*H*)-thione scaffold into compounds with higher modulatory activity and selectivity towards the targets of the present study and potentially enlightening some of the unknown factors regarding binding site localization and function.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Starting material, reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise specified. Dry DMF were obtained by using a solvent purification system. Reactions involving air- and moisture sensitive reagents were performed under nitrogen atmosphere using syringe-septum cap techniques and oven dried glassware. Analytical thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} plates and visualized using UV light (254 nm), $KMnO_4$ or ninhydrin spraying reagents. LC-MS was performed using a Agilent 1100 HPLC systems with a XBridge 3.5 μm C-18 column (100 x 4.60 mm) using gradient elution from buffer A (H_2O:CH_3CN:HCOOH, 95:5:0.1) to buffer B (H_2O:CH_3CN: HCOOH, 5:95:0.05) over 8 min, coupled to an Hewlett Packard 1100 series mass spectrometer with an electrospray ionization source. Analytical HPLC (Anal. HPLC) was performed using a LaChrom HPLC system by Merck (Darmstadt, Germany), consisting of an L-7100 pump (4.0 mL/min), an L-7200 autosampler, and an L-7400 UV-detector (254 nm) using a Chromolith SpeedROD RP-18 column (50 ´ 4.6 mm); gradient elution, 0 to 100 % solvent B (MeCN-H_2O-TFA 90:10:0.1) in solvent A (H_2O-TFA 100:0.1) over 3.5 min. Data were acquired and processed using the EZChromElite Software by Hitachi. All microwave reactions were carried out in a glass reactor using a Biotage Initiator instrument. Dry column vacuum chromatography (DCVC) was performed according to Pedersen and Rosenbohm[34] using Merck silica gel 60 (0.015-0.040 mm). Melting points were determined by OptiMelt from Stanford Research Systems in open capillary tubes and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 400 MHz Bruker Avance spectrometer at room temperature, unless otherwise statet, using MeOD or DMSO as solvents. Solvent residue peak or tetramethylsilane (TMS) were used as reference signals. Chemical shifts (δ) are quoted in ppm and coupling constants in Hertz (Hz). Reactions are not optimized for yields. Anal. HPLC purity is ≥ 95 % unless otherwise stated.

4.1.1. General procedure 1, adapted from ref[25]:

The appropriate β-keto ester (0.75-3.0 mmol, 1.5 equiv.), the appropriate aldehyde (0.5-2.0 mmol, 1 equiv.), the appropriate urea or thiourea (0.5-2.0 mmol, 1 equiv.) and $Yb(OTf)_3$ (0.05-0.20 mmol, 0.10 equiv.) were placed in a microwave oven vial (0.5-2.0 mL) with a magnetic stirring bar and dissolved in THF or MeCN (0.5-1.0 mL). The resulting mixture is heated to 120 °C for 30-60 min

using microwave irradiation. The resulting mixture is poured onto an ice-water mixture and left for precipitation. The resulting crude solids were purified by recrystallization, DCVC or flash chromatography.

4.1.2. General method 2, adapted from ref[26]:

Ethyl acetoacetate (4 mmol, 1 equiv.), the appropriate aldehyde (4 mmol, 1 equiv.), and the appropriate thiourea (4 mmol, 1 equiv.) were dissolved in DMF (10 mL) in a reaction tube. TMSCl (3.0 mL, 24 mmol, 6 equiv.) was added drop wise to the solution. The tube was thoroughly sealed and stirred at room temperature for 16-72 h. The reaction mixture was poured into H_2O (50 mL). The mixture was extracted with Et_2O (3x100 mL). The combined organic phase was washed with brine (50 mL) and H_2O (2x50 mL) and dried with $MgSO_4$. The dry organic phase was evaporated to dryness to give the crude product. The crude product was purified by flash chromatography.

4.1.2.1. Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (Monastrol, 1). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (1.22 g, 10 mmol), ethyl acetoacetate (1.9 mL, 15 mmol), thiourea (0.761 g, 10 mmol), $Yb(OTf)_3$ (0.620 g, 1 mmol), MeCN (5 mL) and 1h of heating. A white solid was isolated in 75% yield (2.19 g). Purity by anal. HPLC (254 nm) > 97%, mp: 184-185 °C, LC-MS(ESP) : m/z = 293 [$M+H^+$]. The spectroscopic data is in agreement with data previously reported in literature[25].

4.1.2.2. Ethyl 6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (2). Synthesized according to general method 1 using benzaldehyde (102 µL, 1.0 mmol), ethyl acetoacetate (191 µL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), $Yb(OTf)_3$ (62 mg, 0.1 mmol), MeCN (1.0 mL) and 45 min of heating. A white solid was isolated in 73% yield (197 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 204-206 °C, LC-MS(ESP) : m/z = 277 [$M+H^+$]. The spectroscopic data is in agreement with data previously reported in literature[35].

4.1.2.3. Ethyl 6-methyl-2-thioxo-4-(m-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (3). Synthesized according to general method 1 using 3-methylbenzaldehyde (60 mg, 0.5 mmol), ethyl acetoacetate (96 µL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A white solid was isolated in 49% yield (71 mg). Purity by

anal. HPLC (254 nm) > 99%, mp: 186-187 °C, LC-MS(ESP): m/z = 291 [M+H$^+$]. The spectroscopic data is in agreement with data previously reported in literature[36].

