thin-layer chromatography on silica gel plates (Merck, ref. 5567, elution with propanol-methanol-34% ammonia-water 45:15:20:20) and measured by radioactivity counting. As cyclic AMP and adenosine have identical R_f values under these conditions, control experiments were carried out and showed that further degradation of [^{3}H]AMP into [^{3}H]adenosine was negligible: No difference was observed in the amount of [^{3}H]AMP in the presence or in the absence of 5'-nucleotidase inhibitors. This result is consistent with the low 5'-nucleotidase activity of PDE (ref. P0134 from Sigma). Inhibition assays were carried out by adding various inhibitor concentrations (or no inhibitor for the blank) in DMSO to the incubation mixture. IC_{50} values were determined by plotting uninhibited velocity/inhibited velocity (V_0/V) vs the inhibitor concentration.

Synthesis and Chemical Characterization of N-Substituted Phenoxazines Directed toward Reversing Vinca Alkaloid Resistance in Multidrug-Resistant Cancer Cells

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A series of 21 N-substituted phenoxazines has been synthesized in an effort to find more specific and less toxic modulators of multidrug resistance (MDR) in cancer chemotherapy. Thus, *N*-(ω-chloroalkyl)- and *N*-(chloroacyl)phenoxazines were found to undergo iodide-catalyzed nucleophilic substitution on reaction with various secondary amines, including *N*,*N*-diethylamine, *N*,*N*-diethanolamine, morpholine, piperidine, pyrrolidine and (β-hydroxyethyl)piperazine. Products were characterized by UV, IR, ^{1}H-, and ^{13}C-NMR, mass spectral data, and elemental analyses. All of the compounds were examined for cytotoxicity and for their ability to increase the accumulation of the vinca alkaloids, vincristine (VCR) and vinblastine (VLB) in multidrug-resistant GC_3/Cl (human colon adenocarcinoma) and KBCh^R-8-5 (HeLa variant) cell lines. Compounds were compared to the standard modulator verapamil (VRP). Substitutions on the phenoxazine ring at position 10 were associated with an increase in antiproliferative and anti-MDR activities. Modification of the length of the alkyl bridge and the type of amino side chain also influenced the potency of these effects. From among the compounds examined, 10 derivatives were found to increase the accumulation of VCR and VLB in GC_3/Cl and KBCh^R-8-5 cells relative to the effect of VRP, suggesting that with the exception of pyrrolidinyl, the tertiary amine attachments to the phenoxazine nucleus linked through a three- or four-carbon alkyl chain resulted in enhanced anti-MDR activity. On the basis of their 50% growth inhibitory (IC_{50}) values, five of the ten compounds, namely, 10-(3'-chloropropyl)phenoxazine, 10-[3'-[*N*-bis(hydroxyethyl)-amino]propyl]phenoxazine, 10-(3'-*N*-morpholinopropyl)phenoxazine, 10-(4'-*N*-morpholinobutyl)phenoxazine and 10-(*N*-piperidinoacetyl)phenoxazine were selected as relatively nontoxic chemosensitizers. These modulators, at nontoxic concentrations, potentiated the cytotoxicity of VCR and VLB in GC_3/Cl and KBCh^R-8-5 cells. Further, two compounds 10-(3'-*N*-morpholinopropyl)phenoxazine, and the butyl derivative, enhanced accumulation of VLB in GC_3/Cl, KBCh^{R}8-5 and highly resistant KB-V1 cells to a level significantly greater than the maximal level achieved with VRP. Additional experiments to understand the mechanism of action of these agents in modulating MDR are in progress.

Introduction

A major reason for failure of treatment of cancer patients is resistance to conventional chemotherapeutic agents. One type of drug resistance, called multidrug resistance (MDR) is characterized by cross-resistance to functionally and structurally unrelated drugs, typically doxorubicin, vincristine (VCR), vinblastine (VLB), colchicine, and actinomycin D. The gene responsible for this form of MDR has been cloned[1] and encodes a glycoprotein called p-glycoprotein (P-gp), which shows homology to a number of bacterial transport proteins.[2-4] Biochemical studies suggest that P-gp may act as an energy-dependent drug efflux pump. The capacity of P-gp to bind cytotoxic drugs has been demonstrated.[5] A number of drugs from various therapeutic and chemical classes appear to be active in modifying MDR in model systems, including the calcium channel blocker, verapamil[6] (VRP), the calmodulin inhibitor, trifluoperazine,[7] the antiarrhythmic drug, quinidine,[8] reserpine,[9] cyclosporin A,[10] vinca alkaloid analogs,[11] dihydropyridines,[12-14] and pyridine analogs.[15] Agents that reverse MDR apparently did not seem to have common features, but putatively each inhibits the pump activity of P-gp.[16,17] When the activity of the pump is inhibited anticancer agents accumulate in MDR cells with resulting

