Allopregnanolone and pregnanolone analogues modified in the C ring: synthesis and activity

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Abstract

(25*R*)-3β-Hydroxy-5α-spirostan-12-one (hecogenin) and 11α-hydroxypregn-4-ene-3,20-dione (11α-hydroxyprogesterone) were used as starting materials for the synthesis of a series of 11- and 12-substituted derivatives of 5ξ-pregnanolone (3α-hydroxy-5α-pregnan-20-one and 3α-hydroxy-5β-pregnan-20-one), the principal neurosteroid acting via γ-aminobutyric acid (GABA). These analogues were designed to study the structural requirements of the corresponding $GABA_A$ receptor. Their biological activity was measured by *in vitro* test with [^{3}H]-flunitrazepam as radioligand in which allopregnanolone and its active analogues stimulated the binding to the $GABA_A$ receptor. Analysis of the SAR data suggests dependence of the flunitrazepam binding activity on the lipophobic-lipophilic balance of the groups at the C-ring edge, rather than on specific interactions between them and the receptor.

Introduction

Although steroids in general are widely understood as a class of natural products, which hold only little potential of a new breakthrough, neurosteroids are still studied with great interest (see the "Neurosteroid" copy[1,2] of *Pharmacol. Ther*. **2007** or recent papers[3-6]). Unlike the family of other steroid hormones, neurosteroids do not bind to nuclear but to membrane receptors for neurotransmitters. One of the neurotransmitters - γ-aminobutyric acid (GABA) elicits the influx of chloride ions into neuronal cells, which changes the membrane potential of a given cell, which in turn prevents the transmission of subsequent neuronal signals.

In agreement with this action, neurosteroids such as allopregnanolone or pregnanolone (**1a** and **1b**, respectively, see Figure 1), 3α,5α-tetrahydrodeoxycorticosterone (**2**), and some of their analogues, acting via the $GABA_A$ receptor, have anxiolytic, anaesthetic, anticonvulsant, and sedative properties.[7,8] Many new types of such analogues were prepared in order to refine the knowledge of relationship between the structure of compounds and their neuronal activity. These analogues either involve compounds with additional substituents (eg., alphaxalone, **3**), or have their skeleton modified by increasing[9] or decreasing[10-12] the flexibility of the molecule or even reversing its chirality.[13] Various changes were also made to the steroid side chain.[14-17] Even heteroatoms (O, N, S) were introduced into the steroid framework with varied success.[18-21]

Almost all the active analogues contained a hydroxyl group in position 3α. Its presence has been considered essential for the activity, even though a 3α-amino analogue, lacking the 3α-hydroxyl (i. e., **4**) retained 56% of the activity of allopregnanolone.[22]

In our search for new analogues of allopreganolone, we prepared a number of 5α-pregnane derivatives designed to exert a higher solubility in water than the principal neurosteroid **1a**. The biological activity of the new products was assessed using *in vitro* tests, which measured

the binding of labelled ligands to $GABA_A$ receptors in the absence and presence of the tested compounds. In our previous paper we reported structure modification of the steroidal B ring.[11] Some anomalies in the structure of the analogues were tolerated by the $GABA_A$ receptor, others not; the biological consequences of the structure modification were rationalized using methods of molecular modelling. A similar study of the effect of C ring substituents upon the activity of neurosteroid analogues is the subject of this paper, which also takes into account earlier results published by other authors.[23-26]

Results and Discussion

Synthesis of 12-substituted analogues. Hecogenin (**5**, see Scheme 1) was used as a readily available synthon with position 20 masked for subsequent transformations. The inversion of configuration of the 3β-hydroxy group in hecogenin (**5**) was first carried out at the beginning of the process using solvolysis of corresponding 3β mesylate **6** and tosylate **7** with potassium nitrite in DMF, which afforded the target 3α alcohol **8** in overall yields 26% and 40%, respectively. The 3α-hydroxy group formed was then protected by esterification, and 12 oxo group in benzoate **9** was reduced with sodium borohydride to yield 12β-alcohol **10**. The expected 12β-configuration of this compound as well as of other products was confirmed by ^{1}H NMR spectroscopy (see the Experimental Section).

The degradation of the spirostane system, using acetic anhydride at 190 ^{o}C, oxidation and pyrolysis, was carried out in 12β alcohol **10**.[27] Crude product was immediately hydrogenated on palladium to afford diester **11**. Its hydrolysis yielded another target - 12β-hydroxy-allopregnanolone (**12**). However, the overall yield from hecogenin was very low (6%) probably due to a low stability of the axial substituent in position 3 during the spirostane degradation step. Partial hydrolysis of the diester **11** in position 12 and subsequent

dehydration and hydrolysis produced another analogue sought - allopregn-11-enolone (**13**). Alternatively, the sequence of hecogenin transformation reaction was reversed: the spirostane system was first oxidised into a pregn-16-ene derivative in much better yields (71%). Standard hydrogenation, hydrolysis, and oxidation produced triketone **14**. The desired 3α-hydroxyl group was then introduced under conditions of the Henbest reaction, i. e., by reduction of the 3-oxo group with hot 2-propanol and phosphorous acid catalyzed with hydrogen hexachloroiridate.[28,29] The reaction yielded another allopregnanolone derivative - 3α,12α-dihydroxy-5α-pregnan-20-one[30] (**15**).

The preparation of 12ξ-acetamino analogues started with the preparation of oxime **16**. Contrary to the above hydride reduction of 12-ketone, the sodium borohydride reduction under catalysis of molybdenum trioxide proceeded in a non-stereospecific manner: both amines **17** and **18** were formed in almost equal yields. Both were subjected to the above four-step degradation of the spirostane system: the equatorial amine **18** yielded 12β-acetamino-allopregnanolone **19**; no 12α-acetamino derivative, however, was isolated from the same treatment of amine **17**.

For the synthesis of free 12β-amino derivative of allopregnanolone, Leuckart-Wallach's reductive amination of 3β-acetoxy-5α-pregnane-12,20-dione[31] (**20**) was used (see Scheme 2): the major product **21** was a *tert*.butoxycarbonyl (BOC) protected 12β-amino derivative **21**, which was hydrolyzed and inverted at carbon 3, yielding compounds **22** and **23**, respectively. On deprotection of the latter with trifluoroacetic acid, the 12β-amino analogue **24** was formed.

Synthesis of 11-substituted analogues. The synthesis of 5β analogues (see Scheme 3) was based on commercial 11α-hydroxyprogesterone (**25**), which was hydrogenated on palladium catalyst in pyridine to yield 11α-hydroxy-5β-pregnane-3,20-dione (**26**) besides some 5α-

isomer **27** (5%). Partial reduction of diketone **26** with sodium borohydride in pyridine produced a 11α-hydroxy analogue of pregnanolone[32-34] **28**. For subsequent reactions, the 3 and 20 oxo groups were protected by treatment with ethylene glycol: compound **26** produced (after oxidation) a diketal **29**, similarly, compound **28** gave (after oxidation) a mono ketal[35] **30**. The latter reacted with lithium aluminum hydride and Grignard reagents to yield 3α,11β-diols **31** to **33,** respectively, or with sodium borohydride in pyridine to produce the known 5β alphaxolone analogue[32] **34**. Analogously, diketal **29** was converted into 3,20-dioxo derivatives **35** and **36**, which were reduced with sodium borohydride to other analogues, compounds **37** and **38**.

The above side product of hydrogenation, 11α-hydroxy-5α-pregnane-3,20-dione (**27**, scheme 4) was utilized for the preparation of 11α-hydroxy allopregnanolone (**39**), although the Henbest reaction (i.e., reduction with 2-propanol catalyzed with iridium salts) had to be used instead of a hydride reduction.[36] Other allopregnanolone analogues were prepared from enol acetate **40**: hydrogenation[37] and hydrolysis produced 3β-hydroxy-5α-pregnane-11,20-dione (**41**) accompanied with a lipophilic product of hydrogenolysis.[38] Solvolysis of the corresponding mesylate **42** and deprotection yielded 3α-hydroxy-5α-pregnane-11,20-dione[39] **(3**). Its more reactive 20-oxo group was protected by ketalization, ketal **43** was then treated with LAH or ethynyl magnesium bromide under the formation (after deprotection) of two other analogues, 11β-alcohols **44** and **45**.[40]

Oximation of 11-ketone **43** produced a mixture of oximino derivatives[41] **46**, which was reduced either with sodium in boiling 2-propanol,[42] selectively yielding equatorial 11α- amine **47** or with $NaBH_4$ in the presence of MoO_3, selectively producing the axial 11β-amine **48**.

Another type of allopregnanolone with a polar functionality below the C-ring was obtained from olefin **13** (Scheme 1). Epoxidation of this compound with a peroxy acid yielded 11α,12α-epoxide **49** only.

Contrary to the above analogues with polar functional groups in the C ring, some lipophilic analogues were prepared and tested too (see Scheme 5). Suitable 11-deoxy analogues were prepared from 5ß- and 5α-diketals **29** and **50** on treatment with methyl lithium. Tertiary alcohols **51** and **52** were dehydrated and de-protected to yield corresponding mixtures of 11-exomethyleno derivatives (**53** and **54**, respectively) and 11-methyl-$\Delta^{9(11)}$- derivatives (**55**). These 3-oxo compounds were reduced as above (i.e., the 5α-isomer **53** was reduced according to Henbest, the 5β isomer **54** was reduced with sodium borohydride) to give unsaturated homologues of allopregnanolone and pregnanolone, compounds **56** to **58.** These were hydrogenated to afford 11β-methyl allopregnanolone and pregnanolone derivatives **59** and **60**, respectively.

Biological Activity at the $GABA_A$ receptor and SAR analysis.

Previously,[11,43,44] we have reported that an increase of [^{3}H]-flunitrazepam binding corresponds to activation/potentiation of the $GABA_A$ receptor, as measured by the chloride anion influx. Both allopregnanolone and pregnanolone increase [^{3}H]-flunitrazepam binding in a dose dependent manner with effective concentration (EC_{50}) around 1 μM and a maximum effect (E_{max}) around 160% of the basal binding.[11] In that work, the [^{3}H]-flunitrazepam binding to the $GABA_A$ receptor was carried out with allopregnanolone analogues modified in the B-ring.[11] 3α-Hydroxy-7-nor-5ξ-pregnan-20-one analogues showed activity at the $GABA_A$ receptor, whereas other analogues carrying electronegative substituents in the B-ring were inactive.

In the present work the effect of modifications in the neurosteroids C-ring on [^{3}H]-flunitrazepam binding has been assessed in primary cultures of intact living cortical neurons (see Table 1). The binding was measured in the presence of the varying concentration of these

compounds. A fixed concentration of allopregnanolone (60 μM, the concentration that produces maximal effect) was included in each assay.

In general, introduction of a double bond into the C-ring (*ie*. **13** and **58**) or small non-polar substituents at C11 (*ie*. **56**, **57**, **58**, **59**, and **60**) rendered compounds active, with similar or moderately higher EC_{50} values than those reported for the parent neurosteroids allopregnanolone (**1a**) and pregnanolone (**1b**) (0.88 μM and 1.18 μM, respectively[11]). The exomethyleno analogue of allopregnanolone **56**, which could be considered as an isostere of alphaxolone, was the most active among the 5α-series. It is noteworthy that the related allopregnanolone derivative containing a C-ring $\Delta^{9(11)}$-double bond (**61**) was reported to be similarly active in a flunitrazepam binding assay.[25] On the other hand, the 5β-analogue **57** showed >30-fold decrease in activity relative to **1b**.

Incorporation of an 11β-OH or $–NH_2$ group into the structure of **1a** led to compounds (**44** and **48**, respectively) with similar efficiency, as represented by the $E_{100\mu M}$ value (Table 1), but again slightly (**48**, 7-fold) or moderately (**44**, 36-fold) reduced potency relative to allopregnanolone. Contrarily, 11β-hydroxylation of the pregnanolone produced the 5β-analogue **31** with very low activity.

Inversely, introduction of an 11α-OH or $–NH_2$ substituent or an 11α,12α epoxy group into the structure of the 5α-steroids lead to compounds with much lower (**47**, 175-fold less active than **1a**) or null activity (**39**, **49**): the 11α amine is 175-fold less active than allopregnanolone (**1a**). In the 5ß series, the effect of an 11α-OH upon the activity is 60 times reduced (compare compounds **28** and **1b**).

The presence of an 11-oxo group had a little effect on the flunitrazepam binding activity in the 5α-series (see alphaxolone, compound **3**) but it caused a 80-fold decrease (relative to **1b**) in the activity of its 5β-analogue **34**. Similar results on flunitrazepam binding had been reported in the literature for 11-oxo derivatives **3** and **34**[26], and 11α-hydroxy derivatives **28**

and **39**[25] whereas the anaesthetic activity of 11ξ-alcohols **31**, **39**, **44,** and amine **47** in mice was found much lower than that of 11-ketone (alphaxolone **3**).[23,24]

The different behaviour of these 5α- and 5β-analogues is in agreement with the evidence for different binding sites for 5α- and 5β-neurosteroids at the $GABA_A$ receptor,[2,11] and implies different tolerance for polar C-ring substituents at each site. In this sense, the incorporation of a non-polar 11α-acetylenic or phenylacetylenic group in addition to the 11β-OH group recovered the activity of the 5β-compounds (compounds **37** and **38** *vs* **31**) but left it unchanged for the 5α-analogue (compound **45** *vs* **44**). It is remarkable that the 11α-phenylethynyl-11β-hydroxyl derivative **38,** the most active in the 5β-series, exhibits essentially the same activity as the parent neurosteroid **1b**. Other neuroactive steroids with relatively large 11α-substituents (ie. minaxolone) have been reported.[45] Interestingly, the presence of an additional 3β-acetylene or phenylacetylene substituent in the 5β-compounds led to a decrease of activity (**32** and **33** *vs* **37** and **38**). This is in contrast with the proposed existence of an auxiliary binding pocket in the 5β-neurosteroid binding site, that could allocate small lipophilic as well as phenylacetylenic 3β-substituents present in several analogues that have shown a high inhibitory activity in TBPS binding assays.[46, 47]

The introduction of polar groups in the equatorial 12β-position of the 5α-neurosteroid series decreased the activity even more than those in 12α-position and than those in position 11β. Thus, 12β-hydroxy, amino or acetamido derivatives **12**, **24,** and **19** were less active than their 11β-counterparts analogues **44** and **48**, while the 12α-hydroxy derivative **15** was more active than the corresponding 12β-hydroxy **12** and 11α-hydroxy **39** derivatives.

In summary, the structure-activity dependence of the compounds analysed in the present study indicates that modifications at the C11-C12 C-ring edge of 5ξ-pregnanolones, which do not imply large local changes of polarity, are relatively well tolerated by the $GABA_A$ neurosteroid receptor, while introduction of polar groups at C11 or C12 is in general more

detrimental for the activity. This negative effect can be compensated, in some cases, by the introduction of additional lipophilic groups in the same region. There are some exceptions to this trend evidenced by compounds such as **48**, which although having a polar 11β-amino substitution showed similar potency and efficiency to other more hydrophobic derivatives, like its isosteric 11β-methyl analogue **59**.

Different studies recently published indicated that the steroid binding sites on the $GABA_A$ receptor are located on transmembrane domains of the protein.[48-50] Accordingly it has been suggested that the effective steroid concentration in the lipid membrane, rather than aqueous concentration, as well as intracellular accumulation, contribute to the potency and longevity of their action.[51-54] Chisari et al. observed a good correlation between the potentiating activity of the neuroactive steroids allopregnanolone (**1a**), (3α5α)-3,21-dihydroxypregnan-20-one (**2**), alphaxolone (**3**), alphadolone (the 11-oxoderivative of **2**) and ZCM41 (the 11α-methyl-11β-benzyloxy derivative of **1a**) and their average logP estimates determined from 11 different algorithms.[52] Similarly, we have determined the logP values for the compounds analysed in this work, as well as their predicted water solubility, using different algorithms (Tables S2 and S3, Supporting Information). The variance of flunitrazepam binding showed a slight correlation with predicted water solubility (average $r^2 \sim 0.4$, Supporting Information), although this parameter might not reflect the real solubility of the compounds under the assay conditions. A slightly better correlation was found with the calculated logP values (average $r^2 \sim 0.5$, Supporting Information). These correlations support the contribution of steroid lipophilicity to $GABA_A$ receptor modulation. However, the contribution from other effects, principally arising from the interaction with the receptor, should be invoked to explain the observations that an increase of hydrophobicity did not always result on an increase of activity (compare **45** vs. **44**, **59** vs. **48**, **32** vs. **37** or **33** vs. **38**), or that compounds very similar in hydrophobicity present very disparate activities. That is the case of the isomeric pairs **39**

and **44**, or **24** and **48** which show activities differing by more than one order of magnitude. Similarly, the 12-keto isomer of alphaxolone (**3**) was >20 fold less potent in our flunitrazepam binding assays (unpublished results), although their logP values are essentially identical. Therefore, in addition to the influence of the global hydrophobicity of the compounds, which would affect their partition between the membrane and the intra- and extracellular aqueous compartments, these results suggested a dependence of the flunitrazepam binding activity on the local lipophobic-lipophilic balance of the groups at the C-ring edge.