4.1.2.4. Ethyl 6-methyl-2-thioxo-4-(p-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carb4xylate (4). Synthesized according to general method 1 using 4-methylbenzaldehyde (120 μL, 1.0 mmol), ethyl acetoacetate (191 μL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), Yb(OTf)$_3$ (62 mg, 0.1 mmol), MeCN (1.0 mL) and 35 min of heating. A yellow solid was isolated in 93% yield (285 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 187-189 °C, LC-MS(ESP): m/z = 291 [M+H$^+$]. The spectroscopic data is in agreement with data previously reported in literature[36]

4.1.2.5. Ethyl 6-methyl-2-thioxo-4-(o-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (5). Synthesized according to general method 1 using 2-methylbenzaldehyde (130 μL, 1.05 mmol), ethyl acetoacetate (201 μL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), Yb(OTf)$_3$ (65 mg, 0.1 mmol), THF (0.5 mL) and 1h of heating. Recrystallized from EtOH to obtain a white solid in 73% yield (223 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 149-150 °C, LC-MS(ESP): m/z = 291 [M+H$^+$]. ^{1}H NMR (DMSO) δ 10.24 (s, 1H), 9.54 (br. s., 1H), 7.13 - 7.23 (m, 4H), 5.41 (s, 1H), 3.93 (q, *J* = 7.03 Hz, 2H), 2.46 (s, 3H), 2.34 (s, 3H), 1.02 (t, *J* = 7.15 Hz, 3H). ^{13}C NMR (DMSO) δ 173.4, 165.0, 144.8, 142.2, 135.0, 130.1, 127.6, 127.1, 126.6, 100.9, 59.4, 50.6, 18.6, 17.0, 13.9.

4.1.2.6. Ethyl 6-methyl-2-thioxo-4-(o-tolyl)-1,2,3,4-tetrahydropyrimidine-5-carboxylate (6). Synthesized according to general method 1 using 3-bromobenzaldehyde (123 μL, 1.05 mmol), ethyl acetoacetate (201 μL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), Yb(OTf)$_3$ (65 mg, 0.1 mmol), THF (1.0 mL) and 1h of heating. The crude product was recrystallized from EtOH to obtain a white solid in 64% yield (237 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 190-191 °C, LC-MS(ESP): m/z = 355. The spectroscopic data is in agreement with data previously reported in literature[37].

4.1.2.7. Ethyl 4-(3-fluorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (7). Synthesized according to general method 1 using 3-flurobenzaldehyde (113 μL, 1.05 mmol), ethyl acetoacetate (201 μL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), Yb(OTf)$_3$ (65 mg, 0.1 mmol), THF (1.0 mL) and 1h of heating. The crude product was recrystallized from EtOH to obtain a white solid in 67% yield (207 mg). Purity by anal. HPLC (254 nm) > 96%, mp: 221-223 °C, LC-MS(ESP) : m/z = 295 [M+H$^+$]. ^{1}H NMR (DMSO) δ 10.39 (s, 1H), 9.68 (br. s., 1H), 7.41

(dt, *J* = 6.15, 7.97 Hz, 1H), 6.90 - 7.21 (m, 3H), 5.20 (d, *J* = 3.51 Hz, 1H), 4.03 (ttd, *J* = 3.76, 7.20, 10.70 Hz, 2H), 2.30 (s, 3H), 1.11 (t, *J* = 7.03 Hz, 3H). ^{13}C NMR (DMSO) δ 174.3, 164.9, 130.7, 130.6, 122.3, 114.5, 113.1, 112.9, 100.1, 59.6, 57.6, 17.1, 13.9. ^{19}F NMR (DMSO) δ 108.08 (s, 1 F).

4.1.2.8. Ethyl 4-(3-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (8). Synthesized according to general method 1 using 3-chlorobenzaldehyde (70, 0.5 mmol), ethyl acetoacetate (96 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A white solid was isolated in 51% yield (81 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 202-204 °C, LC-MS(ESP): m/z = 311 [M+H$^+$]. The spectroscopic data is in agreement with data previously reported in literature[38].

4.1.2.9. Ethyl 6-methyl-4-(3-nitrophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (9). Synthesized according to general method 1 using 3-nitrobenzaldehyde (76 mg, 0.5 mmol), ethyl acetoacetate (96 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The product was isolated as a white solid in 50% yield (81 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 209-210 °C, LC-MS(ESP): m/z = 322 [M+H]$^+$. The spectroscopic data is in agreement with data previously reported in literature[39].

4.1.2.10. Ethyl 4-(4-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (10). Synthesized according to general method 1 using 4-hydroxybenzaldehyde (128 mg, 1.0 mmol), ethyl acetoacetate (191 μL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), $Yb(OTf)_3$ (62 mg, 0.1 mmol), THF (1.0 mL) and 45 min of heating. A white solid was isolated in 94% yield (214 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 196-198 °C, LC-MS(ESP): m/z = 293 [M+H$^+$]. The spectroscopic data is in agreement with data previously reported in literature[40].

4.1.2.11. Ethyl 4-(3,4-dihydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (11). Synthesized according to general method 1 using 3,4-dihydroxybenzaldehyde (552 mg, 4 mmol), ethyl acetoacetate (764 μL, 6 mmol), thiourea (304 mg, 4 mmol), $Yb(OTf)_3$ (248 mg, 0.4 mmol), MeCN (2.0 mL) and 30 min of heating. The crude product was purified by DCVC (heptane:EtOAc) and the product was isolated in 12% yield (144 mg) as colourless crystals.

Purity by anal. HPLC (254 nm) > 99%, mp: 212-213 °C, LC-MS(ESP): m/z = 309 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[41].

