Synthesis, Metabolism, and Pharmacological Activity of 3α-Hydroxy Steroids Which Potentiate GABA-Receptor-Mediated Chloride Ion Uptake in Rat Cerebral Cortical Synaptoneurosomes[1]

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Certain 3α-hydroxy steroids have recently been shown to bind to the γ-aminobutyric acid (GABA) receptor gated chloride ion channel with high affinity and to potentiate the inhibitory effects of GABA when measured both in vitro and in vivo. In the present study, a series of natural and synthetic 3α-hydroxy steroids were tested for their ability to potentiate GABA-receptor-mediated chloride ion (Cl^-) uptake into cerebral cortical synaptoneurosomes. The naturally occurring metabolites 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydroDOC) were found to be the most active in augmenting $GABA_A$-receptor-mediated Cl^- uptake. Pharmacological activity was reduced in the corresponding isomers with the 5β-pregnane configuration and by some, but not all, modifications of the side chain. The ability of these steroids to potentiate muscimol-stimulated Cl^- uptake is lost by acetylation at C_3, introduction of unsaturation at $C_{9(11)}$, inversion to the 3β-hydroxy isomer, or inversion of configuration at C_{17}. A facile procedure is reported for the synthesis of unlabeled and tritium-labeled allopregnanolone and allotetrahydroDOC. The 9α,11α,12α-^{3}H-labeled derivatives of allopregnanolone and allotetrahydroDOC were used to identify the distribution and metabolic products of these active steroids. Uptake of the more hydrophobic [^{3}H]allopregnanolone into brain was significantly greater than that of [^{3}H]allotetrahydroDOC. The principal ^{3}H-labeled metabolites recovered from brain were the 3-ketone derivatives of allopregnanolone and allotetrahydroDOC, which are both inactive on GABA-receptor-mediated Cl^- flux. Molecular modeling of the active steroids based on quantitative structure–activity relationships provides evidence to support the stereospecificity of the binding interactions and suggests that there may be more than one type of steroid binding site associated with the $GABA_A$-receptor-mediated chloride ionophore.

The γ-aminobutyric acid ($GABA_A$)/benzodiazepine receptor–chloride channel complex has been shown to be modulated by several metabolites of the steroid hormones progesterone and deoxycorticosterone (DOC), most notably 3α-hydroxy-5α-prenan-20-one (**2a**, allopregnanolone) and 3α,21-dihydroxy-5α-pregnan-20-one (**4a**, allotetrahydroDOC).[2] These metabolites compete with [^{35}S]-*tert*-butylbicyclophosphorothionate (TBPS) in binding to the $GABA_A$ receptor complex, with an affinity for this recognition site in the high nanomolar range.[2] In the presence of low concentrations of GABA, these metabolites have significantly higher affinity for [^{35}S]TBPS binding,[2c] suggesting an allosteric interaction between the recognition site for GABA and the recognition site for these metabolites. In addition, allopregnanolone and allotetrahydroDOC enhance benzodiazepine receptor binding in vitro in a manner similar to that of barbiturates.[2a] Electrophysiological studies have shown that these metabolites can directly alter GABA-receptor-mediated chloride ion conductance in cultured rat hippocampal neurons.[2a] Moreover, we have recently demonstrated[2e,3] that these steroid hormone metabolites enhance GABA-receptor-mediated chloride uptake into rat cerebral cortical synaptoneurosomes at concentrations between 0.025 and 1 μM. Thus, allopregnanolone and allotetrahydroDOC are extremely potent modulators of GABA-receptor-mediated inhibitory neurotransmission in the central nervous system (CNS), suggesting that they may serve as anxiolytic or hypnotic agents in animals and man.

Behavioral studies have indicated that one or both of these steroid hormone metabolites have anxiolytic, sedative, analgesic, and antiaggressive effects when administered to laboratory animals. AllotetrahydroDOC has been shown to have anticonflict effects in rats,[4] potent dose-dependent sleep-inducing properties in rats,[5] and to reduce aggressive behavior in mice.[6] Allopregnanolone also produces analgesic effects in mice when administered in the cerebroventricular space.[7] These compounds are closely related structurally to anesthetic steroids such as alphaxalone, which have also been shown to have $GABA_A$-receptor-mediated activity in the CNS.[8]

The objectives of the present study were (1) to develop a procedure for the facile synthesis of these steroid hor-

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(1) Portions of this work were presented in November 1988 at the 18th Annual Meeting of the Society for Neuroscience in Toronto, Ontario, Canada.
(2) (a) Majewska, M. D.; Harrison, N. L.; Schwartz, R. D.; Barker, J. L.; Paul, S. M. *Science* **1986**, *232*, 1004. (b) Harrison, N. L.; Majewska, M. D.; Harrington, J. W.; Barker, J. L. *J. Pharmacol. Exp. Ther.* **1987**, *241*, 346. (c) Gee, K. W.; Chang, W.-C.; Brinton, R. E.; McEwen, B. S. *Eur. J. Pharmacol.* **1987**, *136*, 419. (d) Callachan, H.; Cottrell, G. A.; Hather, N. Y.; Lambert, J. J.; Nooney, J. M.; Peters, J. A. *Proc. R. Soc. London* **1987**, *B231*, 359. (e) Morrow, A. L.; Suzdak, P. D.; Paul, S. M. *Eur. J. Pharmacol.* **1987**, *142*, 483. (f) Peters, J. A.; Kirkness, E. F.; Callachan, H.; Lambert, J. J.; Turner, A. J. *Br. J. Pharmacol.* **1988**, *94*, 1257. (g) Gee, K. W.; Bolger, M. B.; Brinton, R. E.; Coirini, H.; McEwen, B. S. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 803. (h) Turner, D. M.; Ransom, R. W.; Yang, J. S.-J.; Olsen, R. W. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 960.
(3) (a) Morrow, A. L.; Suzdak, P. D.; Paul, S. M. *Adv. Biochem. Psychopharmacol.* **1988**, *45*, 247. (b) Morrow, A. L.; Pace, J. R.; Purdy, R. H.; Paul, S. M. *Mol. Pharmacol.* **1990**, *37*, 263.
(4) (a) Crawley, J. N.; Glowa, J. R.; Majewska, M. D.; Paul, S. M. *Brain Res.* **1986**, *398*, 382. (b) Crawley, J. N. Unpublished results.
(5) Mendelson, W. B.; Martin, J. V.; Perlis, M.; Wagner, R.; Majewska, M. D.; Paul, S. M. *Psychopharmacology* **1987**, *93*, 226.
(6) Kavaliers, M. *Psychopharmacology* **1988**, *95*, 488.
(7) Kavaliers, M.; Wiebe, J. P. *Brain Res.* **1987**, *415*, 393.
(8) (a) Simmonds, M. A.; Turner, J. P.; Harrison, N. L. *Neuropharmacology* **1984**, *23*, 877. (b) Cottrell, G. A.; Lambert, J. J.; Peters, J. A. *Br. J. Pharmacol.* **1987**, *90*, 491. (c) Barker, J. L.; Harrison, N. L.; Lange, G. D.; Owen, D. G. *J. Physiol.* **1987**, *386*, 485. (d) Ong, J.; Kerr, D. I. B.; Johnston, G. A. R. *Br. J. Pharmacol.* **1988**, *95*, 33. (e) Turner, J. P.; Simmonds, M. A. *Br. J. Pharmacol.* **1989**, *96*, 409.

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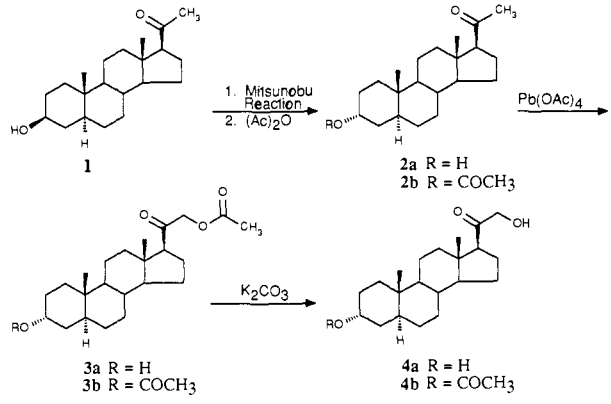
Scheme I



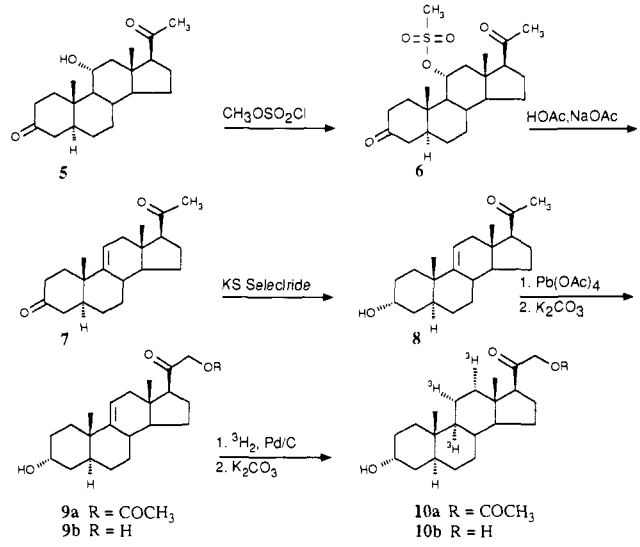
mone metabolites and other closely related compounds, (2) to prepare the tritiated forms of the natural steroids in order to investigate their metabolism and pharmacokinetics, and (3) to determine some of the structural requirements for biological activity by correlating the potentiation of GABA-receptor-mediated Cl^- uptake in synaptoneurosomes with molecular graphic analyses of the steroid structures.

