High Affinity Central Benzodiazepine Receptor Ligands: Synthesis and Biological Evaluation of a Series of Phenyltriazolobenzotriazindione Derivatives

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A series of 2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1,5(6*H*)-diones (PTBTs), **VII**, were prepared and tested at the central benzodiazepine receptor (BzR). The skeleton of these compounds was designed by formally combining the N−C═O moieties of the known BzR ligands, triazoloquinoxalines (**IV**) and triazinobenzimidazoles (ATBIs) (**VI**). Most of the PTBTs displayed submicromolar/nanomolar potency at the BzR. The 9-chloro derivatives (**45**−**49**) were generally found to be more potent than their 9-unsubstituted counterparts (**37**−**44**). Compound **45** turned out to be the most potent of the PTBTs (K_i 2.8 nM). A subset of compounds (**37**, **42**, **45**, **49**), when tested for their affinity on recombinant rat α1β2γ2, α2β2γ2, and α5β3γ2 $GABA_A$/Bz receptor subtypes, showed enhanced affinities for the α1β2γ2 isoform, with compounds **45** and **49** exhibiting the highest selectivity. Moreover, compounds **45** and **49** were found to display a full agonist efficacy profile at α1 and α2 receptor subtypes, and an antagonist efficacy at α5-containing receptors.

Introduction

Benzodiazepine receptor (BzR) ligands are structurally different compounds that bind to the γ-aminobutyric $acid_A$ ($GABA_A$)/BzR complex. They exhibit a wide variety of pharmacological actions, ranging in a continuum from full agonist (agents potentiating the affinity of GABA for the $GABA_A$ receptor, with anxiolytic, anticonvulsant, sedative-hypnotic, and myorelaxant activities), through antagonist (nil efficacy) to inverse agonist (agents reducing the affinity of GABA for the $GABA_A$ receptor, with anxiogenic, somnolytic, proconvulsant, or even convulsant activities).[1−3]

The $GABA_A$/BzR complex is a membrane-bound heteropentameric protein that may be assembled from at least 21 subunits belonging to eight different classes (6α, 4β, 4γ, 1ϵ, 1δ, 3ρ, 1θ, and 1π), which form a chloride channel.[1,4] It has been found that a fully functional $GABA_A$/BzR receptor must contain an α subunit, a β subunit, and a γ subunit, and it is currently accepted that the predominant stoichiometry in vivo is $(\alpha)_2(\beta)_2(\gamma)$.[5] The major Bz-sensitive $GABA_A$ receptor subtypes in the brain are α1βXγ2, α2βXγ2, α3βXγ2, and α5βXγ2, the benzodiazepine binding site being located between the alpha and gamma subunits.[5,6]

Recent studies clearly suggest that a particular pharmacological response might be associated with an action at a specific $GABA_A$ receptor subtype. It has been demonstrated that the alpha subunit is the one which may influence the ligands' efficacy and their selective pharmacological actions. In particular, α1 subtypes containing $GABA_A$ receptors mediate the sedative action; α2 subtypes may be involved in anxiolytic-like activity and in myorelaxation effects; α5 ones might be associated with cognition and memory, while the role of α3-containing receptors remains unclear or, at least, it seems they are partly involved in mediating anxiety behavior.[6−8]

Among the wide variety of nonbenzodiazepine ligands binding to the BzR the following classes are the most potent: 2-arylpyrazoloquinolines (**I**),[9,10] β-carbolines (**II**),[11] pyridodiindoles (**III**)[12] and triazoloquinoxalines (**IV**)[13] (Chart 1). In the past 10 years, our research group has reported several *N*-benzylindolylglyoxylamide derivatives (**V**) (Chart 1) as BzR ligands designed by taking β-carbolines **II** as the reference structure.[14,15] Compounds **V** were generally found to be less potent than ligands of classes **I**−**IV**, probably owing to their greater conformational flexibility. For this reason, we started research aimed at devising new BzR ligands characterized by more rigid molecular scaffolds. As a result of these efforts, we presented 3-aryl[1,2,4]triazino[4,3-*a*]benzimidazol-4(10*H*)-ones **VI** (ATBIs) (Chart 1) as BzR ligands possessing nanomolar affinity values and a higher potency compared with indolylglyoxylamides **V**.[16] We now report the synthesis and the biological evaluation of a series of derivatives of the 2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1,5(6*H*)-dione

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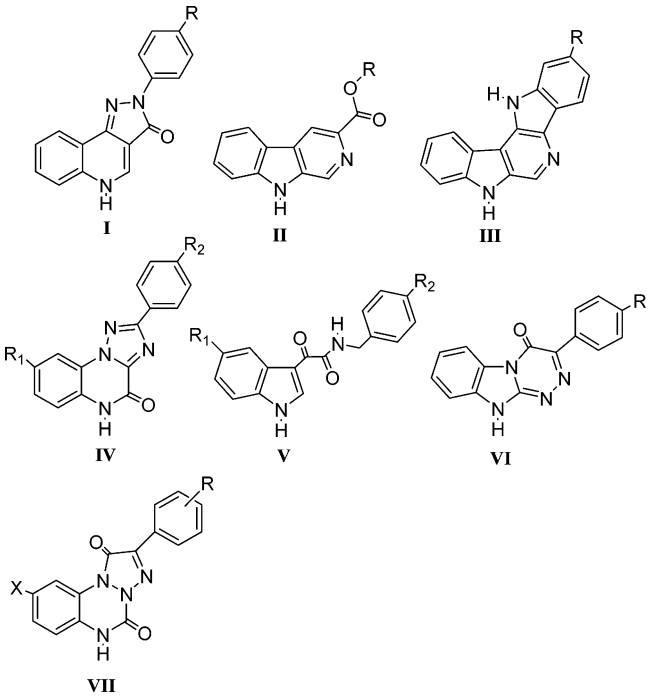
Chart 1



(PTBT) family with the general formula **VII** (Chart 1). These derivatives maintain the 6,6,5-tricyclic heteroaromatic system analogous to that of pyrazoloquinolines (**I**).[9,10] Moreover, the triazolobenzotriazindione skeleton of **VII** was designed by formally combining the N−C=O moieties of triazoloquinoxalines (**IV**)[13] and of ATBIs (**VI**),[16] as shown in Figure 1.

