Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2661–2671

BIOORGANIC & MEDICINAL CHEMISTRY

2-Arylpyrazolo[1,5-*a*]pyrimidin-3-yl Acetamides. New Potent and Selective Peripheral Benzodiazepine Receptor Ligands

Silvia Selleri,[a] Fabrizio Bruni,[a,*] Camilla Costagli,[a] Annarella Costanzo,[a] Gabriella Guerrini,[a] Giovanna Ciciani,[a] Barbara Costa[b] and Claudia Martini[b]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via G. Capponi 9, 50121 Firenze, Italy*
[b]*Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy*

Received 6 February 2001; accepted 23 May 2001

Abstract—A new class of *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamides (**3f**–**y**), as azaisosters of Alpidem, was prepared following a novel synthetic method and their affinities for both the peripheral (PBR) and the central (CBR) benzodiazepine receptors were evaluated. Binding assays were carried out using both [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 as radioligands for PBR, whereas [^{3}H]Ro 15-1788 was used for CBR, in rat kidney and rat cortex, respectively. The tested compounds exhibited a broad range of binding affinities from as low as 0.76 nM to inactivity and most of them proved to be high selective ligands for PBR. The preliminary SAR studies suggested some of the structural features required for high affinity and selectivity; particularly the substituents on the pyrimidine moiety seemed to play an important role in PBR versus CBR selectivity. A subset of the highest affinity compounds was also tested for their ability to stimulate steroid biosynthesis in C6 glioma rat cells and some of these were found to increase pregnenolone formation with potency similar to Ro 5-4864 and PK 11195.

Introduction

It is well known that, besides binding to the $GABA_A$ receptors in the central nervous system (CNS), benzodiazepines (BZs) also bind to various sites located in peripheral tissues. This second class of recognition sites is often called the peripheral benzodiazepine receptor (PBR), since it was originally discovered outside the CNS.[1] Other names have been used to describe this same class of drug binding sites: mitochondrial benzodiazepine receptor (MBR),[2,3] mitochondrial DBI receptor complex (mDRC),[4,5] ω3 receptor,[6] PBS,[7] and p-site.[8] The PBR is pharmacologically distinct from the central benzodiazepine receptor (CBR) which is associated with $GABA_A$-regulated ion-channel in the CNS.[9] Although the Bz GABA-independent receptor was identified in the CNS, mainly in the astrocytes,[10,11] it is particularly plentiful in endrocrine tissues:[12] testis, ovary, and adrenal cortex, but also in kidney, heart,[13] and erytrocytes.[14] Its subcellular location was reported to be mainly in the mitochondrial outer membrane, although non-mitochondrial distribution of the PBR has also been described in various tissues.[15] The PBR is designated as a receptor because of the binding of specific endogenous ligands implicated in several physiological functions,[16,17] including steroidogenesis,[18] calcium homeostasis,[19] cell growth and differentiation,[20] regulation of immune function (chemotaxis, apoptosis),[21,22] mitochondrial respiratory control,[23] and alteration of protooncogene expression.[24] Although the pharmacological role of this receptor remains to be fully clarified, some evidence indicates its involvement in important cellular functions such as the production of neurosteroids (pregnenolone, progesterone, dehydroepiandrosterone, their metabolites and sulfate esters).[25,26] Indeed the PBR, when occupied by the proper ligand, has been shown to facilitate the rate-limiting translocation of cholesterol from the outer to the inner mitochondrial membrane. In the mitochondria, cholesterol is converted by cytochrome $P450_{scc}$ into pregnenolone, which is the parent molecule of endogenous steroids.

It is well known that neurosteroids influence activity and plasticity of neurons and glial cells in their early development and continue to exert trophic effects in the

*Corresponding author. Tel.: +39-55-275-7291x296; fax: +39-55-240776; e-mail: silvia.selleri@unifi.it

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00192-4

adult CNS.[27] Moreover, PBR density alters in several neurological dysfunctions (hepatic encephalopathy, cerebral ischemia, multiple sclerosis, sciatic nerve injury, epilepsy and Alzheimer's disease).[28] The new studies to evaluate the changes in PBR density and expression related to neurological disorders and post-traumatic injury highlight the importance to develop novel strategies for the diagnosis and therapy of the abovementioned pathologies using PBR ligands.

The PBR is characterized by its interaction with isoquinolines such as PK 11195,[29] benzodiazepines as Ro 5-4864,[30] imidazopyridines (Alpidem),[31] indole derivatives (FGIN-1)[4,5] and two potent and selective ligands, DAA1097 and DAA1106, recently identified[32] (Fig. 1). This receptor appears to be a heteromeric complex of at least three different subunits, including an isoquinoline binding subunit (18 kDa), a voltage-dependent anion channel VDAC (32 kDa) and an adenine nucleotide carrier (30 kDa).[33] The isoquinolines that bind specifically to PBR interact with the 18 kDa subunit, whereas the BZ recognition site is supposed to be constituted by an association of 18 kDa and VDAC components[34] or, alternatively, on the 18 kDa subunit in a restrictive conformational state.[35,36] However, the complex is made up of a not yet well-established mitochondrial structure of the mitochondrial permeability transition called (MPT)-pore, on whose opening the mitochondrial permeability depends.[37]

In order to gain further information as to the function of the PBR, the synthesis of potent and selective molecules would appear to be an invaluable tool for investigating the physiological role of the PBR in human tissues. In designing compounds that might exhibit high affinity and selectivity for the PBR, we have thought it interesting to synthesize compounds structurally closely related to Alpidem, which binds with nanomolar affinity

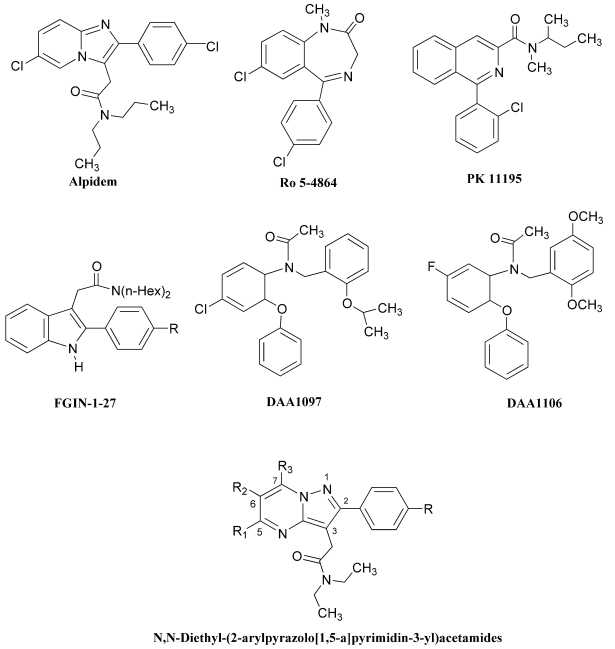


Figure 1. Ligands of peripheral benzodiazepine receptor (PBR).

to both peripheral and central benzodiazepine receptor (PBR $K_i = 0.5–7$ nM; CBR $K_i = 1–28$ nM).[38] Interestingly, extensive SAR studies on the acetamido[1,2-*a*]pyrimidine nucleus of Alpidem have suggested that the introduction of suitable substituents and proper modifications of this nucleus lead to compounds with improved affinity and the PBR versus CBR selectivity.[4,5,39–41] In this paper, we report synthesis, biological evaluations and preliminary SAR studies for a new class of *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamides (Fig. 1) which bind with both high affinity and selectivity to the PBR.

Our attention was focused mainly on the 5-, 6-, and 7-positions to evaluate their influence on selectivity toward the PBR; moreover the effect of *para*-substitutions on the 2-phenyl ring was investigated. However, we maintained in all the series the tertiary acetamide moiety at the 3-position, whose hydrogen bond interaction with the receptor protein is reported to be of paramount importance for the binding to the PBR.[4,5,39–42] In fact, it is known that the substitution of the acetamide moiety with an ester function and the modification of the length of the carbon chain between the amide and the heterocyclic nucleus resulted in a marked loss of affinity.[40] The models proposed in the literature suggest that a hydrogen bond-donating function (δ1) and some lipophilic pockets (PAR, LA, FRA) are the essential pharmacophoric elements for the interaction between ligands and receptor protein. The schematic representation of the interaction models of Alpidem with CBR and PBR proposed by Bourguignon was the hypothesis most frequently reported[38,39,43] (Fig. 2).

Recently, a unique 3-D interaction model has been described for endogenous and synthetic PBR ligands[44] which confirmed two lipophilic regions (L1 and L3 corresponding to PAR and FRA) and one polar group (H2 related to δ1), as essential elements of the PBR interaction, and a lipophilic region modulating the receptor binding (L4 corresponding to LA) into which H2 is expressed.

