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17 β -Nitro-5 α -androstan-3 α -ol and its 3 β -methyl derivative: Neurosteroid analogs with potent anticonvulsant and anxiolytic activities

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Abstract

Many 17-substituted androstan-3 α -ol analogs act as positive allosteric modulators of GABA_A receptors and exert anticonvulsant and anxiolytic-like activity actions in animal models. The endogenous neurosteroid allopregnanolone (17 β -acetyl; **1**) is among the most potent of these. Here we demonstrate that 3 α -hydroxy-17 β -nitro-5 α -androstane (**2b**) and its 3 β -methyl analog (3 α -hydroxy-3 β -methyl-17 β -nitro-5 α -androstane; **2c**) modulate GABA_A receptors as assessed by [³⁵S]*t*-butylbicyclo-phosphorothionate and [³H]flunitrazepam binding with potencies equivalent to or greater than **1**. These compounds also had potencies equivalent to or greater than **1** in the pentylenetetrazol and 6 Hz seizure models in the mouse. Furthermore, **2b** exhibited anxiolytic-like activity in the elevated zero maze. The 3 β -hydroxy, 3 α -desmethyl analog (**2a**) was devoid of activity on GABA_A receptors in vitro but had moderate activity in the seizure models, possibly as a result of epimerization in vivo at the 3-position. This conclusion was supported by the lack of in vivo activity of the 3 β -hydroxy, 3 α -methyl analog (**2d**), which is not expected to undergo epimerization. We conclude that nitro can serve as a bioisostere for acetyl at the 17 β -position of 5 α -androstan-3 α -ol, such that the nitro analog fully retains the bioactivity of the endogenous neurosteroid at GABA_A receptors.

Keywords

neurosteroid; allopregnanolone; GABA_A receptor; anticonvulsant; anxiolytic; bioisosteres

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1. Introduction

Certain endogenous steroids, including allopregnanolone (3 α -hydroxy-5 α -pregnane-20-one; **1**) and the A-ring, reduced metabolite of progesterone are potent stereoselective positive allosteric modulators of GABA_A receptors (Belelli and Lambert, 2005) (Fig. 1). These neurosteroids, like other agents that potentiate the activity of GABA_A receptors, exhibit anxiolytic, sedative-hypnotic, anticonvulsant, and general anesthetic actions.

Allopregnanolone and A-ring-reduced neurosteroids generally lack classical hormonal activity mediated by nuclear hormone receptors. Given their GABA_A receptor modulating activity, natural neurosteroids could potentially be used therapeutically (Gasior et al., 1999). Unfortunately, they do not have ideal properties as drug substances. Natural neurosteroids can be metabolically converted to hormonally active substances (Rupprecht, 2003) and they have poor bioavailability. Consequently, there is an unmet need for synthetic analogs with improved pharmacokinetic and pharmacodynamic properties. Structural modifications at the 3-position inhibit metabolism of the secondary 3-hydroxyl substituent, but permit GABA_A receptor modulating activity to be retained (Hogenkamp et al., 1997). One such analog, ganaxolone, the 3 β -methyl derivative of allopregnanolone, is currently in clinical development for epilepsy (Garofalo et al., 2009; Monaghan et al., 1999; Rogawski, 2006).

Modulation of potency at the GABA_A receptor can also be achieved through substitution of the allopregnanolone 17-position. For example, the naturally occurring neurosteroid androsterone, which has a keto substituent at the 17-position, displays reduced GABA_A receptor modulating potency (Kaminski et al., 2005); whereas allopregnanolone and allotetrahydrodeoxycorticosterone (5 α ,3 α -THDOC), which have acetyl and hydroxyacetyl substituents at the 17-position are the most potent endogenous neurosteroids (Hawkinson et al., 1994). In addition, the synthetic 17 β -carbonitrile (Hawkinson et al., 1994) and 17 β -epoxide (Anderson et al., 2000) analogs exhibit similar GABA_A receptor modulatory potency and efficacy as allopregnanolone (Wittmer et al., 1996). Interestingly, the natural (16–17 unsaturated) pheromone, 3 α -androstanol, lacks a 17-position substituent yet retains GABA_A receptor modulating activity, albeit of reduced potency (Kaminski et al., 2006). Here we show that the 3 α -hydroxy-17 β -nitro analog (**2b**) and its 3 β -methyl derivative (**2c**) influence radioligand binding at GABA_A receptors with potency similar to allopregnanolone and exhibit potent anticonvulsant and anxiolytic-like activity in mouse models.

