EFFECTS OF 3α-AMINO-5α-PREGNAN-20-ONE ON $GABA_A$ RECEPTOR: SYNTHESIS, ACTIVITY AND CYTOTOXICITY+

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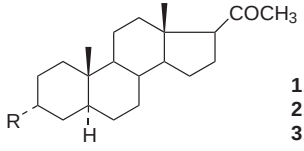
The 3α-hydroxy function has been considered essential for the biological activity of neurosteroids at the $GABA_A$ receptor. It was found that 3α-amino-5α-pregnan-20-one (**3**) increased the binding of [^{3}H]flunitrazepam at the $GABA_A$ receptor in the primary culture of cortical neurons. This derivative did not display cytotoxicity at relevant neuroactive concentrations, and its structure enabled us to gain further insight into possible functional group modifications in position 3α. Various synthetic methods were investigated in search for the most suitable synthetic approach.

Keywords: $GABA_A$ receptor; Steroids; Neurosteroids; Allopregnanolone; [^{3}H]Flunitrazepam; 3α-Amino-5α-pregnan-20-one; Cytotoxicity; MoO_3.

In search[2–8] for new analogues of the natural neurosteroid, 3α-hydroxy-5α-pregnan-20-one (**1**, allopregnanolone), we have tested a number of 5α-pregnane derivatives, mostly using *in vitro* tests, which measured the binding of radiolabelled ligands to γ-aminobutyric acid receptors ($GABA_A$ receptor) in the absence and presence of the tested compounds. The labelled ligands used were [^{3}H]muscimol and [^{35}S]*tert*-butylbicyclo[2.2.2]-phosporothionate (TBPS)[4]. The compounds tested were chosen from the bank of products prepared in our laboratory over the past few decades. One of the compounds – 3α-fluoro-5α-pregnan-20-one (**2**) – exhibited surprising activity in an animal behavioral test[8]. The result seemed to contradict the hitherto held axiom of the essentiality of a 3α-hydroxy group for neuronal

+ Part CDXVII in the Series On Steroids; Part CDXVI see lit.[1]

activity. This prompted us to evaluate the neuronal activity of other 3α-substituted[9–13] pregnan-20-ones by determining their activity on neuronal $GABA_A$ receptors expressed on living cultured neuronal cells first. It has been reported that the active neurosteroid – 3α-hydroxy-5α-pregnan-20-one (**1**) – allosterically increases [^{3}H]flunitrazepam binding in rat brain membranes[14]. We have previously reported that the primary neuronal culture, expressing $GABA_A$ receptors, responds to $GABA_A$ receptor modulators by increasing [^{3}H]flunitrazepam binding[15–17]. Thus, the use of such culture would allow us to test neuroactive compounds acting on $GABA_A$ receptors as well as to assess their cytotoxicity in the same *in vitro* system.