3D-QSAR analysis

A 3D-QSAR approach using the CoMFA and CoMSIA methodologies[55-57] was devised to gain insight into the observed SAR relations. This analysis could only be applied successfully to the 5α-neurosteroid series, since including the compounds in the 5β-series or trying to obtain a model only for those compounds did not provide significant correlations (see Supporting Information). Thus, the best model (CoMSIA-2) was obtained by taking into consideration the hydrophobic and electrostatic CoMSIA fields of compounds **1**, **3**, **12**, **13**, **15**, **24**, **39**, **44**, **45**, **47** to **49**, **56**, and **59** (see Supporting Information for details). No significant improvement was observed by including contributions from the steric, donor and/or acceptor fields. Table 2 summarizes the statistical parameters for this model and the correlation between experimental and calculated EC_{50} values.

Figure 2a shows the electrostatic and hydrophobic* contour maps from model CoMSIA-2. Two main regions are shown for the electrostatic field: one located close to C12 and slightly below the steroid plane (B), which reflects a favoured location for groups of the ligand with positive electrostatic potential, and one located close to C11 above the steroid plane (R),

*NOTE: Due to the relatively small number of available compounds and their high structural similarity except at the C11-C12 edge, no unfavoured hydrophobic region was clearly defined.

which reflects a preferred location for electronegative groups of the ligand. For the hydrophobic field, a region is shown close to the C11-C12 edge (G) where the occupation by hydrophobic groups of the ligand (i.e. compounds **13**, **56** and **59**) is favoured. On the same figure (Figure 2b-d), the actual electrostatic and hydrophobic fields for ligands **3**, **48**, and **24** are also shown. Thus, in addition to several contours that are common to all the compounds because of their structural similarity (i.e. close to the 3-OH or the 17-acetyl group), the similarly active compounds **3** and **48** show electronegative contours around the 11-carbonyl oxygen (R1) and the 11ß-NH_2 (R2) which overlap the R region from the model and, therefore, contribute to increase the predicted activity of these compounds. The 11β-OH group of compounds **44** and **45** also yields electronegative contours (data not shown) close to the R region, which contribute to their activity. In addition, compound **3** shows a hydrophobic region (G1), which partly overlap the favourable contour G from the model and that would correlate with its higher activity. On the contrary, the inactive compound **24** shows an electronegative contour (R3) close to the 12ß-NH_2 group, which overlaps region B of the model, where electropositive groups are preferred and therefore it is a main reason for its decreased activity. Comparable negative contributions are observed for compounds **12**, **15, 39**, **47**, and **49**. Hence, despite that the results from this 3D-QSAR analysis should be taken as preliminary, given the relatively small number of compounds considered, they seem to support the hypothesis that at least for the compounds of the 5α-series most of the changes on their activity can be explained in terms of the different hydrophobic and electrostatic properties at the C11-C12 ring edge of the steroid system, rather than the existence or absence of specific interactions with the receptor (other than the basic pharmacophore, ie. a 3α-hydroxyl and a 20-ketone groups). Based on this, it could be postulated that the region on the $GABA_A$ receptor, which is complementary to the C-ring edge of the steroids has a predominantly hydrophobic character, with residues that tolerate polar substituents only on

certain locations, namely regions R and B on Figure 2a. A more non-specific contribution to the activity of the compounds could also be expected from variations on their lipophilic character, as it has been previously discussed.

On the basis of studies that combine results from electrophysiology, molecular biology and homology modelling, strong evidence of the existence of a neurosteroid potentiation site located on the M1-M4 transmembrane domain of the α-subunit of the $GABA_A$ receptor was obtained.[48,49] On this site, residue Q241 would act as hydrogen-bond acceptor capable of interacting with the 3α-hydroxyl group of neurosteroids, while residues N407 and Y410 are appropriately located to act as hydrogen-bond donors that interact with the C20-keto group. These residues are highly conserved in subunit isoforms α1-α5, therefore revealing the conserved nature of this potentiation site.[50] Similarly, a second binding site that spans the α,β-subunit interface, implying residues αT236 and βY284, and which is exclusively involved in direct receptor activation, was also identified. The existence of additional sites that mediate the action of neurosteroids is still a matter of debate.[49,58,59] Furthermore, the information about which other residues may interact at each site with the steroid scaffold or any groups attached to it, thus modulating also their binding affinity, is much less clear.[48]

Considering that $GABA_A$ receptors present an heteropentameric structure formed by 2 α and 2 β subunits plus an additional subunit, often a γ subunit, it comes out that each receptor must have at least two potentiation sites (at the two α subunits) and two activating sites (at two α/β interfaces).[48,49,60] Such a large number of potential binding sites hampers the elaboration of robust (Q)SAR correlations, since the experimental activity data results from the sum of the effects at all sites, added to other non-specific contributions. Therefore, the model shown in Figure 2a should be interpreted with care since it might not reflect the real nature of a single binding site but rather a combination of the properties required on the ligand to effectively develop its biological activity. However, in the absence of further structural

details about the receptor, building such correlations can still provide information that could be used for the design of new neuroactive steroids with therapeutic potential.

Conclusions

A series of 11- and 12-substituted derivatives of 5ξ-pregnanolone were synthesized and their binding to the $GABA_A$ receptor was measured by *in vitro* test with [^{3}H]-flunitrazepam as radioligand. The highest activity among the 5α-series (EC_{50} ~1 μM) was found in the exomethyleno analogue of allopregnanolone **56**, while the 11α-phenylethynyl-11β-hydroxyl derivative **38**, with similar EC_{50}, was the most active among the 5β-compounds. Several compounds showed activities in the low micromolar range, with EC_{50} values comparable to alphaxolone. Some of them contained modifications that did not alter the local hydrophobicity at the C11-C12 edge but others, like 11β-amino derivative **48**, did. The SAR/QSAR calculations suggest that the changes of activity correlate with the hydrophobic-hydrophilic balance at the C-ring edge.

Experimental

Chemistry: Melting points were determined on a Boetius micro melting point apparatus (Germany). Optical rotations were measured at 25 ^{o}C in chloroform using an Autopol IV (Rudolf Research Analytical, Flanders, USA, $[\alpha]_D$ values are given in 10^{-1}.deg.cm^2.g^{-1}). Infrared spectra (wave numbers in cm^{-1}) were recorded on a Bruker IFS 88 spectrometer. ^{1}H NMR spectra were taken on Bruker AVANCE-400 instruments (400 MHz, FT mode) at 23 ^{o}C in $CDCl_3$ and referenced to TMS as the internal standard. Chemical shifts are given in ppm (δ-scale); coupling constants (*J*) and width of multiplets (*W*) are given in Hz. Thin-layer chromatography (TLC), used for monitoring of reactions, was performed on silica gel G (ICN

Biochemicals, detection by spraying with concentrated sulfuric acid monitored by heating). Preparative TLC (PLC) was carried out on 200 x 200 mm plates coated with a 0.7 mm thick layer of the same material. For column chromatography, Silica gel 60 (Merck, 63–100 μ) was used. Prior to evaporation on a rotary evaporator in vacuo (0.25 kPa, bath temperature 40 ^{o}C), solutions in organic solvents were dried over anhydrous sodium sulfate. Whenever aqueous solutions of hydrochloric acid, potassium hydrogencarbonate, and potassium carbonate were used, their concentration was always 5%. Unless otherwise stated, a usual workup means that extracts were sequentially washed with the potassium hydrogencarbonate solution and brine, dried by filtration through a column of sodium sulfate and evaporated on a rotary evaporator in vacuum (bath temperature 50 ^{o}C).

Purity statement

Purity of all tested compounds possess a purity of at least 95% as documented by TLC and combustion analysis. Unless otherwise stated, the structure of the products was confirmed by IR and ^{1}H NMR spectra.

(25*R*)-12-Oxo-5α-spirostan-3β-yl Methanesulfonate (6). To a cold (0 ^{o}C) solution of compound **5** (351 mg, 0.81 mmol) in pyridine (10 mL), methanesulfonyl chloride (0.17 mL, 251 mg, 2.20 mmol) was added dropwise. The reaction was left at 0 ^{o}C for 2 h, then it was quenched with ice, the organics were taken into chloroform, the extract was worked up as usual. Mesylate **6** (334 mg, 80%) formed white crystals, mp 176-180 ^{o}C (chloroform/ligroin). $[\alpha]_D$ -6 ($CHCl_3$, c 0.27). IR: 1711 (CO); 1368, 1345, 1177 (SO_2). ^{1}H NMR: δ 0.79 d (3H, *J*=6.7, H-27); 0.93 s (3H, H-18); 1.05 s (3H, H-19); 1.07 d (3H, *J*=6.7, H-21); 3.00 s (3H, H-OMs); 3.34 t (1H, *J*=11, H-26ax); 3.35 m (1H, *W*=17, H-26eq); 4.33 q (1H, *J*=5.5, H-16); 4.60 m (1H, *W*=32.4, H-3). Anal. ($C_{28}H_{44}O_6S$) C, H, S.

(25*R*)-12-Oxo-5α-spirostan-3β-yl Toluenesulfonate (7). A solution of hecogenin (**5**) (436.7 mg, 1.01 mmol) in pyridine (10 mL) was cooled to 0 ^{o}C, and then a solution of toluenesulfonyl chloride (577 mg, 3.03 mmol) in pyridine (5.0 mL) was dripped in. After 24 h at rt, the reaction was quenched with ice and organics were taken into chloroform. The extract was washed with the aqueous solution of hydrochloric acid, potassium hydrogencarbonate, and water and dried over sodium sulfate. Evaporation of the solvent yielded tosylate **7** (605 mg, 96%) as white solid, mp 191-193 ^{o}C (chloroform/ligroin). $[\alpha]_D$ -4 ($CHCl_3$, c 0.22). IR: 1711 (CO); 1376, 1178 (SO_2). ^{1}H NMR: δ 0.79 d (3H, *J*=6.6, H-27); 1.03 s (3H, H-18); 0.88 s (3H, H-19); 1.05 d (3H, *J*=7.6, H-21); 3.34 t (1H, *J*=11.0, H-26ax); 3.48 m (1H, *W*=17.1, H-26eq); 4.40 m (H, *W*=29, H-3); 4.36 q (1H, *J*=6.7, H-16). Anal. ($C_{34}H_{48}O_6S$) C, H, S.

(25*R*)-3α-Hydroxy-5α-spirostan-12-one (8). Potassium nitrite (112.0 g, 1.31 mol) was added while stirring to a warm (130 ^{o}C) solution of tosylate **7** (25.0 g, 42.80 mmol) in DMF (250 mL). After for 2 h, the reaction was quenched with 500 mL of water and left in a refrigerator over night. The precipitate was filtered and washed with water. The reaction mixture was separated by column chromatography on silica gel. A mixture of toluene/ethyl acetate (7%) eluted alcohol **8** (8.3 g, 41.5%) as white crystals, mp 209-211 ^{o}C. IR: 1702 (CO), 3616 (OH). $[\alpha]_D$ +15 ($CHCl_3$, c 0.43). ^{1}H NMR: δ 0.79 d (3H, *J*=6.6, H-27); 0.87 s (3H, H-18); 1.05 s (3H, H-19); 1.07 d (3H, *J*=7.6, H-21); 3.35 t (1H, *J*=10.9, H-26ax); 3.46 m (1H, *W*=18.1, H-26eq); 4.06 s (1H, H-3); 4.36 q (1H, *J*=6.7, H-16). Anal. ($C_{27}H_{42}O_4$) C, H.

(25*R*)-12-Oxo-5α-spirostan-3α-yl Benzoate (9). Alcohol **8** (8.3 g, 19.27 mmol) was dried by azeotropic distillation with benzene, dissolved in pyridine (70 mL), and cooled to 0 ^{o}C. Benzoyl chloride (13.0 mL) was added to a stirred solution during 15 min. After 5 h, the reaction was quenched by adding warm water (200 mL, ca 60 ^{o}C). Benzoate **9** was filtered off and washed with the potassium hydrogencarbonate solution. The crude product was dissolved in ethyl acetate and dried. Evaporation of the solvent yielded benzoate **9** as white solid

(9.28 g, 90%), mp 212-214 ^{o}C (ether/methanol). IR: 1717, 1274 (OBz). ^{1}H NMR: δ 0.79 d (3H, *J*=6.1, H-27); 0.94 s (3H, H-19); 1.07 s (3H, H-18); 1.08 d (3H, *J*=7.3, H-21); 3.35 t (1H, *J*=10.4, H-26ax); 3.51 m (1H, *W*=20.0, H-26eq); 4.35 q (1H, *J*=4.9, H-16); 5.30 s (1H, H-3); 7.55, 7.66, 8.15 (Bz). Anal. ($C_{34}H_{46}O_5$) C, H.

(25*R*)-12β-Hydroxy-5α-spirostan-3α-yl Benzoate (10). Sodium borohydride (2.0 g, 53.1 mmol) was gradually added to a solution of (25*R*)-12-oxo-5α-spirostan-3α-yl benzoate **9** (6.9 g, 12.90 mmol) in ethyl acetate (150 mL) and methanol (100 mL). The reaction was monitored quenched after 2 h with 5% HCl. The solvents were evaporated on a vacuum evaporator and the organics were taken into ethyl acetate, washed and dried over sodium sulfate. Evaporation of the solvent yielded white crystals of compound **10** (5.64 g, 81%), mp 178-182 ^{o}C. IR: 3626, 3535 (OH), 3092, 3072, 3064 (BzO), 1717, 1274 (BzO). $[\alpha]_D$ -35.9 ($CHCl_3$, c 0.26). ^{1}H NMR: δ 0.78 s (3H, H-19); 0.80 d (3H, *J*=6.7, H-27); 0.87 d (3H, *J*=2.4, H-21); 0.87 s (3H, H-18); 3.37 t (1H, *J*=11.0, H-26ax); 3.47m (1H, *W*=18.3, H-26eq); 3.75 dd (1H, *J*=3.8, *J*=11.0, H-12); 4.41m (1H, *W*=21.4, H-16); 5.29 s (1H, H-3); 7.46, 7.56, 8.05, 8.07 H (Bz). Anal. ($C_{34}H_{48}O_5$) C, H.

20-Oxo-5α-pregnane-3α,12β-diyl 12-Acetate 3-Benzoate (11). The spirostane derivative **10** (600 mg, 1.0 mmol) was transferred into a thick glass test tube. Acetic anhydride (2.0 mL) and acetic acid (2 drops) were added and the test tube was sealed. The reaction was kept at 180 ^{o}C for 24 h. Then it was cooled to –8 ^{o}C, and water (0.2 mL), acetic acid (10 mL), and dichlorethane (10 mL) were added. A solution of chromium trioxide (200 mg, 2.0 mmol) in acetic acid (10 mL) and water (1 mL) was slowly added during 15 min while stirring. After 1 h, the reaction was quenched with an excess of $Na_2S_2O_3$ dissolved in water (4 mL). The phases were separated, and the aqueous layer was extracted with ether. The extracts were washed, dried over a column of sodium sulfate, and concentrated in a vacuum. Finally, the remainder was refluxed in acetic acid (10 mL). After 2 h, acetic acid was evaporated on a

vacuum evaporator. The remainder was neutralized carefully at 0 ^{o}C with aqueous NH_3. The crude intermediate was hydrogenated in toluene (3.0 mL) in the presence of Pd/C (10%, 50 mg). After 4 h, the catalyst was filtered off and the subsequent preparative chromatography of the crude reaction mixture yielded compound **11** (4.8 mg, 9%). IR: 1736, 1247 (Ac); 1717, 1274 (Bz). ^{1}H NMR: δ 0.85 s (3H, H-19); 0.85 s (3H, H-18); 2.01 s (3H, H-21); 2.08 s (3H, Ac); 4.78 dd (1H, *J*=4.9 and *J*=11.0, H-12); 5.28 s, (1H, H-3); 7.44, 7.47, 7.51, 7.55, 7.58, 8.04, 8.07 (Bz). Anal. ($C_{30}H_{40}O_5$) C, H.

