300 to 500 g were sensitized as described by Klecak.[28] On alternate days, the hapten, emulsified in Freund's complete adjuvant (FCA), was injected intradermally (0.1 mL) in the shaved nuchal region of the animal (in all, three injections, five after boost). The sensitizing molarity used was 0.12 M (FCA/saline (1:1)). After 15 days rest, the elicitation was conducted by an open epicutaneous test (OEt): 25 μL of an ethanol solution of diol (see Table II for amount of diols) was deposited on the shaved flank of the animal (on a 2-cm² surface using a standard circular stamp). Tests were read at the 48th h using the following scale: 0 = no reaction, 0.5 = slight erythema not covering the whole test area, 1 = erythema covering all the test area, 2 = erythema plus swelling of the test area, 3 = erythema plus swelling going well beyond the test area. Before any sensitization, irritation thresholds (primary toxicity) were determined on FCA-injected controls (same procedure as above for elicitation). Concentrations up to 5% in ethanol of cyclohexanediols were *nontoxic*. Control groups of eight animals (FCA treated) were used in each experiment.

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Notes

Pyrazolo[4,5-*c*]quinolines. 2. Synthesis and Specific Inhibition of Benzodiazepine Receptor Binding

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A series of 1-aryl-3,5-dimethyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-ones (**2a–e**) and 1-aryl-3-methyl-1*H*-pyrazolo[4,5-*c*]quinolines (**3–7a–e**) bearing different substituents at position 4 were prepared and tested for their ability to displace specific [³H]flunitrazepam binding from bovine brain membranes. The 5-*N*-methyl derivatives **2a–c,e** were the compounds that bound with the highest affinity within this class. The replacement of the carbonyl group with other substituents and the resulting aromatization of the pyridine moiety greatly decreased the binding affinity. From a Lineweaver–Burk analysis on the most active compound **2b**, it appears that the inhibition is a competitive one.

Since the reports of Squires and Braestrup[1] and Mohler and Okada[2] on the high affinity binding sites for benzodiazepines in rat brain tissues, a number of synthetic compounds with different structures have been found to possess high affinity for the benzodiazepine receptor either as agonists or as antagonists.[3]

Non-benzodiazepine compounds with affinity for the benzodiazepine receptor could be of potential importance as tools for studying the receptor itself and eventually for the introduction into clinical use of new classes of compounds having the same properties of benzodiazepines.

Cain et al.[4] in a study on β-carbolines have reported some of the requirements that affect the affinity for the receptor. They stated that a planar heteroaromatic system containing at least one nitrogen atom is necessary and that a carbonyl group adjacent to the nitrogen atom greatly augments the binding to the receptor.

Guzman et al.[5] recently verified the same requirements in other non-benzodiazepine compounds like canthines, isoquinolines, and imidazoquinolines. In the latter a phenyl substituent provides the necessary hydrophobicity for a better fitting.

Following these findings we have prepared some pyrazoloquinolin-4-ones bearing an aryl substituent at position 1 or at position 2.[6] A preliminary binding study on bovine brain membranes has shown that only the 1-aryl-3-methyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-ones possess activity in displacing specific [³H]flunitrazepam from its receptor site.[6]

In order to establish structure–activity relationships for benzodiazepine receptor binding in this class of compounds, we are now reporting the synthesis and binding properties of some new 1-arylpyrazolo[4,5-*c*]quinoline derivatives.

Chemistry. 1-Phenyl-3-methyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-one (**1a**), already reported in the literature,[7] was synthesized to compare its activity with that of the 1-aryl-3-methyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-ones (**1b–e**) previously reported by us.[6]

Compounds **1a–e** were reacted with methyl iodide to obtain the 5-methyl derivatives **2a–e**.

† Dipartimento di Scienze Farmaceutiche.
‡ Istituto Policattedra di Discipline Biologiche.