PTBTs seem to fulfill all the requirements necessary for a high affinity at the BzR, according to the pharmacophore/topological model proposed by Cook et al.[17] In Figure 2 the PTBT derivative **37** (**VII**, X = R = H) is superimposed on the 2-phenylpyrazoloquinoline (**I**, R = H). In particular, the receptor H-bond sites A_2 (donor), H_1 and H_2 (acceptors) are supposed to interact with NH(6), the 5-C=O oxygen (possibly together with N3 in a three-centered H-bond), and the 1-C=O oxygen, respectively. Additionally, the fused benzene and the pendant phenyl rings might fill the lipophilic pockets L_{Di} and L_1, respectively. Although no structural analogy with benzylindolylglyoxylamides can be invoked, a chlorine was introduced in the 9-position of the PTBT system to verify whether a higher polarization of the NH(6) bond due to electron-withdrawing effects might strengthen the putative H-bond with the receptor acceptor site A_2, as had already been observed for the 5-chloro-substituted benzylindolylglyoxylamides **V**.[14,15] Finally, a small number of substituents were inserted on the 2-phenyl ring (H, F, Cl, Br, MeO) to probe the L_1/L_2 lipophilic pocket and its surroundings.

Chemistry. The general procedure used for the synthesis of [1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1,5(6*H*)-dione derivatives **37**−**49** is shown in Scheme 1.

Ionic 1,3-dipolar cycloaddition of the appropriate azide **1** and **2**,[18,19] to ethyl phenylacetates **3**-**10**,[20,21] furnished the 1-(2-nitrophenyl)-4-aryl-5-oxo[1,2,3]triazoles **11**−**23**.

Catalytic reduction of compounds **11**-**18** in ethanolic solution afforded the corresponding amines **24**−**31**.

Reduction of the nitro group of the chloro derivatives **19**−**23** under the same conditions gave only the starting materials or led to complex mixtures that required tedious purification procedures and provided the corresponding amino derivatives **32**−**36** with poor chemical yields.

To solve this problem, we modified the reduction conditions and found that the desired products **32**−**36** were formed with good yields and a higher purity when the catalyst (10% Pd/C) was first activated overnight on hydrogen.

Due to their low stability on heating, the nitro (**11**−**23**) and the amino (**24**−**31**) compounds were not recrystallized, but were purified by washing with cold petroleum ether 60−80 °C and/or by flash chromatography. The chloro-substituted amino derivatives **32**−**36** were particularly instable, so they were not isolated and characterized, but directly used in the next reaction.

The cyclocondensation of all the amines **24**−**36** to the final tricyclic compounds **37**−**49** was performed using triphosgene[22] in anhydrous tetrahydrofuran solution at room temperature.

The tricyclic derivatives **37**−**49** are stable products, and they were purified by recrystallization from the appropriate solvent. With the exception of the labile products **32**−**36**, the structures of all the newly synthesized compounds were confirmed by analytical and spectral data (Tables1 and 2).

Binding Studies. The binding affinity of each newly synthesized PTBT derivative at the BzR in bovine brain membranes was determined by competition experiments against the radiolabeled antagonist [^{3}H]flumazenil[23] and was expressed as the K_i value only for those compounds inhibiting radioligand binding by more than 80% at a fixed concentration of 10 μM (Table 3).

Subtype selectivity of the most active compounds (**37**, **42**, **45**, **49**) was tested by assaying their ability to displace [^{3}H]flumazenil in membranes from HEK293 cells expressing rat $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, and $\alpha5\beta3\gamma2$ $GABA_A$/Bz receptor subtypes (Table 4).[15]

The functional efficacy of the most potent compounds **45** and **49** was determined on cloned rat $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, and $\alpha5\beta3\gamma2$ $GABA_A$/Bz receptor subtypes expressed in HEK293 cells by measurement of the modulatory effect on the GABA EC_{20} ion current using $^{36}Cl^-$ influx through the ion channel pore, as described by Harris et al.[24] (Figure 3). For each recombinant receptor subtype, we first examined the concentration dependence of the modulatory effects elicited by compounds **45** and **49** on GABA 20% maximum influx (data not shown). In each case concentrations 100 times greater than the compounds' K_i values provided the maximal effect on GABA-evoked $^{36}Cl^-$ influx.[25] These concentrations were used in each experiment. The nonselective full agonist diazepam was included as the standard in the determination of efficacy.

Results and Discussion

The binding results shown in Table 3 indicate that the majority of the PTBTs display from submicromolar to nanomolar potency at the BzR, the 2′-fluorophenyl derivative **43** and the 3′,4′-dimethoxyphenyl derivative **44** being the only compounds devoid of any appreciable affinity.

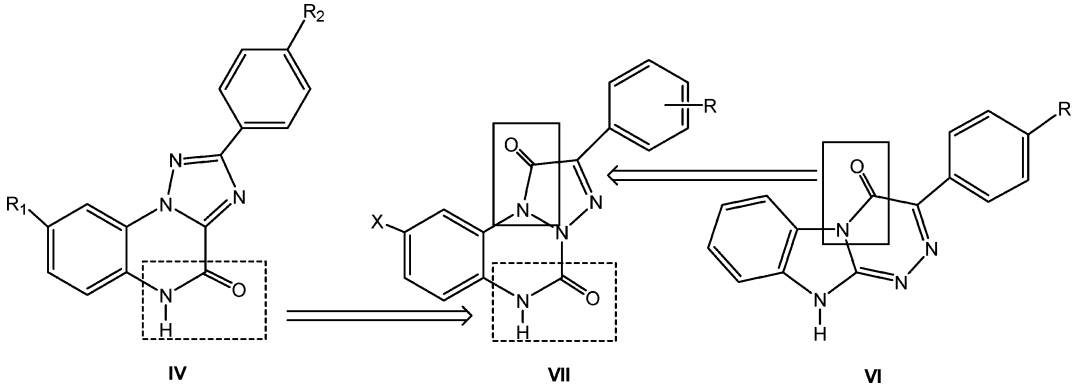


Figure 1. Design of compounds **VII** based on the formal assembly of substructures highlighted in compounds **IV**[13] and **VI**.[16]

