Journal of
Medicinal
Chemistry

Volume 43, Number 17 August 24, 2000

Communications to the Editor

Neurosteroid Analogues. 8. Structure−Activity Studies of *N*-Acylated 17a-Aza-D-homosteroid Analogues of the Anesthetic Steroids (3α,5α)- and (3α,5*β*)-3-Hydroxypregnan-20-one

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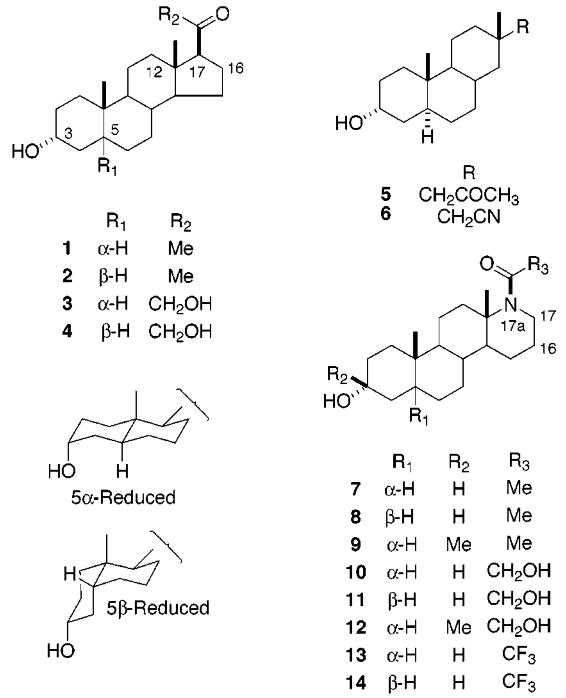
Received June 8, 2000

Introduction. The steroids (3α,5α)- and (3α,5*β*)-3-hydroxypregnan-20-one (**1** and **2**, respectively, Chart 1) are potent anesthetics.[1] Multiple lines of evidence now support the hypothesis that anesthesia results from the ability of these steroids to enhance of the actions of *γ*-aminobutyric acid (GABA) at $GABA_A$ receptors.[2] Structure−activity studies (SAR) have shown that the 3α-OH group is essential for the anesthetic actions of these steroids. The 17*β*-acetyl group can be replaced with other 17*β*-substituents that are hydrogen bond acceptors.[1]

In a previous study, we determined that phenanthrene analogues **5** and **6** were weak modulators of $GABA_A$ receptor function.[3] Apparently, the flexible side chains in these analogues do not have the conformations required for high biological activity. In a further attempt to identify steroids having a modified D-ring and high pharmacological activity at $GABA_A$ receptors, we prepared and evaluated 17a-aza-D-homosteroids **7**−**14**. We report that azasteroid **10** has pharmacological activities comparable to those of neurosteroids **1**−**4** in two different bioassays of $GABA_A$ receptor function. We also report the results of a conformational analysis performed to explain the results of this SAR study.

Chart 1



	R_1	R_2
1	α-H	Me
2	β-H	Me
3	α-H	CH_2OH
4	β-H	CH_2OH

	R
5	CH_2COCH_3
6	CH_2CN

	R_1	R_2	R_3
7	α-H	H	Me
8	β-H	H	Me
9	α-H	Me	Me
10	α-H	H	CH_2OH
11	β-H	H	CH_2OH
12	α-H	Me	CH_2OH
13	α-H	H	CF_3
14	β-H	H	CF_3

Results and Discussion. The 17a-aza-D-homosteroids were readily prepared by the general synthetic pathway shown for the preparation of azasteroid **12** in Scheme 1. The Beckmann rearrangement of the oximes of 17-ketosteroids to give 17a-aza-17-oxo-D-homosteroids is a well-known reaction.[4] The remaining synthetic transformations shown in Scheme 1 involve standard methods. For those steroids not containing the 3*β*-methyl group, the 3α-OH group was protected as either an acetate or a MOM ether prior to the Beckmann rearrangement. Protecting groups were removed either prior to or after addition of the acyl side chains.[5]