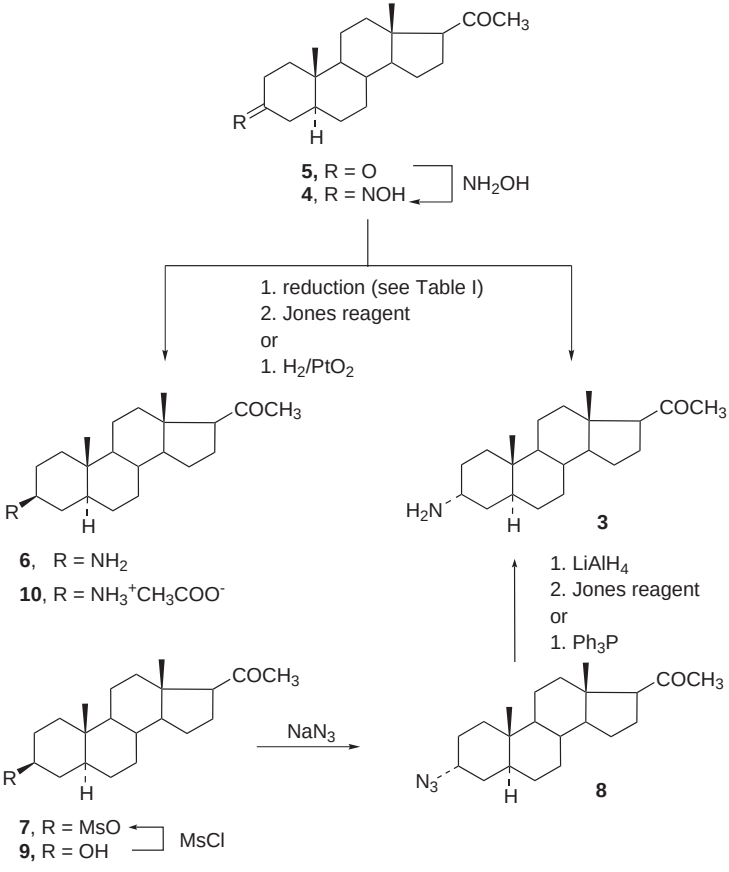
1, R = OH
2, R = F
3, R = NH_2

A 3α-amino group would probably not make the analogue more stable to metabolic processes. However, the group would render it more polar and hence more soluble in aqueous body fluids. 3α-Amino-5α-pregnan-20-one (**3**) is a known alkaloid called funtumin, first isolated from *Funtumia latifola* and *Holarrhena febrifuga*[18,19]. It was prepared from 3-hydroxyimino-5α-pregnan-20-one (**4**; Scheme 1) by catalytic hydrogenation and subsequent oxidation[20]. The hydrogenation reportedly yielded 75% of the 3α-amino 20β-alcohol. Since Dave[21] described the selective conversion of 5α-pregnan-3,20-dione (**5**) into the 3-hydroxyimino derivative **4**, a straightforward reduction of this oxime into funtumin (**3**) appeared to be the best way (Scheme 1). Although other authors (e.g.[22]) have also described the preferential formation of 3α-amino-5α-steroids in the catalytic hydrogenation of 3-oximes, in our hands the desired amine **3** was accompanied by a considerable amount (1:1) of its 3β isomer (amine **6**, as determined by ^{1}H NMR after oxidation of the C-20 hydroxy group).

Alternatively, the reduction of oxime **4** was attempted with other reducing agents (Table I). The reduction was evaluated after oxidation of the C-20 hydroxy group (formed in the course of the reaction) into a mixture of 3α- and 3β-amines **3** and **6**. Table I shows that lithium aluminium hydride yielded mainly equatorial 3β-amine **6**, borohydrides completely failed to reduce the hydroxyimino group, yielding a mixture of hydroxyimino alcohols or hydrolytic products. Eventually, the hydroxyimino derivative was smoothly reduced with sodium borohydride in the presence of some inor-

ganic salts, such as molybdenum oxide[23] or copper chloride. Nevertheless, under these conditions the equatorial isomer **6** prevailed again.

A truly stereospecific route was based on solvolysis of the 3β-methanesulfonyloxy derivative **7**, which was carried out in the presence of sodium azide. ^{1}H NMR spectrum of product **8** (i.e. multiplicity of the H-3 signal, see Experimental) proved the inversion of configuration in the reaction. Azide **8** was then reduced with lithium aluminium hydride and mixture of the amino alcohols (20α and 20β) formed was oxidised with chromium trioxide in aqueous acetic acid into amino ketone **3**. The overall yield of the four-step reaction starting with the 3β-alcohol **9** was around 30%. Compound **9** was taken from the laboratory stocks.



SCHEME 1

A straightforward reduction of azide **8** with triphenylphosphine (i.e. the Staudinger reaction[24]) yielded directly amino ketone **3**; the yield was, however, low (11%).

