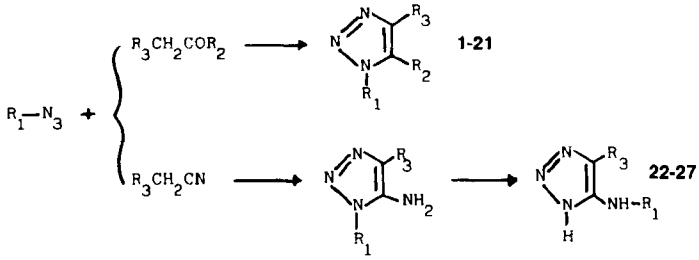
Specific Inhibition of Benzodiazepine Receptor Binding by Some 1,2,3-Triazole Derivatives

C. MARTINI*, W. MARRUCCI*, A. LUCACCHINI*x, G. BIAGI‡, AND O. LIVI‡

Received August 12, 1987, from the *Istituto Policattedra di Discipline Biologiche and the ‡Chimica Farmaceutica dell' Università di Pisa, Pisa, Italy. Accepted for publication June 16, 1988.

Abstract □ Certain 1,2,3-triazole derivatives were prepared and tested for their ability to displace [^{3}H]diazepam from bovine brain membranes. From these compounds, the quinolyltriazole derivatives (**14**, **15**, **16**, **17**) were clearly the most potent, while the naphthyl- and the naphthyridyltriazoles were considerably less active. The *p*-nitrophenyl derivative (**15**) was the compound that bound with the highest affinity within the quinolyltriazole compounds class. The replacement of the *p*-nitrophenyl group with other substituents greatly decreased the binding activity. From a Lineweaver–Burk analysis of **11**, it appears that the inhibition is competitive.



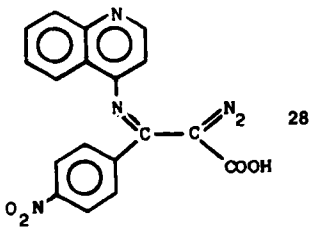
Scheme I

The discovery of pharmacologically relevant, high-affinity, stereospecific binding sites for the benzodiazepines in the central nervous system[1] has prompted studies on the possible physiological significance of these sites and attempts at isolating endogenous ligands.[2] Inosine, hypoxanthine, and nicotinamide were suggested as possible endogenous ligands.[3] In fact, although these compounds are relatively weak competitive inhibitors of [^{3}H]diazepam binding in vitro, they appear to exist in the brain in high concentrations that increase during certain physiological states.[4] Recently we have shown that some intermediates of purine metabolism, such as 5-amino-imidazole-4-carboxamide (AICA), are more potent inhibitors of [^{3}H]diazepam binding than hypoxanthine.[5] Therefore, we studied the effect of substitution of the imidazole ring with a triazole ring and the effect of the triazole substituents on the [^{3}H]diazepam binding to bovine brain membranes. Structural affinity relationships are also discussed.

Experimental Section

Chemistry—All the triazole compounds (**1–21**) were prepared in the usual manner by the 1,3-dipolar cycloaddition reaction of the suitable azide to activated methylenic compounds[6] (Scheme I). Compounds **22–27** were obtained from the corresponding compounds which have the primary amino group in the 5 position of the triazole ring, by the Dimroth isomerization reaction[6] (Scheme I). The syntheses of the triazole derivatives **1–10**, **22–25**,[7] and **11**, **12**, **18–20**, **26**, and **27**[8] have been previously described. The syntheses of **13–17**, **21**, and of the diazo derivative **28**, corresponding to the open structure of the triazole compound **15**, are reported below.

Melting points were determined on a Kofler hot-stage and are uncorrected. The IR spectra in nujol mulls were recorded on a Perkin-Elmer spectrophotometer (model 197). The ^{1}H NMR spectra were recorded on a Jeol C-60 HL spectrometer; all chemical shifts are given in δ from Me_4Si as the internal standard. Elemental analyses (C, H, N) were within ±0.4% of the theoretical values.



