Synthesis of New Molecular Probes for Investigation of Steroid Biosynthesis Induced by Selective Interaction with Peripheral Type Benzodiazepine Receptors (PBR)[†]

Giuseppe Campiani,*[,‡] Anna Ramunno,[‡] Isabella Fiorini,[‡] Vito Nacci,[‡] Elena Morelli,[§] Ettore Novellino,[§] Mara Goegan,[||] Tiziana Mennini,[||] Stephen Sullivan,[⊥] Daniela M. Zisterer,[⊥] and Clive D. Williams[⊥]

Dipartimento Farmaco Chimico Tecnologico, Universita' degli Studi di Siena, via Aldo Moro, 53100 Siena, Italy, Dipartimento di Chimica Farmaceutica e Tossicologica, Universita' degli Studi di Napoli "Federico II", via D. Montesano 49, 80131 Napoli, Italy, Istituto di Ricerche Farmacologiche "Mario Negri", via Eritrea 62, 20157 Milano, Italy, and Department of Biochemistry, Trinity College, Dublin 2, Ireland

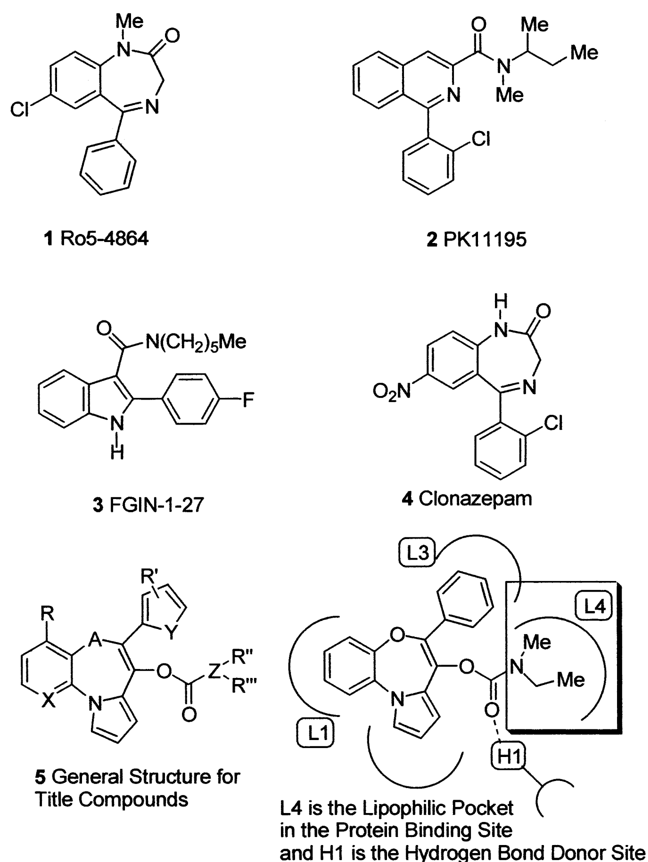
Received February 13, 2002

In the present study, we have synthesized and tested novel pyridopyrrolo- and pyrrolobenzoxazepine derivatives, as novel and selective peripheral type benzodiazepine receptor (PBR) ligands, and their ability to modulate steroid biosynthesis has been investigated. A subset of new ligands bind the PBR (rat brain and testis) with picomolar affinity, representing the most potent ligands that have been identified to date, and elicited effects on endogenous rate of steroidogenesis in MA10 Leydig cells, having similar potency and effect as PK11195. Several compounds, differently substituted at C-7, were used as molecular yardsticks to probe the spatial dimension of the lipophilic pocket L4 in the receptor binding site.

Introduction

In humans, the peripheral-type benzodiazepine receptor (PBR) is almost ubiquitous, with high levels of expression in endocrine tissues and in the central nervous system (CNS).[1,2] Its subcellular localization is mainly mitochondrial, on the outer mitochondrial membrane,[3,4] and is not associated to γ-aminobutyric acid (GABA) receptors. Some benzothiazepine derivatives,[5] the benzodiazepine Ro5-4864 (**1**),[6a] the isoquinoline PK11195 (**2**),[6b] and the indole FGIN-1-27 (**3**),[6c] are selective for the PBR, whereas the prototypic benzodiazepine derivative clonazepam (**4**) exhibits high affinity and selectivity for central benzodiazepine receptors (Chart 1). Among the different mechanisms in which PBR is involved, the regulatory effect on steroid biosynthesis is probably the most intriguing aspect. The PBR, in fact, has been claimed to play a crucial role in steroid biosynthesis regulating the translocation of cholesterol,[7a,b] and high affinity ligands, such as PK11195, facilitate the transport of cholesterol from the outer to the inner mitochondrial membrane, increasing the rate of the pregnenolone synthesis: this is an intriguing aspect, taking into account the possible therapeutic applications.[7c] The initial step of the steroid biosynthetic process is the conversion of cholesterol to pregnenolone. The reaction involved the cytochrome P-450 side chain cleavage ($P450_{scc}$), an enzyme localized at the inner mitochondrial membrane level. The rate-limiting step of the whole process is the translocation of cholesterol, the substrate of the $P450_{scc}$, inside the mitochondria. Neurosteroids[8] (3β-hydroxy-5-androsten-17-one, pregnenolone sulfate, 3α-hydroxy-5α-pregnan-20-one, 5α-pregnane-3α,21-diol-20-one or THDOC, and 3α5βPC[9a]) have received recent wide attention because of their endogenous presence in the CNS[9b] and their modulatory effects on memory, learning, and emotional processes. Neuroactive steroids can positively or nega-

