Synthesis of *N,N'*-Bis(3-substituted Benzylideneaminopropyl)-piperazines and Their Anti-Inflammatory, Antiproteolytic, and Anticonvulsant Properties

ARVIND K. CHATURVEDI *‡, B. V. RAMA SASTRY *, ANSHUMALI CHAUDHARI ‡, and SURENDRA S. PARMAR ‡x

Abstract □ Some *N,N'*-bis(3-substituted benzylideneaminopropyl)piperazines were synthesized and characterized by their sharp melting points and elemental analyses. These substituted piperazines possessed anti-inflammatory activity, and the protection afforded by these compounds against carrageenan-induced edema ranged from 23 to 67%. The antiproteolytic activity of these piperazines was reflected by their ability to inhibit *in vitro* hydrolysis of bovine serum albumin and casein by trypsin. The inhibition of trypsin-induced hydrolysis was concentration dependent and competitive in nature.

Keyphrases □ Piperazines—*N,N'*-substituted, synthesis, anti-inflammatory, antiproteolytic, and anticonvulsant properties screened □ Anti-inflammatory activity—*N,N'*-substituted piperazines synthesized and screened □ Antiproteolytic activity—*N,N'*-substituted piperazines synthesized and screened □ Anticonvulsant activity—*N,N'*-substituted piperazines synthesized and screened □ Structure–activity relationships—*N,N'*-substituted piperazines, anti-inflammatory, antiproteolytic, and anticonvulsant properties

Search for effective antiphlogistics necessitated the evaluation of a wide variety of chemical structures (1, 2). Among these compounds, general piperazine derivatives have been reported to possess significant anti-inflammatory activity (3–7). This observation prompted the synthesis of some piperazine derivatives, which were evaluated for their effectiveness as antiphlogistics.

Furthermore, the role of proteolytic enzymes in the inflammatory processes and the well-known pharmacological effects of piperazine derivatives (8, 9) prompted the investigation of the *in vitro* effects of these new piperazines on protein catabolism and an evaluation of their anticonvulsant effects. In the present study, attempts were made to correlate the anti-inflammatory activity of these substituted piperazines with their antiproteolytic activity for elucidation of their biochemical mechanism of action.

EXPERIMENTAL[1]

***N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines**—To a solution of the appropriate aromatic aldehyde (0.02 mole) in 20 ml of absolute ethanol was added 2.0 g of *N,N'*-bis(3-aminopropyl)piperazine (0.01 mole), and the mixture was refluxed on a steam bath for 2 hr. The reaction mixture was allowed to stand for 24 hr in a refrigerator, and the excess ethanol was removed under reduced pressure. The solid mass which separated was collected, dried, and recrystallized from ethanol or isopropyl alcohol. The substituted piperazines were characterized by their sharp melting points and elemental analyses (Table I).

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with a partial immersion thermometer and are uncorrected.

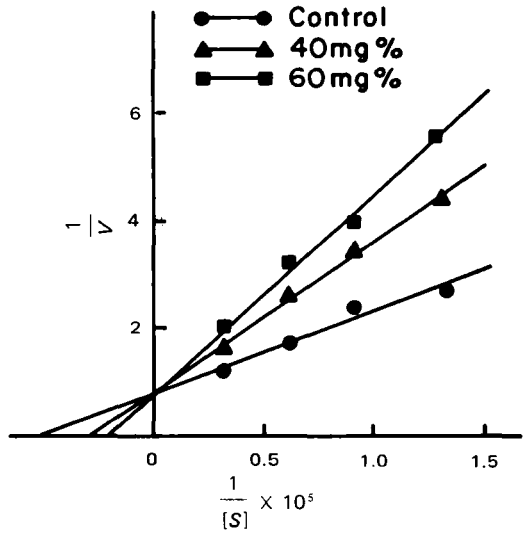


Figure 1—*Inhibition of the trypsin-induced hydrolysis of bovine serum albumin by IV. The* V *represents the change in percent absorbance/0.075 mg of crystalline trypsin/5 min, and* [S] *denotes molar concentration of bovine serum albumin. Key:* ●, *control with no inhibitor;* ▲, *40 mg % of piperazine; and* ■, *60 mg % of piperazine. The* K_m *value for bovine serum albumin is* 2×10^{-5} M; *the* K_i *value for IV is* 0.96×10^{-3} M.

