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Activity of B-Nor Analogues of Neurosteroids on the $GABA_A$ Receptor in Primary Neuronal Cultures

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A $GABA_A$ receptor study of several B-nor analogues of allopregnanolone and pregnanolone has been carried out. B-Norallopregnanolone (i.e., 3α-hydroxy-7-nor-5α-pregnan-20-one) was found comparable to allopregnanolone when measured with labeled TBPS. Analogous results were obtained from their effect on neurons in culture: this time, both 3α-hydroxy-7-nor-5ξ-pregnan-20-ones (**5** and **6**) were found to stimulate [^{3}H]flunitrazepam binding and GABA-induced $^{36}Cl^-$ influx. These effects were inhibited by $GABA_A$ receptor antagonists. Other analogues carrying electronegative substituents (epoxides **9** and **10** and ketone **12**) in the B ring were inactive. Similarly, B-normal ketones **17**, and **18** and 6-azasteroids **20** and **21** were also inactive. B-Nor analogues **5** and **6** did not induce neurotoxicity at relevant concentrations. A computational analysis of active and inactive neurosteroid analogues allowed the proposal of a 3D pharmacophoric hypothesis of their interaction with the $GABA_A$ receptor.

Introduction

Neurons, using neurotransmitter receptor systems, are mainly excited by neurotransmitter glutamate and inhibited by neurotransmitter γ-aminobutyric acid (GABA), which is the commonest message-altering neurotransmitter in the brain. The inhibition of the transport of signals through the neural system reduces sensations of pain, anxiety, and epileptic seizure susceptibility, and thus, compounds modulating GABA neurotransmission are potentially useful in human medicine as analgesic, anesthetic, anxiolytic, and anticonvulsant agents. The major receptor of γ-aminobutyric acid ($GABA_A$ receptor) is known to be modulated by therapeutic drugs, such as benzodiazepines, barbiturates, neurosteroids, and steroidal anesthetics as well as depressant and convulsant toxic agents;[1–6] also, the effect of alcohol on human behavior is interpreted[7] as a GABA receptor matter. The positive modulators of $GABA_A$ receptors potentiate the actions of GABA at the receptor and some of them may also directly gate the channel and act even in the absence of GABA, though only at higher doses.[8,9] Neurosteroids, such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, **1**), 3α-hydroxy-5β-pregnan-20-one (pregnanolone, **2**), and their 21-hydroxy derivatives, that is, 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydrodeoxycorticosterone, **3**) and 3α,21-dihydroxy-5β-pregnan-20-one (tetrahydrodeoxycorticosterone, **4**), have been proven to be anticonvulsant, anesthetic, antidepressant, and neuroprotectant agents.[10–18] In vitro assays reflect this positive modulation by showing an increase of both the Cl^- influx through the $GABA_A$ receptor and the binding of GABA agonists or benzodiazepines.[8,14,15,19–23] Although acting at higher concentrations than benzodiazepines and barbiturates, neurosteroids are valued[24] because they combine the effects of both and have fewer and less severe side effects. Recent studies[25] on the capacitation of human spermatozoa suggest that there are still other prospects for the use of neurosteroids.

New types of neuroactive steroids have been widely sought, and structure modification of allopregnanolone has been examined in light of the vast family of GABA receptor subtypes within the brain.[23,26–30] Additional metabolic arguments[12] are also worthy of consideration because the application of natural neurosteroids is affected by very fast metabolism; the half-time of allopregnanolone in man is about 16 min.[13,31] The enzymatic system for the reduction of progesterone into allopregnanolone (**1**) is present in the body and could convert allopregnanolone back into progestins; if a long-term treatment were required, this metabolism could cause complications in the patient's overall hormone equilibrium. This is the reason why we produced B-nor analogues of allopregnanolone (**5**) and pregnanolone (**6**): their eventual oxidation would lead to dihydro-B-norprogesterone or B-norprogesterone, which do not exert any significant gestagenic activity.[32]

In the present work, the activity of several B-nor synthesized analogues of neurosteroids at the $GABA_A$ receptor was evaluated by determining their activity on $^{36}Cl^-$ flux and [^{3}H]flunitrazepam binding in intact neuronal cells by using primary cultures of mouse cortical neurons. Primary neocortical cultures are enriched in GABAergic neurons and express functional $GABA_A$ receptors both in terms of GABA-induced chloride flux and allosteric modulations among the different[9,32,33] recognitions sites, resembling the native structure of the $GABA_A$ receptor found in vivo. A computational analysis of the structures of the active and inactive neurosteroid analogues reported here and others found in the literature allowed the proposal of pharmacophoric models that contain the features required for neurosteroid interaction with the $GABA_A$ receptor. Finally, the active compounds were also tested for their toxicity in neurons.

Results

Synthesis and Biological Activity. The B-nor analogue of allopregnanolone (**5**) and compounds **17** and **19–21** were prepared according to previous experience.[34–38] Oxygen-containing functionalities were introduced into the molecule[37] to increase the solubility of products in aqueous systems (i.e., epoxides **9** and **10**, dione **12**, and diol **16**). The synthesis of

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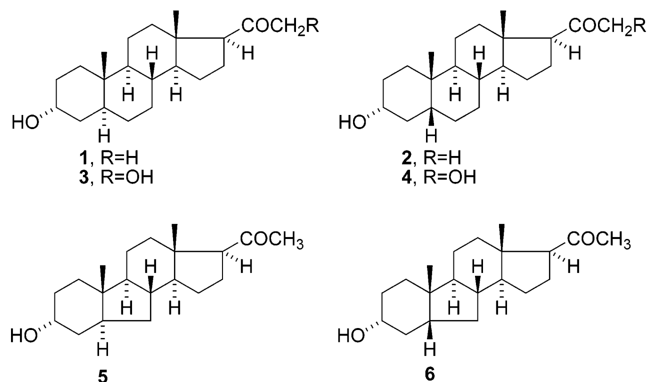


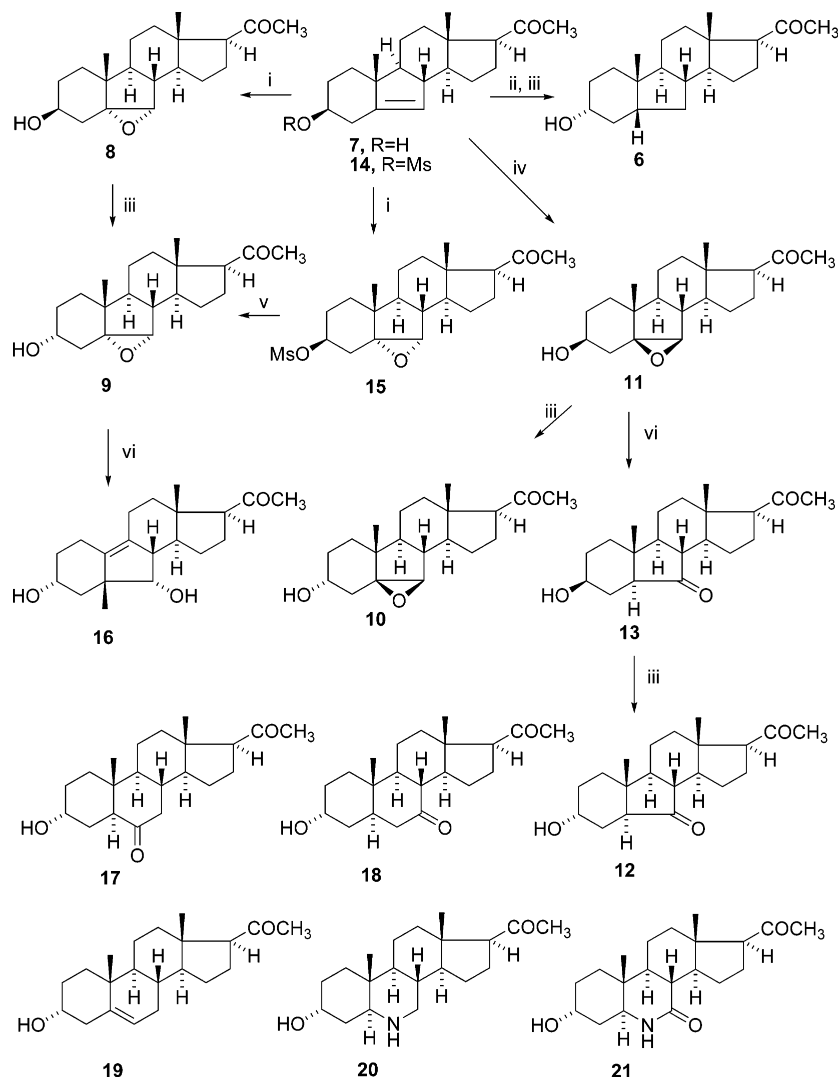
Figure 1. Structures of natural neurosteroids (**1**−**4**) and some of their B-nor analogues (**5** and **6**).

