# Structure-Activity Relationships of Neuroactive Steroids Acting on the $GABA_A$ Receptor

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**Abstract:** The term "neuroactive steroid" (NAS) refers to steroids which, independent of their origin, are capable of modifying neural activities. These steroids positively or negatively modulate the function of members of the ligand-gated ion channel superfamily. Those with positive allosteric actions on the γ-amino butyric acid type A receptor ($GABA_A$ receptor) have been shown to be potent anticonvulsants, anxiolytics, and antistress agents and to possess sedative, hypnotic, and anesthetic activities. New types of neuroactive steroids have been widely sought and structural modifications of the naturally occurring metabolites allopregnanolone, pregnanolone and allotetrahydrodeoxycorticosterone, have been examined in the light of the vast family of GABA receptor subtypes within the brain. Here we review the structure-activity relationship (SAR) of neuroactive steroid analogues obtained by modification of the steroid nucleus, including substitutions at the A, B, C, and D rings and the side chain, with emphasis on the different pharmacophores proposed.

**Keywords:** Neuroactive steroids, Neurosteroids, Structure-activity relationship, $GABA_A$ receptor, anticonvulsant.

## 1. INTRODUCTION

The term "neurosteroid" (NS) was introduced by Baulieu in 1981 to name a steroid hormone, dehydroepiandrostane sulfate (DHEAS) that was found in high levels in the brain long after gonadectomy and adrenalectomy, and shown later to be synthesized by the brain. Currently, all steroids synthesized in the brain are considered neurosteroids. The term "neuroactive steroid" (NAS) on the other hand refers to steroids which, independent of their origin, are capable of modifying neural activities [1]. Some of these steroids and their metabolites can produce immediate changes in neuronal excitability on a time scale that precludes a genomic locus action (within seconds). These non-genomic effects involve modulation of the function of members of the ligand-gated ion channel superfamily, of which the positive allosteric actions on γ-amino butyric acid type A receptor ($GABA_A$ receptor) have been the focus of most of the studies [2-6].

γ-Amino butyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, and rapid synaptic inhibition is mediated through the opening of $GABA_A$ receptor-ionophores. The $GABA_A$ receptor complex can exist in multiple isoforms with a variety of pharmacological profiles that arise from their pentameric structure and the diversity of their subunits. These isoforms are formed from the assembly of two α subunits (from six different gene products, α1-α6), two β subunits (from three different gene products, β1-β3) plus one additional subunit (from γ1-γ3), but sometimes a δ, ε, π, or θ subunit. Fortunately, only a limited number of subunit combinatorial possibilities have been identified, the α1β2γ2 subunit combination being the most widespread combination in mammalian brain. The $GABA_A$ receptor contains many distinct binding sites for a variety of ligands, including sites for GABA, benzodiazepines, barbiturates and convulsant channel antagonists. The indication that steroids act on the $GABA_A$ receptor from within the transmembrane domains is supported by pharmacological and site-directed mutagenesis studies [7].

The interactions of NAS with the $GABA_A$ receptor have been reviewed recently by Akk and colleagues [6]. Briefly, three types of effects can result: potentiation of currents elicited by GABA or another activator, inhibition of these currents, or direct activation of the channel. The most potent effects are the potentiating ones and like many GABA-receptor potentiators, including barbiturates, NAS increase the whole-cell response to low concentrations of GABA. Typical $EC_{50}$ values for $GABA_A$ receptor potentiation by NAS are in the high nanomolar range. Among the endogenously produced steroids (NS), the progesterone reduced metabolites 3α-hydroxy-5α-pregnan-20-one (3α,5α-THPROG or allopregnanolone) (**1**), 3α-hydroxy-5β-pregnan-20 one (3α,5β-THPROG or pregnanolone) (**2**) and the reduced metabolite of deoxycorticosterone, 3α,21-dihydroxy-5α-pregnan-20-one (3α,5α-THDOC or allotetrahydroDOC) (**3**), are potent positive allosteric modulators of the $GABA_A$ receptor (Fig. **1**) [8].

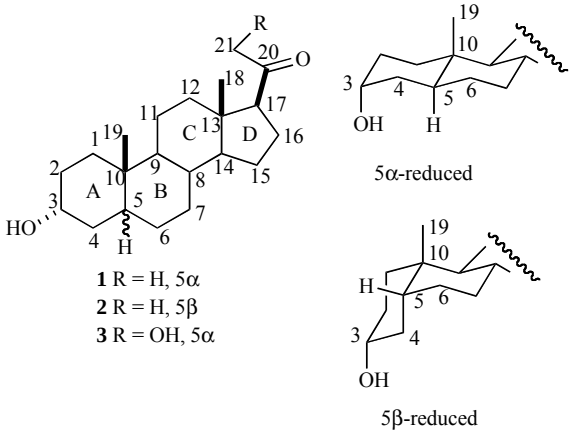


**Fig. (1).** Endogenous steroidal positive allosteric modulators of the $GABA_A$ receptor, allopregnanolone (**1**), pregnanolone (**2**) and allotetrahydrodeoxycorticosterone (**3**) showing carbon atom numbering and overall conformation of the A/B ring fusion for 5α- and 5β-reduced metabolites.

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Somewhat less potent effects correspond to the antagonistic actions of NAS ($IC_{50}$ in the high nanomolar to micromolar range). Typically these are exerted by C-3 sulfated pregnanes with α or β stereochemistry at positions 3 and 5. The fact that when potentiating and inhibitory steroids are applied simultaneously, the resulting effects on receptor kinetics mimic the actions of either steroid applied individually, suggests that they interact with different sites. The direct activation of the channel in the absence of GABA is observed with higher concentrations of steroids and, although small currents result from this effect, they have a significant impact on cellular excitability. Recent results show evidence for at least two sites of neurosteroid action on $GABA_A$ receptors. One site spanning the M1 and M4 transmembrane domains of the α subunit, accounts for the potentiating actions of some NAS. Another site, located between the M1 transmembrane domain of the α subunit and the M3 domain of the β subunit, is responsible for direct gating of the channel by NAS [7]. In addition to modulating receptor functions, there is evidence that treatment with steroids induces receptor plasticity through changes in expression of particular $GABA_A$ subunits [3]. Recently Maguire and coworkers demonstrated that both ovarian and stress hormones are capable of inducing alterations in $GABA_A$ receptor subunit composition by actions through neurosteroid metabolites [9].

Changes in neurosteroid levels are associated with various physiological and pathophysiological conditions, including stress, depression, pregnancy, neuronal development and aging. NAS have been shown to be potent anticonvulsants, anxiolytics, and antistress agents and have been shown to possess sedative, hypnotic, and anesthetic activities. Many of the physiological actions induced by steroids acting at the $GABA_A$ receptor complex and their therapeutic potential have been reviewed [3,6]. This review focuses on structure-activity relationship studies involving the potentiation action of NAS, with emphasis on the different pharmacophores proposed.

## 2. PHARMACOLOGICAL STUDIES

The structure-activity requirements for the interaction of NAS with the steroid binding site on the $GABA_A$ receptor have been studied using binding, electrophysiological and behavioral assays. These have not been used in a systematic way throughout the literature making difficult the comparisons among results. A brief recompilation of the commonly used assays and their significance follows.

### 2.1. Binding Studies

#### *2.1.1. Inhibition of Binding of [$^{35}$S]TBPS on Synaptosomal Membranes of Rat Cerebellum*

The cage convulsant [$^{35}$S]-*t*-butylbicyclophosphorothionate ([$^{35}$S]TBPS) binds to the picrotoxin site of the $GABA_A$ receptor complex, and neurosteroids are known to allosterically displace [$^{35}$S]TBPS binding [10]. In the presence of low concentrations of GABA, these metabolites have significantly higher affinity for binding. As this *in vitro* assay closely reflects the functional state of $GABA_A$ receptors, it may be useful for the characterization of allosteric interactions between various sites on the receptor, being one of the most common of the binding assays [10-15].

#### *2.1.2. Inhibition of Binding of [$^3$H]TBOB and [$^3$H]EBOB on Synaptosomal Membranes of Rat Cerebellum*

Studies using [$^3$H]-*t*-butylbicycloorthobenzoate ([$^3$H]TBOB) to label a chloride ionophore associated binding site within the $GABA_A$ receptor complex are less common than those using [$^{35}$S]TBPS, although the use of this radiolabeled ligand appears to be more convenient due to the longer half-life of tritium compared to $^{35}$S. Since both ligands are supposed to label the same populations of binding sites, studies using the two radioligands may well be compared.[16] Displacement of the specific binding of [$^3$H]-4′-ethynyl-4-*n*-propyl-bicycloorthobenzoate ([$^3$H]EBOB) follows an allosteric binding model that was first applied to glycine receptors which belong to the same superfamily of transmitter-gated ion channels as the $GABA_A$ receptor. Recently this binding assay was successfully used on $GABA_A$ receptors on synaptosomal membranes of rat cerebellum [17].

#### *2.1.3. Stimulation of the Specific Binding of [$^3$H]flunitrazepam to Synaptosomes from Rat Brain*

The benzodiazepine flunitrazepam, is a specific ligand for the benzodiazepine binding site of the $GABA_A$ receptor. The effect on the binding of this ligand has been used as an *in vitro* assay, taking into account that the ability of NAS to stimulate the specific binding of benzodiazepines *in vitro* is proportional to their CNS depressant potencies [10,15].

#### *2.1.4. Stimulation of the Specific Binding of [$^3$H]muscimol to Synaptosomes from Rat Brain*

Muscimol is a specific ligand for the GABA binding site of the $GABA_A$ receptor. NAS increase the apparent affinity of this agonist to rat membranes and this effect can also be used to evaluate NAS analogues [18].

### 2.2. Uptake of $^{36}Cl^-$

Two kinds of experiments are based on the $^{36}Cl^-$ uptake. One measures the direct potentiation of $^{36}Cl^-$ uptake by NAS in rat brain synaptoneurosomes while the other measures the potentiation of muscimol-stimulated $^{36}Cl^-$ uptake. In the latter case, potentiation represents the increase in $^{36}Cl^-$ uptake by steroids compared to the muscimol-stimulated uptake alone in rat brain synaptoneurosomes, the potency and efficacy for this assay being much greater than the direct effects of NAS on chloride flux [19].

### 2.3. Electrophysiological Experiments

The electrophysiological experiments can be divided into two main groups: those which measure the ability of NAS to potentiate GABA-evoked currents, and those which directly elicit membrane currents in the absence of GABA. These experiments have been performed using culture neurons [11] or $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus* oocytes [6]. In some cases the results obtained using culture neurons may differ slightly from those obtained using the cloned $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in oocytes. This may be so because whole cells studies represent average responses recorded from multiple subtypes of $GABA_A$ receptors some of which

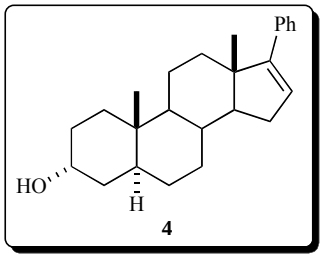
can differ from the cloned receptors in the sensitivity for steroids.

### 2.4. Behavioral Assays

Pharmacological activity of NAS has been evaluated using different behavioral models and a number of review articles have covered the potential therapeutic applications of neuroactive steroids [3,6]. Taking into account that the affinity of a steroid for $GABA_A$ receptors varies with subunit composition, pharmacological activity, e.g. anesthetic activity, is liable to depend on modulation of specific $GABA_A$ receptor-subtypes and *in vitro* and *in vivo* results not always correlate directly. Nonetheless, there is a generally good agreement between the two assay systems, which indicates that the *in vitro* assays described above are useful tools to study steroid structure-activity relationships. For instance, Akk *et al*. evaluated more than 200 compounds and found a very good correlation between TBPS binding inhibition potency ($IC_{50}$) and loss of righting reflex ($EC_{50}$) in *Xenopus* tadpoles [6].

