Material and Methods

I. *Determination of the loss of righting reflex and duration of hypnosis*

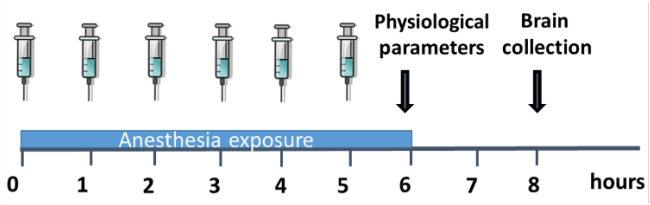
The loss of righting reflex (LORR) in rodents is a widely used behavioral surrogate for assessing the hypnotic effects of drugs. At PND7, rat pups (males and females) received one intraperitoneal injection of either alphaxalone (doses from 1 to 80 mg kg^{-1}), CDNC24 (doses from 0.25 to 60 mg kg^{-1}) or propofol (doses from 0.5 to 80 mg kg^{-1}) and were immediately placed in a supine position to check the animal's ability to right itself, i.e. the ability to turn back over onto all 4 legs within 5-10 seconds (n=4-16 animals per dose). The time to the LORR and the duration of hypnosis were recorded. The percentage of PND7 pups with the LORR at each dose was used to establish dose–response curves and to calculate the ED_{50}. The mortality at each dose was also determined and the LD_{50} was calculated. Both the ED_{50} and LD_{50} were calculated using the Hill-Langumir equation. Therapeutic index was calculated as a ratio of the LD_{50} and ED_{50} for each drug. All curves were generated using Origin 7.0 software (OriginLab, Northhampton, MA). In control experiments, none of the vehicles (intralipid, 15% nor 25% cyclodextrin) caused LORR (data not shown).

II. *Neurotoxicity study design and measurement of physiological parameters*

Exposure protocol

The PND7 rats (males and females) received 6 intraperitoneal injections (i.p) of either alphaxalone (at 10 mg kg^{-1}), CDNC24 (at 10 mg kg^{-1}) or propofol (at 20 mg kg^{-1}). Control groups received the same volume of 15% cyclodextrin, 25% cyclodextrin or intralipid (Sigma Aldrich, MO, USA) solution (vehicles for alphaxalone, CDNC24 and propofol, respectively). Injections were repeated every hour (6 injections) for a total anesthesia duration of 6 hours. The timeline of the exposure protocol and data collection for physiological parameters recording and neurotoxicity study is outlined in Scheme S1.

Scheme S1.



Animal monitoring and the assessment of physiological response during anesthesia exposure

The animals were kept on a heating pad to maintain normal body temperature during all procedures. Animals were carefully obsereved at all times for changes in color and breathing. We measured blood glucose (Countour next EZ, Ascensia Diabetes Care US, Inc., USA) and oxygen saturation levels, as well as heart and respiration rate

(PhysioSuite^{TM}, Kent Scientific Corporation, USA) within the last 15 min of anesthesia exposure .

Immunohistochemical labeling of active caspase-3 (AC-3) and the neurotoxicity analyses

The neurotoxic potential of alphaxalone, CDNC24 and propofol was assessed using AC-3 immunohistochemical staining in the subiculum and four subregions of mPFC in PND7 rats. For immunohistochemical analysis, the animals were briefly anesthetized with 2% isoflurane and perfused with 0.1 M phosphate buffer saline, pH 7.4 (PBS), then with 4% paraformaldehyde in 0.1 M PBS (PFA) two hours after the end of exposure. The extracted brains were immersed in fresh PFA and incubated for 72 hours at 4 °C before embedding in agar. Coronal vibratome sections (50 µm thickness) were mounted on superfrost plus slides, and air-dried at room temperature (RT) for at least 2 hours. Antigen retrieval with sodium citrate buffer (pH 6.0) was performed and slides were washed twice for 5 min each in 0.1 M PBS+0.1% Triton X 100. Endogenous peroxidase quenching was performed by incubating slices in bloxall solution for 10 min. After washing for 5 min in 0.1 M PBS+0.1% Triton X 100, the nonspecific binding was blocked by incubating in 5% normal goat serum in 0.1 M PBS+0.1% Triton X 100 for 60 min at RT before incubating with primary anti-active caspase-3 antibody in 1% normal goat serum in 0.1 M PBS+0.1% Triton X 100 solution (1:100; Cell signaling Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb (Biotinylated), MA, USA) overnight at 4 ˚C. Sections were washed three times for 10 min each in PBS+0.1% Triton X 100 and incubated with Avidin/Biotin based peroxidase reagent (Vectastain Elite ABC HRP kit, PK-6100, CA, USA) for 60 min at RT. After washing for 10 min in 0.1 M PBS, the reaction complexes were developed by incubating