A preliminary *in vitro* test of biological activity of product **3** was first done using $GABA_A$ receptors and [^{3}H]muscimol. While allopregnanolone increased the binding of muscimol to 123.7%, the amino derivative **3** decreased the binding to 88.0%. A different picture, however, was obtained upon using [^{3}H]flunitrazepam in cultured cortical neurons: the title compound **3** increased [^{3}H]flunitrazepam binding in a concentration-dependent manner (Fig. 1; calculated EC_{50} and E_{max} values were 5.1 μM and 119.1%). For comparison, the endogenous neurosteroid allopregnanolone **1** acted with a higher potency (calculated EC_{50} value 0.6 μM) and efficacy (calculated E_{max} value 134.1%). Control specific binding in the absence of steroids was 2518 ± 66 dpm/well (n = 7). γ-Aminobutyric acid (100 μM) increased [^{3}H]flunitrazepam binding up to 150 ± 6% (n = 3).

The amino derivative **3** did not produce cytotoxic effects in cultured cortical neurons after a 30-min exposure (the same conditions as those used in the binding assay), as assessed by optical microscopy (Fig. 2) and by determining the reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-

TABLE I
Reduction of oxime **4** with different agents

Reagent	Temperature, °C	Time, h	Yield, %	
			3 (α)	**7** (β)
L-Selectride	–68; then 25	1; then 1	0	0
$NaBH_4$	0; then 25	1; then 48	0	0
$LiAlH_4$	20	0	13	87
$LiAlH(t\text{-}BuO)_3$	25; then 100	3; then 24	0	0
$NaBH_4 + CeCl_3$	0	1.2	0	0
$NaBH_4 + MoO_3$	0–30	0.1	4	51
$NaBH_4$ + CuCl	25	24	7	12
$NaBH_4 + AgNO_3$	25	24	0	11
$NaBH_4 + CrCl_3$	25	24	0	0
$NaBH_4 + ZnCl_2$	25	24	0	0
H_2, PtO_2	20	20	45	55

2,5-diphenyltetrazolium bromide (MTT) (Fig. 3). In addition, a 24-h exposure to 10 μM concentration of the amino derivative **3** (i.e., the minimum concentration that produced a significant increase of [^{3}H]flunitrazepam binding) did not cause cytotoxicity, even though, a higher concentration (60 μmol/l) resulted in clear cytotoxic effects (Figs 2 and 3). Furthermore, after the [^{3}H]flunitrazepam binding, the protein content did not differ among the wells exposed to different concentrations of the title compound.

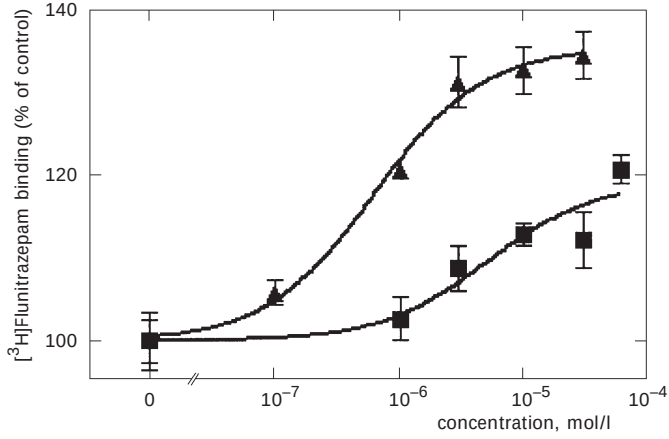


FIG. 1

Effect of pregnane derivatives on [^{3}H]flunitrazepam binding to the $GABA_A$ receptor in primary cultures of cortical neurons. Cultured neurons were incubated at 25 °C for 30 min with [^{3}H]flunitrazepam and allopregnanolone (**1**; reference compound, ▲), or amine **3** (■). Data are means ± sem of 6–7 determinations

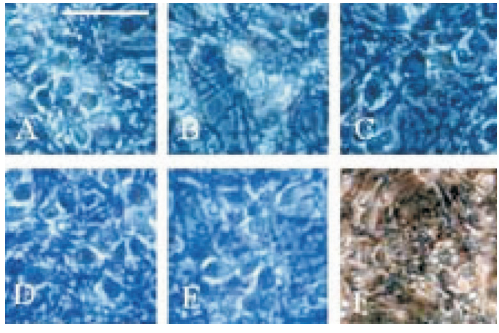


FIG. 2

Photomicrographs of primary cultures of cortical neurons exposed to pregnane derivatives at 25 °C for 30 min (A, B, C) or 24 h at 36 °C (D, E, F). Control (A); 10 μM amine **3** (B and E); 60 μM amine **3** (C and F); 10 μM allopregnanolone (D). The bar represents 5 μm