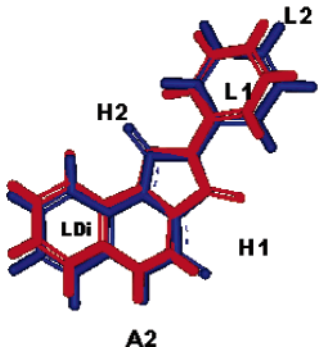
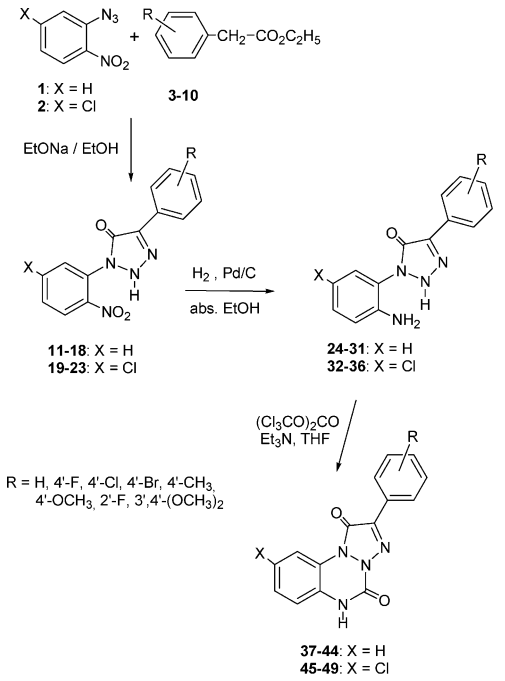


Figure 2. Overlay of the PTBT derivative **37** (blue) on the 2-phenylpyrazoloquinoline CGS 8216[7] of class **I** (red). The two molecular models are embedded within the pharmacophore/topological model proposed by Cook and co-workers[17] for BzR ligands. A_2 is an H-bond donor site, H_1 and H_2 are H-bond acceptor sites, L_1, L_2 and L_{Di} are lipophilic pockets.

Scheme 1



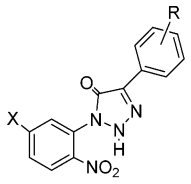
The 9-chloro derivatives are generally more potent than their 9-unsubstituted counterparts to an extent comprised between 2- and 8-fold. Exceptions to this trend are the 4′-fluorophenyl derivatives **38** and **46**, which exhibit almost identical K_i values. The favorable effect of the 9-chloro reaches a maximum when the 2-phenyl ring is not substituted (compare **45** vs **37**). The higher affinity of the 9-chloro derivatives might be attributable to both a stronger H-bond between the NH(6) and the receptor acceptor site A_2, due to a higher polarization of the NH bond, and a better interaction of the 9-chloro with the lipophilic receptor area L_{Di}, which is modulated by the overall fit of the molecule in the Bz-receptor recognition site.

Substituents on the 2-phenyl ring modulate affinity. The highest affinity ligands are 4′-H and 4′-MeO derivatives in the 9-Cl and 9-H subsets: **45** (K_i 2.8 nM) and **37** (K_i 22 nM), and, respectively, **49** (K_i 6 nM) and **42** (K_i 11 nM). In both subsets, insertion in position 4′ of a halogen (**38**−**40**, **46**, **47**) or a methyl (**41**, **48**) lowers affinity. It is worth noticing that Cl, Br and Me are all more lipophilic and bulkier than H, but F has approximately the same lipophilicity and atomic radius as H. Thus, it seems difficult to establish a clear correlation between the affinity and the lipophilic or steric properties of the 4′-substituent. Likewise, electronic effects cannot explain the structure-affinity relationships, since both MeO and Me are classified as electron-donating groups.

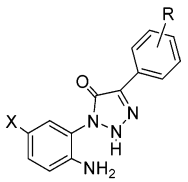
In an attempt to improve the affinity of the 4′-MeO derivative **42**, we prepared compound **44**, featuring an additional MeO group in the 3′-position. This 3′,4′-$(MeO)_2$ derivative turned out to be inactive in the binding assays. No appreciable affinity was displayed by the 2′-F derivative **43**, either. The lack of affinity found for compounds **43** and **44** seems to indicate that the L_1 pocket possesses precise dimensions that can well accommodate only a phenyl group with no ortho- or meta-substituents, as already pointed out by Savini et al. for a series of pyrazolo[4,3-*c*]quinolin-3-one derivatives substituted in the ortho position of the 2-phenyl ring.[26]

The PTBTs were applied to Cook's model,[17] hypothesizing that the 2-phenyl ring and the 4′-substituent might fill the L_1 and L_2 lipophilic pockets, respectively, of the BzR (see Figure 2). In the 2-arylpyrazoloquinoline series **I**,[9,10] it is well-known that H, Cl, and MeO in the 4′-position of the phenyl ring are associated with subnanomolar affinity, while the effects of a 4′-Me have not been disclosed in the literature, although this structure was included in two patents.[27,28] Thus, the low affinity shown by compounds **38**−**41** was unexpected on the basis of the alignment depicted in Figure 2. Actually, the unfavorable effects of the 4′-Me and the 3′,4′-$(MeO)_2$ groups on the pendant phenyl ring had already been observed in the recently reported series of ATBIs **VI**.[16]