the sections in DAB (3,3-diaminobenzidine) HRP substrate (ImmPACT DAB Peroxidase (HRP) Substrate, SK-4105, CA, USA) following manufacturer's instructions. Finally, sections were quick washed in stop solution (1 part 0.1 M PBS + 2 parts ddH_2O) for 5 min and subjected to dehydration in increasing concentrations (70%, 90% and 100%) of ethanol followed by xylene. Slides were mounted with vectamount, coverslipped and examined under a Nikon microscope (Nikon Eclipse E800, NY, USA). The staining was imaged (10x magnification) using a DS-Fi3 camera (Nikon) and analyzed using NLS elements version 5.02 software (Nikon). The number of active-caspase-3 positive cells was quantified in both left and right side of the brain with at least 3 sections per animal. The data were averaged and normalized to a millimeter square of surface to provide a single animal data point. The quantification was performed by a researcher blinded to the experimental condition.

III. Electrophysiology

Brain slice preparation for patch-clamp experiments

Animals were anesthetized briefly with isoflurane and decapitated, and their brains rapidly removed. Horizontal or sagittal brain slices (300 μm) were sectioned at 4 °C in the prechilled solution containing (in mM): sucrose 260, D-glucose 10, $NaHCO_3$ 26, NaH_2PO_4 1.25, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, using a tissue slicer (Leica VT 1200S). Brain slices were immediately incubated for 40-45 minutes at 37 °C in solution containing (in mM): NaCl 124, D-glucose 10, NaHCO3 26, NaH_2PO_4 1.25, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2 prior to use in electrophysiology experiments, which were done at room temperature. During incubation,

slices were constantly perfused with a gas mixture of 95 vol% O_2 and 5 vol% CO_2. Subicular neurons throughout the pyramidal and polymorphic layers between CA1 and presubiculum were selected randomly for electrophysiological recordings.

Electrophysiology Experiments

The external solution for whole-cell patch-clamp electrophysiology experiments consisted of (in mM): NaCl 125, D-glucose 25, $NaHCO_3$ 25, NaH_2PO_4 1.25, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2. This solution was equilibrated with a mixture of 95 vol% O_2 and 5 vol% CO_2 for at least 30 minutes with a resulting pH of 7.3-7.4. For recording of spontaneous inhibitory postsynaptic currents (sIPSCs), we used an internal solution containing the following (in mM): KCl 130, NaCl 4, $CaCl_2$ 0.5, EGTA 5, HEPES 10, $MgATP_2$ 2, Tris-GTP 0.5, and lidocaine *N*-ethyl bromide (QX-314) 5. pH was adjusted with KOH to 7.25. Glass micropipettes (Sutter Instruments O.D. 1.5 mm) were pulled using a Sutter Instruments model P-1000 and fabricated to maintain an initial resistance of 3-5 MΩ. Neuronal membrane responses were recorded using a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA). Voltage current commands and digitization of the resulting voltages and currents were performed with Clampex 8.3 software (Molecular Devices) running on an IBM-compatible computer. Resulting current traces were analyzed using Clampfit 10.5 (Molecular Devices, Foster City, CA).