A subset of the synthesized compounds showing high affinity was also tested for their ability to modulate steroid biosynthesis in C6 glioma cells, since this test is considered so far a reliable method to prove the affinity and/or intrinsic activity of PBR ligands. Some of the

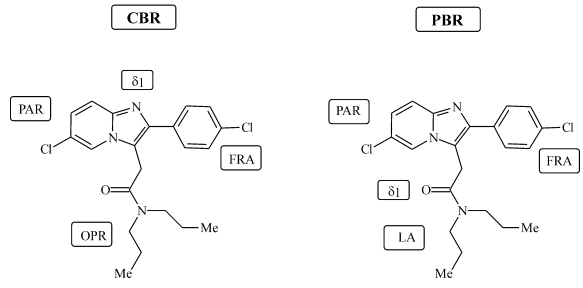


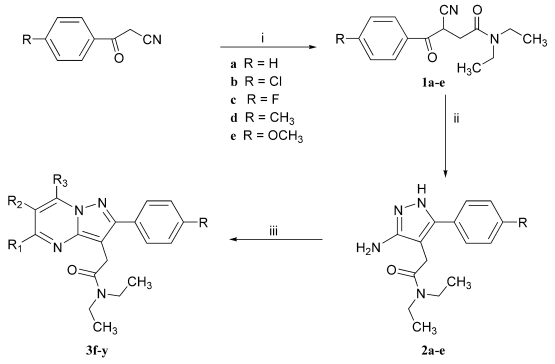
Figure 2. Comparison between the schematic representation of the interaction models of Alpidem with CBR and PBR: PAR, planar aromatic region; FRA, freely rotating aromatic ring region; OPR, outplane region; LA, lipophilic area; δ1, electron-rich zone.

tested compounds were found to stimulate pregnenolone formation with potency comparable to Ro 5-4864 and PK 11195.

Chemistry

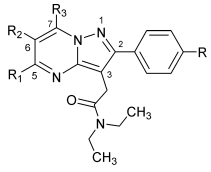
The synthesis of compounds **3f**–**y**, shown in Table 1, was accomplished via the general strategy presented in Scheme 1. The new *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamides were prepared in a three-step procedure starting from the appropriate aroylacetonitriles, which were synthesized following methods previously reported.[45,46] The achievement of the expected *N*,*N*-diethylbutanamides (**1a**–**e**) was performed by exploiting the reactivity of the methylene of aroylacetonitriles which were reacted in alkaline medium with *N*,*N*-diethylchloroacetamide. Unfortunately, following this procedure, the expected *N*,*N*-diethylbutanamides (**1a**–**e**) were obtained together with tars; therefore, a thorough purification by means of column chromatography was mandatory. In the second step, the intermediates **1a**–**e** were reacted at reflux in ethanol with hydrazine hydrate, in the presence of acetic acid, to give the corresponding *N*,*N*-diethyl-(3-amino-5-arylpyrazol-4-yl)acetamides (**2a**–**e**) in good yield.

Finally, the condensation of **2a**–**e** with the suitable electrophilic reagents, reported in Table 1, led to the closure of the pyrimidine ring, affording the expected pyrazolo[1,5-*a*]pyrimidin-3-yl acetamides **3f**–**y**. The condensation of aminopyrazoles with electrophiles such as β-diketones and β-ketoaldehydes or their acetals led generally to regioisomeric mixtures of pyrazolopyrimidines. A simple ^{1}H NMR method, suggested in our previous papers[47,48] for distinguishing isomeric 7-methyl and 5-methyl derivatives, has been used for ascertaining the structure of regioisomers **3f**, **3g**; **3n**, **3o**; **3p**, **3q** and **3s**, **3t**. Convincing data for the structural assignment of the methyl group on the pyrimidine moiety came from the ^{1}H NMR spectra where diagnostic different values are shown by 5- or 7-methyl compounds (δ 2.58–2.63 versus 2.81–2.90 ppm, respectively). Moreover, the coupling constant measured for the H_6 with its vicinal proton ($4.5\,Hz < J_{CH-CH} < 5.0\,Hz$) appears to be in accordance with a =CH–CH= pattern, so that the 7-substitution is ascertained even for **3y**.



Scheme 1. (i) *N*,*N*-diethylchloroacetamide; (ii) hydrazine hydrate; (iii) reagents are reported in Table 1.

Table 1. Experimental data for 2-Arylpyrazolo[1,5-*a*]pyrimidin-3-ylacetamides



Compd	R	R_1	R_2	R_3	Formula (MW)	Analysis[a]	Reagent[b]
3f	H	CH_3	H	H	$C_{19}H_{22}N_4O$ (322.41)	C, H, N	A
3g	H	H	H	CH_3	$C_{19}H_{22}N_4O$ (322.41)	C, H, N	A
3h	H	H	H	H	$C_{18}H_{20}N_4O$ (308.38)	C, H, N	B
3i	H	CH_3	H	CH_3	$C_{20}H_{24}N_4O$ (336.44)	C, H, N	C
3j	Cl	CH_3	H	CH_3	$C_{20}H_{23}N_4OCl$ (370.88)	C, H, N	C
3k	F	CH_3	H	CH_3	$C_{20}H_{23}N_4OF$ (354.43)	C, H, N	C
3l	CH_3	CH_3	H	CH_3	$C_{21}H_{26}N_4O$ (350.46)	C, H, N	C
3m	OCH_3	CH_3	H	CH_3	$C_{21}H_{26}N_4O$ (366.46)	C, H, N	C
3n	F	CH_3	H	H	$C_{19}H_{21}N_4OF$ (340.40)	C, H, N	A
3°	Cl	H	H	CH_3	$C_{19}H_{21}N_4OCl$ (356.85)	C, H, N	A
3p	CH_3	CH_3	H	CF_3	$C_{21}H_{23}N_4OF_3$ (404.43)	C, H, N	D
3q	CH_3	CF_3	H	CH_3	$C_{21}H_{23}N_4OF_3$ (404.43)	C, H, N	D
3r	OCH_3	CF_3	H	CF_3	$C_{21}H_{20}N_4O_2F_6$ (474.41)	C, H, N	E
3s	Cl	CH_3	H	Ph	$C_{25}H_{25}N_4OCl$ (432.95)	C, H, N	F
3t	Cl	Ph	H	CH_3	$C_{25}H_{25}N_4OCl$ (432.95)	C, H, N	F
3u	Cl	CH_3	CH_3	CH_3	$C_{21}H_{25}N_4OCl$ (384.91)	C, H, N	G
3v	Cl	CH_3	COOEt	CH_3	$C_{23}H_{27}N_4O_3Cl$ (442.94)	C, H, N	H
3w	H	H	COOEt	CH_3	$C_{22}H_{26}N_4O_3$ (394.47)	C, H, N	I
3x	CH_3	H	Ph	H	$C_{25}H_{26}N_4O$ (398.51)	C, H, N	J
3y	H	H	H	Ph	$C_{24}H_{24}N_4O$ (384.48)	C, H, N	K

[a]All the compounds were analyzed within ±0.4% of the theoretical values.
[b]A = 4,4-dimethoxy-2-butanone, B = 1,1,3,3-tetraethoxypropane, C = 2,4-pentanedione, D = 1-trifluoromethyl-2,4-pentanedione, E = 1,5-trifluoromethyl-2,4-pentanedione, F = 1-phenyl-1,3-butanedione, G = 3-methyl-2,4-pentanedione, H = ethyl 2-acetyl-3-oxo butanoate, I = ethyl 2-acetyl-3-ethoxyacrylate, J = phenylmalondialdehyde, K = 1-phenyl-3-dimethylamino-2-propen-1-one.

Biological Studies

The ability of the compounds **3f–y** to interact with the peripheral benzodiazepine receptor (PBR) was investigated by means of a binding assay using [^{3}H]PK 11195 and [^{3}H]Ro 5-4864 as radioligands and membranes from rat kidney tissues as receptor source.[40,49] Moreover, in order to examine the PBR versus CBR selectivity of **3f–y**, binding studies were carried out by using [^{3}H]Ro 15-1788 as specific radioligand for benzodiazepine binding site on $GABA_A$ receptor complex and membranes from rat brain tissues as receptor source.[50–52] Binding data for all new pyrazolo[1,5-*a*]pyrimidin-3-yl acetamides are reported in Table 2 and the affinity values of PK 11195, Ro 5-4864 and Alpidem (as reference compounds) are also included. The binding data are expressed as K_i values, which were calculated from the corresponding IC_{50} values according to the equation of Cheng and Prusoff.[53] Most of the synthesized compounds **3f–y** exhibited high affinity and PBR selectivity.

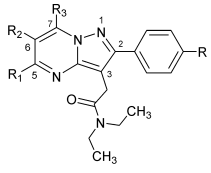
Therefore, with the aim of evaluating thermodynamic similarities with the reference compounds, we performed the binding assay of *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamides (**3f–y**) at two different temperatures. As reported in literature, the Ro 5-4864 binding values are temperature dependent, whereas the PK 11195 values are not.[54,55] The inhibitory effect of Ro 5-4864 has been reported as five times lower at 25 °C than at 4 °C. In the present preliminary study, the inhibitory effect of both the new ligands and PK 11195 on [^{3}H]PK 11195 binding was almost the same at 4 and 25 °C, showing that the binding properties of the new pyrazolopyrimidine ligands and PK 11195 are thermodynamically similar.