new compounds (**6**, **16**, and **18**), based on analogous reactions, is described in the Experimental Section. The key step, that is, the conversion of 3β-alcohols into 3α-alcohols, was mostly carried out using the Mitsunobu reaction or, in some cases, by solvolysis of the corresponding mesylate (epoxide **9** in the Experimental Section). Chemical structures of the compounds used in this work are shown in Figure 1 and Scheme 1. The configuration at individual carbons was controlled by known reaction mechanisms and checked by ^{1}H NMR spectroscopy. Thus, the inversion of the 3β-OH configuration (compounds **9**, **10**, and **12**) or the hydrogenation of olefin **7** was verified by an analysis of the H-3 signals. The Westphalen rearrangement, operating in the formation of compound **16**, was also proved by the inspection of ^{1}H NMR spectra.

Neurosteroids as positive allosteric modulators of GABA$_A$ receptors are known to enhance [^{3}H]muscimol and [^{3}H]-flunitrazepam binding in brain membranes and reduce [^{35}S] TBPS binding.[22,39,40] We have already found that allopregnanolone (**1**) increases [^{3}H]muscimol binding up to 123% and reduces [^{35}S]TBPS binding to 21%. In this work, using rat brain membranes, compound **5** significantly inhibited [^{35}S]TBPS binding to 33% at 10 μM, thus suggesting that B-nor compounds may interact with the GABA$_A$ receptor.[41]

To determine whether B-nor analogues could be positive allosteric modulators of the GABA$_A$ receptor, we measured [^{3}H]-flunitrazepam binding and GABA-induced $^{36}Cl^-$ influx in the presence of different concentrations of these compounds in primary cultures of intact living cortical neurons. Table 1 summarizes the effects of the parent compounds allopregnanolone (**1**) and pregnanolone (**2**) and a series of B-nor

Scheme 1[a]



[a] (i) 3-chloroperoxybenzoic acid in CH_2Cl_2 at 0 °C; (ii) H_2/Pd/EtOH; (iii) HCOOH, N_2COOEt and $(C_6H_5)_3P$ in toluene at 0 °C; then hydrolysis with $KHCO_3$ in methanol; (iv) N-bromoacetamide and $HClO_4$ in dioxane at 15 °C; then K_2CO_3 in methanol; (v) KNO_2 in DMSO at 120 °C for 90 min; (vi) $BF_3.Et_2O$ in THF.

Table 1. Effect of Natural Neurosteroids and the Synthetic Analogs Studied in This Work on [^{3}H]flunitrazepam Binding in Primary Cultures of Cortical Neurons[a]

	log EC_{50}	EC_{50} (μM)	$E_{10\mu M}$ (% of basal)
1, allopregnanolone	-6.06 ± 0.29	0.88	164 ± 11
2, pregnanolone	-5.93 ± 0.16	1.18	162 ± 10
	five-membered B ring (B-nor)		
5	-6.61 ± 0.16	0.24	199 ± 22
9			103 ± 10
12			82 ± 11
6	-6.03 ± 0.39	0.94	172 ± 6
10			104 ± 10
11			99 ± 4
16			76 ± 1
	six-membered B-ring		
17			100 ± 4
18			90 ± 4
19	n.d.[b]	n.d.	131 ± 5
20			86 ± 4
21			81 ± 3

[a] The log EC_{50} values are expressed as mean ± SEM values of three to five independent concentration−response curves. The $E_{10\mu M}$ values represent the effect of 10 μM concentrations with respect to basal binding. [b] n.d.: not determined.

analogues (**5**, **6**, **9**−**12**, **16**) on [^{3}H]flunitrazepam binding. Compounds **1**, **2**, **5**, and **6** increased [^{3}H]flunitrazepam binding in a concentration-dependent manner in primary cultures of cortical neurons (Figure 2), in agreement with data obtained in [^{3}H]muscimol and [^{35}S]TBPS binding assays using brain homogenates.[41] The order of potency for the active compounds was **5** > **1** = **2** and **6**, as evidenced by their EC_{50} values and their maximum effects over basal binding (Table 1 and Figure 2A and B). To demonstrate that the increase of [^{3}H]flunitrazepam binding was $GABA_A$-receptor mediated, compound **5** was tested in the presence of $GABA_A$ receptor antagonists picrotoxinin and bicuculline.

Figure 2C shows that both bicuculline and picrotoxinin completely inhibited the effect of compound **5** on [^{3}H]flunitrazepam binding, as they do on the effect of GABA or other agents that activate the $GABA_A$ receptor.[9,33,42] However, compounds **9**, **10**, **12**, and **16** were inactive on [^{3}H]flunitrazepam binding (Table 1). We also tested the effect of the 3β-alcohol **11**. As expected, because of its 3β configuration, it did not modify the binding of [^{3}H]flunitrazepam[43] (Table 1).

The common feature of the inactive compounds **9**, **10**, **12**, and **16** was the presence of an oxygen atom in the B ring. For comparison, we produced the 6- and 7-oxo derivatives of allopregnanolone (**17** and **18**) as well as the nitrogen-containing analogues **20** and **21**. None of these compounds increased flunitrazepam binding (Table 1). It is worth mentioning that Nicoletti[44] had previously reported a much reduced capacity to modulate flunitrazepam binding to the $GABA_A$ receptor for two closely related 6-oxa-analogues **22** and **23** (Figure 3). However, the introduction of a double bond in the six-membered B ring (**19**) still rendered an active compound (Table 1).

Further assessment of the positive modulatory effects of compounds **5** and **6** on $GABA_A$ receptors was obtained by determining their effects on GABA-induced Cl^- influx in cultured cortical neurons. Figure 4A shows that compounds **5** and **6** potentiated the $^{36}Cl^-$ uptake induced by a submaximal concentration of GABA (5 μM, a concentration that produces about 30% of the maximal effect of GABA in cortical neurons[9,45]). Again, picrotoxinin significantly inhibited the effect of compound **5** on GABA-induced Cl^- influx (Figure 4B). Furthermore, 10 μM compounds **5** and **6** activated Cl^- influx