## 3. STRUCTURAL MODIFICATIONS

Among the most striking structure-activity relationships established in the earlier animal behavioral studies of anesthetic steroids, was one indicating that the stereochemistry of the steroid A/B ring fusion had a relatively minor effect on anesthetic activity. Thus allopregnanolone (**1**) and pregnanolone (**2**) had equivalent anesthetic activity in mice and rats even though the overall conformation of the steroids differs greatly (Fig. **1**) [20]. Recent studies using 17-phenyl-5α-androst-16-en-3α-ol (17PA, **4**), a neurosteroid antagonist with no effect on GABA-evoked reponses *per se*, suggested that compounds **1** and **2** might exert their activity through different transduction mechanisms or binding sites [4,8,21]. Thus, 17PA (**4**) selectively antagonized the GABA-modulatory and GABA-mimetic effects of allopregnanolone (**1**) and related 5α-pregnanes but had little effect on the GABA-enhancing actions of pregnanolone (**2**) and related 5β-pregnanes.

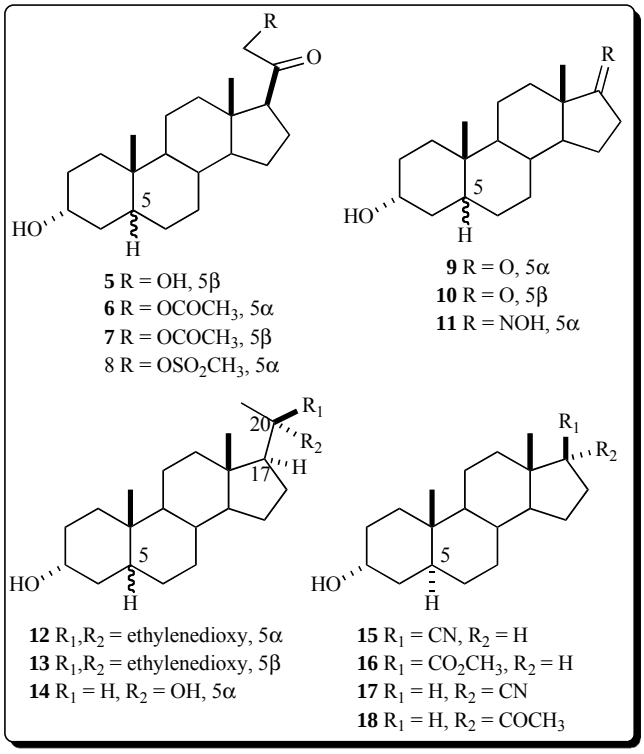



**4**

New types of neuroactive steroids have been widely sought and structure modification of the naturally occurring metabolites allopregnanolone (**1**), pregnanolone (**2**) and allotetrahydroDOC (**3**) (Fig. **1**) has been examined in the light of the vast family of GABA receptor subtypes within the brain. Modifications to the steroid nucleus include substitutions at the A, B, C, and D rings and the side chain and a limited number of modifications involving changes in ring size and additional rings. On the basis of these studies a pharmacophore has been established for the positive modulation of the $GABA_A$ receptor by steroids. The pharmacophore consists of a hydrogen-bond-accepting group such as acetyl or carbonitrile in a pseudoequatorial orientation at the 17β position and a hydrogen-bond-donating hydroxyl group in a 3α configuration.

### 3.1. Modifications at the D Ring and the Side Chain

This part of the steroid framework has been the subject of a considerable number of studies. Most of the work has been focused on functional changes of the 17-substituent and in the search for preferred orientations of the side chain that would help to map the interacting part of the receptor. A series of natural and synthetic 3α-hydroxy steroids (both 5α and 5β) with various side chains (**5**-**18**) were tested for their ability to potentiate muscimol-stimulated $^{36}Cl^-$ uptake (Table **1**) [22].




**5** R = OH, 5β
**6** R = $OCOCH_3$, 5α
**7** R = $OCOCH_3$, 5β
8 R = $OSO_2CH_3$, 5α

**9** R = O, 5α
**10** R = O, 5β
**11** R = NOH, 5α

**12** $R_1$,$R_2$ = ethylenedioxy, 5α
**13** $R_1$,$R_2$ = ethylenedioxy, 5β
**14** $R_1$ = H, $R_2$ = OH, 5α

**15** $R_1$ = CN, $R_2$ = H
**16** $R_1$ = $CO_2CH_3$, $R_2$ = H
**17** $R_1$ = H, $R_2$ = CN
**18** $R_1$ = H, $R_2$ = $COCH_3$

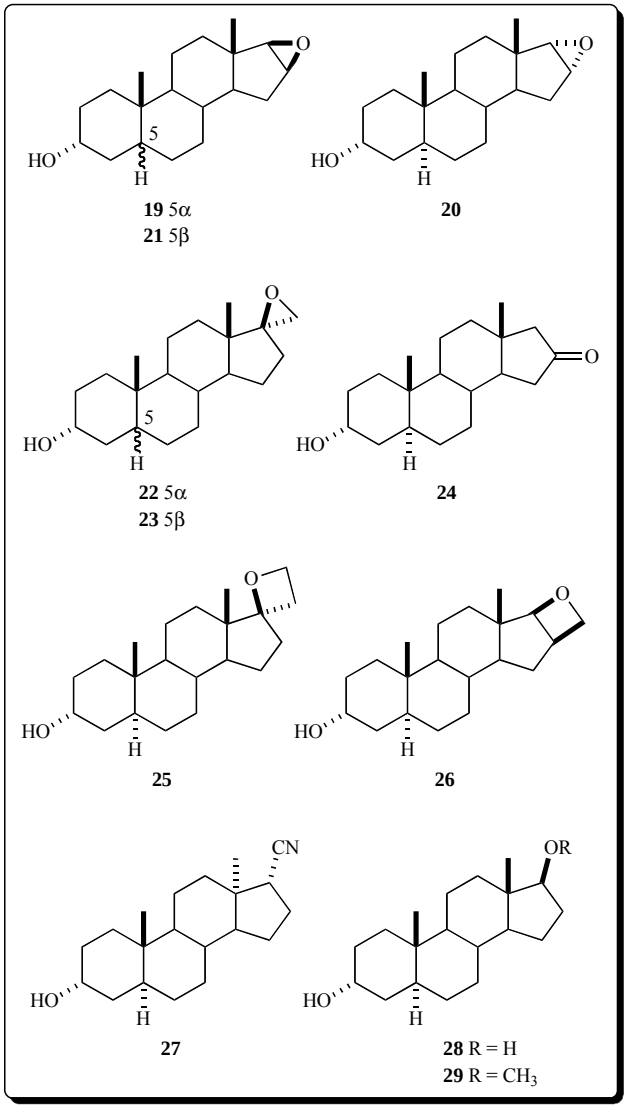
**Table 1. Effect of Side Chain Functional Modifications on the Potentiation by Steroids of Muscimol-Stimulated $^{36}Cl^-$ Uptake in Rat Brain Synaptoneurosomes[a]**

| Steroid | Relative potency[b] |
|---|---|
| **3, 6, 7, 15** | 0.8 to 1.0 |
| **5, 8, 9** | 0.5 to 0.6 |
| **10, 16** | 0.3 to 0.4 |
| **11-14, 17, 18** | Not active |

[a]See reference [22].
[b]Relative potency represents the relative rate of $^{36}Cl^-$ uptake in the presence of steroid and muscimol, compared to that produced by muscimol and allotetrahydroDOC (**3**).

The naturally occurring metabolite allotetrahydroDOC (**3**) was found to be the most active in augmenting $GABA_A$ receptor-mediated $^{36}Cl^-$ uptake. The reduced activity of the A/B-cis fused analogue of tetrahydroDOC (**5**) in this func-

tional assay, was consistent with the comparative anesthetic potency of these steroids in rats [23], but contrasted with the equivalent effects of these two steroids on [$^{35}$S]TBPS binding [24]. While the 21-acetate derivative of allotetrahydroDOC (**6**) or of its 5β-isomer (3α,21-dihydroxy-5β-pregnan-20-one 21-acetate, **7**) were both very effective in potentiating $^{36}Cl^-$ flux, the 21-mesylate of allotetrahydroDOC (**8**) was less active than the parent steroid (**3**). Removal of the side chain as in 3α-hydroxy-5α-androstan-17-one (**9**), reduced the potentiation of $^{36}Cl^-$ uptake and the activity in the [$^{35}$S]TBPS binding assay (see below) compared to allopregnanolone (**1**) but did not substantially affect the ability to enhance [$^3$H]muscimol binding to rat brain membranes [25]. The negligible to null activity found for the synthetic derivatives **11**-**14** supported the importance of the $C_{17}$ or $C_{20}$ ketone group as a presumed hydrogen-bond acceptor in natural steroid metabolites. In apparent agreement with this assumption the 17β-carbonitrile **15**, which was reported to be active as an anesthetic in rats [26], was as active as allotetrahydroDOC (**3**) for potentiation of $^{36}Cl^-$ uptake. However, the synthetic 17β-carboxylic acid methyl ester **16**, in which the ester carbonyl group may also serve as a hydrogen-bond acceptor, had a much lower activity. As expected changing the side-chain configuration, as in compounds **17** and **18** destroyed the activity.




**19** 5α
**21** 5β
**20**
**22** 5α
**23** 5β
**24**
**25**
**26**
**27**
**28** R = H
**29** R = $CH_3$

A number of conformationally constrained steroids bearing a hydrogen bond-accepting substituent on the D-ring were synthesized by Anderson and coworkers, which included epoxides **19**-**23** and oxetanes **25** and **26** [27]. Other related androstanes assayed were ketones **9** and **24**, the nitrile **27** with an inverted configuration at C-13, alcohol **28** and its methyl ether **29**. The anesthetic potency of these analogues and their binding affinity for $GABA_A$ receptor (measured by intravenous administration to mice and inhibition of [$^{35}$S]TBPS binding to rat whole brain membranes respectively), were compared with that of known anesthetic $GABA_A$ receptor modulators **1**-**3** (Table **2**).

**Table 2. Effect of Ring D Substitution on Steroid Activity on the Radioligand [$^{35}$S]TBPS Binding Assay[a]**

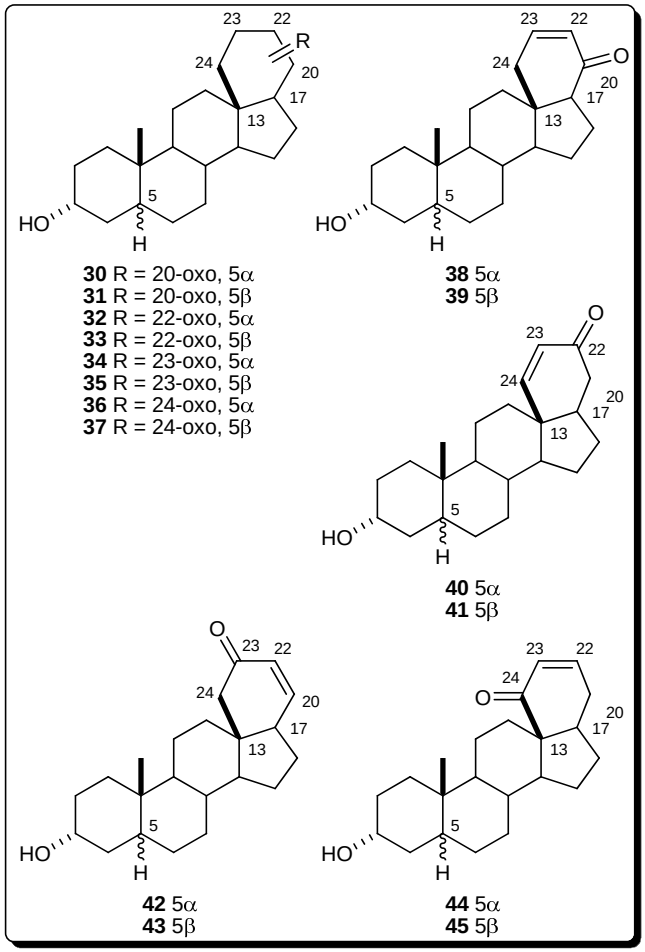
| Steroid | Relative potency[b] |
|---|---|
| **22** | 2.7 |
| **19, 21, 23, 25, 29** | 0.6 to 1.0 |
| **9, 24, 27, 28** | ca. 0.1 |
| **20, 26** | < 0.06 |

[a]See reference [27].
[b]$1/IC_{50}$ relative to compounds **1** and **2**.