Furthermore, a subset of the synthesized compounds showing high affinity for the PBR was examined for the compound's ability to stimulate pregnenolone formation from the mitochondria of C6 glioma cells.[49] All the selected ligands and reference compounds, PK 11195, Ro 5-4864 and Clonazepam, were tested at the same concentration (40 μM) in order to directly compare the efficacies. The references PK 11195 and Ro 5-4864 are known to stimulate steroidogenesis in a concentration-dependent way, whereas the selective CBR ligand, Clonazepam, does not elicit any stimulatory effect at this concentration (Fig. 3).

Results and Discussion

It appears from the results reported in Table 2 that most of the synthesized compounds display high affinity and selectivity for the PBR; only compounds **3f–h**, **3n** and **3o** show a significant affinity for both CBR and PBR. In particular, compound **3h**, devoid of any substituent on

Table 2. Affinities of compounds **3f–y** for PBR and CBR



Compd	R	R_1	R_2	R_3	K_i (nM)[a] PBR [^{3}H]PK 11195	K_i (nM)[a] PBR [^{3}H]Ro 5-4864	K_i (nM)[a] CBR [^{3}H]Ro 15-1788
3f	H	CH_3	H	H	40.0±4.0	21.0±1.0	92.6±7.0
3g	H	H	H	CH_3	155.0±16.0	67.0±3.0	518.0±20.0
3h	H	H	H	H	NT[b]	1200.0±100.0	251.0±18.0
3i	H	CH_3	H	CH_3	29.0±3.0	9.2±0.2	>10,00
3j	Cl	CH_3	H	CH_3	2.4±0.2	1.4±0.2	>10,00
3k	F	CH_3	H	CH_3	9.2±1.0	4.3±0.2	>10,00
3l	CH_3	CH_3	H	CH_3	0.8±0.1	1.7±0.2	>10,00
3m	OCH_3	CH_3	H	CH_3	4.7±0.4	3.1±0.2	>10,00
3n	F	CH_3	H	H	25.0±2.0	67.0±3.0	85.0±3.0
3o	Cl	H	H	CH_3	8.1±0.3	2.9± 0.2	207.0±10.0
3p	CH_3	CH_3	H	CF_3	0.9±0.1	2.3±0.2	5800.0±145.0
3q	CH_3	CF_3	H	CH_3	1.0±0.1	1.8±0.1	6900.0±170.0
3r	OCH_3	CF_3	H	CF_3	16.0±1.0	3.4±0.2	6260.0±250.0
3s	Cl	CH_3	H	Ph	2.4±0.2	2.5±0.2	>10,00
3t	Cl	Ph	H	CH_3	3.4±0.2	2.7±0.2	>10,00
3u	Cl	CH_3	CH_3	CH_3	6.1±0.3	5.2±0.3	>10,00
3v	Cl	CH_3	COOEt	CH_3	8.4±0.4	8.0±0.5	>10,00
3w	H	H	COOEt	CH_3	NT[b]	942.0±5.0	NT[b]
3x	CH_3	H	Ph	H	57.0±5.0	45.0±3.0	>10,00
3y	H	H	H	Ph	NT[b]	160.0±10.0	>10,00
PK 11195					9.3±0.5		>10,00
Ro 5-4864					23.0±3.0		>10,00
Alpidem[c]					0.5–7		1–28

[a] K_i values represent the mean SEM of three determinations.
[b] Not tested.

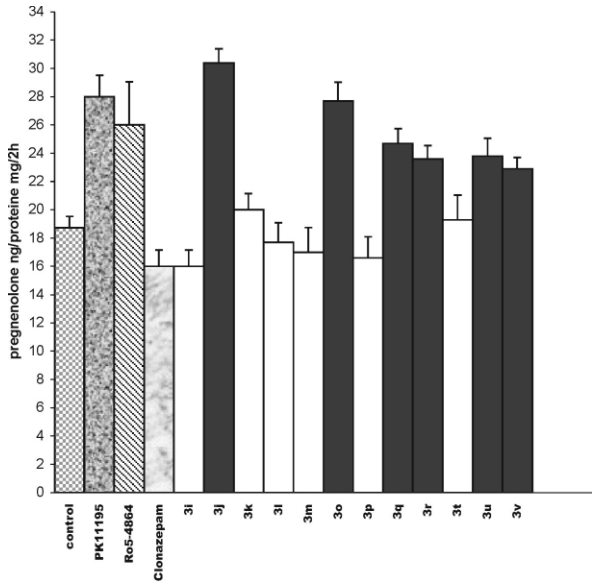


Figure 3. The effect of *N*,*N*-diethyl-(2-arylpyrazolo[1,5-a]pyrimidin-3-yl)acetamides on the pregnenolone accumulation in C6 glioma rat cells. All the compounds were used at the same concentration (40 μM); at the end of incubation period (2 h), the amount of pregnenolone was quantified by radio immunoassay (RIA), using an antibody obtained from ICN Biochemical Inc., CA, USA. The values are the mean ± SEM of at least three determinations. (see Experimental).

the pyrimidine moiety, exhibits the ratio value CBR versus PBR for the CBR, while a marked improvement of PBR affinity is observed for **3f**, **3g**, **3n** and **3o** (**3g** ≤ **3n** < **3f** < **3o**), although their PBR versus CBR selectivity is still modest and comparable to Alpidem. The binding data of the unselective compounds **3f**, **3n** and **3g**, **3o**, bearing a methyl group at the 5- or 7-positions respectively, suggest that the title compounds could be well tolerated into the lipophilic pockets of both peripheral and central receptor proteins probably because of their small lipophilic substituents. Moreover, taking into account the low affinity value of compound **3h**, it seems reasonable to hypothesize that the substitution on the pyrimidine moiety could be the key to promoting the PBR selectivity. This hypothesis is supported by the PBR selectivity of **3y**, bearing the phenyl ring at the 7-position, in comparison to the 7-methyl derivative **3g**. In addition, compounds **3i**–**m** and **3p**–**t**, bearing substituents at the 5- and 7-positions, display high affinity and selectivity for the PBR. On the other hand, as appears in Table 2, the effect of substituents at the 5- and 7-positions, which are endowed with increasing lipophilic nature and steric bulk (**3p**–**t**), produces generally constant or improved affinity values for PBR in comparison to compounds **3i**–**m**. The findings obtained in this study seem to confirm similar results observed in the imidazopyrimidine series, where the substitution at the 6- and 8-positions, corresponding to the 5- and 7-positions of pyrazolopyrimidine series, led to compounds more than 1000-fold selective for PBR versus CBR.[40,41] Therefore, the replacement of imidazo[1,2-*a*]pyridine nucleus of Alpidem with the pyrazolo[1,5-*a*]pyrimidine one appears as a bioisosteric modification. However, the pyrimidine nitrogen (N^4), which could be related to pyrazine nitrogen of Zopiclone (CBR ligand $IC_{50} = 29$ nM) or to N^7 of imidazo[4,5-*b*]pyridines,[39] does not seem to affect PBR binding potency, while its role in CBR versus PBR selectivity remains to be studied in detail.

Encouraged by these preliminary results, we turned our attention to the 6-position of the pyrimidine moiety, corresponding to the 7-position in the imidazopyridine nucleus, that has not been investigated yet. Compounds **3u**–**x**, bearing different substituents at the 6-position, retained the affinity and selectivity for the PBR. This result could suggest that, unlike CBR, a bulky substituent at this position is well tolerated by the peripheral receptor protein. The significant loss of affinity of **3w** is probably due to the spatial orientation of ethoxycarbonyl group with regard to the planar pyrazolopyrimidine nucleus. In fact the lack of substituent at the 5-position might lead to an unfavorable orientation of the ethoxycarbonyl group for the interaction between the ligand and the receptor protein (see affinity values for **3w** and **3v**). As regards the *p*-substitutions at the 2-phenyl ring (**3i**–**m**) a general improvement in PBR affinity values is observed in comparison to the reference compound **3i**; in addition, the chemical features (electrophilicity, lipophilicity and steric hindrance) did not seem to affect either PBR affinity or selectivity. However, further investigations about the position of the substituents on the phenyl ring are required.