28

1-(4'-Quinolyl)-4-carbethoxy-5-methyl-1H-1,2,3-triazole (13)—To an ice-cooled and stirred solution of 1.02 g (6.0 mM) of 4-azidoquinoline[8] and 0.76 mL (6.0 mM) of ethyl acetoacetate in 30 mL of absolute ethanol, a solution of sodium ethoxide [0.14 g (6.0 mM) of sodium in 8 mL of absolute ethanol] was slowly added. After 20 h of stirring at room temperature, the reaction mixture was diluted with H_2O (100 mL) and extracted with $CHCl_3$. From the aqueous alkaline layer, the acid corresponding to the ester **13**[8] was precipitated by acidification (pH 2–3) to yield 0.960 g (63%; mp 192–194 °C). The chloroform extract was evaporated to give a semisolid residue (0.80 g) which was dissolved in benzene and chromatographed through an Al_2O_3 (att. 2–3) column (6 × 1.8 cm). After preparation of a mixture of 4-azidoquinoline and **13** (0.05 g), elution with benzene provided 0.132 g (yield 7.8%) of pure **13** as a white solid with a mp of 90–92 °C; IR: 5.81, 8.04 (COOEt) μ; ^{1}H NMR ($CDCl_3$): δ 9.22 (d, 1, α-quinoline H), 8.53–7.12 (m, 5, aromatic protons), 2.47 (s, 3, CH_3), and 1.47 and 4.53 ppm (t, 3 and q, 2, CH_3CH_2O).

Anal.—Calc. for $C_{15}H_{14}N_4O_2$: C, H, N.

1-(4'-Quinolyl)-4-carbethoxy-5-(p-nitrophenyl)-1H- and 1-(4'-Quinolyl)-4-carboxy-5-(p-nitrophenyl)-1H-1,2,3-triazole (14) and (15)—To an ice-cooled and stirred suspension of 1.02 g (6.0 mM) of 4-azidoquinoline[8] and 1.42 g (6.0 mM) of ethyl *p*-nitrobenzoylacetate in 30 mL of absolute ethanol, a solution of sodium ethoxide [0.14 g (6.0 mM) of sodium in 8 mL of absolute ethanol] was slowly added. The reaction mixture was further stirred at 0–3 °C for 5 h, and then the suspension was diluted with H_2O (≈ 140 mL) and extracted with $CHCl_3$. The chloroform layer was evaporated to give a brown semisolid residue which was crystallized from MeOH: 0.700 g (yield 30%) of **14** as white prisms with a mp of 174–175 °C; IR: 5.79 (COOEt) μ.

Anal.—Calc. for $C_{20}H_{15}N_5O_4$: C, H, N.

Acidification of the aqueous alkaline layer precipitated the corresponding acid **15**, which was collected by filtration (0.805 g, yield 37%). The compound crystallized from MeOH as white needles with a mp of 187–189 °C dec.; IR: 5.95 (COOH) μ; ^{1}H NMR (Me_2SO-d_6): δ 9.16 (d, 1, α-quinoline H), and 8.47–7.63 ppm (m, 9, aromatic protons).

Anal.—Calc. for $C_{18}H_{11}N_5O_4$: C, H, N.

1-(4'-Quinolyl)-5-(p-nitrophenyl)-1H-1,2,3-triazole (16)—A solution of 1.88 g of **15** in 5 mL of DMF was refluxed for 1 h. After cooling, the mixture was diluted with H_2O to precipitate 1.50 g of **15** (yield 91%) as prisms from EtOH with a mp of 199–203 °C.

0022-3549/88/1100-0977$01.00/0

Anal.—Calc. of $C_{17}H_{11}N_5O_2$: C, H, N.

1-(4'-Quinolyl)-4-carbethoxy-5-(p-aminophenyl)-1H-1,2,3-triazole (17)—A solution of 0.200 g of **14** in 30 mL of ethanol was added to 13 mg of 5% Pd/C and hydrogenated at room temperature and pressure. Because the reaction product partially precipitated, the mixture was heated and filtered from the catalyst which was washed with hot ethanol. The combined filtrates were evaporated under reduced pressure to give 0.175 g (yield 95%) of **17**. This compound crystallized from ethanol as needles with a mp of 245–249 °C; IR: 3.03, 3.20 (NH_2) μ.

Anal.—Calc. for $C_{20}H_{17}N_5O_2$: C, H, N.

1-(1'-Napthyl)-4-carbethoxy-5-methyl-1H-1,2,3-triazole (21)—To a solution of 0.250 g of 1-(1'-naphthyl)-4-carboxy-5-methyl-1H-1,2,3-triazole[8] in 30 mL of anhydrous ethanol was added 5 drops of 18 M H_2SO_4, and the solution was refluxed for 24 h. The reaction mixture was concentrated under reduced pressure, diluted with H_2O, and extracted with $CHCl_3$. The chloroform layer, after washing with 2M NaOH, was evaporated to give 0.250 g (yield 90%) of **21** as a viscous yellow oil; IR: 5.86 and 8.09 (COOEt) μ, ^1H NMR ($CDCl_3$): δ 9.46–7.06 (m, 7, aromatic protons), 2.41 (s, 3, CH_3), and 1.48 and 4.54 ppm (t, 3 and q, 2, CH_3CH_2O).

