# *In Vitro* and *In Vivo* Activity of 16,17-dehydro-epipregnanolones: 17,20-Bond Torsional Energy Analysis and D-ring Conformation

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***Purpose.*** Certain neuroactive pregnane steroids (also known as "epalons") are allosteric modulators of the $GABA_A$ receptor and have been shown to be potent anticonvulsants, anxiolytics, sedative/hypnotics, and anesthetic agents. The purpose of this study was to calculate the structural consequences of introduction of a double bond in the 16,17-position and to determine if this modification would selectively reduce sedative activity, but maintain the potent anticonvulsant activity of neuroactive steroids.

***Methods.*** We have studied the biochemical and behavioral effects of introducing a 16,17 double bond into the naturally occurring neuroactive steroids, 3α-hydroxy-5α-pregnan-20-one (3α,5α-P) and 3α-hydroxy-5β-pregnan-20-one (3α,5β-P) and three synthetic neuroactive steroid derivatives, 3α-hydroxy-3β-methyl-5α-pregnan-20-one (3α,3βMe,5α-P), 3α-hydroxy-5α-androstane (3α,5α-A), and alphaxalone (3α,5α-11-one-P).

***Results.*** The 16-ene analogs of most of these neuroactive steroids were found to be 7- and 16-fold less potent in inhibiting [$^{35}$S]TBPS binding to $GABA_A$ receptors and in a similar fashion, had reduced anticonvulsant and sedative potency in proportional amounts. The exception was the androstane (3α,5α-A) without a 17-acetyl group, that had virtually identical $IC_{50}$ and $ED_{50}$ values for the saturated and unsaturated derivatives. Calculation of the torsional energy profile for each of the 17-acetyl side chain conformations showed that the conformational energy minima found in the α,β-unsaturated keto systems, produce an orientation of the 20-keto group that is rotated by 165 degrees when compared to the non-conjugated acetyl group (determined by X-ray crystallography and its minimum energy conformation).

***Conclusions.*** The modified orientation of the 20-keto group of neuroactive steroids containing a 16-ene, provides an explanation for their decreased biological activity overall, but did not lead to an enhanced protective index.

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**ABBREVIATIONS:** 3α,5α-P, 3α-hydroxy-5α-pregnan-20-one (epiallopregnanolone); 3α,5β-P, 3α-hydroxy-5β-pregnan-20-one (epipregnanolone); 3α,5α-A, 3α-hydroxy-5α-androstane; 3α,3β-Me,5α-P, 3α-hydroxy-3β-methyl-5α-pregnan-20-one; 3α,5α-16-en-P, 3α-hydroxy-5α-pregn-16-en-20-one; 3α,5β-16-en-P, 3α-hydroxy-5β-pregn-16-en-20-one; 3α,5α-16-en-A, 3α-hydroxy-5α-androst-16-ene; 3α,3β-Me,5α-16-en-P, 3α-hydroxy-3β-methyl-5α-pregn-16-en-20-one; 3α,5α-11-one-P, 3α-hydroxy-5α-pregn-11,20-dione (alphaxalone); 3α,5β-11-one-P, 3α-hydroxy-5β-pregn-11,20-dione (5β-alphaxalone); TBPS, *t*-Butylbicyclophosphorothionate; GABA, γ-aminobutyric acid; PTZ, pentylenetetrazol.

**KEY WORDS:** neuroactive steroid; epalon; γ-aminobutyric $acid_A$ or $GABA_A$ receptor; 3α-hydroxy-5β-pregnan-20-one; 3α-hydroxy-5α-pregnan-20-one; TBPS or *t*-butylbicyclophosphorothionate; anesthetic; anticonvulsant; sedative; alphaxalone; pregnanolone; molecular mechanics.