Although 3α-amino-5α-pregnan-20-one (**3**) is not as potent in modulating the [^{3}H]flunitrazepam binding on the $GABA_A$ receptor as allopregnanolone itself, the above results clearly present new insight into possible functional group variations[25,26] in position 3α of the steroid skeleton. The discrepancy between the [^{3}H]muscimol and [^{3}H]flunitrazepam tests could be understood in the light of the complex structure of the receptor[27,28] and the idea of the existence of multiple specific steroid binding sites within the $GABA_A$ (see[29–32]). Additional tests have to be performed before any conclusion on the applicability of amine **3** can be made. Nevertheless, the axiom of the 3α-hydroxy group as the essential part of neuroactive steroids has now been put into question.

EXPERIMENTAL

Chemistry

Melting points were determined on a Boetius melting point microapparatus (Germany) and are uncorrected. Analytical samples were dried over phosphorus pentoxide at 50 °C/100 Pa. Optical rotations were measured in chloroform ($[\alpha]_D$ values are given in 10^{-1} deg cm^2/g). IR spectra of chloroform solutions were recorded on a Bruker IFS 88 spectrometer, wavenumbers are given in cm^{-1}. NMR spectra were measured on an FT NMR spectrometer Varian Unity-200 (at 200 MHz) in $CDCl_3$ with tetramethylsilane as internal reference. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) and widths of multiplets (*W*) in Hz. Unless otherwise stated, the data were interpreted as first-order spectra. Thin-

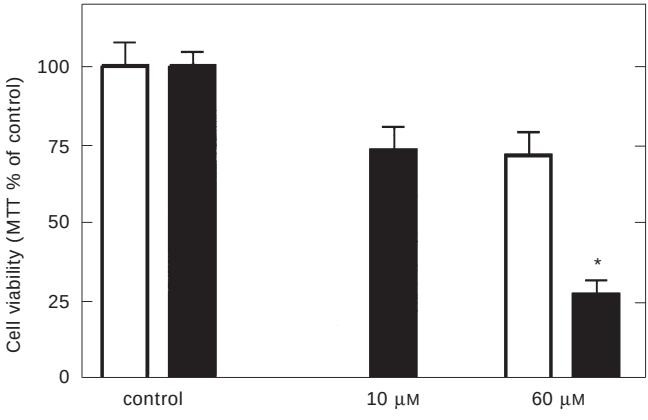


FIG. 3
Cell viability as determined by the MTT assay. Cultured neurons were incubated at 25 °C for 24 h (■) with 10 μM, or at 36 °C for 30 min (□) and 24 h (■) with 60 μM amine **3**. Results are means ± sem of 3–4 determinations. * $p < 0.001$

layer chromatography (TLC) was performed on silica gel (ICN Biochemicals). Preparative TLC (PLC) was carried out on 200 × 200 mm plates coated with a 0.7 mm thick layer of the same material. For column chromatography, 60–120 μm silica gel was used. Whenever aqueous solutions of hydrochloric acid, potassium hydrogencarbonate and potassium carbonate were used, their concentration was always 5%. Before evaporation on a rotary evaporator in vacuum (bath temperature 50 °C), solutions in organic solvents were dried over anhydrous sodium sulfate.

Biology

$GABA_A$ receptor binding. Membranes[12] were preincubated at 37 °C for 10 min in a Tris-HCl buffer (50 mmo/l, pH 7.4) with or without a sample tested. The mixture was incubated with [^{3}H]muscimol (5 nmol/l) and, after 10 min, the reaction was terminated by rapid vacuum filtration through a Whatman GF/B glass-fiber filter. Nonspecific binding was determined in the presence of 1 mM GABA. Preliminary experiments with 5 nM [^{3}H]muscimol were carried out with 100 nM concentration of the steroids tested.