Table 1. Physical Properties and Spectral Data of Compounds **11**−**31**



11-23

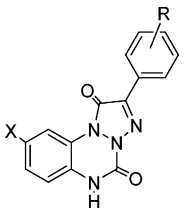


24-31

no.	X	R	yield (%)	mp (°C)	formula[a]	IR (cm^{-1})	^{1}H NMR (δ ppm)	MS *m/e* (%)
11	H	H	70	119−121	$C_{14}H_{10}N_4O_3$	1610, 1520, 1300, 1250, 920	7.24−7.49 (m, 3H, Ar-H); 7.76−7.99 (m, 5H, Ar-H); 8.23 (dd, 1H, Ar-H)	283 (M^+ + 1, 3); 208 (4); 105 (100)
12	H	4′-F	36	115−117	$C_{14}H_9FN_4O_3$	1600, 1580, 1520, 1240, 1160, 780	7.11−7.33 (m, 2H, Ar-H); 7.80−7.97 (m, 5H, Ar-H), 8.17 (dd, 1H, Ar-H)	228 (17); 123 (100)
13	H	4′-Cl	31	108−111	$C_{14}H_9ClN_4O_3$	1600, 1520, 1300, 1140, 940	7.48−7.55 (m, 2H, Ar-H); 7.80−7.99 (m, 5H, Ar-H); 8.24 (dd, 1H, Ar-H)	244 (20); 139 (100)
14	H	4′-Br	39	101−103	$C_{14}H_9BrN_4O_3$	1610, 1540, 1310, 1160, 1020, 950	7.47−7.54 (m, 2H, Ar-H); 7.78−7.96 (m, 5H, Ar-H); 8.23 (dd, 1H, Ar-H)	288 (16); 169 (100)
15	H	4′-CH_3	38	108−111	$C_{15}H_{12}N_4O_3$	1620, 1500, 1340, 1160, 930	2.31 (s, 3H, CH_3); 7.11−7.24 (m, 2H, Ar-H); 7.72−7.97 (m, 5H, Ar-H); 8.16 (dd, 1H, Ar-H)	224 (17), 119 (100)
16	H	4′-OCH_3	56	110−112	$C_{15}H_{12}N_4O_4$	1620, 1560, 1510, 1320, 1160	3.80 (s, 3H, OCH_3); 7.01−7.07 (m, 2H, Ar-H), 7.78−8.01 (m, 5H, Ar-H); 8.23 (dd, 1H, Ar-H)	285 (1); 267 (6); 239 (13); 164 (100)
17	H	2′-F	39	95−97	$C_{14}H_9FN_4O_3$	1600, 1520, 1340, 1160, 740	7.26−7.48 (m, 3H, Ar-H); 7.72−8.02 (m, 4H, Ar-H), 8.23 (dd, 1H, Ar-H)	272 (1); 228 (15); 123 (100)
18	H	3′,4′-$(OCH_3)_2$	63	118−121	$C_{16}H_{14}N_4O_5$	1620, 1500, 1250, 1140, 1010, 840	3.77 (s, 3H, 3′-OCH_3); 3.80 (s, 3H, 4′-OCH_3); 7.00−7.04 (m, 1H, Ar-H); 7.42−7.49 (m, 2H, Ar-H); 7.76−8.00 (m, 3H, Ar-H); 8.19 (dd, 1H, Ar-H)	196 (33); 44 (100)
19	Cl	H	92	111−114	$C_{14}H_9ClN_4O_3$	1615, 1550, 1340, 1160, 780	7.27−7.58 (m, 3H, Ar-H); 7.94−8.11 (m, 4H, Ar-H); 8.17 (dd, 1H, Ar-H); 10.34 (bs, 1H, N-H)	288 (1); 244 (15); 105 (100)
20	Cl	4′-F	47	93−95	$C_{14}H_8ClFN_4O_3$	1620, 1530, 1345, 1160, 740	7.05−7.17 (m, 2H, Ar-H); 7.28−7.48 (m, 2H, Ar-H); 7.59−7.65 (m, 1H, Ar-H); 7.91−8.17 (m, 2H, Ar-H); 10.31 (bs, 1H, N-H)	334 (M^+, 2); 288 (13); 306 (3); 261 (23); 123 (100)
21	Cl	4′-Cl	56	98−100	$C_{14}H_8Cl_2N_4O_3$	1610, 1530, 1300, 1090, 820	7.45−7.56 (m, 3H, Ar-H), 7.85−7.93 (m, 2H, Ar-H); 8.03−8.06 (m, 1H, Ar-H); 8.22 (dd, 1H, Ar-H); 10.38 (bs, 1H, N-H)	317 (M^+ + 1, 10); 289 (5); 244 (23); 228 (100)
22	Cl	4′-CH_3	48	100−102	$C_{15}H_{11}ClN_4O_3$	1620, 1520, 1340, 1140, 930	2.31 (s, 3H, CH_3); 7.12−7.25 (m, 2H, Ar-H); 7.76−8.02 (m, 4H, Ar-H); 8.16 (dd, 1H, Ar-H); 10.32 (bs, 1H, N-H)	330 (M^+, 2); 302 (4); 258 (14); 119 (100)
23	Cl	4′-OCH_3	46	122−125	$C_{15}H_{11}ClN_4O_4$	1620, 1580, 1490, 1150, 810	3.80 (s, 3H, OCH_3); 7.09 (d, 2H Ar-H); 7.35 (dd, 1H, Ar-H); 7.50 (d, 1H, Ar-H); 8.10 (d, 2H, Ar-H); 8.33 (d, 1H, Ar-H); 10.20 (bs, 1H, N-H)	346 (M^+, 4); 318 (38); 301 (49); 273 (15); 135 (100)
24	H	H	39	128−131	$C_{14}H_{12}N_4O$	1600, 1540, 1410, 1260, 760	6.78 (dt, 1H, Ar-H); 6.97 (dd, 1H, Ar-H); 7.24−7.56 (m, 5H, Ar-H); 7.94 (dd, 2H, Ar-H)	222 (71); 194 (100)
25	H	4′-F	26	128−132	$C_{14}H_{11}FN_4O$	1620, 1240, 1150, 940, 830	6.77 (dt, 1H, Ar-H); 6.96 (dd, 1H, Ar-H); 7.21−7.32 (m, 4H, Ar-H); 7.96 (dt, 2H, Ar-H)	272 (M^+ + 2, 1); 240 (68); 212 (100)
26	H	4′-Cl	51	153−155	$C_{14}H_{11}ClN_4O$	1580, 1520, 1400, 1250, 820	6.82 (dt, 1H, Ar-H); 7.00 (dd, 1H, Ar-H); 7.25 (d, 2H, Ar-H); 7.49 (d, 2H, Ar-H); 7.96 (d, 2H, Ar-H)	256 (18); 228 (36); 108 (100)
27	H	4′-Br	33	110−112	$C_{14}H_{11}BrN_4O$	1600, 1270, 1160, 990, 720	6.83 (dt, 1H, Ar-H); 6.99 (dd, 1H, Ar-H); 7.24 (d, 2H, Ar-H); 7.47 (d, 2H, Ar-H); 7.94 (d, 2H, Ar-H)	214 (45); 169 (100)
28	H	4′-CH_3	76	147−160	$C_{15}H_{14}N_4O$	1600, 1460, 1290, 1160, 750	3.34 (s, 3H, CH_3); 6.75 (dt, 1H, Ar-H); 6.94 (dd, 1H, Ar-H), 7.19−7.27 (m, 4H, Ar-H); 7.84 (d, 2H, Ar-H)	236 (31); 208 (51); 119 (100)
29	H	4′-OCH_3	50	128−130	$C_{15}H_{14}N_4O_2$	1610, 1560, 1250, 1140, 760	3.81 (s, 3H, OCH_3); 6.74 (dt, 1H, Ar-H); 6.86−7.22 (m, 5H, Ar-H); 7.86 (d, 2H, Ar-H)	252 (40); 224 (38); 63 (100)
30	H	2′-F	72	100−102	$C_{14}H_{11}FN_4O$	1650, 1620, 1480, 1140, 750	6.73−7.85 (m, 8H, Ar-H); 12.65 (bs, 1H, NH)	240 (48); 212 (100)
31	H	3′,4′-$(OCH_3)_2$	75	127−130	$C_{16}H_{16}N_4O_3$	1570, 1460, 1400, 1190, 640	3.78 (s, 3H, 3′-OCH_3); 3.81 (s, 3H, 4′-OCH_3); 6.74 (dt, 1H, Ar-H); 6.94 (dd, 1H, Ar-H); 7.04 (d, 1H, Ar-H); 7.19−7.27 (m, 2H, Ar-H); 7.46−7.57 (m, 2H, Ar-H)	196 (7); 45 (100)