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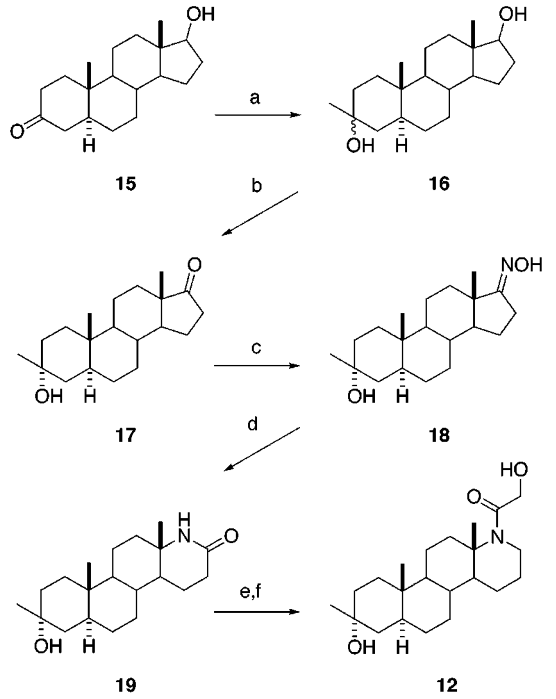
10.1021/jm0002477 CCC: $19.00

Scheme 1[a]



[a] (a) CH_3MgBr, THF, 0 °C during addition, overnight at rt, 94%; (b) (1) Jones reagent, acetone, 30 min at rt, 90%, (2) chromatographic separation of C-3 epimers; (c) $NH_2OH{\cdot}HCl$, NaOAc, MeOH, H_2O, reflux 3 h, 100%; (d) anhydrous pyridine, *p*-TsCl, 4 h at rt, 72%; (e) $BH_3{\cdot}THF$, THF, 8 h at reflux; (f) (1) acetoxyacetyl chloride, Et_3N, THF, (2) MeOH, NaOH, 4 h at rt, 21% overall for steps e and f.

Compounds **1**−**4** and **7**−**14** were evaluated in two different bioassays: (1) noncompetitive displacement of [^{35}S]-*tert*-butylbicyclophosphorothionate (TBPS) from the picrotoxin site of $GABA_A$ receptors found in rat brain membranes and (2) effects on the electrophysiological responses of rat $\alpha1\beta2\gamma2$ $GABA_A$ receptors expressed in *Xenopus* laevis oocytes. For the [^{35}S]TBPS binding, full concentration−response curves were determined and all compounds displaced >90% of specific binding. The electrophysiological data do not represent full concentration−response data, and there may be differences in efficacy as well as potency for the compounds in this bioassay. The results are shown in Table 1.

Although steroids **1** and **2** are equipotent displacers of [^{35}S]TBPS, steroid **1** produces both a greater potentiation of GABA-mediated currents and a greater activation of a chloride current than steroid **2** in the oocyte experiments. Steroids **3** and **4**, which contain a 21-OH group, behave similarly to steroids **1** and **2**. Neither the IC_{50} for [^{35}S]TBPS displacement nor the electrophysiological responses are much changed by the presence of a 21-OH group. Similar results have been reported previously.[2,6]

The results obtained for azasteroids **7**−**14** indicate that whereas the stereochemistry at C-5 has a minor effect on the actions of steroids **1**−**4**, it has a major effect on the actions of the corresponding azasteroid analogues. As shown in Table 1, the 5α-reduced azasteroids are uniformly more potent than the corresponding 5β-reduced azasteroids. The 5α compound **7** is ~17-fold more potent a displacer of [^{35}S]TBPS than the corresponding 5β compound **8**. Additionally, compound **7** (10 μM) causes nearly a 4-fold enhancement of GABA-mediated current, whereas compound **8** (10 μM) causes only ~20% enhancement. The direct activation of a chloride current by either compound (10 μM) is minimal.