The ability of allopregnanolone, allotetrahydroDOC, and their derivatives to potentiate GABA-receptor-mediated stimulation of Cl^- flux provides a sensitive assay for studying the effects of structural changes in these steroids. We have previously shown that the 3α-hydroxy metabolites in the 5α- and 5β-pregnane series have similar, but not identical, activity as modulators of $GABA_A$-receptor-mediated Cl^- flux.[3b] We now demonstrate the activity of the allylic 3α-hydroxypregn-4-en-20-one, another metabolite of progesterone.[9] We also investigated the effects of C_3 acetylation, unsaturation at $C_{9(11)}$, C_{20} ketone modification, C_{21} modification, C_{11} and C_{12} hydroxylation, and several other structural modifications which alter pharmacological activity in vitro. This report provides a description of the structure–activity relationships for the interaction of the $GABA_A$ receptor with these hormonally inactive steroid metabolites and suggests the possibility of "partial agonist" activity by some of these compounds. In addition, we have identified the principal brain metabolites of allopregnanolone and allotetrahydroDOC in vivo and have demonstrated that they are inactive in potentiating GABA-receptor-mediated Cl^- uptake. These studies also indicate that uptake of ^3H-labeled **2a** into the brain is significantly greater than the uptake of ^3H-labeled **4a**.

Results and Discussion

Chemistry. The synthesis of the rare 3α-hydroxy-5α-pregnanes **2a**, **3a**, and **4a** and their 3-acetates was accomplished via the route outlined in Scheme I. The relatively common 3β-hydroxy-5α-pregnan-20-one (**1**) was selected over 5α-pregnane-3,20-dione (**10**) as starting material for the preparation of **2a**. Although stereospecific reduction of **10** to axial alcohol **2a** was reported in about 35% yield after purification with either the Henbest reduction[10] or 1 equiv of potassium trisiamylborohydride,[11] we were unable to obtain **2a** in pure form from **10** without extensive chromatographic purification by HPLC. We therefore used the Mitsunobu reaction[12] employing the very mild

Scheme II



conditions of Varasi et al.[13] for accomplishing the inversion of the 3β-hydroxy group of **1** in 54% yield after final purification. Analogous to the preparation of 3β-hydroxy-5β-pregnan-20-one from 3α-hydroxy-5β-pregnan-20-one, where Varasi et al.[13] found no detectable starting material after crystallization, we were able to remove, by crystallization, the 5% of **1** found in the crude **2a**. In this procedure the trifluoroacetate ester (not isolated) was hydrolyzed without the accompanying formation of 3α-hydroxy-5α,17α-pregnan-20-one. This 17α-isomer is difficult to separate from **2a** and was formed when the more stable formate ester intermediate was prepared by using the general procedure of Bose et al.[14]

Modifying the conditions of Cocker et al.[15] for the 21-acetoxylation of 3β-acetoxy-5α-pregnane-11,20-dione by lead tetraacetate, we were able to obtain **3a** in 35% yield from **2a**. The yield was only 24% with the protected 3-acetate **2b** for similar preparation of the 3,21-diacetate **3b**. Furthermore, hydrolysis of the 3-acetyl group of **3b** does not occur under the mild basic conditions used to remove the 21-acetyl group. Hydrolysis of **4b** required acidic conditions due to the instability of the α-ketol group of **4b** under the more basic conditions required for the hydrolysis of the 3-acetoxy group. Under these acidic conditions, an impurity, presumed to be the 17α-isomer of **4a**, was formed which could be removed only by HPLC. When the *tert*-butyldimethylsilyl ether of **2a** was reacted with lead tetraacetate in an attempt to increase the yield of 21-acetoxylation, this silyl ether did not survive in the presence of the boron trifluoride etherate catalyst.

The sequence of reactions shown in Scheme II was used to obtain the analogous 9(11)-pregnene isosteres. The mesyl group of 11α-hydroxy-5α-pregnane-3,20-dione (**6**) was easily eliminated to provide 5α-pregn-9(11)-ene-3,20-dione (**7**). Stereospecific reduction with trisiamylborohydride gave **8**, followed by 21-acetoxylation to provide **9a**. After separate 6-day catalytic reductions of **8** and **9a** with tritium gas and palladium oxide in dioxane, purification by HPLC gave the desired 9α,11α,12α-^3H-labeled products. The purity of ^3H-labeled **2a** and **4a** as demonstrated by HPLC is illustrated in Figure 1. No radioactive impurities

(9) Wiebe, J. P.; Wood, P. H. *Endocrinology* **1987**, *120*, 2259.
(10) Browne, P. A.; Kirk, D. N. *J. Chem. Soc. C.* **1969**, 1653.
(11) Wiebe, J. P.; Deline, C.; Buckingham, K. D. *Steroids* **1985**, *45*, 39.
(12) Mitsunobu, O. *Synthesis* **1981**, 1.
(13) Varasi, M.; Walker, K. A. M.; Maddox, M. L. *J. Org. Chem.* **1987**, *52*, 4235.
(14) Bose, A. K.; Lal, B.; Hoffman, W. A., III; Manhas, M. S. *Tetrahedron Lett.* **1973**, 1619.
(15) Cocker, J. D.; Henbest, H. B.; Phillipps, G. H.; Slater, G. P.; Thomas, D. A. *J. Chem. Soc.* **1965**, 6.

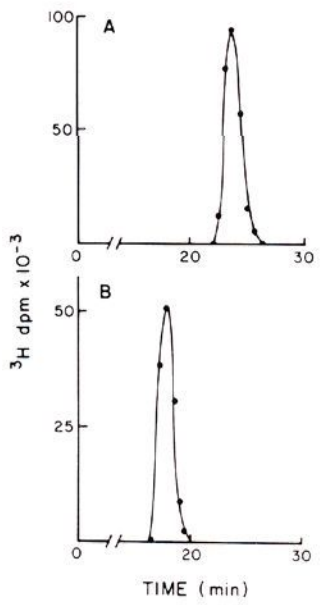


Figure 1. Chromatogram of the separation of 250 × 10^3 dpm [9α,11α,12α-^{3}H]-3α-hydroxy-5α-pregnan-20-one (41 Ci/mmol, allopregnanolone) (A) and 140 × 10^3 dpm [9α,11α,12α-^{3}H]-3α,21-dihydroxy-5α-pregnan-20-one (61 Ci/mmol, allotetrahydroDOC) (B) by HPLC on two 100 × 9.4 mm Partisil 5 ODS-3 RAC columns (Whatman, Inc.) in tandem using the solvent system 75% aqueous methanol with a flow rate of 3 mL/min in A and 2 mL/min in B. Fractions were collected at 0.5-min intervals and measured by liquid scintillation spectrometry for their content of radioactivity. The radioactive steroids eluted with the same retention times as unlabeled authentic standards measured by their absorption at 280 nm (not shown).

Table I. Molecular Modeling of Allopregnanolone: C_{16}–C_{17}–C_{20}–O_{20} Torsion Angles and Relative Energies

τ, deg	relative energy, kcal/mol
−8.5 (minimum)	0
−26.7 (saddle point)	0.177
−30.0 (minimum)	0.162
−140.7 (minimum)	0.284

were detected. A final specific activity of 41 and 63 Ci/mmol of ^{3}H-labeled **2a** and **4a** respectively was achieved with a radiochemical purity of greater than 99%.

21-Mesylate **14** was prepared by the sequence of reactions shown in Scheme III. 21-Acetate **3a** was treated with *tert*-butyldimethylsilyl chloride to give **9c**. Hydrolysis of the 21-acetate group with K_2CO_3 gave the intermediate **9d** which was then reacted with methanesulfonyl chloride in pyridine at 0 °C to provide the protected 21-mesylate **9e**. The hydrolysis of the 3-ether group was achieved in tetrahydrofuran/acetic acid (1:3) at 60 °C for 7 h to give the desired product **14**.

Molecular Modeling

Side-Chain Conformation. The energetically preferred side-chain conformations of allopregnanolone (**2a**) were first investigated to determine a minimum-energy conformation for use in subsequent comparisons with other steroid structures. The C_{17}–C_{20} torsion angle of **2a** was searched at a 10° resolution, to give three minima shown in Table I after minimization (confirmed by eigenvalue test). The minimum at τ = −30° is not physically meaningful since there is only a 0.015 kcal/mol barrier between it and the minimum at τ = −8.5°. The two minima together effectively constitute a single broad minimum with torsion angles between τ = 0° and −40° readily accessible, which fits well with X-ray crystallographic data for a large number of steroids.[16] The minimum at τ = −140.7° is similar to one proposed on the basis of the $^1H_{17\alpha}$–$^{13}C_{21}$ coupling constant,[17] but this conformation is rarely if ever found in crystal structures of related steroids[16] and is somewhat higher in energy by molecular mechanics as well. Thus, the torsional minima corresponding to the τ = −8.5° minimum in allopregnanolone were used for modeling of these steroids.

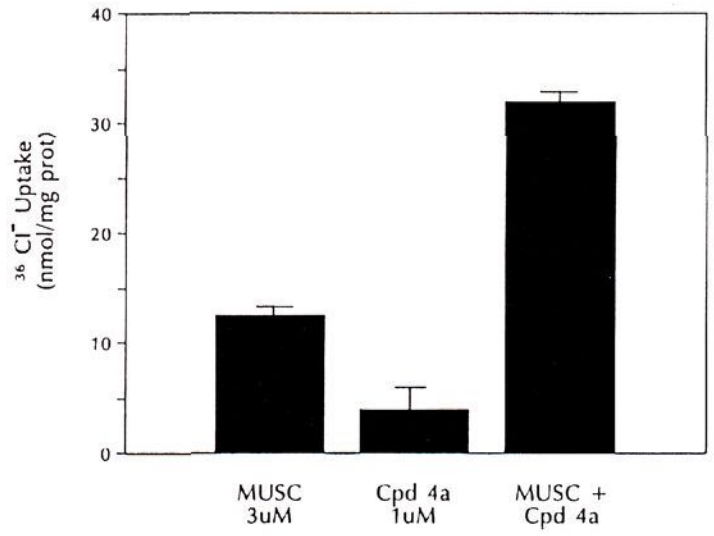


Figure 2. Potentiation of muscimol-stimulated $^{36}Cl^-$ uptake by allotetrahydroDOC. Synaptoneurosomes were incubated for 5 s in the presence of muscimol (3 μM) alone, allotetrahydroDOC (1 μM) alone, or in combination. Muscimol produced a net increase in $^{36}Cl^-$ uptake of 12.8 ± 0.8 nmol/mg of protein. AllotetrahydroDOC had no significant effect on $^{36}Cl^-$ uptake alone; however, it significantly enhanced muscimol stimulation; 31.6 ± 0.6 nmol/mg of protein, $p < 0.01$. Data represent the mean ± SE of five independent experiments, each conducted in quadruplicate.