3α,12β-Dihydroxy-5α-pregnan-20-one (12). Diester **11** (100 mg, 0.21 mmol) was heated in a methanolic solution of potassium hydroxide (5%, 10 mL, 8.91 mmol) in nitrogen atmosphere. After 6 h, the solution was acidified with hydrochloric acid (6.8 mL, 10.21 mmol) and concentrated in a vacuum to a quarter of its volume. The concentrate was diluted with the potassium hydrogencarbonate solution (5 mL) and the product was extracted with chloroform. The extract was worked up yielding a white solid of compound **12** (60 mg, 82%), mp 194-197 ^{o}C (acetone/heptane). $[\alpha]_D$ +8 ($CHCl_3$, c 0.26). ^{1}H NMR: δ 0.71 s (3H, H-18); 0.79 s (3H, H-19); 2.21 s (3H, H-21); 2.43 t (1H, *J*=8.9, H-17); 3.42 dd (1H, *J*=10.6 and 5.2, H-12); 4.05 m (1H, *W*=18, H-3). Anal. ($C_{21}H_{34}O_3$) C, H.

3α-Hydroxy-5α-pregn-11-en-20-one (13). Acetate **11** (20 mg, 0.04 mmol) was hydrolyzed with potassium carbonate (20 mg) in methanol (3 mL) at laboratory temperature. The reaction was quenched after 5 h, the organics were extracted with ethyl acetate, washed with water and dried over sodium sulfate. The crude product was cooled to 0 ^{o}C and methanesulfonyl chloride (0.2 mL, 0.30 mmol) was slowly added. The reaction mixture was kept at 4 ^{o}C and monitored by TLC (10% ether in benzene). After 12 h, the reaction was quenched with ice, the organics were extracted with ethyl acetate, and dried over sodium sulfate. After evaporation of the solvent, additional pyridine (5 mL) was added and the reaction was sealed in a test tube. Elimination occurred at 180 ^{o}C. After 24 h, the reaction mixture was diluted

with ethyl acetate and washed with 5% HCl. The crude unsaturated benzoate was dissolved in a methanolic solution of potassium hydroxide (20 mL, 5%) and stirred for 18 h at laboratory temperature. Preparative chromatography yielded white crystals of **13** (8 mg, 56%), mp 148-150 ^{o}C (acetone/heptane). $[\alpha]_D$ +111 (*c* 0.11). ^{1}H NMR: δ 0.68 s (3H, H-18); 0.74 s (3H, H-19); 2.18 s (3H, H-21); 2.70 t (1H, *J*=9.2, H-17); 4.08 m (1H, *W*=12.2, H-3); 5.62 d (1H, *J*=9.2, H-12); 6.11 dd (1H, *J*=1.8 and 8.5, H-11). Anal. ($C_{21}H_{30}O_2$) C, H.

5α-Pregnane-3,12,20-trione (14). Compound **11** (75 mg, 0.20 mmol) was treated with lithium aluminum hydride (100 mg) in THF (5.0 mL). After 1 h, the excess reagent was carefully quenched with water. The resulting mixture was dissolved in acetic acid (5 mL) and chromium trioxide (40 mg) was added. The oxidation, complete after 2 h (TLC), was quenched with sodium sulfite. The crude organics were extracted with ethyl acetate, washed, and dried. The remainder after evaporation was purified by chromatography (benzene/ether, 4:1) to yield triketone **14** (53 mg, 80%) as white solid, mp 210-212 ^{o}C (AcOEt), Shimizu[61] gives 208-212 ^{o}C. IR: 1652, 1701 (CO). ^{1}H NMR: δ 0.72 s (3H, H-18); 0.78 s (3H, H-19); 2.14 s (3H, H-21).

3α,12α-Dihydroxy-5α-pregnan-20-one (15). In a sealed tube, triketone **14** (50 mg, 0.15 mmol) was heated with a solution of hydrogen hexachloroiridate hydrate (5 mg, 0.01 mmol) and phosphorous acid (50 mg, 0.61 mmol) in water (0.3 mL) and 2-propanol (1.5 mL) at 90 ^{o}C. After 10 h, potassium carbonate (50 mg, 0.36 mmol) was added and the mixture was concentrated in a vacuum. The mixture was diluted with brine and extracted with ethyl acetate. After the usual work-up, the product was purified using thin layer chromatography on silica gel (toluene/ethyl acetate). Extraction of the major zone yielded diol **15** (29 mg, 58%) as white crystals, mp 201-202 ^{o}C (acetone/heptane); $[\alpha]_D$ +165 ($CHCl_3$, c 0.16). IR: 3616, 1005 (OH); 1702, 1362 ($COCH_3$). ^{1}H NMR: δ 0.87 s (3H, H-18); 0.95 s (3H, H-19); 2.27 s (3H, H-21); 3.32 t (1H, *J*=9.1, H-12); 4.10 m (1H, *W*=16.8, H-3). Anal. ($C_{21}H_{34}O_3$) C, H.

(25*R*)-3α-Hydroxy-12-oximino-5α-spirostane (16). Hydroxylamine hydrochloride (1.5 g, 21.70 mmol) was added to a solution of compound **8** (1.13 g, 2.62 mmol) in pyridine (10 mL), the mixture was stirred at 60 ^{o}C for 1 h (the reaction was monitored by TLC developed with 3% of methanol in ammoniacal chloroform). The usual workup yielded oxime **16** (1.11 g, 95%) as white solid, mp 264-266 ^{o}C, IR: 3616 (OH); 3592, 3367 (NOH); 1644 CN. $[\alpha]_D$ +12 ($CHCl_3$, c 0.11). ^{1}H NMR: δ 0.79 d (3H, *J*=6.1, H-27); 0.85 s (3H, H-18); 0.96 s (3H, H-19); 1.05 d (3H, *J*=7.3, H-21); 3.34 t (1H, *J*=11.0, H-26ax); 3.35 m (1H, *W*=17, H-26eq); 4.06 m (1H, *W*=17, H-3); 4.37 q (1H, *W*=30, H-16). Anal. ($C_{27}H_{43}NO_4$) C, H, N.

(25*R*)-12α-Amino-5α-spirostan-3α-ol (17) and **(25*R*)-12β-amino-5α-spirostan-3α-ol (18).** Sodium borohydride (167 mg) was added to a solution of oxime **16** (40.7 mg, 0.09 mmol) and molybdenum trioxide (55 mg) in methanol (5 mL). The reaction mixture turned black immediately. The reaction, followed with TLC (1% methanol in ammoniacal chloroform), was quenched with 10 mL of water. Methanol was evaporated on a vacuum evaporator, the organics were extracted with ammoniacal chloroform, and dried. Analysis of ^{1}H NMR spectra of the reaction mixture revealed that the product (35 mg, 89%) consisted of a 1.2:1 mixture of 12α-and 12ß-amine **17** and **18**, resp. Chromatography yielded:

12α-amine 17 (18 mg, 49%), $[\alpha]_D$ -9 ($CHCl_3$, c 0.09). IR: 3616, 3455 (OH); 3387, 1614, 839 (NH_2). ^{1}H NMR: δ 0.79 d (3H, *J*=6.1, H-27); 0.78 s (3H, H-18); 0.85 s (3H, H-19); 0.97 d (3H, *J*=6.7, H-21); 2.78 s (1H, H-12); 3.37 t (1H, *J*=11.0, H-26ax); 3.47 m (1H, *W*=16, H-26eq); 4.03 s (1H, H-3); 4.37 m (1H, *W*=28, H-16). Anal. ($C_{27}H_{45}NO_3$) C, H, N.

12β amine 18 (16 mg, 40%), $[\alpha]_D$ -33 ($CHCl_3$, c 0.13). IR: 3615, 3453 (OH); 3384, 1610, 842 (NH_2). ^{1}H NMR: δ 0.71 s (3H, H-18); 0.79 s (3H, H-19); 0.79 d (3H, *J*=6.1, H-27); 1.05 d (3H, *J*=6.1, H-21); 2.46 dd, (1H, *J*=3.7, *J*=11.0, H-12); 3.36 t (1H, *J*=11.0, H-26ax); 3.46 m (1H, *W*=17, H-26eq); 4.03 s (1H, H-3); 4.42 q (1H, *J*=22.0, H-16). Anal. ($C_{27}H_{45}NO_3$) C, H, N.

3α-Hydroxy-12β-acetamino-5α-pregnan-20-one (19). The above 12β-aminospirostane derivative **18** (100 mg, 0.23 mmol) was converted into a 5α-pregnane derivative as described in the conversion of compound **10** to **11.** The product was hydrolyzed with methanolic potassium carbonate (10 mL, 10%). The reaction yielded compound **19** (24 mg, 30%) as white solid, mp 296-298 ^{o}C (ether). $[\alpha]_D$ +16 ($CHCl_3$, c 0.36). IR: 3615, 1003 (OH); 3446, 1667, 1504 (CONH); 1698 (C=O). ^{1}H NMR: δ 0.74 s (3H, H-18), 0.78 s (3H, H-19), 2.08 s (3H, H-21), 2.10 s (3H, Ac), 2.83 t (1H, *J*=9.2, H-17), 4.07 m (1H, *W*=18.7, H-3); 4.40 m (1H, *W*=26.3, H-12); 5.82 m (1H, NH). Anal. ($C_{23}H_{37}NO_3$) C, H, N.

12,20-Dioxo-5α-pregnan-3β-yl Acetate (20). Hecogenin (**5**, 10.1 g, 23.45 mmol) was treated with acetic anhydride (250 ml), oxidized, and refluxed in acetic acid as in the preparation of compound **11**. The raw product was hydrogenated in ethyl acetate (150 mL), using palladium on carbon (10%, 400 mg) at laboratory temperature. After 2 h, the catalyst was removed and the solvent evaporated, yielding saturated product **20** (3.3 g, 38%) as white crystals, mp 193-194 ^{o}C (ethyl acetate, reference[62] gives 192-193.5 ^{o}C). IR: 1708, 1737 CO. $[\alpha]_D$ +139 ($CHCl_3$, *c* 0.23). ^{1}H NMR: δ 0.91 s (3H, H-19); 0.95 s (3H, H-18); 2.02 s, (3H, H-21); 2.26 s (3H, Ac); 2.49 t (1H, *J*=13, H-17); 3.31 t (1H, *J*=9.3, H-11); 4.68 m (1H, *W*=32, H-3).

N-(12β-t-Butyloxycarbonyl)-amino-20-oxo-5α-pregnan-3β-yl Acetate (**21**). 12,20-Dioxo derivative **20** (362 mg, 0.97 mmol) was dissolved in hot 2-methyl-2-propanol (12 mL) while stirring. Ammonium acetate (1.8 g, 15.1 mmol) and sodium cyanoborohydride (130 mg, 2.0 mmol) were added and the mixture was allowed to cool to laboratory temperature. After 3 h, the mixture was diluted with chloroform (60 mL), the extract was washed with brine and dried. The solvent was removed in vacuum, the remainder was dissolved in toluene and applied on a short column of silica gel (6 mL). A mixture of toluene and ether (1:1, 40 mL) eluted a neutral admixture (69 mg), while ammoniacal chloroform (60 mL) eluted a mixture

of 12ξ-amino 20ξ-alcohols (328 mg, 0.87 mmol). The mixture was dissolved in pyridine (1.0 mL) and toluene (1.0 mL), and treated with a solution of di-*tert*-butyl dicarbonate (350 mg, 1.6 mmol) in toluene (1.0 mL) at laboratory temperature. After 15 min, the mixture was washed with the solution of potassium hydrogencarbonate and water, and dried. After evaporation of the solvent, the product was dissolved in acetone (2.5 mL) and oxidized with Jones reagent at 0 ^{o}C. After 3 min, the reaction was stopped with the solution of potassium hydrogencarbonate and the product was extracted with ether, washed with water, and dried. PLC yielded 293 mg (81%) of ketone **21** as white crystals, mp 95-98 ^{o}C (acetone/heptane). $[\alpha]_D$ +44 ($CHCl_3$, c=0.2). IR 3444, 3387, 1707, 1169 (NH-CO); 1725, 1360, 1029 (AcO). ^{1}H NMR: δ 0.76 s (3H, H-18); 0.81 s (3H, H-19); 1.41 s (9H, BOC); 2.02 s (3H, AcO); 2.13 s (3H, H-21); 2.72 t (1H, *J*=9.8, H-17); 3.46 m (1H, *W*=28.0, H-12α); 4.70 m (1H, *W*=38.1, H-3). Anal. ($C_{28}H_{45}NO_5$) C, H, N.

N-(12β-t-Butyloxycarbonyl)-amino-3β-hydroxy-5α-pregnan-20-one (**22**). A solution of potassium carbonate (80 mg, 0.58 mmol) in water (0.4 mL) was added into a solution of acetate **21** (248 mg, 0.52 mmol) in methanol (20 mL). The mixture was stirred at 50 ^{o}C for 3 h. The reaction mixture was concentrated in vacuum to a quarter of its volume, brine was added to precipitate the product (224 mg, 99%), which was purified by PLC (toluene/ether, 1:1). The major product **22** (189 mg, 84%) forms white crystals, mp 177-178 ^{o}C (acetone/heptane). $[\alpha]_D$ +57 (chloroform, c=0.24). IR: 3444, 3387 (NH); 1725, 1029 (AcO); 1707, 1360, 1169 (N-C=O and R_2C-O). ^{1}H NMR: δ 0.76 s (3H, H-18); 0.79 s (3H, H-19); 1.41 s (9H, BOC); 2.13 s (3H, H-21); 2.72 t (1H, *J*=9.8, H-17); 3.45 m (1H. (*W*=29.8, H-12α); 3.59 m, 1H (*W*=34.9, H-3), 4.72 d, 1H (*J* =8.8, N-H). Anal. ($C_{26}H_{43}NO_4$) C, H, N.

N-(12β-t-Butyloxycarbonyl)-amino-3α-hydroxy-5α-pregnan-20-one (**23**). 3β-Alcohol **22** (244 mg, 0.56 mmol) was dried by distillation with toluene, dissolved in pyridine (0.6 mL) and the solution was stirred at 0 ^{o}C. Methanesulfonyl chloride (0.3 mL, 3.9 mmol) was

dripped in within 2 min. After 2 h at the same temperature, the mixture was decomposed with ice and product was extracted with chloroform. The extract was washed with the aqueous solutions of hydrochloric acid, water, and potassium hydrogencarbonate. The solution was dried and concentrated in vacuum. The remainder was treated with sodium nitrite (750 mg, 10.9 mmol) in HMPA (5 mL) at 90 ^{o}C under argon. After 4 h, the mixture was cooled, poured onto brine, and precipitate was extracted with ethyl acetate. The extract was washed, dried, and concentrated in vacuum as usual. The product was purified by PLC (6 plates, toluene/ether, 3:1), the major zone was eluted with ether yielding the title compound **23** (146 mg, 60%) as white crystals, mp 154-155 ^{o}C (acetone/heptane). $[\alpha]_D$ +53 (chloroform, c=0.2). IR: 3616, 1001 (OH); 3444, 3388, 1503 (NHCO); 1705 (C=O); 1393, 1367 (*tert*-Bu). ^{1}H NMR: δ 0.76 s (6H, H-18 and H-19); 1.41 s (9H, BOC); 2.13 s (3H, H-21); 2.74 t (1H, *J*=9.9, H-17); 3.49 m (1H, *W*=32.0, H-12α); 4.03 m (1H, *W*=20.0, H-3), 4.69 d (1H, *W*=26, N-H). Anal. ($C_{26}H_{43}NO_4$) C, H, N.