Compounds **19**, **21**, **22**, **23**, **25** and **29**, carrying a hydrogen-bond-accepting ether function on the β-face, had *in vitro* and *in vivo* activities comparable to the endogenous allopregnanolone and pregnanolone or higher (**22**). The activity of steroids **22**, **23** and **25** was somewhat surprising since 17α-substitution was generally deleterious to activity. The authors proposed that the steric constraint within a three or four-membered ring could reduce protrusion of the substituent below the α-face of the steroids thus minimizing the deleterious effect. The negligible *in vitro* activity of the α-epoxide **20** was consistent with the unfavorable orientation of hydrogen bond accepting substituent towards the α-face. Comparison of oxetane **26** with epoxide **19** indicated that the additional methylene unit reduced the *in vitro* and *in vivo* activity. The simultaneous inversion of the D-ring stereochemistry at C-13 and C-17 present in **27** resulted in a significant loss of activity. As already shown in Table **1** inversion of the 17β-carbonitrile alone produces this effect (compare **15** and **17**). The decrease in activity of alcohol **28** compared to methyl ether **29** is in agreement with the low activities conferred by side chain hydroxyls in **11** and **14** and adds further support to the assumption that hydrogen bond-accepting substituents are preferable to hydrogen bond-donating substituents in this region of the molecule. Considering the above results, the authors proposed that for optimal $GABA_A$ receptor modulation the hydrogen bond-accepting substituent should be near perpendicular to the plane of the D-ring on the β-face of the steroid.

In line with the above studies, Covey and coworkers prepared and evaluated a series of 13,24-cyclo-18,21-dinorcholanes containing a ketone or a conjugated ketone group at C-20, C-22, C-23 or C-24 (**30**-**45**) [28]. These ana-

logues with conformationally constrained side chains were used to gain new information concerning the optimal location(s) for a hydrogen bond accepting group on the D-ring. The analogues were evaluated in [$^{35}$S]TBPS binding experiments, in electrophysiological experiments using rat $\alpha_1\beta_2\gamma_{2L}$-type $GABA_A$ receptor expressed in *Xenopus laevis* oocytes and as tadpole anesthetics by determination of the loss of righting response (LRR) and the loss of swimming response (LSR).




Within the 5α-series, none of the cyclosteroids was as potent a displacer of [$^{35}$S]TBPS as the prototype 5α-reduced steroid allopregnanolone (**1**). The order of potency for the radiolabelled ligand displacement by the saturated 13,24-cycloketones was: **36** ≅ **34** > **30** > **32**. The addition of a conjugated double bond in the 13,24-cyclo ring caused a minor enhancement of potency for each compound in the enone series but maintained the relative order of activities with respect to carbonyl position (**44** > **42** > **38** > **40**). The same order of potency was found in the 5β-series and once again, none of the cyclosteroids was as potent a displacer of [$^{35}$S]TBPS as the prototype 5β-reduced steroid pregnanolone (**2**). However a more pronounced loss of activity was observed in this series for the 20-oxo and 22-oxo analogues (**31**, **33**, **39** and **41**) and for the 23-oxo analogue **35**. Overall the unsaturated 24-oxo cyclosteroids **44** and **45** were the most active.

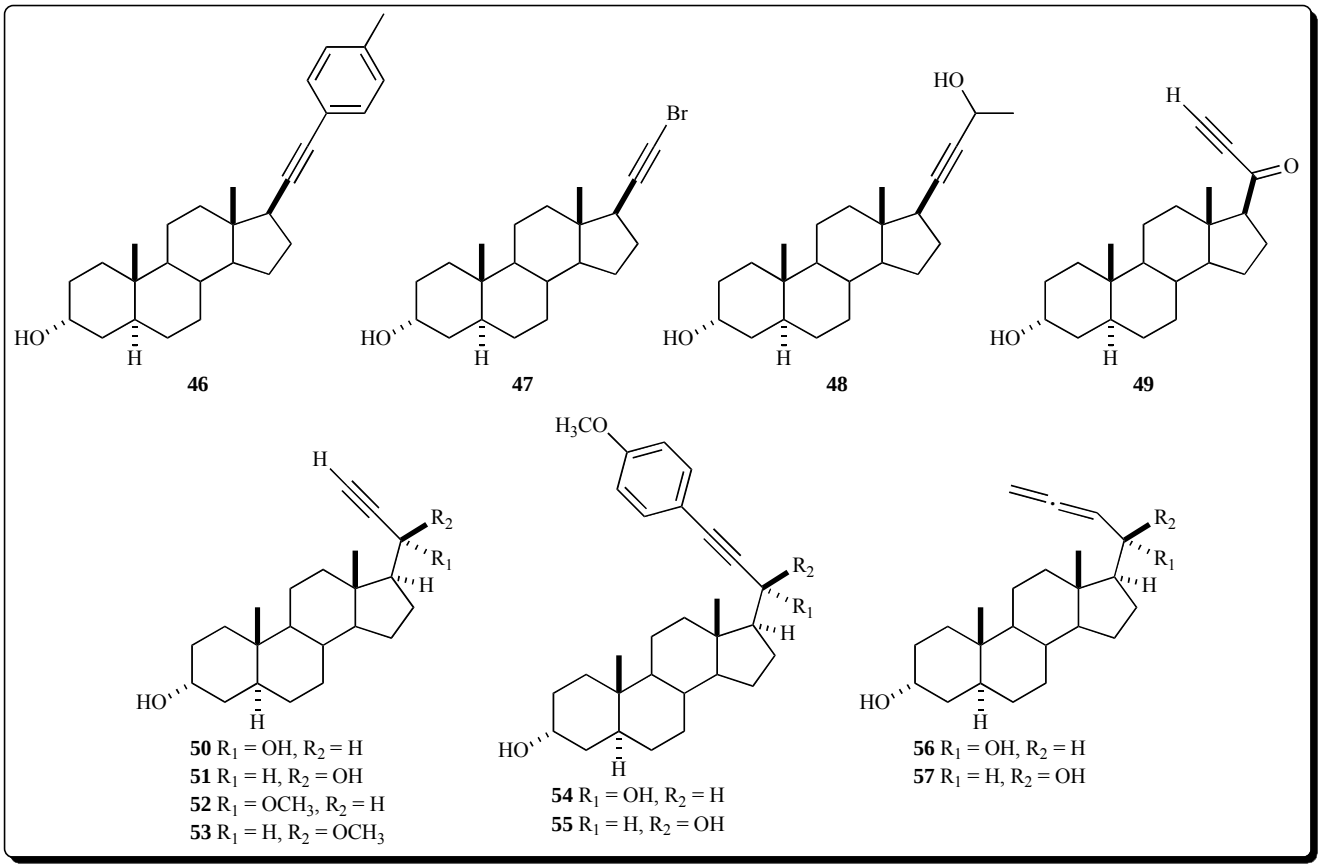
Within each cyclosteroid series, [$^{35}$S]TBPS displacement potency correlated with decreasing intramolecular O-O distance. However, the authors concluded that besides the change in the intramolecular distance between the 3α-hydroxyl and the side chain carbonyl, the spatial relationship between those functional groups may also affect displacement potency [28]. The reference steroids **1** and **2**, have intramolecular O-O distances similar to those of the least potent cyclosteroid analogues when considered in their most stable conformation, but side chain rotation around the C17-C20 bond results in substantial changes in the intramolecular distance and the relative orientation of the functional groups involved, which in some cases may be similar to some of the above compounds (e.g. 23-oxo analogues).

The effect of the cyclosteroids **30**-**45** on $GABA_A$ receptor function was also evaluated by their ability to enhance currents mediated by GABA and to show direct gating effects [28]. In general, the potentiating pattern matched well the rank order of the steroids in the TBPS binding assay, the 24-oxocyclosteroids **36**, **37**, **44** and **45** being the most potent analogues. Regarding the potency as anesthetics, for the ketones in the 5α-series the most potent compounds for causing tadpole LRR were allopregnanolone (**1**) ($ED_{50}$ = 0.42 ± 0.04 μM) and the 24-ketone **36** ($ED_{50}$ = 0.32 ± 0.02 μM). For the ketones in the 5β-series, none of the analogues was as potent in causing LRR as pregnanolone (**2**) [28].

The poor activity showed by the 20-oxo cyclosteroids **30, 31, 38** and **39** was not surprising considering that the carbonyl group in these compounds projected towards the α-face of the steroid nucleus. The activity of the 24-ketone and $\Delta^{22}$-24-ketone analogues was unexpected because their carbonyl group is as far above the D-ring as the carbonyl group of the 22-oxo-cyclosteroids. However, the carbonyl group in 22-oxo and 24-oxo-cyclosteroids projected towards opposite edges of the steroid ring system, and in the case of the 24-oxo steroids, this group was located over the steroid C-ring and oriented towards C-8. The authors explained their results by suggesting that the receptor may have multiple hydrogen bond donors in the binding region close to the D ring, which are able to interact with steroid hydrogen bond acceptor groups located in different positions and orientations. A water molecule may help to supplement the distance change, bridging the steroid to the receptor [28].

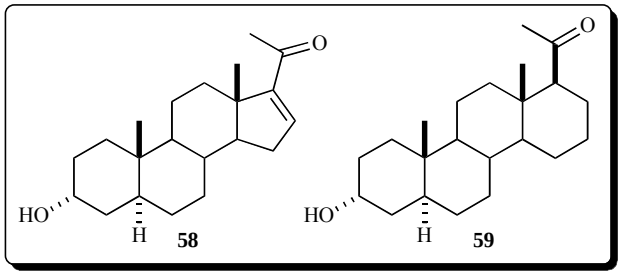
Souli and coworkers, developed another series of allopregnanolone (**1**) analogues with rigid free rotating 17β side chains (**46**-**57**) [17]. Specifically they introduced alkynyl and propadienyl functionalities at positions C-17 and C-20 of the steroid skeleton and also investigated the effects of C-22 and C-20 modifications. The *in vitro* binding affinity for $GABA_A$ receptors of the new analogues was measured by allosteric displacement of the specific binding of [$^3$H]EBOB to $GABA_A$ receptor on synaptosomal membranes of rat cerebellum.

Among the 17β-alkynyl derivatives (**46**-**48**) only the 22-bromoalkynyl derivative (**47**) had potency similar to the control compound **1** as a displacer. The presence of a triple bond in conjugation with the carbonyl group in analogue **49** caused a 4-fold reduction of activity. To study the impact of the orientation (α or β) of the reduced substituent at the C-20 position on the modulating activity of the $GABA_A$ receptor, C-20 epimers were stereoselectively synthesized. The alkynyl pregnanediols **50** and **51** showed higher displacing



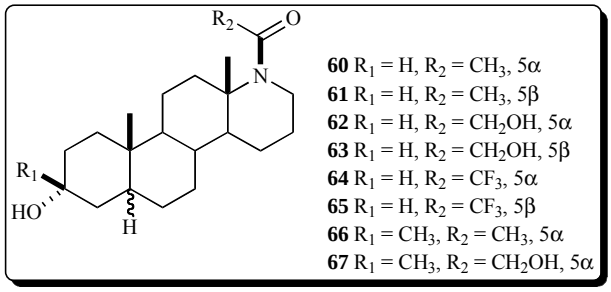

potency than ynone **49** and the activity was dependent on the C-20 stereochemistry, the 20*S* isomer **51** being 3.4 times more active than the corresponding 20*R* epimer **50**. Comparison of the activities of the pregnanediols **50** and **51** with that of the methyl ethers **52** and **53** indicated that methylation of the free hydroxyl group was deleterious for the *in vitro* activity. Furthermore, the substitution of the alkynyl functionality at C-22 in alcohols **54** and **55** by a 4-methoxyphenyl substituent resulted in a more pronounced decrease in activity. The difference in potency showed by the propadienyl derivatives (**56**, **57**) was the most interesting result. In analogue **56**, there was a 1.6-fold decrease in affinity with respect to alcohol **50**, whereas in steroid **57**, there was a 52-fold increase in affinity compared to **51**. Analogue **57** was the most potent compound of this series of derivatives, being ca. 71 times more active than **1**. A conformational search of pregnanediols **50**, **51**, **56** and **57** showed that the C-20 hydroxyl group, presumably acting as a hydrogen-bond acceptor, is positioned above the plane of ring D in all conformers within 6 kcal/mol of the global minimum. Based on these results, the authors proposed that hydroxyl orientation would not be the determining factor for the favorable interaction of **51** and **57** with the receptor binding site [17]. However, careful inspection of the side chain orientation in the four pregnanediols shows that in order to position the hydroxyl group above the plane of ring D, compound **56** also requires orienting the propadienyl group above that plane. This is a major difference with the other three pregnanediols, which could interfere with the interaction of the hydroxyl group and the receptor and better explain the drastic loss of activity observed for compound **56**.