Determination of Anti-Inflammatory Activity—Albino rats, 80–100 g, were allowed food and water *ad libitum;* they were used for the evaluation of the anti-inflammatory activity of these substituted piperazines. The rats were divided into groups of six animals each. A suspension of carrageenan (1.0%) in 0.9% NaCl was prepared fresh 1 hr before use, and 0.5 ml was injected into the plantar side of the right hindpaw (10).

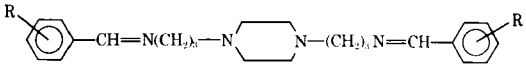
The substituted piperazines (100 mg/kg) were administered intraperitoneally to a group of six rats 1 hr before carrageenan injection, while six control rats received 0.5 ml of 0.9% NaCl solution. Sodium salicylate (100 mg/kg ip) was used as a standard anti-inflammatory agent. The mean increase in paw volume due to carrageenan-induced edema was measured by the micropipet method (11) before and 3 hr after carrageenan treatment.

Assay of Proteolytic Activity[2]—The antiproteolytic activity of substituted piperazines was determined by studying their ability to inhibit *in vitro* trypsin-induced hydrolysis of bovine serum albumin and casein. The reaction mixture consisted of 0.05 *M* tromethamine buffer at pH 8.2, 0.075 mg of crystalline trypsin (1 g sufficient to hydrolyze 250 g of casein), 3.3×10^{-5} *M* of bovine serum albumin (substrate) or casein (substrate), and water in a total volume of 1 ml.

All substituted piperazines were dissolved in dimethylformamide and were used at a final concentration of 40 and 60 mg %. An

[2] Commercial chemicals were used. Bovine serum albumin and casein were obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin was obtained from Serva Chemical Co., Heidelberg, Germany. Sodium salicylate and other common chemicals were obtained from British Drug House, Bombay, India.

Table I—Physical Constants of *N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines



Compound	R	Recrystallization Solvent	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found
I	*m*-NO_2	Ethanol	125°	55	$C_{24}H_{30}N_6O_4$	C 61.80 H 6.43 N 18.02	61.47 6.78 17.94
II	*p*-Cl	Isopropyl alcohol	100°	67	$C_{24}H_{30}Cl_2N_4$	C 64.71 H 6.74 N 12.58	64.52 6.92 12.88
III	*p*-OCH_3	Ethanol	110°	62	$C_{26}H_{36}N_4O_2$	C 71.55 H 8.25 N 12.72	71.38 7.89 13.12
IV	*p*-$N(CH_3)_2$	Ethanol	167°	64	$C_{28}H_{42}N_6$	C 72.72 H 9.09 N 18.18	72.51 9.22 18.45

[a] Melting points were taken in open capillary tubes with a partial immersion thermometer.

equivalent amount of dimethylformamide was added to the control tubes. All piperazines were preincubated with trypsin for 10 min prior to the addition of bovine serum albumin or casein, unless otherwise stated, and the reaction mixture was incubated for another 5 min after the addition of bovine serum albumin or casein.

The reaction was stopped by the addition of 5 ml of 15% trichloroacetic acid solution. The acid-soluble products of protein breakdown, obtained after centrifugation, were determined by the method of Lowry *et al.* (12) as an index of the proteolytic activity of trypsin. A decrease in the degree of trypsin-induced hydrolysis of bovine serum albumin or casein in experiments with the test compound reflected the antiproteolytic activity of these substituted piperazines.

In preincubation studies, trypsin was incubated with or without the substituted piperazines at 37° for 10, 20, and 30 min prior to the addition of bovine serum albumin or casein. The zero-time experiments represent those in which substituted piperazines and bovine serum albumin or casein were added simultaneously to the reaction mixture containing trypsin. In the present study, the nature of the enzyme inhibition by IV was evaluated by the graphic method of Lineweaver and Burk (13) as modified by Dixon (14), using bovine serum albumin and casein as the substrates.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced convulsions in mice of either sex weighing between 25 and 30 g. The mice were divided into groups of 10, keeping the group weights as nearly the same as possible. All compounds were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were administered in a dose of 100 mg/kg ip to a group of 10 animals.