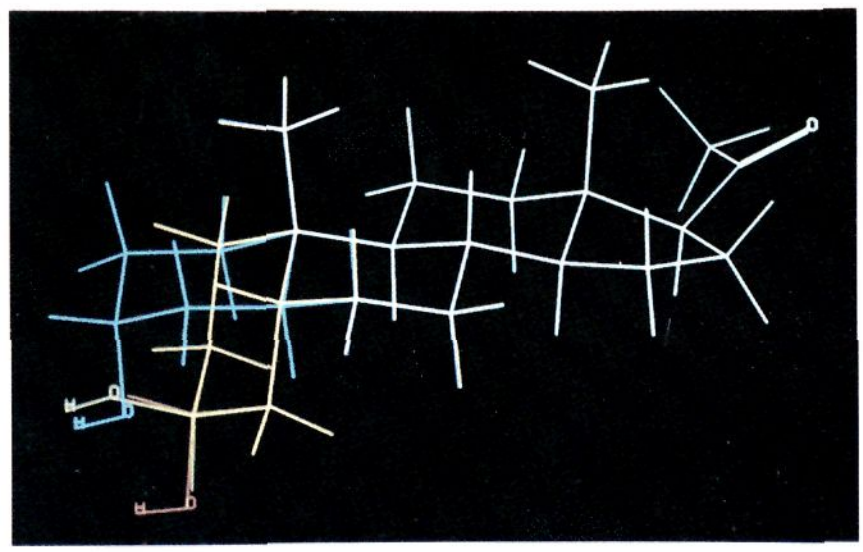


Figure 3. Rigid BCD-ring overlay of allopregnanolone (**2a**, blue), 3α-hydroxy-5β-pregnan-20-one (red), and 3β-hydroxy-5β-pregnan-20-one (green).

Role of A Ring Conformation: Chair vs Boat. The biologically active A ring conformation of analgesic steroids has been proposed by Phillips[18] to be a so-called "α-boat" structure. To test this hypothesis, the A ring of alphaxalone was searched at a 30° resolution with the MULTIC submode, giving structures which included the chair, twist boat, and the putative α- and β-boats. Energy minimization of the structures showed that all boat variants minimized to the expected twist-boat conformation; the α- and β-boats are not minima. The twist-boat conformation was 3.97 kcal/mol higher in energy than the chair conformation. Conformational searching of several of the compounds (**2a**, **3a**, **4a**, **11b**) showed the same behavior for the A rings, with the twist boat at least 4 kcal/mol higher in energy than the chair conformation in each case. These data demonstrate that the normal chair conformation is

(16) Duax, W. L.; Griffin, J. F.; Rohrer, D. C. *J. Am. Chem. Soc.* **1981**, *103*, 6705.

(17) Fesik, S. W.; Makriyannis, A. *Mol. Pharmacol.* **1985**, *27*, 624.

(18) Phillipps, G. H. *J. Steroid Biochem.* **1975**, *6*, 607.

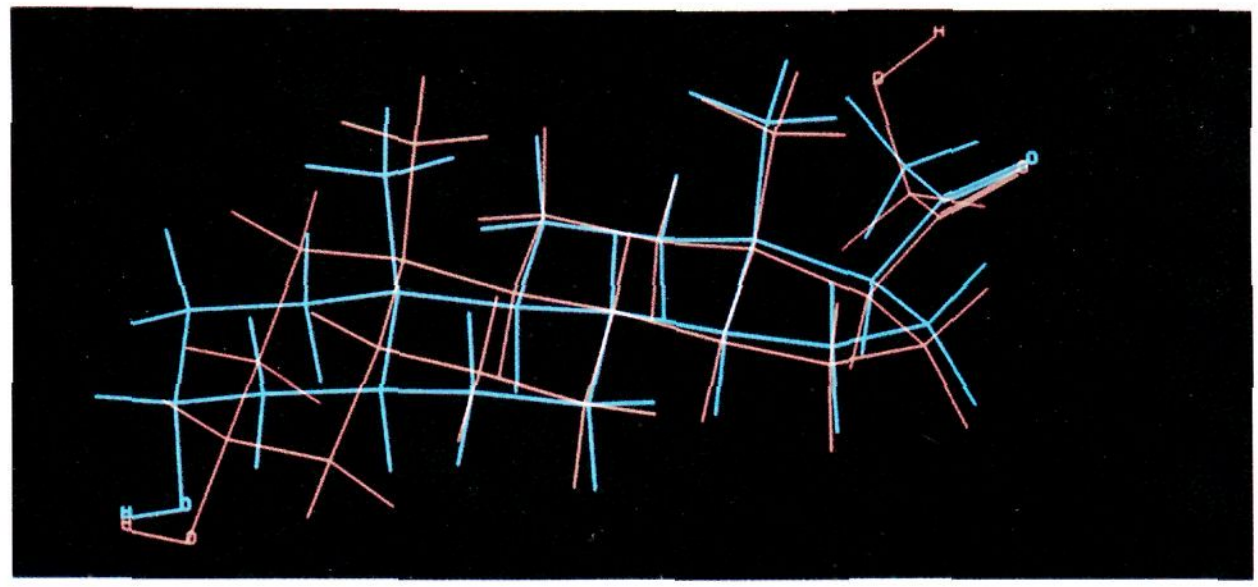


Figure 4. Template forcing of 3α,21-dihydroxy-5β-pregnan-20-one (**11b**, red) onto allopregnanolone (**2a**, blue) matching atoms O_3, C_6, C_8, C_{10}, C_{11}, C_{12}, C_{17} and O_{20} with an added 2.0 kcal/mol per Å potential.

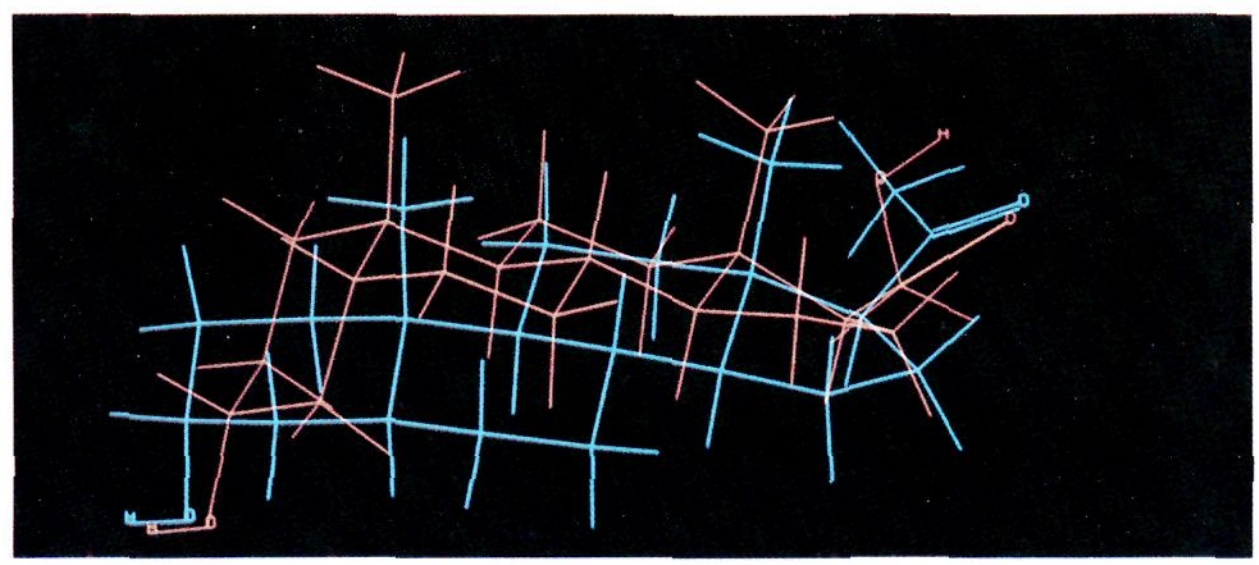
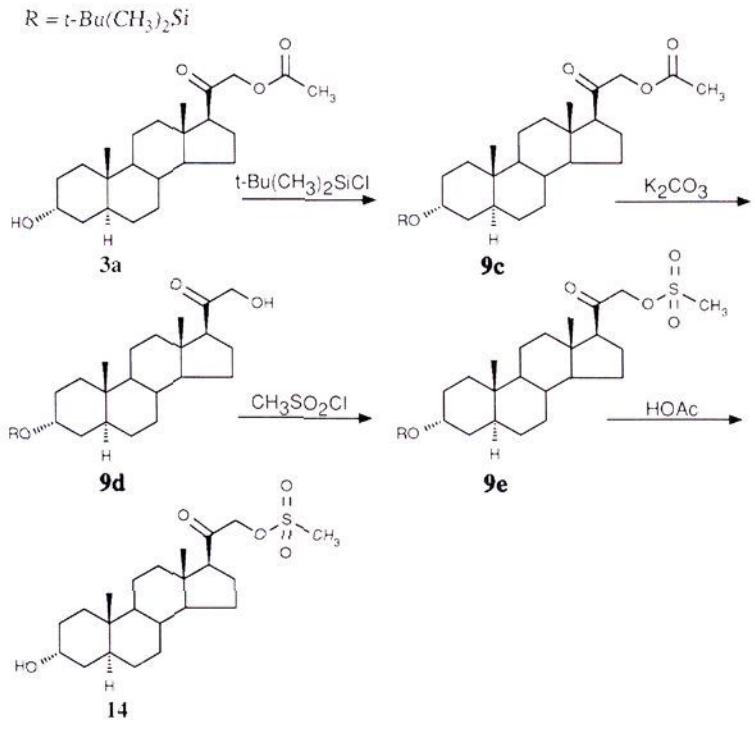


Figure 5. Rigid overlay of allopregnanolone (**2a**, blue) and 3α,21-dihydroxy-5β-pregnan-20-one (**11b**, red) matching atoms O_3, C_{11} and O_{20}. Corresponding carbonyl and hydroxyl groups are closer than in the case of template forcing (Figure 4).