Cell culture preparation. Pregnant (gestational day 17) NMRI rats were obtained from Iffa Credo (St.-Germain-sur-l'Arbreste, France). The animals were handled in compliance with the protocol (No. 95/99 of the University of Barcelona), approved by the Generalitat de Catalunya, Spain, following EU guidelines. Plastic 24- and 48-well plates were purchased from CoStar (Corning Science Products, Acton (MA), U.S.A.) and NUNC Ltd. (Roskilde, Denmark). Foetal Calf Serum was obtained from Gibco (Glasgow, U.K.) and Dulbecco's Minimum Essential Medium (DMEM) from Biochrom (Berlin, Germany). Bicuculline and GABA were purchased from SIGMA Chemical Co. (St. Louis (MO), U.S.A.).

Radioactivity was measured in liquid scintillation cocktail Optiphase "Hisafe" 2, from Wallac Oy (Turku, Finland) on a liquid scintillation counter 1414 Winspectral from Wallac.

Primary cultures of cortical neurons were obtained from the cerebral cortices of 17-day-old rat embryos, following the method described by Hertz et al.[33] In brief, cells were dissociated by mild trypsinisation at 37 °C, followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were then suspended in DMEM (25 mM KCl, 31 mM glucose and 0.2 mM glutamine) supplemented with 4-aminobenzoic acid, insulin, penicillin, and 10% of foetal calf serum. The cell suspension was seeded in 24- or 48-well plates pre-coated with poly-L-lysine and incubated for 7–12 days in a humidified 5% CO_2/95% air atmosphere at 36.8 °C. A mixture of 5 μM 5-fluoro-2′-deoxyuridine and 20 μM uridine was added after 40–48 h in the culture to prevent glial proliferation.

Data analysis. Data are means ± sem. Concentration-response data were fit by non-regression analysis to sigmoid curves by using the GraphPad Prism Program (GraphPad Software Inc., San Diego (CA), U.S.A.). Statistical analysis was performed using the GraphPad Prism Program. One way analysis of variance (ANOVA) followed by Dunnett's test was used. $P < 0.05$ was considered to be significant.

3-Hydroxyimino-5α-pregnan-20-one (**4**)

A solution of dione **5** (289.5 mg, 0.91 mmol) in pyridine (3.0 ml) was treated with hydroxylamine hydrochloride (76 mg, 3.08 mmol) at 0 °C. Hydroxylamine hydrochloride was added stepwise over a period of 1 h. The reaction was followed by TLC (ammoniacal chloroform) and quenched after 3 h with water. The organics were taken up into chloro-

form, the solution was washed and dried. The reaction yielded 280 mg (92%) of **4**. M.p. 235–239 °C (methanol–ether) (lit.[34] 240–241 °C). $[\alpha]_D$ +94.9 (*c* 0.2) (lit.[34] +107 (*c* 0.5)). IR: 1698 (CO), 1654 (CN), 3591 (OH). ^{1}H NMR: 0.60 s, 3 H (H-18); 0.90 s, 3 H (H-19); 2.15 s, 3 H (H-21); 2.53 t, H (H-17). For $C_{21}H_{33}NO_2$ (331.5) calculated: 76.09% C, 10.03% H, 4.23% N; found: 76.54% C, 10.32% H, 4.11% N.

Platinum Catalysed Hydrogenation of Oxime **4**

A solution of oxime **4** (330 mg, 0.99 mmol) in acetic acid (10 ml) was hydrogenated over platinum oxide (100 mg) at room temperature. After 20 h, the catalyst was removed by filtration and washed with acetic acid. The filtrate was concentrated to dryness in vacuum. The remainder was dissolved in acetic acid (90%, 25 ml) and treated at with a solution of chromium trioxide (270 mg, 2.7 mmol) in aqueous sulfuric acid (20%, 0.4 ml) at room temperature. The course of the reaction was monitored by TLC (ammoniacal chloroform). The excess of the oxidant was decomposed with potassium hydrogensulfite (2.0 g, 16.6 mmol). Most of the solvent was removed from the reaction mixture using a rotary evaporator, then ammonia was added under cooling and the precipitate extracted into ethyl acetate. The extract was washed with water, dried and concentrated in vacuum. The solution was applied on 11 PLC plates which were developed with ammoniacal chloroform containing 3% of methanol. Each fraction was evaporated upon the addition of acetic acid (1 ml) and then co-evaporated with an additional dose of toluene (15 ml).