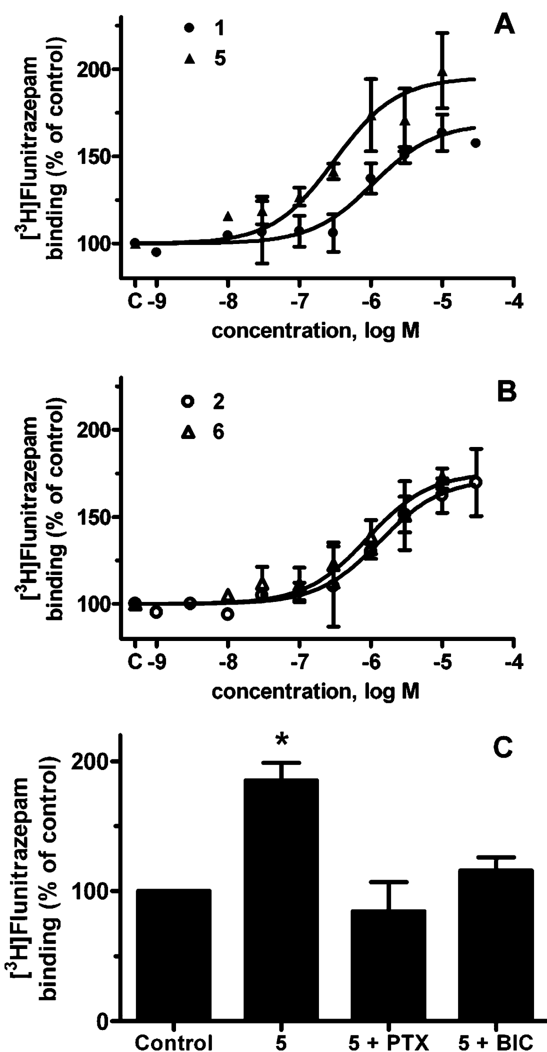


Figure 2. Effect of neurosteroid analogues on [^{3}H]flunitrazepam binding in primary cultures of cortical neurons. (A) Concentration−response curves for allopregnanolone (**1**) and its B-nor analogue (**5**). (B) Concentration−response curves for pregnanolone (**2**) and its B-nor analogue (**6**). (C) Inhibitory effects of bicuculline (BIC) and picrotoxinin (PTX) on the potentiating effect of compound **5**. Data are expressed as percentage of control binding in the absence of neurosteroids. Results are mean ± SEM of three to four independent experiments, each performed in triplicate. *p < 0.01 vs control binding.

in the absence of GABA to 1.7 ± 0.2 and 1.7 ± 0.1 times that of the basal uptake, respectively.

Cell viability was also determined in the above neuronal cultures. They were treated with compounds **1**, **5**, and **6** at a concentration that produced the maximum effect on the $GABA_A$ receptor (10 μM) and also at a concentration that was 10 times higher (100 μM). Figure 5 shows that exposure to 10 μM for up to 72 h and to 100 μM for up to 24 h did not induce the loss of cell viability. However, the latter concentration (100 μM) resulted in significant cell injury after 72 h, even for the endogenous neurosteroid (compound **1**). Nevertheless, it should be realized that this concentration is much higher than those effective at the $GABA_A$ receptor (EC_{50} ~1 μM; Table 1 and Figures 2 and 4).

Computational Studies. The molecular alignment of the above-mentioned active neurosteroids (**1**, **2**, **5**, **6**, and **19**) showed that despite the changes in the configuration of the bridge C-5 atom, the size of the B ring, or the presence of a double bond in the same ring it is possible to achieve a good superposition of the molecules (average RMSD of common heavy atoms ~0.5

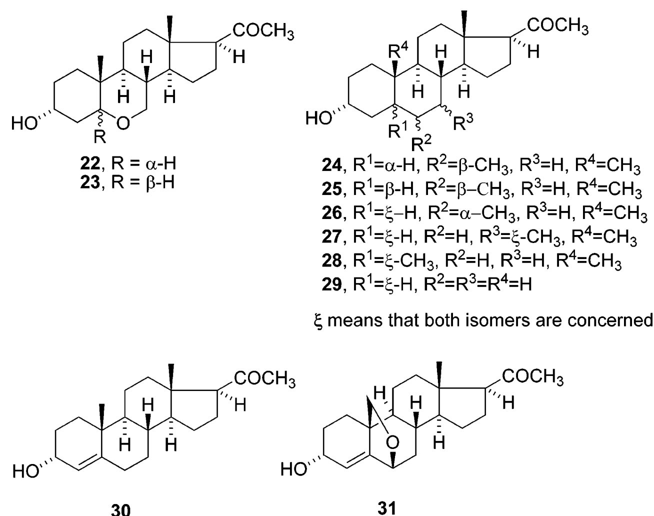


Figure 3. Structures of reported active and inactive neurosteroid analogues considered in this work (see reported activities in Table 1 of Supporting Information).

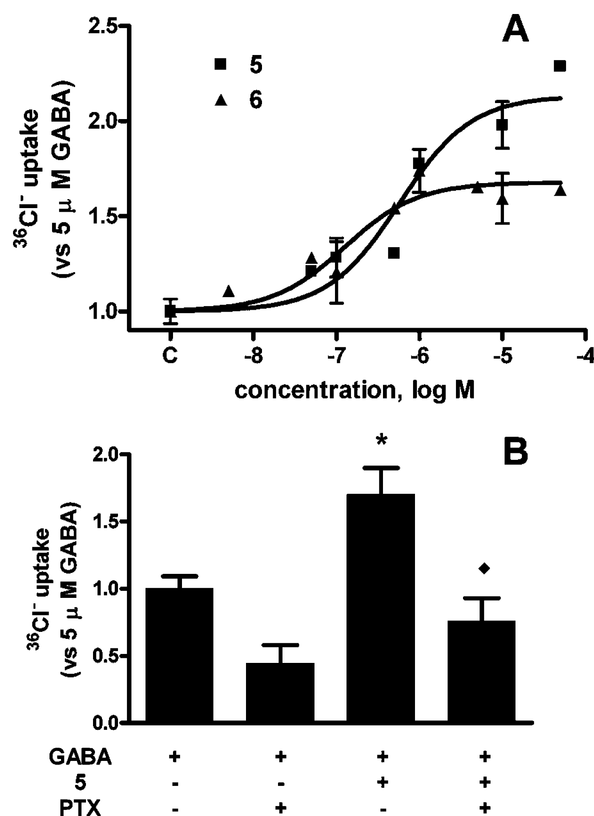


Figure 4. Effect of B-nor neurosteroid analogues **5** and **6** on GABA-induced Cl^- flux in primary cultures of cortical neurons. (A) Concentration−response curves for the potentiating effect of compounds **5** and **6**. (B) Inhibition by picrotoxinin (PTX) of the potentiating effect of compound **5**. Values of Cl^- influx, determined as $^{36}Cl^-$ uptake, were normalized to those corresponding to 5 μM GABA. Results are mean ± SEM of two independent experiments, each performed in triplicate. *$p < 0.05$ vs GABA; ▲p, 0.05 vs compound **5** plus GABA.

Å; see Supporting Information, Figure 1A). The inactive compounds (**9**−**12**, **16**−**18**, **20**, and **21**) could also be aligned reasonably well to the active allopregnanolone (**1**) and pregnanolone (**2**) structures (Supporting Information, Figure 1B and C), particularly in the case of 6-aza-analogue **20**. The isosteric substitution of the 6-methylene group of the pregnane system by nitrogen (**20**) or oxygen (**22**, **23**) does not introduce significant changes in the overall conformation of the steroid nucleus and does not increase its steric bulk. Therefore, this suggests that the lack of activity of these compounds is due, at least in part, to the local changes in hydrophobicity and hydrogen-bonding capacity arising from the presence of the heteroatom.

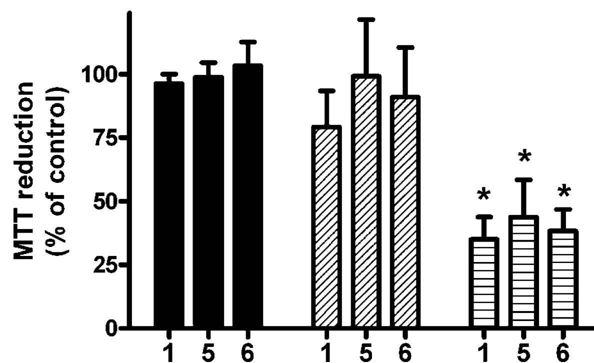


Figure 5. Effect of the exposure to neurosteroid analogues on neuronal viability. The primary cultures of cortical neurons were exposed to 10 μM compounds **1**, **5**, and **6** for 72 h (black bars) or to 100 μM compounds **1**, **5**, and **6** for 24 h (diagonal bars) or 72 h (horizontal bars). Data are expressed as percentage of control values in the absence of neurosteroids. Results are mean ± SEM of two independent experiments, each performed in triplicate. *$p < 0.01$ vs corresponding control cultures.