Scheme III



clearly preferred in comparison to the "α-boat" structure proposed by Phillipps.[18]

Role of AB Ring Fusion: Cis vs Trans. An essential feature in the pharmacologically active 5α- and 5β-pregnane structures is the presence of a 3α-hydroxyl group.[2] This is exemplified in the comparison of allopregnanolone (**2a**) and 3α-hydroxy-5β-pregnan-20-one versus the inactive 3β-hydroxy-5β-pregnan-20-one. A BCD ring rigid overlay of the three structures in Figure 3 shows that the O_3 of the 3β,5β-structure lies within 0.28 Å of the corresponding O_3 of allopregnanolone, whereas the corresponding distance between the O_3's of allopregnanolone and the 3α,5β-structures is 1.96 Å. Nevertheless, it is allopregnanolone and the 3α,5β-compound which are active.

The preceding overlay implicitly assumes that the BCD ring location of the 5β-steroids, when bound to the GABA receptor, is similar to that of the 5α-steroids. To test this assumption, template forcing[19] was used to force the chair and boat forms of 3α,21-dihydroxy-5β-pregnan-20-one (**11b**) to conform to the minimized chair form of allopregnanolone, matching eight sets of atoms (O_3, C_6, C_8, C_{10}, C_{11}, C_{12}, C_{17}, O_{20}) with an added 2.0 kcal/mol per Å potential. After template forcing, the distance between O_3's for the twist-boat conformation of **11b** and allopregnanolone had been reduced to 0.45 Å at a cost of 0.32 kcal/mol in addition to the 4.3 kcal/mol penalty for being in the twist-boat conformation rather than the chair form. The corresponding chair conformation of **11b**, after template forcing to the structure of allopregnanolone, has a distance between the O_3's of 0.60 Å at a cost of 0.59 kcal/mol (Figure 4). Thus neither the chair nor the twist-boat forms of **11b** are able to deform sufficiently to achieve concurrent good overlap of both the oxygens and the steroid skeleton. This is consistent with the reduction of pharmacological activity of **11b** compared to **4a** (Table II).

It was not possible to achieve good overlap of the BCD rings of the 5α- and 5β-structures concurrently with overlap of their 3α-hydroxyl and C_{20} carbonyl groups, so

(19) Duchamp, D. J. In *Computer-Assisted Drug Design*; Olson, E. C., Christoffersen, R. E., Ed.; ACS Symposium Series 112; American Chemical Society: Washington, DC, 1979; p 79.

the overlay requirement was eased to match only O_3, C_{11}, and O_{20}. Rigid overlay of the minimum energy structures (Figure 5) now places the corresponding oxygens within 0.3 Å of each other without needing to deform the 5β-structure. In contrast, the A ring boat conformation proposed by Phillipps[18] requires that the 5β-structure have both the A and B rings in the boat conformation in order to place the corresponding oxygen atoms in reasonable proximity. This would be extremely unfavorable energetically, and it is unnecessary if the rings are left in the chair conformation.

Pharmacology

The potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in cerebral cortical synaptoneurosomes by steroid hormone metabolites provides an accurate and sensitive in vitro assay for the functional activity of these metabolites. The potency and efficacy of allopregnanolone and allotetrahydroDOC for the enhancement of muscimol-stimulated Cl^- flux are much greater than the direct effects of these steroids on Cl^- flux, suggesting that potentiation of GABA receptor-mediated Cl^- conductance may be the physiologically relevant mechanism of action of these steroids.[2e,3] In an initial study the net uptake of $^{36}Cl^-$ into synaptoneurosomes in the presence of muscimol (3 μM) or allotetrahydroDOC (1 μM) alone and in combination is presented in Figure 2. Although allotetrahydroDOC had no significant direct effect on $^{36}Cl^-$ uptake at this concentration, it enhanced the effect of muscimol. The ability of the steroid hormone derivatives prepared in this study to potentiate muscimol-stimulated $^{36}Cl^-$ uptake in subsequent experiments is shown in Table II. Allotetrahydro DOC produced the maximal potentiation, increasing muscimol-stimulated $^{36}Cl^-$ uptake by approximately 120%. The 21-acetate derivative of allotetrahydroDOC (**3a**) or the isomeric 3α,21-dihydroxy-5β-pregnan-20-one 21-acetate (tetrahydroDOC 21-acetate, **11a**) were both very effective in potentiating Cl^- flux. The relatively high potency of the 21-acetate derivative of allotetrahydroDOC has not been observed previously in vitro. Nevertheless, the anesthetic activities of the 21-acetate derivatives in mice are very similar to those of the corresponding alcohols. However, the effect of the 21-acetate group may have only pharmacological significance, since **3a** was not found in the brain as a metabolite of ^3H-labeled **4a** when the latter compound was administered to rats (see below). 3,21-Diacetate **3b**, 3-monoacetates **2b** and **4b**, and 3-oxime **21** were essentially inactive as potentiators of muscimol-stimulated $^{36}Cl^-$ uptake. This further supports the conclusion that a free 3α-hydroxyl group is essential for activity, both in vitro and in vivo.[2,18] 3-Ketone metabolites **20** and **22** either had very low activity or were devoid of activity, consistent with their lack of anesthetic effect in vivo.[18] Progesterone (**23**) is also inactive at 1 μM in vitro (Table II).

Interestingly, the 5β-isomer (tetrahydroDOC, **11b**), as well as the 21-mesylate of allotetrahydroDOC (**14**), were less active than allotetrahydroDOC itself at concentrations of 1 μM. The reduced activity of tetrahydroDOC (**11b**) compared to allotetrahydroDOC in this functional assay is consistent with the comparative anesthetic potency of these steroids in rats,[20] but contrasts with the equivalent effects of these two isomers in comparison with [^{35}S]TBPS binding.[2b] This reduction of activity in the 5β-isomer on GABA-receptor-mediated chloride flux, but not on the affinity for [^{35}S]TBPS binding, suggests that this compound has reduced efficacy compared with that of allotetrahydroDOC.

The ability of 3α-hydroxypregn-4-en-20-one (**12**) to potentiate muscimol-stimulated $^{36}Cl^-$ uptake contrasts with its 3β-hydroxy isomer (**13**), which is inactive. This stereospecificity is consistent with radioligand binding and behavioral studies where 3β-hydroxy-5α-pregnan-20-one was shown to be inactive in competing with [^{35}S]TBPS binding[2] or in producing analgesia.[7] The bioreduction of progesterone by 3α-hydroxysteroid dehydrogenase to form allylic alcohol **12** results in a unique ring A metabolite

Table II. Effect of Structural Modification on the Potentiation of Muscimol-Stimulated $^{36}Cl^-$ Uptake in Rat Brain Synaptoneurosomes by Steroids

no.	steroid[a] (1 μM)	potentiation,[b] nmol/mg of protein (mean ± se)
	Active Compounds	
4a	3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydroDOC)	13.1 ± 1.1
16	3α-hydroxy-5α-androstane-17β-carbonitrile	13.0 ± 2.6
3a	3α,21-dihydroxy-5α-pregnan-20-one 21-acetate	12.2 ± 1.1
2a	3α-hydroxy-5α-pregnan-20-one (allopregnanolone)	10.9 ± 2.2
12	3α-hydroxypregn-4-en-20-one	10.4 ± 1.0
11a	3α,21-dihydroxy-5β-pregnan-20-one 21-acetate	10.3 ± 0.6
14	3α,21-dihydroxy-5α-pregnan-20-one 21-mesylate	7.9 ± 0.7
11b	3α,21-dihydroxy-5β-pregnan-20-one (tetrahydroDOC)	7.7 ± 0.2
15	3α-hydroxy-5α-androstan-17-one	7.0 ± 2.3
18	3α-hydroxy-5β-androstan-17-one	4.9 ± 0.7
19	3α-hydroxy-5α-androstane-17β-carboxylic acid methyl ester	3.6 ± 0.6
	Inactive Compounds	
	A Ring Modification	
2b	3α-hydroxy-5α-pregnan-20-one acetate	2.57 ± 1.65
3b	3α,21-dihydroxy-5α-pregnan-20-one diacetate	1.37 ± 1.10
4b	3α,21-dihydroxy-5α-pregnan-20-one 3-acetate	0.48 ± 0.32
13	3β-hydroxypregn-4-en-20-one	1.43 ± 1.20
20	5α-pregnane-3,20-dione	2.63 ± 1.30
21	5α-pregnane-3,20-dione 3-oxime	−3.26 ± 1.80
22	21-hydroxy-5α-pregnane-3,20-dione	−1.95 ± 0.30
23	progesterone	−1.80 ± 1.20
	C Ring Modification	
8	3α-hydroxy-5α-pregn-9(11)-en-20-one	1.23 ± 1.20
9b	3α,21-dihydroxy-5α-pregn-9(11)-en-20-one	1.40 ± 0.69
9a	3α,21-dihydroxy-5α-pregn-9(11)-en-20-one 21-acetate	0.34 ± 0.32
24	3α,11α-dihydroxy-5α-pregnan-20-one	0.70 ± 0.85
25	3α,11α-dihydroxy-5β-pregnan-20-one	0.98 ± 0.44
26	3α,12α-dihydroxy-5β-pregnan-20-one	−2.58 ± 1.49
	Side-Chain Modification	
27	3α-hydroxy-5α,17α-pregnan-20-one	1.12 ± 0.33
17	3α-hydroxy-5α-androstane-17α-carbonitrile	−2.10 ± 0.98
28	3α-hydroxy-5α-androstan-17-one oxime	−1.91 ± 0.81
29	20-ethylenedioxy-3α-hydroxy-5α-pregnane	0.16 ± 0.90
30	20-ethylenedioxy-3α-hydroxy-5β-pregnane	−0.96 ± 0.86
31	5α-pregnane-3α,20α-diol	1.97 ± 0.50

[a] Steroid (1 μM) and muscimol (3 μM) were added simultaneously. [b] Potentiation represents the relative rate of $^{36}Cl^-$ uptake in the presence of steroid and muscimol, compared to that produced by muscimol alone. Uptake of $^{36}Cl^-$ was measured for 5 s. Data are the mean ±SE of five to 10 separate measurements.