The less polar fraction contained 3α-amino-5α-pregnan-20-one (**3**; 166 mg, 45%). M.p. 119–122 °C (ether–petroleum ether) (lit.[19] 123 °C). $[\alpha]_D$ +94.4 (*c* 0.8) (lit.[35] +95 (*c* 1.7)). ^{1}H NMR: 0.60 s, 3 H (H-18); 0.78 s, 3 H (H-19); 2.11 s, 3 H (H-21); 3.20 s, H (H-3).

The more polar fraction contained 20-oxo-5α-pregnan-3β-yl ammonium acetate (**10**; 158 mg, 42%). M.p. 157–159 °C (ether). $[\alpha]_D$ +11.9 (*c* 0.8). ^{1}H NMR: 0.59 s, 3 H (H-18); 0.81 s, 3 H (H-19); 1.95 s, 3 H (H-21); 2.11 s, 3 H (CH_3COO); 2.52 t, 1 H, J = 8.6 (H-17); 2.88 m, 1 H, W = 43.0 (H-3). For $C_{23}H_{39}NO_3$ (377.6) calculated: 73.17% C, 10.41% H, 3.71% N; found: 73.20% C, 10.70% H, 3.77% N.

Lithium Aluminium Hydride Reduction of Oxime **4**

A solution of oxime **4** (50 mg, 0.15 mmol) in tetrahydrofuran (5.0 ml) was treated with lithium aluminium hydride (approximately 100 mg) at room temperature for 2 h. The excess of the reagent was destroyed with ethyl acetate (10 ml) and the reaction mixture was filtered through a layer of sodium sulfate. The product was oxidised as above (3.0 ml of acetic acid, 60 mg of chromium trioxide in 10 drops of water). Usual work-up yielded a mixture of **3** and **6**, the ratio of **3**:**6** being 1:3, as determined by ^{1}H NMR.

Sodium Borohydride Reduction of Oxime **4** in the Presence of MoO_3

To a solution of oxime **4** (12.2 mg, 0.04 mmol) in methanol (1.0 ml), molybdenum(VI) oxide (50 mg, 0.35 mmol) was added and the solution was carefully heated to dissolve oxime **4**. Sodium borohydride (200 mg, 5.28 mmol) was slowly introduced into the reaction mixture at room temperature. After 10 min, the excess of sodium borohydride was decomposed with water, and methanol was distilled off on a vacuum evaporator. The organics were taken up into ammoniacal chloroform and the solution was dried. The product was oxidised as

above to yield 9.1 mg (78%) of a mixture of **3** and **6**, the ratio of **3**:**6** being 1:12.8, as determined by ^{1}H NMR.

3β-*O*-(Methanesulfonyl)-5α-pregnan-20-one (**7**)

To a solution of hydroxy derivative **9** (204.4 mg, 0.63 mmol) in pyridine (5.0 ml), cooled down to 0 °C, methanesulfonyl chloride (0.15 ml, 1.93 mmol) was slowly added. The reaction was followed by TLC (25% ether in benzene) and quenched with ice after one day. The organics were taken up into chloroform and washed with 5% hydrochloric acid, saturated sodium hydrogencarbonate, water and dried. Derivative **7** was crystallised from chloroform–ether and then from chloroform–petroleum ether to give 63.9 mg (25%) of **7**. The overall yield after chromatography was 193.7 mg (76%). M.p. 139–141 °C (chloroform–petroleum ether). $[\alpha]_D$ +116.5 (*c* 0.4). ^{1}H NMR: 0.6 s, 3 H (H-18); 0.83 s, 3 H (H-19); 2.11 s, 3 H (H-21); 3.00 s, 3 H (CH_3S); 4.63 m, 1 H, *W* = 40.89 (H-3). For $C_{22}H_{36}O_4S$ (396.6) calculated: 66.63% C, 9.15% H, 8.08% S; found: 66.45% C, 9.46% H, 7.81% S.