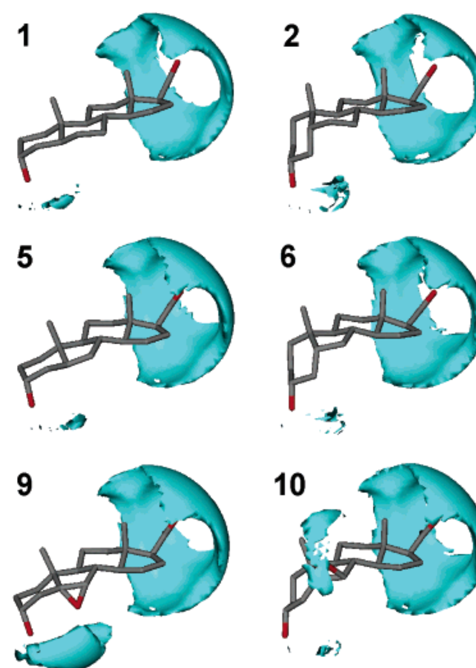


Figure 6. GRID molecular interaction fields determined for compounds **1**, **2**, **5**, **6**, **9**, and **10** with the water probe contoured at −4.5 kcal/mol.

To assess the effects of the presence of oxygen or nitrogen heteroatoms in ring B, or as part of ring B substituents, a 3D molecular interaction field analysis was performed using the program GRID with its standard water probe. The results are illustrated in Figure 6, which shows that the isopotential surface for active neurosteroids **1**, **2**, **5**, and **6**, contoured at −4.5 kcal/mol, is mainly located around the 20-oxo group, with just a small patch close to the 3α-hydroxy group. However, inactive compounds **9** and **10** show, as expected, an additional extended surface close to the C6−C7 edge of ring B, where the interaction with water is favored. Similar distributions of the isopotential surfaces were determined for other active and inactive steroids considered in this work (Supporting Information, Figures 2 and 3). Desolvation energies were calculated to further assess the

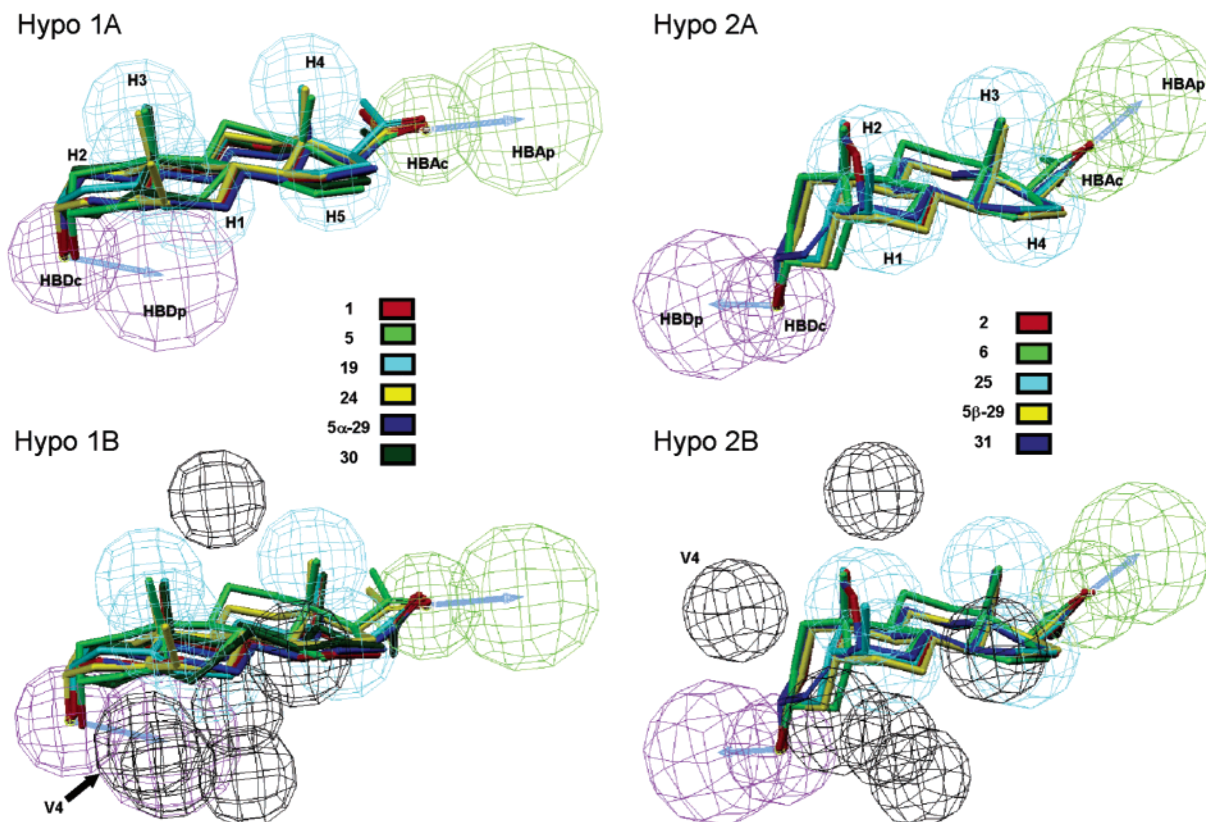


Figure 7. Best alignments of compounds in sets 1 and 2 to pharmacophoric hypotheses Hypo 1A, 1B, 2A, and 2B. The compounds are colored as shown in the figure. (Some are apparently hidden because of the superposition of the structures.) The pharmacophoric features are color coded: hydrophobic features (H1−H5) in cyan, hydrogen bond acceptor (HBA) in green, hydrogen bond donor (HBD) in violet, and exclusion volumes (only the label of V4 is shown for the sake of clarity) in black. Suffixes c and p after HBA and HBD stand for center and projection points, respectively.

hydrophobic profiles of the compounds considered. This resulted in an average estimated desolvation energy cost of about 4.0 kcal/mol for inactive compounds **9**−**12**, **16**−**18**, **20**−**22**, and **23** relative to the active and more hydrophobic steroids **1**, **2**, **5**, **6**, and **19** (Supporting Information, Table 2).

Thus, according to the above observations, a minimal pharmacophoric model for the neurosteroid binding site in the $GABA_A$ receptor should include a hydrophobic feature appropriately located to interact with the C6−C7 edge of ring B as well as the two hydrogen bond forming groups close to the 3α and C-20 positions.[46] The presence of these three features seems essential for activity; however, they are not sufficient to allow discrimination between active and inactive compounds. In this sense, Zeng[30] recently reported the $GABA_A$ modulatory activity for the 6α-, 6*β*-, 7α-, and 7*β*-methyl analogues of allopregnanolone and pregnanolone (**24**−**27**, Figure 3). Their results show that only the 6*β*-methyl substitution (**24**, **25**) yields compounds with activities comparable to or higher than the parent neurosteroids, whereas the other analogues (**26**, **27**) have a much reduced activity. Similarly, the activity of 5-methyl derivative **28** is 2−4 times lower than that of corresponding neurosteroids **1** and **2**, whereas 19-nor derivative **29** is only slightly less active than those.[26] Because these modifications do not introduce significant changes in the hydrophobicity of the molecules (Supporting Information, Figures 2 and3 and Table 2), the differences in the activities of compounds **24**−**29** should probably arise from other factors, that is, unfavorable steric interactions with the receptor. However, compounds with a Δ^4-double bond (as in **30** and **31**) show a similar $GABA_A$ potentiation of activity to compounds **1** and **2**.[39,47] Although the hydrophobicity of compound **30** is similar to that of the active steroids considered here, the presence in compound **31** of an oxygen atom bound to C-6 yields a hydrophobicity profile that is closer to that of the inactive compounds (Supporting Information, Figure 3 and Table 2).