Chart 1



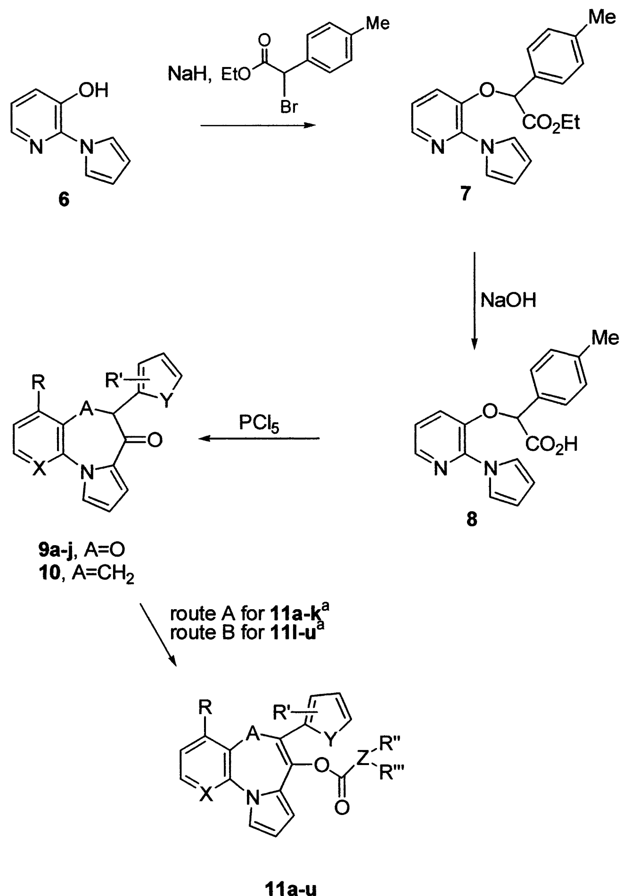
* To whom correspondence should be addressed. Tel: 0039-0577-234172. Fax: 0039-0577-234333. E-mail: campiani@unisi.it.

† In honor of Raffaele Giuliano's 90th birthday.

‡ Universita' degli Studi di Siena.
§ Universita' degli Studi di Napoli "Federico II".
|| Istituto di Ricerche Farmacologiche "Mario Negri".
⊥ Trinity College.

10.1021/jm020849l CCC: $22.00 © 2002 American Chemical Society
Published on Web 08/20/2002

Scheme 1[a]



[a] Route A: KH, triphosgene, THF, R″R‴NH, room temperature. Route B: KH, THF, R″R‴ZCOCl, room temperature.

tively modulate the $GABA_A$ receptor function and NMDA receptor function.[11a,b] In the present work, we investigated the role played by PBR through the synthesis of potent and selective pyridopyrrolo- and pyrrolobenzoxazepines as high affinity ligands for PBR. Several analogues showed K_i values in the low nanomolar range, and the oxazepines **11a**,**b**,**r** were found to be among the most potent PBR ligands developed to date (K_i = 110, 180, and 260 pM, respectively). A subset of potent ligands (**11a**,**g**–**j**,**p**) was further tested on mitochondrial suspension of rat testes and was subjected to in vitro assays to prove the ability of the new compounds to promote steroid biosynthesis in MA10 Leydig cells and to test the hypothesis of a direct role of PBR in this process. The structure–activity relationships (SAR) are discussed taking into account our previously developed pharmacophore model.[5] Three lipophilic pockets and a hydrogen bond donor site are available to our ligands at the binding site level. To gain further insights into the topology of the PBR binding site, in the present work, we discuss the development of ligands for optimum binding to the receptor subsite L4 (Chart 1).

Chemistry

The target compounds **11a**–**u** were synthesized as shown in Scheme 1. The new pyridopyrrolooxazepinone **9i** was synthesized as shown in Scheme 1 following the procedure reported for already known oxazepinone intermediates **9a**–**h**,**j**.[10] Compound **10** was obtained according to ref 10c. 2-(1H-Pyrrol-1-yl)-3-pyridinol **6**[10b] was converted into the ester **7** by O-alkylation with the appropriate α-bromoaryl acetate. Hydrolysis (**8**) followed by an intramolecular Friedel–Crafts cyclization reaction gave the pyridooxazepinone **9i**. Treatment of the corresponding potassium enolate of ketones **9** with triphosgene and successively with suitable amines provided the oxazepines **11a**–**k**. Compounds **11l**,**n**–**s**,**u** were obtained in good yields by treatment of the corresponding potassium enolate of the ketones **9a**,**b**,**e**,**g**–**i** and **10** with the appropriate *N*,*N*-dialkylcarbamyl chloride, while the enol esters **11m**,**t** were obtained by using acetyl chloride.