Regarding changes within ring D, the effect of introducing a 16,17-double bond in allopregnanolone (**1**), pregnanolone (**2**) and allotetrahydroDOC (**3**) (e.g. $\Delta^{16}$-allopregnanolone, **58**), was deleterious for *in vitro* and *in vivo* activities [29]. On the other hand, expansion of the D-ring to give D-homosteroids resulted in active analogues, in particular D-homoallopregnanolone (**59**) is very active and has been the subject of a patent as an anesthetic [30].




Covey and coworkers prepared a series of 17a-aza-D-homosteroids (**60**-**67**) and evaluated their activity in binding experiments using [$^{35}$S]TBPS displacement and in electrophysiological experiments measuring the effects on the responses of rat $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes [31]. Except for compound **62**, there was a substantial decrease in [$^{35}$S]TBPS displacement activity compared to the corresponding reference compounds **1**-**3**, the deleterious effect being much stronger in the 5β series (up to two orders of magnitude).

While the substitution of the N-acetyl group in **60** and **61** by an α-hydroxyacetyl (azasteroids **62** and **63**) increased the




**60** $R_1$ = H, $R_2$ = $CH_3$, 5α
**61** $R_1$ = H, $R_2$ = $CH_3$, 5β
**62** $R_1$ = H, $R_2$ = $CH_2OH$, 5α
**63** $R_1$ = H, $R_2$ = $CH_2OH$, 5β
**64** $R_1$ = H, $R_2$ = $CF_3$, 5α
**65** $R_1$ = H, $R_2$ = $CF_3$, 5β
**66** $R_1$ = $CH_3$, $R_2$ = $CH_3$, 5α
**67** $R_1$ = $CH_3$, $R_2$ = $CH_2OH$, 5α

potency as displacers of [$^{35}$S]TBPS, the substitution by a trifluoroacetyl group (compounds **64** and **65**) decreased the potency. Although it was shown that the presence of a 3β-methyl substituent did not have a negative effect on the pharmacological activities of **1** and **2** (see A and C ring modifications below), the presence of this substituent in azasteroids **66** and **67** markedly decreased the biological activity compared to **60** and **62** respectively. These results suggest that the D-ring modification affected in some way how the A-ring interacts with the receptor, this effect being more pronounced in the 5β series. Electrophysiological responses followed mostly the same tendency observed in the binding experiments when comparing the 5α and 5β series but showed a sizeable response for the three steroids with a hydroxyacetyl side chain (**62**, **63**, **67**) and for the trifluoroacetyl analogue **64**.

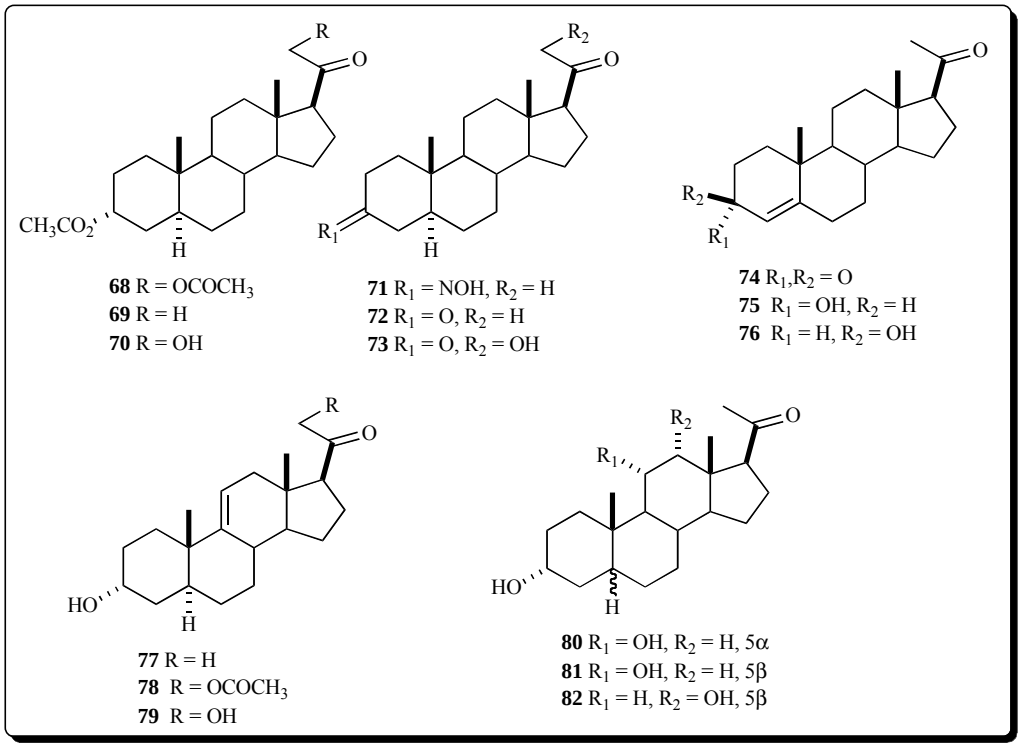
### 3.2. A and C Ring Modifications

Modifications in the rest of the steroid nucleus have been mostly focused on ring A, especially the region around the 3α-hydroxyl. Purdy and colleagues compared the enhancement of muscimol-stimulated $^{36}Cl^-$ flux by allopregnanolone (**1**) and pregnanolone (**2**), with the potencies of several analogues with modified substitution at C-3 [22]. Essentially inactive compounds resulted from either acetylation of the 3-hydroxyl (e.g. diacetate **68** and monoacetates **69** and **70**) or oxidation to the 3 ketone (as in progesterone **74** and metabolites **72** and **73**). The 3-oxime **71** with the N-hydroxyl in the plane of ring A, was also inactive as potentiator. While 3α-hydroxypregn-4-en-20-one (**75**) potentiated muscimol-stimulated $^{36}Cl^-$ uptake, its 3β-hydroxy isomer (**76**) was inactive. These results supported the conclusion that a free 3α-hydroxyl group was essential for activity.

Regarding the C ring, the 5α-pregn-9(11)-ene series (**77-79**) were essentially inactive as potentiators of muscimol-stimulated $^{36}Cl^-$ flux [22]. The introduction of an 11α-hydroxy group in allopregnanolone (compound **80**) or pregnanolone (compound **81**) or at the 12α position in pregnanolone (compound **82**), also gave inactive compounds.

Although **1** and **2** are potent allosteric modulators of the $GABA_A$ receptor, their therapeutic potential as anticonvulsant, anxiolytic and sedative-hypnotic agents is limited by rapid metabolism to inactive compounds, presumably by conjugation of the 3α-hydroxyl or oxidation to the corresponding ketones. Hogenkamp and coworkers prepared a series of 3β-alkyl-substituted derivatives of allopregnanolone (**1**) and pregnanolone (**2**) that increase their *in vivo* half-life as anesthetics [32]. The ability of these derivatives to allosterically modulate the binding of [$^{35}$S]TBPS was compared to the endogenously occurring metabolites **1** and **2**. The potencies of the synthetic steroids (**83-113**) varied from more potent than **1** and **2** to completely inactive (Table **3**).

The data in Table **3** show that steric bulk at the 3β position is of secondary importance while substituent lipophilicity appears as a significant determinant of activity. Thus, lipophilic substituents were generally well accommodated at the 3β-position, while the polar hydrogen bond-donating




**68** R = $OCOCH_3$
**69** R = H
**70** R = OH

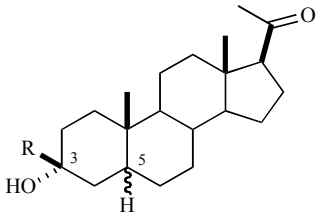
**71** $R_1$ = NOH, $R_2$ = H
**72** $R_1$ = O, $R_2$ = H
**73** $R_1$ = O, $R_2$ = OH

**74** $R_1$,$R_2$ = O
**75** $R_1$ = OH, $R_2$ = H
**76** $R_1$ = H, $R_2$ = OH

**77** R = H
**78** R = $OCOCH_3$
**79** R = OH

**80** $R_1$ = OH, $R_2$ = H, 5α
**81** $R_1$ = OH, $R_2$ = H, 5β
**82** $R_1$ = H, $R_2$ = OH, 5β

**Table 3. Effects of 3β-Substitution on 1 and 2 in the Radioligand [$^{35}$S]TBPS Binding Assay[a]**

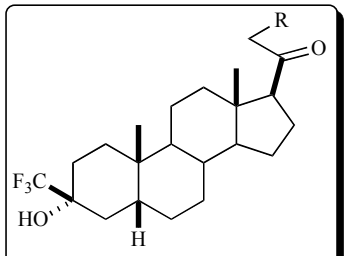



| Steroid | 5-H | R | $IC_{50}$ (nM) |
|---|---|---|---|
| **1** | α | H | 51 ± 5 |
| **83** | α | Me | 80 ± 18 |
| **84** | α | Et | 257 ± 24 |
| **85** | α | Pr | 173 ± 33 |
| **86** | α | $H_2C$=CH | 120 ± 7 |
| **87** | α | HC≡C | 64 ± 8 |
| **88** | α | $H_2C$=$CHCH_2$ | 231 ± 25 |
| **89** | α | HC≡$CCH_2$ | 50 ± 7 |
| **90** | α | $H_2C$=$CHCH_2CH_2$ | 325 ± 65 |
| **91** | α | $H_2C$=C=$CHCH_2$ | 365 ± 47 |
| **92** | α | $MeOCH_2$ | 76 ± 9 |
| **93** | α | $EtOCH_2$ | 230 ± 51 |
| **94** | α | n-$PrOCH_2$ | 655 ± 77 |
| **95** | α | $BnOCH_2$ | 376 ± 50 |
| **96** | α | $HOCH_2$ | 2134 ± 79 |
| **97** | α | $MeSCH_2$ | inactive |
| **98** | α | $NCCH_2$ | 614 ± 54 |
| **99** | α | $N_3CH_2$ | 27 ± 2 |
| **100** | α | $Me_2NCH_2$ | inactive |
| **101** | α | $F_3C$ | 266 ± 43 |
| **102** | α | $FCH_2$ | 228 ± 51 |
| **103** | α | $ClCH_2$ | 71 ± 11 |
| **104** | α | $BrCH_2$ | 224 ± 45 |
| **105** | α | $ICH_2$ | 702 ± 143 |
| **106** | α | $H_2NCOCH_2$ | inactive |
| **2** | β | H | 44 ± 11 |
| **107** | β | Me | 37 ± 10 |
| **108** | β | Et | 135 ± 7 |
| **109** | β | $H_2C$=CH | 43 ± 4 |
| **110** | β | HC≡C | 39 ± 5 |
| **111** | β | HC≡$CCH_2$ | 214 ± 20 |
| **112** | β | $ClCH_2$ | 243 ± 52 |
| **113** | β | $MeOCH_2$ | 40 ± 5 |

[a]Data taken from reference [32].

groups tested, i.e. the 3β-hydroxymethyl (**96**), the tertiary amine (**100**) and the 3β-carboxamidomethyl (**106**) reduced or abolished activity. In the series 3β-methyl, 3β-ethyl, and 3β-propyl (**83-85**) **83** was the most potent, being almost as active as allopregnanolone (**1**). The alkyne **87** and its homologue **89**, were also equipotent with **1**. The ethers **92-94** showed a 3-fold loss of activity for each methylene added and the thioether (**97**) was inactive. Among the halogenated derivatives (**101-105**) only compound **103** showed a good activity. The most potent compound in this series was the azide **99**, which had a 2-fold potency increase compared to **1**. For the 5β-pregnanes the limited number of substituents tested mostly showed tendencies similar to those observed in the 5α-series. Thus while the 3β-methylpregnane **107** was equipotent with **2**, the addition of a methylene spacer in **108** resulted in a 3.6-fold loss in activity. Unsaturated groups directly attached to the steroid nucleus at the 3β-position (**109** and **110**) and the methoxymethyl derivative **113** exhibited no loss in potency compared to **2**. Contrary to the results in the 5α-series, the 3β-(2-propyne) derivative (**111**) and the 3β-chloromethyl alcohol (**112**) resulted in ca. 5-fold loss in activity.