Some of the title compounds (**3i**–**m**, **3o**–**r**, **3t**–**v**) have been examined for their ability to increase steroid biosynthesis (Fig. 3) and some of these stimulate steroidogenesis with potency similar to Ro 5-4864 and PK 11195. Surprisingly, these results show that there is no correlation between PBR affinity and steroidogenic activity. In fact, compounds **3i**, **3k**–**m**, **3p** and **3t**, although showing high affinity, do not produce any effect on the pregnenolone level. A moderate increase in pregnenolone formation can be observed for the 5-trifluoromethyl-7-methyl derivative, **3q**, and the 5,7-trifluoromethyl derivative, **3r**, in comparison to the corresponding 5,7-dimethyl-substituted compounds, **3l** and **3m**. An analogous moderate increase is observed for the 5, 6, 7-substituted **3u** and **3v**; while the most steroidogenic active compounds appear to be **3j** and **3o**, bearing a *p*-chlorine atom on the 2-phenyl ring. In a preliminary evaluation, PK 11195, the partial agonist of the steroidogenic action of PBR ligands,[5] was used in the presence of the most active ligand, **3j**; it moderately inhibits the increase in pregnenolone accumulation elicited by compound **3j** per se (PK 11195 + **3j** = 24.3 ± 0.2 pregnenolone ng/mg protein; PK 11195 = 28.0 ± 1.5 pregnenolone ng/mg protein). Moreover, compound **3i**, endowed with PBR affinity but devoid of steroidogenic action, was used together with Ro 5-4864, a well-known modulator in steroid biosynthesis. The inefficiency of **3i** to have any effect on the Ro 5-4864 activity could suggest that this compound fails to antagonize the steroidogenic action of PBR ligand in C6 glioma cells (Ro 5-4864 + **3i** = 29.5 ± 0.6 pregnenolone ng/mg protein; Ro 5-4864 = 26.0 ± 3.1 pregnenolone ng/mg protein). Further investigations and more detailed study are required to define if tissue-specificity of PBR ligands exist and/or

if PBR ligands display different behavioral profiles in different tissues.

Conclusions

In this paper, we report synthesis and biological evaluation of a novel class of high affinity and selective PBR ligands, *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-ylacetamides (**3f**–**y**). The preliminary SAR study suggests that the new pyrazolopyrimidines are bioisteres of imidazopyridine derivatives[40,41] and, in particular, substitutions on the pyrimidine moiety seem to be the key factor promoting the PBR versus CBR selectivity. In addition, introduction of substituents at the *para* position of the 2-phenyl ring is able to increase the affinity values for the PBR. To clarify the role of the amide moiety at the 3-position and the main structural features concerning the number and length of the alkyl substituents on the amide nitrogen, a targeted study is currently in progress. Some of the highest affinity compounds (**3i**–**m**, **3o**–**r**, **3t**–**v**) were also tested for their ability to stimulate steroid biosynthesis in C6 glioma rat cells, and some of these increased the pregnenolone formation with potency similar to Ro 5-4864 and PK 11195. The effects of these compounds on steroid production take on further interest in the light of recent reports showing that neuroactive steroids seem to elicit anxiolytic, anticonvulsant, sedative, hypnotic, and even memory modulating effects.[56,57] On the other hand, the results of our preliminary steroidogenic assay show that there is no correlation between PBR affinity and steroidogenic activity; in fact, some of the tested compounds, although showing high affinity, did not produce any effect on pregnenolone level. This observation prompted us to continue our investigations since no PBR ligands with antagonistic properties on steroidogenesis have been described yet. The availability of such molecules related to Alpidem structure and endowed with different intrinsic activities may afford new opportunities to understand the PBR function, its localization, and the proposed existence of PBR subtypes, in addition to giving new tools for evaluating PBR pathological alterations. Further studies of the biological properties of these compounds, together with an expansion of their SAR and molecular modeling efforts, are underway.

Experimental

The structures of all compounds were supported by their ^{1}H NMR data (measured with a Varian Gemini at 200 MHz, chemical shifts expressed in δ (ppm) using DMSO-d_6 or $CDCl_3$ as solvent). The following abbreviations are used: b = broad, d = doublet, m = multiplet, q = quartet, t = triplet and s = singlet. Melting points were determined with a Gallenkamp apparatus and were uncorrected. Elemental analyses were performed by the laboratories of Dipartimento Farmaco-Chimico-Tecnologico of Università di Siena, Italy, with a Perkin-Elmer model 240C, elemental analyzer, and their results are within ±0.4% of theoretical values. The purity of samples was determined by means of TLC, which was performed using Machery-Nagel Duren, Alugram silica gel plates. Silica gel 60 (Merck 70–230 mesh) was used for column chromatography.

General procedure for synthesis of compounds 1a–e. To a solution of NaOH (10 mmol, 0.39 g) in 80% EtOH (80 mL) the suitable aroylacetonitrile (10 mmol) and *N*,*N*-diethylchloroacetamide (10 mmol, 1.37 mL), with NaI (30 mmol, 4.49 g), were added under magnetic stirring. The reaction mixture was stirred at reflux for 8 h. After cooling the suspension was filtered to eliminate the inorganic materials and the filtrate was concentrated; the residue was then purified by silica gel column chromatography (toluene/EtOAc, 8:3 v/v, as eluent) to give the corresponding *N*,*N*-diethylamides.

***N*,*N*-Diethyl-(3-cyano-4-oxo-4-phenyl)butanamide (1a).** This compound was obtained from 3-oxo-3-phenylpropanenitrile[45] as yellow liquid; yield 12%; ^{1}H NMR ($CDCl_3$) δ 1.09 (t, 3H, $N(CH_2CH_3)_2$), 1.22 (t, 3H, $N(CH_2CH_3)_2$), 2.90 (dd, 1H, CH_2), 3.25–3.49 (m, 5H: 4H, $N(CH_2CH_3)_2$; 1H, CH_2), 5.01–5.1 (m, 1H, CH), 7.48–7.52 (m, 3H, Ph), 8.01–8.12 (m, 2H, Ph).

***N*,*N*-Diethyl-[3-cyano-4-(4-chlorophenyl)-4-oxo]butanamide (1b).** This compound was obtained from 3-(4-chlorophenyl)-3-oxopropanenitrile[45] as yellow liquid; yield 20%; ^{1}H NMR ($CDCl_3$) δ 1.09–1.24 (m, 6H, $N(CH_2CH_3)_2$), 2.88 (dd, 1H, CH_2), 3.22–3.52 (m, 5H: 4H, $N(CH_2CH_3)_2$; 1H, CH_2), 4.92–5.01 (m, 1H, CH), 7.52 (d, 2H, Ph), 8.09 (d, 2H, Ph).

***N*,*N*-Diethyl-[3-cyano-4-(4-fluorophenyl)-4-oxo]butanamide (1c).** This compound was obtained from 3-(4-fluorophenyl)-3-oxopropanenitrile[46] as yellow liquid; yield 13%; ^{1}H NMR ($CDCl_3$) δ: 1.1–1.35 (m, 6H, $N(CH_2CH_3)_2$), 2.89 (dd, 1H, CH_2), 3.22–3.52 (m, 5H: 4H, $N(CH_2CH_3)_2$; 1H, CH_2), 4.98–5.10 (m, 1H, CH), 7.19–7.30 (m, 2H, Ph), 8.03–8.09 (m, 2H, Ph).

***N*,*N*-Diethyl-[3-cyano-4-(4-methylphenyl)-4-oxo]butanamide (1d).** This compound was obtained from 3-(4-methylphenyl)-3-oxopropanenitrile[45] as yellow liquid; yield 42%; ^{1}H NMR ($CDCl_3$) δ 1.14–1.31 (m, 6H, $N(CH_2CH_3)_2$), 2.42 (s, 3H, CH_3), 2.86 (dd, 1H, CH_2), 3.28–3.48 (m, 5H: 4H, $N(CH_2CH_3)_2$; 1H, CH_2), 4.99–5.12 (m, 1H, CH), 7.31 (d, 2H, Ph), 7.98 (d, 2H, Ph).

***N*,*N*-Diethyl-[3-cyano-4-(4-methoxyphenyl)-4-oxo]butanamide (1e).** This compound was obtained from 3-(4-methoxyphenyl)-3-oxopropanenitrile[45] as yellow liquid; yield 10%; ^{1}H NMR ($CDCl_3$) δ 1.03–1.371 (m, 6H, $N(CH_2CH_3)_2$), 2.85 (dd, 1H, CH_2), 3.22–3.52 (m, 5H: 4H, $N(CH_2CH_3)_2$; 1H, CH_2), 3.98 (s, 3H, OCH_3), 4.96–5.04 (m, 1H, CH), 7.00 (d, 2H, Ph), 8.03 (d, 2H, Ph).

General procedure for synthesis of compounds 2a–e. To a solution of **1a**–**e** (10 mmol) in EtOH (50 mL) hydrazine hydrate (20 mmol, 0.97 mL) and acetic acid (1 mL) were added and the reaction mixture was refluxed for 6 h.

After cooling, evaporation of the solvent, under reduced pressure, gave a residue that was purified by silica gel column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent).

***N*,*N*-Diethyl-(3-amino-5-phenylpyrazol-4-yl)acetamide (2a).** Ivory crystals obtained from compound **1a** (10 mmol, 2.58 g); yield 18%; mp 157–158 °C; ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, 3H, $N(CH_2CH_3)_2$), 1.07 (t, 3H, $N(CH_2CH_3)_2$), 3.05 (q, 2H, $N(CH_2CH_3)_2$), 3.33 (q, 2H, $N(CH_2CH_3)_2$), 3.52 (s, 2H, CH_2), 5.65 (bs, 2H, NH_2 exchangeable), 7.34–7.41 (m, 5H, Ph).