[a] Elemental analyses for C, H, N were within ±0.4% of the calculated values.

Table 2. Physical Properties and Spectral Data of Compounds **37**−**49**



no.	X	R	yield (%)	mp[a] (°C)	formula[b]	IR (cm^{-1})	^{1}H NMR (δ, ppm)	MS *m/e* (%)
37	H	H	41	240−241 (EtOH)	$C_{15}H_{10}N_4O_2$	3080, 1750, 1650, 1600, 1260, 1110, 960	7.16−7.41 (m, 3H, H-7, H-8, H-9); 7.49−7.60 (m, 3H, H-3′, H-4′, H-5′); 8.29 (dd, 2H, H-2′, H-6′); 8.67 (dd, 1H, H-10)	278 (M^+, 18); 250 (35); 222 (52); 194 (40); 90 (100)
38	H	4′-F	35	228−230 (EtOH)	$C_{15}H_9FN_4O_2$	3100, 1750, 1660, 1600, 1300, 1160, 770	7.16−7.45 (m, 5H, Ar-H); 8.34 (dd, 2H, H-2′, H-6′); 8.66 (dd, 1H, H-10); 11.69 (bs, 1H, NH)	296 (M^+, 78); 268 (46); 240 (68); 212 (100)
39	H	4′-Cl	67	225−227 (DMF/H_2O)	$C_{15}H_9ClN_4O_2$	3350, 1710, 1660, 1600, 1340, 1280, 750	7.17−7.38 (m, 3H, H-7, H-8, H-9); 7.63 (d, 2H, H-3′, H-5′); 8.30 (d, 2H,H-2′, H-6′); 8.67 (dd, 1H, H-10); 11.72 (bs, 1H, NH)	312 (M^+, 18); 284 (42); 256 (67); 228 (54); 89 (100)
40	H	4′-Br	33	237−238 (EtOH)	$C_{15}H_9BrN_4O_2$	3100, 1740, 1640, 1600, 1320, 1260, 740	7.17−7.38 (m, 3H, H-7, H-8, H-9); 7.77 (d, 2H, H-3′, H-5′); 8.23 (d, 2H, H-2′, H-6′); 8.66 (dd, 1H, H-10); 11.75 (bs, 1H, NH)	356 (M^+,3); 328 (19); 300 (32); 272 (10); 89 (100)
41	H	4′-CH_3	45	243−245 (EtOH)	$C_{16}H_{12}N_4O_2$	3150, 1740, 1650, 1590, 1340, 1260, 720	2.38 (s, 3H, CH_3); 7.16−7.39 (m, 5H, Ar-H); 8.19 (d, 2H, H-2′, H-6′); 8.66 (dd, 1H, H-10); 11.61 (bs, 1H, NH)	292 (M^+, 84) 264 (64); 236 (90); 208 (100)
42	H	4′-OCH_3	38	253−254 (EtOH)	$C_{16}H_{12}N_4O_3$	3050, 1740, 1630, 1590, 1290, 1160, 750	3.83 (s, 3H, OCH_3); 7.09−7.39 (m, 5H, Ar-H); 8.24 (d, 2H, H-2′, H-6′); 8.65 (dd, 1H, H-10); 11.60 (bs, 1H, NH)	308 (M^+, 100); 280 (49); 252 (89); 224 (37)
43	H	2′-F	72	220−222 (EtOH)	$C_{15}H_9FN_4O_2$	3100, 1750, 1620, 1600, 1320, 1260, 770	7.17−7.59 (m, 6H, Ar-H); 8.24−8.32 (m, 1H, Ar-H); 8.65 (dd, 1H, H-10); 11.77 (bs, 1H, NH)	296 (M^+, 11); 268 (58); 240 (92); 212 (89); 107 (100)
44	H	3′,4′-$(OCH_3)_2$	70	235−238 (DMF/H_2O)	$C_{17}H_{14}N_4O_4$	3075, 1720, 1670, 1600, 1380, 1260, 760	3.83 (s, 3H, 3′-OCH_3); 3.85 (s, 3H, 4′-OCH_3); 7.10−7.32 (m, 4H, Ar-H); 7.75 (s, 1H, H-2′); 7.97 (dd, 1H, H-6′); 8.65 (dd, 1H, H-10); 11.58 bs,1H, NH)	338 (M^+, 34); 310 (43); 282 (97); 251 (7); 178 (100)
45	Cl	H	33	228−230 (EtOH)	$C_{15}H_9ClN_4O_2$	1730, 1680, 1610, 1310, 730	7.18 (d, 1H, H-7); 7.44 (dd, 1H, H-8); 7.52−7.61 (m, 3H, H-3′, H-4′, H-5′); 8.27 (dd, 2H, H-2′, H-6′); 8.65 (d, 1H, H-10); 11.78 (bs, 1H, NH)	312 (M^+, 100); 284 (35); 278 (47); 256 (29); 228 (51)
46	Cl	4′-F	59	208−210 (EtOH)	$C_{15}H_8ClFN_4O_2$	1735, 1670, 1600, 1320, 1220, 730	7.17−7.45 (m, 4H, Ar-H); 8.32 (dd, 2H, H-2′, H-6′); 8.66 (d, 1H, H-10); 11.70 (bs, 1H, NH)	330 (M^+, 9); 296 (63); 268 (26); 240 (44); 212 (79); 103 (100)
47	Cl	4′-Cl	49	168−170 (EtOH)	$C_{15}H_8Cl_2N_4O_2$	1730, 1665, 1610, 1340, 1260, 760	7.16−7.64 (m, 4H, Ar-H); 8.28 (d, 2H, H-2′, H-6′); 8.65 (d, 1H, H-10); 11.67 (bs, 1H, NH)	348 (M^+ + 1,7); 312 (60); 284 (39); 278 (100); 256 (25); 228 (45)
48	Cl	4′-CH_3	54	245−247 (EtOH)	$C_{16}H_{12}N_4O_3$	1720, 1675, 1600, 1290, 1180, 760	2.38 (s, 3H, CH_3); 7.17−7.38 (m, 5H, Ar-H); 8.19 (d, 2H, H-2′, H-6′); 8.66 (d, 1H, H-10); 11.44 (bs, 1H, NH)	326 (M^+, 3); 292 (100); 264 (27); 236 (17); 208 (63)
49	Cl	4′-OCH_3	49	239−241 (DMF/H_2O)	$C_{16}H_{11}ClN_4O_3$	1650, 1600, 1330, 1030, 750	3.83 (s, 3H, OCH_3); 7.12 (d, 2H, H-3′, H-5′); 7.19−7.39 (m, 2H, H-7, H-8); 8.24 (d, 2H, H-2′, H-6′); 8.65 (d, 1H, H-10); 12.60 (bs, 1H, N−H)	342 (M^+, 8); 308 (100); 280 (21); 252 (21); 224 (29)