Table 1. Biological Activities of Steroids **1**−**4** and 17a-Aza-D-homosteroids **7**−**14**

compd	TBPS IC_{50}[a] (μM)	oocyte electrophysiology[b] 0.1 μM	1 μM	10 μM
1	0.074 ± 0.0074	1.26 ± 0.14	3.89 ± 1.34	9.65 ± 3.87 0.37 ± 0.07 (G)
2	0.071 ± 0.018	0.97 ± 0.08	1.92 ± 0.46	4.3 ± 1.4 0.01 ± 0.01 (G)
3	0.039 ± 0.003	1.30 ± 0.07	4.55 ± 0.55	11.83 ± 2.10 1.67 ± 1.22 (G)
4	0.12 ± 0.02	1.21 ± 0.02	2.37 ± 0.17	5.43 ± 0.28 0.19 ± 0.02 (G)
7	0.20 ± 0.01	1.21 ± 0.06	1.66 ± 0.13	3.74 ± 0.28 0.05 ± 0.02 (G)
8	3.47 ± 0.28	1.37 ± 0.17	1.04 ± 0.08	1.18 ± 0.09 0.01 ± 0.01 (G)
9	2.61 ± 0.28	0.96 ± 0.06	1.04 ± 0.06	1.99 ± 0.16 0.05 ± 0.02 (G)
10	0.045 ± 0.02	1.17 ± 0.10	1.73 ± 0.13	8.09 ± 1.29 0.49 ± 0.07 (G)
11	0.93 ± 0.13	1.36 ± 0.27	1.57 ± 0.22	3.87 ± 1.50 0.10 ± 0.06 (G)
12	0.55 ± 0.041	1.00 ± 0.09	1.22 ± 0.14	6.50 ± 0.98 0.20 ± 0.04 (G)
13	0.43 ± 0.04	1.11 ± 0.03	1.91 ± 0.21	10.12 ± 1.49 0.50 ± 0.16 (G)
14	6.22 ± 1.28	0.98 ± 0.01	0.96 ± 0.02	1.12 ± 0.06 0.02 ± 0.02 (G)

[a] [^{35}S]TBPS binding was carried out as described previously.[14] Results presented are from duplicate experiments performed in triplicate. Error limits are calculated as standard error of the means. The Hill coefficients for displacement of [^{35}S]TBPS varied from 0.57 to 1.43. [b] The electrophysiological methods were as described previously.[15] The GABA concentration used for the control response was 2 μM. Each steroid or azasteroid was evaluated ($N = 3-6$, except compound **1** where $N = 12$) at the 3 concentrations indicated, and the results reported are the ratio of the currents measured in the presence/absence of added steroid. (G) represents the direct current gated by 10 μM compound in the absence of GABA, and this current is reported as the ratio of steroid only current/2 μM GABA current. Error limits are calculated as standard error of the means.

Changing the 17a-acetyl group to an α-hydroxyacetyl group as in azasteroids **10** and **11** increases the potency (or possibly the efficacy in the oocyte experiments) of both compounds in both bioassays. By contrast, changing the 17a-acetyl group to a trifluoromethylacetyl group as in compounds **13** and **14** decreases the potency of both compounds as displacers of [^{35}S]TBPS. In the oocyte experiments, the trifluoromethylacetyl group increases the activity of the 5α compound **13** (compare with azasteroid **7**) but does not enhance the activity of the 5β compound **14** (compare with azasteroid **8**). Finally, addition of a 3β-methyl group, as in azasteroids **9** and **12**, adversely affects the activity of the azasteroids in both bioassays (compare compound **9** with **7** and **12** with **10**).