(20) Norberg, L.; Wahlström, G.; Bäckström, T. *Pharmacol. Toxicol.* 1987, *61*, 42.

which has been reported in brain,[11] and found to suppress follicle stimulating hormone secretion in the pituitary without also inhibiting luteinizing hormone secretion.[9] The potentiation of muscimol-stimulated $^{36}Cl^-$ uptake in rat cerebral cortical synaptoneurosomes by **12** suggests that this metabolite may also have anxiolytic or sedative properties. With use of molecular graphics, the structure of **12** can be rigidly fitted to allopregnanolone (matching O_3, C_{11}, C_{17}, O_{20}) with a root mean square (RMS) difference of 0.20 Å. Template forcing (k = 2.0 kcal/mol per Å) reduces the RMS difference to only 0.11 Å at a cost of 0.07 kcal/mol (Figure 6).

In contrast to the 3α-hydroxy metabolites with 5α-pregnane or 5β-pregnane steroid skeletons, the 3α-hydroxy derivatives in the 5α-pregn-9(11)-ene series (**8**, **9a**, and **9b**) were essentially inactive as potentiators of muscimol-stimulated Cl^- flux (Table II). These results demonstrate that the interaction of steroids with this chloride ion channel complex is markedly altered when the 9(11)-double bond is introduced in ring C, making this ring more planar. Comparing structure **8** by molecular graphics (built from the alphalaxone crystal structure and minimized as usual) with allopregnanolone shows that C_{11} and C_{12} of **8** protrude well into the α-face of the steroid, relative to the active structure (Figure 7). There are no other significant differences between these two structures.

In previous studies it has been shown that a steroidal C_{20} ketone group is not absolutely required for significant interaction of a steroid with the GABA-receptor complex. Thus 3α-hydroxy-5α-androstan-17-one (**15**) was shown to effectively enhance [^{3}H]muscimol binding to rat brain membranes,[2h] and 3α,17β-dihydroxy-5α-androstane weakly displaced [^{35}S]TBPS binding to these membrane preparations.[2g] For natural C_{21} steroid metabolites, reduction of the C_{20} ketone group led to a decrease in binding potency, but did not abolish [^{35}S]TBPS binding activity in the 3α-hydroxy-5α-pregnane series.[2g] We found that the 17-oxime (**28**) of 3α-hydroxy-5α-androstan-17-one (**15**) was inactive in the potentiation of Cl^- flux in vitro (Table II). Similarly, the synthetic 20-ethylenedioxy derivatives (**29**, **30**) of allopregnanolone and pregnenolone were also inactive, as was 5α-pregnane-3α,20α-diol (**31**), in contrast to other results[21] for this metabolite of progesterone. This supports the importance of a C_{17} or C_{20} ketone group as a presumed hydrogen-bond acceptor in natural steroid metabolites. The synthetic 17β-carboxylic acid methyl ester **19** contains the ester carbonyl group, which can also serve this function; molecular modeling of the ester shows the same two minima for the C_{17}–C_{20} torsion angle as for structure **2a**, and rigid overlay of **19** and **2a** matching the ABCD ring atoms places the carbonyl oxygens within 0.11 Å of each other. However, this methyl ester (**19**) has much less pharmacological activity than **2a**.

Since the 17β-carbonitrile of 3α-hydroxy-5α-androstan-11-one was reported to be active as an anesthetic in rats,[18] we prepared the 17α- and 17β-carbonitrile derivatives of 3α-hydroxy-5α-androstane. As expected from the importance of the side-chain configuration, 17α-carbonitrile **17** was inactive, analogous to the inactive 17α-epimer 3α-hydroxy-5α,17α-pregnan-20-one (**25**). However, the synthetic 17β-carbonitrile **16** was as active at 1 μM as allotetrahydroDOC in the potentiation of Cl^- flux, and 17-ketone **15** was less active. The ABC ring overlay of compounds **2a**, **15**, and **16** in Figure 8 shows that these compounds differ primarily in the location of the side-chain heteroatom (O_{20} for **2a**, O_{17} for **15**, nitrile N for **16**).

(21) Belelli, D.; Gee, K. W. *Eur. J. Pharmacol.* **1989**, *167*, 173.

Table III. Recovery of Radioactivity 20 min after Administration of ^{3}H-Labeled Steroids to Rats[c]

plasma dpm/mL × 10^3	brain dpm/g × 10^3	ratio brain/plasma[b]	% of total tritium in extracts of brain	
Treatment: 100 μCi [9α,11α,12α-^{3}H]-3α,21-Dihydroxy-5α-pregnan-20-one (**4a**)				
			4a	**22**
316 ± 14	212 ± 13	0.68 ± 0.05	48.1 ± 3.6	46.1 ± 2.3
Treatment: 100 μCi [9α,11α,12α-^{3}H]-3α-Hydroxy-5α-pregnan-20-one (**2a**)				
			2a	**20**
176 ± 1	342 ± 11	1.95 ± 0.05	50.4 ± 3.9	40.6 ± 1.1

[a] Adult male Sprague–Dawley rats (240–260 g) administered ^{3}H-labeled steroid by tail vein injection. [b] Column 3 divided by column 2. [c] All values are means ± SE.

Structure **2a** (O_3–O_{20} distance = 11.15 Å) can be reconciled with **15** (O_3–N_{20} distance = 11.09 Å) by a slight reorientation to align the side-chain heteroatoms. Since both **15** (O_3–O_{17} distance = 9.66 Å) and **16** are rigid, the 1.43 Å difference in O_3–heteroatom distance suggests that these compounds may interact with different binding sites on the $GABA_A$ receptor complex, although this possibility or the existence of multiple conformational states of a single binding site or the interaction of these ligands with multiple $GABA_A$ isoreceptors cannot be distinguished by molecular modeling of the ligands. The presence of different 3α-hydroxysteroid binding sites is strongly implied from recent neurophysiological evidence demonstrating differences in the effects of these steroids on membrane current recorded from cells with recombinantly expressed human $GABA_A$ isoreceptors (S. Vincini et al., unpublished results).

The formation of progesterone metabolites (**2a** and **12**) and DOC metabolites (**4a** and **11b**) in vivo may be an important mechanism in the regulation of GABA-mediated inhibition in the central nervous system. The comparative hypnotic effects of **2a** and **4a** were studied by Mendelson et al.[5] in the rat at doses of 5 and 10 mg/kg, where **4a** but not **2a** had potent sleep-inducing properties and increased non-REM sleep. Similarly, **4a** but not **2a** was shown by Crawley et al.[4a] to have anxiolytic activity in rodents in the range of 5–15 mg/kg. These comparative results in vivo might be explained by a more rapid metabolism of **2a** compared to that of **4a**. These possibilities were explored in a preliminary experiment in rats following the administration by tail vein of 100 μCi of ^{3}H-labeled **2a** or **4a**. From the data in Table III, the concentration of radioactivity in plasma obtained 20 min after the labeled steroid was injected was 80% higher for allotetrahydroDOC (**4a**) than the level obtained from rats given allopregnanolone (**2a**). It is therefore possible that an increased metabolic clearance rate of **2a** compared to that of **4a** might account for the greater anxiolytic and sedative effects of **4a** in vivo.[4,5] However, in brain tissue obtained 20 min after the radioactive compound was administered, there was a 60% higher level of total radioactivity/g of brain in the group receiving **2a** compared to the group treated with **4a** (Table III). After purification by solvent extraction and HPLC, there was an equal amount (50.4% ± 3.9%) of **2a** and (48.1% ± 3.6%) of **4a** as the mean percent of total radioactivity in brain tissue in each group. A more extensive pharmacokinetic study is required to resolve this dilemma.

The principal metabolites of the ^{3}H-labeled 3α-hydroxysteroids in brain were the 3-ketones, with 5α-pregnane-3,20-dione (**20**) as 40.6% ± 1.1% of total radioactivity in brain tissue from **2a** and 21-hydroxy-5α-preg-

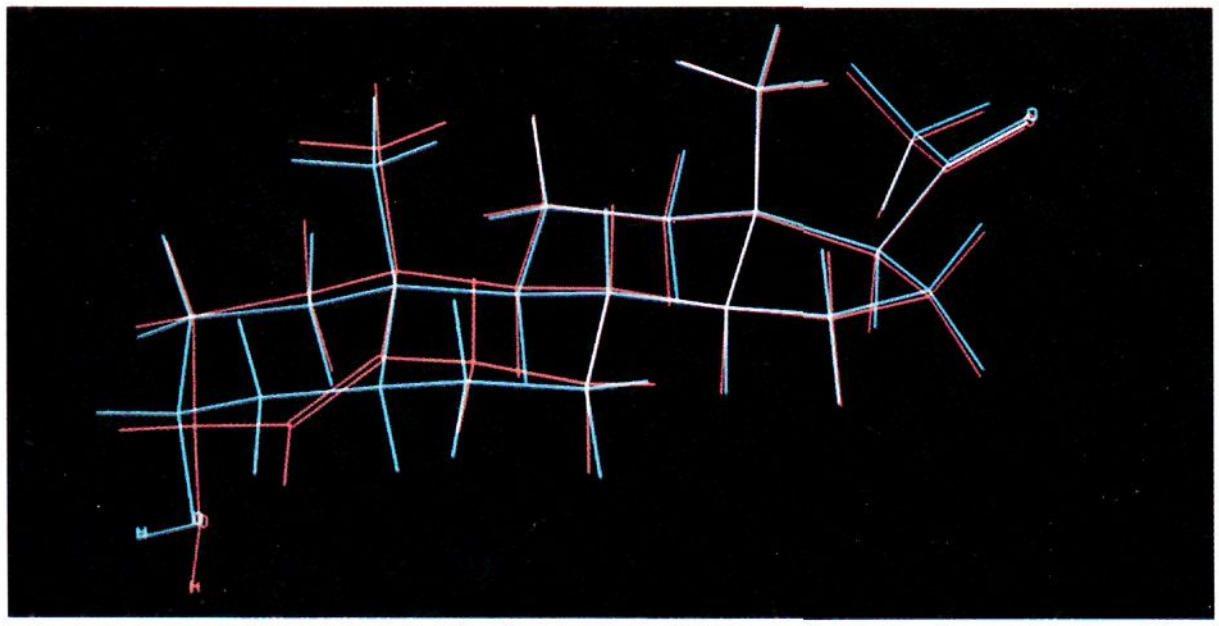


Figure 6. Template forcing of 3α-hydroxypregn-4-en-20-one (**12**, red) onto allopregnanolone (**2a**, blue) matching atoms O_3, C_{11}, C_{17}, O_{20} with an added 2.0 kcal/mol per Å potential.