## INTRODUCTION

Anesthetic actions of certain pregnane steroids have been previously described (1–3). Several recent studies have focused on the anticonvulsant (4,5), anxiolytic (6) (7–10), and sleep-inducing properties (11–12) of a class of neuroactive steroids. These neuroactive steroids are referred to as "epalons", an acronym for epiallopregnanolone (3α-hydroxy-5α-pregnan-20-one)(1c) (13) which demonstrates high potency for the $GABA_A$ receptor. The term epalon is used to define this class of neuroactive steroids which share the recognition site(s) with epiallopregnanolone. Two drugs, Althesin® (a mixture of alphaxalone (3α-hydroxy-5α-pregnan-11,20-dione)(1a) and alphadalone acetate (21-acetoxy-3α-hydroxy-5α-pregnan-11,20-dione) in Cremophor EL) and hydroxydione (21-hydroxy-5β-pregnan-3,20-dione sodium hemisuccinate), from this class reached the clinic as anesthetic agents. Hydroxydione was used clinically as an anesthetic from 1956 until 1959, when it was removed from market due to slow onset of action (5 to 10 minutes) and marked venous irritation and frequent thrombophlebitis. Althesin® was introduced to the clinic in 1971 (14) and removed from the market in 1975 because of suspected allergic complications arising from the Cremophor EL vehicle (15). At least one report of the successful use of Althesin® for status epilepticus has also appeared (16). New found interest in the potential clinical usefulness of neuroactive steroids for indications other than anesthetics has resulted from the discovery of specific high affinity receptor sites for these steroids on the $GABA_A$ receptor chloride channel complex (17–22).

Decreased anesthetic potency upon introduction of a double bond in the D-ring at the 16-position of alphaxalone was first reported by Gyermek in 1968 (2,3). Since that time, several studies have exploited the properties of alphaxalone(1a) and its 16-ene-analog(1b) (Fig. 1) in order to determine the molecular basis of steroid anesthetic action. ESR spectra obtained from liposomes (containing 69% lecithin, 30% cholesterol, and 1% nitroxide labeled dipalmitoyl-lecithin) in the presence of 0.48 moles of alphaxalone per mole of lecithin indicated a strong decrease of phospholipid order (order parameter S = 0.447) compared to no drug controls (S = 0.734) (23). In accord with its anesthetic potency, the disordering effect was completely absent when the 16-ene analog of alphaxalone was used at the same concentration. Richards and Hesketh studied the inhibition of the excitatory postsynaptic potential (E.P.S.P.) in isolated guinea pig olfactory cortex, and found that at a concentration of 50 μM, alphaxalone was two to four times as effective as the 16-ene analog (24). In a similar fashion, alphaxalone, at a concentration of 90 μM produced four-fold greater inhibition of anion transport associated with the band 3 protein in erythrocytes than the 16-ene analog (25). Makriyannis et al. concluded that because these two compounds display similar activity profiles in very different membrane systems, the mechanism of neuroactive steroid action at these very high concentrations does not involve interaction with a specific receptor, but rather reflects a structurally specific perturbation of the phospholipid

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bilayer for alphaxalone that is absent in the 16-ene analog. Additional studies using $^{1}H$, $^{2}H$, and $^{13}C$ high resolution NMR spectroscopy and differential scanning calorimetry confirmed that alphaxalone perturbs the phospholipid bilayer more effectively than its inactive 16-ene analog when used at high concentrations (26–30).

In contrast to the physical studies described above, our current work compares the *in vivo* and *in vitro* properties of neuroactive steroids at low concentrations that predominately affect the $GABA_A$ receptor and attempts to explain the relative inactivity of 16-ene analogs in terms of differences in 17,20-bond torsional energies and D-ring conformations.

## MATERIALS AND METHODS

### Drugs

3α,5α-P (1c) and 3α, 3βMe, 5α-P (1i) were prepared at CoCensys as previously described [22,31]. The androstanes,