***N*,*N*-Diethyl-[3-amino-5-(4-chlorophenyl)pyrazol-4-yl]-acetamide (2b).** Ivory crystals obtained from compound **1b** (10 mmol, 2.92 g); yield 38%; mp 180–181 °C; ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, $N(CH_2CH_3)_2$), 1.09 (t, 3H, $N(CH_2CH_3)_2$), 3.1 (q, 2H, $N(CH_2CH_3)_2$), 3.33 (q, 2H, $N(CH_2CH_3)_2$), 3.49 (s, 2H, CH_2), 4.82 (bs, 2H, NH_2 exchangeable), 7.34–7.42 (m, 4H, Ph).

***N*,*N*-Diethyl-[3-amino-5-(4-fluorophenyl)pyrazol-4-yl]-acetamide (2c).** Ivory crystals obtained from compound **1c** (10 mmol, 2.76 g); yield 40%; mp 137–138 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, $N(CH_2CH_3)_2$), 1.12 (t, 3H, $N(CH_2CH_3)_2$), 3.10 (q, 2H, $N(CH_2CH_3)_2$), 3.40 (q, 2H, $N(CH_2CH_3)_2$), 3.53 (s, 2H, CH_2), 6.1 (bs, 2H, NH_2 exchangeable), 7.01–7.20 (m, 2H, Ph), 7.35–7.43 (m, 2H, Ph).

***N*,*N*-Diethyl-[3-amino-5-(4-methylphenyl)pyrazol-4-yl]acetamide (2d).** Light yellow crystals obtained from compound **1d** (10 mmol, 2.72 g) and recrystallized from 80% EtOH; yield 44%; mp 210–211 °C; ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, $N(CH_2CH_3)_2$), 1.12 (t, 3H, $N(CH_2CH_3)_2$), 2.40 (s, 3H, CH_3), 3.1 (q, 2H, $N(CH_2CH_3)_2$), 3.33 (q, 2H, $N(CH_2CH_3)_2$), 3.52 (s, 2H, CH_2), 4.41 (bs, 2H, NH_2 exchangeable), 7.27–7.40 (m, 4H, Ph).

***N*,*N*-Diethyl-[3-amino-5-(4-methoxyphenyl)pyrazol-4-yl]-acetamide (2e).** Yellow crystals obtained from compound **1e** (10 mmol, 2.88 g); yield 72%; mp 140–141 °C; ^{1}H NMR ($CDCl_3$) δ 0.86–1.18 (m, 6H, $N(CH_2CH_3)_2$), 3.18–3.23 (m, 4H, $N(CH_2CH_3)_2$), 3.5 (s, 2H, CH_2), 3.83 (s, 3H, OCH_3), 6.99 (d, 2H, Ph), 7.31 (d, 2H, Ph).

General procedure for synthesis of compounds 3f–y. To a solution of the suitable *N*,*N*-diethyl-(2-arylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (**2a**–**e**) (1 mmol) in EtOH (5 mL) the appropriate electrophilic reagent (1 mmol) was added and the mixture was refluxed under stirring for 4 h. The progress of reaction was monitored by TLC. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography to give the amides **3f**–**y**.

***N*,*N*-Diethyl-(2-phenyl-5-methylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (3f).** From **2a** (1 mmol, 272 mg) and 4,4-dimethoxy-2-butanone (1 mmol, 0.14 mL); ivory crystals purified by silica gel column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent); yield 32%; mp 135–136 °C; ^{1}H NMR ($CDCl_3$) δ 1.08–1.26 (m, 6H, $N(CH_2CH_3)_2$), 2.58 (s, 3H, 5-CH_3), 3.36–3.57 (m, 4H, $N(CH_2CH_3)_2$), 3.92 (s, 2H, CH_2), 6.62 (d, J_{H6-H7} = 6.9 Hz, 1H, H-6), 7.42–7.49 (m, 3H, Ph), 7.76–7.81 (m, 2H, Ph), 8.49 (d, J_{H7-H6} = 6.9 Hz, 1H, H-7).

***N*,*N*-Diethyl-(2-phenyl-7-methylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (3g).** From **2a** (1 mmol, 272 mg) and of 4,4-dimethoxy-2-butanone (1.1 mmol, 0.15 mL) in the presence of 37% HCl (0.3 mL); the reaction mixture was refluxed under magnetic stirring for 30 min, poured into ice-water and extracted with Et_2O (3×5 mL). The combined extracts were washed with water (3×10 mL), dried over sodium sulfate, and concentrated under reduced pressure. Purification of the residue was accomplished by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent). Ivory crystals; yield 45%; mp 127–128 °C, ^{1}H NMR ($CDCl_3$) δ 1.12–1.31 (m, 6H, $N(CH_2CH_3)_2$), 2.82 (s, 3H, CH_3) 3.39–3.58 (m, 4H, $N(CH_2CH_3)_2$) 4.02 (s, 2H, CH_2), 6.72 (d, J_{H6-H5} = 4.8 Hz, 1H, H-6), 7.38–7.50 (m, 3H, Ph), 7.80–7.83 (m, 2H, Ph), 8.39 (d, J_{H5-H6} = 4.8 Hz, 1H, H-5).

***N*,*N*-Diethyl-(2-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (3h).** From **2a** (1 mmol, 272 mg) and 1,1,3,3 tetraethoxypropane (1.1 mmol, 0.32 mL) in the presence of 37% HCl (0.2 mL); yellow crystals purified by silica gel column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent); yield 30%; mp 133–134 °C; ^{1}H NMR ($CDCl_3$) δ 1.10–1.30 (m, 6H, $N(CH_2CH_3)_2$), 3.30–3.61 (m, 4H, $N(CH_2CH_3)_2$), 4.04 (s, 2H, CH_2), 6.88–6.92 (m, 1H, H-6), 7.26–7.50 (m, 3H, Ph), 7.77–7.82 (m, 2H, Ph), 8.50–8.53 (m, 1H, H-5), 8.84 (dd, 1H, H-7).

***N*,*N*-Diethyl-(2-phenyl-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (3i).** From **2a** (1 mmol, 272 mg) and 2,4-pentanedione (1 mmol, 0.1 mL); light yellow crystals purified by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent); yield 30%; mp 143 °C; ^{1}H NMR ($CDCl_3$) δ 1.15–1.28 (m, 6H, $N(CH_2CH_3)_2$), 2.55 (s, 3H, 5-CH_3), 2.75 (s, 3H, 7-CH_3), 3.48–3.62 (m, 4H, $N(CH_2CH_3)_2$), 3.93 (s, 2H, CH_2), 6.52 (s, 1H, H-6), 7.41–7.45 (m, 3H, Ph), 7.80–7.84 (m, 2H, Ph).

***N*,*N*-Diethyl-[2-(4-chlorophenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3j).** From **2b** (1 mmol, 306 mg) and 2,4-pentanedione (1 mmol, 0.1 mL); light yellow crystals purified by column chromatography (CH_2Cl_2/MeOH, 20:1 v/v, as eluent); yield 34%; mp 129–130 °C; ^{1}H NMR ($CDCl_3$) δ 1.05–1.30 (m, 6H, $N(CH_2CH_3)_2$), 2.57 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.38–3.61 (m, 4H, $N(CH_2CH_3)_2$) 3.99 (s, 2H, CH_2), 6.58 (s, 1H, H-6), 7.41 (d, 2H, Ph), 7.81 (d, 2H, Ph).

***N*,*N*-Diethyl-[2-(4-fluorophenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3k).** From **2c** (1 mmol, 290 mg) and 2,4-pentanedione (1 mmol, 0.1 mL); white crystals purified by column chromatography (CH_2Cl_2/MeOH, 20:1 v/v, as eluent); yield 33%; mp 113–114 °C; ^{1}H NMR ($CDCl_3$) δ 1.11–1.31 (m, 6H, $N(CH_2CH_3)_2$), 2.58 (s, 3H, 5-CH_3), 2.80 (s, 3H, 7-CH_3), 3.40–3.62 (m, 4H, $N(CH_2CH_3)_2$), 3.98 (s, 2H, CH_2), 6.58 (s, 1H, H-6), 7.10–7.21 (m, 2H, Ph), 7.80–7.88 (m, 2H, Ph).

***N,N*-Diethyl-[2-(4-methylphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3l).** From **2d** (1 mmol, 286 mg) and 2,4-pentanedione (1 mmol, 0.1 mL); white crystals purified by column chromatography (CH_2Cl_2/MeOH, 20:1 v/v, as eluent); yield 80%; mp 120 °C; ^{1}H NMR ($CDCl_3$) δ: 1.10–1.25 (m, 6H, $N(CH_2CH_3)_2$), 2.41 (s, 3H, CH_3), 2.60 (s, 3H, 5-CH_3), 2.79 (s, 3H, 7-CH_3), 3.38–3.58 (m, 4H, $N(CH_2CH_3)_2$) 3.98 (s, 2H, CH_2), 6.55 (s, 1H, H-6), 7.25 (d, 2H, Ph), 7.80–7.88 (d, 2H, Ph).