3α-Azido-5α-pregnan-20-one (**8**)

A solution of compound **7** (402.1 mg, 1.01 mmol) in dimethylformamide (4.0 ml) was treated with sodium azide (200 mg, 3.08 mmol) at room temperature, and the mixture was then heated to 80 °C for 5 h. The organics were taken up into ethyl acetate and the solution was dried. Crystallisation from ethyl acetate yielded 164.6 mg (47%) of **8**. M.p. 162–164 °C (ethyl acetate). $[\alpha]_D$ +67.3 (c 0.8). IR: 1710 (CO), 2110 (N_3). ^{1}H NMR: 0.60 s, 3 H (H-18); 0.79 s, 3 H (H-19); 2.12 s, 3 H (H-21); 2.53 t, H (H-17); 3.89 m, 1 H, *W* = 24.41 (H-3). For $C_{21}H_{33}N_3O$ (343.5) calculated: 73.43% C, 9.68% H, 12.23% N; found: 72.54% C, 9.75% H, 11.59% N.

Triphenylphosphine Reduction of Azide **8**

A solution of azide **8** (75 mg, 0.22 mmol) in tetrahydrofuran (2.0 ml) was treated with triphenylphosphine (234 mg, 0.88 mmol) at room temperature. The reaction was quenched with water after two days. Tetrahydrofuran was evaporated on a vacuum evaporator. The organics were taken up into ammoniacal chloroform. The reaction mixture was separated using preparative TLC (20% methanol in chloroform) to afford 21.6 mg (30%) of compound **3**.

[^{3}H]Flunitrazepam Binding Assay

A sample of amino derivative **3** was dissolved in DMSO. A concentration scale of 0.1, 1, 3, 10, 30, and 60 μmol/l of derivative **3** in a HEPES (0.5% DMSO) was prepared. As an internal standard, GABA (100 μmol/l) in HEPES was used. Diazepam (20 μmol/l) was used to determine the nonspecific binding. A 6-day-old culture of cortical neurons cultivated on a 48-well plate (for preparation, see above) was washed 3 times with approximately 0.5 ml of HEPES buffer prewarmed to 37 °C. After washing, the cells were maintained in 0.5 ml of HEPES buffer until the assay solution was added. The HEPES buffer was replaced with 150 μl of the assay solutions (steroid, 100 μM GABA or 20 μM diazepam). 0.5% DMSO was present in HEPES buffer control cells. [^{3}H]Flunitrazepam (50 μl) was added into each well. The final concentration of [^{3}H]flunitrazepam in a well, after fine-tuning the method, was 5 nmol/l. The plate was then incubated at 25 °C for 30 min and the wells were then washed 4 times

with 0.5 ml of HEPES buffer. NaOH (0.2 mol/l, 150 µl) was added into each well and the plates were stirred for 4 h. The whole content of each well was transferred into a vial and scintillation liquid (4 ml) was added. The vials were agitated, and radioactivity measured.

Cell Viability Assay

Cultured cells were exposed to pregnane derivatives in HEPES buffer at 25 °C for 24 h (the same conditions as in the [^{3}H]flunitrazepam binding assay) or at 36 °C for 30 min and 24 h in the culture medium. DMSO (0.5%) was present in the incubation medium of control cells. At the end of the exposure, the cultures were rinsed with HEPES buffer and incubated at 37 °C for 15 min with HEPES buffered saline containing 250 µg/ml of oxidised 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is incorporated into viable cells and reduced by mitochondria to a coloured tetrazolium compound. Following a careful removal of excess MTT, the cells were disintegrated with 5% sodium dodecylsulfate. The coloured solution was measured at 560 nm. The results are expressed as the percentage of the control values after blank subtraction. The MTT assay measures mitochondrial activity and is currently used as the cell viability assay[36].

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