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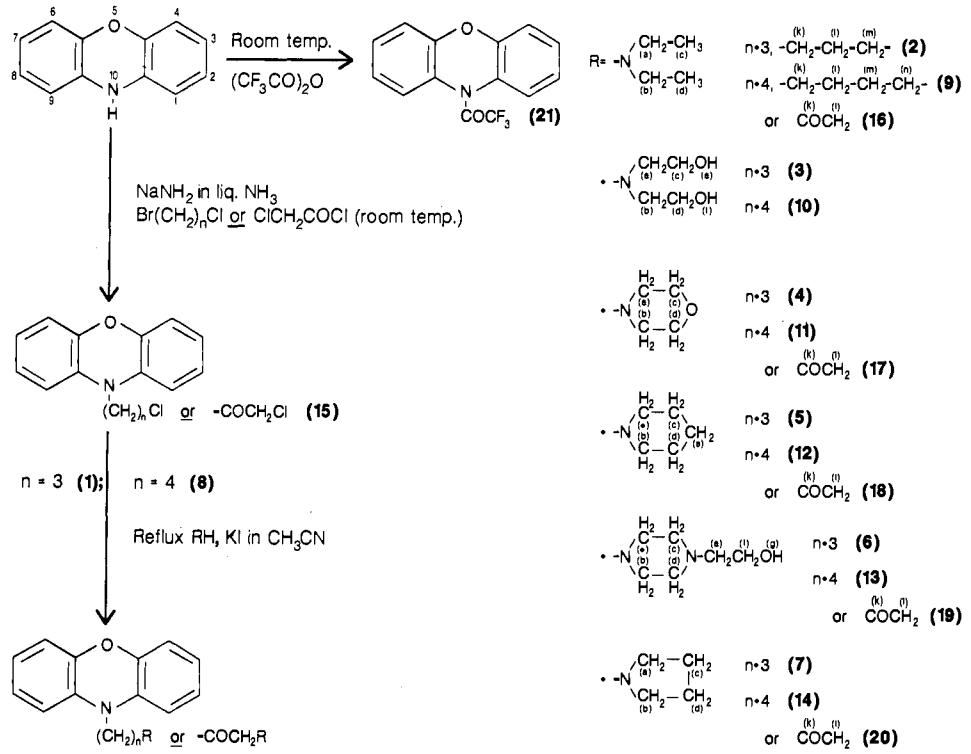
Scheme I



cytotoxicity. Although several of these MDR-reversing agents have been or are now being tested clinically in cancer patients, they have largely failed to enhance drug sensitivity. Instead, serious toxicities develop at or below plasma drug levels required for MDR reversal in vitro.

In a recent publication,[18] we have reported that a tricyclic compound, phenoxazine, potentiated the uptake of VCR and VLB in MDR GC_3/Cl and $KBCh^R$-8-5 cells to a greater extent than verapamil. Kinetic analysis suggested that phenoxazine may enhance vinca uptake rather than inhibit efflux, as with verapamil. However, there was a poor correlation between the level of accumulation, over 2 h, of vinca alkaloids in the presence of phenoxazine and chemosensitization, under conditions of continuous exposure. This discrepancy was due to the breakdown of phenoxazine in serum containing medium. We were interested, therefore, in synthesizing more stable and less toxic derivatives of phenoxazine, which may modulate MDR by novel mechanisms. We report the synthesis, chemical characterization, and anti-MDR activities of 21 N-substituted phenoxazines.

Chemistry

Compounds of pharmacological interest have been found among phenoxazine derivatives and they have been

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claimed to be nervous system depressants, in particular with sedative, antiepileptic, tranquilizing activity,[19-24] spasmolytic activity,[25] antitubercular activity,[26] and anthelminthic activity.[27]

Phenoxazine resists alkylation with alkyl halides because the nitrogen atom of the phenoxazine nucleus is not sufficiently basic for direct action. However, N-alkylation can be achieved in the presence of basic condensing agents like sodium amide. The general procedure for preparing *N*-alkyl derivatives consists of the condensation of phenoxazines with the requisite alkyl halide in the presence of sodium amide, either in liquid ammonia or in anhydrous solvents such as toluene or benzene. The reaction of phenoxazine with mixed chlorobromoalkanes in the presence of sodium amide gave reactive *N*-(chloroalkyl)-phenoxazines. Phenoxazine can be easily acylated with acetic anhydride or by heating with acyl chlorides in benzene.