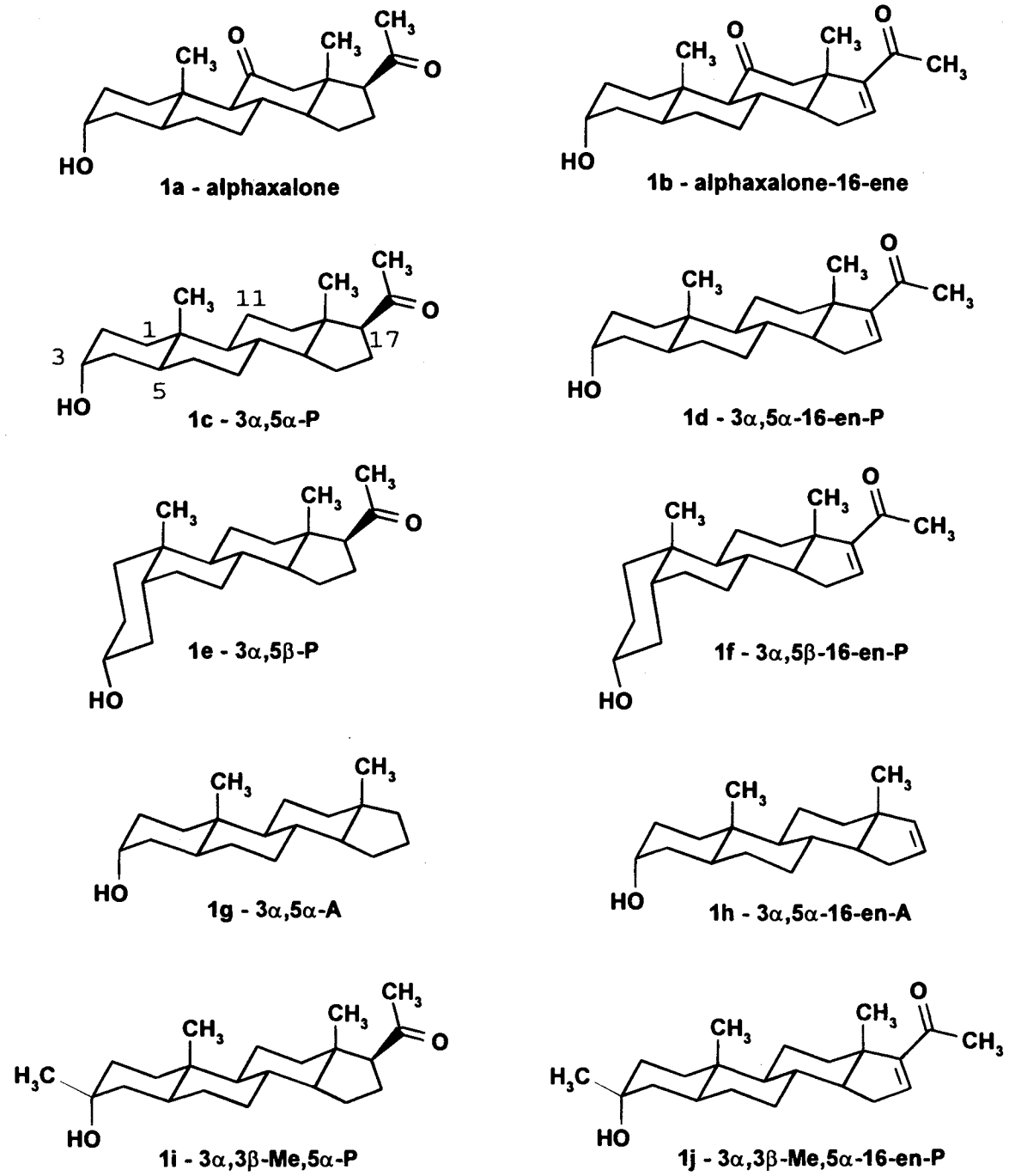


**Fig. 1.** Structural formula of epalon derivatives. Figure 1a is alphaxalone and 1b is alphaxalone-16-ene. Figure 1c is 3α,5α-P and 1d is 3α,5α-16-en-P. Figure 1e is 3α,5β-P and 1f is 3α,5β-16-en-P. Figure 1g is 3α,5α-A and 1h is 3α,5α-16-en-A. Figure 1i is 3α,3β-Me,5β-P and 1j is 3α,3β-Me,5β-16-en-P.

3α, 5α-A (1g) and 3α, 5α-16-en-A (1h), were obtained from Steraloids (Wilton, NH) and 3α, 5β-P (1e) from Diosynth (Oss, Netherlands). Alphaxalone and its 16-ene derivative were a generous gift of Dr. B. M. Bain at Glaxo Research and Development Ltd. (Greenford, U.K.). Unlabeled *t*-butylbicyclophosphorothionate (TBPS) was purchased from RBI (Natick, MA). Pentylenetetrazol (PTZ) and γ-aminobutyric acid (GABA) were purchased from Sigma (St. Louis, MO) and dissolved in saline (0.9% NaCl). The 16-ene derivatives of 3α,5α-P, 3α,5β-P, and 3α,3βMe,5α-P were prepared at CoCensys by the following general two step method.