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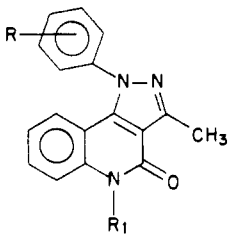
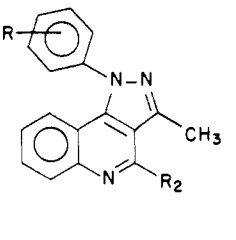
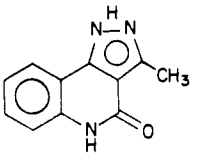
Table I. Inhibition of [^{3}H]Flunitrazepam Binding



1, 2



3-7



8

no.	R	R_1	inhibn,[a] % (34 μM)	IC_{50}[b] μM	no.	R	R_2	inhibn,[a] % (34 μM)
1a	H	H	62 ± 5		3b	3-CH_3	Cl	21 ± 1
1b	3-CH_3	H	97 ± 7[c]	9.0 ± 0.8	3c	3-Cl	Cl	21 ± 2
1c	3-Cl	H	94 ± 5[c]	4.4 ± 0.1	4c	3-Cl	OCH_3	18 ± 2
1d	4-CH_3	H	65 ± 6[c]		6a	H		41 ± 3
1e	4-Cl	H	70 ± 4[c]	22.0 ± 2.0	6b	3-CH_3		44 ± 4
2a	H	CH_3	85 ± 6	3.4 ± 0.2	6c	3-Cl		49 ± 3
2b	3-CH_3	CH_3	95 ± 8	0.26 ± 0.01	6e	4-Cl		23 ± 2
2c	3-Cl	CH_3	96 ± 7	1.0 ± 0.2	7a	H		8 ± 1
2d	4-CH_3	CH_3	63 ± 5		7b	3-CH_3		7 ± 1
2e	4-Cl	CH_3	78 ± 4	2.7 ± 0.2	7c	3-Cl		24 ± 2
8			47 ± 2					

[a] Percent of inhibition of specific [^{3}H]flunitrazepam binding at 34 μM compound concentration are means ± SEM of five determinations. [b] Concentrations necessary for 50% inhibition (IC_{50}) are means ± SEM of four determinations. [c] See ref 6. Compounds **3a**, **4a,d–e**, **5a–e**, **6d**, and **7d,e** were devoid of any binding inhibition of specific [^{3}H]flunitrazepam binding.

When compounds **1a–e** were allowed to react with phosphorus oxychloride, the 4-chloropyrazoloquinolines **3a–e** were isolated. The latter treated with sodium methoxide or sodium ethoxide gave rise to the 3-methoxy derivatives **4a–e** or 4-ethoxy derivatives **5a–e**, respectively.

Compounds **6a–e** and **7a–e** were prepared by refluxing a solution of the 4-chloro derivatives **3a–e** in pyridine with 1-methylpiperazine or morpholine (see Scheme I).

Moreover, 3-methyl-4,5-dihydro-1(2)*H*-pyrazolo[4,3-*c*]-quinolin-4-one (**8**)[8] was prepared to fully evaluate the influence of the hydrophobic phenyl substituent.

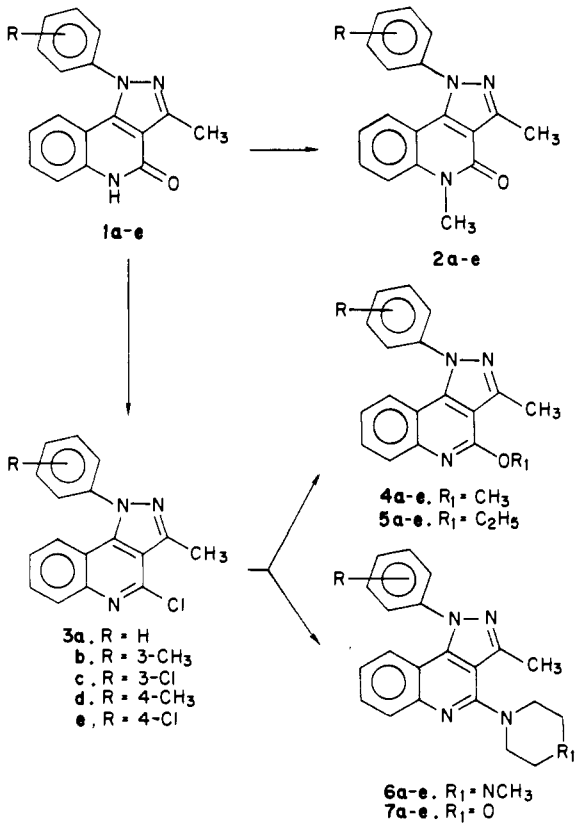
Binding to the Benzodiazepine Receptor. All the synthesized compounds were tested for their abiility to displace specific [^{3}H]flunitrazepam binding from bovine brain membranes.