4.1.2.12. Ethyl 4-(3,5-dihydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (12). Synthesized according to general method 1 using 3,5-dihydroxybenzaldehyde (552 mg, 4.0 mmol), ethyl acetoacetate (765 µL, 6.0 mmol), thiourea (304 mg, 4.0 mmol), $Yb(OTf)_3$ (248 mg, 0.4 mmol), MeCN (2.0 mL) and 1h of heating. Purified by flash chromatography (heptane:EtOAc, 2:1). The product was obtained as white crystals though recrystelization of the purified compound yielding 12 mg (1%) of pure product. Purity by anal. HPLC (254 nm) > 99%, mp: 144-145 °C, LC-MS(ESP): m/z = 309 $[M+H]^+$. ^{1}H NMR (MeOD): δ 6.25 (d, *J* = 2.2 Hz, 2H), 6.17 (t, *J* = 2.2 Hz, 1H), 5.17 (s, 1H), 4.11 (q, *J* = 7.1 Hz, 2H), 2.33 (s, 3H), 1.20 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO): δ 174.7, 166.0, 158.4, 145.4, 144.1, 104.8, 101.80, 101.66, 59.9, 55.0, 16.2, 13.1

4.1.2.13. Ethyl 6-methyl-4-(pyridin-3-yl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (13). Synthesized according to general method 1 using nicotinaldehyde (214 mg, 2.0 mmol), ethyl acetoacetate (382 µL, 3.0 mmol), thiourea (152 mg, 2.0 mmol), $Yb(OTf)_3$ (124 mg, 0.2 mmol), MeCN (1.0 mL) and 30 min of heating. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a yellow solid in 15% yield (36 mg). Purity by anal. HPLC (254 nm) > 95%, mp: 204-205 °C, LC-MS(ESP): m/z = 278 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[40].

4.1.2.14. Ethyl 4-(3-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (14). Synthesized according to general method 1 using 3-methoxybenzaldehyde (100 µL, 0.8 mmol), ethyl acetoacetate (153 µL, 1.2 mmol), thiourea (62 mg, 0.8 mmol), $Yb(OTf)_3$ (48 mg, 0.08 mmol), MeCN (1.0 mL) and 45 min of heating. The crude product was recrystallized from EtOH and H_2O to obtain a yellow solid in 83% yield (209 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 143-145 °C, LC-MS(ESP) : m/z = 307 $[M+H^+]$. The spectroscopic data is in agreement with data previously reported in literature[42].

4.1.2.15. Ethyl 6-methyl-4-(1-methyl-1*H*-pyrazol-5-yl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (15). Synthesized according to general method 1 using 1-methyl-1*H*-pyrazole-5-carbaldehyde (55 mg, 0.5 mmol), ethyl acetoacetate (96 µL, 0.75 mmol), thiourea (38 mg, 0.5

mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The product was isolated as a white solid in 29% yield (41 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 185-187 °C, LC-MS(ESP): m/z = 281 $[M+H]^+$. ^{1}H NMR (DMSO): δ 10.42 (s, 1H), 9.66 (s, 1H), 7.28 (d, *J* = 1.7 Hz, 1H), 6.00 (d, *J* = 1.9 Hz, 1H), 5.34 (d, *J* = 3.3 Hz, 1H), 3.99 (q, *J* = 7.1 Hz, 2H), 3.87 (s, 3H), 2.31 (s, 3H), 1.07 (t, *J* = 7.1 Hz, 3H). ^{13}C-NMR (DMSO): δ 174.6, 165.2, 145.8, 144.3, 138.0, 104.5, 99.9, 60.1, 45.9, 36.8, 17.5, 14.5.

4.1.2.16. Ethyl 6-methyl-4-(1-phenyl-1*H*-1,2,3-triazol-4-yl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (16). Synthesized according to general method 1 using 1-phenyl-1*H*-1,2,3-triazole-4-carbaldehyde (87 mg, 0.5 mmol), ethyl acetoacetate (96 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The product was isolated as a white solid in 65% yield (112 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 223-224 °C, LC-MS(ESP): m/z = 344 $[M+H]^+$. ^{1}H NMR (DMSO): δ 10.39 (d, *J* = 1.5 Hz, 1H), 9.65 (dd, *J* = 3.8, 1.8 Hz, 1H), 8.60 (s, 1H), 7.90 (q, *J* = 1.5 Hz, 1H), 7.88 (dt, *J* = 1.5, 0.9 Hz, 1H), 7.61-7.56 (m, 2H), 7.50-7.46 (m, 1H), 5.42 (d, *J* = 3.9 Hz, 1H), 4.04 (qd, *J* = 7.1, 0.9 Hz, 2H), 2.32 (s, 3H), 1.12 (t, *J* = 7.1 Hz, 3H). ^{13}C-NMR (DMSO): δ 175.3, 165.3, 150.3, 146.6, 137.0, 130.3, 129.1, 120.6, 120.5, 99.4, 60.0, 47.0, 17.7, 14.6.

4.1.2.17. Ethyl 4,6-dimethyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (17). Synthesized according to general method 1 using acetoaldehyde (59 μL, 1.05 mmol), ethyl acetoacetate (201 μL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), $Yb(OTf)_3$ (65 mg, 0.1 mmol), THF (0.5 mL) and 1h of heating. A yellow solid was isolated in 97% yield (219 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 189-191 °C, LC-MS(ESP): m/z = 215 $[M+H^+]$. The spectroscopic data is in agreement with data previously reported in literature[43].