Biological Studies

In Vitro SAR Study. The affinity of the new compounds **11a**–**u** for the PBR in rat cortex homogenate is illustrated in Table 1. Binding studies for a subset of compounds (**11a**,**g**–**j**,**p**), using [^{3}H]PK11195 and [^{3}H]-Ro5-4864, were also carried out using rat testicular mitochondria, and the results are summarized in Table 2. Selected oxazepines were found to be inactive on $GABA_A$ and central benzodiazepine receptors (not active at 10^{-5} M concentration, data not shown). We have previously reported an extensive SAR study on a benzothiazepine class of PBR ligands,[5] and later, we have discussed the improved PBR affinity of benzoxazepine analogues.[10a] In general, the most active compounds were characterized by a phenyl ring at C-6 and symmetric dimethyl/diethylcarbamoyl moieties at C-7.[10a] A pharmacophore model, previously described and refined by us, consists of the following features: two aromatic rings (L1 and L3 sites) and a receptor hydrogen bond donor site (H1).[5c] The presence of an additional lipophilic region L4 was also previously deduced from SAR analysis on the small set of benzoxazepines initially synthesized.[10a] Though modifications at the L3 site were extensively investigated with the synthesis of a number of benzothiazepines and benzoxazepines, the L1 site and the influence on affinity of different alkylcarbamoyl groups at position 7 (L4 site) remained to be investigated. Accordingly, to assess the predictivity of the previously described three-dimensional (3D) pharmacophore model (L4 site)[5d,10a] and to improve the inhibitory potency of the [^{3}H]PK11195 binding, in the present work, the SAR study was carried on as a function of (i) the nature of the substituents at position 7 (L4 site),[5d,10a] (ii) the nature of the aromatic system at C-6, and (iii) the modification of the aromatic-fused system. Furthermore, the effect of the replacement of the oxygen-bridged atom by a methylene was evaluated.

Effect of "Unsymmetrical" Dialkycarbamoyl Substituents at C-7 (L4 Site). In previous studies, we have observed that dimethyl- (IC_{50} = 5.1 nM), diethyl- (IC_{50} = 0.7 nM), and diisopropylcarbamoyl derivatives (IC_{50} = 8 nM) showed nanomolar affinity for PBR with the following rank order of magnitude: diEt > diMe > di*i*-Pr.[5] The ethyl and the *i*-propyl groups attached to carbamoyl nitrogen lie apart in order to minimize intramolecular steric repulsion, and the increased affinity of the diethylcarbamoyl derivative, with respect to the dimethyl analogue, could be explained hypothesizing that at least one of the two *N*-ethyl fragments is positioned in a lipophilic subsite of the PBR (L4

Table 1. Receptor Binding Affinity of Compounds **11a**–**u** Calculated on Rat Cortex Homogenate

compd	X	A	Y	Z	R	R′	R″	R‴	K_i (nM)[a]
11a	CH	O	CH=CH	N	H	H	H	Et	0.11 ± 0.02
11b	CH	O	CH=CH	N	Cl	H	H	Et	0.18 ± 0.01
11c	CH	O	CH=CH	N	H	H	Me	Et	0.78 ± 0.31
11d	CH	O	CH=CH	N	H	H	Me	*n*-Pr	0.69 ± 0.2
11e	CH	O	CH=CH	N	H	H	H	*n*-Pr	0.91 ± 0.11
11f	CH	O	CH=CH	N	H	H	Me	*n*-Bu	0.92 ± 0.16
11g	CH	O	CH=CH	N	H	H	H	-CH(CH_2)$_2$	2.21 ± 0.73
11h	CH	O	CH=CH	N	H	H	H	Me	2.26 ± 0.26
11i	CH	O	CH=CH	N	H	H	-	-(CH_2CH_2)$_2$NMe	7.20 ± 0.03
11j	CH	O	CH=CH	N	H	H	-	-(CH_2)$_3$	6.03 ± 0.2
11k	CH	O	CH=CH	N	H	*p*-CH_3	H	Et	0.68 ± 0.05
11l	CH	O	CH=CH	N	H	*m*-CH_3	Et	Et	1.20 ± 0.15
11m	CH	O	CH=CH	CH	H	*m*-CH_3	H	H	2.50 ± 0.85
11n	CH	O	2-S	N	H	H	Et	Et	11.5 ± 4.9
11o	CH	O	CH=CH	N	H	3,5-diF	Et	Et	1.26 ± 0.26
11p	N	O	CH=CH	N	H	H	Et	Et	1.77 ± 0.54
11q	N	O	CH=CH	N	H	*m*-CH_3	Et	Et	0.56 ± 0.1
11r	N	O	2-S	N	H	H	Et	Et	0.23 ± 0.07
11s	N	O	CH=CH	N	H	*p*-CH_3	Me	Me	0.44 ± 0.02
11t	N	O	CH=CH	CH	H	*p*-CH_3	H	H	13.5 ± 1.3
11u	CH	CH_2	CH=CH	N	H	H	Et	Et	5.7 ± 0.89

[a] The concentration of tested compounds that inhibited [^{3}H]PK11195 binding to rat cortex homogenate by 50% was determined with 8–12 concentrations of the displacers, each performed in triplicate. Values are the mean ± SEM of at least three separate experiments.