At first a single concentration (34 μM) of the compounds to be tested was examined, followed by the determination of the IC_{50} values from log–probit plots of the more active ones. The resulting data are listed in Table I.

From our results it appears that, in this series of compounds, a prerequisite for retaining some activity to displace specific [^{3}H]flunitrazepam from the benzodiazepine receptor is the presence of a carbonyl group near a nitrogen atom. In fact the replacement of the carbonyl group with other substituents and the resulting aromatization of the pyridine moiety dramatically affected the inhibitory potency of the compounds. Slight activity was observed in derivatives **4a–c,e** which bore a 4-methylpiperazinyl group.

On the other hand, the replacement of the hydrogen atom in position 5 with a methyl group led to a significant increase of the ability to displace the radioligand, as shown by the IC_{50} values.

Scheme I



Two of the requirements for binding reported in the literature,[4] namely the presence of a carbonyl group ad-

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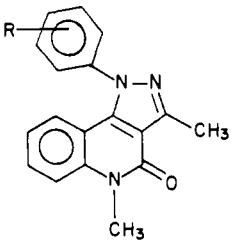
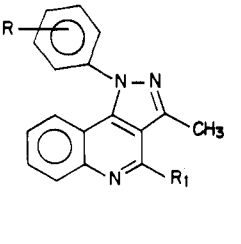
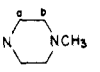
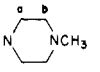
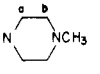
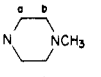
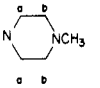
Table II. Physical Properties of Pyrazolo[4,5-c]quinolines



2a-e



3-7a-e

no.	formula	R	R_1	mp, °C	solv crystn	yield, %
2a	$C_{18}H_{15}N_3O$	H		181–183	$EtOH/H_2O$	10
2b	$C_{19}H_{17}N_3O$	3-CH_3		177–179	$EtOH/H_2O$	70
2c	$C_{18}H_{14}ClN_3O$	3-Cl		175–177	$EtOH/H_2O$	35
2d	$C_{19}H_{17}N_3O$	4-CH_3		220–222	$EtOH/H_2O$	15
2e	$C_{18}H_{14}ClN_3O$	4-Cl		218–220	$EtOH/H_2O$	20
3a	$C_{17}H_{12}ClN_3$	H	Cl	202–205	acetone	52
3b	$C_{18}H_{14}ClN_3$	3-CH_3	Cl	182–184	acetone	42
3c	$C_{17}H_{11}Cl_2N_3$	3-Cl	Cl	219–221	acetone	62
3d	$C_{18}H_{14}ClN_3$	4-CH_3	Cl	189–191	acetone	44
3e	$C_{17}H_{11}Cl_2N_3$	4-Cl	Cl	222–224	acetone	47
4a	$C_{18}H_{15}N_3O$	H	OCH_3	179–181	methanol	54
4b	$C_{19}H_{17}N_3O$	3-CH_3	OCH_3	155–157	$MeOH/H_2O$	90
4c	$C_{18}H_{14}ClN_3O$	3-Cl	OCH_3	158–159	$MeOH/H_2O$	40
4d	$C_{19}H_{17}N_3O$	4-CH_3	OCH_3	168–170	$MeOH/H_2O$	66
4e	$C_{18}H_{14}ClN_3O$	4-Cl	OCH_3	198–200	$MeOH/H_2O$	20
5a	$C_{19}H_{17}N_3O$	H	OC_2H_5	134–136	$EtOH/H_2O$	45
5b	$C_{20}H_{19}N_3O$	3-CH_3	OC_2H_5	138–140	ethanol	53
5c	$C_{19}H_{16}ClN_3O$	3-Cl	OC_2H_5	166–168	ethanol	30
5d	$C_{20}H_{19}N_3O$	4-CH_3	OC_2H_5	159–161	$EtOH/H_2O$	45
5e	$C_{19}H_{16}ClN_3O$	4-Cl	OC_2H_5	151–153	$EtOH/H_2O$	40
6a	$C_{22}H_{23}N_5$	H		209–212	$EtOH/H_2O$	65
6b	$C_{23}H_{25}N_5$	3-CH_3		180–182	$EtOH/H_2O$	60
6c	$C_{22}H_{22}ClN_5$	3-Cl		154–155	$EtOH/H_2O$	42
6d	$C_{23}H_{25}N_5$	4-CH_3		179–182	$EtOH/H_2O$	50
6e	$C_{22}H_{22}ClN_5$	4-Cl		166–170	cyclohexane	25
7a	$C_{21}H_{20}N_4O$	H		164–166	$EtOH/H_2O$	60
7b	$C_{22}H_{22}N_4O$	3-CH_3		170–172	$EtOH/H_2O$	70
7c	$C_{21}H_{19}ClN_4O$	3-Cl		137–139	$EtOH/H_2O$	65
7d	$C_{22}H_{22}N_4O$	4-CH_3		173–175	$EtOH/H_2O$	73
7e	$C_{21}H_{19}ClN_4O$	4-Cl		208–210	$EtOH/H_2O$	65