Because there is compelling evidence of the existence of independent binding sites on the $GABA_A$ receptor for pregnanolone and allopregnanolone,[48−50] two sets of active analogues of each neurosteroid were chosen to generate pharmacophoric hypotheses for both sites, which correlate with the activity data mentioned above. A minimum activity criterion was established to ensure that the selected analogues in both sets were the most active of the 5α and 5*β* series of compounds. Thus, set 1 consisted of compounds **1**, **5**, **19**, **24**, **5α-29**, and **30**, whereas set 2 included compounds **2**, **6**, **25**, **5*β*-29**, and **31**. The program Catalyst (Accelrys, Inc.) was used to obtain pharmacophoric models Hypo 1A and Hypo 2A, derived from sets 1 and 2, respectively. The alignments of the compounds in each set to these models are shown in Figure 7, and the fit values of all the compounds considered here are given in Table 2. Hypo 1A includes the three essential pharmacophoric groups mentioned above (i.e., a hydrogen bond donor, HBD, a hydrogen bond acceptor, HBA, and a hydrophobic feature properly located to match the C6−C7 edge of the active aligned neurosteroids, H1) as well as four additional hydrophobic groups (H2−H5) (3D coordinates in Supporting Information, Table 3). However, Hypo 2A is similar but contains only four hydrophobic groups (H1−H4). The fit values from the alignment of molecules in set 1 to model Hypo 1A are close to or higher than 90% of the maximum value (average fit value, AFV = 93%), in good correlation with the observed activities. In particular, compound **24** shows the best fit to the model, being higher than that of allopregnanolone

Table 2. Best Fit Values of Alignment to the Pharmacophoric Models[a]

compd	Hypo 1A	Hypo 1B	Hypo 2A	Hypo 2B
1[b]	9.47	9.24	9.42	7.22
2[c]	8.76	8.06	9.48	9.48
5[b]	8.82	8.79	8.66	8.15
6[c]	8.45	6.59	9.29	9.28
9	7.37	5.97	8.84	5.58
10	6.82	5.84	8.67	6.31
11	7.41	6.41	8.78	5.11
12	7.66	6.29	8.88	5.70
16	6.97	4.75	8.76	5.12
17	7.58	6.27	8.86	5.15
18	7.70	6.27	8.80	6.45
19[b]	9.31	9.12	9.02	5.90
20	7.70	6.33	8.85	5.68
21	7.53	5.70	8.79	4.66
22	7.60	6.33	8.88	5.73
23	7.31	4.97	8.91	6.50
24[b]	10.00	10.00	9.49	8.29
25[c]	9.15	6.74	10.00	10.00
5α-26	9.38	6.34	8.94	6.31
5β-26	8.47	4.63	9.49	6.63
5α,7α-27	9.39	7.70	9.27	7.07
5α,7β-27	9.26	6.62	9.30	6.32
5β,7α-27	8.45	6.42	9.39	7.84
5β,7β-27	7.70	6.27	9.24	6.43
5α-28	9.11	5.90	9.13	5.64
5β-28	8.41	6.61	9.42	4.49
5α-29[b]	8.94	8.81	9.38	6.31
5β-29[c]	8.36	7.28	9.47	9.47
30[b]	9.39	9.24	9.42	9.00
31[c]	4.66	no fit	6.48	6.48

[a] Values are normalized to a maximum fit of 10. [b] The compounds belonging to set 1. [c] The compounds belonging to set 2.

(**1**), which is in agreement with the activities reported for both compounds.[30] Similarly, the fits obtained from the alignment of molecules in set 2 to Hypo 2A are higher than 90% for compounds **2**, **6**, **25**, and **5β-29** (AFV = 96%). In this case, compound **25** shows the best fit to the model. However, compound **31** obtains a much lower fit value, although its alignment is very similar to that obtained for the rest of the compounds in set 2. This is a consequence of the presence of the oxygen atom bound to C-6, which, under the algorithm used by Catalyst, causes a large reduction on the hydrophobicity of the region close by (C6−C7 edge), with a negative contribution to the fit value of the molecule.

The less hydrophobic and inactive analogues of the 5α (**9**, **12**, **17**, **18**, and **20**−**22**) and 5β series (**10**, **11**, **16**, and **23**) could only be partially aligned to Hypo 1A and Hypo 2A (AFV = 76 and 88%, respectively). In addition, their mappings significantly differ from those obtained for reference neurosteroids **1** and **2** (Supporting Information, Figure 4 panels A and D). However, inactive methylated analogues **26**−**28** mapped very well (AFV > 90%) to most of the features present in each hypothesis, as anticipated from their similarity to compounds **1** and **2**. Finally, the cross-alignment of compounds in sets 1 and 2 to models Hypo 2A and Hypo 1A, respectively, yielded AFV values close to ~90%. These results indicated that these pharmacophoric models could not discriminate, in many cases, between active and inactive compounds and between 5α and 5β neurosteroids. This failure was mainly because of the lack of information contained in the models about the steric requirements of the receptor. For this reason, the models were refined to improve their selectivity by the addition of exclusion volumes that reflect regions of space occupied by the receptor and which cannot be occupied by any atom of the ligands. Figure 7 illustrates the result of adding six exclusion volumes (V1−V6) to Hypo 1A and 2A, which yielded models Hypo 1B and 2B (3D coordinates in Supporting Information, Table 3). The location of V1−V4 was adjusted to simulate the steric repulsion of the receptor cavity on the methyl substituents at C5, C6, and C7 of compounds **26**−**28**, which could be responsible for the lack of activity of these neurosteroid analogues. Furthermore, Han[26] rationalized the existence of unfavorable interactions between the C-19 methyl as well as the region of space below the A ring of different neurosteroid analogues and the $GABA_A$ receptor. These were now simulated by the introduction into the hypotheses of two additional exclusion volumes (V5−V6).

The fitting of the above neurosteroid analogues to the new models Hypo 1B and 2B is shown in Table 2. The active analogues in sets 1 and 2 (with the exception of compound **31**) still show good fits to the respective hypothesis (AFV = 92 and 96%, minimum fits = 88 and 93%, respectively); however, their cross-alignment is now worse (set 2 (except **31**) to Hypo 1B: AFV = 72%, maximum fit = 81%; set 1 to Hypo 2B: AFV = 75%, maximum fit = 90%). This is mainly achieved by the presence of one exclusion volume (V4, Figure 7). Furthermore, all of the inactive compounds now have fit values (AFV < 70%, maximum fit = 78%) clearly lower than that of most of the active ones. Attempts to obtain such a discrimination using a smaller number of exclusion volumes were only partially successful. Thus, pharmacophoric hypotheses Hypo 1B and 2B show a much better discrimination between the active and inactive compounds and further suggest a possible model for the existence of similar but different binding sites for neurosteroids **1** and **2** in the $GABA_A$ receptor.

Discussion

The positive modulation of the binding sites for 5α and 5β neurosteroids in the $GABA_A$ receptor by B-norallopregnanolone (**5**) and B-norpregnanolone (**6**) could eventually be useful if a long-term treatment with allopregnanolone were considered. Although the formation of progesterone from allopregnanolone as well as its influence at the hormone status of a patient are conceivable, the possible formation of B-norprogesterone would pose no such danger. Furthermore, these compounds compare very well with allopregnanolone in terms of drug safety for neuronal viability at concentrations that are relevant for their activity at the $GABA_A$ receptor.