### Preparation of 17α-bromo-3α-hydroxy-3β-substituted-5α[β]-pregnan-20-one

A solution of 3α-hydroxy-3β-substituted-5α[β]-pregnan-20-one (8 mmol) and benzoyl peroxide (20 mg) in $CCl_4$ (120 mL) was treated at reflux in parts with N-bromosuccinimide (3.53 g) over a period of 4.5 h. The mixture was cooled and diluted with more $CCl_4$ (15 mL). The organic layer was separated, washed with water, sodium bisulfite solution, sat. $NaHCO_3$ solution, water, and dried over anhydrous $MgSO_4$. Evaporation of the solvent gave the titled bromo compound as a yellow oil. This crude oil was dissolved in 130 mL of toluene and concentrated to 80 mL. This solution was then used for the next step.

### Preparation of 3α-hydroxy-3β-substituted-5α[β]-pregn-16-en-20-one

The above solution of the bromo compound in toluene (25 mL) was treated with 2.6 mL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and was refluxed for 2 h. More DBU (2 mL) was added and the heating continued for 2.5 h. After cooling to room temperature, the mixture was passed through a small column of Florisil and eluted with ether (300 mL). The ether solution was washed with dil. HCl, water, dil. $NaHCO_3$, water, dried over anhydrous $MgSO_4$ and evaporated to yield the crude product. The pure compounds were isolated by chromatography on silica gel (8% acetone, 8% methylene chloride in hexane). This product was crystallized from acetone:hexane (1:1) to yield pure 3α-hydroxy-3β-substituted-5α[β]-pregn-16-en-20-one as colorless needles (between 37% and 73% yield). Melting points for the previously reported synthetic derivatives were the following: 3α,5α-16-en-P, mp 224–229°C (lit. 219–222°C (32)); 3α,5β-16-en-P, mp 199–201°C (lit. 194–196°C (33)). In addition, we report here a new chemical entity, 3α-hydroxy-3β-methyl-5α-pregn-16-en-20-one, mp 163–166°C. $^1H$ NMR (300 MHz, $CDCl_3$) δ 6.68 (bs, 1H), 2.26 (s, 3H), 1.21 (s, 3H), 0.89 (s, 3H), 0.79 (s, 3H). Mass Spectrum (electron impact), m/z $M^+$ 330, $-CH_3$ (315 m/z), $-H_2O$ (297 m/z), $-H_2O$ (279 m/z). Mass Spectrum (chemical ionization), m/z $MH^+$ 331, $-H_2O$ (313 m/z), $-H_2O$ (295 m/z). *Analytical Analysis*: Calculated for $C_{22}H_{34}O_2$: C, 79.93%; H, 10.4%. Found: C, 79.93%; H, 10.91%.

### Radioligand Binding Assay

#### *Membrane Preparation*

Rat brain cortical membranes were prepared as previously described (17). Briefly, cortices were rapidly removed following decapitation of carbon dioxide-anesthetized Sprague-Dawley rats (200–250 g), homogenized in 10 volumes of ice-cold 0.32 M sucrose using a glass/teflon homogenizer, and centrifuged at 1500 × *g* for 10 min at 4°C. The resultant supernatants were centrifuged at 10,000 × *g* for 20 min at 4°C to obtain the P2 pellets. The P2 pellets were resuspended in 200 mM NaCl/50 mM Na-K phosphate pH 7.4 buffer and centrifuged at 10,000 × *g* for 20 min at 4°C. This washing procedure was repeated twice and the pellets were resuspended in 10 volumes of buffer.

#### *[$^{35}$S]TBPS Binding Assay*

Aliquots (100 μL) of the membrane suspensions were incubated with 2 nM [$^{35}$S]TBPS (60–100 Ci/mmol; NEN, Boston, MA) and 5 μL aliquots of test drug (9 concentrations ranging from 1 nM to 10 μM final) dissolved in dimethyl sulfoxide (DMSO) (final 0.5%) in the presence of 5 μM GABA. The incubation was brought to a final volume of 1.0 mL with buffer. Nonspecific binding was determined in the presence of 2 μM unlabeled TBPS and ranged from 15 to 25%. Following a 90 min incubation at room temperature, the assays were terminated by filtration through glass fiber filters (Schleicher and Schuell No. 32, Keene, NH) using a cell harvester (Brandel, Gaithersburg, MD) and rinsed three times with ice-cold buffer. Filter bound radioactivity was measured by liquid scintillation spectrometry.

#### *Data Analysis*

The concentration of steroid producing 50% inhibition of specific binding ($IC_{50}$) was calculated for each individual experiment by fitting the data to the sigmoidal function using Prism (GraphPad, San Diego, CA).