The general synthesis of *N*-propyl, *N*-butyl, and *N*-acyl series of phenoxazines listed in Table I is shown in Scheme I.

Results and Discussion

Compounds 1–14 listed in Table I were prepared in good yield in two steps. The first step consisted of alkylating phenoxazine with 1-bromo-3-chloropropane or 1-bromo-4-chlorobutane to produce 10-(3′-chloropropyl)phenoxazine (1) or 10-(4′-chlorobutyl)phenoxazine (8), alkylation being accomplished by first converting phenoxazine to the anionic species using the strong base, sodium amide. Iodide-catalyzed nucleophilic substitution of the 10-propyl or -butyl chloride of phenoxazine with various secondary amines (*N*,*N*-diethylamine, *N*,*N*-diethanolamine, morpholine, piperidine, pyrrolidine, and (β-hydroxyethyl)-piperazine) by refluxing overnight with potassium carbonate in anhydrous acetonitrile afforded the free bases.

The acyl derivatives (Table I) were synthesized by the reaction of secondary amines under reflux conditions with 10-(chloroacetyl)phenoxazine (15) in anhydrous acetonitrile containing potassium iodide. 10-(Trifluoroacetyl)phenoxazine (21) was prepared by the route shown in Scheme I. All these compounds were separated and purified by column chromatography or recrystallization and dried under high vacuum. The structures were established by UV, IR, ^{1}H- and ^{13}C-NMR, EIMS, and elemental analyses, and the data are given in the Experimental Section.

Table I. Effects of N^{10}-Substituted Phenoxazines on Accumulation of Vinca Alkaloids

	vinca accumulation (% control)			
	KB8-5		GC_3/Cl	
compd no.	VCR	VLB	VCR	VLB
1	454	342	846	570
2	546	2123	439	1025
3	473	1666	464	1070
4	742	1717	634	960
5	435	1227	282	633
6	343	824	368	879
7	408	969	250	757
8	398	792	317	361
9	211	697	325	737
10	92	403	382	1165
11	702	2684	477	1175
12	196	1071	416	1121
13	91	188	543	1340
14	198	477	412	1315
15	138	236	171	284
16	184	953	160	305
17	290	674	213	298
18	326	2023	177	446
19	280	776	157	426
20	188	776	151	296
21	415	827	230	222
verapamil	402	1124	178	238

[a] Vinca uptake with modulator/vinca uptake without modulator × 100. [b] Compounds were tested at 100 μM. All values represent the mean of two separate experiments with a SD of less than 10% of the mean; each experiment was done in triplicate.

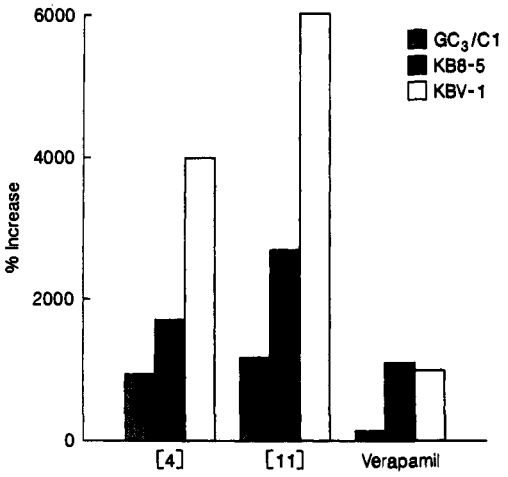

Figure 1. Effect of modulators on the accumulation of VLB in GC_3/C1, KB-8-5, and KB-V1 cells. Monolayers were incubated with compounds 4, 11 or verapamil each at 100 μM for 2 h in the presence of [^{3}H]VLB (49 nM) washed, and the radiolabel was determined, as described in text. The increase in accumulated radiolabel, relative to that in cells exposed only to VLB, is shown. Each result is the mean of triplicate determinations (standard deviations <5% mean).