jacent to the nitrogen atom and the existence of a planar heteroaromatic system, are both satisfied in our series of pyrazoloquinolines. In fact, in spite of the presence of two sp^3 nitrogen atoms, the system is fully planar, as may easily be seen from the Dreiding models.

Moreover, comparison of the binding affinity of compound 8 with that of compound **1a** points to the importance of the phenyl substituent. This finding agrees with what was observed by Guzman et al.[5] in the imidazopyridine series. The latter compounds have been found to have a moderate affinity for the receptor unless they bear a hydrophobic phenyl substituent.

Our previous findings[6] have shown that, to have affinity for the receptor site, the phenyl substituent ought to be at position 1.

Some 2-arylpyrazolo[4,3-c]quinolin-3-ones (CGS series[3]) have been reported to have an extremely high affinity for the benzodiazepine receptor binding sites.

On the other hand, our previously reported 2-aryl-3-methyl-4,5-dihydro-2*H*-pyrazolo[4,3-c]quinolin-4-ones,[6] more closely related to the CGS series, lack any binding affinity.

It follows that the displacement of the carbonyl group from position 3 to position 4 leads to a lack of affinity in

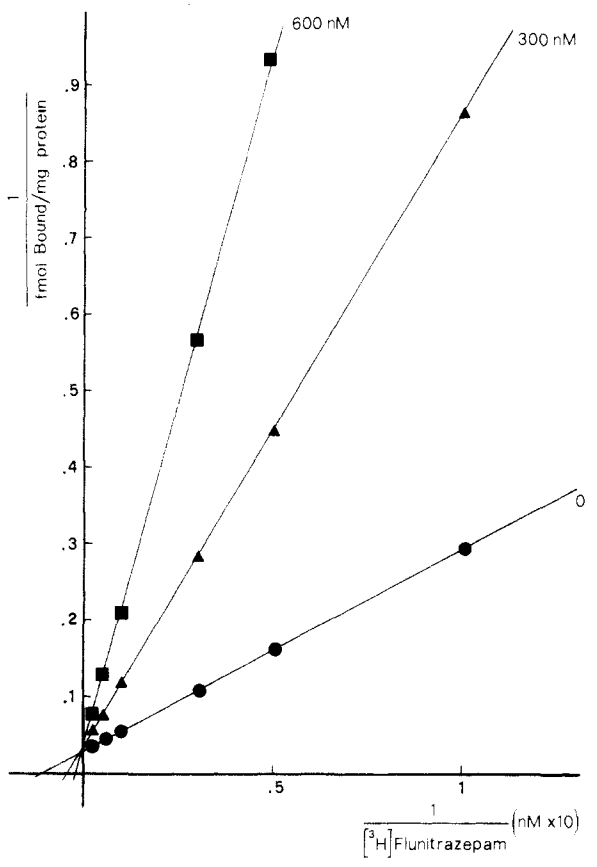


Figure 1. Lineweaver–Burk analysis of compound **2b** inhibition of specific [^{3}H]flunitrazepam binding (K_i = 0.2 μM). Compound **2b** was incorporated at the indicated concentrations in the [^{3}H]flunitrazepam receptor binding assay. The experiments were performed three times and gave the same results.

the 2-arylpyrazoloquinoline series and to a decreased affinity in the 1-arylpyrazoloquinoline series.