4.1.2.18. Ethyl 4-(3-hydroxyphenyl)-1,6-dimethyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (18). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1-methylthiourea (361 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 16h of stirring. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a white solid in 75% yield (916 mg). Purity by anal. HPLC (254 nm) > 95%, mp: 135-136 °C, LC-MS(ESP): m/z = 307. ^{1}H NMR (DMSO): δ 9.78 (d, *J* = 4.7 Hz, 1H), 9.43 (s, 1H), 7.11 (t, *J* = 8.0 Hz, 1H), 6.66-6.61 (m, 3H), 5.13 (d, *J* = 4.7 Hz,

1H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.48 (s, 3H), 1.18 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO): δ 178.5, 165.8, 157.9, 148.0, 144.1, 130.0, 116.9, 115.0, 113.4, 106.1, 60.6, 52.6, 36.7, 16.7, 14.5.

4.1.2.19. Ethyl 1-ethyl-4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (19). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1-ethylthiourea (417 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 16h of stirring. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a white solid in 74% yield (949 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 149-150 °C, LC-MS(ESP): m/z = 321 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[44].

4.1.2.20. Ethyl 1-butyl-4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (20). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1-butylthiourea (529 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 72h of stirring. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a white solid in 69% yield (955 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 141-142 °C, LC-MS(ESP): m/z = 349 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[44].

4.1.2.21. Ethyl 4-(3-hydroxyphenyl)-6-methyl-1-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (21). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1-phenylthiourea (609 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 16h of stirring. Purified by flash chromatography (heptane:EtOAc, 3:1) to obtain the pure product as a white solid in 74% yield (1097 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 66-68 °C, LC-MS(ESP): m/z = 369 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[44].

4.1.2.22. Ethyl 1-benzyl-4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (22). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1-benzylthiourea (665 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 16h of stirring. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a white solid in 70% yield (1072 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 159-160 °C, LC-MS(ESP): m/z = 383

$[M+H]^+$. ^{1}H NMR (DMSO): δ 10.03 (d, *J* = 4.6 Hz, 1H), 9.48 (s, 1H), 7.20 (t, *J* = 3.2 Hz, 3H), 7.15-7.11 (m, 1H), 6.99-6.97 (m, 2H), 6.70 (ddd, *J* = 8.0, 2.2, 1.1 Hz, 1H), 6.66-6.63 (m, 2H), 6.07 (s, 1H), 5.20 (d, *J* = 4.5 Hz, 1H), 5.15 (s, 1H), 4.09 (q, *J* = 6.7 Hz, 2H), 2.35 (s, 3H), 1.15 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO): δ 178.9, 165.8, 157.9, 146.9, 144.2, 138.2, 129.9, 128.8, 127.3, 126.6, 117.0, 115.1, 113.6, 107.5, 60.7, 58.2, 52.4, 51.0, 16.7, 14.5

4.1.2.23. Ethyl 4-(3-hydroxyphenyl)-1,3,6-trimethyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (23). Synthesized according to general method 2 using 3-hydroxybenzaldehyde (488 mg, 4 mmol), ethyl acetoacetate (524 μL, 4 mmol), 1,3-dimethylthiourea (417 mg, 4 mmol), TMSCl (3 mL, 24 mmol), DMF (10 mL) and 16h of stirring. Purified by flash chromatography (heptane:EtOAc, 2:1) and recrystallization to obtain the pure product as a white crystals in 5% yield (63 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 127-131 °C, LC-MS(ESP): m/z = 321 $[M+H]^+$. ^{1}H NMR (DMSO): δ 9.46 (s, 1H), 7.13-7.09 (m, 1H), 6.66 (ddd, *J* = 8.1, 2.4, 1.1 Hz, 1H), 6.58-6.56 (m, 2H), 5.49 (s, 1H), 4.19-4.10 (m, 2H), 3.50 (s, 3H), 3.34 (s, 3H), 2.47 (d, *J* = 0.4 Hz, 3H), 1.22 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO): δ 178.7, 165.0, 157.6, 147.2, 141.1, 129.7, 116.4, 114.9, 112.8, 105.0, 60.4, 60.1, 42.4, 37.7, 16.3, 14.1.

4.1.2.24. Ethyl 6-ethyl-4-(3-hydroxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (24). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), ethyl 3-oxopentanoate (107 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A white solid was isolated in 37% yield (57 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 160-161 °C, LC-MS(ESP): m/z = 307 $[M+H]^+$. ^{1}H NMR (MeOD): δ 7.15 (t, *J* = 7.8 Hz, 1H), 6.80-6.70 (m, 3H), 5.27 (s, 1H), 4.12 (q, *J* = 6.3 Hz, 2H), 2.85-2.70 (m, 2H), 1.24 (t, *J* = 5.7 Hz, 3H), 1.21 (t, *J* = 5.4 Hz, 3H).
13-C NMR (DMSO): δ 174.4, 164.8, 157.4, 150.3, 144.8, 129.4, 116.9, 114.6, 113.2, 99.9, 59.6, 53.8, 23.4, 13.9, 13.0

4.1.2.25. Ethyl 4-(3-hydroxyphenyl)-6-propyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (25). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), ethyl 3-oxohexanoate (120 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A white solid was isolated in 69% yield (111 mg). Purity by anal. HPLC (254 nm) > 96%, mp: 149-151 °C, LC-MS(ESP): m/z = 321 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[44].