Spontaneous IPSCs

In the patch-clamp configuration at a holding potential of −70 mV, spontaneous $GABA_A$-mediated currents (sIPSCs) were isolated in the presence of the NMDA channel blocker d-APV (50 μM) and AMPA channel blocker NBQX (10 μM). All events were

visually detected and analyzed off-line using MiniAnalysis software (Synaptasoft Inc, USA), and sIPSC frequency, amplitude and decay time were calculated. The detection threshold for sIPSCs was set at two times the root mean square of the baseline noise. In our analysis of kinetics of spontaneous synaptic currents, we included only isolated (i.e. nonoverlapping) events. All sIPSCs were analyzed with respect to peak amplitude and decay time course, which was analyzed only in the case of a near perfect fit ($R^2 \geq 0.990$) using either a mono- or bi-exponential function. Plots describing the cumulative distribution of inter-event interval or decay time of sIPSC events were derived empirically using GraphPad Prism 7.02 software (GraphPad Software Inc., San Diego, CA). These plots were derived from all recorded neurons, and they represent a portion of events whose apparent inter-event intervals or decay times are less than a certain value. The tonic GABA current was calculated by subtracting the holding current before and after application of a potent $GABA_A$ receptor antagonist picrotoxin (20 μM) using at least 30-s-long epochs, which is a well-established method for measuring the tonic conductance[1].

IV. Data analysis

Results are presented as mean (SEM). The results of neurotoxicity study were statistically analyzed by one-way ANOVA followed by Tukey post hoc test, while statistical analysis for electrophysiology experiments was performed using two-tailed paired t-test. The data presented in Fig. 6 were analyzed using one-way ANOVA followed by Dunnett's post hoc test, as we aimed to compare neurosteroid effects on phasic and tonic inhibition to the ones of propofol. Degrees of freedom were reported next to the t- and F-values for t-test and ANOVA, respectively. Significance was accepted as $P<0.05$. Statistical and graphical

analyses were performed using GraphPad Prism 7.02 software (GraphPad Software, La Jolla, CA) and Origin 2018 (OriginLab, Northhampton, MA).