Four hours after the administration of the substituted piperazines, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol produced convulsions in almost all untreated mice, which died within 24 hr. No mortality was observed during 24 hr in animals treated with 100 mg/kg of the substituted piperazine alone.

The mice were observed 60 min for the occurrence of seizures. An episode of clonic spasm persisting for at least 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during the 60-min period were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of the substituted piperazines was represented as percent protection. The animals were then observed for 24 hr, and their mortality was recorded.

Table II—Anti-Inflammatory Activity of *N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines

Compound	Mean Volume of Edema[a], ml ± *SE*	Anti-Inflammatory Activity, % Protection	*p*
Saline control	0.48 ± 0.04	—	—
I	0.18 ± 0.04	62.5	<0.001
II	0.29 ± 0.06	49.5	0.05–0.02
III	0.37 ± 0.03	23.0	0.02
IV	0.16 ± 0.03	66.6	0.01–0.001
Sodium salicylate	0.32 ± 0.01	33.3	0.01–0.001

[a] The experimental procedures are as indicated in the text. All substituted piperazines and sodium salicylate were administered in a dose of 100 mg/kg ip. The decrease in the volume of carrageenan-induced edema was used to calculate the anti-inflammatory activity of substituted piperazines. Statistical tables of Fisher and Yates (15) were used to determine *p* values.

Table III—Anticonvulsant Activity of *N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines

Compound	Anticonvulsant Activity[a], % Protection	Pentylenetetrazol Mortality[a], % Protection after 24 hr
I	20	70
II	30	50
III	20	40
IV	30	50

[a] Screening procedures for the determination of anticonvulsant activity are as described in the text. Mortality in pentylenetetrazol-treated animals was observed during 24 hr.

RESULTS AND DISCUSSION

All substituted piperazines were evaluated for anti-inflammatory activity against carrageenan-induced edema in albino rats in a dose of 100 mg/kg (Table II). All piperazines possessed anti-inflammatory activity, and their degree of protection against carrageenan-induced edema ranged from 23 to 67%. Maximum protec-

Table IV—Effect of *N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines on the Proteolytic Activity of Trypsin

Compound	Bovine Serum Albumin: Piperazine Concentration 40 mg %	Bovine Serum Albumin: Piperazine Concentration 60 mg %	Casein: Piperazine Concentration 40 mg %	Casein: Piperazine Concentration 60 mg %
	Inhibition[a], %			
I	33.9 ± 1.0	51.6 ± 0.4	14.0 ± 0.9	25.4 ± 0.7
II	28.0 ± 0.8	43.4 ± 0.9	13.3 ± 0.4	23.4 ± 0.8
III	16.8 ± 0.4	34.4 ± 0.7	11.6 ± 0.7	17.3 ± 0.8
IV	40.2 ± 0.7	63.5 ± 0.4	26.0 ± 0.9	31.1 ± 0.7
Sodium salicylate	65.5 ± 0.9	74.5 ± 0.6	28.7 ± 2.0	32.8 ± 0.9

[a] Assay procedures are as indicated in the text. Each experiment was done in triplicate, and the values are the mean values of three separate experiments with ± standard error of the mean.

Table V—Preincubation Studies with *N,N'*-Bis(3-substituted Benzylideneaminopropyl)piperazines to Study Trypsin-Induced Hydrolysis of Bovine Serum Albumin and Casein

Compound	Bovine Serum Albumin, Preincubation Time, min				Casein, Preincubation Time, min			
	0	10	20	30	0	10	20	30
	Inhibition[a], %							
I	32.7 ± 0.7	32.9 ± 0.8	33.8 ± 0.9	34.1 ± 1.0	14.0 ± 1.2	14.0 ± 0.9	13.9 ± 1.0	14.3 ± 0.7
II	28.4 ± 1.0	27.5 ± 0.7	28.9 ± 0.4	27.9 ± 0.7	13.2 ± 1.2	13.2 ± 1.1	13.9 ± 1.0	12.9 ± 0.8
III	16.7 ± 0.5	17.2 ± 0.4	17.0 ± 0.8	16.5 ± 0.6	10.7 ± 1.0	12.0 ± 0.5	11.9 ± 0.7	12.2 ± 0.8
IV	41.1 ± 1.0	39.0 ± 1.2	40.0 ± 0.9	40.9 ± 0.7	26.0 ± 0.9	25.7 ± 0.9	25.8 ± 0.4	26.2 ± 1.5