2. Materials and Methods

2.1. Materials

All reagents and chemicals unless otherwise specified were purchased from Sigma-Aldrich (Milwaukee, WI). Radioligands were obtained from PerkinElmer Life Sciences (Boston, MA).

2.2. Chemistry

Compounds **2a–2d** were synthesized according to the methods described in Fig. 2. Briefly, 5 α -androstane-3 β -hydroxy-17-oxime (**4**) (Pettit et al., 1966), 5 α -androstane-3 α -hydroxy-17-oxime (**5**) (Anderson et al., 2000), and 5 α -androstane-3 α -hydroxy-3 β -methyl-17-oxime (**6**)

(Covey et al., 2000) were oxidized using *N*-bromosuccinimide, and potassium carbonate in dioxane/water according to the method of Patchett and colleagues (Patchett et al., 1962). The resulting 17-bromo, 17-nitro intermediate was immediately dehalogenated with sodium borohydride to provide **2a**, **2b**, and **2c** in good yield. The 3 α -methyl derivative **2d** was synthesized in a similar manner from the respective oxime (**7**) (Fig. 2). 5 α -Androstan-3 β -hydroxy-3 α -methyl-17-one (**3**) (Cook et al., 1968) in pyridine was treated with hydroxylamine hydrochloride and allowed to stir for 8 h at room temperature. The resulting oxime (**7**) was oxidized using the conditions described for **2a**, **2b**, and **2c** to afford compound **2d**.

3 β -Hydroxy-17 β -nitro-5 α -androstane (2a)—Potassium bicarbonate (2.09 g, 21 mol) in H₂O (10 ml) was added to a solution of *N*-bromosuccinimide (1.84 g, 10 mmol) in dioxane (15 ml). The suspension was allowed to stir at room temperature for 0.25 h and a solution of compound **4** (1.0 g, 3.5 mmol) in dioxane (15 ml) was added. A pale green color rapidly developed, and the reaction was allowed to stir at room temperature for 8 h. The solution was cooled to 0 °C and sodium borohydride (0.96 g, 24 mmol) was added in portions. A large amount of gas evolution was observed and the reaction was allowed to stir for 8 h, gradually warming to room temperature. The reaction was quenched with saturated aqueous ammonium chloride (50 ml) and concentrated to ¼ its original volume. Water (50 ml) was added and the biphasic mixture was extracted with ethyl acetate (3 $\times$ 50 ml). The organic layers were combined, washed with brine (50 ml), dried over sodium sulfate, and concentrated under reduced pressure. The product was purified on silica using medium pressure column chromatography (hexane/ethyl acetate, 7:3) to provide **2a** (0.63 g, 56%) as a white solid. mp. 198–201 °C. ¹H-NMR (300 MHz, CDCl₃) δ 0.73 (s, 3 H), 0.81 (s, 3 H), 1.85–0.61 (m, 21 H), 2.14–2.00 (m, 2 H), 2.56–2.52 (m, 1 H), 3.63–3.56 (m, 1 H), 4.36 (dd, *J* = 9, 9 Hz, 1 H). Elemental Anal. for C₁₉H₃₁NO₃ Calcd. C: 70.99, H: 9.72, N: 4.36. Found. C: 70.65, H: 9.72, N: 4.35.

3 α -Hydroxy-17 β -nitro-5 α -androstane (2b)—The title compound was prepared from compound **5** in a manner analogous to that of **2a**. The resulting semisolid was purified on silica using medium pressure column chromatography (hexane/ethyl acetate, 7:3) to provide **2b** (1.05 g, 49%) as a pale yellow solid. mp. 150–152 °C. ¹H-NMR (300 MHz, CDCl₃) δ 0.73 (s, 3 H), 0.79 (s, 3 H), 2.05–0.86 (m, 23 H), 2.70–2.52 (m, 1 H), 4.05 (br s, 1 H), 4.37 (dd, *J* = 9, 9 Hz, 1 H). Elemental Anal. for C₁₉H₃₁NO₃ • 0.40 H₂O Calcd. C: 69.43, H: 9.75, N: 4.26. Found. C: 69.47, H: 9.49, N: 4.43.