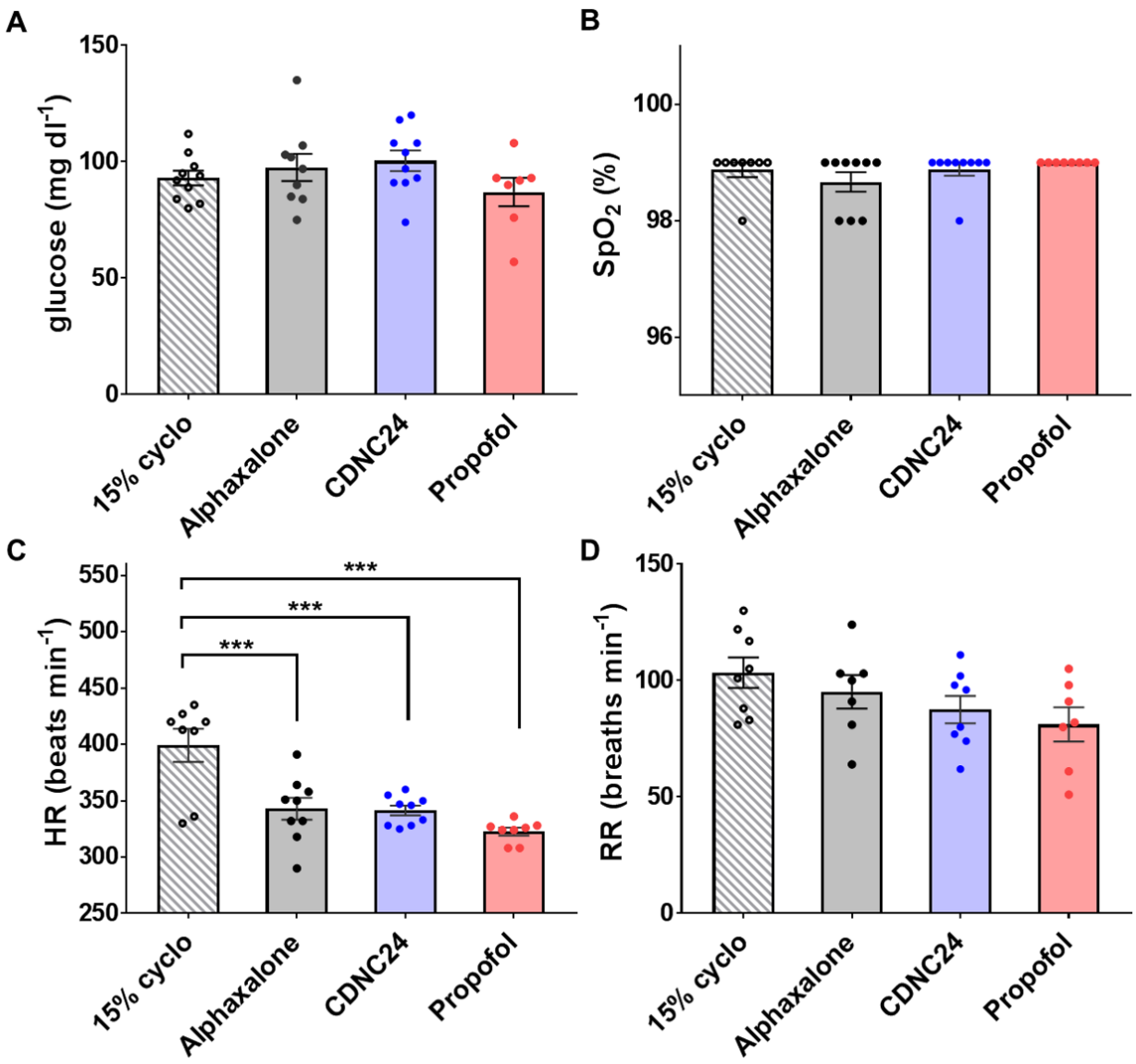


Figure S1. Alphaxalone, CDNC24 and propofol decrease heart rate, but do not change glucose or oxygen saturation levels, nor the respiration rate after six hours of exposure when compared to their respective vehicle controls. Physiological parameters [glucose level (**A**), oxygen saturation (**B**), heart rate (**C**) and respiration rate (**D**)] are assessed after 6 i.p. injections of alphaxalone (filled grey bar), CDNC24 (filled blue bar) or propofol (filled red bar) and compared to 15% cyclodextrin treated animals (patterned gray bar) as control. All data are presented as mean (SEM) (n= 7-10 animals

per each data point). Statistical analysis was done using one-way ANOVA with Tukey's post-hoc test (***, p<0.001).