Table 2. K_i Values for Tested Compounds **11a**,**g**–**j**,**p** for Displacing [^{3}H]PK11195 and [^{3}H]Ro5-4864 from Mitochondrial Rat Testis PBR[a] and Their Stimulatory Effect on Progesterone Biosynthesis in MA10 Leydig Cells

compd	X	Z	R″	R‴	K_i (nM) (±SEM)		MA10 Leydig cells
					[^{3}H]Ro5-4864	[^{3}H]PK11195	progesterone production (ng/2 h/1 × 10^5 cells)
11a	CH	N	H	Et	0.10 ± 0.05	0.20 ± 0.14	3.3 ± 0.4
11g	CH	N	H	CH(CH_2)$_2$	0.47 ± 0.16	0.31 ± 0.12	3.5 ± 0.2
11h	CH	N	H	Me	0.76 ± 0.62	0.96 ± 0.73	3.9 ± 0.4
11i	CH	N	(CH_2CH_2)$_2$NMe		15 ± 8	29 ± 19	3.8 ± 0.3
11j	CH	N	(CH_2)$_3$		4.3 ± 3.5	6.0 ± 5.1	3.5 ± 0.2
11p	N	N	Et	Et	22 ± 7	11 ± 3	3.5 ± 0.2
control							2.1 ± 0.1
clonazepam							2,3 ± 0.2
PK11195							3.4 ± 0.1

[a] K_i values represent the mean ± SEM of three determinations. The K_i values for each compound were generated from the IC_{50} values by the computer programs EBDA and LIGAND. The values for progesterone production represent the mean ± SEM of three determinations. For comparison purposes, the effect of PK11195 on steroid production at a concentration of 10 μM is also included.

site).[5d] The drop in affinity observed with the *N,N*-diisopropyl groups could be related to steric bulk. According to these results, in the present paper, we have synthesized and tested a series of compounds unsymmetrically disubstituted or monosubstituted at the carbamoyl nitrogen at C-7. Introduction of a monomethyl group (**11h**, K_i = 2.2 nM) provided an oxazepine analogue similarly potent to previously described dimethylcarbamoyl analogues (K_i = 2 nM).[10] Replacement of the methyl by an ethyl chain determined a 20-fold improvement on affinity, providing one of the most potent PBR ligands described to date (**11a**, K_i = 110 pM) (NHEt > N(Et)$_2$ ≫ NHMe ≥ N(Me)$_2$). Insertion of a chlorine atom at C-4 (**11b**) did not increase affinity, as previously seen in the diethylcarbamoyl series,[5,10a] but provided a compound equipotent to **11a** (**11b**, K_i = 180 pM). Among the different carbamoyl nitrogen substituents, the ethyl group was found to be optimal for PBR affinity, fitting a relatively large lipophilic receptor domain L4. Replacing the N-hydrogen atom of **11a** by a methyl group provided the high affinity ligand **11c**. Anyway, despite what was observed with previously described "symmetric" dialkylcarbamoyl derivatives, by increasing the hindrance with larger lipophilic chains, such as *n*-propyl or *n*-butyl (**11d**–**f**, K_i = 0.69, 0.91, and 0.92 nM, respectively), only a slight (but progressive) drop in PBR affinity was observed in the new series (**11e**,**f** vs **11a**,**b**). The *n*-butylmethyl analogue **11f** is by far more potent than the dipropylcarbamoyl analogue previously described.[5] The extra affinity showed by **11f** is probably linked to the possibility for the longer alkyl chain to assume a partially folded arrangement corresponding to an optimum of ligand–receptor shape complementarity. Positioning of a cyclic substituent such as a cyclopropyl group or introduction of a constrained four-membered heterocyclic ring determined a drop in affinity (**11g**,**j**, K_i = 2.21 and 6.03 nM, respectively). The effect of substituents on the phenyl group at C-6 (**11k**,**l**,**o**) and the effect of a different electron-rich system at C-6, such as a 2-thienyl group (**11n**), was also evaluated. In the *m*-tolyl series (**11l**,**m**), the introduction of an ester moiety provided similarly active compounds. Lower affinity was also found for the 3,5-difluoro analogue **11o**. Positioning of the methyl group in para position provided a monosubstituted analogue (**11k**, K_i = 0.68 nM) slightly less potent than **11a**.