Changing the number of carbon atoms in the B ring from six to five does not seem to be critical for the neuronal activity of allopregnanolone analogues at the $GABA_A$ receptor. Further substitution of the B ring was found to play an important role; not a single compound from the tested B-oxygenated analogues was active in any of the tests. The middle part of the molecule was found to be more than an unimportant construction lump; the presence of a lipophilic part in this area obviously has its role in the hydrophobic interactions of a ligand with the $GABA_A$ receptor. It was interesting to compare the situation with the normal six-membered B ring. In agreement with the above statement, neither of the 6- and 7-oxo derivatives **17** and **18** stimulated [^{3}H]flunitrazepam binding. Other compounds with an oxo group in the B ring have been reported to present very low activity at the $GABA_A$ receptor.[44] Contrarily, the modifications of the B ring that do not include the incorporation of heteroatoms such as the B-unsaturated analogue **19** do not preclude its activity at the $GABA_A$ receptor.

The activity of the neurosteroid analogues tested in this work and other reported analogues in the References can be explained in terms of their alignment to two proposed pharmacophoric hypotheses (Hypo 1B and 2B). These two models would reflect some of the structural features required for a good interaction with the proposed two nonequivalent neurosteroid binding sites

present on the $GABA_A$ receptor, namely, the site where the 5α steroids preferentially interact and the site where the 5β steroids preferentially do so.[49,50] The pharmacophoric hypotheses described in this work include three essential requirements, specifically one hydrophobic group, one hydrogen bond donor, and one hydrogen bond acceptor; these must be fulfilled by complementary groups present in the ligands. In addition, the hypotheses include three or four additional hydrophobic features that contribute to binding and up to six exclusion volumes. The latter are required to achieve a good discrimination between the active compounds and the inactive ones and between the active 5α and 5β analogues. The two hypotheses are quite similar in terms of the number and distribution of features. This is in agreement with the observations reported up to date about similar structure−activity relationships (SAR) for the action of 5α- and 5β-anesthetic steroids on $GABA_A$ receptors. Indeed, it is mainly the presence of one of the exclusion volumes in each model that allows some discrimination between the 5α and 5β configurations. Therefore, these pharmacophoric models provide hints about the functional requirements and topology of the binding sites at the $GABA_A$ receptors, particularly in the region that matches with the B ring of the steroid skeleton. Furthermore, they provide a qualitative criterion for high activity, that is, compounds with fit values close to or higher than 90% to any of the models will probably show similar or higher activities than that of reference compounds **1** and **2**. They also suggest the probable site of interaction with the $GABA_A$ receptor, that is, compounds that have a better fit to Hypo 1A than to Hypo 1B probably bind at the same site as allopregnanolone, whereas those with a better fit to Hypo1B probably bind at the pregnanolone site. However, these models should be accepted as preliminary because the diversity of the compounds used to build them was mainly restricted to the B ring area of the steroid system. One exception to the validity of the proposed models was the results obtained with compound **31**, which clearly differ from those obtained for the rest of the active compounds studied. The activity of this compound is already somewhat surprising, given the SAR data available for other neurosteroid analogues.[30] Although this may reflect the fact that further refinement of the models will be necessary to obtain a better and global picture of the 5α- and 5β-neurosteroid binding sites at the $GABA_A$ receptor, it could also suggest a different mechanism of action for compound **31**. Work to improve these models is currently being carried out in our group.

Experimental Section

Chemistry. The melting points were determined on a Boetius micromelting point apparatus (Germany). Optical rotations were measured at 25 °C in chloroform using Autopol IV (Rudolf Research Analytical, Flanders, U.S.A.; $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}). Infrared spectra (wavenumbers in cm^{-1}) were recorded on a Bruker IFS 88 spectrometer. ^1H NMR spectra were taken on Bruker AVANCE-400 instruments (400 MHz, FT mode) at 23 °C in $CDCl_3$ and referenced to TMS as the internal standard. Chemical shifts are given in ppm (δ-scale); the coupling constants (*J*) and width of multiplets (*W*) are given in Hz. Thin-layer chromatography (TLC) was performed on silica gel G (ICN Biochemicals; detection by spraying with concentrated sulfuric acid followed by heating). For column chromatography, Silica gel 60 (Merck, 63−100 μ) was used. Prior to evaporation on a rotary evaporator in vacuo (0.25 kPa, bath temperature 40 °C), solutions in organic solvents were dried over anhydrous $MgSO_4$. 3α-Hydroxy-7-nor-5α-pregnan-20-one (**5**) was prepared from 3β-hydroxy-7-norpregn-5-en-20-one (**7**) by reduction with azodiimide.[36] The epoxidation of olefin **7** yielded epoxide **8**,[37] which was treated under conditions of the Mitsunobu reaction to produce 3α-hydroxy-5,6α-oxido-7-nor-5α-pregnan-20-one (**9**). 3α-Hydroxy-5,6β-oxido-7-nor-5β-pregnan-20-one (**10**) was also prepared from olefin **7**, this time via the corresponding bromohydrin and epoxide **11**.[37] 3α-Hydroxy-7-nor-5α-pregnane-6,20-dione (**12**) was prepared from epoxide **11** via diketone **13**.[37] 3α-Hydroxy-5α-pregnane-6,20-dione (**17**) was prepared according to ref 35. ^1H NMR: δ 0.62 (s, 3H, H-18), 0.74 (s, 3H, H-19), 2.13 (s, 3H, H-21), 2.32 (dd, 1H, *J* = 4.6 and 13.2 Hz, H-7), 2.56 (t, 1H, *J* = 9.0 Hz, H-17), 2.74 (dd, 1H, *J* = 6.1 and 9.4 Hz), 4.18 (m, 1H, *W* = 16.5 Hz, H-3). 3α-Hydroxypregn-5-en-20-one (**19**) was prepared according to ref 34.[34] ^1H NMR: δ 0.64 (s, 3H, H-18), 1.01 (s, 3H, H-19), 2.13 (s, 3H, H-21), 2.55 (t, 1H, *J* = 9.9 Hz, H-17), 2.59 (m, 1H, *W* = 15.8 Hz, H-7), 4.03 (m, 1H, *W* = 21.5 Hz, H-3), 5.41 (m, 1H, *W* = 15.5 Hz, H-6). 6-Aza-3α-hydroxy-5α-pregnan-20-one (**21**) and 6-aza-3α-hydroxy-5α-pregnane-7,20-dione (**20**) were prepared according to ref 38.[38]

3α-Hydroxy-7-nor-5β-pregnan-20-one (6). 3β-Hydroxy-7-nor-pregn-5-en-20-one[34] (**7**, 200 mg, 0.66 mmol) was hydrogenated in ethanol (10 mL) in the presence of palladium on carbon (5%, 50 mg) at laboratory temperature. After 5 h, the product was purified by TLC (20% ether in benzene), and a more lipophilic fraction, the 5β-product (δ 4.04, W = 31.0 Hz, H-3; 113 mg), was then submitted to the Mitsunobu reaction[51] using triphenylphosphine (609 mg, 2.32 mmol), diethyl azodicarboxylate (20 drops, 2.87 mmol) in toluene (10 mL), and formic acid (6 drops, 2.63 mmol). After 2 h, the formate formed was hydrolyzed with $KHCO_3$ (100 mg, 1.0 mmol) in methanol (16 mL) and water (1 mL) at 60 °C. After 1 h, the product was purified by preparative thin-layer chromatography (toluene/ether, 3:1). Elution of the more lipophilic zone afforded compound **6** as white crystals. Yield: 99 mg (49%). Mp 105−107 °C (acetone and heptane). $[\alpha]_D$ +88° ($CHCl_3$, c 0.7). IR: 3609, 3459, 1050 (OH), 1697, 1358 ($COCH_3$). ^1H NMR: δ 0.60 (s, 3H, H-18); 0.80 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.55 (t, 1H, *J* = 9.3 Hz, H-17); 3.69 (m, 1H, *W* = 35.0 Hz, H-3). ^{13}C NMR: δ 13.82 (C-18), 21.23 (C-11), 23.16 (C-16), 23.98 (C-19), 24.69 (C-15), 30.36 (C-2), 31.55 (C-21), 31.93 (C-1), 35.19 (C-6), 39.06 (C-12), 39.68 (C-8), 40.25 (C-10), 41.16 (C-4), 43.93 (C-9), 45.84 (C-13), 52.47 (C-5), 57.49 (C-14), 63.19 (C-17), 69.35 (C-3), 209.85 (C-20). Anal. ($C_{20}H_{32}O_2$) C, H.