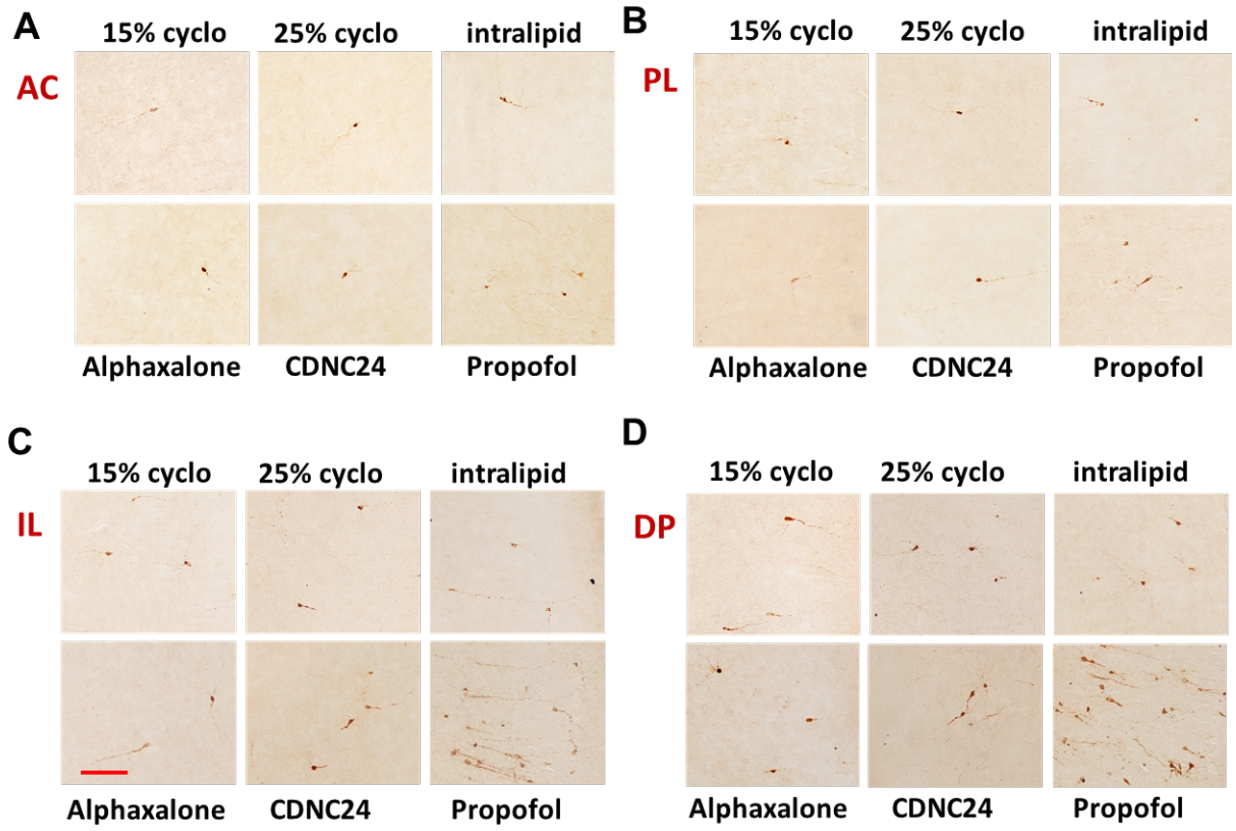
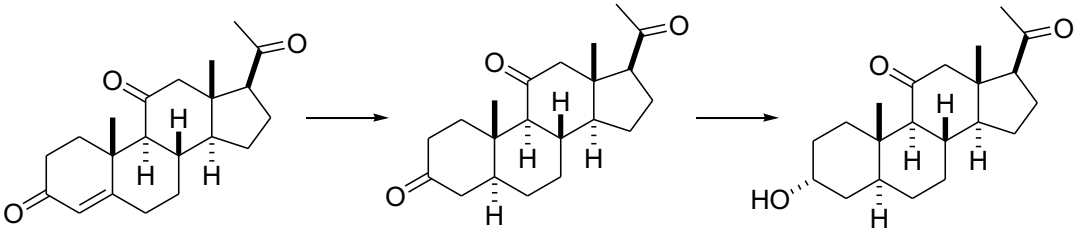


Figure S2. Representative images of Active caspase-3 immunostaining in mPFC of PND7 rat pups. Representative images of Activated Caspase-3 immuno-staining in (**A**) anterior cingulate cortex (AC), (**B**) prelimbic (PL), (**C**) infralimbic (IL), and (**D**) dorsal peduncular cortex (DP) after six i.p. injections of alphaxalone, CDNC24 or propofol and 15% cyclodextrin, 25% cyclodextrin or intralipid as their controls, respectively are depicted on the panels (scale bar 100 μm).

Table S1. The number of Activated caspase-3 labeled cells per mm^2 in the subiculum and different regions of mPFC presented as mean (SEM).

	Subiculum	AC	PL	IL	DP
15% cyclodextrin	16.01 (1.25)	5.72 (0.66)	7.06 (0.82)	13.67 (1.41)	23.90 (2.65)
25% cyclodextrin	18.07 (1.75)	7.83 (1.58)	7.18 (1.20)	14.07 (2.02)	42.25 (3.27)
intralipid	18.44 (1.28)	10.64 (1.60)	8.62 (1.20)	17.58 (1.49)	23.02 (5.44)
Alphaxalone	26.87 (2.69)	6.80 (0.55)	6.44 (0.43)	13.10 (1.39)	23.65 (2.95)
CDNC24	34.68 (2.90)	7.48 (0.46)	10.56 (1.04)	21.09 (2.50)	42.91 (4.65)
Propofol	88.73 (7.49)	19 (3.43)	21.31 (5.53)	63.53 (11.72)	109.20 (18.30)

Synthesis of Alphaxalone (3)