Pyrido-Fused vs Benzo-Fused Analogues (L1 Site). Effect on PBR Affinity. The introduction of an extra nitrogen on the benzo-fused ring of our benzoxazepines is well-tolerated and led to a new class of potent PBR ligands based on a pyridopyrrolooxazepine skeleton (**11p**–**t**). The presence of an endocyclic nitrogen

does not introduce changes in the overall 3D arrangement of the tricyclic system and does not disfavor interactions involving other parts of the ligand. The lower affinity of the diethylcarbamoyl derivative (**11p**, K_i = 1.8 nM) with respect to the benzoxazepine analogue (K_i = 0.3[10a]) and the higher potency of the thienyl analogue **11r** with respect to **11n** ($K_{i\mathbf{11r}}$ = 0.23 nM vs $K_{i\mathbf{11n}}$ = 11.5 nM) could be explained by assuming a slightly different binding mode of this series of ligands within the PBR binding site probably due to extra interactions of the heterocyclic nitrogen atom.

Azepine-Based PBR Ligands vs Oxazepines. Previously,[10a] a comparison of the IC_{50} values relative to some pairs of benzoxazepines and benzothiazepines revealed that replacement of the bridged sulfur by an oxygen increased the PBR affinity by 2–3-fold. Because the order of magnitude was not large enough to account for a different ability for O/S to give hydrogen bonding with the receptor site, we investigated the effect on PBR affinity of the introduction of a methylene-bridged system. Replacement of the O-bridged atom by a methylene group (**11u**, K_i = 5.7 nM) caused a 20-fold drop in affinity with respect to the oxazepine counterpart (K_i = 0.3 nM[10a]). The lower affinity of the benzazepine analogue is mainly due to a partial rotation of the phenyl ring at C-6 (about 30 degrees) in order to minimize steric repulsions, induced by the presence of the hydrogens of the methylene-bridged group.

Binding studies for a subset of compounds (**11a**,**g**–**j**,**p**), using [^{3}H]PK11195 and [^{3}H]Ro5-4864, were also carried out using rat testicular mitochondria (Table 2). In these experiments, the tested compounds showed a different behavior confirming slight differences among PBR receptors in different tissues. The benzoxazepine analogue **11a** was confirmed as the most potent PBR ligand described to date ($K_{i\,PK11195}$ = 100 pM and $K_{i\,Ro5-4864}$ = 200 pM). Taking into account the activity of compounds **11e**,**f** that proved a steric tolerance at the L4 subsite, to further explore any specific interaction at this lipophilic subsite, we synthesized compound **11i**, which bears an extra basic center, namely, the distal protonatable nitrogen of the piperazine ring. Accordingly, it is noteworthy that the binding affinity was displayed by **11i** in both tissues. This compound showed a nanomolar affinity for central and peripheral PBR, indicating that the receptor may tolerate an extra basic function, which is capable of giving rise to a favorable interaction with **11i**.

Modulation of Steroidogenesis in MA10 Leydig Cells. Investigations were carried out to see whether this subset of PBR ligands elicited effects on the endogenous rate of steroidogenesis in MA10 Leydig cells. Both PK11195 and Ro5-4864 have previously been shown to stimulate steroid production in MA10 Leydig cells[7] and to elicit similar effects in Y-1 cells, in C6-2B glioma cells, and in freshly isolated rat adrenal cells, albeit at much higher concentrations than theoretically required to saturate the PBR. All of the derivatives tested caused a 1-fold increase in progesterone production over the endogenous levels when MA10 Leydig cells were incubated for 2 h in the presence of 10 μM concentrations of the tested compounds (Table 2). The stimulation of the endogenous steroidogenic rate observed with 10 μM tested compounds was not significantly different from that seen with an identical concentration of PK11195 and the centrally acting clonazepam (Table 2). These data confirm that the high affinity new pyridopyrrolo- and pyrrolobenzoxazepine derivatives and other related PBR ligands modulate the endogenous rate of steroidogenesis at micromolar concentrations. Evaluation of in vivo activity of these novel PBR ligands will be the object of future work.

Conclusions

In summary, we have described herein a further development of benzoxazepine derivatives and their SARs for PBR affinity, investigating the topography of the L4 receptor subsite. Starting from these compounds, a new class of potent ligands was identified, based on a pyridopyrrolooxazepine skeleton. Among the synthesized compounds, analogues **11a**,**b**,**r** showed picomolar affinity for rat brain and mitochondrial rat testis PBR. A subset of these compounds caused a stimulation of steroidogenesis in MA10 Leydig cells, similar to the effect caused by PK11195 and Ro5-4864, thus demonstrating the biological efficacy of these novel PBR ligands.

Experimental Section

For general experimental information, see ref 10.

General Procedure for Preparation of Compounds 11a–k. This procedure is illustrated for the preparation of 6-phenylpyrrolo[2,1-*d*][1,5]benzoxazepin-7-yl ester of cyclopropylcarbamic acid (**11g**). To a suspension of potassium hydride (76 mg, 0.66 mmol, 35% in oil) in dry tetrahydrofuran (THF, 1 mL) was added a solution of ketone **9a** (183 mg, 0.66 mmol) in dry THF (1 mL). The reaction mixture was stirred at room temperature for 1 h and then was added dropwise to a solution of triphosgene (180 mg, 0.66 mmol) in dry THF (1 mL). After the mixture was stirred at room temperature for 5 min, cyclopropylamine (90 μL, 1.3 mmol) and *N*,*N*-diisopropylethylamine (226 μL, 1.3 mmol) were added and the mixture was stirred for an additional 10 min, and then, 5% $NaHCO_3$ solution (1 mL) was added. The mixture was concentrated in vacuo, then the residue was taken up in dichloromethane, and the organic phase was washed with brine, dried, and evaporated. The residue was purified by flash chromatography using dichloromethane as eluent and recrystallized to yield **11g** as colorless prisms. IR (KBr): 1770 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.51 (s, 2H), 0.71 (d, 2H, J = 6.0 Hz), 2.62 (br s, 1H), 5.28 (br s, 1H), 6.43 (m, 2H), 7.21–7.37 (m, 8H), 7.79 (d, 2H, J = 7.4 Hz).