5,6α-Oxido-20-oxo-7-nor-5α-pregnan-3α-ol (9). Methanesulfonate **15** (48 mg, 0.12 mmol) and potassium nitrite (340 mg, 3.4 mmol) in DMSO (1.5 mL) were stirred at 120 °C for 90 min. The mixture was diluted with brine, and the product was extracted with ethyl acetate, washed with water, and dried. The solution was partly concentrated in a vacuum and applied to a PLC plate, which was developed in 25% ether in benzene. Elution of the major zone produced alcohol **9**. Yield: 25 mg (65%). Mp and IR were found to be identical with those of compound **9**, which was prepared earlier by using a different route. ^1H NMR: δ 0.61 (s, 3H, H-18); 0.87 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.27 (dd, 1H, *J* = 14.4 and 3.2, H-4); 2.59 (t, 1H, *J* = 8.9, H-17); 3.24 (s, 1H, H-6); 4.18 (m, 1H, *W* = 25, H-3). ^{13}C NMR: δ 13.53 (C-18), 15.08 (C-19), 20.63 (C-11), 23.30 (C-16), 24.47 (C-15), 28.39 (C-1), 28.82 (C-2), 31.58 (C-21), 31.60 (C-4), 38.87 (C-12), 39.18 (C-10), 41.58 (C-8), 45.99 (C-13), 48.88 (C-9), 51.01 (C-14), 58.32 (C-6), 62.90 (C-17), 68.05 (C-5), 68.10 (C-3), 209.49 (C-20).

20-Oxo-7-norpregn-5-en-3β-yl Methanesulfonate (14). A solution of compound **7** (1.2 g, 3.15 mmol) in pyridine (5 mL) was stirred in an ice bath and treated with methanesulfonyl chloride (1 mL, 12.92 mmol). After 2 h, crushed ice (10 mL) was added, and after 2 h, the white precipitate was filtered off and washed with water. Yield: 1.32 g (72%). Mp 148−150 °C ($CHCl_3$-ether). $[\alpha]_D$ −21° (c 1.2). IR: 1699 (C=O); 1635 (C=C); 1358, 1333, 1173, 931, 561, 528 (SO_2). ^1H NMR: δ 0.64 (s, 3H, H-18); 0.90 (s, 3H, H-19); 2.14 (s, 3H, H-21); 2.56 (t, 1H, *J* = 8.7, H-17 Hz); 3.03 (s, 3H, OMs); 4.56 (m, 1H, *W* = 40.0 Hz, H-3); 5.45 (s, 1H, H-6). Anal. ($C_{21}H_{34}O_4S$) C, H.

5,6α-Oxido-20-oxo-7-nor-5α-pregnan-3β-yl Methanesulfonate (15). 3-Chloroperoxybenzoic acid (2.0 g, 14.5 mmol) was added to a cooled (0 °C) solution of olefin **14** (1.54 g, 4.03 mmol) in $CHCl_3$ (20 mL). After 1 h, the solution was washed with a solution

of $KHCO_3$ and water. The solution was stripped of most of the solvent in a vacuum. Yield of epoxide **15**: 1.17 g (73%); mp 108−109 °C (ether). $[\alpha]_D$ +27° (c 1.3). IR: 1700 (C=O); 1358, 1342, 1176, 942, 562, 536, 526 (SO_2). ^{1}H NMR: δ 0.60 (s, 3H, H-18); 0.92 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.57 (t, 1H, J = 8.9 Hz, H-17); 3.00 (s, 3H, OMs); 3.31 (s, 1H, H-6); 4.88 (m, 1H, W = 38.0 Hz, H-3). Anal. ($C_{21}H_{34}O_5S$) C, H.

3α,6α-Dihydroxy-5-methyl-7,19-dinor-5*β*-pregn-9-en-20-one (16). A solution of boron trifluoride etherate (1.8 mL, 14.4 mmol) in ether (18 mL) was dripped into a stirred and cooled (0 °C) solution of epoxide **9** (450 mg, 1.4 mmol) in THF (3 mL). After 20 h, the mixture was made alkaline with a solution of $KHCO_3$ (70 mL). The solvents were removed on a rotary evaporator, and the precipitate formed was extracted with $CHCl_3$. Upon the evaporation of the solvent, the residue (350 mg) was subjected to flash chromatography on silica gel (10% acetone in ether). The major zone afforded compound **16** as white solid. Yield: 190 mg (42%). Mp 179−181 °C (toluene and heptane). $[\alpha]_D$ +126° (c 0.7). IR: 3615, 3415, 1032, 1062, 980 (OH); 1699, 1359 ($COCH_3$). HRMS (EI) for $C_{20}H_{32}O_3$: calcd. 318.21949; found: 318.21934. ^{1}H NMR: δ 0.69 (s, 3H, H-18), 0.99 (s, 3H, H-19), 2.15 (s, 3H, H-21), 2.57 (t, 1H, J = 8.9 Hz, H-17), 3.75 (d, 1H, J = 4.8 Hz, H-6), 4.00 (m, 1H, W = 25.0 Hz, H-3). ^{13}C NMR: δ 12.33 (C-18), 21.05 (C-1), 21.52 (C-11), 22.39 (C-19), 23.40 (C-16), 25.26 (C-15), 31.70 (C-21), 35.23 (C-2), 37.43 (C-12), 40.49 (C-4), 44.76 (C-13), 48.51 (C-14), 48.66 (C-8), 51.60 (C-5), 63.06 (C-17), 68.43 (C-3), 79.19 (C-6), 129.29 (C-9), 134.61 (C-10), 209.73 (C-20). Anal. ($C_{20}H_{30}O_3$) C, H.

3α-Hydroxy-5α-pregnane-7,20-dione (18). 3*β*-Hydroxy-5α-pregnane-7,20-dione (265 mg, 0.80 mmol) was treated, as above, with diethyl azodicarboxylate, triphenylphosphine, and formic acid. PLC of the product yielded a formate of 3α-alcohol **18**. Yield: 230 mg (80%). ^{1}H NMR: δ 0.63 (s, 3H, H-18); 1.08 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.51 (t, 1H, J = 9.3, H-17); 5.21 (m, 1H, W = 15.5, H-3); 8.03 (s, 1H, HCOO). The formate (225 mg, 0.62 mmol) was hydrolyzed as above and yielded 3α-alcohol **18** as white crystals. Yield: 190 mg (92%). Mp 160−161 °C (acetone/heptane). $[\alpha]_D$ −25° (c 0.15). ^{1}H NMR: δ 0.63 (s, 3H, H-18), 1.05 (s, 3H, H-19), 2.12 (s, 3H, H-21), 2.51 (t, 1H, J = 9.4 Hz, H-17), 4.10 (m, 1H, W = 16.2 Hz, H-3). ^{13}C NMR: δ 10.70 (19), 13.41 (18), 21.13 (11), 23.39 (16), 25.17 (15), 28.66 (2), 31.62 (21). 31.85 (1), 35.98 (4), 36.51 (10), 37.77 (12), 41.31 (9), 43.96 (13), 45.74 (6), 49.13 (14), 49.93 (8), 55.49 (5), 62.54 (17), 65.91 (3), 209.69 (20), 211.38 (7). Anal. ($C_{21}H_{32}O_3$) C, H.