1 **2** **3 (Alphaxalone)**

Steroid 2

Anhydrous NH_3 gas (200 mL) was condensed into a cold (–78 °C) three-necked 500 mL flask equipped with a Dewar condenser and overhead stirrer. Li metal (280 mg, 40 mmol) was added in small pieces and the resulting blue solution was stirred for 30 minutes. Steroid **1** (11-ketoprogesterone purchased from Steraloids, 3.28 g, 10 mmol) in THF (100 mL) was added and the mixture was stirred vigorously for 3 hours. Solid NH_4Cl (~5 g) was then added to quench the reaction and the NH_3 was allowed to slowly evaporate at room temperature. Water (300 mL) was added to the residue. The product mixture was extracted into ethyl acetate (3x100 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4 and the solvent removed to give a mixture of steroid **2** and other steroids in which one or more of the three ketone groups was reduced to the alcohol group.

The crude product mixture was dissolved acetone (100 mL), stirred and Jones reagent was added until an orange color persisted. The excess Jones reagent was consumed by the addition of a few drops of isopropyl alcohol and the resulting green solution was extracted with ethyl acetate (3x70 mL). The combined organic extracts were washed with

brine, dried over anhydrous Na_2SO_4 and the solvent removed to give an off-white solid. The crude product was purified by flash column chromatography (silica gel eluted with 20-40% ethyl acetate in hexanes) to give steroid **2** as a solid (2 g, 60%).^{1}H NMR ($CDCl_3$): δ 2.82-1.10 (m), 2.10 (s, 3H), 1.19 (s, 3H), 0.60 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 211.06, 209.07, 207.75, 63.64, 62.05, 56.49, 55.45, 47.02, 46.85, 44.17, 37.85, 36.99, 36.50, 35.15, 32.21, 31.16, 28.14, 23.83, 23.28, 14.19, 11.00.

Alphaxalone (3)

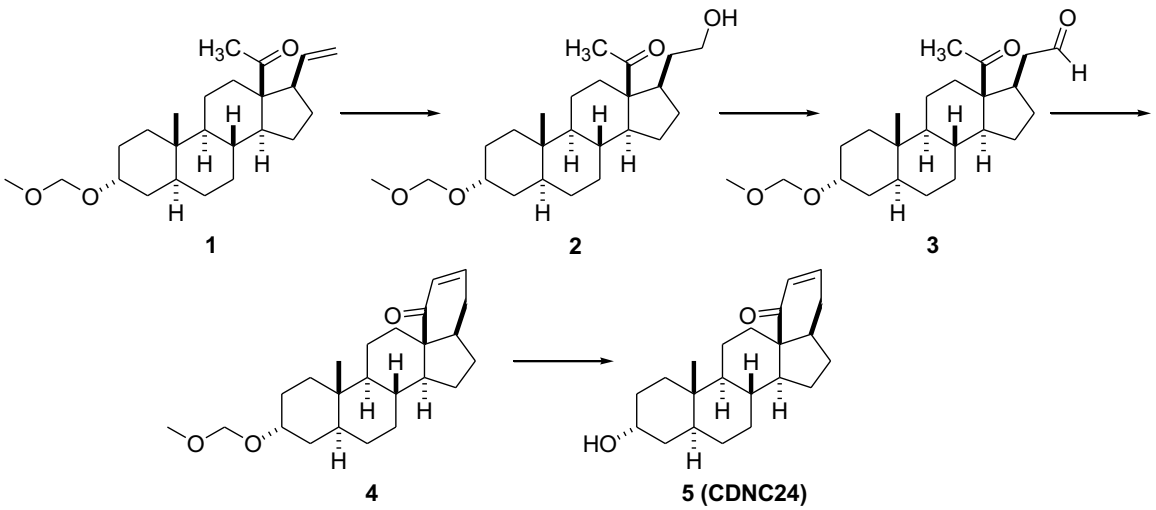
1 M K-Selectride in THF (6.6 mL, 1.1 eq) was added to a cold (–78 °C) solution of steroid **2** (2 g, 6 mmol) in dry THF (100 mL) and the reaction was stirred at that temperature for 1.5 h. The reaction was quenched by adding few drops of water and slowly warmed to 0 °C. 3 M NaOH (12 mL) and 50% H_2O_2 (12 mL) were added and the reaction was stirred at room temperature for 2 h. Water (200 mL) was added and the product was extracted into ethyl acetate (3 x 80 mL). The combined organic extracts were washed with water followed by brine, dried over anhydrous Na_2SO_4 and solvent removed give a viscous liquid which was purified by flash column chromatography (silica gel eluted with 20-40% ethyl acetate in hexanes) to give alphaxalone as a white solid (1.5 g, 75%) which contained 10-15% of the 3β-OH epimer. The 3β-OH epimer was removed as follows.