[a] Recrystallization solvents are indicated in parentheses. [b] Elemental analyses for C, H, N were within ±0.4% of the calculated values.

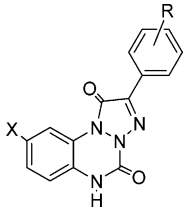
However, the puzzling structure-affinity relationships in this class of ATBIs were ascribed to tautomeric equilibria involving the NH(10) proton responsible for the bond with the receptor A_2 site. It is worth noting that in the case of the PTBT series, among compounds with different affinity, we did not observe the establishment of any tautomerism either by NMR or IR spectroscopy.

The most potent compounds (**37**, **42**, **45**, **49**), when tested for their affinity at $\alpha1\beta2\gamma2$, $\alpha2\beta2\gamma2$, and $\alpha5\beta3\gamma2$ $GABA_A$/Bz receptor subtypes,[15] showed enhanced affinities for the $\alpha1\beta2\gamma2$ isoform and exhibited a certain selectivity over both $\alpha2\beta2\gamma2$, and $\alpha5\beta3\gamma2$ subtypes (Table 4).

It has been proposed that one of the features of a BzR ligand necessary to display α1 selectivity is that of allowing a favorable occupation of the lipophilic L_{Di} region of the receptor binding site.[29] The moderate α1 selectivity shown by PTBTs is consistent with the binding mode proposed in Figure 2 whereby the L_{Di} site is occupied by the fused benzene ring.

Compounds **45** and **49** were selected to be screened for functional efficacy and, as shown in Figure 3, their efficacy on coapplication with GABA at the $\alpha1\beta2\gamma2$ and $\alpha2\beta2\gamma2$ receptor subtypes was comparable to that of standard diazepam (110 ± 9%, 118 ± 10%, and 113 ± 9%, for compounds **45**, **49**, and diazepam at $\alpha1\beta2\gamma2$, respectively; 105 ± 10%, 108 ± 9% and 110 ± 6%, for compounds **45**, **49** and diazepam at $\alpha2\beta2\gamma2$, respectively), showing that the main feature of compounds **45** and **49** is their full agonist efficacy profile at both these receptor subtypes. By contrast, in comparison to the modulation induced by standard diazepam, compounds **45** and **49** did not substantially

Table 3. Inhibition of [^{3}H]Flumazenil Specific Binding to Bovine Brain Membranes of PTBT Derivatives **37**−**49**



no.	X	R	K_i[a] (nM)
37	H	H	22 ± 2
38	H	4′-F	54 ± 3
39	H	4′-Cl	169 ± 10
40	H	4′-Br	224 ± 15
41	H	4′-CH_3	616 ± 59
42	H	4′-OCH_3	11 ± 1
43	H	2′-F	>10000
44	H	3′,4′-$(OCH_3)_2$	>10000
45	Cl	H	2.8 ± 0.15
46	Cl	4′-F	56 ± 3
47	Cl	4′-Cl	38 ± 2
48	Cl	4′-CH_3	185 ± 15
49	Cl	4′-OCH_3	6 ± 0.5
diazepam			10 ± 1
flumazenil			0.90 ± 0.05
clonazepam			0.85 ± 0.02

[a] K_i values are means ± SEM of three determinations.

Table 4. Inhibition of [^{3}H]Flumazenil Specific Binding of Selected Compounds on Rat α1β2γ2, α2β2γ2 and α5β3γ2 $GABA_A$/Bz Receptor Subtypes[a]

	K_i (nM)[b] or % inhibition (10 μM)[c]		
no.	α1β2γ2	α2β2γ2	α5β3γ2
37	9.14 ± 1	27.6 ± 2	24.1 ± 2
42	3.76 ± 0.3	14.3 ± 1	17.2 ± 2
45	1.74 ± 0.1	13.9 ± 1	10.3 ± 1
49	1.82 ± 0.1	18.0 ± 2	7.57 ± 0.5
zolpidem	50 ± 3	765 ± 63	35% ± 3

[a] The ability of the compounds to displace [^{3}H]flumazenil was measured in membranes from HEK293 cells expressing the α1β2γ2, α2β2γ2 and α5β3γ2 subtypes, as described in the Experimental Section. [b] K_i values are means ± SEM of three determinations. [c] Percentage inhibition values of specific [^{3}H]flumazenil binding at 10 μM concentration are means ± SEM of three determinations.