Anal.—Calc. for $C_{16}H_{15}N_3O_2$: C, H, N.

3-(Quinolyl-4'-imino-3-(p-nitrophenyl)-2-diazopropionic Acid (28)—To an ice-cooled and stirred solution of sodium ethoxide [76 mg (3.3 mM) of Na in 9 mL of absolute EtOH], a solution of 0.736 g (3.3 mM) of ethyl *p*-nitrobenzoylacetate and 0.510 g (3.0 mM) of 4-azidoquinoline[8] in 20 mL of absolute ethanol was added in a dropwise manner. After 1.5 h, the ice bath was removed, stirring was continued for 1.5 h, and the reaction mixture was kept at room temperature for 18 h. To the obtained suspension, 100 mL of H_2O was added and the mixture was heated to complete solution. Acidification precipitated a white solid (0.982 g) consisting of **15** and a small amount of **28**, which was collected and washed with H_2O. Crystallization of this solid from DMF gave 0.140 g (yield 13%) of **28** which was collected and washed with H_2O to yield an orange solid with a mp of 215–220 °C dec.; IR: 4.66 (N_2) and 5.97 (COOH) μ; ^1H NMR (Me_2SO-d_6): δ 9.15 (m, 1, α-quinoline H), and 8.63–7.50 ppm (m, 9, aromatic protons).

Anal.—Calc. for $C_{18}H_{11}N_5O_4$: C, H, N.

Dilution of the mother liquors with H_2O provided 0.790 g (73%) of the triazole derivative **15**.

Receptor Binding Assay—The ability of these triazole derivatives to displace specific [^{3}H]diazepam binding was tested. Initially, a single concentration (250 μM) of the potential displacing agent was examined, followed by the determination of the concentration able to displace 50% of the specific [^{3}H]diazepam binding (IC_{50}) from log-probit plots for the most active compounds. The data generated are shown in Table I.

[^{3}H]Diazepam and [3H]Ro 5-4864 were obtained from New England Nuclear (Dreieichenhain, West Germany) and had a specific activity of 76.9 and 77.9 Ci/mM, respectively, and a radiochemical purity >99%. [^{3}H]Clonazepam was a gift of Hoffmann-La Roche (Basel, Switzerland). All other chemicals were of reagent grade and obtained from commercial suppliers.

Bovine cerebral cortex was dissected over ice and homogenized in 10 vol of ice-cold 0.32 M sucrose, containing protease inhibitors,[9] in an Ultra-turrax for 30 s. The homogenate was centrifuged at 1000 × *g* for 5 min at 4 °C and the supernatant was recentrifuged at 50,000 × *g* for 30 min at 4 °C. The pellet was osmotically shocked by suspension in 20 vol of 50 mM tris-HCl buffer (pH 7.4) containing protease inhibitors and recentrifuged at 50,000 × *g* for 30 min at 4 °C. The pellet was resuspended in 10 vol of 50 mM tris-HCl buffer at pH 7.4.

The estimation of proteins was based on the method of Lowry et al.[10] after solubilization with 0.75 M NaOH. Bovine serum albumin was utilized as the standard.

The membrane suspension (0.4–0.6 mg of proteins) was incubated in triplicate with ~ 1.2 nM [^{3}H]diazepam and various concentrations of displacers for 45 min at 0 °C in 500 μL of 50 mM tris-HCl buffer at pH 7.4. After incubation, the samples were diluted with 5 mL of assay buffer and immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B) and then washed with 5 mL of the same buffer. Radioactivity on the filters was determined in 8 mL of HP Beckman scintillation cocktail in a liquid scintillation counter. Nonspecific binding was determined by parallel experiments containing diazepam (10 μM) and accounted for <10% of total binding. [^{3}H]Clonazepam and [^{3}H]Ro 5-4864 binding assays were carried out as described previously.[11,12] Water insoluble derivatives were dissolved in dimethylsulfoxide (<1% in the assay) and the same concentration was present in blank experiments. The concentration of the triazole derivatives that inhibits specific [^{3}H]diazepam binding by 50% (IC_{50}) was determined by log-probit analysis, with four to six concentrations of the displacers, and each analysis was performed in triplicate.