Conformational analysis of the 17a-aza-D-homosteroids was performed as an initial step to correlate the above results with the active conformation of the compounds. Minimum energy conformations of azasteroids **7**−**14** were identified by molecular mechanics calculations (MM2 parameters and the Dihedral Driver option contained in the CS Chem3D Pro program). In every case, and as shown in Figure 1A−D for azasteroid **9**, four minimum energy conformers (two pairs in which the piperidine ring is in either the chair or boat conformation) were found. Only one of these conformers has the carbonyl oxygen of the side chain located above

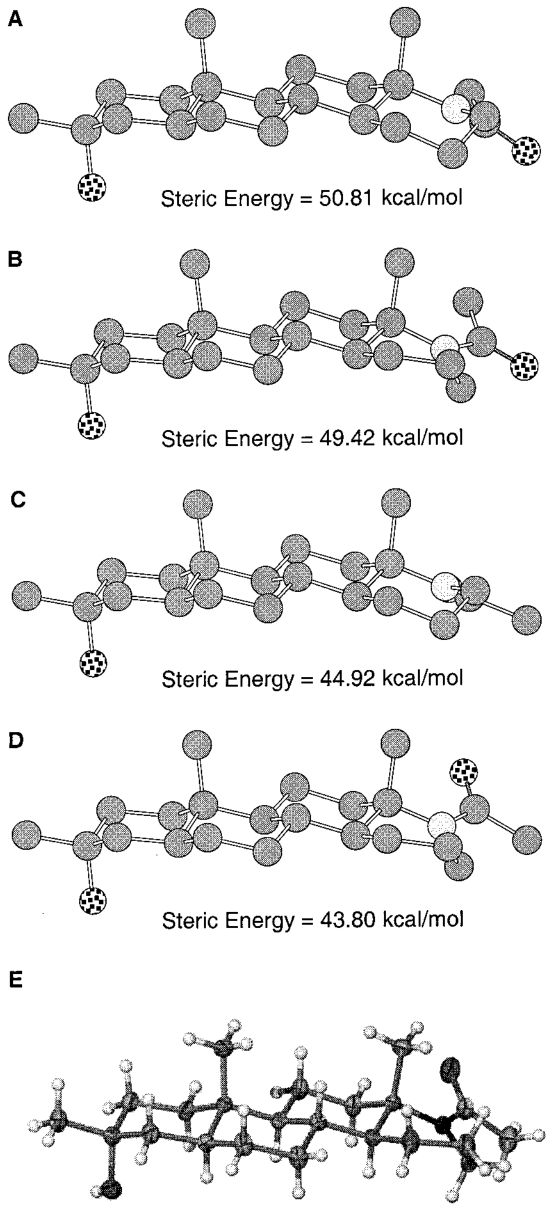


Figure 1. Minimum energy conformations (A−D) and X-ray crystal structure (E) of compound **9**. The hydrogen atoms are not displayed in panels A−D.

a plane defined by atoms C-5, C-7, and C-12 of the steroid rings. This conformer is also the minimum energy conformer. On the basis of steric energy considerations, neither conformer in which the carbonyl group is oriented away from C-12 (Figure 1A,B) is expected to be significantly populated at physiologic temperatures. Although the conformer having a steric energy of 44.92 kcal/mol cannot be eliminated from consideration as a biologically active conformation on the basis of its steric energy, it is unlikely to be biologically relevant because the carbonyl group is below the plane (C-5, C-7, and C-12) of the steroid. Steroids containing either a 17α-acetyl or a 17α-cyano group, as well as those in which the acetyl group is pulled down into the plane of the D-ring by a Δ^{16}-double bond, are known to lack anesthetic activity and to be weak modulators of $GABA_A$ receptors.[1,7−9] Thus, the modulatory actions of azasteroids **7**−**14** can be reasonably associated with a conformation analogous to that shown for the minimum energy conformer of azasteroid **9**.