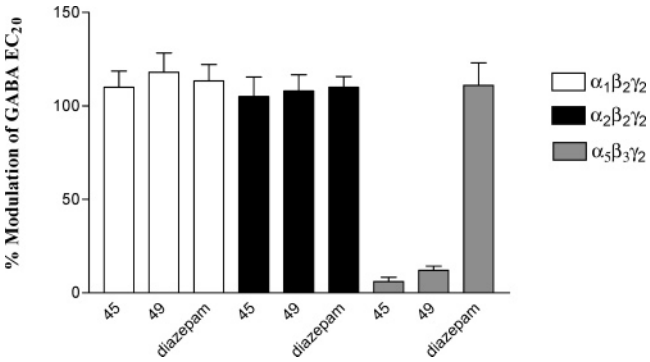


Figure 3. Maximal efficacy of compounds **45** and **49** on rat recombinant α1β2γ2, α2β2γ2 and α5β3γ2 $GABA_A$ receptor subtypes. Results are expressed as percentages of increase in response to GABA at EC_{20} (mean ± SEM) from at least five independent experiments.

affect GABA-evoked Cl^- influx at the α5β3γ2 $GABA_A$/Bz receptor subtype, suggesting an intrinsic antagonist activity (6 ± 2%, 12 ± 2%, and 111 ± 12%, for compounds **45**, **49** and diazepam at α5β3γ2, respectively). These products should consequently exert their effects only through modulation of the α1 and α2 subtypes, bearing a functional agonist efficacy at these subtypes and an antagonist behavior at the α5 subtype. Thus, these results might indicate hypnotic with anxiolytic properties for compounds **45** and **49**, possibly without any effect on learning or memory processes.

In conclusion, we have described 2-phenyl[1,2,3]-triazolo[1,2-*a*][1,2,4]benzotriazin-1,5(6*H*)-diones (PTBTs) as a novel class of BzR ligands. Compounds **45** (X = Cl and R = 4′−H) and **49** (X = Cl and R = 4′-OCH_3) displayed the highest potency at the BzR (K_i 2.8 nM and 6 nM, respectively). When tested for their affinity on recombinant rat α1β2γ2, α2β2γ2, and α5β3γ2 $GABA_A$/Bz receptor subtypes, these ligands proved to be about 4−10-fold selective for the α1β2γ2 over the α2β2γ2, and α5β3γ2 subtypes. Moreover, they showed a full agonist efficacy on the α1 and α2 receptor subtypes, and an antagonist efficacy on α5-containing receptors. This affinity and efficacy selectivity profile makes compounds **45** and **49** a valuable starting point for further research aimed at obtaining novel zolpidem-like[30] sedative agents.

Experimental Section

Chemistry. Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Infrared spectra were recorded with a Nicolet/Avatar FT-IR spectrometer in Nujol mulls. Routine nuclear magnetic resonance spectra were recorded in DMSO-d_6 solution on a Varian Gemini 200 spectrometer operating at 200 MHz. Mass spectra were obtained on a Hewlett-Packard 5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Silica gel 60 (230−400 mesh) was used for column chromatography. Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

Ethyl phenylacetate **3**, ethyl 4-methylphenylacetate **7**, ethyl 4-methoxyphenylacetate **8** and ethyl 3,4-dimethoxyphenylacetate **10** are commercially available products. The following compounds were prepared in accordance with reported procedures: 2-azidonitrobenzene **1**,[18] 2-azido-4-chloronitrobenzene **2**,[19] ethyl 4-fluorophenylacetate **4**,[20] ethyl 4-chlorophenylacetate **5**,[20] ethyl 4-bromophenylacetate **6**,[20] ethyl 2-fluorophenylacetate **9**.[21]

General Procedure for the Synthesis of 1-(2-Nitro-5-substituted-phenyl)-4-(substituted-phenyl)-5-oxo-1,2,3-triazole Derivatives 11−23. The appropriate ethyl phenylacetate **3**−**10** (8.5 mmol) was added dropwise, at −10 °C, under stirring and in a nitrogen atmosphere, to an ethanolic solution of sodium ethoxide (0.196 g, 8.5 mmol of sodium in 4.5 mL of absolute ethanol). Then, the azido derivatives **1** and **2** (8.2 mmol) was added in a single portion to the resulting mixture. Stirring was continued at −10 °C overnight, and then at room temperature for 1−6 h (TLC analysis). The suspension obtained was evaporated to dryness at reduced pressure and at a temperature not higher than 30−40 °C; the resulting semisolid residue was treated with ice and water. The aqueous mixture was washed three times with dichloromethane, cooled at 0 °C and acidified to pH 5 with dilute hydrochloric acid. The precipitate which formed, made up of the crude products **11**−**23**, was collected and purified by washing with cold petroleum ether 60−80 °C. Yields, melting points and analytical and spectral data are reported in Table 1.

General Procedure for the Synthesis of 1-(2-Aminophenyl)-4-(substituted-phenyl)-5-oxo-1,2,3-triazole Derivatives 24−31. A mixture of the appropriate 1-(2-nitrophenyl)-4-(substituted-phenyl)-5-oxo-1,2,3-triazole derivative

11−**18** (0.285 mmol) and 10% Pd/C (0.02 g) in 150 mL of absolute ethanol was hydrogenated at room temperature and pressure. Once hydrogen absorption ceased (2−4 h), the catalyst was filtered off and the solution was evaporated to dryness at reduced pressure and at a temperature not higher than 30−40 °C. The resulting residue made up of the crude amines **24**−**31** was purified by flash-chromatography (eluting system: toluene/acetonitrile/glacial acetic acid in various ratios). Yields, melting points and analytical and spectral data are reported in Table 1.