Results and Discussion

Table I shows that the most active compounds are **7**, **11**, **13**, **14**, **15**, **16**, and **1**. Therefore, these data indicate that the introduction of quinoline in the 1 position of the triazole ring produces more active compounds than naphthyridine and naphthalene compounds. Among the more active compounds, the substituents in the 4 and 5 positions of the triazole ring produce important differences in the inhibitory activity; in fact, the most active compound presents a *p*-nitrophenyl substituent in the 5 position and a carboxyl in the 4 position. Esterification of the carboxyl group or its substitution with a hydrogen atom causes a decrease in the inhibitory potency. These results may be explained by the presence of a positive charge in the receptor binding site, as suggested in a previous paper[13] concerning the structure–activity requirements of β-carboline analogues. In that paper, the presence of an arginine residue or two lysine residues bearing basic functions was hypothized. Therefore, the possibility that the reduction of the NO_2 group to a NH_2 group led to a decrease in the inhibitory activity (see **14** and **17**) seems in accordance with this hypothesis. Also the substitution of a methyl group in the 5 position reduces the activity. No structure–activity relationship (SAR) may be deduced from the inhibition of the naphthyridine compounds owing to their low activity; the substitution at the 4 or 5 positions gave the same results as quinoline compounds. These data indicate that the substitutions in the 1, 4, and 5 positions gave more active compounds than hypoxanthine and AICA.

The nature of [^{3}H]diazepam binding inhibition was determined by Lineweaver–Burk analysis in the presence of a fixed concentration of **11**. The results in Figure 1 show that the K_m for [^{3}H]diazepam binding is increased while the

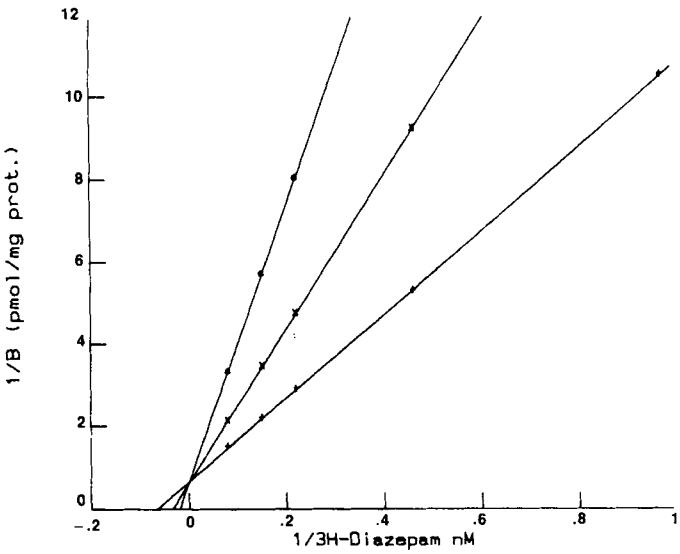
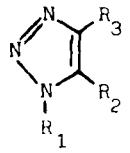
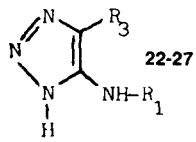


Figure 1—*Lineweaver–Burk analysis of inhibition of [^{3}H]diazepam binding by **11**. Membranes were incubated with 0 μM (+), 10 μM (×), or 20 μM (*) concentrations of compound and used for the [^{3}H]diazepam receptor binding assay at five concentrations of [^{3}H]diazepam. The assays were performed as described in the text.*

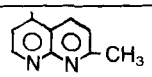
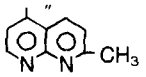
Table I—Inhibition of [^{3}H]Diazepam Binding