Furthermore, even if at the moment we cannot give a logical explanation of this fact, a substituent on the phenyl and its position seem to play an important role. The most active compound, **2b**, bears a meta substituent. A decrease in binding affinity is observed when the 1-phenyl ring carries no substituents or when the substituent is displaced in the para position.

The kinetics of [^{3}H]flunitrazepam binding inhibition were determined by Lineweaver–Burk analysis in the presence of a fixed concentrations of **2b**.

The results, shown in Figure 1, indicate that the K_m for [^{3}H]flunitrazepam binding is increased, while its maximal binding (B_{max}) remains unchanged. It follows that compound **2b**, and most likely the others too, inhibits specific [^{3}H]flunitrazepam binding in a competitive manner.

Experimental Section

Chemistry. All melting points were determined on a Büchi 510 capillary melting point apparatus and are uncorrected. The ^{1}H NMR spectra were recorded with a Varian EM 360 instrument; chemical shifts are reported in δ (ppm) downfield from internal Me_4Si. Silica gel plates (Merck F_{254}) and silica gel 60 (Merck; 70–230 mesh) were used for analytical and column chromatography, respectively. Elemental analyses were performed for C, H, N with a Perkin-Elmer 260C elemental analyzer and results with within ±0.4% of the theoretical values.

Synthesis of 1-Aryl-3,5-dimethyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-ones 2a–e. To a solution of 1-aryl-3-methyl-4,5-dihydro-1*H*-pyrazolo[4,5-*c*]quinolin-4-one[6] (**1a–e**) (0.7 mmol) in DMF (5–10 mL) were added anhydrous K_2CO_3 (1 mmol) and CH_3I (1.4 mmol). The mixture was stirred at room temperature for 24 h. The filtered clear solution was diluted with water to give rise to a residue, which was purified by column chromatography and eluted with cyclohexane/EtOAc (1:1); the product was present in the first running band.

Compound **2a** displayed the following ^{1}H NMR ($CDCl_3$): δ 7.7–7.2 (m, 9 H, aromatics), 3.77 (s, 3 H, NCH_3), 2.77 (s, 3 H, CH_3).

Synthesis of 1-Aryl-3-methyl-4-chloro-1*H*-pyrazolo[4,5-*c*]quinolines 3a–e. A solution of compound **1a–e**[6] (3.5 mmol) in an excess of $POCl_3$ (10 mL) was refluxed for 36 h. After cooling, the mixture was poured onto crushed ice. The resulting precipitate was collected and washed with diethyl ether.

Compound **3a** displayed the following ^{1}H NMR ($CDCl_3$): δ 8.2–8.0 (m, 1 H, aromatic), 7.8–7.2 (m, 8 H, aromatics), 2.84 (s, 3 H, CH_3).

Synthesis of 1-Aryl-3-methyl-4-methoxy-1*H*-pyrazolo[4,5-*c*]quinolines 4a–e and 1-Aryl-3-methyl-4-ethoxy-1*H*-pyrazolo[4,5-*c*]quinolines 5a–e. To a solution of sodium methoxide in methanol or sodium ethoxide in ethanol, compound **3a–e** (3 mmol) was added. The mixture was refluxed for 2–3 h. Evaporation of the solvent afforded a residue.

Compound **4a** displayed the following ^{1}H NMR ($CDCl_3$): δ 8.1–7.8 (m, 1 H, aromatic), 7.7–7.2 (m, 8 H, aromatics), 4.21 (s, 3 H, OCH_3), 2.71 (s, 3 H, CH_3).

Synthesis of 1-Aryl-3-methyl-4-(4-methyl-1-piperazinyl)-1*H*-pyrazolo[4,5-*c*]quinolines 6a–e and 1-Aryl-3-methyl-4-(4-morpholinyl)-1*H*-pyrazolo[4,5-*c*]-quinolines 7a–e. To a solution of compound **3a–e** (0.7 mmol) in pyridine (25 mL) was added an excess (1:4) of 1-methylpiperazine or morpholine. The mixture was refluxed for 24 h. Dilution of the cooled solution with water afforded a solid residue, which was collected and washed with water.

Compound **6a** displayed the following ^{1}H NMR ($CDCl_3$): δ 8.1–7.8 (m, 1 H, aromatic), 7.7–6.9 (m, 8 H, aromatics), 3.7–3.4 (m, 4 H, $2CH_2$a), 2.77 (s, 3 H, CH_3), 2.7–2.5 (m, 4 H, $2CH_2$b), 2.42 (s, 3 H, NCH_3).