### *In Vivo* Pharmacology

#### *Animals*

Male NSA mice (Harlan, Indianapolis, IN) weighing 20–30 g were used in all experiments. Animals were group housed (4 per cage) in a light (0600–1800 h) and temperature (22 ± 2°C) controlled vivaria. Food and water were available *ad libitum*, except during testing. The experiments were run from 0700–1500 h and groups were counterbalanced for time of day effects. Mice were only administered drug or vehicle once. For behavioral tests, all of the drugs were administered intravenously by tail vein in a vehicle containing 0.35% hydroxypropyl cellulose and 4% Tween 80 in 0.9% NaCl (micronizing solution). The drugs were placed in a glass mill jar with glass beads and micronized for 24 hrs prior to administration. This procedure results in drug particles approximately 7–35 micrometers in size as determined by video microscopy.

#### *Anti-PTZ Test*

NSA mice were treated with various doses of neuroactive steroid, 10 min prior to PTZ. The test compounds were administered intravenously (*iv*) in a tail vein in a volume equivalent to 5 ml/kg. PTZ (85 mg/kg) was administered subcutaneously (*sc*) in a volume equivalent to 5 ml/kg. Following PTZ administration, mice were placed in individual cages for observation of clonic seizures during a 30 min test period.

*Rotorod Test*

The rotorod apparatus consisted of a variable speed rotating rod 1″ in diameter, divided into four equivalent spaces. Photocells located below the rotating drum automatically measured the time spent on the drum. The rotating drum was set for 6 rotations per min.

NSA mice were trained to stay on the rotating drum for 2 min prior to testing. Test compounds were administered *iv* in a tail vein (5 ml/kg) 10 min prior to testing. During testing each animal was given 3 opportunities to stay on the rotating drum for a continuous 60 sec. Animals failing to stay on the rotating drum for 60 sec were recorded as failing.

*Determination of $ED_{50}$, $TD_{50}$, and Protective Index (PI)*

The effective dose of neuroactive steroid test drug required to prevent myoclonic seizures in 50% of the test animals ($ED_{50}$) was determined using the anti-PTZ assay. The dose of neuroactive steroid test drug needed to cause 50% of the test animals to display signs of generalized sedation ($TD_{50}$) was determined using the rotorod test. The protective index (PI), a relative measure of the ability of a neuroactive steroid test drug to protect the test animal from seizures compared to the sedative properties of the drug, was determined by calculating the ratio $TD_{50}/ED_{50}$.

*Molecular Mechanics Calculations*

All calculations were performed using HyperChem (Hypercube, Waterloo, Ontario, Canada). The initial coordinates for energy minimization were obtained by editing the X-ray crystallographic coordinates of alphaxalone (34) or 3α-, 3β-Me, 5α-P (unpublished data) or by using the model building procedures of HyperChem. For each of the neuroactive steroids, a master structure was constructed and partial atomic charges were calculated using the semi-empirical quantum mechanical program MOPAC (AM1) (35). The molecules were then subjected to geometry optimization using the MM+ force field (HyperChem by Hypercube, Waterloo, Ontario, Canada) with convergence criterion equal to a force gradient of less than 0.1 Kcal/mol/Å. The MM+ force field is a commercial implementation of the public domain force field MM2(1977) (36). MM+ has been extended to include Urey-Bradley 1–3 interaction terms that are critical for simulating the energies of rotation for the 17–20 bond in the neuroactive steroids. The initial values of the 16–17–20–21 torsion angles were −180 degrees for both the saturated and un-saturated derivative master molecules. The rotational energy barriers were calculated for torsional angles from −180 to 180 degrees in 15 degree steps by first rotating the 16–17–20–21 torsion of the master molecule to the desired torsion value. At each step the torsion was restrained and a geometry optimization was performed for the whole molecule with the same convergence criterion of less than 0.1 Kcal/mol/Angstrom force gradient. The final energies were plotted and a minimum energy conformation determined.