***N,N*-Diethyl-[2-(4-metoxyphenyl)-5,7-dimethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3m).** From **2e** (1 mmol, 302 mg) and 2,4-pentanedione (1 mmol, 0.1 mL); ivory crystals purified by column chromatography (CH_2Cl_2/MeOH, 20:1 v/v, as eluent); yield 44%; mp 212–213 °C; ^{1}H NMR ($CDCl_3$) δ 1.04–1.23 (m, 6H, $N(CH_2CH_3)_2$), 2.58 (s, 3H, 5-CH_3), 2.78 (s, 3H, 7-CH_3), 3.40–3.56 (m, 4H, $N(CH_2CH_3)_2$), 3.83 (s, 2H, CH_2), 3.97 (s, 3H, OCH_3), 6.50 (s, 1H, H-6), 6.99 (d, 2H, Ph), 7.80 (d, 2H, Ph).

***N,N*-Diethyl-[2-(4-fluorophenyl)-5-methylpyrazolo[1,5-*a*]-pyrimidin-3-yl]acetamide (3n).** From **2c** (1 mmol, 290 mg) and 4,4-dimethoxy-2-butanone (1 mmol, 0.14 mL); ivory crystals purified by column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent); yield 34%; mp 103–104 °C; ^{1}H NMR ($CDCl_3$) δ 1.05–1.25 (m, 6H, $N(CH_2CH_3)_2$), 2.60 (s, 3H, 5-CH_3), 3.38–3.60 (m, 4H, $N(CH_2CH_3)_2$), 3.95 (s, 2H, CH_2), 6.64 (d, J_{H6-H7} = 7.0 Hz, 1H, H-6), 7.10–7.24 (m, 2H, Ph), 7.78–7.95 (m, 2H, Ph), 8.55 (d, J_{H7-H6} = 7.0 Hz, 1H, H-7).

***N,N*-Diethyl-[2-(4-chlorophenyl)-7-methylpyrazolo[1,5-*a*]-pyrimidin-3-yl]acetamide (3o).** Starting from **2b** (1 mmol, 306 mg) the title compound was prepared as described for **3g**. Purification by column chromatography ($CHCl_3$/MeOH, 20:1 v/v, as eluent) gave pure **3o** as white crystals; yield 32%; mp 126–127 °C; ^{1}H NMR ($CDCl_3$) δ 1.10–1.32 (m, 6H, $N(CH_2CH_3)_2$), 2.81 (s, 3H, 7-CH_3) 3.38–3.60 (m, 4H, $N(CH_2CH_3)_2$) 3.99 (s, 2H, CH_2), 6.70 (d, J_{H6-H5} = 4.8 Hz, 1H, H-6), 7.41 (d, 2H, Ph), 7.80 (d, 2H, Ph), 8.39 (d, J_{H5-H6} = 4.8 Hz, 1H, H-5).

***N,N*-Diethyl-[2-(4-methylphenyl)-5-methyl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3p) and *N,N*-diethyl-[2-(4-methylphenyl)-5-trifluoromethyl-7-methylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3q).** These compounds were obtained from **2d** (1 mmol, 286 mg) and 1-trifluoromethyl-2,4-pentanedione (1 mmol, 0.12 mL) following the general procedure. The isomeric compounds **3p** and **3q** were separated by column chromatography with toluene/EtOAc, 4:1 v/v as eluent; in particular the first material eluted was compound **3p** and the second **3q**.

3p: yellow crystals; yield 57%; mp 170 °C, ^{1}H NMR ($CDCl_3$) δ 1.08–1.31 (m, 6H, $N(CH_2CH_3)_2$), 2.41 (s, 3H, CH_3), 2.63 (s, 3H, 5-CH_3), 3.42–3.48 (m, 4H, $N(CH_2CH_3)_2$), 3.98 (s, 2H, CH_2), 6.99 (s,1H, H-6), 7.24 (d, 2H, Ph), 7.68 (d, 2H, Ph).

3q: ivory crystals; yield 18%; mp 138 °C, ^{1}H NMR ($CDCl_3$) δ 1.04–1.37 (m, 6H, $N(CH_2CH_3)_2$), 2.42 (s, 3H, CH_3), 2.9 (s, 3H, 7-CH_3), 3.47–3.52 (m, 4H, $N(CH_2CH_3)_2$), 3.99 (s, 2H, CH_2), 6.97 (s,1H, H-6), 7.28 (d, 2H, Ph), 7.82 (d, 2H, Ph).

***N,N*-Diethyl-[2-(4-methoxyphenyl)-5,7-trifluoromethylpyrazolo[1,5-*a*]pyrimidin-3-yl] acetamide (3r).** From **2e** (1 mmol, 302 mg) and 1,5-trifluoromethyl-2,4-pentanedione (1 mmol, 0.14 mL) in absolute EtOH (5 mL); yellow crystals purified by column chromatography with $CHCl_3$/MeOH, 10:1 v/v, as eluent; yield 10%; mp 157 °C; ^{1}H NMR ($CDCl_3$) δ 1.18 (t, 3H $N(CH_2CH_3)_2$), 1.36 (t, 3H, $N(CH_2CH_3)_2$), 3.44 (q, 2H, $N(CH_2CH_3)_2$) 3.60 (q, 2H, $N(CH_2CH_3)_2$) 3.88 (s, 3H, OCH_3), 4.04 (s, 2H, CH_2), 7.02 (d, 2H, Ph), 7.40 (s,1H, H-6), 7.89 (d, 2H, Ph).

***N,N*-Diethyl-[2-(4-chlorophenyl)-5-methyl-7-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl] acetamide (3s) and *N,N*-diethyl-[2-(4-chlorophenyl)-5-phenyl-7-methylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3t).** These compounds were obtained from **2b** (1 mmol, 306 mg) and 1-phenyl-1,3-butanedione (1 mmol, 156 mg) following the general procedure. After cooling, compound **3s** was obtained as a yellow solid in 40% yield by filtration and recrystallized from EtOH. Otherwise the evaporation of the filtrate gave a residue mainly consisting of compound **3t**, which was purified by column chromatography with toluene/EtOAc, 8:3 v/v, as eluent.

3s: yellow crystals; yield 40%; mp 185 °C; ^{1}H NMR ($CDCl_3$) δ 1.16 (t, 3H, $N(CH_2CH_3)_2$), 1.24 (t, 3H, $N(CH_2CH_3)_2$), 2.62 (s, 3H, 5-CH_3), 3.48–3.52 (m, 4H, $N(CH_2CH_3)_2$), 3.99 (s, 2H, CH_2), 6.79 (s,1H, H-6), 7.4 (d, 2H, Ph), 7.58–7.61 (m, 2H, Ph), 7.81 (d, 2H, Ph), 8.1-8.21 (m, 3H, Ph).

3t: yellow crystals; yield 18%; mp 177–178 °C; ^{1}H NMR ($CDCl_3$) δ 1.15 (t, 3H, $N(CH_2CH_3)_2$), 1.22 (t, 3H, $N(CH_2CH_3)_2$), 2.82 (s, 3H, 7-CH_3), 3.41 (q, 2H, $N(CH_2CH_3)_2$), 3.62 (q, 2H, $N(CH_2CH_3)_2$), 4.05 (s, 2H, CH_2), 7.09 (s,1H, H-6), 7.41–7.58 (m, 5H, Ph), 7.93 (d, 2H, Ph), 8.1–8.20 (m, 2H, Ph).

***N,N*-Diethyl-[2-(4-chlorophenyl)-5,6,7-trimethylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3u).** From **2b** (1 mmol, 306 mg) and 3-methyl-2,4-pentanedione (1 mmol, 0.14 mL); ivory crystals purified by silica gel column chromatography ($CHCl_3$/MeOH, 10:1 v/v, as eluent); yield 58%; mp 146–147 °C; ^{1}H NMR ($CDCl_3$) δ 1.05–1.29 (m, 6H, $N(CH_2CH_3)_2$), 2.30 (s, 3H, 6-CH_3), 2.59 (s, 3H, 5-CH_3), 2.81 (s, 3H, 7-CH_3), 4.48–5.52 (m, 4H, $N(CH_2CH_3)_2$) 3.98 (s, 2H, CH_2), 7.44 (d, 2H, Ph), 7.81 (d, 2H, Ph).

***N,N*-Diethyl-[2-(4-chlorophenyl)-5,7-methyl-6-ethoxycarbonylpyrazolo[1,5-*a*]pyrimidin-3-yl]acetamide (3v).** From **2b** (1 mmol, 306 mg) and ethyl 2-acetyl-3-oxo butanoate (1 mmol, 172 mg); ivory crystals purified by silica gel column chromatography (toluene/EtOAc, 8:3 v/v, as eluent); yield 70%; mp 116–117 °C; ^{1}H NMR ($CDCl_3$) δ 1.05–1.25 (m, 6H, $N(CH_2CH_3)_2$), 1.42 (t, 2H, OCH_2CH_3), 2.53 (s, 3H, 5-CH_3), 2.90 (s, 3H, 7-CH_3), 3.38–3.60 (m, 4H, $N(CH_2CH_3)_2$) 3.93 (s, 2H, CH_2), 4.42 (q, 2H, OCH_2CH_3), 7.42 (d, 2H, Ph), 7.82 (d, 2H, Ph).