The crystal structure of azasteroid **9** was determined (see Figure 1E).[10] As expected from the molecular mechanics calculations, the molecule has the piperidine ring in a boat conformation with the side chain carbonyl group oriented toward C-12. The O−O distance in the molecule is 9.82 Å, which is in excellent agreement with the value of 9.85 Å from the molecular mechanics calculation. The relevance of this solid-state structure to the solution conformation of azasteroids **7**−**14** is supported by ^{1}H NMR ($CDCl_3$) data. The two C-17 hydrogens in the amine precursors to the acylated azasteroids **7**−**14** occur in the chemical shift range of δ 2.9−2.7. For compounds **7**−**14**, these C-17 hydrogen resonances occur in the region of δ 3.6−3.3. In every case, however, an additional hydrogen resonance in the region of δ 3.0−3.3 is observed for compounds **7**−**14**. This additional downfield resonance can only be attributed to a hydrogen on either C-12 or C-16 being oriented within the deshielding cone of the newly introduced side chain carbonyl group.

For the 3β-OH epimer (structure not shown) of 5α-reduced azasteroid **7**, the C-17 protons are observed as overlapping multiplets at δ 3.45−3.31. An additional resonance is centered at δ 3.28 (broadened dd, $J = 3.3$, 9.3 Hz). Replacement of the C-17 hydrogens with deuteriums (achieved by $LiAlD_4$ reduction of the appropriate lactam precursor) has no effect on the splitting pattern of the δ 3.28 resonance. This indicates that the δ 3.28 resonance is not the result of a C-16 resonance residing within the deshielding cone of the side chain carbonyl group. The result is consistent with the C-12 equatorial hydrogen residing within the deshielding cone of the carbonyl group when it is oriented as it is in the solid-state structure of azasteroid **9**. In the solid state, this hydrogen is 2.27 Å from the carbonyl oxygen and it is located along the axis of the carbonyl bond as required for it to be deshielded by the carbonyl group.[11]

On the basis of this conformational analysis, we hypothesize that the receptor-bound conformation of the 17a-aza-D-homosteroids when bound to $GABA_A$ receptors is analogous to the conformation found for azasteroid **9** in the solid state. Consequently, we propose that the 5β-reduced azasteroid **8** is less active than the 5α-reduced azasteroid **7** because of the shorter distance between the hydrogen-bonding groups found in the former compound. The calculated O−O distance in 5α-reduced azasteroid **7** is 9.88 Å, whereas it is only 9.43 Å in 5β-reduced azasteroid **8**. The ranges of O−O distances (calculated by rotation of the 17β-acetyl group in 10° increments) for conformers of steroids **1** and **2** in which the carbonyl group remains above the plane (defined by C-5, C-7, and C-12) of the steroid are calculated to be 10.19−11.22 Å (5α-reduced ring system) and 9.72−10.59 Å (5β-reduced ring system), respectively. Hence, only the O−O distance of 5α-reduced azasteroid **7** is within the range of the corresponding distance values found in anesthetic steroids **1** and **2**.

Substitution of the 17a-acetyl group with an α-hydroxyacetyl group in the C-5 epimeric azasteroids (compare compound **7** to **10** and **8** to **11**) increased activity in both cases. Azasteroid **10** has activities comparable to those of steroids **1** and **2**, while azasteroid

11 has activities similar to those of azasteroid **7**. We propose that the side chain OH group of the hydroxyacetyl group in compounds **10** and **11**, which is likely to be intramolecularly hydrogen bonded to the adjacent carbonyl group, is also acting as a hydrogen bond acceptor in an interaction with the receptor. Hence, the increased activities for azasteroids **10** and **11** are explained by the more favorable distances between the hydrogen-bonding groups (3α-OH, donor; side chain OH, acceptor) in these compounds (11.73 and 10.97 Å, respectively). Since the biological activities of steroids **1** and **2** are not substantially increased by this structural modification (compare with steroids **3** and **4**), the O−O distances are presumably optimal for the bound conformations of steroids **1** and **2** and nothing is gained by introducing an alternative hydrogen bond acceptor group in the side chain.