[a] Contents of the reaction mixture and the assay procedures are as indicated in Table IV. Trypsin was incubated with substituted piperazines for varying times before the addition of the substrate (bovine serum albumin or casein). Zero-time experiments indicate that both the substrate and the piperazines were added to the reaction mixture containing trypsin simultaneously. Each experiment was done in triplicate, and the values are the mean values of three separate experiments with ± standard error of the mean. The various substituted piperazines were used at a final concentration of 40 mg %.

tion of about 67% was observed with IV, having a p-$N(CH_3)_2$ group as the substituent on the phenyl nucleus of this piperazine.

The compound possessing the p-OCH_3 substituent on the phenyl nucleus (III) showed minimum protection against carrageenan-induced edema. In the present study, I, II, and IV possessed greater anti-inflammatory activity than sodium salicylate, which produced about 33% protection of carrageenan-induced edema under similar experimental conditions.

All piperazines possessed low anticonvulsant activity (Table III). The protection offered by these compounds in a dose of 100 mg/kg against pentylenetetrazol-induced convulsions ranged from 20 to 30%. Data on the anticonvulsant activity of these piperazines indicate that there is no relationship between the increased protection from convulsions and the decreased mortality in experimental animals.

All piperazines exhibited antiproteolytic activity (Table IV). These results indicated that, with the exception of IV during casein hydrolysis, sodium salicylate exhibited a higher degree of inhibition of the hydrolysis of bovine serum albumin and casein by trypsin than the substituted piperazines. Among the new compounds, IV exhibited the highest degree of inhibition against trypsin. All of the new compounds as well as sodium salicylate were better inhibitors of trypsin with bovine serum albumin as the substrate than with casein as the substrate.

The results of preincubation studied to evaluate the nature of the inhibition by substituted piperazines are recorded in Table V. Preincubation of trypsin with the piperazines for varying times (0, 10, 20, and 30 min) before the addition of bovine serum albumin or casein *in vitro* did not alter the degree of hydrolysis. Therefore,

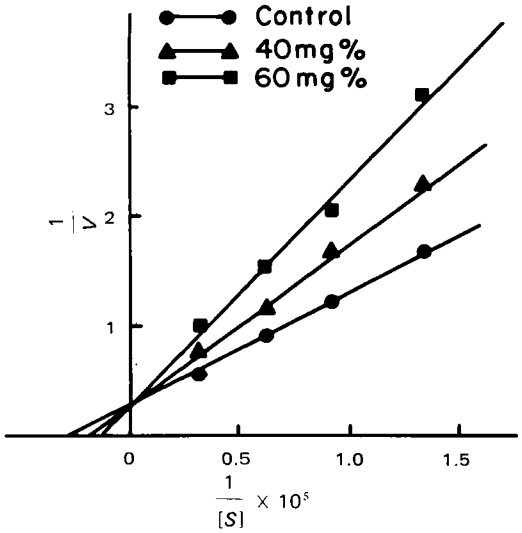


Figure 2—*Inhibition of trypsin-induced hydrolysis of casein by IV. The* [S] *denotes the molar concentration of casein, and* V *represents the change in percent absorbance/0.075 mg of crystalline casein/5 min. Key:* ●, *control with no inhibitor;* ▲, *40 mg % of piperazine; and* ■, *60 mg % of piperazine. The* K_m *value for casein is* 3.6×10^{-5} M; *the* K_i *value for IV is* 1.06×10^{-3} M.

these studies indicated the reversible and/or competitive nature of inhibition of trypsin-induced hydrolysis by substituted piperazines. These findings were further supported by kinetic studies with IV, where the nature of its inhibition of trypsin was evaluated. Compound IV was a competitive inhibitor of the hydrolysis of bovine serum albumin (Fig. 1) and casein (Fig. 2) by trypsin.

In the present series of piperazines, there is a direct relationship between the inhibition of trypsin-induced hydrolysis and anti-inflammatory activity. A similar relationship was reported between the anti-inflammatory and antiproteolytic activities of amyrin acetates (16–18).