3 α -Hydroxy-3 β -methyl-17 β -nitro-5 α -androstane (2c)—The title compound was prepared from 3 α -hydroxy-3 β -methyl-5 α -androstane-17-oxime (Covey et al., 2000) in a manner analogous to **2a**. The solid was purified on silica using medium pressure column chromatography (petroleum ether/acetone, 8.5:1.5). The resulting white solid was recrystallized from ethyl acetate/hexane to provide white needles (0.10 g, 33%). mp. 211–213 °C. ¹H-NMR (300 MHz, CDCl₃) δ 0.72 (s, 3H), 0.76 (s, 3H), 0.79–1.75 (m, 25H), 2.03–2.08 (m, 2H), 2.48–2.58 (m, 1H), 4.34–4.40 (dd, *J* = 9, 9 Hz, 1H). Elemental Anal. for C₂₀H₃₃NO₃ Calcd. C: 71.6, H: 9.91, N: 4.17. Found. C: 71.71, H: 9.97, N: 4.21.

3 β -Hydroxy-3 α -methyl-17 β -nitro-5 α -androstane (2d)—The title compound was prepared from **7** in a manner analogous to **2a**. The resulting solid was purified on silica using medium pressure column chromatography (petroleum ether/acetone, 8.5:1.5). The white solid was then recrystallized from ethyl acetate/hexane to provide white needles (0.24 g, 33%). mp. 216–219 °C. ¹H-NMR (300 MHz, CDCl₃) δ 0.73 (s, 3H), 0.81 (s, 3H), 1.25 (s, 3H), 0.82–1.67 (m, 25H), 2.03–2.08 (m, 2H), 2.52–2.60 (m, 1H), 4.33–4.40 (dd, *J* = 9, 9 Hz, 1H). Elemental Anal. for C₂₀H₃₃NO₃ Calcd. C: 71.6, H: 9.91, N: 4.17. Found. C: 71.65, H: 9.99, N: 4.19.

3 β -Hydroxy-5 α -androstan-17-oxime (4)—Hydroxylamine hydrochloride (0.5 g, 7 mmol) was added under nitrogen to a solution of epiandrosterone (0.5 g, 1.7 mmol) in anhydrous pyridine (25 ml) and the solution was allowed to stir at room temperature for 24 h. The solution was then concentrated under reduced pressure to 10% of its original volume and was purified on silica using medium pressure column chromatography (hexane/ethyl acetate, 1:1) to provide **4** as a white solid. The product was used in the next step without additional purification. ¹H-NMR (300 MHz, CDCl₃) δ 0.82 (s, 3 H), 0.90 (s, 3 H), 1.72–0.93 (m, 21 H), 2.55–2.49 (m, 2 H), 3.63–3.56 (m, 1 H).

3 β -Hydroxy-3 α -methyl-5 α -androstan-17-oxime (7)—The title compound was prepared from 3 β -hydroxy-3 α -methyl-5 α -androstan-17-one (Cook et al., 1968) in a manner analogous to compound **4**. ¹H-NMR (300 MHz, CDCl₃) δ 0.83 (s, 3 H), 0.90 (s, 3 H), 1.25 (s, 3H), 0.70–1.95 (m, 21 H), 2.50 (m, 2 H), 8.09 (br-s).

2.3. Radioligand binding assays

Tissue preparation—Rat brain cerebral cortex homogenates and the binding assays were conducted essentially as described (Carter et al., 1997). Briefly, adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were killed by excess CO₂ asphyxiation, decapitated, and their brains rapidly removed and frozen on dry ice. The frozen cortices were placed in 10 volumes of an ice-cold 0.32 M sucrose solution and homogenized with a Teflon-glass homogenizer. The homogenate was centrifuged at 1500 $\times$ g (10 min at 4 °C). The supernatant was retained and centrifuged at 10,000 $\times$ g (20 min at 4 °C), yielding the P2 pellet. The P2 pellet was resuspended in an equal volume of a 50 mM Na⁺/K⁺ phosphate buffer containing 200 mM NaCl (pH 7.4) and centrifuged at 10,000 $\times$ g (10 min at 4 °C). The pellet was washed twice more, resuspended in 1/10 volume of buffer and stored at -76 °C until the time of assay.