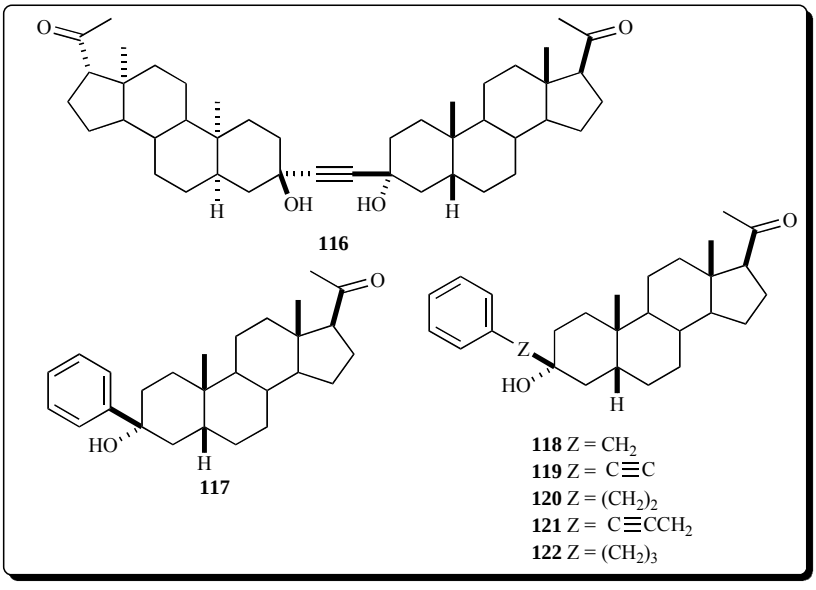
Electrophysiological measurements using $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in oocytes showed that 3β-methyl and 3β-(azidomethyl)-3α-hydroxy-5α-pregnan-20-one (**83** and **99** respectively) were potent modulators, confirming the results obtained from [$^{35}$S]TBPS binding studies. In animal studies, while the naturally occurring progesterone metabolites **1** and **2** were inactive as anticonvulsants when administered orally, compound **83** (CCD 1042) known clinically as ganaxolone was an orally active anticonvulsant [33]. Ganaxolone is used to treat epilepsy, currently being the steroid compound which has progressed the most clinically. In line with the above, the 3β-trifluoromethyl analogues of pregnanolone, CCD 3693 (**114**), and Co26749 (**115**) were tested as a therapeutic entity for insomnia and as an anxiolytic respectively [34]. These results showed that 3β-substitution slowed metabolism of the 3-hydroxyl by blocking oxidation to the 3-ketone, resulting in orally bioavailable steroid modulators of the $GABA_A$ receptor.




**114** R = H
**115** R = OH

During the synthesis of the potent 3β-ethynyl-3α-hydroxy-5β-pregnan-20-one (**110**), the dimer **116** was isolated as a byproduct [35]. Although this steroid derivative had relatively low potency in the [$^{35}$S]TBPS assay, even this level of activity was surprising considering the bulk of the 3β-substituent. On this basis, the analogue **117** with the smaller phenyl group at the 3β position was prepared. This compound was more potent than steroid **116** but had a 10-fold loss in affinity compared to the corresponding 3β-methyl derivative **107**. Therefore, a further modification of the 3β-position was carried out by adding a spacer between the phenyl ring and the steroid nucleus to evaluate whether a bridging unit could provide enhanced potency. The spacers used were flexible or rigid chains of 1-3 carbon atoms [35].

The resulting derivatives with a spacer (**118-122**) were more active than the 3β-phenyl compound **117**. The optimal spacer was the two carbon rigid ethynyl group (**119**) although there were no large differences among the spacers tested. These results suggested the presence of an auxiliary binding pocket for hydrophobic groups in the neuroactive steroid binding site located close to the position occupied by the steroid A ring. In order to provide systematic variations in lipophilic, electronic, steric and hydrogen-bonding properties, a set of 3β-substituted steroids containing the ethynyl spacer was prepared and tested in the [$^{35}$S]TBPS binding assay (Table **4**).

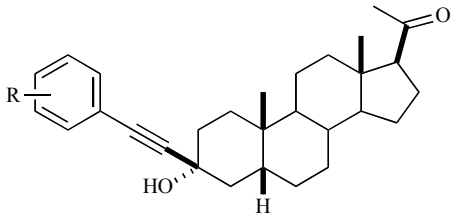



**116**

**117**

**118** Z = $CH_2$
**119** Z = C≡C
**120** Z = $(CH_2)_2$
**121** Z = C≡$CCH_2$
**122** Z = $(CH_2)_3$

**Table 4. Effects of Functionalized 3β-(arylethynyl) Substituents in the Radioligand [$^{35}$S]TBPS Binding Assay[a]**

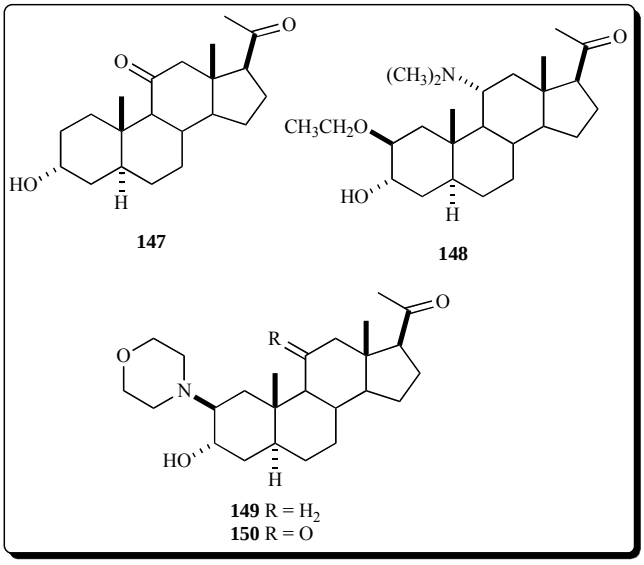



| Steroid | R | $IC_{50}$ (nM) |
|---|---|---|
| **119** | H | 100 ± 10 |
| **123** | 4-OMe | 42 ± 4 |
| **124** | 4-Cl | 70 ± 15 |
| **125** | 4-$NMe_2$ | 40 ± 5 |
| **126** | 4-CN | 60 ± 9 |
| **127** | 4-$CONH_2$ | 1800 ± 400 |
| **128** | 4-Ph | 54 ± 12 |
| **129** | 4-OH | 2300 ± 200 |
| **130** | 4-$NO_2$ | 42 ± 9 |
| **131** | 2-$OCH_3$ | 170 ± 20 |
| **132** | 3,4-$(OMe)_2$ | 290 ± 10 |
| **133** | 3,4-$OCH_2O$ | 68 ± 2 |
| **134** | 2-OH | 82 ± 31 |
| **135** | 3-OH | 420 ± 100 |
| **136** | 4-Me | 83 ± 16 |
| **137** | 4-$NH_2$ | 11800 ± 1800 |
| **138** | 4-$CF_3$ | 56 ± 7 |
| **139** | 4-COMe | 10 ± 2 |
| **140** | 4-$CO_2Et$ | 12 ± 3 |
| **141** | 4-CHO | 67 ± 44 |
| **142** | 3-COMe | 45 ± 9 |
| **143** | 4-$CONEt_2$ | 93 ± 13 |
| **144** | 4-COPh | 55 ± 11 |
| **145** | 4-COMe, 19nor | 12 ± 3 |
| **146** | 4-$CO_2Et$, 19nor | 26 ± 3 |

[a]Data taken from reference [35].

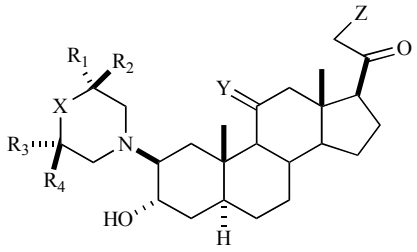
The activity of these 3β-(arylethynyl) derivatives of pregnanolone was found to be highly dependent on the substitution on the phenyl ring. Thus, addition of hydrogen bond accepting groups at the 4-position of the phenyl ring generally increased the affinity (**123**, **125**, **126**, **139-141** and **143-146**) while addition of hydrogen bond donating groups (**129**, **137**) decreased the affinity. However, a gradual increase in activity was observed upon moving the hydroxyl group closer to the steroid nucleus. Hence, the 3-hydroxy derivative **135** was about 5-fold more potent than the 4-hydroxy-substituted compound **129** and the 2-hydroxy-substituted compound **134**, with the hydroxyl group adjacent to the steroid nucleus, was 28 times more potent than compound **129**. On the other hand an unfavorable effect was observed upon moving the methoxy and the acetyl substituents (compounds **131** and **142**). Additional support for the importance of hydrogen bond accepting ability of *para*-substituents was the fact that the derivative **143** was 19 times more active than compound **127**. The potency of selected steroids was confirmed electrophysiologically in oocytes expressing cloned human $GABA_A$ $\alpha_1\beta_2\gamma_{2L}$ receptors. Consistent with their *in vitro* activity, some of the 3β-(phenylethynyl)-substituted steroids displayed anticonvulsant activity in the pentylenetetrazol (PTZ) and maximal electroshock (MES) tests following ip administration in mice. Remarkably, the 3β-[(4-acetylphenyl)ethynyl] derivative **145** has demonstrated an attractive anticonvulsant profile.

Though intravenous administration of any of the endogenous steroids **1-3** to mice caused anesthesia, the poor aqueous solubility of these compounds requires that they be formulated as emulsions or solubilized in water with the aid of a surfactant or complexing agent such as cyclodextrin. For instance, pregnanolone **2** was formulated in a lipid emulsion as an intravenous anesthetic [36], but it was abandoned after Phase III trials indicated a higher than anticipated incidence of urticaria [37]. Considering the anesthetic properties of alfaxolone (**147**) and minaxolone (**148**), two steroids introduced to clinic but later withdrawn because of adverse side effects [38], Anderson and coworkers prepared a series of water-soluble analogues bearing a 2β-morpholinyl substituent [39].