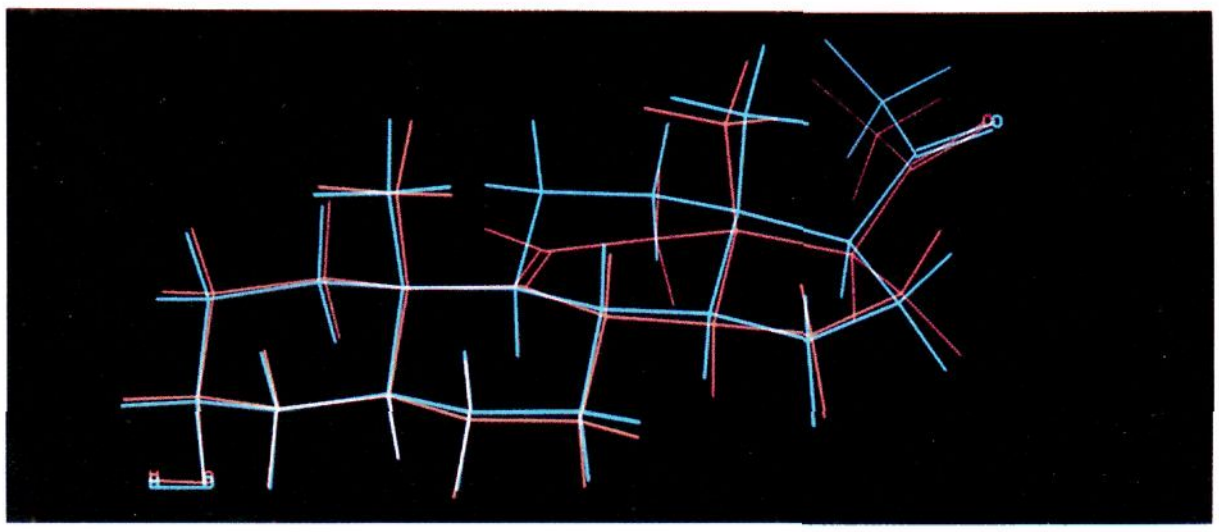


Figure 7. Rigid overlay of allopregnanolone (**2a**, blue) and 3α-hydroxy-5α-pregn-9(11)-en-20-one (**8**, red) showing the added steric hindrance on the α-face at C_{11} and C_{12} of structure **8**.

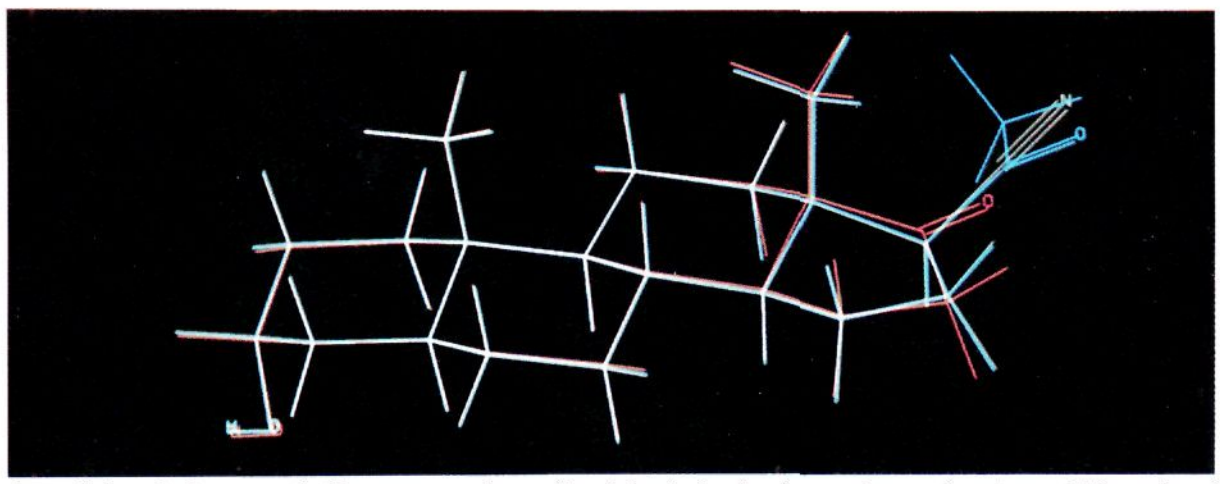


Figure 8. Rigid overlay of the skeletons of allopregnanolone (**2a**, blue), 3α-hydroxy-5α-androstane-17β-carbonitrile (**16**, green), and 3α-hydroxy-5α-androstan-17-one (**15**, red).

nane-3,20-dione (**22**) as 46.1 ± 2.3% of total radioactivity in the brain after administration of **4a**. After acetylation, the retention time of radioactive **20** remained unchanged and radioactive **22** was converted to a derivative with the retention time of 21-hydroxy-5α-pregnane-3,20-dione acetate. This provides additional confirmation of the identification of **20** and **22** as metabolites. No evidence was found for the presence of the 21-acetate of **4a** as a metabolite of **4a** in the brain extracts.

The availability of the tritium-labeled compounds will be of assistance in measuring physiological concentrations of **2a** and **4a** in blood and brain tissue by radioimmunoassay. In fact, we have recently demonstrated the presence of **2a** in both blood and brain tissue of rats at concentrations capable of potentiating muscimol-stimulated Cl^- flux.[22] The formation of these metabolites in vivo may therefore, be a significant mechanism in regulating $GABA_A$ receptor-mediated inhibition in the central nervous system. The synthesis of a bioavailable analogue of **2a** or **4a** may be a useful starting point for the development of novel anxiolytic/hypnotic compounds.

Experimental Section

Steroid Synthesis. Melting points were determined with a Thomas-Hoover Model 6406-H apparatus. IR spectra were recorded in KBr with a Perkin-Elmer 1600 FTIR spectrometer. ^1H NMR spectra were recorded in $CDCl_3$ solutions with a Varian EM-390 90-MHz spectrometer and are reported in ppm downfield from Me_4Si as the internal reference. Elemental microanalyses

(22) Purdy, R. H.; Moore, P. H., Jr.; Rao, P. N.; Hagino, N.; Yamaguchi, T.; Schmidt, P.; Rubinow, D. R.; Morrow, A. L.; Paul, S. M. *Steroids*, in press.

were performed by Midwest Microlab, Ltd., Indianapolis, IN, and are within ±0.3% of the theoretical values. Emission spectroscopy was carried out by Huffman Laboratories, Inc., Golden, CO. Chromatographic purification of products was performed by using the technique of Still et al.[23] with Merck silica gel (grade 60, 230–400 mesh, Aldrich Chemical Co.). High-performance liquid chromatography (HPLC) was performed with a Waters Associates Model 6000A pump, two Partisil 5 ODS-3 RAC columns (9.4 × 100 mm, Whatman, Inc.) in tandem using 75–85% aqueous methanol as solvent system at a flow rate of 1–3 mL/min. A Milton Roy SpectroMonitor 3100 detector was used to monitor the chromatographic effluent at 205 and 280 nm. A SuperRac Model 2211 LKB fraction collector was used to collect the effluent for determination of radioactivity after the addition of 8 mL ScintiVerse LC scintillation fluid (Fisher Scientific Co.). Radioactivity was measured with a Beckman Model LS 7500 liquid-scintillation spectrometer.

The preparations of 3α-hydroxypregn-4-en-20-one (**12**) and 3β-hydroxypregn-4-en-20-one (**13**) were carried out by the method of Wiebe et al.[11] The 3-oxime of 5α-pregnane-3,20-dione was synthesized by the procedure of Nace and Watterson.[24] 3α-Hydroxy-5α,17α-pregnan-20-one (**27**) was synthesized by isomerization of allopregnanolone (**2a**).[25] The 17-oxime of 3α-hydroxy-5α-androstan-17-one (**28**) and the 20-ethylenedioxy derivatives (**29**, **30**) were prepared in the usual manner. The starting material 3β-hydroxy-5α-pregnan-20-one (**1**) was a gift from Dr. John S. Baran, G. D. Searle Co., and 11α-hydroxy-5α-pregnane-3,20-dione (**5**)[26] was purchased from Steraloids, Inc.

3α-Hydroxy-5α-pregnan-20-one (2a). The reaction of 3β-hydroxy-5α-pregnan-20-one (**1**) with trifluoroacetic acid in the presence of triphenylphosphine and diethyl azodicarboxylate without isolation of the intermediate trifluoroacetate[13] yielded **2a** in 74% yield after the usual workup and crystallization from ethyl acetate and aqueous ethanol free of detectable **1** by HPLC, mp 168–170 °C (lit.[10] mp 170–173 °C). The mother liquor from the first crystallization contained 5% of total steroids as **1** by HPLC analysis. The final product was purified to homogeneity with dry column chromatography on silica gel with 2% acetone in CH_2Cl_2 and crystallized from aqueous ethanol; yield 54%, mp 174–176 °C.

3α-Acetoxy-5α-pregnan-20-one (2b). Treatment of **2a** in the usual manner gave **2b**, mp 135–138 °C (lit.[27] mp 139–140 °C).

21-Acetoxy-3α-hydroxy-5α-pregnan-20-one (3a). The reaction of 5.23 g of **2a** in 300 mL of benzene, 16 mL of methanol, and 29 mL of boron trifluoride etherate with 10.9 g of lead tetraacetate was carried out under argon for 3 h at 0 °C. The reaction mixture was diluted with 300 mL of saturated aqueous sodium bicarbonate and extracted twice with 300 mL of ether at 0 °C. The combined ether extracts were washed with 300 mL of water, dried over Na_2SO_4, and crystallized from ethyl acetate to afford 2.07 g (35%) of **3a**, mp 210–216 °C (lit.[28] mp 216–218 °C). Recrystallization gave product, mp 215–219 °C, which was free of detectable impurities by HPLC.