The UV-spectral data of N-substituted phenoxazines are in close agreement with the spectral characteristics of analogous heterocycles.[28-30] The IR bands also indicate

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Table II. Cytotoxicity of N^{10}-Substituted Phenoxazines[a]

	KB8-5		GC_3/Cl	
compd no.	IC_{50}	IC_{10}	IC_{50}	IC_{10}
1	57	20	83	50
2	15	ND[b]	10	ND
3	38	25	37	25
4	73	25	40	25
10	<10	ND	16	ND
11	18	6	17	10
12	≤7	ND	7	ND
13	≤7	ND	7	ND
14	≤7	ND	7	ND
18	73	40	88	40

[a] IC_{50} and IC_{10} are the concentrations (μM) required to produce 50% and 10% reduction, respectively, in clonogenic survival of GC_3/Cl and KB8-5 cells under the conditions described in the Experimental Section. [b] ND = not determined.

the presence of characteristic functional groups, and peaks at 1670–1695 cm^{-1} indicated the presence of C=O group in the acyl derivatives. The ^{1}H-NMR, typical of phenoxazine compounds, showed eight aromatic protons, and the data are in accordance with the structures assigned. The assignment of protons is fully supported by the integration curves. The ^{13}C-NMR spectrum of each N-substituted phenoxazine exhibited six signals representing 12 aromatic carbons. The GC–mass spectrum showed an intense molecular ion peak (M^+) for each of the compounds characteristic of phenoxazine type of structure. The spectral data are consistent with the assigned structures.

Cellular Pharmacology

Vinca Alkaloid Accumulation. Initial evaluation (at 100 μM) showed that 20/21 compounds (Table I) significantly increased accumulation of VLB in $KBCh^R$-8-5 cells, with compounds 2, 3, 4, 5, 11, and 18 being more effective than verapamil. Compounds 2, 3, and 10–14 increased accumulation of VLB >10-fold in GC_3/Cl cells, whereas the prototype modulator, verapamil, had little effect in these cells. Of note was that compounds 4 and 11 (propyl- and butylmorpholino derivatives) had activity in enhancing accumulation of VCR and VLB in both cell lines. This was examined in an additional cell line, KB-V1, selected for several hundred-fold resistance to VLB. Accumulation of VLB, over 2 h, was examined in the presence of 100 μM modulator. Results, shown in Figure 1, demonstrate that in each of the three cell lines both compounds 4 and 11 had greater effect than the maximal effect achieved by exposure to VRP. In addition, compounds 3 and 18 increased accumulation of VLB (Table I), but did not inhibit efflux.[33] These results suggest that certain phenoxazine derivatives may have a novel mechanism(s) of reversing drug resistance.

Toxicity and Reversal of Vinca Alkaloid Resistance. On the basis of their activity to increase the accumulation of VCR and VLB, compounds were selected to determine their cytotoxic activity against each cell line. Cytotoxicity was assessed by colony forming assay after 7 days exposure to each agent. Concentrations of agent that reduced colony formation by 50% (IC_{50}) were calculated, and results are given in Table II. For compounds that were relatively nontoxic, dose–response curves were established and the concentration of modulator reducing colony formation by 10% was determined (Table II). Compounds 1, 3, 4, 11, and 18 were subsequently evaluated as reversing agents in cells exposed to VCR or VLB continuously for 7 days. IC_{50} values were assessed by colony formation and compared to that for treatment of cells with vinca alkaloid alone. Each modulator was tested at a concentration that reduced colony formation by 10% (given in Table II). As shown in Table III, each modulator tested had significant activity in sensitizing both $KBCh^R$-8-5 and GC_3/Cl cells to VCR and VLB. Compound 4 demonstrated the greatest effect and had good activity against both cell lines. The activity, and perhaps novel mechanism of action of these compounds, will be further investigated for therapeutic modulation in vivo.

Table III. Potentiation of VCR and VLB Cytotoxicity by Phenoxazines

	IC_{50} (nM)			
	KB8-5		GC_3/Cl	
compd no.[a]	VCR	VLB	VCR	VLB
no modulator	55.9	15.0	20.5	7.4
1	11.8 (4.7)[b]	5.3 (2.8)	2.0 (10.3)	1.7 (4.3)
3	5.9 (9.5)	2.7 (5.6)	4.8 (4.3)	2.0 (3.7)
4	1.9 (29.5)	1.6 (9.4)	2.1 (9.8)	2.0 (3.7)
11	4.5 (12.4)	2.0 (7.5)	2.4 (8.5)	2.0 (3.7)
18	6.0 (9.3)	2.3 (6.3)	2.9 (7.1)	2.2 (3.4)

[a] Modulators used at the IC_{10} concentration (Table II). [b] Fold potentiation.