These analogues were prepared taking into account that the morpholino steroids **149** and **150** were known to cause a loss of righting reflex in mice upon intravenous administration of their water-soluble hydrochloride salts [40]. They included modifications of the 2β-amino function and the introduction of substituents at the C-21 position (**151-193**) (Table **5**). In order to enhance aqueous solubility and assist pharmacological studies, a salt of each compound was prepared prior to evaluation. The anesthetic potency of the steroids was determined upon their intravenous administration to mice using the dose required to cause a loss of righting reflex for a minimum period of 30 s in 50% of treated mice ($HD_{50}$,

**Table 5. Hypnotic Activity of Substituted 2β-Morpholino and 2β-Thiomorpholino Allopregnanolone Analogues with Enhanced Water Solubility[a]**




| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | X | Y | Z | Salt[b] | $HD_{50}$ (μmol/kg)[c] |
|---|---|---|---|---|---|---|---|---|---|
| **149** | H | H | H | H | O | $H_2$ | H | HCl | 22.2 (21.9-24.8) |
| **150** | H | H | H | H | O | O | H | HCl | 34.6 |
| **151** | Me | H | Me | H | O | $H_2$ | H | HCl | 26.0 |
| **152** | Me | H | Me | H | O | O | H | cit | 14.4 (13.2-15.7) |
| **153** | Me | H | H | H | O | O | H | MS | 17.0 (15.5-18.6) |
| **154** | Et | H | H | H | O | O | H | MS | 8.7 (7.8-9.6) |
| **155** | H | Et | H | H | O | O | H | MS | 15.1 (12.9-18.0) |
| **156** | Pr | H | H | H | O | O | H | cit | 6.4 (5.7-7.1) |
| **157** | H | Pr | H | H | O | O | H | cit | 10.8 (9.1-12.3) |
| **158** | Bn | H | H | H | O | O | H | cit | 12.6 (11.5-13.8) |
| **159** | H | Bn | H | H | O | O | H | cit | 12.8 (11.7-14.2) |
| **160** | *i*-Pr | H | H | H | O | O | H | cit | 3.4 (2.8-4.7) |
| **161** | *i*-Bu | H | H | H | O | O | H | cit | 6.5 (6.2-7.3) |
| **162** | Me | Me | H | H | O | O | H | cit | 9.8 (8.9-10.6) |
| **163** | Et | Et | H | H | O | O | H | cit | 12.0 (10.7-13.1) |
| **164** | Bu | Bu | H | H | O | O | H | MS | 31.1 (27.8-34.9) |
| **165** | Ph | Ph | H | H | O | O | H | MS | 49.9 (46.7-52.4) |
| **166** | Me | Me | Me | Me | O | O | H | CD | 8.3 (7.6-9.6) |
| **167** | Me | H | H | H | O | $H_2$ | H | MS | 15.0 |
| **168** | H | Me | H | H | O | $H_2$ | H | MS | 29.0 |
| **169** | Et | H | H | H | O | $H_2$ | H | cit | 11.2 (10.0-13.0) |
| **170** | H | Et | H | H | O | $H_2$ | H | cit | 22.7 (20.6-24.9) |
| **171** | Me | Me | H | H | O | $H_2$ | H | MS | 17.4 (16.6-18.3) |
| **172** | H | H | H | H | S | $H_2$ | H | MS | 40.5 |
| **173** | H | H | H | H | S | O | H | MS | 20.0 (15.9-23.1) |
| **174** | Me | Me | H | H | S | $H_2$ | H | MS | 51.9 (44.1-58.8) |
| **175** | Me | Me | H | H | S | O | H | cit | 13.7 (11.5-15.9) |
| **176** | Me | Me | Me | Me | S | O | H | C-EL | 61.8 |
| **177** | H | H | H | H | O | $H_2$ | OH | MS | 31.6 (18.0-50.0) |
| **178** | H | H | H | H | O | $H_2$ | OAc | MS | 31.0 (23.1-37.8) |
| **179** | H | H | H | H | O | $H_2$ | SAc | MS | 33.8 |
| **180** | H | H | H | H | O | $H_2$ | Cl | MS | 8.5 (7.6-9.5) |
| **181** | H | H | H | H | O | $H_2$ | Br | MS | 22.8 (21.2-27.4) |
| **182** | H | H | H | H | O | O | OH | MS | 198.2 |

**(Table 5). Contd…..**

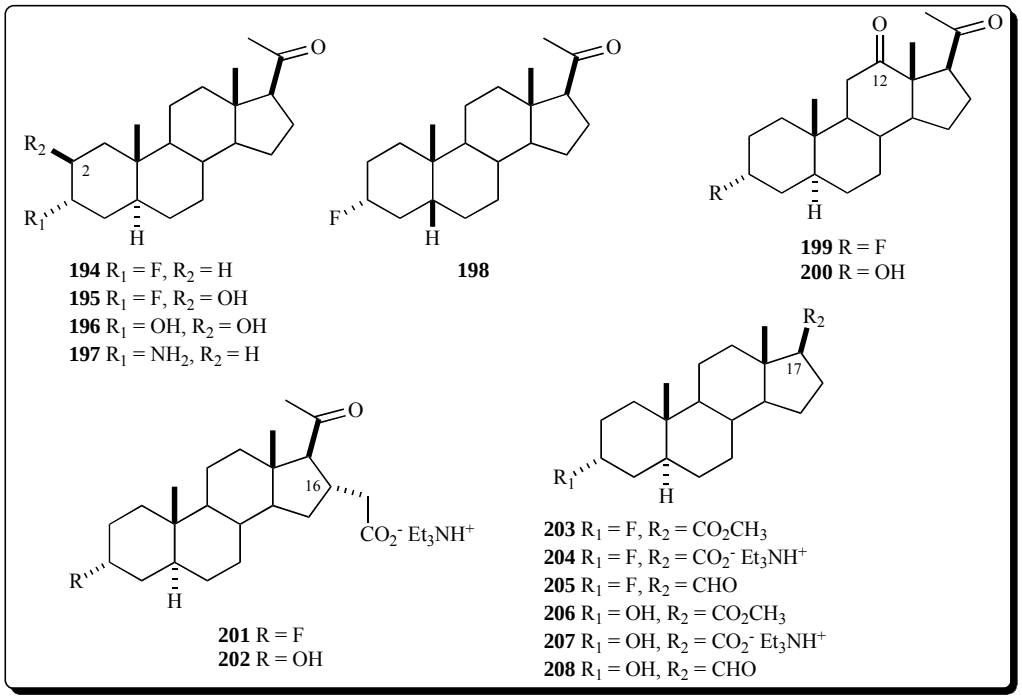
| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | X | Y | Z | Salt[b] | $HD_{50}$ (μmol/kg)[c] |
|---|---|---|---|---|---|---|---|---|---|
| **183** | H | H | H | H | O | O | Cl | MS | convulsant |
| **184** | Me | Me | H | H | O | $H_2$ | OH | MS | 18.4 (9.0-24.5) |
| **185** | Me | Me | H | H | O | $H_2$ | OAc | MS | 16.9 (14.2-18.6) |
| **186** | Me | Me | H | H | O | $H_2$ | SAc | MS | convulsant |
| **187** | Me | Me | H | H | O | $H_2$ | Cl | MS | 17.6 |
| **188** | Me | Me | H | H | O | O | OH | MS | 42.3 |
| **189** | Me | Me | H | H | O | O | OAc | MS | 21.1 (18.6-24.3) |
| **190** | Me | Me | H | H | O | O | SAc | MS | 7.9 (6.6-9.7) |
| **191** | Me | Me | H | H | O | O | Cl | MS | 13.4 (11.9-14.7) |
| **192** | Me | Me | H | H | O | O | SCN | MS | 10.0 (9.4-10.5) |
| **193** | Me | Me | H | H | O | O | $N_3$ | MS | 10.7 (8.9-12.1) |

[a]Data taken from reference [39]; [b]cit, citrate; HCl, hydrochloride; MS, methanesulfonate; CD, free base dissolved in 20% w/v aqueous (hydroxylpropyl)-β-cyclodextrin; C-EL, free base dissolved in aqueous Cremophor EL; [c]Dose required to cause a loss of righting reflex for a minimum period of 30 s in 50% of treated mice when administered i.v.

hypnotic $dose_{50}$). The *in vitro* effect of several compounds at $GABA_A$ receptors was assessed through determination of their ability to inhibit [$^{35}$S]TBPS binding to rat whole brain membranes and to potentiate GABA-evoked currents.

Comparison of diastereomers such as compounds **154** and **155** illustrated that, for monoalkylated morpholinyl derivatives, better potency resided in isomers with *R* configuration at the 2-position of the morpholine. Thus, the (2*R*)-2-isopropylmorpholine derivative (**160**) was more potent *in vivo* than either alfaxalone (**147**) or minaxolone (**148**). While compounds with small alkyl substituents, such as **162** and **166** retained excellent activity, compounds with larger substituents on the 2β-morpholine such as **164** and **165** were less potent *in vivo*. Alkylation of the 2β-thiomorpholinyl substituent with two or four methyl groups (**174**-**176**) did not always improve anesthetic activity. Comparison of the activity of some 21-substituted derivatives (**177**-**193**) showed that, with the exception of the convulsant thioacetate **186** and the 21-chloro compound **187** the alkylated 2β-morpholinyl derivatives **184**-**193** were more potent than analogues in which the 2β-morpholine was unsubstituted.

Examination of the *in vitro* results showed that all the morpholino and thiomorpholino steroids tested proved to be relatively potent inhibitors of TBPS binding at $GABA_A$ receptor complexes. The results of the electrophysiological assay showed that in most cases the steroids were more potent in the *in vitro* assay than the anesthetic agents propofol and thiopentone. There was not a good correlation between the *in vivo* and *in vitro* results as evidenced, for example, by the poor anesthetic activity of the dibutyl-substituted mor-



pholinyl derivative **164**, despite being one of the most potent morpholino-steroids inhibitors of [$^{35}$S]TBPS binding. Such a discrepancy could be accounted for by pharmacokinetic factors such as the ability of a compound to penetrate the blood-brain barrier or bind to plasma proteins. Other factors that may influence the *in vivo* profile are different selectivities of the morpholino steroids for $GABA_A$ receptor complexes with various subunit combinations and the relative importance of each of these complexes, both within the CNS and for producing anesthesia. The most promising new compounds, **162** and **180**, were selected for development as potential water soluble steroidal intravenous anesthetics. Compound **162** (Org 21465) progressed to the clinic but was discontinued due to initial excitatory effects upon i.v. administration [34].

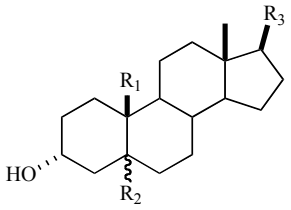
Kasal and coworkers prepared the 3α-fluoro analogues (**194** and **198**) of allopregnanolone (**1**) and pregnanolone (**2**) respectively, taking into account that the strength of the C-F bond yielded products with a high metabolic stability [41,42]. Although the results of binding assays *in vitro* (using [$^3$H]muscimol and [$^{35}$S]TBPS) showed that the replacement of the 3α-hydroxyl by a 3α-fluoro gave a small loss in activity, compound **194** showed stronger antiaggressive effects than allopregnanolone (**1**) in a behavioral test with mice [42]. The authors proposed that the slightly reduced $GABA_A$-receptor binding ability of the 3-fluoro analogue **194** when compared with the 3α-hydroxy compound **1**, was more than compensated by its metabolic stability resulting in an enhanced *in vivo* activity.

To increase the hydrophilicity of the products, the structure of the fluorosteroid **194**, was modified at positions 2, 12, 16 and 17 (compounds **195**, **199**, **201**, **203-205**). The activity of these 3α-fluoro compounds was compared with that of the corresponding 3α-hydroxy analogues (**196**, **200**, **202**, **206-208**) in binding assays *in vitro* (using [$^3$H]muscimol and [$^{35}$S]TBPS) [41]. No general rule could be formulated on the basis of these results, in some cases the 3α-fluoro analogues were slightly more active than the corresponding 3α-alcohol, whereas in other cases they showed a small decrease in activity. However, it is important to emphasize the antiagressive effects shown by the fluoride **194**, which contrast with the generally accepted belief that the presence of a 3α-hydroxy group is essential for the GABA–like activity. Kasal and coworkers have also found that 3α-amino-5α-pregnan-20-one (**197**) increased the binding of [$^3$H]flunitrazepam at the $GABA_A$ receptor in a primary culture of cortical neurons [43].

