istered by intraperitoneal (ip) injection in a volume of 2.0 mL kg^{-1}, dissolved in saline containing 0.1% sodium bisulfite. After 40 min, during which time the behavioral effect was scored according to the method of Costall et al.,[9] the rats were killed by cervical dislocation.

The corpora striata were rapidly dissected, frozen on dry ice, and stored at −80 °C. Following weighing of the frozen samples, homogenization in 0.1 M perchloric acid, and centrifugation (3000 g, 7 °C, 15 min), the amount of dopamine and its metabolites HVA and DOPAC in the supernatants was determined according the method of Westerink and Mulder[8] by use of purification on Sephadex G 10, separation on a reverse-phase (RP 18) high-performance liquid chromatographic column, and amperometric detection.

Binding Experiments. The binding studies were carried out according to the procedure of Leysen and Gommeren.[6] Striata of female Wistar rats (C.D.L., Groningen) were dissected and homogenized in 40 volumes of ice cold 15 mM Tris-chloride buffer (1 mM EDTA and 0.01% ascorbic acid added, pH 7.5 at 5 °C) with an ultra-turrax (IKA, 165 rpm, 45 s). The homogenate was centrifuged at 35000*g* for 10 min, washed with buffer, and recentrifuged at the same speed two times. The final pellet was rehomogenized at a final concentration of 50 mg of original wet weight per millimeter in cold fresh 15 mM Tris buffer containing 1 mM EDTA and 0.01% ascorbic acid. In the competition experiments, to incubation tubes in triplicate were added 100 μL of [^{3}H]NPA (final concentration 0.46 nM), increasing concentrations of competing drugs, soluted in the buffer (10^{-11} to 10^{-5} M) or 200 μl of (+)-butaclamol (final concentration 10^{-6} M), and 100 μL of the tissue suspension, giving a final volume of 1 mL.

All tubes were incubated at 25 °C for 30 min and the contents rapidly filtered under vacuum through Whatman glass fiber GF/B filters and washed three times with 5 mL of ice-cold buffer. The filters were collected and placed in glass vials with 6 mL of scintillation cocktail, left overnight, shaken for 20 min, and counted by liquid scintillation spectrometry (Beckman LS 18) at a counting efficiency of 45–50%. Saturable or specific binding was defined as the difference between the binding in the absence and in the presence of 10^{-6} M *d*-butaclamol (total binding 200 dps, nonspecific binding 40 dps as typical values were found).

Registry No. (+)-**2**·DB, 94844-52-7; (−)-**2**·DB, 94844-54-9; (−)-**3**·HCl, 94903-51-2; (−)-**3**, 82730-71-0; (+)-**3**·HCl, 94903-52-3; (+)-**3**, 82730-70-9; (±)-**3**, 72189-85-6; (+)-2-amino-5,6-dimethoxytetralin, 94903-49-8; (−)-2-amino-5,6-dimethoxytetralin, 94903-50-1.

Synthesis and Interaction of 5-(Substituted-phenyl)-3-methyl-6,7-dihydropyrazolo[4,3-*e*][1,4]diazepin-8(7*H*)-ones with Benzodiazepine Receptors in Rat Cerebral Cortex

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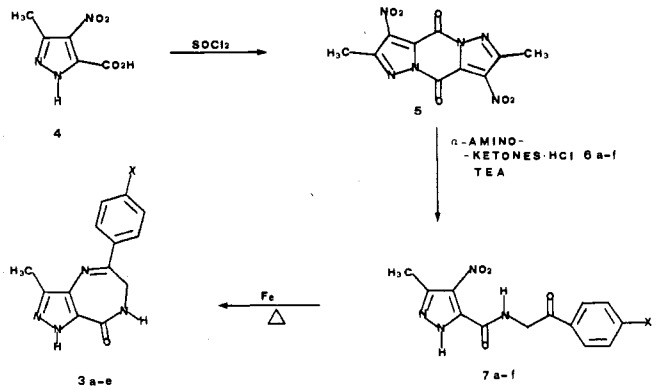
On the basis of the anxiolytic property of ripazepam, 1-ethyl-4,6-dihydro-3-methyl-8-phenylpyrazolo[4,3-*e*][1,4]-diazepin-5(1*H*)-one (**1**), a series of isomeric 5-(phenyl-substituted)pyrazolo[4,3-*e*][1,4]diazepin-8-ones **3a–f** were prepared and tested for their ability to bind to the benzodiazepine receptor. All compounds **3a–f** display affinities for the benzodiazepine receptor in the μM range of concentration; in particular 5-phenyl-3-methyl-6,7-dihydropyrazolo-[4,3-*e*][1,4]diazepin-8(7*H*)-one (**3a**) is 2 orders of magnitude less potent in inhibiting [^{3}H]flunitrazepam binding than diazepam and displays an affinity for the benzodiazepine receptor practically comparable to that of its structural isomer, ripazepam, and to that of chlordiazepoxide.