Binding Studies. Tritiated flunitrazepam was obtained from New England Nuclear (Dreieichenhain, West Germany) and had a specific activity of 76.9 Ci/mmol and a radiochemical purity >99%. All the other chemicals were reagent grade and obtained from commercal suppliers.

Bovine brains were obtained from a local slaughterhouse and were stored at −20 °C after dissection of the cortex. Membranes were prepared by homogenization in 20 volumes of ice-cold 0.32 M sucrose in an Ultra-Turrax homogenizer for 30 s. The homogenate was centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was recentrifuged at 48000*g* for 30 min at 4 °C. The pellet was osmotically shocked by suspension in 20 vol of 50 mM Tris-HCl buffer and recentrifuged at 48000*g* for 30 min at 4 °C. The homogenization media contained protease inhibitors according to Martini et al.[9] Benzodiazepine receptor binding activity was determined as follows: 100 μL of diluted membranes (0.4–0.5 mg of proteins) were incubated in triplicate with 1.0 nM [^{3}H]flunitrazepam at 0 °C (90 min) in 50 mM Tris-HCl buffer in a final volume of 500 μL. After incubation the samples were diluted at 0 °C with 5 mL of the assay buffer and were immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B). Afterwards the samples were washed with 5 mL of the same buffer, dried, and added to 8 mL of HP Beckman scintillation liquid containing 0.4 mL of a solution of 0.01 M KOH in plastic vials.

The pyrazoloquinoline derivatives were dissolved in ethanol and added to the assay mixture to a final volume of 500 μL. Blank experiments were carried out to determine the effect of ethanol (2%) on the binding.

Specific binding was obtained by subtracting nonspecific binding from total binding and approximated to 85–90% of the total binding. The amount of nonspecific binding was determined by incubating membranes and [^{3}H]flunitrazepam in the presence of 10 μM diazepam.

The estimation of proteins was based on the method of Lowry et al.[10] after membrane solubilization with 0.75 N NaOH. Bovine

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serum albumin was utilized as the standard.

The concentrations of the pyrazoloquinolines that inhibit specific [^{3}H]flunitrazepam binding by 50% (IC_{50}) were determined by log–probit analysis with four to six concentrations of the displacers, each performed in triplicate.

Registry No. **1a**, 97789-05-4; **1b**, 97993-50-5; **1c**, 97993-47-0; **1d**, 97993-49-2; **1e**, 97993-46-9; **2a**, 71814-52-3; **2b**, 98990-72-8; **2c**, 98990-73-9; **2d**, 98990-74-0; **2e**, 98990-75-1; **3a**, 98990-76-2; **3b**, 98990-77-3; **3c**, 98990-78-4; **3d**, 98990-79-5; **3e**, 98990-80-8; **4a**, 98990-81-9; **4b**, 98990-82-0; **4c**, 98990-83-1; **4d**, 98990-84-2; **4e**, 98990-85-3; **5a**, 98990-86-4; **5b**, 98990-87-5; **5c**, 98990-88-6; **5d**, 98990-89-7; **5e**, 98990-90-0; **6a**, 98990-91-1; **6b**, 98990-92-2; **6c**, 98990-93-3; **6d**, 98990-94-4; **6e**, 98990-95-5; **7a**, 98990-96-6; **7b**, 98990-97-7; **7c**, 98990-98-8; **7d**, 98990-99-9; **7e**, 98991-00-5; 8, 37638-10-1.

Ketomethylene Pseudopeptide Analogues of Substance P: Synthesis and Biological Activity†

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Two pseudopeptide analogues [Bz-(*RS*)Phe$^8\psi$(COCH$_2$)Gly^9]SP$_{8-11}$ (I) and [pGlu^6,(*RS*)Phe$^8\psi$(COCH$_2$)Gly^9]SP$_{6-11}$ (II) of the substance P related C-terminal hexapeptide [pGlu^6]SP$_{6-11}$ were prepared as follows. The pseudodipeptidic unit H(*RS*)Pheψ(COCH$_2$)GlyOH was synthesized by using a modified Dakin–West reaction between Bz-Phe-OH and monomethyl succinoyl chloride. The N$^\alpha$-protected pseudopeptidic unit was then incorporated into the appropriate peptide by using various coupling methods. The two pseudopeptide analogues were purified, characterized, and tested for their biological activity and inhibitory effect on SP degrading enzymes. Analogue II was a full agonist contracting the isolated guinea pig ileum with a potency of 70% compared to the parent hexapeptide [pGlu^6]SP$_{6-11}$. It was also a potent inhibitor of SP degrading activity in rat diencephalon membranes with a K_i of 20 μM whereas analogue I was a weak inhibitor.