### 3.3. B Ring Modifications

To study the influence of steric bulk around the A,B ring fusion on the pharmacological actions of 5α and 5β-reduced steroids, a series of 20-ketopregnanes and 17β-cyanoandrostanes (**15**, **209-219**) were prepared with different

**Table 6. Influence of Substitution at the A/B Ring Junction on Electrophysiological Effects on $GABA_A$ Receptor Function[a]**

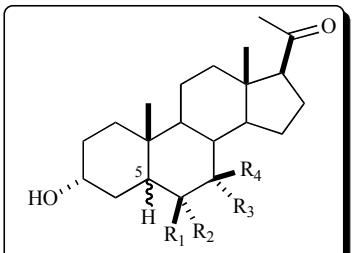



| Steroid | $R_1$ | $R_2$ | $R_3$ | Potentiation[b] (steroid 10μM) |
|---|---|---|---|---|
| **1** | $CH_3$ | αH | $COCH_3$ | 1111 ± 178 |
| **209** | H | αH | $COCH_3$ | 926 ± 101 |
| **210** | H | α$CH_3$ | $COCH_3$ | 110 ± 6 |
| **211** | $CH_3$ | α$CH_3$ | $COCH_3$ | 294 ± 19 |
| **15** | $CH_3$ | αH | CN | 1829 ± 272 |
| **212** | H | αH | CN | 1721 ± 182 |
| **213** | H | α$CH_3$ | CN | 92 ± 3 |
| **2** | $CH_3$ | βH | $COCH_3$ | 1023 ± 192 |
| **214** | H | βH | $COCH_3$ | 754 ± 84 |
| **215** | H | β$CH_3$ | $COCH_3$ | 652 ± 83 |
| **216** | $CH_3$ | β$CH_3$ | $COCH_3$ | 498 ± 121 |
| **217** | $CH_3$ | βH | CN | 1297 ± 251 |
| **218** | H | βH | CN | 1823 ± 360 |
| **219** | H | β$CH_3$ | CN | 452 ± 75 |

[a]Data taken from reference [44].
[b]Percent response relative to current produced by GABA (2 μM).

methyl group substitution patterns at C-5 and C-10 [44]. Each compound was evaluated on potentiation of the GABA-mediated current (Table **6**) and for its ability to initiate a current in the absence of GABA.

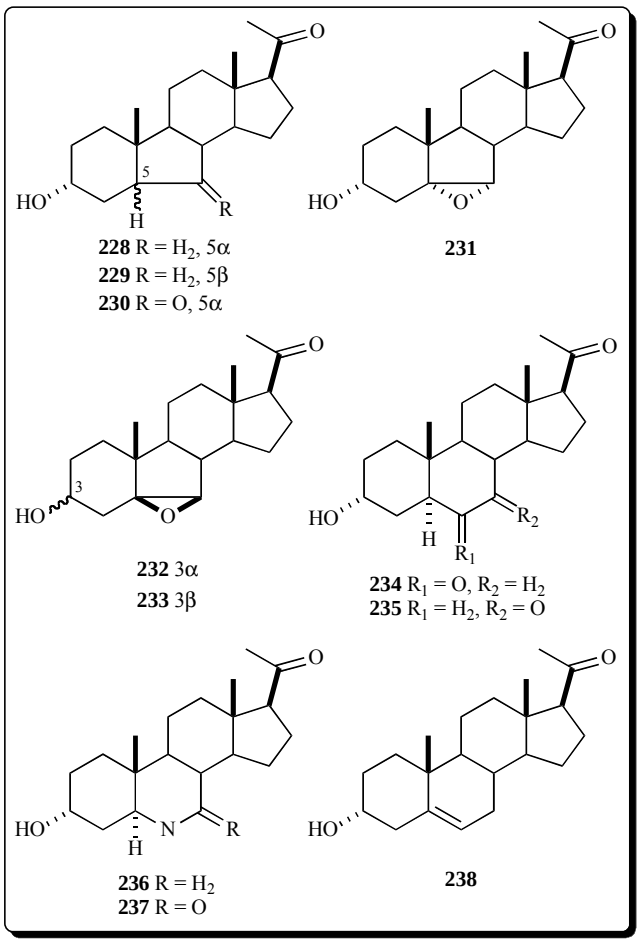
Removal of the methyl group at C-10 had no significant effect on the activity of 5α- or 5β-reduced 20-ketosteroids (**209** and **214**), whereas the presence of a methyl group at C-5 decreased this activity (**211** and **216**). The latter effect was less evident in the 5β series. The potentiating effects of 17β-carbonitriles were closely parallel to those found for the corresponding 20-ketosteroids. These results suggested that there are steric restrictions for the space that can be occupied in 5α-and 5β-reduced steroid modulators of $GABA_A$ receptors in the region of space around C-5, with the 5α substitution being the most unfavorable. To determine how structural modifications at the C-6 and C-7 positions affected the activities of allopregnanolone (**1**) and pregnanolone (**2**), an axial or equatorial methyl group was introduced at these positions, and the analogues were evaluated in [$^{35}$S]TBPS binding experiments, in electrophysiological experiments and as tadpole anesthetics [45].



**220** $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, 5α
**221** $R_2 = CH_3$, $R_1 = R_3 = R_4 = H$, 5α
**222** $R_3 = CH_3$, $R_1 = R_2 = R_4 = H$, 5α
**223** $R_4 = CH_3$, $R_1 = R_2 = R_3 = H$, 5α
**224** $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$, 5β
**225** $R_2 = CH_3$, $R_1 = R_3 = R_4 = H$, 5β
**226** $R_3 = CH_3$, $R_1 = R_2 = R_4 = H$, 5β
**227** $R_4 = CH_3$, $R_1 = R_2 = R_3 = H$, 5β

The results of the binding experiments showed that introduction of a 6β-methyl group on allopregnanolone (**220**) caused a 13-fold enhancement in potency as an inhibitor. The same substitution in the 5β series (**224**) had a smaller but still favorable effect. By contrast, an additional 6α-, 7β- or 7α-methyl group caused a large decrease in activity in both series (**221-223**, **225-227**). The largest decrease in inhibitory potency was found for the 7α-methyl steroid **222** in the 5α series (81-fold). In the 5β series introduction of a 6α-methyl had the most deleterious effect (**225**). These results suggest a remarkable similarity in the shape of the $GABA_A$ receptor binding sites for steroids **1** or **2** in the region interacting with the outer part of ring B.

Taking into account that the enzymatic system for the reduction of progesterone (**74**) into allopregnanolone (**1**) is present in the body and could convert allopregnanolone back into progestins causing complications in the overall hormone equilibrium, Kasal and coworkers prepared the B-nor analogues of allopregnanolone (**1**) and pregnanolone (**2**), compounds **228** and **229** respectively [46]. Their eventual oxidation at position 3 would lead to dihydro-B-norprogesterone or B-norprogesterone, which do not exert any significant gestagenic activity [47].



**228** R = $H_2$, 5α
**229** R = $H_2$, 5β
**230** R = O, 5α

**231**

**232** 3α
**233** 3β

**234** $R_1$ = O, $R_2$ = $H_2$
**235** $R_1$ = $H_2$, $R_2$ = O

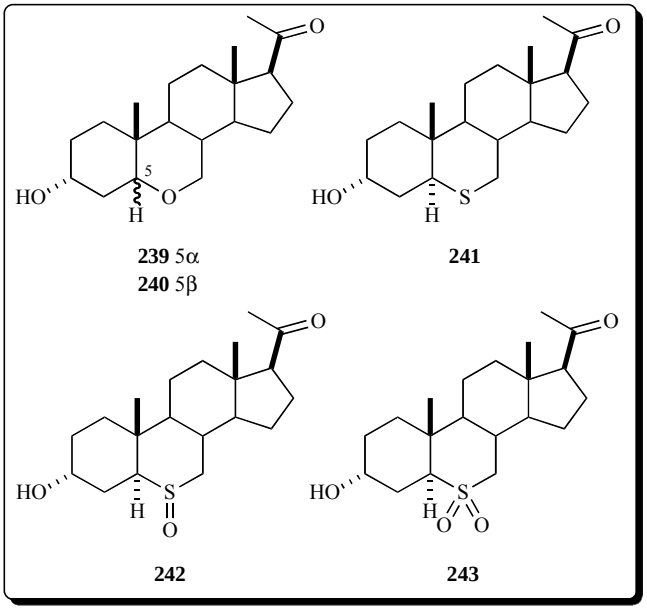
**236** R = $H_2$
**237** R = O

**238**

These compounds were found to stimulate [$^3$H]flunitrazepam binding and GABA-induced $^{36}Cl^-$ influx. However, other B-nor-analogues carrying electronegative substituents in the B ring (compounds **230-233**) were inactive. This was in agreement with the results obtained with B-normal ketones **234** and **235** and the 6-azasteroids **236** and 2**37**, which showed a poor activity. On the other hand, the B-unsaturated analogue **238** potentiated the binding of [$^3$H]flunitrazepam.

The results obtained with the 6-azasteroids **236** and **237** are consistent with the effect of 6-oxasteroids **239** and **240** on the binding of [$^3$H]flunitrazepam, [$^3$H]muscimol and [$^{35}$S]TBPS [48]. Compound **239**, identical in all respects to allopregnanolone (**1**) except for the replacement of the 6-methylene group by an oxygen atom, showed a potency for the stimulation of [$^3$H]flunitrazepam, [$^3$H]muscimol or the inhibition of [$^{35}$S]TBPS binding that was about two orders of magnitude smaller than that of **1**. Compared with pregnanolone (**2**), compound **240** also exhibited at least a 100-fold reduced potency in all the bindings mentioned above. On the other hand the 6-thiasteroids **241-243** showed a displacement pattern for [$^3$H]flunitrazepam, [$^3$H]muscimol and [$^{35}$S]TBPS similar to that observed for allopregnanolone (**1**) [49]. On the basis of these results, the decrease in activity observed for the 6-aza (**236-237**) and 6-oxa (**239-240**) analogues may be related to their hydrogen bonding acceptor

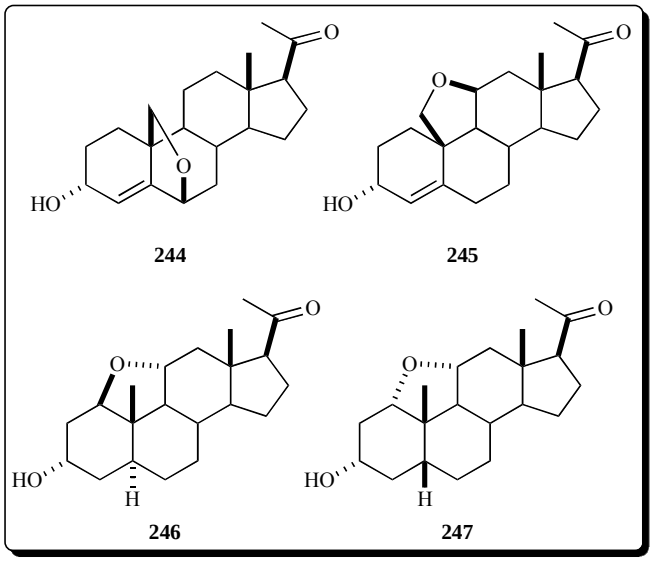
capacity at position 6 that could favor an alternate futile binding mode. The small change in activity observed upon oxidation to the sulfoxide and sulfone analogues **242** and **243** indicated that lipophilicity and electrostatic potential changes in the vicinity of position 6 were not critical for $GABA_A$ receptor activity.




**239** 5α
**240** 5β
**241**
**242**
**243**

### 3.4. Bridged Analogues

In the search for conformationally restricted analogues that could mimic the molecular shapes of allopregnanolone (**1**) and pregnanolone (**2**), compounds **244-247** were prepared [50,51]. These incorporate oxygen bridges involving selected carbons of the steroid nucleus that can change in a controlled way its overall shape.