Substitution of the 17a-acetyl group with the trifluoromethylacetyl group in a 5α-reduced azasteroid (compare compound **7** to **13**) adversely affected binding but enhanced electrophysiological activity. For a 5β-reduced azasteroid, this same substitution has a slightly negative effect on both activities (compare compound **8** to **14**). We expected that this structural modification would lead to a decrease in activities for both fluorinated analogues in both bioassays. The trifluoromethyl group is expected to be, at best, a poor hydrogen bond acceptor.[12] Additionally, for inductive reasons the trifluoromethyl group should make the carbonyl group a poorer hydrogen bond acceptor. An explanation for why the activity of azasteroid **13** is increased in the electrophysiological assay is not apparent to us.

Finally, the 3β-methyl group present in azasteroids **9** and **12** decreased the biological activity of these compounds (compare compound **9** to **7** and **12** to **10**). Since it is known that this structural modification does not have a negative effect on the pharmacological activities of steroids **1** and **2**,[1,13] this result indicates that the D-ring modification has affected the way that the steroid A-ring interacts with the receptor. This result is not surprising when one considers the difference in the overall shape and size of the steroid and 17a-aza-D-homosteroid ring systems.

In conclusion, these studies demonstrate that 17a-aza-D-homosteroid analogues can potently increase the actions of GABA at GABA_A receptors. For example, azasteroid **10** has pharmacological activities comparable to those of anesthetic steroids **1**−**4**. Additionally, the facile manner in which these azasteroids can be prepared and modified makes the 17a-aza-D-homosteroid ring system an attractive core structure for additional SAR studies.

Acknowledgment. The authors thank Eric C. Zorumski for technical assistance and Melissa Sauter for teaching D.N. and M.C. the TBPS binding method. This work was supported by NIH Grant GM 47969 and Grant NS 14834, Research Scientist Development Award MH 00964 (C.F.Z.), and the Bantly Foundation.

Supporting Information Available: X-ray crystallographic data for (3α,5α)-17a-acetyl-3-methyl-17a-aza-D-homoandrostan-3-ol (**9**). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Phillipps, G. H. Structure−Activity Relationships in Steroidal Anesthetics. In *Molecular Mechanisms in General Anaesthesia*; Halsey, M. J., Millar, R. A., Sutton, J. A., Eds.; Churchill Livingston: New York, 1974; pp 32−47. (b) Phillips, G. H. Structure−Activity Relationships in Steroidal Anesthetics. *J. Steroid Biochem*. **1975**, *6*, 607−613.

(2) (a) Lambert, J. J.; Belelli, D.; Hill-Venning, C.; Peters, J. A. Neurosteroids and GABA_A Receptor Function. *Trends Pharmacol. Sci.* **1995**, *16*, 295−303. (b) Lambert, J. J.; Belelli, D.; Pistis, M.; Hill-Venning, C.; Peters, J. A.; The Interaction of Intravenous Anesthetic Agents with Native and Recombinant GABA_A Receptors: an Electrophysiological Study. In *GABA Receptors*, 2nd ed.; Enna, S. A., Bowery, N. G., Eds.; Humana Press: Totowa, NJ, 1997; pp 121−156.

(3) Covey, D. F.; Hu, Y.; Bouley, M. G.; Holland, K. D.; Rodgers-Neame, N. T.; Isenberg, K. E.; Zorumski, C. F. Modulation of GABA_A Receptor Function by Benz[*e*]indenes and Phenanthrenes. *J. Med. Chem*. **1993**, *36*, 627−630.

(4) Revelli, G. A.; Gros, E. G. Synthesis of 17a-Alkyl and 17a-Aryl-3β-acetoxy- and 3 β-Methoxy-17a-aza-D-homoandrost-5-en-17-one. *Steroids* **1993**, *58*, 181−184.

(5) Compounds **7**−**19** gave satisfactory elemental analyses for C, H, and (when present) N. The spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, ^{19}F NMR) were consistent with the structures given.