12β-Amino-3α-hydroxy-5α-pregnan-20-one (**24**) Trifluoroacetic acid (0.4 mL, 5.2 mmol) was dripped into a solution of the BOC-derivative **23** (65 mg, 0.15 mmol) in aqueous acetone (50%, 2.0 mL). After 1 h, the mixture was concentrated in vacuum and alkalized with ammonia (3.0 mL). The product was extracted with chloroform, washed with water, dried, and applied onto two PLC plates. The plates were developed with ammoniacal $CHCl_3$/propan-2-ol, 95/5. The major zone was eluted with ethyl acetate which yielded amine **24** (45 mg, 90%) as colourless solid, mp 134-136 ^{o}C (chloroform/heptane). $[\alpha]_D$ +6 (c 0.10). IR: 3616, 999 (OH); 3376, 3309, 1603 (NH_2); 1700, 1358, 597 (C=O). ^{1}H NMR: δ 0.72 s (3H, H-18); 0.77 s (3H, H-19); 2.25 s (3H, H-21); 2.54 t (1H, *J*=9.7, H-17); 2.58 dd (1H, *J*=11.2 and 4.3, H-12α); 4.04 m (1H, *W*=19.7, H-3). Anal. ($C_{21}H_{35}NO_2$) C, H, N.

11α-Hydroxy-5β-pregnane-3,20-dione (26) 11α-Hydroxyprogesterone (**25**, 2.1 g, 6.36 mmol) was hydrogenated in pyridine (15 mL) in the presence of palladium on carbon (10%,

180 mg). After 5 h, the catalyst was filtered off and the solvent was evaporated in a vacuum. On addition of ether (10 mL) to the remainder, crystals of a mixture of 5ξ-dihydro derivatives (505 mg) were swiftly formed. Recrystallization from ethyl acetate yielded the 5β-dihydro product **26** (333 mg, 16%). Additional product (1.54 g, 73%) was obtained by chromatography of mother liquor on silica gel with toluene/ether, 1:1, mp 173-175 ^{o}C (acetone/heptane). $[\alpha]_D$ +103 (c 0.3) (Mancera[32] gives 169-171 ^{o}C and +105). ^{1}H NMR: δ 0.66 (s, 3H, H-18); 1.14 (s, 3H, H-19); 2.14 (s, 3H, H-21); 2.57 (t, 1H (*J*=9.0, H-17); 3.99 (m, 1H (*W*=36, H-11);. HRMS (ESI): M^- found 331.2267; for $C_{21}H_{32}O_3$ calc.331.2273.

11α-Hydroxy-5α-pregnane-3,20-dione (27). The side product of the above chromatography was identified as the 5α-isomer **27** (95 mg, 5%), mp 188-192 ^{o}C (acetone/heptane; Peterson[33] gives 198-201 ^{o}C).

3α,11α-Dihydroxy-5β-pregnan-20-one (28). Sodium borohydride (5.5 mg, 0.15 mmol) was added to a solution of diketone **26** (73 mg, 0.22 mmol) in methanol (0.2 mL) and pyridine (1.8 mL) under stirring at 0 ^{o}C. After 2 h, the mixture was kept at laboratory temperature for 18 h. Excess reagent was destroyed with hydrochloric acid (5%, 8 mL), the solvents were partly evaporated in a vacuum and brine was added. Product was extracted with ethyl acetate, the extract was washed with brine, dried and concentrated in a vacuum. TLC (2 plates) was run in toluene/ethyl acetate, 1:3. The most lipophilic component (5 mg, 7%) was the 3β-isomer, the most polar component (6 mg, 8%) was the 17α-isomer. The major component, compound **28** (52 mg, 71%), was obtained as white crystals, mp 180-181 ^{o}C (acetone/heptane, reference[34] gives 181.4 - 182.4 ^{o}C). $[\alpha]_D$ +82 (c 0.3). ^{1}H NMR: δ 0.61 s, 3H (H-18); 1.05 s, 3H (H-19); 2.13 s, 3H (H-21); 2.56 t, 1H (*J*=8.8, H-17); 3.70 m, 1H (*W*=32, H-3); 3.88 dt, 1H (*J*=10.2 and 5.1, H-11).

3,3,20,20-Bisethylenedioxy-5β-pregnan-11-one (29) was prepared by oxidation and ketalisation of ketone **26** according to Kalvoda[35]. ^{1}H NMR: δ 0.71 s, 3H (H-18); 1.17 s, 3H (H-19); 1.25 s, 3H (H-21); 1.31 t, 1H (*J*=7.2, H-17), 3.93 m, 8H, *W*=59.4, $(CH_2O)_2$.

20,20-Ethylenedioxy-5β-pregnane-3,11-dione (30) was prepared by ketalisation and oxidation of ketone **28** according to Mancera[32]. ^{1}H NMR: δ 0.74 s (3H, H-18); 1.24 s (3H, H-19); 1.26 s (3H, H-21); 3.89 m (2H, *W*=36.1, OCH_2); 3.97 m (2H, *W*=32.8, OCH_2).

General Method for the Grignard Reaction. A solution of the Grignard reagent in THF (0.5M, 24 mL, 12 mmol) was added to a ketone (1.20 mmol) under stirring. The reaction mixture, monitored by TLC, was kept at 65 ^{o}C for 10-18 h. When complete, the mixture was poured into a saturated aqueous ammonium chloride solution. The precipitate was extracted with ether, washed with brine, and dried over magnesium sulfate. The solvent was evaporated and the residue was purified by preparative thin-layer chromatography:

3α,11β-Dihydroxy-5β-pregnan-20-one (**31**) was prepared from ketal **30** by reduction with LAH in THF and deprotection according to ref.[35]. ^{1}H NMR: δ 0.84 (s, 3H, H-18); 1.17 (s, 3H, H-19); 2.12 (s, 3H, H-21); 2.46 (t, *J*=8.9, H-17); 3.69 (m, *W*=38 Hz, 1H, H-3); 4.26 (m, 1H, *W*=18, H-11).

3β,11α-Bis-ethynyl-3α,11β-dihydroxy-5β-pregnan-20-one (**32**). Following the general procedure, 20,20-ethylenedioxy-5β-pregnane-3,11-dione (ref.,[16] **30**, 80 mg, 0.22 mmol) was treated with ethynyl magnesium bromide (0.5 *M* solution in THF, 40 mL) for 16 h. After the usual work up and chromatography (toluene/ethyl acetate, 6:4), the ketal obtained was hydrolyzed in position 20 by the treatment with *p*-toluenesulfonic acid (15 mg) in acetone (15 mL) at 55 ^{o}C for 15 min. The usual work up and chromatography on two preparative plates (toluene/ethyl acetate, 6:4) gave compound **32** (25 mg, 30%) as white crystals, mp 235-238 ^{o}C (acetone/heptane). $[\alpha]_D$ +109 ($CHCl_3$, c 0.2). IR: 3594, 1106 (OH); 3305, 2223, 651

(C≡C); 1702, 594 (CO). ^{1}H NMR: δ 0.81 (s, 3H, H-18); 1.26 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.50 (s, 1H, H-C≡C) and 2.53 (s, 1H, H-C≡C). Anal. ($C_{25}H_{34}O_3$) C, H.

3α,11β-Dihydroxy-3β,11α-bis-phenylethynyl-5β-pregnan-20-one (**33**). Following the general procedure, ketal **30** (100 mg, 0.27 mmol) was treated with phenylethynyl magnesium bromide (1.0 M solution in THF, 2.4 mL) for 10 h. After the usual work up and deprotection, chromatography (elution with toluene/ethyl acetate, 6:4) gave compound **33** (49 mg, 34%) as white crystals, mp 220-222 ^{o}C (acetone/heptane). $[\alpha]_D$ +33 ($CHCl_3$, c 0.1). IR: 3593, 1105 (OH); 2223 (C≡C); 1701,1356, 594 (CO); 1490, 1444, 1329, 1028, 692 (phenyl). ^{1}H NMR: δ 0.86 (s, 3H, H-18); 1.32 (s, 3H, H-19); 2.16 (s, 3H, H-21); 7.31 (m, 5H, phenyl), and 7.37 (m, 5H, phenyl). Anal. ($C_{37}H_{42}O_3$) C, H.

3α-Hydroxy-5β-pregnane-11,20-dione (**34**). Ketal **30** (90 mg, 0.24 mmol) was reduced with sodium borohydride in pyridine and deprotected as in the preparation of compound **28** PLC yielded white crystals of compound **34** (31 mg, 39%), mp 172-174 ^{o}C (acetone/heptane. Mancera[32] gives 168-171 ^{o}C). ^{1}H NMR: δ 0.57 (s, 3H, H-18); 1.15 (s, 3H, H-19); 2.10 (s, 3H, H-21); 2.47 and 2.57 (2H, AB system, *J*=11.5, H-12); 2.76 (t, 1H, *J*=9.1, H-17); 3.65 (m, 1H, *W*=36, H-3).

11α-Ethynyl-11β-hydroxy-5β-pregnane-3,20-dione (**35**). Following the general procedure, 3,3,20,20-bisethylenedioxy-5β-pregnan-11-one (**29**, 0.5 g, 1.20 mmol) was treated with ethynyl magnesium bromide (0.5 *M* solution in THF, 24 mL, 12 mmol) for 18 h. The product was deprotected by treatment with *p*-toluenesulfonic acid (30 mg) in acetone (30 mL) at 55 ^{o}C for 15 min. The usual work up and chromatography (elution with toluene/ethyl acetate, 7:3) gave compound **35** (102 mg, 24%) as white crystals. mp 192-195 ^{o}C (acetone/heptane). $[\alpha]_D$ +150 ($CHCl_3$, c 0.1). IR: 3627, 3593, 1112 (OH); 3303, 2103, 637 (C≡C); 1703, 1422, 1359, 594 (CO). ^{1}H NMR: δ 1.00 (s, 3H, H-18); 1.45 (s, 3H, H-19); 2.28 (s, 3H, H-21); 2.69 (s, 1H, H-C≡C). Anal. ($C_{23}H_{32}O_3$) C, H.

11β-Hydroxy-11α-phenylethynyl-5β-pregnane-3,20-dione (**36**). Analogously, ketone **29** (0.5 g, 1.20 mmol) was treated with phenylethynyl magnesium bromide (1.0 *M* solution in THF, 12 mL, 12 mmol) for 10 h and deprotected as above. After the usual work up and chromatography (toluene/ethyl acetate, 9:1), compound **36** (145 mg, 28%) was obtained as white crystals, mp 181-183 ^{o}C (acetone/heptane). $[\alpha]_D$ +190 ($CHCl_3$, c 0.2). IR: 3593, 1112, 1236 (OH); 2220 (C≡C); 1703, 594 (CO); 1491, 1444, 1161, 691 (phenyl). ^{1}H NMR: δ 0.90 (s, 3H, H-18); 1.35 (s, 3H, H-19); 2.16 (s, 3H, H-21); 2.72 (t, 1H, *J*=14.4, H-5); 7.32 (m, 5H, phenyl). Anal. ($C_{29}H_{36}O_3$) C, H.

11α-Ethynyl-3α,11β-dihydroxy-5β-pregnan-20-one (**37**) . A solution, prepared from methanol (1.5 mL), a methanolic solution of sodium hydroxide (2.5 N, 0.05 mL) and a pyridine solution of sodium borohydride (0.18 M, 1.4 mL), was added to a solution of diketone **35** (61 mg, 0.17 mmol) in methanol (4.5 mL) at 0 ^{o}C under nitrogen. After 10 min, an excess of hydrochloric acid was added and the solution was extracted with ether. The extract was washed with an aqueous solution of potassium hydrogencarbonate, water, and dried over sodium sulfate. Evaporation of the solvent and chromatography of the residue on three preparative plates (toluene/ethyl acetate, 6:4) gave compound **37** (40 mg, 66%), mp 195-197 ^{o}C (acetone/heptane). $[\alpha]_D$ +133 ($CHCl_3$, c 0.1). IR: 3599, 3457, 1030, 1115 (OH); 1701, 1359, 593 (CO); 3304, 2105, 636 (C≡C). ^{1}H NMR: δ 0.81 (s, 3H, H-18); 1.22 (s, 3H, H-19); 2.14 (s, 3H, H-21); 2.53 (s, 1H, H-C≡C); 3.72 (m, 1H, *W*=32, H-3). Anal. ($C_{23}H_{34}O_3$) C, H.

3α,11β-Dihydroxy-11α-phenylethynyl-5β-pregnan-20-one (**38**). Diketone **36** (74 mg, 0.17 mmol) was reduced in position 3 as described above. Compound **38** (46 mg, 62%) forms white crystals. mp 199-202 ^{o}C (acetone/heptane). $[\alpha]_D$ +207 ($CHCl_3$, c 0.1). IR: 3603, 1235, 1114 (OH); 2222 (C≡C); 1700, 1358, 594(CO); 1491, 1448, 1179, 1164, 1073, 1029, 691 (phenyl). ^{1}H NMR: δ 1.00 (s, 3H, H-18); 1.40 (s, 3H, H-19); 2.30 (s, 3H, H-21); 3.87 (m, 1H, *W*=31, H-3); 7.43 and 7.52 (2x m, 5H, phenyl). Anal. ($C_{29}H_{38}O_3$) C, H.

3α,11α-Dihydroxy-5α-pregnan-20-one (39). Diketone **27** (85 mg, 0.26 mmol) was treated with hydrogen hexachloroiridate hydrate and 2-propanol as in the preparation of compound **15**. The product was purified by PLC, the major component was identified as the diol **39** (ref.[63], 40 mg, 47%). ^{1}H NMR: δ 0.62 (s, 3H, H-18); 0.93 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.56 (t, 1H, J =8.0, H-17); 3.92 (m, 1H, W=36, H-11); 4.02 (m, 1H, W=16, H-3).

11,20-Dioxo-pregna-3,5-dien-3-yl Acetate (40) was prepared from 11α-hydroxyprogesterone (**25**) according to ref.[36] ^{1}H NMR: δ 0.63 (s, 3H, H-18); 1.19 (s, 3H, H-19); 2.12 (s, 3H, H-21); 2.13 (s, 3H, OAc); 2.70 (t, 1H, *J*=9.0, H-17); 5.35 (m, 1H, *W*=7.3, H-4); 5.68 (m, 1H, *W*=2.0, H-6).

3β-Hydroxy-5α-pregnane-11,20-dione (**41**). Enol acetate **40** (6.0 g, 16.2 mmol) was hydrogenated in a mixture of ethanol (200 mL) and ethyl acetate (50 mL) in the presence of palladium on charcoal (2.5 g, 10%). The product was dissolved in methanol (300 mL) and boiled with a solution of potassium carbonate (6.0 g, 43.4 mmol) in water (30 mL). After 1 h, acetic acid was added (5 mL) and the solvent was partly evaporated in a vacuum and the product precipitated after addition of water. Flash chromatography of the product on silica gel in a mixture of toluene/ether, 4:2 yielded compound **41** (2.2 g, 41%) as white crystals, mp 192-194 ^{o}C (acetone/heptane, Djerassi[64] recorded the same value). ^{1}H NMR δ 0.57 (s, 3H, H-18); 1.01 (s, 3H, H-19); 2.09 (s, 3H, H-21); 2.71 (t, 1H, *J*=9.2, H-17); 3.57 (m, 1H, *W*=35.4, H-3).

11,20-Dioxo-5α-pregnan-3β-yl Mesylate (**42**). Methanesulfonyl chloride (4 mL, 51.7 mmol) was slowly added to a cold (0 ^{o}C) solution of compound **41** (2.1 g, 6.3 mmol) in pyridine (8 mL) under stirring. After 2 h, crushed ice and water (50 g) was added. After 2 h, solid was filtered off, washed with water, and dissolved in CH_2Cl_2. The extract was dried over sodium sulfate and concentrated in a vacuum. On addition of ether, compound **42** (2.1 g, 81%) crystallized as white needles, mp 155-156 ^{o}C, $[\alpha]_D$ +86 (c 0.4, $CHCl_3$). IR ($CHCl_3$):

1704 (C=O); 1357, 1172, 935, 926 (MsO). ^{1}H NMR: δ 0.57 (s, 3H, H-18); 1.03 (s, 3H, H-19); 2.10 (s, 3H, H-21); 2.72 (t, 1H, *J*=9.1, H-17); 3.00 (s, 3H, Ms); 4.60 (m, 1H, *W*=33.1, H-3). Anal. ($C_{22}H_{34}O_5S$) C, H, S.