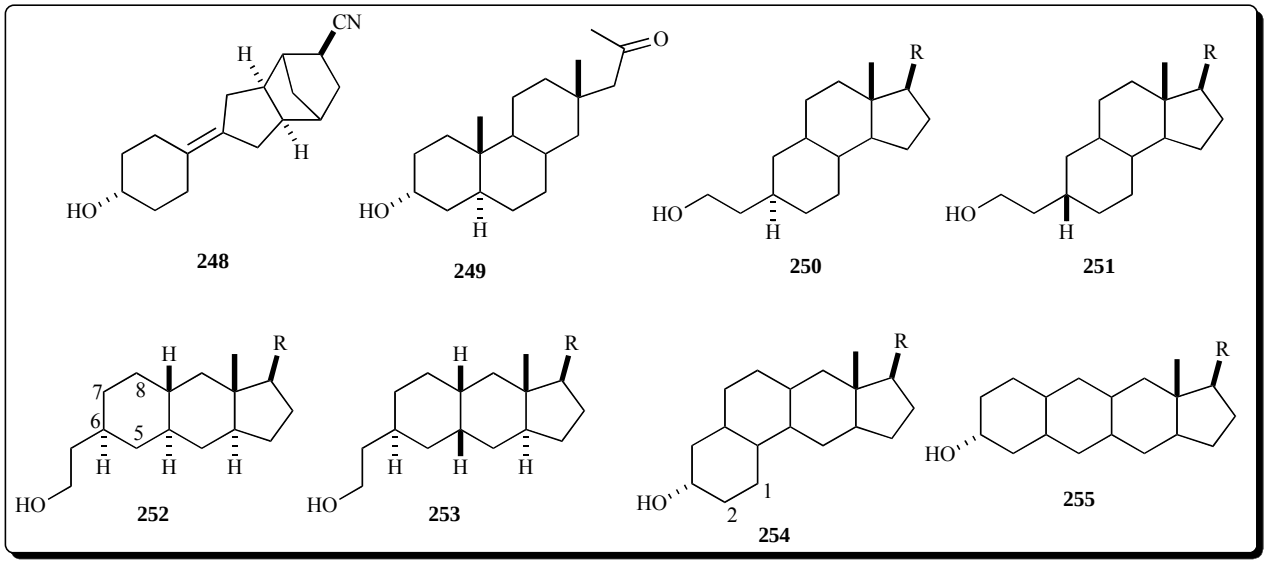

**244**
**245**
**246**
**247**

The 6,19-epoxysteroid **244** has ring A highly torsioned towards the α-face and an overall shape close to that of pregnanolone. This compound significantly increased GABA induced $^{36}Cl^-$ influx in hamster cerebral cortex synaptoneurosomes being more active than **2**. At 20 mg/kg, compound **244** decreased the percentage of hamsters showing seizures induced by 3-mercaptopropionic acid [50]. The enhanced activity of **244** is in consonance with the favorable effects observed for 6β-substitution in the conformationally analogous 5β series (e.g. **224**). $GABA_A$ receptor activity for compounds **245-247** was evaluated by assaying their effect on the binding of [$^3$H]TBOB [51]. Compound **245** ($IC_{50}$ = 518 ± 68 nM), displayed an activity similar to allopregnanolone (**1**; $IC_{50}$ = 329 ± 13 nM); although conformationally related to **1**, the 11,19-epoxypregnane **245** was flatter, had the 3-hydroxyl displaced from the axial position and a larger distance between the 3-hydroxyl and the C-20 carbonyl. C-11/C-1 epoxy bridges shift the A ring towards the C ring of the steroid nucleus with C-3 moving to a position close to that of C-2 in a normal non-bridged steroid. Ab initio calculations showed that in spite of this shift, the overall conformation as well as the conformation of ring A is similar to the corresponding non-bridged pregnanes. Compound **246** ($IC_{50}$ = 741 ± 300 nM), structurally related to **1** and **245,** was less active than these compounds. On the other hand, compound **247** ($IC_{50}$ = 420 ± 48 nM), structurally related to **2** ($IC_{50}$ = 830 ± 11 nM), was more active than the latter steroid with values similar to those of **1**. Thus in this case, the more compact and rigid structure produced by the additional ring, resulted in a more favorable spatial arrangement for binding to the pregnanolone site.

### 3.5. Nonsteroidal Analogues

An attempt to replace the steroid skeleton with an alicyclic framework gave compound **248** which retained weak potentiating activity on the binding of the $GABA_A$ receptor agonist [$^3$H]muscimol to rat synaptic membranes [52]. Covey and coworkers prepared and examined a series of nonsteroidal analogues to assess the importance of the steroid ring system in mediating hydrophobic interactions with the $GABA_A$ receptor. These nonsteroidal analogues included perhydro benz[*e*]indenes (**250-251**), benz[*f*]indenes (**252-253**), phenanthrenes (**254**) and anthracenes (**255**) with the typical side chains known to give favorable activity (e.g. R = acetyl, cyano, ketone) [6,54]. Perhydro phenanthrene derivatives are steroid analogues in which ring D is missing (e.g. compound **249**); these compounds had reduced potency relative to the corresponding steroids [53].

Perhydro benz[*e*]indenes (**250-251**) are steroid-like molecules in which the A-ring has been opened and partially removed, giving the molecule considerable flexibility at the position occupied by the critical 3α hydrogen bond donor. Certain benz[*e*]indenes were potent and effective modulators of $GABA_A$ receptor function, showing in some cases potentiating and inhibitory effects. The authors explained these dual effects considering that, due to its greater flexibility, the 3-hydroxy group in benz[*e*]indenes could mimic steroids having either 3α or 3β hydroxyls. Perhydro benz[*f*]indenes (**252-253**) are tricyclic steroid analogues in which the rings are aligned in a linear fused carbocyclic structure.[54] While certain *trans-trans* benz[*f*]indenes (e.g. **252**) showed activity as GABA potentiators, nonplanar *cis-trans* compounds (e.g. **253**) had little activity on the $GABA_A$ receptor. Interestingly, benz[*e*]indenes were effectively antagonized by 17PA whereas the *trans-trans* benz[*f*]indenes were not, suggesting that they interacted with different binding sites. The authors




considered that several factors could contribute to the diminished actions of the *trans-trans* benz[*f*]indenes in comparison with benz[*e*]indenes: a) the flexibility of the hydroxylated chain on C-6, which could be assuming conformations that were not adequate for interacting with the receptor; b) the location of C-7 and C-8 in a forbidden region of space; or c) the loss of a favorable hydrophobic contact at the C-6/C-7 edge of the steroid, which was considered a critical interaction with the receptor or the lipids surrounding it [46]. To better understand the behavior of *trans-trans* benz[*f*]indenes the authors synthesized a series of cyclopenta[*b*]phenanthrene (**254**) and cyclopenta[*b*]anthracene analogues (**255**) [54]. Cyclopenta[*b*]phenanthrenes are tetracyclic compounds with a nonlinear ring system different from that of steroids, and cyclopenta[*b*]anthracenes are tetracyclic molecules with a linear 6-6-6-5 carbocyclic ring system. These tetracyclic compounds were properly substituted to satisfy the pharmacophore requirements of the critical hydrogen-bond donor and acceptor groups found in neuroactive steroids. Cyclopenta[*b*]phenanthrene analogues are similar to benz[*f*]indenes (they have the same linear 6-6-5 carbocyclic framework), but instead of the flexible hydroxyethyl side chain, they have a fourth staggered ring that compensates for the steroid A ring. Though the hydrophobic framework of the cyclopenta[*b*]anthracene analogues was different from that of steroids, the geometrical relationships between the hydrogen-bonding groups within each molecule could be similar when both compounds were properly aligned. Cyclopenta[*b*]phenanthrene or cyclopenta[*b*]anthracene analogues had potent pharmacological activity in the electrophysiological, binding and tadpole anesthesia assays. In comparative studies with steroids, benz[*e*]indenes and benz[*f*]indenes, the rank order for positive modulation of $GABA_A$ receptor was: steroids = benz[*e*]indenes = cyclopenta[*b*]phenanthrenes = cyclopenta[*b*]anthracenes > benz[*f*]indenes. Comparing the high activity of the cyclopenta[*b*]phenanthrene system (**254**) with the low activity of the benz[*f*]indene system (**252**, **253**), the authors suggested that the space occupied by the C-7 and C-8 carbons of the benz[*f*]indenes (carbons C-5 and C-6 for the cyclophenanthrenes) could be accommodated by the receptor. This finding is in agreement with the activities observed for 1,11-epoxysteroids (**246**, **247**) that have oxygen bridges occupying that region of space [51]. In addition, the C-6/C-7 edge of the steroid B ring did not appear to provide a critical hydrophobic interaction with the receptor or the lipid surrounding the receptor, as the potent cyclopenta[*b*]phenanthrene system and the weak benz[*f*]indene system both lack this region [54]. Once again these results are consistent with those obtained with steroidal analogues having a modified ring B [46,49]. The low activity of benz[*f*]indenes compared with the activity of cyclopenta[*b*]phenanthrenes and benz[e]indenes could originate in the bottom portion of the steroid A ring, i.e. carbons C-4 and C-5. If these carbons were important in the interaction with the $GABA_A$ receptor, the benz[*e*]indene analogues, with their flexible hydroxyethyl side chain, may assume a conformation that closely mimics the bottom portion of the steroid A ring. On the other hand, the hydroxyethyl side chain on carbon C-6 of the benz[*f*] indenes could not occupy the same space as steroid ring carbons C-4 and C-5. In the cyclopenta[*b*]phenanthrenes carbons C-1 and C-2 correspond to positions 4 and 5 in the steroid, respectively [54].

## 4. FINAL REMARKS

Although the existence of defined and specific binding sites for NAS on the $GABA_A$ receptor is now a settled issue, the exact number and specific functions of these sites are still standing questions. Current evidence points to three or possibly four NAS binding sites, two of which would be related to positive modulation of the receptor and the others to inhibitory effects [55]. The former sites have been further classified into a potentiating site and an activating site with distinct pharmacophores which nevertheless share common features. The parallelism in structure-activity relationships observed for 5α and 5β-reduced steroids point to a common binding site with alternative anchor points for the region around ring A, at least for the potentiating effects. In consonance with this, mutations to the putative steroid binding site in the $GABA_A$ receptor diminished channel potentiation by both types of steroids [7].

Despite the large number of analogues synthesized, our knowledge of the binding mode of NAS is still limited. Structure-activity relationships are still our best source for understanding the interactions of NAS with the $GABA_A$ receptor. The basic pharmacophore derived from them, consisting of a hydrogen-bond-accepting group such as acetyl or carbonitrile in a pseudoequatorial orientation at the 17β position and a hydrogen-bond-donating hydroxyl group in a 3α configuration, has been refined with a more detailed understanding of the hydrophobic interactions elicited by the steroid ring system. Thus it has been shown that the hydrophobic contacts involving the C-6/C-7 edge of the steroid B ring are not necessary while those provided by the steroid A ring, especially the C-4/C-5 portion, are critical for interaction with the $GABA_A$ receptor [46,54]. Activity data from several series of nonsteroidal derivatives (benz[*e*]indenes, cyclopenta[*b*]phenanthrenes and cyclopenta[*b*]anthracenes) showed that the steroid binding pocket does not require interactions with a full steroid backbone, and this is consistent with results obtained on bridged steroids with distorted skeletons [51]. On the other hand, the lack of activity observed for 6-aza and 6-oxa (but not 6-thia) analogues and the different effects of substituents at positions 6 and 7, indicate that interactions (favorable or unfavorable) of parts of the receptor in the neighborhood of this region of the steroid nucleus, although not critical, can eventually gain importance in determining the activity of synthetic analogues. Further structure-activity relationship studies are needed to better characterize these interactions.

The lack of any crystal structures for the $GABA_A$ receptor has hampered more detailed studies of the steroid binding modes. Recently, homology models have been generated based on the nicotinic acetylcholine receptor that are being used as a first approach to model ligand binding [55-57]. Future work in this direction will help to identify and characterize the NAS binding sites, thus providing a molecular template that can be used for developing new entities with therapeutical potential and for manipulating receptor function.

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