3α,21-Diacetoxy-5α-pregnan-20-one (3b). In a similar manner 1.71 g of **2b** was converted to 0.46 g (24%) of **3b** after crystallization from ether/petroleum ether, mp 162–163 °C (lit.[29] mp 164–165 °C).

3α,21-Dihydroxy-5α-pregnan-20-one (4a). A suspension of 0.68 g of **3a** in 21 mL of methanol was treated with 1.80 mL of aqueous 10% K_2CO_3 under argon, stirred at room temperature for 30 min, cooled to 0 °C, and acidified (pH 5) with 0.7 mL of 2 M acetic acid. Methanol was removed in vacuo until crystallization occurred, giving 0.58 g (96%) of **4a**, mp 162–164 °C (lit.[28] mp 163–166 °C). To remove all traces of lead, **3a** was purified by dry column chromatography on silica gel using CH_2Cl_2/ethyl acetate (9:1) prior to hydrolysis. This provided **4a** with less than 10 ppm lead by emission spectroscopy.

3α-Acetoxy-21-hydroxy-5α-pregnan-20-one (4b). A methanolic suspension of **3b** was hydrolyzed by the procedure described for compound **4a** to provide 125 mg (93%) of **4b**. An analytical sample was crystallized from aqueous methanol: mp 168–172 °C; IR (KBr) 3501 (OH), 1721 (CO), 1703 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.62 (s, 3 H, 18-CH_3), 0.77 (s, 3 H, 19-CH_3), 2.05 (s, 3 H, $COCH_3$), 4.20 (d, 2 H, 21-CH_2), 5.05 (b s, 1 H, 3β-H). Anal. ($C_{23}H_{36}O_4$) C, H.

11α-[(Methylsulfonyl)oxy]-5α-pregnan-20-one (6). A solution of 1.02 g of 11α-hydroxy-5α-pregnane-3,20-dione (**5**)[26] in 5 mL of dry pyridine at 0 °C was treated with 0.4 mL of methanesulfonyl chloride for 2 h. The mixture was poured on ice, and the precipitate was collected and washed with cold 1 N HCl to afford 1.22 g (97%) of crude **6**, mp 145–146 °C dec. An analytical sample was crystallized from acetone/hexane: mp 146–148 °C dec; IR (KBr) 1165 (SO), 1321 (SO), 1700 (CO), 1715 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.70 (s, 3 H, 18-CH_3), 1.19 (s, 3 H, 19-CH_3), 2.13 (s, 3 H, 21-CH_3), 3.00 (s, 3 H, CH_3SO_2), 5.13 (m, 1 H, 11β-H). Anal. ($C_{22}H_{34}O_5S$) C, H, S.

5α-Pregn-9(11)-ene-3,20-dione (7). A solution of 1.22 g of **6** and 1.7 g of anhydrous sodium acetate in 15 mL of glacial acetic acid was refluxed for 30 min, cooled to 0 °C, and diluted with 15 mL of H_2O to give 0.86 g (92%) of **7**: mp 203–204 °C; IR (KBr) 1695 (CO), 1715 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.59 (s, 3 H, 18-CH_3), 1.15 (s, 3 H, 19-CH_3), 2.12 (s, 3 H, 21-CH_3), 5.43 (b t, 1 H, 11-H). Anal. ($C_{21}H_{30}O_2$) C, H.

3α-Hydroxy-5α-pregn-9(11)-en-20-one (8). To a stirred solution of 0.86 g of **7** in 60 mL of dry tetrahydrofuran under argon at −78 °C was added 3.0 mL of 1 M K-Selectride (Aldrich Chemical Co.). After 1 h the solution was warmed to 0 °C and added with stirring to 55 mL of 0.5 M Tris HCl buffer (pH 7). After 45 min of stirring at 0 °C, the mixture was extracted twice with 100 mL of ethyl acetate and dried in vacuo to give 0.97 g of crude **8**. The product was purified by HPLC on a Magnum 20 Partisil 10 ODS-3 column (2.25 × 50 cm, Whatman, Inc.) with a 0.94 × 15 cm ODS-3 guard column using 75% aqueous methanol at a flow rate of 9.9 mL/min, eluting between 56 and 70 min. Crystallization from aqueous methanol provided 0.22 g (25%) of **8**: mp 172–173 °C (lit.[30] mp 169–170 °C); IR (KBr) 3277 (OH), 1706 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.57 (s, 3 H, 18-CH_3), 0.90 (s, 3 H, 19-CH_3), 2.12 (s, 3 H, 21-CH_3), 4.10 (b s, 1 H, 3β-H), 5.38 (b t, 1 H, 11-H). Anal. ($C_{21}H_{32}O_2$) C, H.

21-Acetoxy-3α-hydroxy-5α-pregn-9(11)-en-20-one (9a). By the procedure described for **3a**, 0.158 g of **8** in 9.5 mL of benzene, 0.5 mL of methanol, and 0.9 mL of boron trifluoride etherate was reacted with 0.334 g of lead tetraacetate to give 98 mg (52%) of **9a** after crystallization from ethyl acetate: mp 236–238 °C; IR (KBr) 3558 (OH), 1753 (CO), 1715 (CO) cm^{-1}; NMR ($CDCl_3$/CD_3OD, 1:1) δ 0.57 (s, 3 H, 18-CH_3), 0.90 (s, 3 H, 19-CH_3), 2.13 (s, 3 H, $COCH_3$), 3.98 (b s, 1 H, 3β-H), 4.66 (dd, 2 H, 21-CH_2), 5.30 (b t, 1 H, 11-H). Anal. ($C_{23}H_{34}O_4$) C, H.

3α,21-Dihydroxy-5α-pregn-9(11)-en-20-one (9b). Under the conditions of hydrolysis described for **4a**, 66 mg of **9a** suspended in 4 mL of methanol and treated with 0.33 mL of aqueous 10% K_2CO_3 gave 50 mg of crude **9b**, which was purified by HPLC on a Magnum 9 Partisil ODS-3-column (2.25 × 25 cm, Whatman, Inc.) using 75% aqueous methanol at a flow rate of 5 mL/min, eluting between 13 and 16 min. Crystallization from aqueous methanol gave 31 mg (53%) of **9b**: mp 152–154, 183–185 °C; IR (KBr) 3281 (OH), 1717 (CO) cm^{-1}; NMR ($CDCl_3$/CD_3OD, 1:1) δ 0.56 (s, 3 H, 18-CH_3), 0.91 (s, 3 H, 19-CH_3), 4.06 (b s, 1 H, 3β-H), 4.20 (b m, 2 H, 21-CH_2), 5.29 (b t, 1 H, 11-H). Anal. ($C_{21}H_{32}O_3$) C, H.

21-Acetoxy-3α-[(*tert*-butyldimethylsilyl)oxy]-5α-pregnan-20-one (9c). A solution of 1.55 g of **9a** and 0.73 g of imidazole in 12 mL of dry dimethylformamide was treated with 0.81 g of *tert*-butyldimethylsilyl chloride overnight at 24 °C.[31] After extraction with ether the crude product was purified by dry column chromatography on silica gel using petroleum ether/ether (5:1) to give 1.05 g (51%) of **9c**. An analytical sample was

(23) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(24) Nace, H. R.; Watterson, A. C., Jr. *J. Org. Chem.* **1966**, *31*, 2109.
(25) Eriksson, H.; Gustafsson, J. A.; Sjövall, J. *Eur. J. Biochem.* **1968**, *6*, 219.
(26) Nussim, J.; Sandheimer, F. *Chem. Ind.* **1960**, 400.
(27) Marker, R. E.; Kamm, O.; McGrew, R. V. *J. Am. Chem. Soc.* **1937**, *59*, 616.
(28) Harnik, M. *Steroids* **1964**, *3*, 359.
(29) Schneider, J. J. *J. Biol. Chem.* **1952**, *199*, 235.
(30) Ger. Offen. DE 2,526,373, 1976; *Chem. Abstr.* **1977**, *86*,
(31) Corey, E. J.; Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190.

crystallized from petroleum ether: mp 110–112 °C; IR (KBr) 1748 (CO), 1730 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.62 (s, 3 H, 18-CH_3), 0.73 (s, 3 H, 19-CH_3), 0.88 [s, 6 H, $Si(CH_3)_2$ $C(CH_3)_3$], 2.13 (s, 3 H, $COCH_3$), 3.96 (b s, 1 H, 3β-H), 4.55 (dd, 2 H, 21-CH_2). Anal. ($C_{29}H_{50}O_4Si$) C, H.

3α-[(*tert*-Butyldimethylsilyl)oxy]-21-hydroxy-5α-pregnan-20-one (9d). A suspension of 1.0 g of **9c** in 75 mL of methanol was hydrolyzed with 1.9 mL of aqueous 10% K_2CO_3 as described for **4a** to give 0.88 g of crude **9d**. The product was purified by dry column chromatography on silica gel with petroleum ether/ether (5:1) to give 0.71 g (78%) of **9d**, which crystallized from ether: mp 106–108 °C; IR (KBr) 3490 (OH), 1711 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.60 (s, 3 H, 18-CH_3), 0.72 (s, 3 H, 19-CH_3), 0.87 [s, 6 H, $Si(CH_3)_2C(CH_3)_3$], 3.97 (b s, 1 H, 3β-H), 4.17 (d, 2 H, 21-CH_2). Anal. ($C_{27}H_{48}O_3Si$) C, H.

3α-[(*tert*-Butyldimethylsilyl)oxy]-21-[(methylsulfonyl)oxy]-5α-pregnan-20-one (9e). A solution of 0.71 g of **9d** in 3.5 mL of pyridine was treated with 0.35 mL of methanesulfonyl chloride as described for the preparation of **6** to give 0.84 g of crude **9e**. The product was purified by dry column chromatography on silica gel using petroleum ether/ether (2:1) to yield 0.70 g (82%) of **9e** as a white gum which failed to crystallize: NMR ($CDCl_3$) δ 0.64 (s, 3 H, 18-CH_3), 0.74 (s, 3 H, 19-CH_3), 0.88 [s, 6 H, $Si(CH_3)_2C(CH_3)_3$], 3.22 (s, 3 H, SO_2CH_3), 3.98 (b s, 1 H, 3β-H), 4.80 (dd, 2 H, 21-CH_2).