Since the discovery of benzodiazepines (BZ) in 1960, extensive research efforts in the field led to the development of a variety of modified derivatives.[1] Since almost all BZs display a wide pharmacological spectrum, e.g., anxiolytic, sedative-hypnotic, anticonvulsive, and muscle relaxant properties, an important goal of these investigations is to identify analogues that possess a more selective action.[2] Much attention has been paid to the role of the replacement of the fused benzo ring by an heterocyclic ring, so 1,4-diazepines fused to thiophenes,[3a] imidazoles,[3b] pyrroles,[3c] isoxazoles,[3d] pyrazines,[3e] and pyrazoles[3f] have been reported. However, of all these systems, pyrazolodiazepines have proven to be the most important ones since two derivatives, ripazepam (**1**) and zometapine (**2**) are in an advanced phase of clinical investigation.[3f]

Scheme I



Taking these results into consideration, we decided to synthesize a new series of pyrazolo[4,3-*e*][1,4]diazepin-8-ones (**3a–f**) isomeric with respect to the ring system of

(1) Hamor, T. A.; Martin, I. L. "Progress in Medicinal Chemistry"; Ellis, G. P., West, G. B., Eds.; Elsevier: Amsterdam, 1983; Vol. 20, pp 157–223. Sternbach, L. H. "The Benzodiazepines"; Garattini, S., Mussini, E., Randall, L. O., Eds.; Raven Press: New York, 1973; pp 1–26.
(2) Williams, M. *J. Med. Chem.* **1983**, *26*, 619.
(3) (a) Tinney, F. J.; Sanchez, J. P.; Nogas, J. A. *J. Med. Chem.* **1974**, *17*, 624. (b) Edenhofer, A. *Helv. Chim. Acta* **1974**, *58*, 2192. (c) Fontanella, L.; Mariani, L.; Tarzia, G. *Eur. J. Med. Chem.* **1976**, *11*, 217. (d) Jaunin, R. *Helv. Chem. Acta* **1974**, *57*, 1934. (e) U.S. Patent 3 880 840, 1975. (f) De Wald, H. A.; Nordin, I. C.; L'Italien, Y. J.; Parcell, R. F. *J. Med. Chem.* **1973**, *16*, 1346. De Wald, H. A.; Lobbestael, S.; Butler, D. E. *J. Med. Chem.* **1977**, *20*, 1562. De Wald, H. A.; Lobbestael, S.; Poschel, B. P. H. *J. Med. Chem.* **1981**, *24*, 982.

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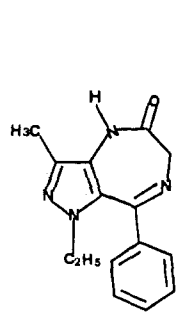
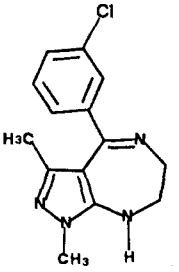


1



2

ripazepam. Herein we have described a straightforward synthesis of **3a–f** as well as preliminary [^{3}H]flunitrazepam binding assays of rat cortex membrane preparations for the determination of IC_{50} values of the synthesized compounds.

Chemistry. The synthesis of the various analogues **3a–f** is outlined in the Schemes I and II, and the properties of the compounds are summarized in Table I. The key intermediate, diketopiperazin **5**, was prepared with 3-methyl-4-nitropyrazole-5-carboxylic acid (**4**) as the starting material according to the procedure of Musante.[4] Recently, we reported that diketopiperazine **5**, showing an excellent reactivity toward nucleophilic reagents such as aliphatic and aromatic amines[5] and substituted phenylhydrazines,[6] can be considered a versatile intermediate in heterocyclic chemistry.