***N*,*N*-Diethyl-(2-phenyl-6-ethoxycarbonyl-7-methylpyrazolo[1,5-*a*]pyrimidin-3-yl)acetamide (3w).** From **2a** (1 mmol, 272 mg) and ethyl 2-acetyl-3-ethoxyacrylate (1 mmol, 200 mg); white crystals purified by silica gel column chromatography (toluene/EtOAc, 4:1 v/v, as eluent); yield 16%; mp 85–87 °C; ^{1}H NMR ($CDCl_3$) δ 1.07–1.29 (m, 6H, $N(CH_2CH_3)_2$), 1.44 (t, 2H, OCH_2CH_3), 3.22 (s, 3H, 7-CH_3), 3.40–3.46 (m, 4H, $N(CH_2CH_3)_2$) 3.93 (s, 2H, CH_2), 4.42 (d, 2H, OCH_2CH_3), 7.41–7.51 (m, 3H, Ph), 7.82–7.90 (m, 2H, Ph), 8.97 (s, 1H, 5-H).

***N*,*N*-Diethyl-[2-(4-methylphenyl)-6-phenylpyrazolo[1,5-*a*]-pyrimidin-3-yl]acetamide (3x).** This compound was obtained from **2d** (1 mmol, 286 mg) and phenylmalondialdehyde (1 mmol, 161 mg)[58] in glacial acetic acid (2 mL) following the general procedure. After cooling, concentration of the solvent under reduced pressure gave a residue that was diluted with H_2O (5 mL) and then extracted with Et_2O (2×5 mL). The extracts were dried over sodium sulfate and evaporated under reduced pressure to give compound **3x**. Ivory crystals; yield 89%; mp 141–142 °C; ^{1}H NMR ($CDCl_3$) δ 1.04–1.29 (m, 6H, $N(CH_2CH_3)_2$), 2.41(s, 3H, CH_3), 3.41–3.58 (m, 4H, $N(CH_2CH_3)_2$) 4.01(s, 2H, CH_2), 7.30 (d, 2H, Ph), 7.48–7.61 (m, 5H, Ph), 7.72 (d, 2H, Ph), 8.72 (s, 1H, 5-H), 8.84 (s, 1H, 7-H).

***N*,*N*-Diethyl-(2,7-phenylpyrazolo[1,5-*a*]pyrimidin-3-yl)-acetamide (3y).** Following the general procedure, this compound was obtained from **2a** (1 mmol, 272 mg) and 1-phenyl-3-dimethylamino-2-propen-1-one (1 mmol, 166 mg), synthesized according to a procedure described for a similar compound,[59] in glacial acetic acid (3 mL) as solvent. Compound **3y** was purified by column chromatography (toluene/EtOAc/AcOH, 8:2:1 v/v/v, as eluent). Yellow crystals; yield 43%; mp 131–132 °C; ^{1}H NMR ($CDCl_3$) δ: 1.08–1.31 (m, 6H, $N(CH_2CH_3)_2$), 3.38–3.59 (m, 4H, $N(CH_2CH_3)_2$) 4.03 (s, 2H, CH_2), 6.89 (d, J_{H6-H5} = 4.5 Hz, 1H, 6-H), 7.40–7.53 (m, 2H, Ph), 7.54–7.60 (m, 3H, Ph), 7.78–7.86 (m, 2H, Ph), 8.10–8.22 (m, 3H, Ph), 8.50 (d, J_{H5-H6} = 4.5 Hz, 1H, 5-H).

Biological methods

Materials. [^{3}H]PK 11195 (sp.act. 87.0 Ci/mmol), [^{3}H]Ro5-4864 (sp.act. 84.5 Ci/mmol) and [^{3}H]Ro15-1788 (sp.act. 84.2 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA, USA). Culture medium, Fetal Bovine Serum (FBS), L-glutamine and antibiotics were purchased from Bio-Whittaker Italia. PK11195, Ro 5-4864 and clonazepam were obtained from Sigma. 1,2,3,4-tetrahydro-4-oxo-7-chloro-2-naphthylpyridine (SU10603) and 2α,4α,5α,17β-4,5epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile (trilostane) were gifts from Novartis Farma S.p.a. and Dr. D. Zister, University of Dublin, respectively. All other reagents were obtained from commercial suppliers.

[^{3}H]PK 11195 and [^{3}H]Ro 5-4864 binding to rat kidney mitochondrial membranes. For binding studies, mitochondria were prepared as previously described[40,49] with minor modification, from kidneys of male Wistar rats killed by cervical dislocation. Kidneys were homogenized in 20 volumes of ice-cold 50 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA (buffer A), containing protease inhibitors (160 μg/mL benzamidine, 200 μg/mL bacitracine and 20 μg/mL soybean trypsin inhibitor) and centrifuged at 600*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 10,000*g* for 10 min at 4 °C. The pellet was then suspended, homogenized in 20 volumes of ice-cold 50 mM Tris–HCl, pH 7.4 (buffer B), and centrifuged at 10,000*g* for 10 min at 4 °C. The crude mitochondrial pellet was frozen at −20 °C until the time of assay or incubated with either 0.6 nM [^{3}H]PK11195 or 1 nM [^{3}H]Ro 5-4864 in buffer B, with a range of concentrations of the tested compounds (0.1 nM–10 μM) in a total volume of 0.5 mL for 90 min at 4 °C. The incubation was terminated by dilution to 5 mL with ice-cold buffer B, followed immediately by rapid filtration through glass fiber Whatman GF/C filters. The filters were then washed (2×5 mL) with buffer B and the amount of radioactivity retained on the filters was determined by Packard 1600 TR liquid scintillation counter at 66% efficiency. Non-specific binding was estimated in each case in the presence, respectively, of unlabeled 1 μM PK 11195 or Ro 5-4864. For the active compounds, the IC_{50} values were determined and K_i values were derived according to the equation of Cheng and Prusoff.[53] Protein concentration was estimated by the method of Lowry et al.,[60] with bovine serum as standard.

[^{3}H]Ro15-1788 binding to rat cerebral cortex membranes. Rat cerebral cortex membranes were prepared as previously described.[50] After differential centrifugation, the obtained crude membrane fraction was subjected to washing procedures to remove endogenous GABA.[51] The washed membranes were incubated with 0.2 nM [^{3}H]Ro 15-1788 for 90 min at 0 °C in 500 μL of buffer 50 mM Tris–citrate buffer pH 7,4, as previously described.[52]

Cell culture. Rat glioma C6 cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

Steroid biosynthesis. C6 cells were seeded in 24-well plates at a density of ∼1×10^6 cells/well in a final volume of 1 mL. Prior to measurement of pregnenolone production, the cells were washed three times with a simple salts medium consisting of 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES/NaOH, pH 7.4, plus 0.1% BSA. During experiments, cells were incubated with this simple salts medium in air incubator at 37 °C. In order to measure pregnenolone secreted into the medium, its further metabolism was blocked by the addition of trilostane (25 μM) and SU 10603 (10 μM) (inhibitors of 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase, respectively) to the simple salts medium, as previously described.[49] The addition of the novel compounds and

of PK 11195, Ro 5-4864, or clonazepam to the C6 cells was made by the complete change of the simple salts medium to medium containing the appropriate concentration (40 μM) of compound. The final concentration of ethanol was constant for all the wells within each experiment and did not exceed 0.5% (v/v), a concentration, which on its own had no effect on steroid production. At the end of the incubation period (2 h) the cell medium was retained and centrifuged at 1500*g* for 10 min. The amount of pregnenolone secreted into the medium was quantified by radio immunoassay (RIA), using an antibody obtained from ICN Biochemical Inc., CA, USA, under the conditions recommended by the supplier. Cell protein concentration was measured according to the method of Lowry et al.[60]