The epimeric steroid mixture (1.5 g, 4.6 mmol) was dissolved in dichloromethane (25 mL), pivaloyl chloride (0.12 mL, 1mmol) was added and the reaction was stirred for 2 h at room temperature. During this time the reaction was carefully monitored until ~20-25% of the

epimeric 3-hydroxysteroid mixture was converted to the 3-pivaloyl esters. The majority of the alphaxalone remained unesterified while the 3β-epimer was fully esterified and thus readily removed by subsequent flash column chromatography. Aqueous $NaHCO_3$ was added and the steroids were extracted into dichloromethane (3 x 60 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and solvent removed to give the products as a viscous liquid. The crude product was purified by flash column chromatography (silica gel eluted with 20-35% ethyl acetate in hexanes to obtain pure alphaxalone (1.05 g, 70%): mp 168–169°C; $[\alpha]_D$ = +115 ($CHCl_3$, *c* = 0.1); ^{1}H NMR ($CHCl_3$): δ 4.05 (s, 1H), 2.74 (t, *J* = 8.1 Hz), 2.57 (d, 1H, *J* = 12.2 Hz), 2.50 (d, 1H, *J* = 11.8 Hz), 2.26 (m, 2H), 2.10 (s, 3H), 1.80-1.10 (m), 1.01 (s, 3H), 0.58 (s, 3H). ^{13}C NMR ($CHCl_3$): δ 209.83, 208.16, 65.89, 64.04, 61.97, 56.55, 55.62, 47.04, 38.69, 36.43, 35.53, 35.08, 32.41, 31.16, 30.70, 28.62, 27.63, 23.64, 23.08, 14.09, 10.7.

Synthetic Scheme for the preparation of CDNC24

A synthetic route for the preparation of CDNC24 has been reported previously[2]. The following synthetic route was used for the preparation of the CDNC24 used in this study.



Steroid 2

Steroid **1** was prepared as described previously[3]. A solution of steroid **1** (748 mg, 2 mmol) in THF (30 mL) and 0.5 M 9-BBN in THF (4.4 mL, 1.1 equiv) was stirred at room temperature. The reaction was monitored by TLC for the disappearance of steroid **1**. Upon completion, the reaction was cooled (0°C), 3 M NaOH (8 mL) and 50% H_2O_2 (8 mL) were added and the reaction was stirred for 2 h. The reaction was extracted with ethyl acetate (3 x 70 mL) and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. Removal of solvents and purification of the product by flash column chromatography (silica gel eluted with 20-45% ethyl acetate in hexanes) gave steroid **2** as a viscous oil (560 mg, 71%). ^{1}H NMR ($CDCl_3$): δ 4.64 (AB q, *J* = 7Hz), 3.81 (s, 1H), 3.70 (m, 1H), 3.55 (m, 1H), 3.35 (s, 3H), 2.48 (d, 1H, *J* = 13Hz), 2.11 (s, 3H), 2.0-0.70 (m), 0.67 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 213.83, 94.42, 71.63, 61.93, 61.46, 57.21, 55.10, 54.83, 46.85, 39.69, 36.31, 35.82, 35.09, 34.48, 33.43, 32.78, 31.83, 30.44, 28.77, 28.31, 26.22, 25.07, 22.96, 11.42.