When **5** was allowed to react with α-amino ketone hydrochlorides **6a–f** in the presence of TEA, only poor yields of **7a–f** contaminated with colored byproducts could be isolated. However, when a diluted solution of TEA was slowly added to the reaction mixture at 0 °C, good yields of **7a–f** were obtained. In this way pyrazine formation, a complicating side reaction due to the tendency of α-amino ketones to dimerize,[7] was minimized. The nitro amides **7a–f**, so prepared, served as intermediate precursors to the following reduction–cyclization reaction sequence. When the amides **7a–e** were refluxed in a 3:1 mixture of 2-methoxyethanol–water in the presence of iron powder, reduction of the nitro group followed by in situ cyclodehydratation took place in good overall yield, resulting in the construction of the seven-membered ring of **3a–e** (Scheme I). Attempted extension of this route to the amide **7f** led instead to an inseparable reaction mixture.

Our second approach required the intermediacy of 4-amino-3-methylpyrazole-5-carboxylic acid[8] (**8**) as depicted in Scheme II. Protection of the amino group of **8** with di-*tert*-butyl carbonate[9] resulted in formation of Boc derivatives **9** in 79% yield. Condensation of the α-amino ketones **6a,f** with **9** by using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide as coupling reagents yielded the blocked amides **10a,f**. Removal of the Boc protecting group by treatment with trifluoroacetic acid (TFA), followed by base-promoted (TEA) cyclization, gave **3a,f** in good yield.

Compound **3a** obtained by both the routes showed identical analytical and spectral data.

Analytical and spectral data (IR, NMR) of all intermediates and final products were consistent with the proposed structures.

Scheme II

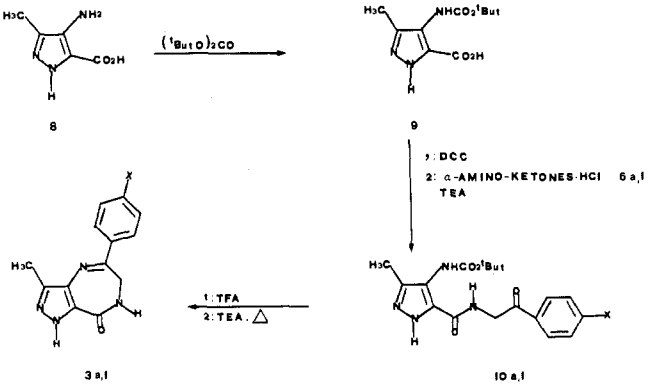


Table I. Physical Constants of **7a–f** and **3a–f**

compd	X	mp, °C	yield, %	formula	anal.
7a	C_6H_5	225–227	68	$C_{13}H_{12}N_4O_4$	C, H, N
7b	*p*-ClC_6H_4	233–235	58	$C_{13}H_{11}ClN_4O_4$	C, H, N
7c	*p*-$CH_3OC_6H_4$	219–221	62	$C_{14}H_{13}N_4O_5$	C, H, N
7d	*p*-$C_6H_4C_6H_4$	233–235	61	$C_{19}H_{16}N_4O_4$	C, H, N
7e	*p*-BrC_6H_4	232–234	63	$C_{13}H_{11}BrN_4O_4$	C, H, N
7f	*p*-$NO_2C_6H_4$	224–226	55	$C_{13}H_{12}N_5O_6$	C, H, N
3a	C_6H_5	249–251	53	$C_{13}H_{12}N_4O$	C, H, N
3b	*p*-ClC_6H_4	258–260	48	$C_{13}H_{11}ClN_4O$	C, H, N
3c	*p*-$CH_6OC_6H_4$	234–236	61	$C_{14}H_{14}N_4O_2$	C, H, N
3d	*p*-$C_6H_5C_6H_4$	281–283	49	$C_{19}H_{16}N_4O$	C, H, N
3e	*p*-BrC_6H_4	266–268	53	$C_{13}H_{11}BrN_4O$	C, H, N
3f	*p*-$NO_2C_6H_4$	>310	56	$C_{13}H_{12}N_5O_3$	C, H, N