3α-Hydroxy-5α-pregnane-11,20-dione (3). Mesylate **42** (2.05 g, 5.0 mmol) was solvolyzed as in the preparation of compound **8**. The mixture was separated using column chromatography, the major fraction (0.93 g, 56%) crystallized from acetone, mp 170-171 ^{o}C. $[\alpha]_D$ +97 (c 0.2). (Nagata[65] recorded 172 ^{o}C and +113.4). ^{1}H NMR: δ 0.57 (s, 3H, H-18); 1.00 (s, 3H, H-19); 2.10 (s, 3H, H-21); 2.57 and 2.50 (AB system, 2H, *J*=11.6, 2H-11); 2.73 (t, 1H, J =9.0, H-17); 4.05 (m, 1H, *W*=8.0, H-3).

20,20-Ethylenedioxy-3α-hydroxy-5α-pregnan-11-one (43). Diketone **3** was converted into its mono ketal **43** (ref.[66]) as described above. IR: 3616, 3492, 999 (OH); 1700 (C=O). ^{1}H NMR: δ 0.71 (s, 3H, H-18); 1.00 (s, 3H, H-19); 1.25 (s, 3H, H-21); 2.02 (t, *J*=9.5, H-17); 3.87 (m, *W*=28.5, 4H, OCH_2CH_2O); 3.96 (m, *W*=25.0, 4H, OCH_2CH_2O); 4.03 (m, 1H, *W*=14.0, H-3).

3α,11β-Dihydroxy-5α-pregnan-20-one (44). Ketal **43** (120 mg, 0.36 mmol) was treated with LAH in THF as in the preparation of compound **31**. After deprotection and PLC purification, diol **44** (85 mg, 70%) was obtained as white crystals, mp 202-204 ^{o}C (acetone/heptane; ref.[67] records 204-206 ^{o}C). ^{1}H NMR: δ 0.84 (s, 3H, H-18); 1.03 (s, 3H, H-19); 2.12 (s, 3H, H-21); 2.43 (t, *J*=9.2, H-17); 4.06 (m, 1H, *W*=14.0, H-3), 4.40 (m, 1H, *W*=14.0, H-3).

11α-Ethynyl-3α,11β-dihydroxy-5α-pregnan-20-one (45) Ketal **43** (65 mg, 0.20 mmol) was treated with a solution of ethynylmagnesium bromide (0.5 *M* solution in THF, 6 mL) for 16 h as in the preparation of compound **32**. After deprotection and PLC purification, diol **45** (23 mg, 33% or 90% after allowance for the recovered starting material) was obtained as white crystals, mp 150-152 ^{o}C (aqueous alcohol). IR ($CHCl_3$): 3615, 3596, 1050, 1000 (OH);

3305, 640, 620 (C≡C); 1700, 593 (C=O). $[\alpha]_D$ +94 (c 0.1). ^{1}H NMR: δ 0.80 (s, 3H, H-18); 1.08 (s, 3H, H-19); 2.14 (s, 3H, H-21); 2.46 (t, *J*=9.4, H-17); 2.59 (s, 1H, H-C≡C); 4.03 (m, 1H, *W*=14.0, H-3). HRMS (ESI), *m/z* calcd. for $C_{23}H_{34}O_3Na$: 381.2400; found: 381.2390. Anal. ($C_{23}H_{34}O_3$) C,H.

20,20-Ethylenedioxy-11-oximino-5α-pregnan-3α-ol (46). Compound **43** (618 mg, 1.64 mmol) was added to a solution of $NH_2OH.HCl$ (2.11 g, 30.3 mmol) and KOH pellets (2.58 g, 39.1 mmol) in EtOH (15 mL) and the mixture was heated in a sealed tube at 85 ^{o}C. After 5 d, the reaction mixture was diluted with brine (50 mL), ethanol was removed in vacuum on a rotary evaporator, and product was extracted with chloroform. Amorphous product consisting of crude oxime **46** (523 mg, 81%) was used as such without purification. IR ($CHCl_3$): 3616, 1002 (OH); 3594 (C=NOH). ^{1}H NMR: δ 0.71 (s, 3H, H-18); 1.05 (s, 3H, H-19); 1.33 (s, 3H, H-21); 3.92 (m, 4H, *W*=58.0, ketal); 4.03 (m, 1H, *W*=14.0, H-3); 7.18 (m, 1H, oxime).

11α-Amino-3α-hydroxy-5α-pregnan-20-one (47). Sodium (1.4 g, 60.9 mmol) was added stepwise to a boiling solution of oxime **46** (75 mg, 0.19 mmol) in 2-propanol (15 mL). After 2 h, the excess sodium was decomposed with 2-propanol, the mixture was concentrated in vacuum, diluted with brine, and extracted with chloroform. The extract was washed, dried and concentrated in vacuum as usual. A mixture of THF (3 mL) and dilute hydrochloric acid (5%, 0.2 mL) was used to de-protect the ethylenedioxy intermediate. After 2 h, ammonia (2 mL) was added, solvents were evaporated and the remainder purified by TLC ($CHCl_3$ pretreated with ammonia, two elutions). The most polar component consisted of compound **47** (31 mg, 49%), mp 110-113 ^{o}C (acetone/heptane). $[\alpha]_D$ +28 (*c* 0.2). ^{1}H NMR: δ 0.61 (s, 3H, H-18); 0.93 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.53 (t, 1H, *J*=8.8, H-17); 3.04 (m, 1H, W=33.6, H-11); 4.02 (m, 1H, *W*=16.0, H-3). MS-ESI: M^+ 334.2. Anal. ($C_{21}H_{35}NO_2$) C, H, N.

11β-Amino-3α-hydroxy-5α-pregnan-20-one (48). Within 2 h, sodium borohydride (120 mg, 3.17 mmol) was added in portions to a slurry of oxime **46** (38 mg, 0.1 mmol) and MoO_3

(100 mg, 0.78 mmol) in MeOH (2.0 mL) while stirring at laboratory temperature. After additional 2 h, the mixture was alkalized with a solution of KOH (66 mg, 1.18 mmol) in water (0.7 mL) at laboratory temperature. After 18 h, inorganic material was filtered off and washed with methanol. The filtrate was concentrated in vacuum, the remainder was treated with hydrochloric acid (5%, 0.3 mL) in THF (3 mL) to free the protecting group. After addition of ammonia (6 mL), volatile components were evaporated in vacuum and the remainder was purified by TLC (chloroform pretreated with ammonia/2-propanol, 96:4). The most polar component **48** (13 mg, 40 mmol) formed white crystals (MeOH/H_2O), mp 153-156 ^oC. $[\alpha]_D$ 113.7 (*c* 0.3). IR: 3615, 3444, 1002 (OH); 1700, 1358, 594 (C=O); 3412, 1623, 1237 (NH_2). ^1H NMR: δ 0.85 (s, 3H, H-18); 1.02 (s, 3H, H-19); 2.13 (s, 3H, H-21); 2.45 (t, 1H, *J*=9.0, H-17); 3.60 (m, 1H, W=21.4, H-11); 4.06 (m, 1H, *W*=19.0, H-3). HRMS (ESI): M^+ calc. 334.2741; found: 334.200. Anal. ($C_{21}H_{35}NO_2.H_2O$) C,H,N.

3α-Hydroxy-11α,12α-oxido-5α-pregnan-20-one (49). Standard oxidation of olefin **13** (46 mg, 0.14 mmol) with 4-chloroperoxybenzoic acid (30 mg in 2 ml of chloroform) gives a single product (**49**) which was purified with TLC (toluene/ether, 2+1), mp 166-168 ^oC (acetone/heptane). Anal. ($C_{21}H_{32}O_3$) C,H.

3,3,20,20-Bis-ethylenedioxy-5α-pregnan-11-one (50). 5α-Pregnane-3,11,20-dione (1.28 g, 3.87 mmol) was treated with ethylene glycol and p-toluenesulfonic acid in toluene as in the preparation of compound **29**, mp 205-206 ^oC (ref.[23] records 207-210 ^oC). $[\alpha]_D$ +50 (*c* 0.2). IR ($CHCl_3$): 1701 (C=O); 1100, 1074, 1056, 948 (OCH_2CH_2O). ^1H NMR: δ 0.71s (3H, H-18); 1.02 s (3H, H-19); 1.25 s (3H, H-21); 2.02 t (1H, *J*=9.7, H- 17α); 2.25 d and 2.58 d (2H, AB system, *J*=12.4, H-12), 3.92 m (8H, *W*=73.7, ethylene dioxy groups).

3,3,20,20-Bis-ethylenedioxy-11α-methyl-5α-pregnan-11β-ol (51). Ketone **50** (150 mg, 0.45 mmol) was treated with a solution of methyl lithium in ether (1 M, 2.0 mL) at laboratory temperature. After 2 h, the mixture was worked up as usual. Compound **51** was obtained as

white crystals (150 mg, 96%), mp 137-138 °C (acetone/heptane; ref.[68] records 135-136 °C). $[\alpha]_D$ +26 (*c* 25.7). IR (CHC_3): 3604, 1082 (OH); 1474, 1372, 1296, 1099, 948 (ketal). ^{1}H NMR: δ 0.94 s (3H, H-18); 1.12 s (3H, H-19); 1.28 s (3H, H-21); 1.41 s (3H, H-11a); 1.98 d (1H, *J*=14.2); 2.12 dt (1H, *J*=12.6 and 3.4); 3.94 m (8H, *W*=76.3, ethylene dioxy groups).

3,3,20,20-Bis-ethylenedioxy-11α-methyl-5β-pregnan-11β-ol (52). Ketone **29** (450 mg, 1.05 mmol) was treated with methyl lithium as above. The crude product (466 mg, 100%), not characterised, was immediately used to produce olefins **54** and. **55**.

11-Methylene-5α-pregnane-3,20-dione (53). 11β-Alcohol **51** (450 mg, 1.1 mmol) was dissolved in formic acid (10 mL) and kept at rt for 20 h. The solution was concentrated on a rotary evaporator and the remainder was dissolved in toluene (20 mL) and washed with a solution of potassium hydrogencarbonate. Usual workup yielded white crystals of crude dione **53** (220 mg, 63%). After three crystallisations, mp was 216-218 °C (toluene/heptane). $[\alpha]_D$ +95 (*c* 0.2). IR ($CHCl_3$): 3121, 1638, 1420, 907 (C=C); 1703, 597 (C=O). ^{1}H NMR: □δ 0.56 s (3H, H-18); 1.25 s (3H, H-19); 2.13 s (3H, H-21); 2.58 t (1H, *J*=8.97, H- 17α); 2.71 m (1H, *W*=22.8, H-12β); 4.98 d (2H, *J* = 9.1, C=CH_2). Anal. ($C_{22}H_{32}O_2$) C, H.

11-Methyl-5β-pregn-9(11)-ene-3,20-dione (55). Alcohol **52** (465 mg, 1.07 mmol) was dehydrated with formic acid as above. Flash chromatography of the product (silica gel, toluene, ethyl acetate, 9:1) yielded white crystals of the lipophilic component (**55**, 177 mg, 49%), mp 141-143 °C (acetone/heptane). $[\alpha]_D$ +119 (*c* 0.2). IR ($CHCl_3$): 1703, 1418, 597 (C=O); 1638 (C=C). ^{1}H NMR: δ 0.56 s (3H, H-18); 1.45 s (3H, H-19); 1.85 bs (1H, *W*=2.6, H-11a); 2.15 s (3H, H-21); 2.57 t (1H, *J*=9.3, H-17α). Anal. ($C_{22}H_{32}O_2$) C, H.

11-Methylene-5β-pregnane-3,20-dione (54). The more polar fractions of the above chromatography yielded compound **54** as white crystals (90 mg, 25%), mp 138-139 °C (acetone/heptane). $[\alpha]_D$ +104 (*c* 0.15). IR ($CHCl_3$): 1703, 1358, 603 (C=O); 3116, 1639, 910 (C=C). ^{1}H NMR: δ 0.57 s (3H, H-18); 1.29 s (3H, H-19); 2.14 s (3H, H-21); 2.49 d (1H,

J=11.6, H-9α); 2.60 t (1H, *J*=9.0, H-17α); 2.68 m (1H, *W*=24.4, H-12β); 4.96 s (1H, H-11a); 5.06 s (1H, H-11a'). Anal. ($C_{22}H_{32}O_2$) C, H.

3α-Hydroxy-11-methyleno-5α-pregnan-20-one (56). Diketone **53** (150 mg, 0.46 mmol) was treated with hydrogen hexachloroiridate hydrate and 2-propanol as in the preparation of compound **15**. PLC yielded the target compound **56** (90 mg, 65%) as white crystals, mp 153-155 ^{o}C (acetone/heptane). $[\alpha]_D$ +102.3 ($CHCl_3$, c 0.3). IR ($CHCl_3$): 3615, 3465, 3596, 1005, (OH); 3112, 1637, 1420, 904, 529 (C=C); 1699, 597 (C=O). ^{1}H NMR: δ 0.54 s (3H, H-18); 1.02 s (3H, H-19); 2.12 s (3H, H-21); 4.05 m (1H, *W*=15.2, H-3); 4.92 d (2H, *J*=10, H-11a). Anal. ($C_{22}H_{34}O_2$) C, H.

3α-Hydroxy-11-methylene-5β-pregnan-20-one (57). In analogy with the preparation of compound **34**, 3,20-diketone **54** (70 mg, 0.21 mmol) was reduced with sodium borohydride in pyridine to yield compound **57** (53 mg, 75%), mp 203-204 ^{o}C (acetone/heptane). $[\alpha]_D$ +86.5 (*c* 0.2). ^{1}H NMR: δ 0.53 s (3H, H-18); 1.19 s (3H, H-19); 2.12 s (3H, H-21); 2.43 d (1H, *J*=11.9, H-12β); 2.58 t (1H, *J*=9.0, H-17α); 3.68 m (1H, *W*=31.6, H-3); 4.90 s (1H, H-11a); 5.00 s (1H, H-11a'). Anal. ($C_{22}H_{34}O_2$) C, H.

3α-Hydroxy-11-methyl-5β-pregn-9(11)-en-20-one (58). Analogously, 3,20-Diketone **55** (150 mg, 0.45 mmol) was selectively reduced to yield compound **58** (46 mg, 31%), mp 145-147 ^{o}C (acetone/heptane). $[\alpha]_D$ +56.4 (*c* 0.2). IR ($CHCl_3$) 3609, 3455, 1037 (OH); 1698, 586, 1359 ($COCH_3$); 1389, 1379 (CH_3). ^{1}H NMR: δ 0.50 s (3H, H-18); 1.09 s (3H, H-19); 1.81 s (3H, H-11a); 2.14 s (3H, H-21); 2.58 t (1H, *J*=9.2, H-17α); 3.72 m (1H, *W*=36.2, H-3). Anal. ($C_{22}H_{34}O_2$) C, H.

3α-Hydroxy-11β-methyl-5α-pregnan-20-one (59). Olefin **56** (35 mg, 0.11 mmol) was hydrogenated in ethanol (5 mL) in the presence of palladium on charcoal (30 mg, 10%). Compound **59 (**34 mg, 97%) forms white crystals, mp 193-195 ^{o}C (acetone/heptane). $[\alpha]_D$ +142.8 ($CHCl_3$, c 0.2). IR ($CHCl_3$): 3616, 3468, 1002 (OH); 593 (C=O). ^{1}H NMR: δ 0.69 s

(3H, H-18); 0.88 s (3H, H-19); 1.08 d (3H, *I*=7.6, H-11a); 2.15 s (3H, H-21); 2.44 t (1H, *J*=9.0, H-17α); 4.04 m (1H, *W*=12.2, H-3). Anal. ($C_{22}H_{36}O_2$) C, H.