4.1.2.26. Ethyl 4-(3-hydroxyphenyl)-6-isopropyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (26). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), ethyl 4-methyl-3-oxopentanoate (121 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The crude product was recrystallized from MeOH and H_2O to obtain the product as white crystals in 43% yield (68 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 117-119 °C, LC-MS(ESP): m/z = 321 $[M+H]^+$. ^{1}H NMR (DMSO): δ 9.72 (s, 1H), 9.57 (d, *J* = 2.4 Hz, 1H), 9.44 (s, 1H), 7.12 (t, *J* = 7.8 Hz, 1H), 6.66-6.63 (m, 3H), 5.08 (d, *J* = 3.9 Hz, 1H), 4.09 (m, 1H), 4.01 (q, *J* = 7.0 Hz, 2H), 1.20 (d, *J* = 7.1 Hz, 3H), 1.13 (d, *J* = 7.1 Hz, 3H), 1.10 (t, *J* = 7.1 Hz, 3H). ^{13}C-NMR (DMSO): δ 175.2, 165.6, 157.9, 153.1, 145.0, 130.0, 117.4, 115.1, 113.7, 100.4, 60.2, 54.4, 27.2, 19.5, 19.0, 14.4.

4.1.2.27. Ethyl 6-(*tert*-butyl)-4-(3-hydroxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (27). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), ethyl 4,4-dimethyl-3-oxopentanoate (130 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A yellow solid was isolated in 11% yield (19 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 155-157 °C, LC-MS(ESP): m/z = 335 $[M+H]^+$. ^{1}H NMR ($CDCl_3$): δ 9.42 (s, 1H), 9.31 (s, 1H), 8.48 (s, 1H), 7.12 (t, *J* = 7.7 Hz, 1H), 6.72-6.61 (m, 3H), 4.90 (d, *J* = 3.2 Hz, 1H), 4.08-3.99 (m, 2H), 1.28 (s, 9H), 1.12 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (DMSO): δ 174.5, 167.4, 157.4, 145.5, 143.3, 129.4, 117.1, 114.8, 113.4, 103.7, 60.4, 55.9, 35.2, 28.0, 13.7.

4.1.2.28. Ethyl 4-(3-hydroxyphenyl)-6-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (28). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), ethyl 3-oxo-3-phenylpropanoate (130 μL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. A white solid was isolated in 56% yield (99 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 196-197 °C, LC-MS(ESP): m/z = 355 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[35].

4.1.2.29. Methyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (29). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (128 mg, 1.05 mmol), methyl acetoacetate (170 μL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), $Yb(OTf)_3$

(65 mg, 0.1 mmol), THF (1.0 mL) and 30 min of heating. A white solid was isolated in 70% yield (203 mg). Purity by anal. HPLC (254 nm) > 98%, mp: 217-218 °C, LC-MS(ESP) : m/z = 279 [M+H$^+$]. The spectroscopic data is in agreement with data previously reported in literature[45].

4.1.2.30. 4-(3-Hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (30). KOH (0.10 g, 1.8 mmol) was dissolved in H_2O (5 mL). Monastrol (180 mg, 0.62 mmol) was added and the mixture was stirred for 72 h at room temperature. The mixture was acidified with 1M HCl. The water was extracted with EtOAc. The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness. The crude white product was washed with Et_2O to obtain the product in 22% yield (35 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 163-165 °C, LC-MS(ESP) : m/z = 264. The spectroscopic data is in agreement with data previously reported in literature[41].

4.1.2.31. Benzyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (31). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (128 mg, 1.05 mmol), benzyl acetoacetate (273 µL, 1.6 mmol), thiourea (80 mg, 1.05 mmol), $Yb(OTf)_3$ (65 mg, 0.1 mmol), MeCN (1.0 mL) and 30 min of heating. The oily crude product was purified by DCVC (EtOAc:Heptane). Re-crystallised from EtOH to obtain pure white crystals in 28% yield (191 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 168-169 °C, LC-MS(ESP) : m/z = 355 [M+H$^+$]. ^{1}H NMR (DMSO) δ 10.41 (s, 1H), 9.69 (d, *J* = 2.01 Hz, 1H), 9.50 (s, 1H), 7.34 - 7.39 (m, 3H), 7.24 (dd, *J* = 2.64, 6.90 Hz, 2H), 7.18 (t, *J* = 7.65 Hz, 1H), 6.69 - 6.76 (m, 3H), 5.20 (d, *J* = 3.76 Hz, 1H), 5.09 - 5.19 (m, 2H), 2.38 (s, 3H). ^{13}C NMR (DMSO) δ 174.0, 164.9, 157.5, 145.6, 144.6, 136.3, 129.5, 128.3, 127.7, 127.5, 117.1, 114.7, 113.3, 100.2, 65.1, 53.9, 17.2.

4.1.2.32. *N*,*N*-diethyl-4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide (32). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), *N*,*N*-diethyl-3-oxobutanamide (118 µL, 0.75 mmol), thiourea (38 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The product was isolated as a white solid in 38% yield (60 mg). Purity by anal. HPLC (254 nm) > 98%, mp: decomp. > 250 °C, LC-MS(ESP): m/z = 320 [M+H]$^+$. The spectroscopic data is in agreement with data previously reported in literature[46].

4.1.2.33. 1-(4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethanone (33). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (122 mg, 1.0 mmol), pentane-2,4-dione (103 µL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), $Yb(OTf)_3$ (62 mg, 0.1 mmol), MeCN (0.5 mL) and 30 min of heating. The product was isolated as a white solid in 48% yield (127 mg). Purity by anal. HPLC (254 nm) > 95%, decomp > 250°C, LC-MS(ESP): m/z = 263 $[M+H]^+$. The spectroscopic data is in agreement with data previously reported in literature[47].

4.1.2.34. Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (34). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 µL, 0.5 mmol), ethyl acetoacetate (96 µL, 0.75 mmol), urea (30 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), THF (1.0 mL) and 40 min of heating. A white solid was isolated in 78% yield (107 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 184-185 °C, LC-MS(ESP) : m/z = 277 $[M+H^+]$. The spectroscopic data is in agreement with data previously reported in literature[47].