[³⁵S]-t-Butylbicyclopophosphorothionate binding assays—These assays were carried out in 1.4 ml polypropylene tubes (Matrix Technologies, Hudson, NH). In a final volume of 0.5 ml, each tube contained 100 μ l cortical membrane suspension (40 μ g of protein, added last), 2 nM [³⁵S]-t-butylbicyclopophosphorothionate (64–165 Ci/mmol), 6.25 μ M GABA, and one of six different concentrations of test compound added as 5 μ l in 100% DMSO (1% final DMSO concentration). The addition of GABA to the [³⁵S]-t-butylbicyclopophosphorothionate assay permits sufficient channel opening to occur during the assay to permit pseudo-equilibrium conditions to be achieved, thus allowing us to measure the effect of the neurosteroids on radioligand binding during the assay incubation period. The test

compounds were then added to the assay tubes using a BiomekFX automated liquid handler with 96-well head (Beckman-Coulter, Fullerton, CA). Nonspecific binding was determined in the presence of 200 μ M picrotoxin. The assays were incubated for 2 h at room temperature before being terminated by vacuum filtration through 96-well GF/B glass filter plates using a 96-well harvester (Brandel Scientific, Gaithersburg, MD). Prior to harvesting, the filter plates were pre-soaked for 20 min in buffer containing 0.15% bovine serum albumin and 0.1% polyethylene imine. The radioactivity remaining on the filter was determined in a TopCount 12-detector scintillation counter (Packard Instruments, Meriden, CT) using standard liquid scintillation counting techniques.

[³H]Flunitrazepam binding assay—This assay was conducted as described for the [³⁵S]*t*-butylbicyclopophosphorothionate assay except that the assays contained 20 μ g tissue homogenate, 1.0 μ M GABA and 1.0 nM [³H]flunitrazepam (74.1–85 Ci/mmol). Clonazepam (1.0 μ M) was used to determine nonspecific binding. A submaximal concentration of GABA was employed in this assay in order to determine if our compounds had a positive modulatory effect on GABA-stimulated [³H]flunitrazepam binding.

Data analysis—The IC₅₀ or EC₅₀ and E_{max} values for allopregnanolone and the test compounds were calculated from a three-parameter logistic equation fit to the binding data using Prism (version 3, GraphPad Software, San Diego, CA).

The E_{max} values for allopregnanolone and the test compounds are presented as the percentage of E_{max} determined for allopregnanolone. The average enhancement of [³H]flunitrazepam measured in the presence of allopregnanolone was approximately 60%. In cases where limited inhibition of [³⁵S]*t*-butylbicyclopophosphorothionate or enhancement of [³H]flunitrazepam occurred at the top concentration of 10,000 nM, the IC₅₀ or EC₅₀ values are given as >10,000 nM. The data for the active compounds are reported as the mean $\pm$ S.E.M. from at least three independent experiments.

2.4. Testing in mouse models

Male NIH Swiss mice (25–30 g) were housed two per cage. Animals were kept in a vivarium (temperature 22–26 °C; humidity 40–50%) with an artificial 12-h light/dark cycle and free access to food and water. Animals were allowed to acclimate to the vivarium for at least 5 days. The experiments were performed during the light phase of the light/dark cycle (between 9:30 AM and 3:30 PM), after at least a 30-min period of acclimation to the experimental room. Animals were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and experiments were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington, DC; <http://www.nap.edu/readingroom/books/labrats/>).

Solutions of the steroids were made fresh daily in 40% hydroxypropyl- β -cyclodextrin in sterile 0.9% saline. Further dilutions were made using sterile saline. The steroids were injected intraperitoneally. The convulsant agent pentylenetetrazol (Sigma-Aldrich) was

dissolved in saline immediately before use. All drug solutions were administered in a volume equaling 0.01 ml/g of the animal's body weight.

The pentylenetetrazol seizure test was carried out as described (Kokate et al., 1994). In brief, mice were injected subcutaneously with pentylenetetrazol (80 mg/kg) 15 min after injection of the test steroid and were observed for a 30-min period. Mice failing to show clonic seizures lasting longer than 5 s were scored as protected. To construct dose-response curves, steroids were tested at 3 doses spanning the dose producing 50% protection (ED₅₀). Six to eight mice were tested at each dose. ED₅₀ values and corresponding 95% confidence limit were determined by log-probit analysis.