## RESULTS

Biochemical, behavioral, and conformational analysis data are presented in Table I. The neuroactive steroids 3α,5α-P, 3α,5β-P, and 3α,3β-Me, 5α-P are 7 to 16 times more potent inhibitors of [$^{35}$S]TBPS binding to rat brain cortical $GABA_A$ receptors than the corresponding 16-ene compounds. In a similar fashion, these compounds are 8 to 18 times more potent than their 16-ene derivatives *in vivo*. These compounds were synthesized in order to test the hypothesis that the 16-ene analogs would have anticonvulsant potency similar to the parent molecules with much less sedative activity. The results show that, following *iv* administration, the protective index (PI = ratio of $TD_{50}/ED_{50}$) is never greater than 4.4 and the PI values for the 16-ene compounds are equal to or less than the corres-

**Table I.** Biochemical, Behavioral, and Conformational Results on Epalons and Their 16-ene Analogs

| | [$^{35}$S]TBPS $IC_{50}$ | AntiPTZ $ED_{50}$ | Roto-rod $TD_{50}$ | Protective Index | Min. Energy Torsional Angle(°) |
|---|---|---|---|---|---|
| | nM[a] | *iv* mg/kg | *iv* mg/kg | Ratio $TD_{50}/ED_{50}$ | C16-C17-C20-C21 |
| 3α,5α-P | 51 ± 5 | 1.1 | 4.8 | 4.4 | 165 |
| 3α,5α-16-en-P | 348 ± 68 | 20.0 | 48.8 | 2.4 | 0 |
| Ratio (16-ene/sat.) | 7 | 18 | 10 | | |
| 3α,5β-P | 44 ± 11[b] | 1.8 | 5.0 | 2.8 | 165 |
| 3α,5β-16-en-P | 689 ± 89 | 28.0 | 35.0 | 1.3 | 0 |
| Ratio (16-ene/sat.) | 16 | 16 | 7 | | |
| 3α,3β-Me,5α-P | 80 ± 18 | 1.8 | 4.8 | 2.7 | 165 |
| 3α,3β-Me,5α-16-en-P | 933 ± 102 | 13.5 | 41.5 | 3.1 | 0 |
| Ratio (16-ene/sat.) | 12 | 8 | 9 | | |
| Alphaxalone | 303 ± 37 | 13.1 | 15.9 | 1.2 | 165 |
| Alphaxalone-16-ene | 2956 ± 239 | >40 | >60 | 1.5 | 0 |
| Ratio (16-ene/sat.) | 10 | 3 | 4 | | |
| 3α,5α-A | 593 ± 143 | 4.1 | n/d[c] | | n/a[d] |
| 3α,5α-16-en-A | 541 ± 43 | 4.7 | n/d[c] | | n/a[d] |
| Ratio (16-ene/sat.) | 0.9 | 1.1 | | | |

[a] TBPS $IC_{50}$ values represent the mean ± sem of at least three independent experiments.
[b] 44 nM is the high affinity $IC_{50}$ based on a two site displacement curve with individual $IC_{50}$s of 44 nM and 12 μM, Hawkinson et al. [37].
[c] Experimental values have not been determined (n/d).
[d] Rotational torsion for the androstane is not applicable (n/a) because there is no 17β-substituent.

ponding saturated compounds, indicating that the 16-ene analogs are not relatively less sedating than the saturated analogs. Rather there seems to be a common aspect of the 16-ene analogs that gives rise to lowered potency in all of the pharmacological and behavioral tests. In contrast, the androstane derivative (3α,5α-A) has no side chain in the 17-position and has equal potency both *in vitro* and *in vivo* to the 16,17-ene derivative.

In order to test the hypothesis that orientation of the carbonyl group of the 17-acetyl side chain might be correlated with diminished potency for unsaturated vs. saturated neuroactive steroids, a series of molecular mechanics calculations were performed. Figure 2 is a plot of the total potential energy following geometry optimization with the torsion angle of the 16–17–20–21 carbons restrained (100 Kcal force constant) to values from −180 to 180 degrees. It is clear that a torsional angle of 0 degrees is preferred for the 16-ene analogs and a torsional angle of 165 degrees is the minimum energy for the saturated analogs. These torsions cause the carbonyl oxygen to point in opposite directions for the two classes of compounds. In the case of the 16-ene derivatives, a torsional angle of 0 degrees is preferred by approximately 5 Kcal/mol with insurmountable energy barriers of 30 Kcal/mol between the *cis* and *trans* oriented side chain. The 16,17-saturated analogs, with higher potency, have a calculated energy minimum that matches the X-ray crystallographic orientation of the side chain in which the carbonyl oxygen is pointing above the β-face of the steroid ring system (Figure 3).