4.1.2.35. Methyl 4-(3-hydroxyphenyl)-1,6-dimethyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (35). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (61 mg, 0.5 mmol), methyl 3-oxobutanoate (91 µL, 0.75 mmol), 1-methylthiourea (45 mg, 0.5 mmol), $Yb(OTf)_3$ (31 mg, 0.05 mmol), MeCN (0.5 mL) and 30 min of heating. The crude product was recrystallized from MeOH and H_2O to obtain the product as a yellow solid in 15% yield (22 mg). Purity by anal. HPLC (254 nm) > 97%, mp: 213-214 °C, LC-MS(ESP): m/z = 293 $[M+H]^+$. ^{1}H NMR (DMSO): δ 9.80 (d, *J* = 4.7 Hz, 1H), 9.42 (s, 1H), 7.10 (t, *J* = 8.0 Hz, 1H), 6.66-6.63 (m, 1H), 6.62-6.60 (m, 2H), 5.13 (d, *J* = 4.8 Hz, 1H), 3.65 (s, 3H), 3.47 (s, 3H). ^{13}C NMR (DMSO): δ 178.0, 165.8, 157.5, 147.9, 143.4, 129.5, 116.4, 114.6, 112.8, 105.2, 52.0, 51.5, 36.2, 16.3.

4.1.2.36. Ethyl 6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (36). Synthesized according to general method 1 using benzaldehyde (107 µL, 1.05 mmol), methyl acetoacetate (170 µL, 1.6 mmol), urea (63 mg, 1.05 mmol), $Yb(OTf)_3$ (65 mg, 0.1 mmol), THF (0.5 mL) and 45 min of heating. The crude product was recrystallized from EtOH and H_2O to obtain a yellow solid in 78% yield (214 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 205-206 °C, LC-MS(ESP): m/z = 261 $[M+H]^+$. ^{1}H NMR (DMSO) δ 9.17 (s, 6H), 7.71 (br. s., 6H), 7.29 - 7.35 (m, 12H), 7.21 - 7.26 (m, 18H), 5.15 (d, J = 3.39 Hz, 1H), 3.98 (q, J = 7.11 Hz, 2H), 2.25 (s, 3H), 1.09 (t, J = 7.09 Hz, 3H). ^{13}C NMR (DMSO) δ 165.3, 152.1, 148.3, 144.8, 128.4, 127.2, 126.2, 99.2,

59.1, 53.9, 17.7, 14.0. The spectroscopic data is in agreement with data previously reported in literature[48].

4.1.2.37. Methyl-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate (37). Synthesized according to general method 1 using benzaldehyde (107 μL, 1.05 mmol), methyl acetoacetate (170 μL, 1.6 mmol), urea (63 mg, 1.05 mmol), $Yb(OTf)_3$ (65 mg, 0.1 mmol), THF (0.5 mL) and 45 min of heating. A white solid was isolated in 74% yield (190 mg). Purity by anal. HPLC (254 nm) >99%, mp: 210-211 °C, LC-MS(ESP): m/z = 247 $[M+H^+]$. ^{1}H NMR (DMSO) δ 9.19 (s, 1H), 7.73 (s, 1H), 7.21 - 7.35 (m, 5H), 5.14 (d, *J* = 3.51 Hz, 1H), 3.53 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (DMSO) δ 165.8, 152.1, 148.6, 144.6, 128.4, 127.2, 126.1, 99.0, 67.0, 53.8, 50.7, 25.1, 17.8. The spectroscopic data is in agreement with data previously reported in literature[49].

4.1.2.38. 1-(6-ethyl-4-(3-hydroxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)propan-1-one (38). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (122 mg, 1.0 mmol), heptane-3,5-dione (203 μL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), $Yb(OTf)_3$ (62 mg, 0.1 mmol), MeCN (0.5 mL) and 30 min of heating. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a yellow solid in 87% yield (253 mg). Purity by anal. HPLC (254 nm) > 95%, mp: 187-189 °C, LC-MS(ESP): m/z = 291 $[M+H]^+$. ^{1}H NMR (DMSO): δ 10.18 (d, *J* = 0.9 Hz, 1H), 9.63 (dd, *J* = 3.4, 1.3 Hz, 1H), 9.47 (s, 1H), 7.14 (t, *J* = 7.7 Hz, 1H), 6.69-6.65 (m, 3H), 5.22 (d, *J* = 3.9 Hz, 1H), 2.70-2.53 (m, 3H), 2.14 (dq, *J* = 17.7, 7.1 Hz, 1H), 1.14 (t, *J* = 7.3 Hz, 3H), 0.82 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (DMSO): δ 197.7, 173.9, 157.6, 148.8, 144.1, 129.7, 117.3, 114.9, 113.5, 108.2, 53.9, 33.2, 23.7, 13.1, 8.0.

4.1.2.39. Methyl 4-(3-hydroxyphenyl)-6-(methoxymethyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (39). Synthesized according to general method 1 using 3-hydroxybenzaldehyde (122 mg, 1.0 mmol), methyl 4-methoxy-3-oxobutanoate (194 μL, 1.5 mmol), thiourea (76 mg, 1.0 mmol), $Yb(OTf)_3$ (62 mg, 0.1 mmol), MeCN (0.5 mL) and 4h of heating. Purified by flash chromatography (heptane:EtOAc, 2:1) to obtain the pure product as a yellow solid in 39% yield (121 mg). Purity by anal. HPLC (254 nm) > 99%, mp: 165-167 °C, LC-MS(ESP): m/z = 309 $[M+H]^+$. ^{1}H NMR (DMSO): δ 9.72 (dd, *J* = 3.6, 1.8 Hz, 1H), 9.58 (d, *J* = 1.6 Hz, 1H), 9.46 (s, 1H), 7.15-7.11 (m, 1H), 6.68-6.64 (m, 3H), 5.13 (d, *J* = 3.8 Hz, 1H), 4.60 (d, *J* = 12.9 Hz,

1H), 4.40 (d, *J* = 12.9 Hz, 1H), 3.60 (s, 3H), 3.32 (s, 3H). ^{13}C NMR (DMSO): δ 174.1, 165.0, 157.5, 144.0, 143.4, 129.6, 116.8, 114.8, 113.1, 101.8, 66.7, 58.1, 53.9, 51.4.