The 6-Hz seizure test was carried out 15 min after injection of the test steroid as described (Kaminski et al., 2004). In brief, 3-s corneal stimulation (200 μ s-duration, 32-mA monopolar rectangular pulses at 6 Hz) was delivered by a constant current device (ECT Unit 5780; Ugo Basile, Comerio, Italy). Ocular anesthetic (0.5% tetracaine) was applied to the corneas 15 min before stimulation. In response to stimulation, the animals exhibited a "stunned" posture associated with rearing and automatic movements that lasted from 60–120 s in untreated animals. Animals resumed their normal exploratory behavior after the seizure. The experimental endpoint was protection against the seizure. An animal was considered to be protected if it resumed its normal exploratory behavior within 10 s of stimulation. The statistical comparisons were the same as described for the pentylenetetrazol test.

Anxiolytic activity was assessed with the elevated zero maze as described (Kaminski et al., 2006). The maze (Hamilton-Kinder, Poway, CA) was positioned in the center of a room under dim lighting. Each mouse was individually removed from its home cage and placed just inside a closed arm. Five-min test sessions were video-recorded with a tripod-mounted camcorder. Percent time in open areas and number of entries into open areas were scored from the taped records using Observer 3.0 software (Noldus, Wageningen, The Netherlands). An animal was considered to have entered an open area if all four paws had left the closed areas. Open-area time was considered terminated once a single paw was placed back into the closed area. Increases in these measures reflect anxiolytic activity. Dose-response data were analyzed by one-way analysis of variance. Differences from control (vehicle-treatment) values for individual doses were identified by post-hoc comparisons using the Dunnett's test.

3. Results

3.1. Radioligand binding evidence for allosteric modulation of GABA_A receptors

The ability of the compounds to interact with the GABA_A receptor was assessed using [³⁵S]*t*-butylbicyclopophosphorothionate and [³H]flunitrazepam binding assays in rat cerebral cortex homogenates. Both ligands bind specifically to GABA_A receptors. Neurosteroid-like compounds that positively modulate GABA_A receptors inhibit [³⁵S]*t*-butylbicyclopophosphorothionate binding and enhance [³H]flunitrazepam binding (Carter et al., 1997). In keeping with their known activities (Gee et al., 1988; Harrison and Simmonds, 1984; Reddy et al., 2004), allopregnanolone and pregnanolone inhibited [³⁵S]*t*-butylbicyclopophosphorothionate binding and enhanced [³H]flunitrazepam binding in a concentration-dependent fashion. Allopregnanolone completely inhibited [³⁵S]*t*-

butylbicyclophosphorothionate binding and increased [^3H]flunitrazepam binding by approximately 60% (Table 1). In contrast, pregnanolone did not completely inhibit [^{35}S]-butylbicyclophosphorothionate binding at concentrations as high as 1000 nM (maximum percent inhibition $90 \pm 2.0\%$). The 3 α -hydroxy derivatives, **2b** and **2c**, inhibited [^{35}S]-butylbicyclophosphorothionate binding with IC_{50} values comparable to that obtained for allopregnanolone (Fig. 3). Moreover, **2b** and **2c** also enhanced [^3H]flunitrazepam binding to the same degree and with the same potency as allopregnanolone (Fig. 3). The 3 α -configuration was required for this activity since **2a** and **2d**, the 3 β -hydroxy analogs, were inactive in the two binding assays, as is the analogous 5 α -pregnan-3 β -hydroxy-20-one.

3.2. In vivo anticonvulsant activity

Allopregnanolone and pregnanolone have previously been shown to be protective in the pentylenetetrazol and 6 Hz models (Kaminski et al., 2004; Kokate et al., 1994). In contrast, the 3 β -hydroxy analog 5 α -pregnan-3 β -hydroxy-20-one was inactive in both models. We found **2b** to confer protection in a dose-dependent fashion in both models (Fig. 4). Compound **2b** had similar potency to allopregnanolone in the pentylenetetrazol model and was more potent in the 6 Hz model. Similarly, **2c** also conferred protection in both models. It was slightly less potent in the pentylenetetrazol model and slightly more potent in the 6 Hz model than **2b**, but these differences did not reach statistical significance. Interestingly, although it was inactive in the binding assays, compound **2a**, which has a 3 β -hydroxy group showed modest in vivo activity in the pentylenetetrazol and 6 Hz models. We postulated that the activity of **2a** resulted from epimerization in vivo at the 3-position to form the corresponding 3 α -hydroxy form **2b**. This prompted us to prepare and evaluate the analog **2d** with a 3 α -methyl substitution to block epimerization. This analog was inactive in the [^3H]flunitrazepam binding assay (Table 1) and consistent with the possibility that epimerization accounts for the activity of **2a**, was also inactive in the seizure models (Table 2).