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NMR Study of Amphetamines Using Europium Shift Reagents

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Abstract □ Amphetamine and certain of its methoxylated derivatives show a high degree of interaction with NMR shift reagents of the type tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione)europium(III). The shifts are not accompanied by appreciable line broadening, and both the aliphatic and aromatic protons can be resolved. The strong interaction between amine and shift reagent diminishes rapidly as the amine function is alkylated. For derivatives containing *ortho*-methoxyl groups, a weaker interaction with this functionality also becomes evident as the amine is alkylated. The stereospecificity of the shifting process was investigated by employing tris[3-(trifluoroacetyl)-*d*-camphorato]europium(III), a chiral shift reagent, with stereochemically pure enantiomers and known enantiomeric mixtures. Although certain (*R*)-enantiomers showed greater downfield C-methyl group shifts, these shift differences from the corresponding (*S*)-enantiomers were small and not well resolved.

Keyphrases □ Amphetamine and methoxylated derivatives—interaction with NMR europium shift reagents, stereospecificity of shifting process □ NMR spectroscopy—study of amphetamines and methoxylated derivatives, interaction with europium shift reagents, stereospecificity of shifting process □ Europium—NMR shift reagents, interaction with amphetamines and methoxylated derivatives

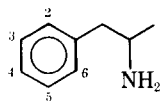
Methods for the use of lanthanide NMR shift reagents in spectral simplification and interpretation of molecules possessing a single functional group are well established (1–3). Their use with polyfunctional compounds and the techniques employing chiral shift reagents, however, are not as well characterized (4–6). Korver and Van Gorkom (7) referred to the scarcity of data defining the scope of application of chiral reagents in determining enantiomeric purity, while Armitage *et al.* (8) referred to the highly neglected problem of employing shift reagents in the determination of molecular configuration.

An interest in these analytical tools and a desire for an efficient assay of enantiomeric composition in certain amphetamine derivatives (9) were the bases for this investigation. The NMR spectra of amphetamine (I) and the derivatives II–VII were examined in the presence of increasing amounts of tris-(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione)europium(III) (VIII). Certain enantiomers and enantiomeric mixtures of amphetamine, *p*-methoxyamphetamine, and 2,5-dimethoxyamphetamine were also studied with the chiral reagent tris[3-(trifluoroacetyl)-*d*-camphorato]europium(III) (IX).

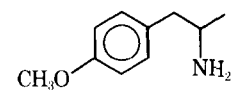
EXPERIMENTAL

Chemistry—The enantiomers of I–V were prepared by a modification of the method of Weinges and Graab (10) as described previously (9). The optical purity of these compounds is in the 96–99% range (9). Racemic samples were prepared by reaction of the appropriate benzaldehyde with nitroethane (11), followed by lithium aluminum hydride reduction of the resulting 1-phenyl-2-nitropropenes (12). Racemates were purified by distillation or by recrystallization of the hydrochloride salts.

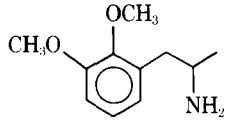
NMR Studies—Free amines were generated by first dissolving 100-μmole quantities of hydrochloride salts in 7 ml of water. These solutions were placed in separators, 5-ml portions of ether were added, and the aqueous phases were made basic with 0.5 ml of 3 *N* sodium hydroxide. After separation of the ether layers, the aqueous phases were extracted with a second 5-ml portion of ether. The



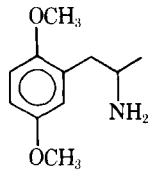
I: amphetamine



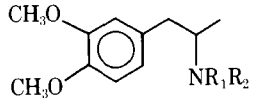
II: *p*-methoxyamphetamine



III: 2,3-dimethoxyamphetamine



IV: 2,5-dimethoxyamphetamine



V: 3,4-dimethoxyamphetamine, $R_1 = R_2 = H$

VI: 3,4-dimethoxy-*N*-methylamphetamine, $R_1 = CH_3$, $R_2 = H$

VII: 3,4-dimethoxy-*N*,*N*-dimethylamphetamine, $R_1 = R_2 = CH_3$