3α-Hydroxy-21-[(methylsulfonyl)oxy]-5α-pregnan-20-one (14). A solution of 0.60 g of **9e** in 15 mL of a mixture of tetrahydrofuran/acetic acid/water (1:3:1) under argon was heated at 60 °C for 7 h. After evaporation of the solvent in vacuo, the crude product was purified by dry column chromatography on silica gel using 5% acetone in CH_2Cl_2 and crystallized from acetone/heptane to give 0.39 g (82%) of **14**: mp 171–172 °C; IR (KBr) 3558 (OH), 1723 (CO) cm^{-1}; NMR ($CDCl_3$) δ 0.67 (s, 3 H, 18-CH_3), 0.78 (s, 3 H, 19-CH_3), 3.23 (s, 3 H, SO_2CH_3), 4.08 (b s, 1 H, 3β-H), 4.80 (dd, 2 H, 21-CH_2). Anal. ($C_{22}H_{36}O_5S$) C, H, S.

3α-Hydroxy-5α-androstane-17β-carbonitrile (16). An approximate 2:1 mixture of the 17-epimers **16** and **17**, respectively, was prepared from 3 g of 3α-hydroxy-5α-androstan-17-one (**15**) with tosylmethyl isocyanide.[32] The epimeric mixture was separated by dry-column chromatography on silica gel using hexane/ethyl acetate (2:1), to afford 0.73 g (24%) of **16** as the more polar epimer after crystallization from ethyl acetate: mp 156–158 °C; IR (KBr) 3550 (OH), 2237 (CN) cm^{-1}; NMR ($CDCl_3$) δ 0.79 (s, 3 H, 18-CH_3), 0.90 (s, 3 H, 19-CH_3), 4.05 (m, 1 H, 3β-H). Anal. ($C_{20}H_{31}NO$) C, H, N.

3α-Hydroxy-5α-androstane-17α-carbonitrile (17). The less polar epimer separated chromatographically in the preparation of **16** above was crystallized from ethyl acetate/hexane to give an analytical sample: mp 183–185 °C; IR (KBr) 3550 (OH), 2237 (CN) cm^{-1}; NMR ($CDCl_3$) δ 0.78 (s, 3 H, 18-CH_3), 0.80 (s, 3 H, 19-CH_3), 4.05 (m, 1 H, 3β-H). Anal. ($C_{20}H_{31}NO$) C, H, N.

Steroid Modeling. Interactive molecular modeling was performed with the Mosaic molecular modeling program running on a VAX 8800 and an Evans and Sutherland PS300 or IBM PC-AT display terminal. Mosaic is derived from the MacroModel program.[33] Energy calculations used the MM2 force field of MacroModel 2.1 in either Mosaic (interactive) or BatchMin (batch) with a distant dependent dielectric ($\epsilon = R$) and default nonbonded cutoffs. The Mosaic/MacroModel/BatchMin implementation of the MM2 force field differs from authentic MM2[34] primarily in the treatment of electrostatics; the Mosaic/MacroModel implementation uses a point-charge model whereas the MM2 program uses a dipole–dipole model. Energy minimizations were generally performed with the block diagonal Newton Raphson minimizer; the full matrix Newton Raphson minimizer was used to minimize to saddle point structures. The crystal structure of alphaxalone[35] was used as a starting point for building models of the other steroid structures. Conformational searching was performed with MULTIC submode,[36] using 1.0–2.0 Å ring closures when searching rings, and 60° torsional resolution unless otherwise stated.

Steroid Potentiation of Chloride Flux. Synaptoneurosomes were prepared from fresh cerebral cortices of adult male Sprague–Dawley rats (180–200 g) as previously described.[2e] Briefly, cerebral cortices were homogenized in ice-cold (4 °C) Tris–Hepes buffer (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM D-glucose. The homogenate was filtered through three layers of nylon mesh (160 μm) and then through a 10-μm Millipore filter. The filtrate was washed twice by centrifugation at 1000*g* for 15 min and the final pellet was resuspended in buffer at a concentration of 10 mg of protein per mL. In the present experiments the effect of the various steroids in potentiating muscimol-stimulated $^{36}Cl^-$ uptake of synaptoneurosomes was measured as this has been previously shown to be a sensitive method to detect activity at the $GABA_A$ receptor coupled Cl^- ion channel.[2a,e] A muscimal concentration of 3 μM to approximate the ED_{50} was used in all experiments. Chloride-36 uptake was measured in quadruplicate by the simultaneous addition of 0.5 μCi $^{36}Cl^-$ (Du Pont New England Nuclear) to the synaptoneurosomes (1 mg of protein per tube) which had been preincubated at 30 °C for 10 min. All drugs or steroids were added for the preincubation time of 10 min. Uptake was terminated after 5 s by dilution in 5 mL of ice-cold buffer containing 100 μM picrotoxin, followed by rapid filtration over glass-fiber filters (Schleicher and Schuell No. 30) under vacuum. The filters were washed twice with 5 mL of buffer and the radioactivity was determined by liquid-scintillation spectroscopy in 5 mL of RediSolve (Beckman Instruments). Net uptake was calculated by subtracting $^{36}Cl^-$ uptake observed in the absence of steroid (basal level) from that observed in the presence of steroid. Potentiation represents the increase in $^{36}Cl^-$ uptake by steroids compared to the muscimol-stimulated uptake alone. Statistical comparisons are made with Student's *t* test. Protein determinations were performed by the method of Lowry et al.[37]

Steroid Metabolism. Groups of four adult male Sprague–Dawley rats (240–260 g) were administered 100 μCi of ^3H-labeled allopregnanolone or allotetrahydroDOC in (70 μL) ethanol–(130 μL) saline solution by tail vein injection. After 20 min the animals were killed by decapitation, and blood and brain tissue were collected and immediately cooled on ice. After aliquots were taken for determination of radioactivity, the tissue was frozen at −40 °C. Prior to extraction, the brain tissue (1.7–1.9 g) was homogenized in 10 mL/g of chloroform/methanol (2:1, v/v) at 0–4 °C with a Polytron (Brinkman Instruments) at setting 2 for five 5-s intervals. Internal standards of 100 μg of deoxycorticosterone were added for calculation of recoveries and the samples were centrifuged at 3000*g* for 15 min. The pellet was washed with an additional 10 mL/g ethanol at 0 °C and centrifuged, and the combined supernatant fractions were evaporated in vacuo. The residues were partitioned between hexane and 90% aqueous methanol, 100 mL of each phase, using a three transfer countercurrent distribution with stripping. The 90% methanol phases containing over 95% of the radioactivity were combined and evaporated in vacuo. The residues were similarly partitioned between ethyl acetate and water, 100 mL of each phase. The combined ethyl acetate phases, containing over 95% of the radioactivity, were evaporated in vacuo. The resulting samples were purified by HPLC using the solvent system 75% aqueous methanol at 2 mL/min on two Whatman ODS-3 RAC columns in tandem. Fractions were collected every 0.4 min and 10% aliquots were used for the measurement of radioactivity. Internal standards of allopregnanolone (**2a**) or allotetrahydroDOC (**4a**), 3-ketone derivatives **20** or **22**, and other metabolites were injected with the radioactive samples and their elution was monitored by ultraviolet absorption at 280 nm. The recovery standards of deoxycorticosterone were measured by absorption at 254 nm. For further identification, half of the fractions containing the purified radioactivated compounds were evaporated in vacuo and acety-

(32) Bull, J. R.; Tuinman, A. *Tetrahedron* **1975**, *30*, 2151.
(33) Still, W. C.; Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Hendrickson, T. MacroModel V2.0, Batchmin V2.1; Department of Chemistry, Columbia University: New York.
(34) Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127.
(35) (a) Cambridge Crystallographic Database, reference code HXPRDO. (b) Midgley, J. M.; Whalley, W. B.; Ferguson, G.; Marsh, W. C. *J. Chem. Soc., Perkin Trans. 2* **1978**, 1042.
(36) Lipton, M.; Still, W. C. *J. Comput. Chem.* **1988**, *9*, 343.
(37) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

lated with acetic anhydride (0.1 mL) in pyridine (0.1 mL) overnight at room temperature. After evaporation of the solvents under a stream of nitrogen, the acetylated residues were purified by HPLC as described above using the solvent system 85% aqueous methanol. Internal standards of the authentic acetates were located in the eluates by ultraviolet absorption at 280 nm.

Acknowledgment. This work was supported in part by a grant from the Upjohn Co. We are indebted to Perry H. Moore, Jr. for his excellent technical assistance in the synthetic work and in the metabolism study, to John R. Pace for some of the chloride-uptake determinations, and to Dr. Donald E. Ayer and Dr. Douglas C. Rohrer for assistance and helpful discussions. The formation of the ^{3}H-labeled compounds was performed at Du Pont NEN Products, Boston, MA. We are grateful to Dr. Mayo Cabell, Du Pont NEN for providing the ^{3}H NMR and specific activity data of these products.

Registry No. 1, 516-55-2; **2a**, 516-54-1; **2b**, 6003-24-3; **3a**, 96611-83-5; **3b**, 96038-59-4; **4a**, 567-02-2; **4b**, 126451-95-4; **5**, 565-96-8; **6**, 126296-19-3; **7**, 111464-63-2; **8**, 38393-03-2; **9a**, 126296-20-6; **9b**, 102917-20-4; 11, 126296-21-7; **12**, 126296-22-8; **13**, 126296-23-9; **14**, 126296-24-0; **15**, 53-41-8; **16**, 51872-53-8; **17**, 126296-25-1; **18**, 53-42-9; **19**, 85611-15-0; **20**, 566-65-4; **21**, 6750-73-8; **22**, 298-36-2; **23**, 57-83-0; **24**, 38398-44-6; **25**, 600-52-2; **26**, 6951-91-3; **27**, 21788-58-9; **28**, 5953-71-9; **29**, 126451-96-5; **30**, 18000-89-0; **31**, 566-58-5; Cl^-, 16887-00-6; 3α-hydroxypregn-4-en-20-one, 25680-68-6; 3α,21-dihydroxy-5β-pregnan-20-one 21-acetate, 2402-24-6; 3α,21-dihydroxy-5β-pregnan-20-one, 567-03-3; 3β-hydroxypregn-4-en-20-one, 566-66-5.