3α-Hydroxy-11β-methyl-5β-pregnan-20-one (60). Analogously, olefin **57** yielded compound **60** as white crystals (67%), mp 162-164 ^{o}C (acetone, heptane). $[\alpha]_D$ +99 (c 0.2). IR ($CHCl_3$): 3609, 3455, 1037 (OH); 1698, 586 (C=O). ^{1}H NMR: δ 0.68 s (3H, H-18); 1.04 s (3H, H-19); 1.08 d (3H, d, *J*=7.8, H-11a); 2.12 s (3H, H-21); 2.45 t (1H, *J*=9.2, H-17α); 3.67 m (1H, *W*=39.9, H-3). Anal. ($C_{22}H_{36}O_2$) C, H.

Biological Evaluation

In vitro test using intact neurons in culture. Primary cultures of cortical neurons were obtained from cerebral cortices of 16-day-old mouse embryos.[43,44,69] The dissociated cells were suspended in Dulbecco's Minimum Essential Medium supplemented with sodium 4-aminobenzoate, insulin, penicillin, and 10% fetal calf serum, and seeded in 24-multiwell plates precoated with poly-*L*-lysine. Cultured cells were incubated for 7-9 days in a humidified 5% CO_2/95% air atmosphere at 36.8 °C. To prevent glial proliferation, a mixture of 5 μM 5-fluoro-2'-deoxyuridine and 20 μM uridine was added into the culture 48 hr earlier.

[^{3}H]Flunitrazepam Binding. Benzodiazepine binding to intact cultured cortical neurons was determined as previously described[43,44,70] using 1.3–2.0 nM [^{3}H]flunitrazepam. Prior to incubation with the radioligand, the plates were washed three times with 1 mL/well of HEPES buffer (136 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 10 mM HEPES, and 9 mM glucose adjusted to pH 7.3) and the binding assay took place in the culture well in the presence of the HEPES buffer, [^{3}H]flunitrazepam, and drug solutions. After 30 min incubation at 25 ^{o}C without shaking, a cold buffer was added, and rapidly removed by suction. The cells were rinsed three times with the cold buffer, then they were disaggregated in 0.2 N NaOH overnight, and their radioactivity was determined by liquid

scintillation counting (with cocktail Optiphase 'Hisafe'2). Non-specific binding was determined in the presence of 20 µM diazepam. All the experiments were simultaneously run with a parallel experiment that determined the increase of the [^{3}H]flunitrazepam binding induced by 100 µM GABA or 60 µM allopregnanolone, which were used as positive assay control. Solutions of tested compounds were prepared in dimethylsulfoxide (DMSO) and diluted in HEPES buffer to the assay concentrations (1 nM–300 µM). The final DMSO concentration in HEPES buffer was < 0.5%. Occasionally, 2% DMSO was used when the compounds were tested at concentrations > 100 µM. DMSO was also present in the HEPES control solutions.

Data analysis. Data shown represent the mean ± standard deviation (sd). Sigmoid curves were fitted to concentration-response data, and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). At least six concentrations of tested compounds were used in the concentration-response curves, and each point was determined in triplicate.

Supporting Information Available: Results of combustion analysis, full details of 3D-QSAR analysis and computational methods are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations Used: TBPS, *tert*-butyl-bicyclo[2.2.2]phosphorothionate.

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Tables, Figures, Schemes

Table 1. Effect of the synthetic analogues synthesized in this work on [^{3}H]flunitrazepam binding in primary cultures of cortical neurons

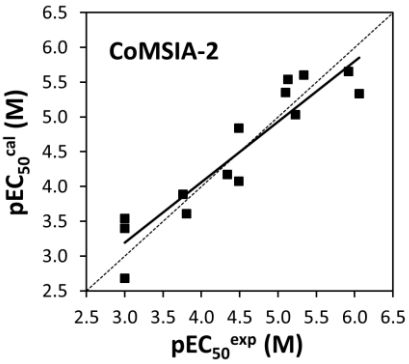
#	EC50 (μM)	$E_{100\ \mu M}$
3	4.6 ± 1.6	92 ± 11
12	173 ± 38	28 ± 15
13	7.4 ± 3.5	67 ± 27
15	46 ± 3	60 ± 4
19	> 100	0
24	> 300	0
28	71 ± 30	43 ± 19
31	> 300	25 ± 4
32	75 ± 55	61 ± 22
33	> 100	0
34	94 ± 25	42 ± 12
37	14 ± 10	91 ± 23
38	1.2 ± 0.7	116 ± 20
39	> 300	28 ± 24
44	32 ± 7	117 ± 9
45	32.0 ± 16.0	87 ± 7
47	154 ± 78	45 ± 19
48	5.9 ± 2.8	140 ± 33
49	> 300	9 ± 12

56	1.2 ± 0.7	102 ± 22
57	37.5 ± 0.05	28 ± 14
58	3.0 ± 1.9	97 ± 16
59	8 ± 5	97 ± 41
60	14 ± 12	71 ± 23

Values are mean ± sd (n = 2-4). The effect of 100 µM neurosteroid was related to the maximum effect on [^{3}H]flunitrazepam binding obtained with 60 µM allopregnanolone. $E_{100\ \mu M}$ of a compound is expressed in % of E_{max} of allopregnanolone.

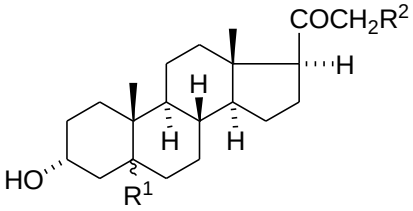
Table 2. Most relevant statistical parameters and correlation between experimental and calculated EC_{50} values (expressed as pEC_{50} = –log EC_{50} in molar units) for the best CoMSIA model obtained for compounds **1a**, **3**, **12**, **13**, **15**, **24**, **39**, **44**, **45**, **47** to **49**, **56** and **59**.

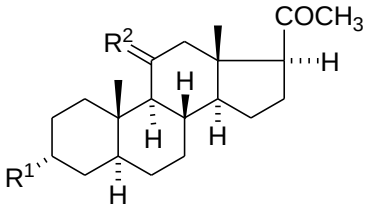
	CoMSIA-2
PC [a]	2
q^2 (CV) [b]	0.60 ± 0.04
PRESS (CV) [c]	0.72 ± 0.03
SEE [d]	0.41
r^2	0.87



[a] Number of principal components. [b] Average q^2 from 10 runs of random groups cross validation (5 groups). [c] Average predictive error sum of squares from 10 runs of random groups cross validation (5 groups). [d] Standard error of estimate.

Figures





1a, allopregnanolone, R^1= 5α-H, R^2=H
1b, pregnanolone, R^1= 5β-H, R^2=H
2, R^1= 5α-H, R^2=OH, 3α,5α-tetrahydro-deoxycorticosterone

3, alphaxolone, R^1=OH, R^2=O
4, R^1=NH_2, R^2=H_2

Figure 1. Basic $GABA_A$-receptor modulating steroids and some of their analogues

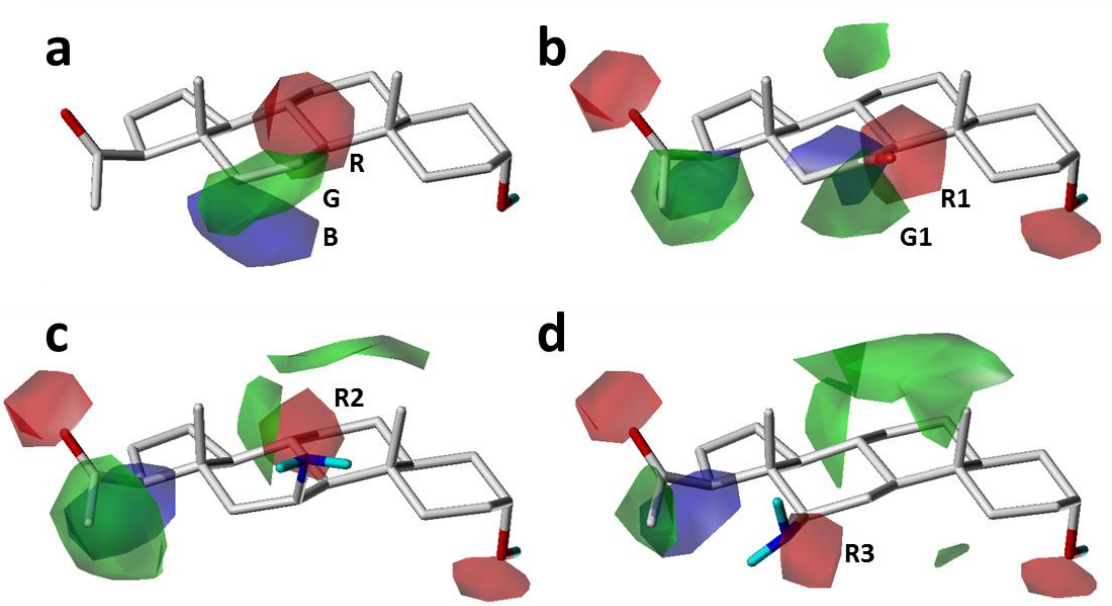
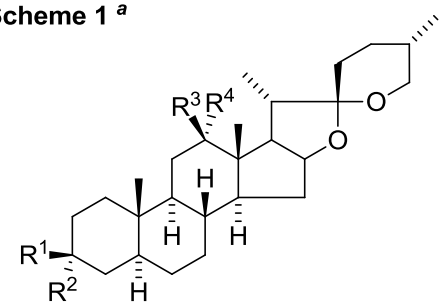


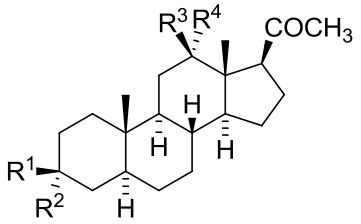
Figure 2. a: STDev*Coeff contour plots for CoMSIA-2 model overlaid on the structure of compound **1**: Blue (B) and red (R) surfaces represent the electrostatically favoured (contour at 75% contribution level) and disfavoured (contour at 25% contribution level) regions, i.e. regions where positive potential on the ligand atoms increase or decrease the activity of the compounds; a green (G) surface represents the hydrophobically favoured region (contour at 80% contribution), where hydrophobic groups of the ligand increase the activity of the compound; **b-d**: Positive (blue surface, contour at 90% level) and negative (red surface, contour at 10% level) electrostatic fields and favoured hydrophobic field (green surface, contour at 90% level) contour maps of compounds **3** (**b**), **48** (**c**), and **24** (**d**).

Schemes

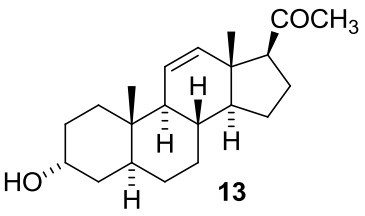
Scheme 1 *a*



	R^1	R^2	R^3	R^4	
5	OH	H	=O		(hecogenin)
6	OMs	H	=O		
7	OTs	H	=O		
8	H	OH	=O		
9	H	OBz	=O		
10	H	OBz	OH	H	
16	H	OH	=NOH		
17	H	OH	H	NH_2	
18	H	OH	NH_2	H	



	R^1	R^2	R^3	R^4
11	H	OBz	OAc	H
12	H	OH	OH	H
14	=O		=O	
15	H	OH	H	OH
19	H	OH	NHAc	H



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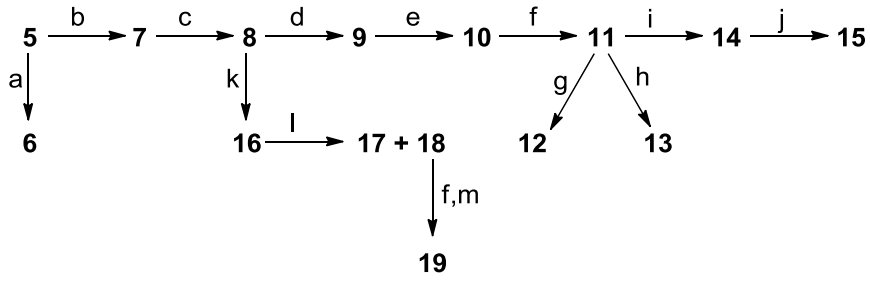
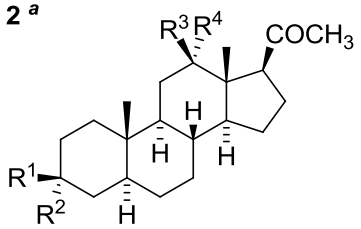


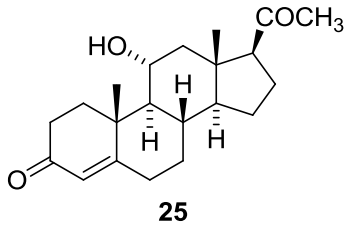
Scheme 2 *a*



	R^1	R^2	R^3	R^4
20	OAc	H	=O	
21	OAc	H	NH-BOC	H
22	OH	H	NH-BOC	H
23	H	OH	NH-BOC	H
24	H	OH	NH_2	H

5 —a→ **20** —b→ **21** —c→ **22** —d→ **23** —e→ **24**

Scheme 3 [a]



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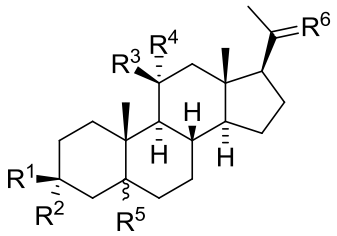
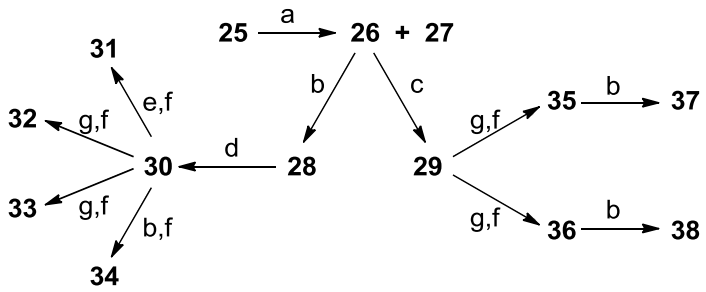
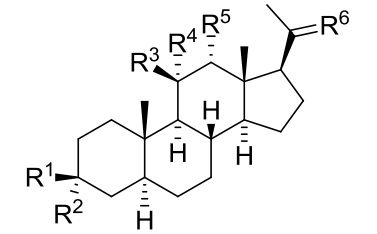


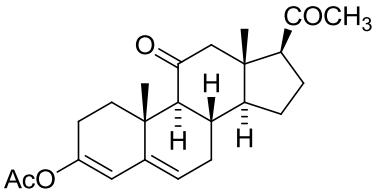
	R^1	R^2	R^3	R^4	R^5	R^6
26	=O		H	OH	β-H	=O
27	=O		H	OH	α-H	=O
28	H	OH	H	OH	β-H	=O
29	$-O(CH_2)_2O-$		=O		β-H	$-O(CH_2)_2O-$
30	=O		=O		β-H	$-O(CH_2)_2O-$
31	H	OH	OH	H	β-H	=O
32	CCH	OH	OH	CCH	β-H	=O
33	CCPh	OH	OH	CCPh	β-H	=O
34	H	OH	=O		β-H	=O
35	=O		OH	CCH	β-H	=O
36	=O		OH	CCPh	β-H	=O
37	H	OH	H	CCH	β-H	=O
38	H	OH	H	CCPh	β-H	=O



Scheme 4 [a]



	R^1	R^2	R^3	R^4	R^5	R^6
39	H	OH	H	OH	H	=O
41	OH	H	=O		H	=O
42	OMs	H	=O		H	=O
43	H	OH	=O		H	$-O(CH_2)_2O-$
44	H	OH	OH	H	H	=O
45	H	OH	OH	CCH	H	=O
46	H	OH	=NOH		H	$-O(CH_2)_2O-$
47	H	OH	H	NH_2	H	=O
48	H	OH	NH_2	H	H	=O
49	H	OH	H	-O-		=O