Table II. Affinities of **3a–f** (Expressed as IC_{50} Values) for [^{3}H]Flunitrazepam Binding Sites in Membranes from Rat Cerebral Cortex

compd	IC_{50}[a]	compd	IC_{50}[a]
3a	4.4 ± 0.3	**3f**	30.5 ± 2.1
3b	10.0 ± 0.8	ripazepam	0.67 ± 0.08
3c	46.5 ± 3.0	diazepam	0.03 ± 0.002
3d	>100	chlordiazepoxide	0.91 ± 0.07
3e	22.3 ± 1.8		

[a] IC_{50} values (μM) are mean values (±SE) of at least three determinations. Binding experiments were performed, as described in the Biological Investigations section with 0.5 nM [^{3}H]flunitrazepam in the presence of 200 nM NaCl.

Results and Discussion

The data in Table II show the relative potencies of the tested compounds **3a–f**, expressed as IC_{50} values, in displacing [^{3}H]flunitrazepam receptor binding from synaptic membranes in rat cerebral cortex. The IC_{50} values of ripazepam (as a typical representative of pyrazolodiazepines) and of diazepam and chlordiazepoxide (two classical benzodiazepines in clinical use) are also included, as reference compounds.

The IC_{50} value for compound **3d** could be determined only as exceeding an upper limit owing to its low degree of solubilization. From examination of Table II, the following preliminary conclusions can be drawn.

(a) All compounds can inhibit [^{3}H]flunitrazepam binding at relatively high concentrations (in the range 4–100 μM); however, compound **3a**, the most potent of the series, which is 2 orders of magnitude less potent in inhibiting

(4) Musante, C. *Gazz. Chim. Ital.* **1945**, *75*, 112.
(5) Baraldi, P. G.; Simoni, D.; Periotto, V.; Manfredini, S.; Guarneri, M.; Manservigi, R.; Cassai, E.; Bertolasi, V. *J. Med. Chem.* **1984**, *27*, 986 and references herein quoted.
(6) Baraldi, P. G.; Guarneri, M.; Simoni, D.; Moroder, F.; Vicentini, C. B. *Synthesis* **1982**, 70. Baraldi, P. G.; Vicentini, C. B.; Simoni, D.; Menziani, E. *Farmaco, Ed. Sci.* **1983**, *38*, 508.
(7) La Mattina, J. L.; Suleska, R. T. *Synthesis* **1980**, 329.
(8) Musante, C.; Mugnaini, E. *Gazz. Chim. Ital.* **1947**, *77*, 182.
(9) Moroder, L.; Hallet, A.; Wünsch, E.; Keller, O.; Wersin, G. *Z. Physiol. Chem.* **1976**, *357*, 1651.

[^{3}H]flunitrazepam binding than diazepam, displays an affinity for the BDZ receptor practically comparable to that of chlordiazepoxide and to that of its structural isomer, ripazepam (IC_{50} = 4.4 vs. 0.81 and 0.67 μM, respectively).

(b) Any para substitution in the phenyl ring at position 5, irrespective of the electronic or lipophilic features, appears to be detrimental to BZ receptor binding. In particular, it would appear that bulkier substituents (see, e.g., **3d**) may prevent the interaction with BZ receptor sites by a steric hindrance effect. This fact is similar to that observed in BZs in which any kind of substitution at the para position of the 5-phenyl ring provokes a dramatic decrease both in biological activity[1] and in receptor binding affinity.[11]

Experimental Section

Melting points were determined in open capillary tubes on a Tottoli-Buchi apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 257 spectrometer. NMR spectra were taken on a Perkin-Elmer R32 instrument; chemical shifts are reported as δ (ppm) relative to Me_4Si as internal standard; J values are in hertz. All compounds were analyzed for C, H, N; analytical results were within ±0.4% of the theoretical values.

Starting Materials. The amino ketone **6a** is commercially available (Fluka); **6b–f** were prepared following the literature methods.[10]

General Procedure for *N*-(Substituted-phenacyl)-3-methyl-4-nitropyrazole-5-carboxamides 7a–f. To an ice-cooled and stirred suspension of diketopiperazine **5** (10 mmol) in chloroform (50 mL) and the appropriate α-amino ketone hydrochloride **6a–f** (20 mmol) was added dropwise a solution of TEA (20 mmol) in chloroform (50 mL) in 2 h. The suspension was stirred at room temperature overnight and filtered to give a solid, which was purified by crystallization from 1:1 2-methoxyethanol/1 N HCl. All physical data are reported in Table I.