4.1.2.40. Ethyl 4-(3-((*tert*-butyldimethylsilyl)oxy)phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (40). Monastrol (1.46 g, 5 mmol), TBDMSCl (1.51 g, 10 mmol) and imidazole (1.02 g, 15 mmol) was dissolved in dry DMF (5 mL) under N_2 and stirred over night. The reaction mixture was diluted with MeOH (10 mL) and stirred for 30 min and thereafter concentrated under vacuum. The residue was dissolved in EtOAc (100 mL) and the organic phase was washed with water (3 x 30 mL), dried with $MgSO_4$, filtered and evaporated. The crude product was purified by DCVC (heptane:EtOAc) to give the product as a white solid on 80% yield (1.63 g). Purity by anal. HPLC (254 nm) > 97%, LC-MS(ESP): m/z = 407 $[M+H]^+$. ^{1}H NMR (DMSO): δ 10.34 (d, *J* = 0.8 Hz, 1H), 9.63 (dd, *J* = 3.5, 1.5 Hz, 1H), 7.22 (t, *J* = 7.8 Hz, 1H), 6.82 (d, *J* = 7.8 Hz, 1H), 6.76-6.71 (m, 2H), 5.12 (d, *J* = 3.8 Hz, 1H), 4.02 (q, *J* = 7.1 Hz, 2H), 2.27 (s, 3H), 1.11 (t, *J* = 7.1 Hz, 3H), 0.93 (s, 9H), 0.17 (s, 6H). ^{13}C NMR (DMSO): δ 174.4, 165.1, 155.2, 144.97, 144.94, 129.7, 119.2, 119.0, 117.6, 100.7, 59.6, 53.6, 17.9, 17.1, 14.0, -4.5.

4.1.2.41. Ethyl 3-(3-hydroxyphenyl)-1-thioxo-1,2,3,5,6,7-hexahydropyrrolo[1,2-*c*]pyrimidine-4-carboxylate (41). TBDMS protected monastrol (40) (0.500 g, 1.23 mmol) was dissolved in dry THF (12.5 mL) under N_2 and cooled to -10°C in an ice-salt bath. n-BuLi (2.7 mL, 1.6 M solution in hexanes, 4.3 mmol) was added dropwise at this temperature. The reaction mixture was allowed to heat to room temperature and stirred for 3h. The mixture was then cooled to -10°C and dibromoethane (318 µL, 3.69 mmol) in dry THF (2.5 mL) was added dropwise at this temperature. The reaction mixture was stirred over night at room temperature. Sat. NH_4Cl (25 mL) was added to the reaction mixture and the mixture was extracted with EtOAc (3 x 25 mL). The combined organic phases were washed with brine (25 mL) and water (2 x 25 mL), dryed over $MgSO_4$ and evaporated to dryness. The residue was purified by flash chromatography (heptane:EtOAc, 4:1) to give the product as a colorless oil (44 mg, 8%). Purity by anal. HPLC (254 nm) > 85%, LC-MS(ESP): m/z = 433 $[M+H]^+$. ^{1}H NMR (MeOD): δ 7.18 (t, *J* = 7.9 Hz, 1H), 6.91 (dt, *J* = 7.7, 0.5 Hz, 1H), 6.79 (t, *J* = 2.1 Hz, 1H), 6.74 (ddd, *J* = 8.1, 2.4, 0.9 Hz, 1H), 5.27 (s, 1H), 4.16-4.10 (m, 2H), 4.06-4.01 (m, 2H), 3.40-3.34 (m, 1H), 3.15-3.08 (m, 1H), 2.14-2.06 (m, 1H), 2.01-1.94 (m, 1H), 1.22 (t, *J* = 7.1 Hz, 3H), 0.97 (s, 9H), 0.19 (s, 6H). Used without further purification. This compound (40 mg, 0.1 mmol) was dissolved in 0.5 M MeONA in MeOH (2 mL) and stirred at room temperature over

night. The reaction mixture was evaporated to dryness and purified by flash chromatography (heptane:EtOAc, 4:1) to give the product as an oily solid (8 mg, 28%). Purity by anal. HPLC (254 nm) > 98%, LC-MS(ESP): m/z = 319 $[M+H]^+$. ^{1}H NMR (MeOD): δ 7.14 (t, *J* = 7.8 Hz, 1H), 6.78 (d, *J* = 7.9 Hz, 1H), 6.75 (t, *J* = 2.0 Hz, 1H), 6.70 (ddd, *J* = 8.1, 2.4, 0.7 Hz, 1H), 5.26 (s, 1H), 4.14 (qd, *J* = 7.1, 1.8 Hz, 2H), 4.05 (ddd, *J* = 8.5, 5.8, 2.7 Hz, 2H), 3.39 (ddd, *J* = 18.3, 8.5, 4.3 Hz, 1H), 3.17-3.08 (m, 1H), 2.13-2.02 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (MeOD): δ 176.6, 167.2, 158.8, 151.8, 146.2, 130.6, 118.8, 115.8, 114.4, 101.4, 61.4, 56.0, 53.1, 33.2, 22.1, 14.6.