Experimental Section

Melting points were recorded on a Kofler hot-stage with microscope and are uncorrected. IR spectra were recorded on a Perkin-Elmer Model 1320 spectrophotometer, as KBr pellets; UV spectra were recorded in MeOH on a Perkin-Elmer Lambda 3B spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN; found values are within 0.4% of theoretical unless otherwise noted. Reactions were monitored by TLC. For TLC, Analtech silica gel GF plates (20 × 20 cm, 250 μm, glass-backed), with petroleum ether–ethyl acetate (9.7:0.3 by volume, system A), and ethyl acetate–methanol (9.9:0.1 by volume, system B) as solvents were used. Column chromatography utilized silica gel Merck grade 60 (230–400 mesh, 60 Å). ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solution in a 5-mm tube on an IBM NR 200 AF Fourier transform spectrometer with tetramethylsilane as internal standard. Chemical shifts are expressed as "δ" (ppm) values. The spectrometer was internally locked to the deuterium frequency of the solvent. All the derivatives showed the characteristic chemical shifts for the phenoxazine nucleus: ^{1}H-NMR δ 6.36–6.86 (m, 8 H, Ar-H, H_1–H_4 and H_6–H_9) for alkyl derivatives and 7.05–7.63 (m, 8 H, Ar-H, H_1–H_4 and H_6–H_9) for acyl derivatives; ^{13}C-NMR δ (^{1}H-decoupled) 111.38 (C_1 and C_9), 115.35 (C_4 and C_6), 121.02 (C_3 and C_7), 123.66 (C_2 and C_8), 133.05 ($C_{1'}$ and $C_{9'}$), and 144.84 ($C_{4'}$ and $C_{6'}$) for alkyl derivatives and 116.90[34] (C_1 and C_9), 123.50 (C_4 and C_6), 124.70 (C_3 and C_7), 127.10 (C_2 and C_8), 128.90[35] ($C_{1'}$ and $C_{9'}$), and 150.95 ($C_{4'}$ and $C_{6'}$) for acyl derivatives. Electron-impact mass spectra (EIMS) were recorded on a Ribermag R10-10C GC-mass spectrometer with an upper mass limit of 1500 AMU.

Materials. All the chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Phenoxazine, secondary amines indicated in the text, and anhydrous organic solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Vincristine sulfate (oncovin) was purchased from Eli Lilly and Co. (Indianapolis, IN), and vinblastine sulfate

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(34) Except for 15 (δ 110.04).
(35) Except for 21 (δ 151.04).

was from Cetus Corporation (Emeryville, CA). [G-^{3}H]Vincristine (sp act. 7.1 Ci/mmol) and [G-^{3}H]vinblastine (sp act. 10.1 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). Verapamil hydrochloride, colchicine, RPMI-1640 medium, or medium with glutamine and without sodium bicarbonate were purchased from the Sigma Chemical Co. (St. Louis, MO).

Synthesis. 10-(3′-Chloropropyl)phenoxazine (1). To a suspension of sodium amide (1.72 g) in 50 mL of liquid ammonia was added 7 g (0.04 mol) of phenoxazine. After stirring for 30 min, 6.3 g (0.04 mol, 3.96 mL) of 1-bromo-3-chloropropane was added slowly with constant stirring. After one more hour, ammonia was allowed to evaporate and solid ice pieces were added carefully followed by cold water. When the reaction ceased, the mixture was extracted three times with ether. The ether solution was washed three times with water, dried over anhydrous sodium sulfate, and evaporated. The residue was chromatographed on silica gel. Petroleum ether–ethyl acetate (9 mL + 3 mL) eluted pure 1 (7.94 g, 80%) as white crystals: mp 53 °C; UV λ_{max} (ϵ) (MeOH) 218 (39 125), 238 (53 290), 321 (32 530) nm; IR 3070, 2860, 1630, 1490, 1380, 1275, 920, 815, 740 cm^{-1}; ^{1}H-NMR δ 2.11 (m, 2 H, H$_j$), 3.63 (m, 2 H, H$_k$), 3.69 (m, 2 H, H$_m$); ^{13}C-NMR δ 27.82, 41.09, 42.63; EIMS (m/z) 259 (M$^+$). Anal. ($C_{15}H_{14}ClNO$) C, H, N.