Steroid 3

2-Iodoxy benzoic acid (1.24g, 2, mmol, 45% by weight) in DMSO (5 mL) was added to a stirred solution of steroid **2** (560 mg, 1.42 mmol) in DMSO (5 mL) and the reaction was stirred at room temperature for 3 h. Water was added and the product was extracted into ethyl acetate (3 x 50 mL). The combined organic extracts were washed with aqueous $NaHCO_3$ followed by brine and dried over anhydrous Na_2SO_4. Removal of solvents and purification by flash column chromatography (silica gel eluted with 5-15% ethyl acetate in hexanes) gave steroid **3** (481 mg, 87%) as a viscous liquid, which was immediately converted to steroid **4**. Steroid **3** had: ^{1}H NMR: δ 9.75 (s, 1H), 4.67 (AB q, *J* = 7.1 Hz), 3.82 (s, 1H), 3.37 (s, 3H), 2.60-0.70 (m), 2.12 (s, 3H), 0.69 (s, 3H).

Steroid **4**

A mixture of steroid **3** (481 mg, 1.2 mmol), powdered 90% potassium hydroxide (102 mg, 1.8 mmol), dibenzo-18-crown-6 (360 mg, 1 mmol) and benzene (100 mL) was refluxed with a Dean-Stark apparatus attached to a condenser for 4 h. The reaction was cooled to room temperature and washed with water followed by brine and the organic layer was dried over anhydrous Na_2SO_4. The solvent was removed to give the product which was purified by flash column chromatography (silica gel eluted with 15-25% ethyl acetate in hexanes) to give steroid **4** (403mg, 88%). ^{1}H NMR ($CDCl_3$): δ 6.61 (m, 1H), 5.78 (m, 1H), 4.66 (AB q, *J* = 6.6 Hz), 3.83 (s, 1H), 3.37 (s, 3H), 2.61 (m, 2H), 2.58 (m, 1H), 2.07-0.64 (m), 0.86 (3H, s).

Steroid 5 (CDNC24)

Steroid **4** (400 mg, 1.07 mmol) was dissolved in THF (50 mL) and 3 N HCl (5 mL) was added and the reaction was stirred at room temperature until TLC monitoring showed complete removal of the methoxymethyl protecting group. Aqueous $NaHCO_3$ was added and the product was extracted into dichloromethane (3 x 70 mL). The combined extracts were washed with brine, dried over anhydrous Na_2SO_4 and the solvent removed to yield the product as an off-white solid which was purified by flash column chromatography (silica gel eluted with 20-40% ethyl acetate in hexanes) to give steroid **5** (250 mg, 71%). Steroid **5** (**CDNC24**) was recrystallized from ethyl acetate-hexanes to obtain the pure product (200 mg): mp 142-143° C; ^{1}H NMR ($CDCl_3$): δ 6.61 (m, 1H), 5.78 (m, 1H), 4.03 (s, 1H), 2.56 (m, 2H), 2.26 (m, 1H), 2.05-0.65 (m), 0.84 (s, 3H). ^{13}CNMR ($CDCl_3$): δ 204.15 144.51, 129.40, 66.67, 57.19, 54.41, 53.53, 45.11, 39.37, 36.32, 35.94, 35.46, 32.75, 32.72, 32.40, 29.04, 28.51, 27.70, 26.78, 24.33, 20.29, 11.35.

References

1. Bright DP, Smart TG. Methods for recording and measuring tonic GABAA receptor-mediated inhibition. Front Neural Circuits. 2013;7:193. doi: 10.3389/fncir.2013.00193.

2. Jiang, X., Manion, B.D., Benz, A., Rath, N.P., Evers, A.S., Zorumski, C.F., Mennerick, S., and Covey, D.F. Neurosteroid analogues. 9. Conformationally constrained pregnanes: structure–activity studies of 13,24-cyclo-18-21-dinorcholane analogues of the GABA modulatory and anesthetic steroids (3α,5α)- and (3α,5β)-3-hydroxypregnan-20-one. J. Med. Chem. 2003;46:5334-5348.

3. Covey D.F. and Jiang, X. Neuroactive 13,17-substituted steroids as modulators for GABA Type-A receptors. United States Patent 10,160,783. Issued December 25, 2018.