Biological Evaluation. In vitro Test Using Rat Brain Membranes and [^{35}S]TBPS Binding. The membranes were isolated from whole brains of adult male Wistar rats in accordance with our previous study[41] and re-suspended in a buffer (20 mM KH_2PO_4, 200 mM KCl, pH = 7.4). Aliquots were incubated with 2 nM [^{35}S]-*tert*-butyl-bicyclo[2.2.2]phosphorothionate (TBPS, Perkin-Elmer), 1 μM GABA, and 1 nM−10 μM steroids for 60 min at 37 °C. The nonspecific binding was estimated using 200 μM picrotoxinin. The results were related to the control samples containing DMSO and expressed in percent form.

In vitro Test Using Intact Neurons in Culture. Primary cultures of cortical neurons were obtained from cerebral cortices of 16-day-old mouse embryos.[33,45,52] The dissociated cells were suspended in Dulbecco's minimum essential medium supplemented with *p*-amino benzoate, insulin, penicillin, and 10% fetal calf serum and seeded in 24-multiwell plates precoated with poly-L-lysine. The cultured cells were incubated for 7−9 days in a humidified 5% CO_2/95% air atmosphere at 36.8 °C. A mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added before 48 h in culture to prevent glial proliferation.

[^{3}H]Flunitrazepam Binding. The benzodiazepine binding to intact cultured cortical neurons was determined as previously described[9,42,52] using 1.3−2.0 nM [^{3}H]flunitrazepam. Prior to incubation with the radioligand, the plates were washed three times with 1 mL/well of HEPES buffer (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 9 mM glucose adjusted to pH 7.3), and the binding assay took place in the culture well in the presence of the HEPES buffer, [^{3}H]-flunitrazepam, and drug solutions. After 30 min of incubation at 25 °C without shaking, a cold buffer was added, and it was rapidly removed by suction. The cells were rinsed three times with the cold buffer, then they were disaggregated in 0.2 N NaOH overnight, and their radioactivity was determined by liquid scintillation counting (with cocktail Optiphase Hisafe2). Nonspecific binding was determined in the presence of 20 μM diazepam. All of the experiments were simultaneously run with a parallel experiment that determined the increase of [^{3}H]flunitrazepam binding induced by 100 μM GABA, which was used as a positive assay control. The solutions of tested compounds were prepared in dimethyl sulfoxide (DMSO) and diluted in HEPES buffer to the assay concentrations (1 nM−30 μM). The final DMSO concentration in HEPES buffer was <0.5%. DMSO was also present in the HEPES control solutions. Bicuculline (100 μM) and picrotoxinin (100 μM) were used as $GABA_A$ receptor antagonists.

Cl^- Flux Determination. Cl^- flux was measured by the $^{36}Cl^-$ uptake assay in intact cellular cultures.[9,33] Briefly, the culture medium was replaced with an Earle's balanced salt solution containing (in mM) NaCl (116.0), $MgSO_4$ (0.8), $CaCl_2$, (1.8), NaH_2PO_4 (1.0), $NaHCO_3$ (15.2), and glucose (5.5) adjusted to pH 7.4. After 45 min of preincubation in a humidified 5% CO_2 /95% air atmosphere at 36.8 ° C, the cells were preincubated for an additional period of 10 min at room temperature. The cultures were then incubated for 7 s with HEPES buffer containing ^{36}Cl (0.2−0.7 μCi/mL) in the absence or presence of 5 μM GABA and the neurosteroids. Basal $^{36}Cl^-$ uptake was determined in the absence of GABA and the neurosteroids. Each well was rinsed three times with 1 mL of ice-cold buffer. The cells were lysed in distilled water, and the lysates were used for the determination of radioactivity by liquid scintillation counting.

Cell Viability. Cell viability was determined by measuring the reduction of 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a colored formazan salt by mitochondrial reducing activity, as described previously.[52,53] Briefly, the cultures were rinsed and incubated for 15 min with a solution of MTT (250 μg/mL) dissolved in a HEPES buffer solution at 37 °C. After washing off the excess MTT, the cells were disaggregated with 5% SDS, and the colored formazan salt was measured at 560 nm in a spectrophotometer plate reader.

Data Analysis. Data shown represent the mean ± standard error of mean (SEM). The concentration−response data were fitted to sigmoid curves, and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA). At least six concentrations of tested compounds were used in the concentration−response curves, and each point was determined in triplicate. A two-tailed Student's *t*-test as well as one-way analysis of variance (ANOVA) was used.

Computational Methods. The molecular alignments were generated with the Flexible Alignment module included in the program MOE (v. 2004.03, Chemical Computing Group, Montreal, Canada; http://www.chemcomp.com). The structures of the molecules studied were generated and minimized using the MMFF94x force field, a modified version of the MMFF94s force field,[54,55] with the default parameters implemented in MOE, and then they were aligned to maximize the superposition of the heavy atoms. Molecular interaction fields (MIFs) between the water probe and the compounds considered in this work were calculated with the program GRID[56,57] (v. 22a, Molecular Discovery Ltd., Oxford, England; http://moldiscovery.com) by using a grid box with a 0.33 Å spacing, which extended 5 Å beyond the analyzed molecules. The desolvation energetic costs were estimated as described by Wei.[58] Briefly, free solvation energies in water and in hexadecane were calculated with the program Amsol v. 7.1 (http://comp.chem.umn.edu/amsol/)[59,60] using the AM1 semiempiric Hamiltonian to optimize the structures of each compound, the CM2A charge model, and the SM5.42 solvation model. The solvent hexadecane (dielectric 2.06) is used to simulate the hydrophobic environment inside the receptor. The desolvation cost was then calculated as the difference between the free solvation energy in hexadecane and

water. Therefore, a lower value means a lower amount of energy required to bring the compound from bulk water to the inside of the protein. Pharmacophoric models were created using the program Catalyst (v. 4.10, Accelrys Inc., Burlington, MA; http://www.accelrys.com) on a Silicon Graphics Octane workstation operating under IRIX 6.5. Hypothesis generation was performed using the HipHop[61] module implemented in Catalyst. Briefly, this approach tries to identify common pharmacophoric features present in a set of active compounds. Molecules are described as a set of chemical functions distributed in the 3D space. Molecular flexibility is taken into account for each compound by sampling the conformational space to pick a number of conformations (maximum of 255) that are accessible to the molecule within a given energy range. This range was fixed at 10 kcal/mol relative to the lowest energy conformer.[62] The rest of the software parameters used were chosen according to Accelrys recommendations and are summarized in the Supporting Information. Molecules selected to generate the hypotheses satisfied a minimum activity criteria that was either (1) a TBPS IC_{50} or flunitrazepam EC_{50} value not less than 10% of those measured for compounds **1** or **2** or (2) a GABA-induced chloride current at least 0.7-fold of those measured for compounds **1** or **2**. Two sets of active molecules were chosen: set 1 comprised compounds with 5α sterochemistry (**1**, **5**, **24**, and **5α-29**) or shapes that are close to that of 5α steroids (**19** and **30**), whereas set 2 consisted of compounds with 5β sterochemistry (**2**, **6**, **25**, and **5β-29**) or whose shape is close to that of 5β steroids (**31**[47]). The molecules were aligned to the hypotheses generated within Catalyst to maximize their superposition to the pharmacophoric features that constitute each model. Catalyst then assigned a fit value, which increases with the quality of the superposition, to every possible mapping and ranked the mappings for each compound according to it. Weights for each feature were manually assigned to normalize the maximum fit values to 10 (Supporting Information, Table 3). Larger weights were assigned to the essential pharmacophoric features (HBA, HBD, and H1) such that the alignment process gave preference to satisfying them. The best pharmacophoric hypotheses obtained were further refined by the manual addition of exclusion volumes, which were located in the hypotheses according to data reported in the literature,[26,30] to obtain a better discrimination between active and inactive compounds.

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Supporting Information Available: Supplementary data on reported activities of the compounds considered, molecular alignments, molecular interaction field analysis, desolvation cost results, pharmacophoric hypothesis definition, alignment of the compounds to the pharmacophoric models and software parameters used. This material is available free of charge via the Internet at http://pubs.acs.org.

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