(6) (a) Majewska, M. D.; Harrison, N. L.; Schwartz, R. D.; Barker, J. L.; Paul, S. M. Steroid Hormone Metabolites Are Barbiturate-Like Modulators of the GABA Receptor. *Science* **1986**, *232*, 1004−1007. (b) Harrison, N. L.; Majewska, M. D.; Harrington, J. W.; Barker, J. L. Structure−Activity Relationships for Steroid Interaction with the γ-Aminobutyric Acid_A Receptor Complex. *Mol. Pharmacol.* **1987**, *241*, 346−353.

(7) (a) Purdy, R. H.; Morrow, A. L.; Blinn, J. R.; Paul, S. M. Synthesis, Metabolism, and Pharmacological Activity of 3α-Hydroxy Steroids Which Potentiate GABA-Receptor-Mediated Chloride Ion Uptake in Rat Cerebral Cortical Synaptoneurosomes. *J. Med. Chem*. **1990**, *33*, 1572−1581. (b) Hu, Y.; Zorumski, C. F.; Covey, D. F.; Neurosteroid Analogues: Structure−Activity Studies of Benz[*e*]indene Modulators of GABA_A Receptor Function. 1. The Effect of 6-Methyl Substitution on the Electrophysiological Activity of 7-Substituted Benz[*e*]indene-3-carbonitriles. *J. Med. Chem*. **1993**, *36*, 3956−3967.

(8) Fesik, S. W.; Makriyannis, A. Geometric Requirements for Membrane Perturbation and Anesthetic Activity. Conformational Analysis of Alphaxalone and Δ^{16}-Alphaxalone and ^{2}H NMR Studies on Their Interactions with Model Membranes. *Mol. Pharmacol.* **1985**, *27*, 624−629.

(9) Bolger, M. B.; Wieland, S.; Hawkinson, J. E.; Xia, H.; Upasani, R.; Lan, N. C. In Vitro and In Vivo Activity of 16,17-Dehydroepipregnanolones: 17,20-Bond Tortional Energy Analysis and D-Ring Conformation. *Pharm. Res.* **1996**, *13*, 1488−1494.

(10) Intensity data were collected on a Siemens P4 diffractometer, and structure solution and refinement were performed using SHELX-97 software.

(11) Silverstein, R. M.; Bassler, G. C. *Spectrometric Identification of Organic Compounds*, 2nd ed.; Wiley: New York, 1967; pp 116−117.

(12) (a) Shimoni, L.; Glusker, J. P. The Geometry of Intermolecular Interactions in Some Crystalline Fluorine-containing Organic Compounds. *Struct. Chem*. **1994**, *5*, 383−397. (b) Howard, J. A. K.; Hoy, V. J.; O'Hagen, D.; Smith, G. T. How Good is Fluorine as a Hydrogen Bond Acceptor? *Tetrahedron* **1996**, *52*, 12613−12622.

(13) Hogenkamp, D. J.; Tahir, S. H.; Hawkinson, J. E.; Upasani, R. B.; Alauddin, M.; Kimbrough, C. L.; Acosta-Burruel, M.; Whittemore, E. R.; Woodward, R. M.; Lan, N. C.; Gee, K. W.; Bolger, M. B. Synthesis and in Vitro Activity of 3β-Substituted-3α-hydroxypregnan-20-ones: Allosteric Modulators of the GABA_A Receptor. *J. Med. Chem*. **1997**, *40*, 61−72.

(14) Covey, D. F.; Nathan, D.; Kalkbrenner, M.; Nilsson, K. R.; Hu, Y.; Zorumski, C. F.; Evers, A. S. Enantioselectivity of Pregnanolone-Induced γ-Aminobutyric Acid_A Receptor Modulation and Anesthesia. *J. Pharmacol. Exp. Ther.* **2000**, *293*, 1009−1016.

(15) Shen, W.; Mennerick, S.; Zorumski, E. C.; Covey, D. F.; Zorumski, C. F. Pregnenolone Sulfate and Dehydroepiandrosterone Sulfate Inhibit GABA-Gated Chloride Currents in *Xenopus* Oocytes Expressing Picrotoxin-Insensitive GABA_A Receptors. *Neuropharmacology* **1999**, *38*, 267−271.

JM0002477