General Procedure for Preparation of Compounds 11l–u. This procedure is illustrated for the preparation of 6-phenylpyrido[3,2-b]pyrrolo[1,2-*d*][1,4]oxazepin-7-yl ester of diethylcarbamic acid (**11p**). To a suspension of potassium hydride (115 mg, 1.00 mmol, 35% in oil) in dry THF (1 mL) was added a solution of ketone **9d** (250 mg, 0.90 mmol) in dry THF (2 mL). The reaction mixture was stirred at room temperature for 2 h, and then, diethylcarbamyl chloride (139 μL, 1.10 mmol) was added. After it was stirred overnight at room temperature, the solvent was removed in vacuo and the residue was taken up in dichloromethane. The organic layer was washed with NH_4Cl and brine, dried, and concentrated. The residue was purified by flash chromatography using petroleum ether (40–60 °C)/chloroform (9:1) and recrystallized to give **11p** as colorless prisms. IR (KBr): 1650 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.16 (t, 6H, J = 7.1 Hz), 2.35 (s, 3H), 3.38 (q, 4H, J = 7.1 Hz), 6.40 (m, 2H), 7.13 (m, 1H), 7.28 (m, 2H), 7.57 (m, 1H) 7.69 (m, 1H), 8.22 (d, 1H, J = 4.9 Hz).

Binding Assays. A. Binding Affinity on Rat Cortex Homogenate. Male CRL:CD(SD)BR rats (Charles River Italia, Calco, Italy), weighing about 150 g, were used in these experiments. Procedures involving animals and their care were

conducted in conformity with the institutional guidelines that are in compliance with national (D:L: n. 116, G. U., suppl. 40, Feb.18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec.12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Before they were killed by decapitation (unanesthetized), the rats hed been housed in groups of five in plastic cages, kept under standard conditions (room temperature, 21 ± 1 °C; relative humidity, 55 ± 10%; 12/12 h light−dark cycle), and given tap water and food pellets ad libitum. After they were decapitated, the brains were rapidly removed from the skulls and the cortex was dissected out, frozen on dry ice, and stored at −80 °C until used. Cortices were homogenized in about 50 volumes of ice cold phosphate-buffered saline, 50 mM, pH 7.4, using an Ultra Turrax TP-1810 (2 × 20 s) and centrifuged at 50 000*g* for 10 min. The pellet was then washed three times more by resuspensions in fresh buffer and centrifugations as before. The last pellet was resuspended just before the binding assay.

For binding assay, 10 mg of original wet tissue weight was incubated with 1 nM [^{3}H]PK11195 (sa 85.8 Ci/mmol; NEN) in 1 mL (final volume) for 120 min at 4 °C in the presence of 8−12 increasing concentrations of drugs. Nonspecific binding was determined by using 1 μM clonazepam.

Incubation was stopped by rapid filtration under vacuum through GF/B fiber filters, which were then washed with 12 mL of ice-cold buffer and counted in 4 mL of Ultima Gold MV (Packard) in a liquid scintillation spectrometer WALLAC 1409, with a counting efficiency of about 56%. IC_{50} values were determined by nonlinear fitting of binding inhibition curves, using the Allfit program running on an IBM AT personal computer. The K_i values were derived from the IC_{50} values.[11] Each point was the mean of triplicate samples.

Rat Testicular Mitochondrial Preparation and [^{3}H]-PK11195 and [^{3}H]Ro5-4864 Binding. For binding studies, mitochondria were prepared from the testes of Wistar rats (200−250 g) killed by cervical dislocation. Testes were removed and homogenized in buffer A (50 mM Tris/Cl, pH 7.4, containing 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA) with a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 600*g* for 10 min, and the resulting supernatant was then centrifuged at 10 000*g* for 10 min. The mitochondrial pellet was then suspended in buffer A and centrifuged at 10 000*g* for a further 10 min. The resulting washed mitochondrial pellet was resuspended in 50 mM Tris/Cl, pH 7.4, at a concentration of 2 mg/mL as determined by the method of Markwell et al.,[12] for use in binding assays. All procedures were performed at 4 °C. Testicular mitochondria (20 μg of protein) were incubated with either [^{3}H]PK11195 (2 nM) or [^{3}H]Ro5-4864 (2 nM) (NEN) in 50 mM Tris/Cl, pH 7.4, with a range of concentrations of the tested compounds (0.1 nM−1 μM) dissolved in this buffer containing 0.1% ethanol, in a total volume of 0.5 mL at 4 °C. Total and nonspecific binding in each case were determined in the absence and presence, respectively, of unlabeled PK11195 and Ro5-4864 (1 μM). All samples were incubated in triplicate for 60 min. Assays were terminated by filtration through Whatman glass fiber filters (GF/B, 2.5 cm) using a Brandel cell harvester. Radioactivity trapped on the filters was determined by liquid scintillation counting. The IC_{50} values and subsequent K_i values for each compound were generated by the use of the computer programs EBDA and LIGAND.