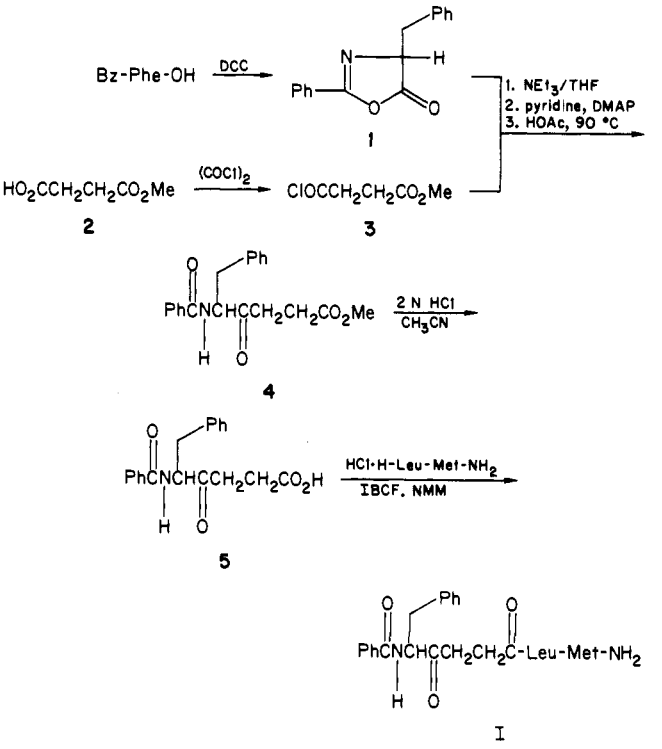
Substance P (SP), an undecapeptide with the sequence H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$, is a putative neurotransmitter or neuromodulator.[1] It has been shown that C-terminal hexapeptide fragments and analogues are equipotent to the parent compound in most bioassays. For example, the hexapeptide [pGlu^6]SP$_{6-11}$ is more potent than SP in contracting the guinea pig ileum[2] and in depolarizing spinal cord motoneurons;[3] it is equipotent to SP in inducing K^+ release from rat parotid slices.[4]

The transient nature of most in vitro and in vivo pharmacological effects stimulated by SP and [pGlu^6]-SP$_{6-11}$ is due to fast proteolytic degradation by various enzymes.[5] Lee and co-workers have reported the isolation of a membrane-bound neutral metalloendopeptidase from human brain, which cleaves SP between the residues Gln^6-Phe^7, Phe^7-Phe^8, and Phe^8-Gly^9.[5] We have recently reported the characterization of the degradation patterns of the hexapeptide [pGlu^6]SP$_{6-11}$ by rat parotid and hypothalamic slices.[6]

It has been reported that other membrane-bound brain metalloendopeptidases like enkephalinase and angiotensin converting enzyme (ACE) are capable of cleaving SP, yet it is not clear at present whether these peptidases also play significant roles in the inactivation of SP at the synaptic level.[7,8]

In order to extend the duration of substance P activity, degradation resistant analogues thereof have been designed and prepared. These were found to elicit more sustained biological responses.[9-11] An alternative approach to extend substance P action involves preparation of specific inhib-

Scheme I



1 2 3 4 5 I

† Abbreviations according to IUPAC–IUB Commission (1972), *Biochemistry*, *11*, 1726–1732, and Specialist Periodical Reports, "Amino Acids, Peptides and Proteins", Volume 11 (The Chemical Society, London, 1980, R. C. Sheppard, Ed.), are used throughout. The following special abbreviations are used for the ketomethylene peptides and fragments. The standard three-letter notation for amino acid residues preceded by the symbols ψ(COCH$_2$) represents the ketomethylene-modified residue of the pseudodipeptide.

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