3.3. In vivo anxiolytic activity

Compound **2b** was tested in the elevated zero maze at doses of 1, 3, and 10 mg/kg. At a dose of 10 mg/kg, there was a significant increase in percent time in open areas, and at doses of 3 and 10 mg/kg there were significant increases in number of entries to open arms indicative of decreased anxiety (Fig. 5).

4. Discussion

In this report we describe the synthesis and biological activity of a new series of 17 β -nitro androstan-3 α -ol analogs, some of which influence radioligand binding at GABA $_A$ receptors with potencies similar to allopregnanolone. Taylor and colleagues (Taylor et al., 1990) noted similarities in the preferred intermolecular environments of nitro and carbonyl as well as a resemblance in their electronic properties. Therefore, we postulated that nitro substitution for the 17 β -acetyl group of allopregnanolone would maintain biological activity and serve as a bioisostere. We have now demonstrated this is the case and, indeed, substitution results in the full retention of biological activity. In particular, compound **2b** and its 3 β -methyl analog **2c** have in vitro and in vivo potencies equivalent to the naturally occurring neurosteroid

allopregnanolone (Kaminski et al., 2004; Kokate et al., 1994). Compound **2c** is analogous to the 3 β -methyl analog of allopregnanolone (ganaxolone), which has roughly equivalent potency to allopregnanolone as a GABA_A receptor modulator (Carter et al., 1997). As measures of in vivo biological activity we used the pentylenetetrazol and 6 Hz seizure models, both of which are highly sensitive to positive modulators of GABA_A receptors, including neurosteroids (Kokate et al., 1994; Kaminski et al., 2004). In addition, we had the opportunity to evaluate **2b** in the elevated zero maze, a test paradigm used to evaluate anxiolytic activity in rodents that is similar to the elevated plus maze but has certain advantages (Kaminski et al., 2006; Kulkarni et al., 2007). At doses comparable to those that exhibited protective activity in the seizure models, **2b** displayed an anxiolytic profile.

During the course of this investigation, we also observed moderate activity in the pentylenetetrazol and 6 Hz models for the 3 β -hydroxy-17 β -nitro derivative (**2a**). This result was unexpected, given that our in vitro binding experiments showed this 3 β -hydroxy compound to be inactive as a modulator of GABA_A receptors. To explain the observation, we postulated that epimerization occurs in vivo at the 3-position to form the active 3 α -hydroxy isomer **2b**.

Metabolic conversion of 3 β -hydroxy steroids to 3 α -hydroxy steroids is known to occur in vivo (Freudenthal et al., 1971) and until recently, a mechanism for the converse 3 α –3 β conversion in vivo has remained undefined. Metabolic enzymes such as retinol/sterol dehydrogenases have been shown to catalyze the transformation of androsterone and allopregnanolone to their 3 β -epimers in vitro via the 3-keto intermediate. Recent studies (Belyaeva et al., 2007) have provided evidence suggesting in vivo epimerization of **2a** is plausible. NAD⁺ dependent 3 α –3 β -OH epimerase activity for allopregnanolone and androsterone has now been identified in human brain tissue and is attributed to a class of short chain dehydrogenase/reductases having dual specificity retinol/sterol dehydrogenase activity. Importantly, catalytic efficiency is 2.5-fold lower for epimerization of allopregnanolone than for androsterone suggesting steroid substructure plays a key role in determining the rate of epimerization.