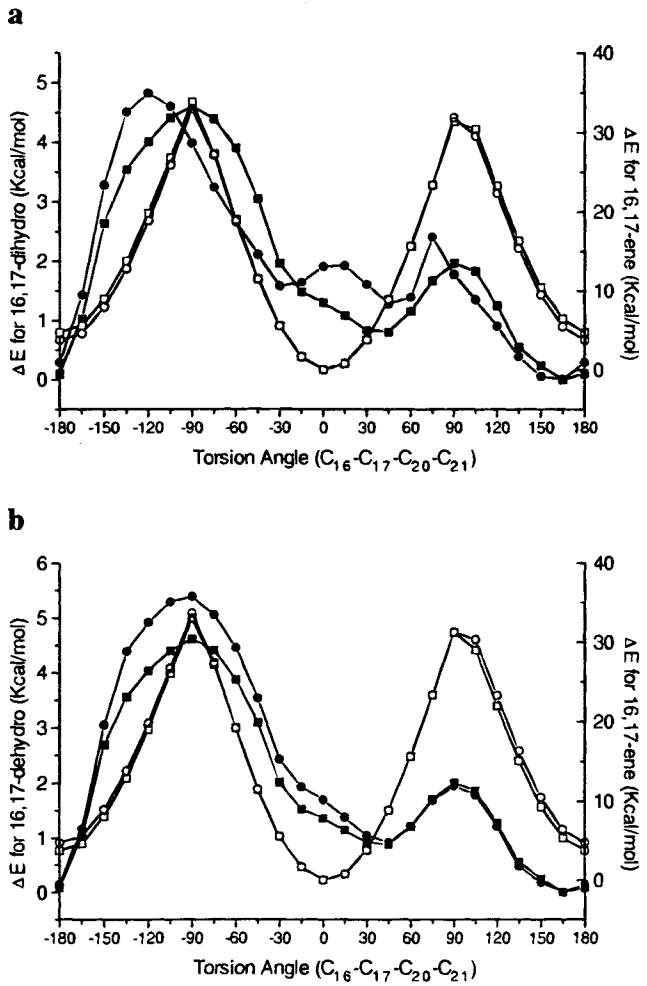


**Fig. 2.** Force field torsional energies for epalon derivatives. Plot of change in total calculated potential energy as a function of $C_{16}$-$C_{17}$-$C_{20}$-$C_{21}$ torsional angle scaled to zero for the lowest energy conformer. For each point from −180° to 180° in 15 degree steps, the desired torsion angle was introduced into a master molecule and restrained during geometry optimization by conjugate gradient energy minimization using the MM+ force field to a convergence criterion of 0.1 Kcal/mol/Angstrom force gradient. The torsional energy profiles were identical regardless of starting conformation. Figure 2a compares 3α,5α-P (-■-); 3α,5β-P (-●-); 3α,5α-16-en-P (-□-); 3α,5β-16-en-P (-○-). Figure 2b compares 3α,3β-Me,5α-P (-■-); alphaxalone (-●-); 3α,3β-Me,5α-16-en-P (-□-); alphaxalone-16-ene (-○-).

## DISCUSSION AND CONCLUSIONS

Five sets of neuroactive steroid analogs were examined in biochemical and behavioral assays to test the hypothesis that a double bond in the 16-position would enhance the protective index which compares the separation between the anticonvulsant and sedative properties. The results show that the 16-ene analogs are inherently less potent than the saturated analogs in all tests. Theoretical calculations were used to determine the preferred conformation of the 17-acetyl side chain and indicate that 16-ene analogs adopt a conformation in which the carbonyl oxygen of the 20-keto group is oriented, S-trans planar, in conjugation with the 16,17-double bond in a direction that is opposite to both the calculated and the X-ray crystallographically determined conformation of the saturated analogs. This predicted orientation of the 17-acetyl side chain of the