General Procedure for the Synthesis of 1-(2-Amino-5-chlorophenyl)-4-(substituted-phenyl)-5-oxo-1,2,3-triazole Derivatives 32−**36.** The amino derivatives **32**−**36** were prepared following the same procedure described for the unsubstituted analogues **24**−**31**, but the catalyst (10% Pd/C) was first activated overnight on hydrogen. The usual workup of the reaction mixture gave compounds **32**−**36**, which were particularly unstable; they were not isolated and characterized, and they were utilized in the following reaction without any further purification.

General Procedure for the Synthesis of 9-Substituted-2-(substituted-phenyl)-[1,2,3]triazolo[1,2-*a*][1,2,4]-benzotriazin-1,5(6*H*)-dione Derivatives 37−**49.** Triphosgene (0.060 g, 0.2 mmol) and triethylamine (0.17 mL, 1.22 mmol) were successively added to a stirred and ice-cooled solution of the appropriate 1-(2-amino-5-substituted-phenyl)-4-(substituted-phenyl)-5-oxo-1,2,3-triazole derivative **24**−**36** (0.5 mmol) in 9.0 mL of anhydrous tetrahydrofuran. The mixture was stirred under a nitrogen atmosphere at 0 °C for 1 h and then at room temperature for 12−36 h (TLC analysis). The suspension obtained was filtered, and the solid was washed with water to give a first portion of crude tricyclic product. The reaction mother liquors were reduced to half volume to precipitate an additional amount of crude product. The quantities of tricyclic derivatives obtained from the initial insoluble precipitate and from the concentrated tetrahydrofuran solution were variable, depending upon the solubility of the particular compound. All the newly synthesized tricyclic products **37**−**49** were purified by recrystallization from the appropriate solvent. Yields, recrystallization solvents, melting points and analytical and spectral data are reported in Table 2.

Biological Procedures. 1. Radioligand Binding Studies. [^{3}H]Flumazenil (specific activity 70.8 Ci/mmol) was obtained from NEN Life Sciences Products. All other chemicals were of reagent grade and were obtained from commercial suppliers.

Bovine cerebral cortex membranes were prepared in accordance with ref 31. The membrane preparations were subjected to a freeze−thaw cycle, washed by suspension and centrifugation in 50 mM Tris-citrate buffer pH 7.4 (T1) and then used in the binding assay. Protein concentration was assayed by the method of Lowry et al.[32]

[^{3}H]Flumazenil binding studies were performed as previously reported.[15]

HEK293 cells stably expressing rat $GABA_A$ receptor subtypes ($\alpha1\beta2\gamma2$, $\alpha3\beta2\gamma2$, $\alpha5\beta3\gamma2$) were maintained, as previously described,[33] in DMEM/Nut Mix F-12 with Glut-I (GIBCO), supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells were harvested and then centrifuged at 500*g*. The crude membranes were prepared after homogenization in 10 mM potassium phosphate, pH 7.4, and differential centrifugation at 48 000*g* for 30 min at 4 °C. The pellets were washed twice in this manner before final resuspension in 10 mM potassium phosphate, pH 7.4, containing 100 mM potassium chloride.[33]

[^{3}H]Flumazenil binding assays to transfected cell membranes were carried out as previously described.[33] In brief, the cell line membranes were incubated in a volume of 500 μL which contained [^{3}H]flumazenil at a concentration of 1−2 nM and the test compound in the range 10^{-9}−10^{-5} M. Nonspecific binding was defined by 10^{-5} M diazepam. Assays were incubated to equilibrium for 1 h at 4 °C.

2. Functional Efficacy Studies. $^{36}Cl^-$ uptake was measured in transfected HEK293 cells by minor modification of a previously described method.[24] In brief, coverlips were placed on 8−10/cell culture plates (100 mm) and were incubated overnight under germicidal light in a solution containing 0.01 mg/mL poly-lysine in 0.1 M boric acid (pH 8.4). The following day, coverlips were washed twice with phosphate-buffered saline and were placed on individual six-well cell culture plates. DMEM/Nut Mix F-12 with Glut-I (GIBCO), supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/mL), were added to each well. Cells were then plated into each well at a density of 50×10^5 cells/well and were grown on the coverlips for 3 days at 37 °C with 5% CO_2. Cells were washed twice (10 s/wash) in wash buffer 136 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, adjusted to a pH of 7.4 with Tris base. All steps were conducted at room temperature. Cells were then dipped into 2 mL of a $^{36}Cl^-$ solution (2 μCi/mL) containing the drug(s) to be studied, with or without GABA. Influx was terminated after 8 s by transfer of coverlips to 500 mL ice-cold assay buffer (with 0.1 mM picrotoxin). Coverlips were drained rapidly and placed in 1.6 mL of 0.2 N NaOH in 20 mL scintillation vials and were left overnight. A 0.1 mL aliquot was removed and assayed for protein determination. The remaining 1.5 mL was neutralized with 0.3 mL of 1 N acetic acid and 20 mL of BioSafe II was added for counting by liquid scintillation spectrometry. Values for $^{36}Cl^-$ influx were expressed as nanomol/mg protein.

Molecular Modeling. Molecular modeling was performed using the software package SYBYL version 6.5 (Tripos Inc.) running on a Silicon Graphics R8000 Indigo 2 workstation. The model of the ligand CGS 8216 (Figure 2) corresponds to the crystal structure retrieved from the Cambridge Structural Database[34] (CSD) with the entry code COVLII using ConQuest version 1.6. The model of PTBI derivative **37** (Figure 2) was constructed using the SYBYL fragment library and optimizing the trial geometry with the semiempirical quantum-mechanics AM1 method[35] available within MOPAC version 6.00 (QCPE). MOPAC was run under default settings using the keyword "MMOK". To evaluate the reliability of the geometry-optimization, we searched the CSD looking for compounds similar to **37** through structure-based queries. The results of the searches were consistent with the overall coplanar geometry of **37** yielded by the AM1 method. Examples of hits similar to **37** are two compounds filed in the CSD with the entry codes DMOCDO10[36] and GOZLUC,[37] both featuring entirely coplanar geometries. Overlay of the molecular models was performed as described in a previous study.[38]

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Supporting Information Available: Microanalytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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