Previous studies in our laboratory with 5 α ,3 β -THDOC and 5 α ,3 β -pregnanolone failed to detect in vivo anticonvulsant activity, indicating that if epimerization occurs at the 3-position of these compounds, it is not substantial (Kokate et al., 1994). Our present results suggest that the 17 β -nitro substitution may enhance the rate of epimerization, but this conclusion requires direct comparisons with the other 3 β -hydroxy steroids. In an attempt to limit metabolic conversion at the 3-position, we synthesized and tested compound **2d** having a 3 α -methyl group. In vitro binding experiments confirmed that **2d** was devoid of activity at GABA_A receptors as is the corresponding *des*-methyl analog **2a**. Compound **2d** was similarly inactive in in vivo anticonvulsant testing. These observations support the concept that the in vivo activity of **2a** results from epimerization, but conclusive proof requires chemical identification of the metabolic product in animals receiving **2a**.

In conclusion, our results expand the range of 17 β -substituents in 3 α -hydroxy-androstanes that retain GABA_A receptor modulating activity to include nitro. In fact, the nitro bioisostere is the first 17 β -substituent that has been found to enhance receptor activity beyond that of

the acetyl in naturally occurring allopregnanolone. It remains to be determined whether these analogs will exhibit enhanced pharmacokinetic or pharmacodynamic properties, such as improved bioavailability or metabolic stability, which would confer advantages as pharmacological tools or as therapeutic agents for the treatment of epilepsy or other neuropsychiatric conditions.

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Fig. 1.
Structures of androstan-3 α -ol analogs.

Fig. 2.

Synthesis of compounds **2a–2d**: *Reagents and conditions*: (a) $\text{NH}_2\text{OH} \cdot \text{HCl}$, pyridine, 8 h, (b) KHCO_3 , *N*-bromosuccinimide, H_2O , dioxane, (c) sodium borohydride.

Fig 3.
Radioligand binding data for allopregnanolone, pregnanolone, **2b**, and **2c**.

Fig. 4.

Dose-response curves for seizure protection by compounds **2a**, **2b**, and **2c** in pentylenetetrazol (A) and 6 Hz (B) models. Compounds were administered intraperitoneally 15 min before testing. Each data point indicates the percent of animals protected out of the 6–8 tested. Vehicle injections failed to provide protection in either model.

Fig. 5.

Evaluation of compound **2b** at various doses in the elevated zero maze. Bars indicate mean ± S.E.M. of percent of time spent in open areas (A) and number of entries into open areas (B) for the indicated number of animals. Compound **2b** increased the percent time in open areas [$F(3,29)=4.6$, $P<0.05$, one-way ANOVA] and number of entries into open areas [$F(3,29)=14.0$, $P<0.001$]. *Different from vehicle (0 mg/kg) by Dunnett's test ($P<0.05$).

Table 1

Summary of radioligand binding data

Test Compound	³⁵ S]- <i>t</i> -butylbicyclo-phosphorothionate		³ H]Flunitrazepam	
	IC ₅₀ (nM)	% Max Inhibition	EC ₅₀ (nM)	E _{max}
1 (Allopregnanolone)	17 ± 2	100 ± 2	26.1 ± 9.9	100 ± 6
Pregnanolone	20.9 ± 6.4	90 ± 2	19.5 ± 5.2	100 ± 1
5α-Pregnan-3β-ol-20-one	>10,000	–	>10,000	ND
2a	>10,000	–	>10,000	ND
2b	12.2 ± 0.1	94 ± 6	22.9 ± 3.5	98 ± 6
2c	17.9 ± 6.2	93 ± 3	33.5 ± 4.9	99 ± 7
2d	ND	–	>10,000	ND

ND = not determined.

Table 2

Summary of results from anticonvulsant testing in mice

Test Compound	Pentylentetrazol	6 Hz
	ED ₅₀ (95% C.L.), mg/kg	ED ₅₀ (95% C.L.), mg/kg
1 (Allopregnanolone)	13.7 ^a (10.1–18.7)	14.2 ^b (10.3–19.4)
Pregnanolone	18.2 ^a (14.7–22.6)	14.4 ^b (10.4–19.9)
5 α -Pregnan-3 β -ol-20-one	>100 ^a	>100 ^b
2a	89.4 (63.8–125.3)	83 (74.5–92.7)
2b	12.9 (9.8–16.9)	5.3 (4.6–6.1)
2c	10 (8.9–11.2)	8.8 (7.1–10.8)
2d	>100	>100

^aFrom Kokate et al. (1994).^bFrom Kaminski et al. (2004). All compounds were administered intraperitoneally 15 min before testing.