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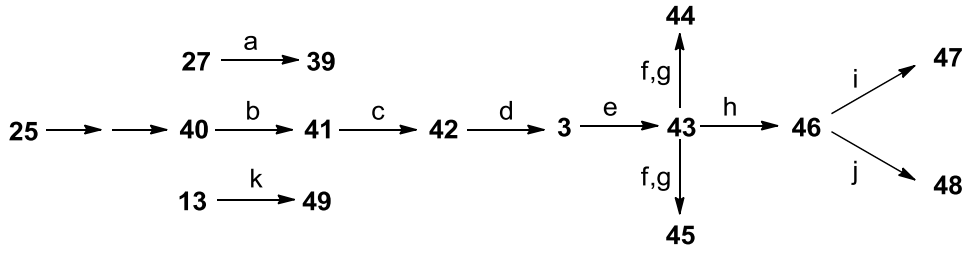
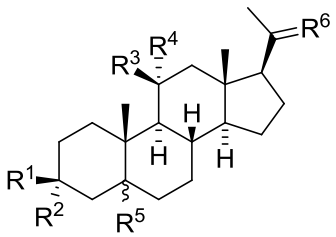
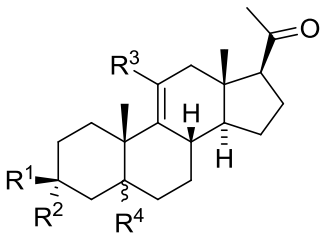


Scheme 5 [a]





	R^1	R^2	R^3	R^4	R^5	R^6
50	$-O(CH_2)_2O-$		=O		α-H	$-O(CH_2)_2O-$
51	$-O(CH_2)_2O-$		OH	Me	α-H	$-O(CH_2)_2O-$
52	$-O(CH_2)_2O-$		OH	Me	β-H	$-O(CH_2)_2O-$
53	=O		$=CH_2$		α-H	=O
54	=O		$=CH_2$		β-H	=O
56	H	OH	$=CH_2$		α-H	=O
57	H	OH	$=CH_2$		β-H	=O
59	H	OH	Me	H	α-H	=O
60	H	OH	Me	H	β-H	=O

	R^1	R^2	R^3	R^4
55	=O		Me	β-H
58	H	OH	OH	β-H
61	H	OH	H	α-H

50 $\xrightarrow{a}$ **51** $\xrightarrow{b}$ **53** $\xrightarrow{c}$ **56** $\xrightarrow{d}$ **59**

29 $\xrightarrow{a}$ **52** $\xrightarrow{b}$ **54** $\xrightarrow{e}$ **57** $\xrightarrow{d}$ **60**; **55** $\xrightarrow{e}$ **58**

Legends

Ad Scheme 1

[a] Reagents and Conditions: (a) MsCl, py; (b) TsCl, py; (c) KNO_2, DMF, 130 ^oC; (d) BzCl, py; (e) $NaBH_4$, EtOAc-MeOH; (f) Ac_2O, AcOH, 180 ^oC; CrO_3, AcOH-dichlorethane; AcOH, reflux; H_2, Pd/C, toluene; (g) KOH, MeOH; (h) K_2CO_3, MeOH; MsCl, AcOEt; py, 180 ^oC; KOH, MeOH (i) $LiAlH_4$, THF; CrO_3, AcOH; (j) $H_2IrCl_6 \cdot 6H_2O$, H_3PO_3, ^{i}PrOH-H_2O, 90 ^oC; (k) $NH_2OH \cdot HCl$, py, 60 ^oC; (l) $NaBH_4$, MoO_3, MeOH; (m) K_2CO_3, MeOH.

Ad Scheme 2

[a] Reagents and Conditions: (a) Ac_2O, AcOH, 180 ^oC; CrO_3, AcOH-dichlorethane; AcOH, reflux; H_2, Pd/C, EtOAc; (b) NH_4OAc, $NaCNBH_3$, ^{t}BuOH; $(t\text{-}BuO)_2CO$, py; CrO_3, MeCOMe; (c) K_2CO_3, MeOH; (d) MsCl, py; $NaNO_2$, HMPA, 90 ^oC; (e) TFA, acetone.

Ad Scheme 3

[a] Reagents and Conditions: (a) H_2, Pd/C, py; (b) $NaBH_4$, py; (c) CrO_3, MeCOMe; ethylene glycol, p-TsOH; (d) CrO_3, MeCOMe; ethylene glycol, p-TsOH; (e) $LiAlH_4$, THF; (f) AcOH, MeOH, H_2O, 60 ^oC; (g) HCCMgBr or $C_6H_5CCMgBr$, THF.

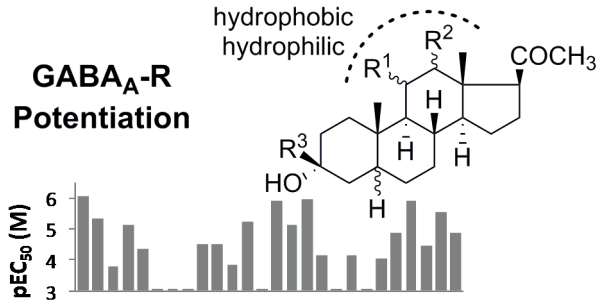
Ad Scheme 4

[a] Reagents and Conditions: (a) $H_2IrCl_6 \cdot 6H_2O$, ^{i}PrOH; (b) H_2, Pd/C, EtOH-AcOEt; K_2CO_3, MeOH-H_2O, reflux; (c) MsCl, py; (d) KNO_2, DMF, 130 ^oC; (e) ethylene glycol, p-TsOH; (f) $LiAlH_4$ or HCCMgBr, THF; (g) AcOH, MeOH, H_2O, 60 ^oC; (h) $NH_2OH \cdot HCl$, KOH, EtOH, 86 ^oC; (i) Na, ^{i}PrOH; HCl aq., THF; (j) MoO_3, $NaBH_4$, MeOH; HCl aq., THF. (k) mCPBA, $CHCl_3$.

Ad Scheme 5

[a] Reagents and Conditions: (a) MeLi, Et_2O; (b) HCOOH, 20 ^oC; (c) $H_2IrCl_6 \cdot 6H_2O$, H_3PO_3, ^{i}PrOH; (d) H_2, Pd/C, EtOH; (e) $NaBH_4$, py.

Table of Contents Graphic



Supporting Information

Allopregnanolone and pregnanolone analogues modified in the C ring: synthesis and activity

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Table S1. Results of combustion analyses of newly prepared compounds*

Subst.	Formula	Calculated	Found
6	$C_{28}H_{44}O_6S$	66.11 %C, 8.72 %H, 6.30 %S	59.88 %C, 8.76 %H, 6.03 %S
7	$C_{34}H_{48}O_6S$	69.83 %C, 8.27 %H, 5.48 %S	69.59 %C, 8.35 %H, 5.73 %S
8	$C_{27}H_{42}O_4$	75.31 %C, 9.83 %H	75.29 %C, 9.86 %H
9	$C_{34}H_{46}O_5$	76.37 %C, 8.67 %H	76.21 %C, 8.53 %H
10	$C_{34}H_{48}O_5$	76.08 %C, 9.01 %H	76.13 %C, 9.11 %H
11	$C_{30}H_{40}O_5$	74.97 %C, 8.39 %H	75.18 %C, 8.28 %H
12	$C_{21}H_{34}O_3$	75.41 %C, 10.25 %H	75.28 %C, 10.21 %H
13	$C_{21}H_{30}O_2$	79.70 %C, 10.19 %H	79.52 %C, 10.26 %H
15	$C_{21}H_{34}O_3$	75.41 %C, 10.25 %H	75.36 %C, 10.28 %H
16	$C_{27}H_{43}NO_4$	72.77 %C, 9.73 %H, 3.14 %N	72.50 %C, 9.64 %H, 3.15 %N
17	$C_{27}H_{45}NO_3$	75.13 %C, 10.51 %H, 3.24 %N	74.90 %C, 10.61 %H, 3.53 %N
18	$C_{27}H_{45}NO_3$	75.13 %C, 10.51 %H, 3.24 %N	74.95 %C, 10.55 %H, 3.49 %N
19	$C_{23}H_{37}NO_3$	73.56 %C, 9.93 % H, 3.73 % N	73.49 %C, 9.98 % H, 3.85 % N
21	$C_{28}H_{45}NO_5$	70.70 %C, 9.54 %H, 2.94 %N	70.49 %C, 9.64 %H, 3.05 %N
22	$C_{26}H_{43}NO_4$	72.02 %C, 10.00 %H, 3.23 %N	71.78 %C, 10.08 %H, 3.32 %N
23	$C_{26}H_{43}NO_4$	72.02 %C, 10.00 %H, 3.23 %N	72.07 %C, 10.19 %H, 3.42 %N
24	$C_{21}H_{35}NO_2$	75.63 %C, 10.58 %H, 4.20 %N	75.54 %C, 10.47 %H, 4.42 %N
32	$C_{25}H_{34}O_3$	78.49 %C, 8.96 %H	78.31 %C, 9.01 %H
33	$C_{37}H_{42}O_3$	83.11 %C, 7.92 %H	83.19 %C, 8.04 %H
35	$C_{23}H_{32}O_3$	77.49 %C, 9.05 % H	77.34 %C, 9.11 % H
36	$C_{29}H_{36}O_3$	80.52 %C, 8.39 %H	80.48 %C, 8.46 %H
37	$C_{23}H_{34}O_3$	77.05 %C, 9.56 %H	77.16 %C, 9.75 %H
38	$C_{29}H_{38}O_3$	80.14 %C, 8.81 %H	79.95 %C, 8.86 %H
42	$C_{22}H_{34}O_5S$	64.36 %C, 8.35 %H, 7.81 %S	64.18 %C, 8.52 %H, 7.55 %S
45	$C_{23}H_{34}O_3$	77.05 %C, 9.56 %H	76.79 %C, 9.72 %H
47	$C_{21}H_{35}NO_2$	75.63 %C, 10.58 %H, 4.20 %N	75.42 %C, 10.39 %H, 4.27 %N
48	$C_{21}H_{35}NO_2.H_2O$	73.64 %C, 10.59 %H, 4.09 %N	73.25 %C, 10.77 %H, 4.22 %N
49	$C_{21}H_{32}O_3$	75.86 %C, 9.70 %H	75.85 %C, 9.67 %H
50	$C_{25}H_{38}O_5$	71.74 %C, 9.15 %H	71.49 %C, 9.26 %H
53	$C_{22}H_{32}O_2$	80.44 %C, 9.82 %H	80.39 %C, 9.85 %H
54	$C_{22}H_{32}O_2$	80.44 %C, 9.82 %H	80.48 %C, 9.91 %H
55	$C_{22}H_{32}O_2$	80.44 %C, 9.82 %H	80.21 %C, 9.79 %H
56	$C_{22}H_{34}O_2$	79.95 %C,10.37 %H	79.68 %C,10.19 %H
57	$C_{22}H_{34}O_2$	79.95 %C,10.37 %H	79.79 %C,10.44 %H
58	$C_{22}H_{34}O_2$	79.95 %C,10.37 %H	79.98 %C,10.52 %H
59	$C_{22}H_{36}O_2$	79.46 %C, 10.91 %H	79.19 %C, 11.20 %H
60	$C_{22}H_{36}O_2$	79.46 %C, 10.91 %H	79.29 %C, 10.96 %H

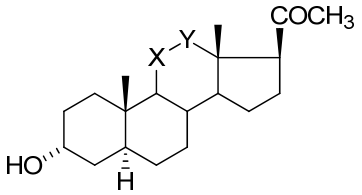
*Combustion analyses were carried out using Perkin Elmer 2400 in the Analytical Laboratory of the Institute of Organic Chemistry and Biochemistry, Dr. S. Matějková, Head.

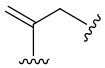
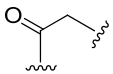
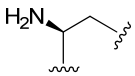
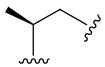
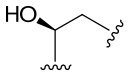
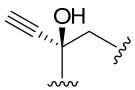
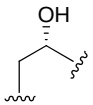
Selection of 3D-QSAR methodology and compounds

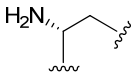
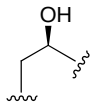
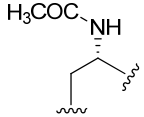
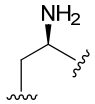
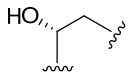
The CoMFA and CoMSIA methodologies[1] were explored in order to obtain 3D-QSAR models that would correlate the experimental flunitrazepam binding activity with the structural properties of the compounds. Both methods assume that the compounds interact with the receptor at the same place and with similar relative geometry. Briefly, they start from a set of prealigned conformations, which ideally reflect the "active" conformation of each compound, and imply the following steps: (1) a cubic grid is defined around the molecules, (2) different atomic probes are used to calculate field values at each grid point, (3) the resulting table of values, normally constituted by thousands of columns, is analysed using the Partial Least Squares regression method (PLS) to correlate biological activities using an optimum number of latent variables (or principal components, PC), (4) the model is validated by determining the internal predictivity (crossvalidation) and, if possible, by predicting the biological activity of an external set of molecules. CoMFA and CoMSIA methods differ mainly on the types of fields considered and their potential functional forms. Thus, CoMFA normally relies on the calculation of steric and electrostatic fields derived from Lenard-Jones and Coulomb interaction potentials, while CoMSIA is based on the calculation of steric, electrostatic, hydrophobic, donor and acceptor similarity indexes.

Concerning the compounds, in the present work two series have been synthesised, namely a 5α- and a 5β-collection, which can be considered analogues of the parent neurosteroids allopregnanolone (1a) and pregnanolone (**1b**) (Tables S1 and S2). Given the small number of compounds on each series (15 and 11, respectively) the separation of a test set to carry out an external validation was discarded from the beginning. However, it was assumed that models supported by a strong enough internal validation (ie. $q^2 \geq 0.6$) could provide some information about the main structural features that determine the activity of the compounds considered. To that end, both the CoMFA and CoMSIA methods were attempted for the generation of 3D-QSAR models from the whole set of 5α- and 5β-compounds as well as from each series separately.

Table S2. Structures of 5α-steroids with their activities expressed as pEC_{50} (ie. -log EC_{50} (M)), logP and logS (log solubility in water (M)) molecular descriptors calculated by different methods,[a] and calculated squared correlation coefficient (r^2) of activity vs. each descriptor.