4.2. Pharmacology

4.2.1. NCS-382 binding assay

Preparation of rat cortical membranes and the [^{3}H]NCS-382 binding assay was conducted exactly as described previously.[18] In brief, membrane homogenate (protein concentration of 50-70 µg per well) was incubated with increasing concentrations of monastrol analogue together with 16 nM [^{3}H]NCS-382 for 1 hr at 0-4 °C, followed by rapid filtration through GF/C filter plates using a 96-well harvester (PerkinElmer). The filter plates were dried, added scintillation liquid and CPM values determined using a TopCount NXT Microplate Scintillation counter (PerkinElmer).

4.2.2. Molecular biology

DNA constructs encoding the human $GABA_A$ receptor subunits α_1, β_2 and γ_{2S} were in the pcDNA3 vector. The human δ subunit, originally in the pcDNA1 vector, was subcloned into the pUNIV vector (Addgene, Cambridge, MA, USA) reported to enable high oocyte expression levels.[50]
To do so, a forward primer 5'-CGCGC-
TCGAGGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACCATGAAGAA-
AAGTCCGG-3', containing the alfalfa mosaic virus (AMV) sequence and a XhoI restriction site and a reverse primer 5'-CGCGA-CGCGTTCACATGGCGTATGCCGCCC-3', containing a MluI restriction site, were used to amplify δ cDNA in a standard polymerase chain reaction. The sequence of the cDNA and the absence of mutations was confirmed by full cDNA sequencing (GATC Biotech AB, Konstanz, Germany).

4.2.2.1. Xenopus laevis oocytes and two-electrode voltage clamp electrophysiology

For expression in *Xenopus laevis* oocytes, defolliculated stage V–VI oocytes (EcoCyte Bioscience, Germany) were co-injected with cRNA constructs encoding the $GABA_A$ receptor subunits in the

following combinations and ratios: $\alpha_1\beta_2$ (1:1), $\alpha_1\beta_2\delta$ (5:1:5) and $\alpha_1\beta_2\gamma_{2S}$ (5:1:5). The cRNAs were prepared by linearization of DNA plasmids using NotI restriction enzyme (Thermo Fischer Scientific, West Palm Beach, FL). The linearized cDNA was purified by extraction using buffer-saturated phenol, phenol:chloroform:isoamyl alcohol and chloroform followed by precipitation from the aqueous phase using sodium acetate and ethanol. The purified linearized cDNAs were used as templates to synthetize cRNA using the Message Machine T7 transcription kit (Ambion, Life Technologies, Paisley, UK). The quality of the cRNA was evaluated by 0.8% agarose gel electrophoresis, and the concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific).

The oocytes were injected with 0.9–1.8 ng cRNA in a final volume of 18–36 nL and were stored at 18 °C in Barth's solution (88 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 100 IU/ml penicillin, 100 mg/ml streptomycin, 0.1 mg/ml gentamycin, pH 7.5).

Two-electrode voltage-clamp (TEVC) were performed at room temperature 3–7 days after cRNA injection at a holding potential of -60 mV using an OC-725C oocyte clamp amplifier (Warner Instruments, Hamden, CT). Signals were low-pass filtered at 10 Hz (950L8L, Frequency Devices, Ottawa, IL) and digitized using USB-6212 BNC data acquisition board (National Instruments, Austin, TX). Voltage and current electrodes were pulled from thin-walled glass capillary tubes (World Precision Instruments, Hertfordshire, UK) using a PC-10 puller (Narishige, East Meadow, NY) and were agar-plugged, filled with 3 M KCl and had a tip resistance of ~2–4 MΩ. The oocyte recoding solution was composed of 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH adjusted to 7.4 with NaOH) and was continuously perfused to the oocytes placed in a recording chamber. Compound solutions were diluted in the recoding solution and applied to the recoding chamber using a gravity-driven VC3-8xG perfusion system (ALA Scientific Instruments, Inc., Farmingdale, NY).

4.2.3 Data analysis

For binding experiments analysis was performed using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA, USA). Data were fitted according to the *Allosteric modulator titration* equation based on the allosteric ternary complex model developed for G protein-coupled receptors,[51] but formally in agreement with the two-state allosteric model also used for ionotropic receptors.[52]

$$Y = \frac{[A]}{[A] + \frac{K_A(1+[B]/K_B)}{(1+\alpha[B]/K_B)}}$$

where Y denotes the fractional specific binding, [A] denotes the concentration of [^{3}H]NCS-382 (fixed as constant) and [B] the concentration of monastrol. K_A and K_B denote the equilibrium dissociation constants of [^{3}H]NCS-382 and monastrol, respectively, and K_A was fixed as constant (based on the previously published [^{3}H]NCS-382 K_d value of 430 nM[53]). α is the cooperativity factor reflecting the direction and magnitude of the modulation. An α value of 1.0 indicates no modulatory effect, α<1.0 indicates negative modulation and α>1.0 positive allosteric modulation. K_B and α were fitted by non-linear regression. All data are summarized as means ± S.E.M. of at least two independent experiments performed in triplicate.

The currents from the TEVC recordings were analysed using Clampfit 10 (pCLAMP Software, Molecular Devices, Sunnyvale, CA). To evaluate the modulatory effect of a given compound, the data were normalized and expressed as the mean fold potentiation ± S.E.M. as shown in the following equation: $I_A/I_{control}$ ± S.E.M., where I_A represents the amplitude of the current mediated by the co-application of GABA and the modulator, and $I_{control}$ represents the amplitude of the current elicited by GABA.

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