Steroid Biosynthesis in MA10 Leydig Cells. The MA10 Leydig cells were gratefully obtained from Mario Ascoli, Department of Pharmacology, University of Iowa College of Medicine, Iowa. They were cultured in Waymouth's medium supplemented with horse serum (15% v/v) and gentamycin (50 mg/mL). Cultures were maintained in a humidified atmosphere at 5% CO_2/95% air at 37 °C. MA10 Leydig cells were seeded in 24 well plates at a density of 1 × 10^5 cells per well in a final volume of 0.5 mL. The test compounds, PK11195 and Ro5-4864, were then added to the cells at a final concentration of 10 μM. The final concentration of ethanol was constant for all of the wells in each experiment and did not exceed 1% (v/v), a concentration that on its own had no effect on steroid production. Following a 2 h incubation with the compounds, the incubation medium was acidified by the addition of perchloric acid (107 mM), which lysed the cells. The incubation plate was then frozen and thawed, and the contents were neutralized by the addition of K_3P0_4. The amount of progesterone produced by the cells was quantified by radioimmunoassay (RIA) using an antibody from ICN Biochemical Inc. (California), under the conditions described by the manufacturer. Analysis of the RIA data was performed using the Apple Macintosh ASSAYZAP program obtained from Biosoft.

Acknowledgment. We thank MURST-Rome, Italy (PRIN 99), for financial support.

Supporting Information Available: Experimental details (chemistry and pharmacology, Tables 1 and 2). This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Anholt, R. R. H.; De Souza, E. B.; Oster-Granite, M. L.; Snyder, S. H. Peripheral-Type Benzodiazepine Receptors: Autoradiographic Localisation in Whole-Body Sections of Neonatal Rats. *J. Pharmacol. Exp. Ther.* **1985**, *233*, 517−526.

(2) De Souza, E. B.; Anholt, R. R. H.; Murphy, K. M. M.; Snyder, S. H.; Kuhar, M. J. Peripheral-Type Benzodiazepine Receptors in Endocrine Organs: Autoradiographic Localisation in Rat Pituitary, Adrenal and Testis. *Endocrinology* **1985**, *116*, 567−573.

(3) Papadopoulos, V. Peripheral-Type Benzodiazepine/Diazepam Binding Inhibitor Receptor: Biological Role in Steroidogenic Cell Function. *Endocr. Rev.* **1993**, *14*, 222−240.

(4) Anholt, R. R. H.; Pedersen, P. L.; De Souza, E. B.; Snyder, S. H. The Peripheral-Type Benzodiazepine Receptor: Localisation to the Mitochondrial Outer Membrane. *J. Biol. Chem.* **1986**, *261*, 576−583.

(5) (a) Fiorini, I.; Nacci, V.; Ciani, S. M.; Garofalo, A.; Campiani, G.; Savini, L.; Novellino, E.; Greco, G.; Bernasconi, P.; Mennini, T. Novel Ligands Specific for Mitochondrial Benzodiazepine Receptors: 6-Arylpyrrolo[2,1-*d*][1,5]benzothiazepine Derivatives. Synthesis, Structure−Activity Relationships, and Molecular Modelling Studies. *J. Med. Chem.* **1994**, *37*, 1427−1438. (b) Greco, G.; Novellino, E.; Fiorini, I.; Nacci, V.; Campiani, G.; Ciani, S. M.; Garofalo, A.; Bernasconi; Mennini, T. A Comparative Molecular Field Analysis Model for 6-Arylpyrrolo[2,1-*d*][1,5]-benzothiazepines Binding Selectively to the Mitochondrial Benzodiazepine Receptor. *J. Med. Chem.* **1994**, *37*, 4100−4108. (c) Dalpiaz, A.; Bertolasi, V.; Borea, P. A.; Nacci, V.; Fiorini, I.; Campiani, G.; Mennini, T.; Manzoni, C.; Novellino, E.; Greco, G. A Concerted Study Using Binding Measurements, X-ray Structural Data, and Molecular Modeling on the Stereochemical Features Responsible for the Affinity of 6-Arylpyrrolo[2,1-*d*][1,5]-benzothiazepines Toward Mitochondrial Benzodiazepine Receptors. *J. Med. Chem.* **1995**, *38*, 4730−4738. (d) Campiani, G.; Nacci, V.; Fiorini, I.; De Filippis, M. P.; Garofalo, A.; Ciani, S. M.; Manzoni, C.; Mennini, T. New Pyrrolobenzothiazepine Derivatives as Molecular Probes of the "Peripheral-type" Benzodiazepine Receptor (PBR) Binding Site. *Eur. J. Med. Chem.* **1997**, *32*, 241−251.