General Procedure for 5-(Substituted-phenyl)-3-methyl-6,7-dihydropyrazolo[4,3-*e*][1,4]diazepin-8(7*H*)-ones 3a–e. The appropriate nitro amide **7a–e** (5 mmol) was dissolved in 2-methoxyethanol (40 mL), diluted with 10 mL of water, and cooled to 30 °C. Iron powder (reduced, 3 g) was added, followed by 0.5 mL of concentrated HCl, and the mixture was stirred and heated to 70 °C. An exothermic reaction resulted in a rapid reflux for 10 min; after the reaction subsided, the mixture was stirred and refluxed for another hour. The cooled reaction mixture was filtered through a pad of Celite 503. The filter cake was washed well with hot 2-methoxyethanol and the filtrate was diluted with water to precipitate a solids which was purified by crystallization from 1:1 DMF/H_2O. All physical data are reported in Table I.

Preparation of 4-[(*tert*-Butyloxycarbonyl)amino]-3-methylpyrazole-5-carboxylic Acid (9). To a stirred solution of 3-methyl-4-aminopyrazole-5-carboxylic acid[8] (**8**; 2.63 g, 18.6 mmol) in a mixture of 10% NaOH (7.15 mL, 20 mmol) and dioxane (14 mL) was added di-*tert*-butyl carbonate (4.32 g, 19 mmol) at room temperature. After the solution had been stirred overnight, acidification with 10% citric acid precipitated the Boc derivative **9** as a white crystalline solid, which was filtered and purified by crystallization from 2-methoxyethanol to give 3.4 g (79%). An analytical sample was prepared by recrystallization from 2-methoxyethanol and obtained as white needles: mp 208–209 °C; IR (KBr) 3400, 3260, 1740, 1700 cm^{-1}; NMR (Me_2SO-d_6) δ 1.4 (s, 9 H), 2.1 (s, 3 H), 8.0 (br s, 1 H), 13.2 (br s, 2 H).

General Procedure for *N*-(Substituted-phenacyl)-4-[(*tert*-butyloxycarbonyl)amino]-3-methylpyrazole-5-carboxamides 10a,f. To an ice-cooled and stirred solution of 4-[(*tert*-butyloxycarbonyl)amino]-3-methylpyrazole-5-carboxylic acid (**9**; 2.41 g, 10 mmol) and of *N*-hydroxysuccinimide (1.15 g, 10 mmol) in a mixture 20:1 of THF/DMF (63 mL) was added dicyclohexylcarbodiimide (2.06 g, 10 mmol). The mixture was stirred overnight at room temperature and then filtered to remove dicyclohexylurea. The filtrate was evaporated to dryness to give an oil, which was used in the following condensation without any purification. To an ice-cooled and stirred suspension of this ester (10 mmol) and of the appropriate α-amino ketone hydrochloride (10 mmol) in chloroform (50 mL) was added dropwise a solution of TEA (10 mmol) in chloroform (50 mL) in 2 h. After the mixture was stirred overnight at room temperature, the suspension was filtered to give a solid, which was purified by crystallization from 1:1 DMF/H_2O. According to this procedure **10a** and **10f** were prepared. **10a**: 68% yield; mp 230–231 °C, IR (KBr) 3400, 3220, 1730, 1700, 1650, 1570 cm^{-1}; NMR (Me_2SO-d_6) δ 1.40 (s, 9 H), 2.2 (s, 3 H), 4.8 (d, J = 6 Hz, 2 H), 7.6 (m, 3 H), 8.1 (m, 2 H), 8.3 (br s, 2 H), 13.1 (br s, 1 H). **10f**: 67% yield; mp 235–237 °C; IR (KBr) 3400, 3240, 1740, 1700, 1650, 1570, 1520, 1350 cm^{-1}; NMR (Me_2SO-d_6) δ 1.40 (s, 9 H), 2.2 (s, 3 H), 4.9 (d, J = 6 Hz, 2 H), 7.9 (m, 4 H), 8.2 (br s, 2 H), 13.5 (br s, 1 H).