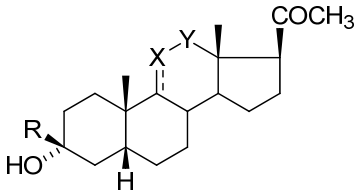


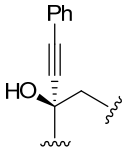
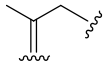
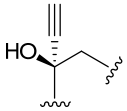
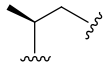
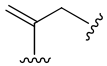
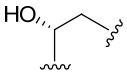
	X, Y	pEC_{50}	cLogP	VG	KLOP	PHYS	ALOGPs	AC_logP	ALOGP	MLOGP	KOWWIN	XLOGP2	XLOGP3	miLogP	Mean ±SD	ALOGpS	AClogS	Mean
1a		6.06 [b]	4.51	4.22	3.80	3.94	4.28	3.71	3.73	4.25	3.97	4.83	4.86	4.18	4.19 ±0.39	-5.37	-4.76	-5.07
56		5.92	4.55	4.15	3.63	4.00	3.60	3.93	3.79	4.36	4.39	4.42	4.30	4.49	4.13 ±0.34	-5.46	-4.82	-5.14
3		5.34	3.73	3.60	2.85	2.74	3.23	3.04	2.38	3.31	2.63	3.60	3.24	3.07	3.12 ±0.42	-4.14	-4.41	-4.28
48		5.23	2.52	2.88	2.35	2.48	2.71	2.34	2.28	3.42	2.43	3.95	3.26	2.77	2.78 ±0.52	-4.07	-4.43	-4.25
13		5.13	4.03	3.96	3.35	3.57	3.96	3.61	3.29	4.15	3.76	4.11	4.38	3.67	3.82 ±0.33	-5.12	-4.53	-4.83
59		5.10	5.03	4.55	4.07	4.21	4.28	3.90	3.99	4.47	4.39	5.34	5.12	4.65	4.50 ±0.46	-5.54	-4.91	-5.23
44		4.49	3.58	2.93	2.40	2.70	3.00	2.87	2.57	3.42	2.84	4.01	3.52	3.26	3.09 ±0.47	-3.84	-4.36	-4.10
45		4.49	4.17	2.94	2.72	2.83	2.93	2.67	3.61	3.74	3.02	4.03	3.42	3.49	3.30 ±0.52	-4.71	-4.79	-4.75
15		4.34	3.12	3.00	2.49	2.78	3.23	2.87	2.63	3.42	2.84	4.01	3.52	3.26	3.10 ±0.42	-3.89	-4.36	-4.13

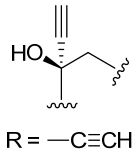
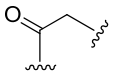
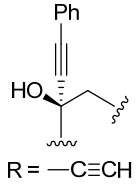
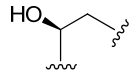
47		3.81	2.52	2.88	2.35	2.48	2.71	2.34	2.28	3.42	2.43	3.95	3.26	2.77	2.78 ±0.52	-4.07	-4.43	-4.25
12		3.76	3.12	3.00	2.49	2.78	3.23	2.87	2.63	3.42	2.84	4.01	3.52	3.26	3.10 ±0.42	-3.89	-4.36	-4.13
19		<4.2 [c]	3.02	2.78	2.09	2.53	3.26	2.89	2.37	3.34	2.37	4.22	3.52	3.04	2.95 ±0.59	-4.69	-4.63	-4.66
24		<3.5 [c]	3.20	2.95	2.44	2.55	3.07	2.34	2.34	3.42	2.43	3.95	3.26	2.77	2.89 ±0.51	-4.12	-4.43	-4.28
39		<3.5 [c]	3.58	2.93	2.40	2.70	3.00	2.87	2.57	3.42	2.84	4.01	3.52	3.26	3.09 ±0.47	-3.84	-4.36	-4.10
49		<3.5 [c]	2.86	3.15	2.79	2.82	2.71	2.51	2.50	3.42	2.46	3.58	3.54	3.45	2.98 ±0.43	-4.23	-4.44	-4.34
	r^2 [d]		0.37	0.54	0.51	0.45	0.35	0.45	0.29	0.40	0.40	0.17	0.34	0.35	0.42	0.49	0.31	0.46

[a] logP calculations: clogP calculated with Sybyl 8.0 (Tripos International); VG, KLOP and PHYS logP values calculated with Marvin 5.11.4 (2012, ChemAxon, http://www.chemaxon.com); ALOGPs, AC_logP, ALOGP, MLOGP, KOWWIN, XLOGP2 and XLOGP3 calculated on-line with the ALOGPS 2.1 web applet[2] (http:// http://www.vcclab.org/lab/alogps/); miLogP calculated on-line with the Molinspiration Property Calculation Service (http://www.molinspiration.com). logS calculations: ALOGpS and AClogS calculated on-line with the ALOGPS 2.1 web applet[2] (http:// http://www.vcclab.org/lab/alogps/). [b] From reference [3b]. [c] Only a low limit for the EC_{50} could be experimentally determined. [d] Calculated from compounds with a defined pEC_{50} value (ie. **1a**, **56**, **3**, **48**, **13**, **59**, **44**, **45**, **15**, **47** and **12**).

Table S3. Structures of 5β-steroids with their activities expressed as pEC_{50} (ie. -log EC_{50} (M)), logP and logS (log water solubility (M)) molecular descriptors calculated by different methods,[a] and calculated squared correlation coefficient (r^2) of activity vs. each descriptor.



	X, Y	pEC_{50}	cLogP	VG	KLOP	PHYS	ALOGPs	AC_logP	ALOGP	MLOGP	KOWWIN	XLOGP2	XLOGP3	miLogP	Mean ±SD	ALOGpS	AClogS	Mean
1b		5.93 [b]	4.51	4.22	3.80	3.94	4.28	3.71	3.73	4.25	3.97	4.83	4.86	4.18	4.19 ±0.39	-5.37	-4.76	-5.07
38		5.92	5.29	4.74	4.81	4.84	4.80	5.14	4.60	4.69	4.78	6.14	5.20	5.15	5.02 ±0.42	-5.76	-6.39	-6.08
58		5.52	4.55	3.93	3.44	4.10	4.46	4.20	3.93	4.36	4.44	3.62	3.85	4.54	4.12 ±0.37	-4.34	-4.61	-4.48
37		4.85	4.17	2.94	2.72	2.83	2.93	2.67	3.61	3.74	3.02	4.03	3.42	3.49	3.30 ±0.52	-4.71	-4.79	-4.75
60		4.85	5.03	4.55	4.07	4.21	4.28	3.90	3.99	4.47	4.39	5.34	5.12	4.65	4.50 ±0.46	-5.54	-4.91	-5.23
57		4.43	4.55	4.15	3.63	4.00	3.60	3.93	3.79	4.36	4.39	4.42	4.30	4.49	4.13 ±0.34	-5.46	-4.82	-5.14
28		4.15	3.58	2.93	2.40	2.70	3.00	2.87	2.57	3.42	2.84	4.01	3.52	3.26	3.09 ±0.47	-3.84	-4.36	-4.10

32		4.12	4.76	2.95	3.05	2.95	2.90	2.47	4.66	4.07	3.19	4.05	3.33	3.72	3.51 ±0.74	-4.66	-5.22	-4.94
34		4.03	3.73	3.60	2.85	2.74	3.23	3.04	2.38	3.31	2.63	3.60	3.24	3.07	3.12 ±0.42	-4.14	-4.41	-4.28
33		<4.0 [c]	7.00	6.56	7.23	6.98	5.71	7.41	6.63	5.85	6.72	8.26	6.88	7.05	6.86 ±0.67	-5.66	-8.43	-7.05
31		<3.5 [c]	3.58	2.93	2.40	2.70	3.00	2.87	2.57	3.42	2.84	4.01	3.52	3.26	3.09 ±0.47	-3.84	-4.36	-4.10
	r^2 [d]		0.33	0.42	0.51	0.58	0.71	0.54	0.23	0.47	0.50	0.33	0.50	0.50	0.56	0.32	0.24	0.34

[a] logP calculations: clogP calculated with Sybyl 8.0 (Tripos International); VG, KLOP and PHYS logP values calculated with Marvin 5.11.4 (2012, ChemAxon, http://www.chemaxon.com); ALOGPs, AC_logP, ALOGP, MLOGP, KOWWIN, XLOGP2 and XLOGP3 calculated on-line with the ALOGPS 2.1 web applet[2] (http:// http://www.vcclab.org/lab/alogps/); miLogP calculated on-line with the Molinspiration Property Calculation Service (http://www.molinspiration.com). logS calculations: ALOGpS and AClogS calculated on-line with the ALOGPS 2.1 web applet[2] (http:// http://www.vcclab.org/lab/alogps/). [b] From reference [3b]. [c] Only a low limit for the EC_{50} could be experimentally determined. [d] Calculated from compounds with a defined pEC_{50} value (ie. **1b**, **38**, **58**, **37**, **60**, **57**, **28**, **32** and **34**).

3D-QSAR analysis

The structures of the 5α- and 5β-steroids were respectively aligned to the lowest energy conformations of allopregnanolone and pregnanolone (Figures S1 and S2), previously determined by conformational analysis, and the best global alignment of the two series was also determined.

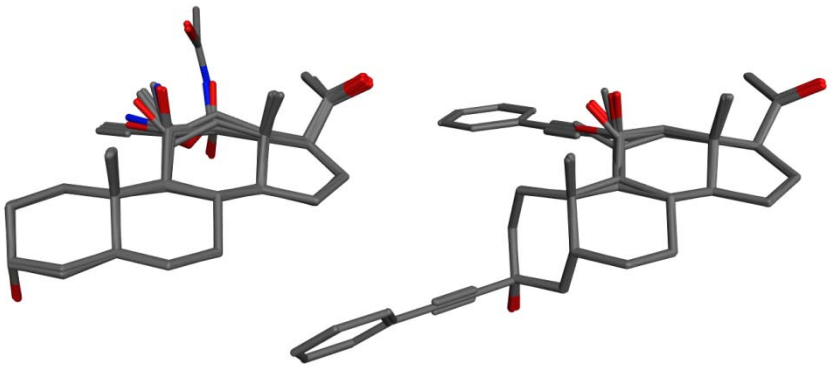


Figure S1. Alignments of the 5α- and 5β-neurosteroid analogs (left and right, respectively).

Attempts to obtain a CoMFA or CoMSIA model using the global alignment of all the compounds were unsuccessful (i.e. statistical parameters showed low significance, results not shown), as expected considering the hypothesis that 5α- and 5β-neurosteroids could have different binding-sites at the $GABA_A$ receptor.[3] Poor results were also obtained when using only the compounds from the 5β-series or the CoMFA fields for compounds of the 5α-series (results not shown). However, good COMSIA models (Table S2) were obtained when using the electrostatic and hydrophobic fields of the compounds from the 5α-series **1a**, **3**, **12**, **13**, **15**, **24**, **39**, **44**, **45**, **47**, **48**, **49**, **56**, and **59**.

Hence, CoMSIA models -1 and -2 showed statistically significant leave-one-out q^2 values of 0.61 (1 Principal Component, PC) and 0.67 (2 PC), respectively. Further crossvalidation with 5 random groups yielded q^2 values of 0.57 and 0.60, while non-crossvalidated PLS resulted on r^2 values of 0.82 and 0.87, respectively, indicating a good correlation between the observed and calculated EC_{50} values, particularly for model CoMSIA-2 (Figure S2). Taking also into account the steric and/or donor fields did not result in any improvement of the models, while considering the acceptor field resulted on similar or slightly improved models (CoMSIA-3, -4 and -5, see Table S2). However, analysis of the acceptor field contribution indicated that this improvement was most likely derived from overfitting the data.

Table S4. Statistical parameters of the different CoMSIA models built from the alignment of compounds **1a**, **3**, **12**, **13**, **15**, **24**, **39**, **44**, **45**, **47**, **48**, **49**, **56** and **59**.

	CoMSIA-1	CoMSIA-2	CoMSIA-3	CoMSIA-4	CoMSIA-5
q^2 (LOO) [a]	0.61	0.65	0.60	0.69	0.72
PRESS (LOO) [b]	0.68	0.67	0.69	0.64	0.64
PC [c]	1	2	1	2	3
q^2 (CV) [d]	0.57 ± 0.07	0.60 ± 0.04	0.52 ± 0.12	0.63 ± 0.05	0.70 ± 0.07
PRESS (CV) [e]	0.71 ± 0.06	0.72 ± 0.03	0.75 ± 0.09	0.69 ± 0.05	0.65 ± 0.08
SEE [f]	0.47	0.41	0.39	0.32	0.29
r^2	0.82	0.87	0.87	0.92	0.94
F-value	53.6	36.5	79.8	63.4	53.6
Fraction:					
Hydrophobic	0.35	0.40	0.25	0.27	0.27
Electrostatic	0.65	0.60	0.45	0.42	0.42
Acceptor			0.30	0.31	0.31

[a] Leave-one-out q^2. [b] Leave-one-out predictive error sum of squares. [c] Number of principal components. [d] Average q^2 from 10 runs of random groups cross validation (5 groups). [e] Average predictive error sum of squares from 10 runs of random groups cross validation (5 groups). [f] Standard error of estimate.

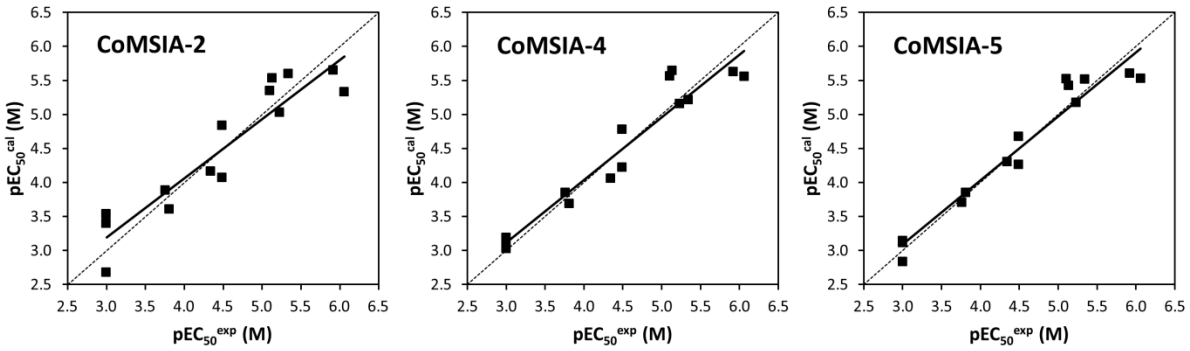


Figure S2. Correlations between experimental and calculated EC_{50} values (expressed as $pEC_{50} = -\log EC_{50}$ in molar units) for the best CoMSIA models.

Computational Methods

Molecular modelling and structural alignment. Compounds were built in their neutral state (i.e. unprotonated amine groups for compounds **24**, **47** and **48**) and energy minimized using the program suite MOE.[4] The implemented MMFF94s force field[5] was used for all energy calculations. Conformational searches were carried out using the LowModeMD method[6] implemented in the same program with its default parameters. Compounds were structurally aligned using the lowest energy conformations of allopregnanolone and pregnanolone as templates for the 5α- and 5β-series, respectively, and the Flexible Alignment application implemented in MOE. This alignment algorithm flexibly aligns molecules by maximizing steric and feature overlap while minimizing internal ligand strain.[7] Each alignment is given a score that quantifies the quality of the alignment as the sum of the average strain energy of the molecules (calculated as the sum of the individual forcefield potential energies divided by the number of molecules) plus a similarity measure of the alignment (calculated as a *P*-density overlap function that scores the degree of feature overlap among compounds). Thus, parameters were adjusted to achieve the best superposition of the steroid skeleton, as well as the hydrogen bond donor and acceptor groups. The best scored alignments were then refined with the same algorithm but this time allowing the free movement of all the compounds, including the templates.

Dataset for 3D-QSAR. The compounds from the 5α-series **1a**, **3**, **12**, **13**, **15**, **24**, **39**, **44**, **45**, **47**, **48**, **49**, **56** and **59** were chosen as training-set to develop the 3D-QSAR models. Compound **19** was excluded from this study since it was only tested at concentrations up to 100 μM and its EC_{50} could not be determined. Compounds **24**, **39** and **49** were arbitrarily assigned an EC_{50} value of 1 mM (other values were tried up to 10 mM that yielded similar results) based on the experimentally determined low limit (>300 μM) for their real EC_{50}. Given the relatively small number of compounds, a test-set to carry out an external validation of the models could not be selected and only an internal validation of the models was carried out (see below).

3D-QSAR methodology.

Generation of CoMFA and CoMSIA fields. The program Sybyl[8] and the aligned compounds with MMFF94s partial charges were used for the 3D-QSAR analysis. Potential fields for CoMFA (steric and electrostatic) and CoMSIA (steric, electrostatic, hydrophobic, donor and acceptor) were calculated using the default Sybyl settings. Thus, a 3D-cubic grid with 1 Å grid spacing, enclosing the aligned compounds and extending 4 Å beyond any of their atoms, was defined. The steric and electrostatic CoMFA fields were calculated from the Lennard-Jones and coulombic terms of the interaction potential with the default sp3 charged C^+ atom probe. The CoMSIA fields were derived according to Klebe et al.[1b] using the default probe with charge +1, radius 1 Å, hydrophobicity +1 and H-bond donor and acceptor property +1.

PLS-based CoMFA and CoMSIA model derivation. Model derivation employing partial least-squares (PLS) regression was carried out as implemented in Sybyl. Leave-one-out (LOO) cross-validation, as determined by the SAMPLS method,[9] was used for the fast determination of statistical significance (i.e. q^2 and standard error of prediction, PRESS) of different combination of fields and parameters. Random groups cross validation using 5 groups was repeated 10 times and average statistical values were calculated to further assess the robustness and statistical confidence of the best models. The final non cross-validated models were developed using the optimal number of components that had both the highest q^2 and smallest PRESS values. Quality of these models was assessed through calculation of their statistical parameters (i.e. squared correlation coefficient (r^2), standard error of estimate (SEE) and F-value).

References

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