References and Notes

1. Braestrup, C.; Squires, R. F. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 3805.
2. Anholt, R. R.; Pedersen, P. L.; De Souza, E. B.; Snyder, S. H. *J. Biol. Chem.* **1986**, *261*, 576.
3. Hirsch, J. D.; Beyer, C. F.; Malkowitz, L.; Loullis, C. C.; Blume, A. *J. Mol. Pharmacol.* **1988**, *34*, 164.
4. Romeo, E.; Auta, J.; Kozikowski, A. P.; Ma, D.; Papadopoulos, V.; Puia, G.; Costa, E.; Guidotti, A. *J. Pharmacol. Exp. Ther.* **1992**, *262*, 971.
5. Kozikowski, A. P.; Ma, D.; Brewer, J.; Sun, S.; Costa, E.; Romeo, E.; Guidotti, A. *J. Med. Chem.* **1993**, *36*, 2908.
6. Langer, S. Z.; Arbilla, S. *Pharmacol. Biochem. Behav.* **1988**, *29*, 763.
7. Basile, A. S.; Skolnick, P. *J. Neurochem.* **1986**, *46*, 305.
8. Taupin, V.; Toulmond, S.; Serrano, A.; Benavides, J.; Zavala, F. *J. Neuroimmunol.* **1993**, *42*, 177.
9. Costa, E.; Guidotti, A. *Annu. Rev. Pharmacol. Toxicol.* **1979**, *19*, 531.
10. Gallager, D. W.; Mallorga, P.; Oertel, W.; Henneberry, R.; Tallman, J. F. *J. Neurosci.* **1981**, *1*, 218.
11. Syapin, P. J.; Skolnick, P. *J. Neurochem.* **1979**, *32*, 1047.
12. Papadopoulos, V. *Endocr. Rev.* **1993**, *14*, 222.
13. Saano, V. *Acta Pharmacol. Toxicol.* **1986**, *58*, 333.
14. Olson, J. M.; Ciliax, B. J.; Mancini, W. R.; Young, A. B. *Eur. J. Pharmacol.* **1988**, *152*, 47.
15. Woods, M. J.; Williams, D. C. *Biochem. Pharmacol.* **1996**, *52*, 1805.
16. Krueger, K. E. *Biochim. Biophys. Acta* **1995**, *1241*, 453.
17. Gavish, M.; Bachman, I.; Shoukrun, R.; Katz, Y.; Veenman, L.; Weisinger, G.; Weizman, A. *Pharmacol. Rev.* **1999**, *51*, 629.
18. Culty, M.; Li, H.; Boujrad, N.; Amri, H.; Vidic, B.; Bernassau, J. M.; Revers, J. L.; Papadopoulos, V. *J. Steroid Biochem. Mol. Biol.* **1999**, *69*, 123.
19. Mestre, M.; Carriot, T.; Belin, C.; Uzan, A.; Renault, C.; Dubroeucq, M. C.; Gueremy, C.; Doble, A.; Le Fur, G. *Life Sci.* **1985**, *36*, 391.
20. Miccoli, L.; Poirson-Bichat, F.; Sureau, F.; Bras Gonçalves, R.; Bourgeois, Y.; Dutrillaux, B.; Poupon, M. F.; Oudard, S. *J. Natl. Cancer Inst.* **1998**, *90*, 1400.
21. Torres, S. R. R.; Nardi, G. M.; Ferrara, P.; Ribeiro-do-Valle, R. M.; Farges, R. C. *Eur. J. Pharmacol.* **1999**, *385*, R1.
22. Zorov, D. B.; Krasnikov, B. F.; Kuzminova, A. E.; Vysokikh, M. Y.; Zorova, L. D. *Biosci. Rep* **1997**, *17*, 507.
23. Moreno-Sánchez, R.; Houge, B. A.; Bravo, C.; Newman, A. H.; Basile, A. S.; Chang, P. K. *Biochem. Pharmacol.* **1991**, *41*, 1479.
24. Curran, T.; Morgan, J. I. *Science* **1985**, *229*, 1265.
25. Brown, R. C.; Cascio, C.; Papadopoulos, V. *J. Neurochem.* **2000**, *74*, 847.
26. Serra, M.; Madau, P.; Chessa, M. F.; Caddeo, M.; Sanna, E.; Trapani, G.; Franco, M.; Liso, G.; Purdy, R. H.; Barbaccia, M. L.; Biggio, G. *Br. J. Pharmacol.* **1999**, *127*, 177.
27. Schumacher, M.; Akwa, Y.; Guennoun, R.; Robert, F.; Labombarda, F.; Desarnaud, F.; Robel, P.; De Nicola, A. F.; Baulieu, E. E. *J. Neurocytol* **2000**, *29*, 307.
28. Rao, V. L. R.; Dogan, A.; Bowen, K. K.; Dempsey, R. *J. Exp. Neurol.* **2000**, *161*, 102.
29. Le Fur, G.; Perrier, M. L.; Vaucer, N.; Imbault, F.; Flamier, A.; Benavides, J.; Uzan, A.; Renault, C.; Dubroeucq, M. C.; Gueremy, C. *Life Sci.* **1983**, *32*, 1839.
30. Marangos, P. J.; Patel, J.; Boulenger, J. P.; Clark-Rosenberg, R. *Mol. Pharmacol.* **1982**, *22*, 26.
31. Langer, S. Z.; Arbilla, S.; Benavides, J.; Scatton, B. *Adv. Biochem. Psychopharmacol.* **1990**, *46*, 61.
32. Okuyama, S.; Chaki, S.; Yoshikawa, R.; Ogawa, S.; Suzuki, Y.; Okubo, T.; Nakazato, A.; Nagamine, M.; Tomisawa, K. *Life Sci.* **1999**, *64*, 1455.
33. McEnery, M. W.; Snowman, A. M.; Trifiletti, R. R.; Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3170.
34. Garnier, M.; Dimchev, A. B.; Boujrad, N.; Price, J. M.; Musto, N. A.; Papadopoulos, V. *Mol. Pharmacol.* **1994**, *45*, 201.
35. Joseph-Liauzun, E.; Farges, R.; Delmas, P.; Ferrara, P.; Loison, G. *J. Biol. Chem.* **1997**, *272*, 28102.
36. Joseph-Liauzun, E.; Delmas, P.; Shire, D.; Ferrara, P. *J. Biol. Chem.* **1998**, *273*, 2146.
37. Salvetti, F.; Chelli, B.; Gesi, M.; Pellegrini, A.; Giannaccini, G.; Lucacchini, A.; Martini, C. *Life Sci.* **2000**, *66*, 1165.
38. Bourguignon, J. J. In *Peripheral Benzodiazepine Receptors*; Giesen-Crouse, E., Ed.; Academic: London, 1993; pp 59–85.
39. Anzini, M.; Cappelli, A.; Vomero, S.; Giorgi, G.; Langer, T.; Bruni, G.; Romeo, M. R.; Basile, A. S. *J. Med. Chem.* **1996**, *39*, 4275.
40. Trapani, G.; Franco, M.; Ricciardi, L.; Latrofa, A.; Genchi, G.; Sanna, E.; Tuveri, F.; Cagetti, E.; Biggio, G.; Liso, G. *J. Med. Chem.* **1997**, *40*, 3109.
41. Trapani, G.; Franco, M.; Latrofa, A.; Ricciardi, L.; Carotti, A.; Serra, M.; Sanna, E.; Biggio, G.; Liso, G. *J. Med. Chem.* **1999**, *42*, 3934.
42. Cappelli, A.; Anzini, M.; Vomero, S.; De Benedetti, P. G.; Menziani, M. C.; Giorgi, G.; Manzoni, C. *J. Med. Chem.* **1997**, *40*, 2910.
43. Lentini, G.; Bourguignon, J. J.; Wermuth, C. G. In *QSAR: Rational Approaches to the Design of Bioactive Compounds*; Silipo, C., Vittoria, A., Eds.; Elsevier Science: Amsterdam, 1991; pp 257–260.
44. Cinone, N.; Höltje, H. D.; Carotti, A. *J. Comput.-Aided Mol. Des.* **2000**, *14*, 753.
45. Long, R. S. *J. Am. Chem. Soc.* **1947**, *69*, 990.
46. Kuthan, J.; Paleèek, J.; Valihrach, J. *Collect. Czech. Chem. Commun.* **1981**, *46*, 748.
47. Chimichi, S.; Cosimelli, B.; Bruni, F.; Selleri, S. *Can. J. Chem.* **1992**, *70*, 1093.
48. Chimichi, S.; Cosimelli, B.; Bruni, F.; Selleri, S. *Magn. Reson. Chem.* **1992**, *30*, 1117.
49. 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. *J. Med. Chem.* **1996**, *39*, 3435.
50. Squires, R. F.; Braestrup, C. *Nature* **1997**, *266*, 732.
51. Karobath, M.; Sperk, G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 1004.
52. Costanzo, A.; Guerrini, G.; Ciciani, G.; Bruni, F.; Selleri, S.; Costa, B.; Martini, C.; Lucacchini, A.; Aiello, P. M.; Ipponi, A. *J. Med. Chem.* **1999**, *42*, 2218.

53. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
54. Le Fur, G.; Vaucher, N.; Perrier, M. L.; Flamier, A.; Benavides, J.; Renault, C.; Dubroeucq, M. C.; Gueremy, C.; Uzan, A. *Life Sci.* **1983**, *33*, 449.
55. Chaki, S.; Funakoshi, T.; Yoshikawa, R.; Okuyama, S.; Okubo, T.; Nakazato, A.; Nagamine, M.; Tomisawa, K. *Eur. J. Pharmacol.* **1999**, *371*, 197.
56. Rupprecht, R.; Holsboer, F. *Steroids* **1999**, *64*, 83.
57. Tsutsui, K.; Ukena, K.; Usui, M.; Sakamoto, H.; Takase, M. *Neurosci. Res.* **2000**, *36*, 261.
58. Coppola, G. M.; Hardtmann, G. E.; Huegi, B. S. *J. Heterocyclic Chem.* **1974**, *11*, 51.
59. Zaleplon *Drugs Future* **1996**, *21*, 37.
60. Lowry, O. H.; Rosenburg, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.