(6) (a) Marangos, P. J.; Patel, J., Boulenger, J. P.; Clarke-Rosenberg, R. Characterization of Peripheral-Type Benzodiazepine Binding Sites in Brain Using [^{3}H]Ro5-4864. *Mol. Pharmacol.* **1982**, *22*, 26−32. (b) Le Fur, G.; Guilloux, F.; Rufat, P.; Benavides, J.; Uzan, A.; Renault, C.; Dubroeucq, M. C.; Guérémy, C. Peripheral Benzodiazepine Binding Sites: Effects of PK11195, 1-(2-Chlorophenyl)-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide I: In Vitro Studies. *Life Sci.* **1983**, *32*, 1849−1856. (c) Romeo, E.; Auta, J.; Kozikowski, A.; Ma, A.; Papadopoulos, V.; Puia, G.; Costa, E.; Guidotti, A. 2-Aryl-3-indoleacetamides (FGIN-1): a New Class of Potent and Specific Ligands for the Mitochondrial DBI Receptor. *J. Pharmacol. Exp. Ther.* **1992**, *262*, 971−978.

(7) (a) Papadopoulos, V.; Mukhin, A. G.; Costa, E.; Kreuger, K. E. The Peripheral-Type Benzodiazepine Receptor is Functionally Linked to Leydig Cell Steroidogenesis. *J. Biol. Chem.* **1990**, *265*, 3772−3779. (b) Papadopoulos, V.; Amri, H.; Li, H.; Boujrad, N.; Vidic, B.; Garnier, M. Targeted Disruption of the Peripheral-Type Benzodiazepine Receptor Gene Inhibits Steroidogenesis in the R2C Leydig Tumor Cell Line. *J. Biol. Chem.* **1997**, *272*, 32129−35. (c) Papadopoulos, V.; Amri, H.; Li, H.; Boujrad, N.; Vidic, B.; Garnier, M. Targeted Disruption of the Peripheral-Type Benzodiazepine Receptor Gene Inhibits Steroidogenesis in the R2C Leydig Tumor Cell Line. *J. Biol. Chem.* **1997**, *272*, 32129−32135 and references therein.

(8) Baulieu, E.-E.; Robel, P. J. Neurosteroids: a New Brain Functions? *J. Steroid Biochem. Mol. Biol.* **1990**, *37*, 395−403.

(9) (a) Tsutsui, K.; Ukena, K.; Usui, M.; Sakamoto, H.; Takase, M. Novel Brain Function: Biosynthesis and Action of Neurosteroids in Neurons. *Neurosci. Res.* **2000**, *36*, 261−273 and references therein. (b) Mennerick, S.; Zeng, C.-M.; Benz, A.; Shen, W.; Izumi, I.; Evers, A. S.; Covey, D. F.; Zorumski, C. F. Effects on γ-Aminobutyric Acid (GABAA) Receptors of a Neuroactive Steroid That Negatively Modulates Glutamate Neurotransmission And Augment GABA Neurotransmission. *Mol. Pharmacol.* **2001**, *60*, 732−741.

(10) (a) Campiani, G.; Nacci, V.; Fiorini, I.; De Filippis, M. P.; Garofalo, A.; Ciani, S. M.; Greco, G.; Novellino, E.; Williams, D. C.; Zisterer, D. M.; Woods, M. J.; Mihai, C.; Manzoni, C.; Mennini, T. Synthesis, Biological Activity, and SARs of Pyrrolobenzoxazepine Derivatives, a New Class of Specific "Peripheral-Type" Benzodiazepine Receptor Ligands. *J. Med. Chem.* **1996**, *39*, 3435−3450. (b) Campiani, G.; Morelli, E.; Fabbrini, M.; Nacci, V.; Greco, G.; Novellino, E.; Ramunno, A.; Maga, G.; Spadari, S.; Caliendo, G.; Bergamini, A.; Faggioli, E.; Uccella, I.; Bolacchi, F.; Marini, S.; Coletta, M.; Nacca, A.; Caccia, S. Pyrrolobenzoxazepinone Derivatives as Non-Nucleoside HIV-1 RT Inhibitors: Further Structure−Activity Relationship Studies and Identification of More Potent Broad-Spectrum HIV-1 RT Inhibitors with Antiviral Activity. *J. Med. Chem.* **1999**, *42*, 4462−4470. (c) Garofalo, A.; Ragno, G.; Campiani, G.; Brizzi, A.; Nacci, V. Polycondensed Heterocycles. Part 11: Preparation and Regioselective Reductions of 5-Phenyl-4*H*-pyrrolo[1,2-a][1]-benzazepin-4-one. *Tetrahedron* **2000**, *56*, 9351−9355.

(11) Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, 3099−3108.

(12) Markwell, M.; Haas, S. M.; Bieker, L.; Tolbert, N. A. A Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein Samples. *Anal. Biochem.* **1980**, *87*, 206−210.

JM020849L