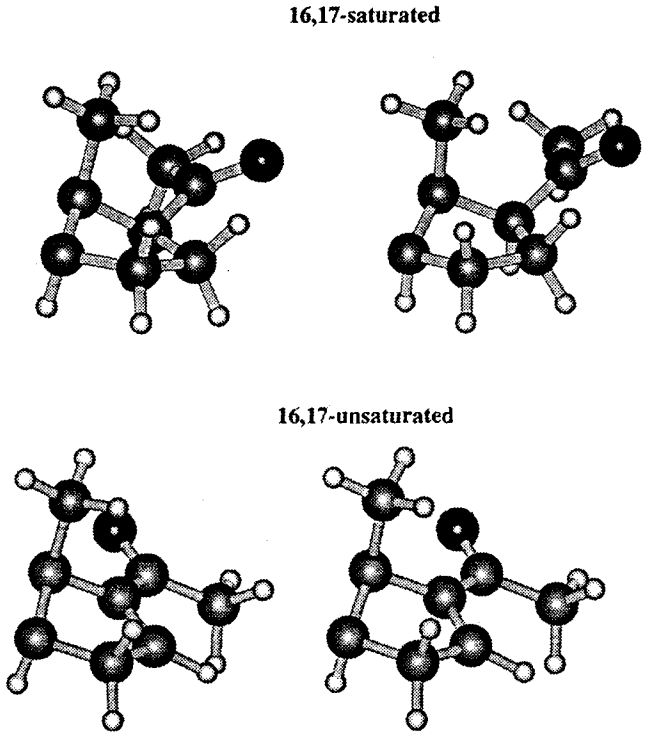


**Fig. 3.** Preferred conformations of epalon derivatives. Stereodiagram of the D-ring of 3α-hydroxy-5α-pregnan-20-one (3α,5α-P)(epiallopregnanolone) and 3α-hydroxy-5α-pregn-16-en-20-one (3α,5α-16-en-P) showing the preferred orientation of the 20-keto group in each case.

16-ene analogs is in agreement with measurement of a significant 8% nuclear Overhauser enhancement (NOE) of the C-16 vinylic proton following irradiation of the 21-carbon. These NMR studies were carried out on the 16-ene analog of alphaxalone, which was also predicted to adopt an S-trans planar conformation (27). Our calculations indicate that a large energy barrier (30 Kcal/mol) prevents the 16-ene analogs from adopting the higher energy S-cis planar conformation. A much smaller energy barrier (2 Kcal/mol) separates the minimum energy conformation of the saturated analogs from adopting an orientation that is similar to the 16-ene analogs. Fesik and Makriyannis found a negligible NOE between C-21 and H-17 of alphaxalone indicating a torsional angle (H-17, C-17, C-20, C-21) of approximately 90 degrees in agreement with the present calculations (27). However, the diagram of the side chain orientation in their Figures 2 and 5 (27) indicate an orientation of the carbonyl carbon that is 180 degrees opposite to the minimum energy conformation presented here. Essentially, their data suggest two possible orientations of the 17-acetyl side chain and they arbitrarily selected to illustrate the one that is the higher energy conformer based on our present calculations. They concluded that the puckered D-ring of alphaxalone compared to the flattened planar D-ring of the 16-ene analog is responsible for the observed differences in anesthetic potency and that the mechanism of anesthetic activity is due to perturbation of membrane phospholipids by alphaxalone and not by the 16-ene analog. It is possible that such a mechanism is active at the very high concentrations used in their studies. However, the present results indicate that when low concentrations of neuroactive steroids are tested in *in vivo* and *in vitro* models, the differential activity of saturated and 16-ene analogs can be explained by the conformational orientation of the 20-keto group, which might be able to make a more favorable contact with the $GABA_A$ receptor complex when it points in a direction that is upwards above the β-face of the steroid. Thus, rotation of the 17,20-bond from the most favorable conformation of saturated analogs to that favored by the 16-enes, results in an average potency loss of 11-fold due to loss of the favorable contact with the $GABA_A$ receptor complex. Comparing the average $IC_{50}$ (519 nM) of the pregn-16-ene derivatives, 3α,5α-16-en-P and 3α,5β-16-en-P, to the average $IC_{50}$ (567 nM) of the androstane derivatives (3α,5α-A) and (3α,5α-16-en-A), illustrates that removal of the 17-acetyl substituent can explain all of the loss in activity demonstrated by the 16-ene derivatives. In other words, the increased potency conferred by the 17β-acetyl substituent in the D-ring saturated neuroactive steroids is completely negated by introduction of a 16,17 double bond. In addition, there is no change in activity of the androstane derivatives due to a flattening of the D-ring as a result of oxidation of the 16,17-bond to form the 16-ene suggesting that introduction of the double bond reduces neuroactive steroid potency solely by affecting a change in the conformation of the 17b-acetyl group and not by reducing the effects of D-ring puckering on membrane fluidity.

In conclusion, introduction of a 16,17 double bond into neuroactive epalon analogs results in markedly reduced *in vitro* and *in vivo* (anticonvulsant and sedative) activities. This decreased activity can be explained by a conformation of the 20-one that is induced by conjugation with the 16,17 double bond and has greatly reduced affinity for the $GABA_A$ receptor complex.

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