General Procedure for the Removal of the 4-[(*tert*-Butyloxycarbonyl)amino] Group Followed by Cyclization to 3a,f. The appropriate 4-[(*tert*-butyloxycarbonyl)amide] **10a,f** (2 mmol) was stirred with trifluoroacetic acid (10 mL) at room temperature for 30 min, and then the solution was evaporated to dryness and the residue taken up with chloroform and evaporated several times. The oily residue (TLC), consisting of a mixture of deblocked and cyclized compounds, was dissolved in 2-methoxyethanol (20 mL) containing TEA (2 mmol) and the mixture was refluxed for 20 min. The cooled solution was diluted with water (10 mL) to give the cyclized compound, which was purified by crystallization from 1:1 DMF/H_2O. According to this procedure, **3a** and **3f** were prepared. **3a**: 62% yield; this compound showed all analytical and spectral data identical with those of the same compound obtained through the reaction sequence of Scheme I. **3f**: 56% yield; IR (KBr) and NMR (Me_2SO-d_6) are reported in Table III (supplementary material).

Biological Investigation. Since the discovery by means of radioligand binding techniques of high affinity, stereospecific and saturable recognition sites for benzodiazepines in the mammalian central nervous system,[11,12] structure–activity studies of BZ-like compounds have been enormously simplified.

[^{3}H]Flunitrazepam binding experiments were carried out essentially according to Karobath and Supavilai.[13] Briefly, membranes from cerebral cortex of male Wistar rats (150–200 g) were prepared at 0–4 °C in 50 mM Tris-citrate buffer, pH 7.1. The membranes were washed five times in order to remove endogenous GABA and stored frozen. Membranes equivalent to 6 mg wet weight tissue were incubated at 0 °C in 1 mL of 50 mM Tris-citrate buffer, pH 7.1, which contained 200 mM NaCl, with the drugs to be tested, and the radioligand [^{3}H]flunitrazepam binding assay was terminated after 90 min of incubation. Bound and free ligands were separated by rapid filtration through Whatman GF/B glass fiber filters and washed two times with 5 mL of ice-cold buffer. Radioactivity on the filters was determined by conventional scintillation counting. Binding assays were usually performed at least in triplicate. Unspecific binding was determined with use of 2 μM methyl β-carboline-3-carboxylate.

Registry No. **3a**, 94993-75-6; **3b**, 94993-76-7; **3c**, 94993-77-8; **3d**, 94993-78-9; **3e**, 94993-79-0; **3f**, 94993-80-3; **5**, 80030-73-5; **6a**, 5468-37-1; **6b**, 5467-71-0; **6c**, 3883-94-1; **6d**, 71350-68-0; **6e**, 5467-72-1; **6f**, 5425-81-0; **7a**, 94993-69-8; **7b**, 94993-70-1; **7c**, 94993-71-2; **7d**, 94993-72-3; **7e**, 94993-73-4; **7f**, 94993-74-5; **8**, 94993-81-4; **9**, 94993-82-5; **10a**, 94993-83-6; **10f**, 94993-84-7.

Supplementary Material Available: Two tables containing the absorption characteristics and the ^{1}H NMR chemical shifts and coupling constants for the nitro amides **7a–f** and pyrazolo[4,3-*e*][1,4]diazepin-8(7*H*)-ones **3a–f** (2 pages). Ordering information is given on any current masthead page.

(10) Long, L. M.; Troutman, H. D. *J. Am. Chem. Soc.* **1949**, *71*, 2473. Cheng, S. S.; Jonsson, S.; Semenink, F. T. *J. Pharm. Sci.* **1962**, *51*, 108. Temple, G. P., Elliott, R. D.; Rose, J. D.; Kussner, C. L.; Comber, R. N.; Montgomery, J. A. *J. Med. Chem.* **1982**, *25*, 1045.
(11) Squires, R. F.; Braestrup, C. *Nature (London)* **1977**, *266*, 732.
(12) Mohler, H.; Okada, T. *Science* **1977**, *198*, 849.
(13) Karobath, M.; Supavilai, P. *Neurosci. Lett.* **1982**, *31*, 65.