1-21



22-27

Compound	R_1	R_2	R_3	Reference	Inhibition (250 μM),%[a]	IC_{50}, μM[b]
1		—CH_3	—COOH	7	17 ± 1	—
2	"	"	—COOEt	7	60 ± 5	185 ± 21
3	"	"	—C_6H_5	7	55 ± 3	200 ± 18
4	"	"	—CONH-C_6H_5	7	27 ± 3	—
5	"	—NH_2	—CN	7	22 ± 2	—
6	"	—C_6H_5	—COOH	7	6 ± 0.3	—
7	"	"	—$COCH_3$	7	68 ± 5	30 ± 5
8	"	"	—CO-C_6H_5	7	59 ± 3	180 ± 15
9	"	—$C_6H_4NO_2$-*p*	—H	7	17 ± 2	—
10	"	"	—COOEt	7	12 ± 1	—
11		—NH_2	—COOEt	8	86 ± 5	10 ± 0.8
12	"	—CH_3	—C_6H_5	8	60 ± 3	175 ± 14
13	"	"	—COOEt		78 ± 5	30 ± 2
14	"	—$C_6H_4NO_2$-*p*	"	8	100	7.5 ± 0.5
15	"	"	—COOH		100	0.45 ± 0.03
16	"	"	—H		100	2 ± 0.1
17	"	—$C_6H_4NH_2$-*p*	—COOEt		72 ± 5	33 ± 4
18		—NH_2	—$CONH_2$	8	60 ± 4	190 ± 13
19	"	"	—COOEt	8	89 ± 3	70 ± 5
20	"	—CH_3	—C_6H_5	8	—[b]	80 ± 8
21	"	"	—COOEt		—[b]	90 ± 6
22		—	—CN	7	—	—
23	"	—	—COOH	7	—[b]	30 ± 2
24	"	—	—COOEt	7	48 ± 3	—
25	"	—	—C_6H_5	7	40 ± 4	—
26		—	—COOEt	8	—[b]	90 ± 6
27		—	—COOEt	8	100	45 ± 3
28	see Introduction				100	6 ± 0.4
Diazepam						0.041 ± 0.004
Hypoxanthine						1350 ± 120
5-Amino-imidazole-4-carboxamide						630 ± 55

[a] Percent of inhibition of specific [^{3}H]diazepam binding at 250 μM compound concentration are means ± SEM of five determinations. [b] The compound was not soluble at 250 μM concentration in Me_2SO. [c] Concentrations necessary for 50% inhibition (IC_{50}) are means ± SEM of four determinations.

maximal binding (B_{max}) remains unchanged for the tested compound, indicating that it inhibits the specific [^{3}H]diazepam binding in a competitive manner.

To evaluate if the tested compounds displace [^{3}H]diazepam from the central or peripheral type benzodiazepine receptors, we used [^{3}H]clonazepam as a specific ligand for central type receptors and [^{3}H]Ro 5-4864 for the peripheral ones.[14] The results obtained suggest that the compounds displace [^{3}H]clonazepam with identical affinity as [^{3}H]diazepam. Also, at 250 μM concentration, no inhibition of [^{3}H]Ro 5-4864 binding was observed, indicating that these compounds are specific for the benzodiazepine central type receptors.

References and Notes

1. Braestrup, C.; Squires, R. F. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3805–3809.
2. Marangos, P. J.; Paul, S. M.; Goodwin, F. K.; Skolnich, P. *Life Sci.* **1979**, *25*, 1093–1098.
3. Asano, T.; Spector, S. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 977–981.
4. Kleihues, P.; Kobeyeshi, K.; Hossemann, K. A. *J. Neurochem.* **1974**, *23*, 417–420.
5. Bazzichi, L.; Martini, C.; Lucacchini, A. 4th Meeting of the European Society for Neurochemistry; Catania (Italy) Sept. 1982; Abstract 393.

6. Gilchrist, T. L.; Gymer, G. E. In *Advances in Heterocyclic Chemistry;* Katritzky, A. R.; Boulton, A. J., Eds.; Academic: New York, 1974; pp 33–85; and Finley, K. T. *The Chemistry of Heterocyclic Compounds;* Montgomery, J. A., Ed.; Wiley: New York, 1980; Vol. 19.
7. Livi, O.; Ferrarini, P. L.; Tonetti, I. *Farmaco, Ed. Sci.* **1976**, *31*, 797–808.
8. Livi, O.; Ferrarini, P. L.; Tonetti, I.; Smaldone, F.; Zefola, G. *Farmaco, Ed. Sci.* **1979**, *34*, 217–228.
9. Martini, C.; Lucacchini, A.; Ronca, G.; Rossi, C. A. *J. Neurochem.* **1982**, *38*, 15–19.
10. Lowry, O. H.; Roserbrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
11. Martini, C.; Gervasio, T.; Lucacchini, A.; Da Settimo A.; Primofiore, G.; Marini, A. M. *J. Med. Chem.* **1985**, *28*, 506–509.
12. Martini, C.; Giannaccini, G.; Lucacchini, A. *Biochim. Biophys. Acta* **1983**, *728*, 289–292.
13. Loew, G. H.; Nienow, J.; Lawson, J. A.; Toll, L.; Uyeno, E. T. *Mol. Pharm.* **1986**, *28*, 17–31.
14. Martini, C.; Lucacchini, A.; Hrelia, S.; Rossi, C. A. In *Advances in Biochemical Psychopharmacology;* Biggio, G.; Costa, E., Eds.; Raven: New York, 1986; pp 1 and 10.