10-[3′-(*N*-Diethylamino)propyl]phenoxazine (2). One gram (3.86 mmol) of 1 was dissolved in 150 mL of anhydrous acetonitrile, and 1.5 g of KI, 2.10 g of K_2CO_3, and 1.6 mL (15.4 mmol) of *N,N*-diethylamine were added. The mixture was refluxed overnight until a substantial amount of product was formed. The reaction mixture was diluted with water and extracted with ether (3 × 100 mL). The ether layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness on the rotary evaporator. The crude oil was subjected for column chromatography on silica gel for purification. Ethyl acetate–petroleum ether (1:1) eluted the free base 2 as a colorless oil. An ethereal solution of the free base was treated with an excess of tartaric acid to separate the hygroscopic tartrate salt (1.2 g, 69%): UV λ_{max} (ϵ) (MeOH) 215 (45 675), 238 (89 250), 320 (51 735) nm; IR ($CHCl_3$) 3378, 2974, 2838, 1453, 1375, 1155, 973, 722 cm^{-1}; ^{1}H-NMR δ 1.16 (t, 6 H, H$_c$ and H$_d$), 1.70 (m, 2 H, H$_j$), 2.50 (q, 4 H, H$_a$ and H$_b$, J = 7 Hz), 3.42–3.63 (m, 4 H, H$_k$ and H$_m$); ^{13}C-NMR δ 8.21, 19.90, 40.72, 45.87, 48.50; EIMS (m/z) 296 (M$^+$).

10-[3′-[*N*-Bis(hydroxyethyl)amino]propyl]phenoxazine (3). The procedure used for 2 was repeated with 1 g (3.86 mmol) of 1, 1.5 g of KI, 2.10 g of K_2CO_3, and 1.62 g (15.4 mmol, 1.5 mL) of diethanolamine. Recrystallization of the solid in ethyl acetate and petroleum ether gave pure 3 (1.14 g, 90%): mp 83–84 °C; UV λ_{max} (ϵ) (MeOH) 218 (48 795), 239 (62 385), 322 (53 125) nm; IR 3300, 2960, 2880, 1590, 1490, 1440, 1375, 1270, 1190, 1075, 890, 840, 740 cm^{-1}; ^{1}H-NMR δ 1.71–1.82 (m, 2 H, H$_j$), 2.54–2.61 (t, 4 H, H$_a$ and H$_b$, J = 6 Hz), 2.95 (s, H$_e$ and H$_f$, disappearing on D_2O exchange), 3.39–3.68 (m, 8 H, H$_k$, H$_c$, H$_d$, and H$_m$); ^{13}C-NMR δ 22.42, 41.83, 52.38, 55.91, 59.64; EIMS (m/z) 328 (M$^+$). Anal. ($C_{19}H_{24}N_2O_3$) C, H, N.

10-(3′-*N*-Morpholinopropyl)phenoxazine (4). Repeated the procedure used for 2 with 1 g of 1, 1.5 g of KI, 2.0 g of K_2CO_3, and 1.40 g (15.40 mmol, 1.34 mL) of morpholine. The oily residue was purified by column chromatography to give the brown oil 4. An ethereal solution of the free base was treated with ethereal hydrochloride to give the hydrochloride salt (1.07 g, 80%, mp 198 °C); UV λ_{max} (ϵ) (MeOH) 216 (48 225), 239 (77 535), 320 (61 305) nm; IR 3200, 1495, 1380, 1280, 1230, 1135, 1100, 1020, 980, 870, 760, 735 cm^{-1}; ^{1}H-NMR δ 1.78 (m, 2 H, H$_j$), 2.40 (t, 4 H, H$_a$ and H$_b$, J = 12 Hz), 3.45–3.80 (m, 8 H, H$_k$, H$_m$, H$_c$, and H$_d$); ^{13}C-NMR δ 20.06, 40.93, 51.91, 55.20, 63.50; EIMS (m/z) 310 (M$^+$). Anal. ($C_{19}H_{22}N_2O_2HCl$) C, H, N.

10-(3′-*N*-Piperidinopropyl)phenoxazine (5). The experimental procedure used for 2 is applicable with 1.12 g (4.31 mmol) of 1, 1.5 g of KI, 2.4 g of K_2CO_3, and 1.5 g (17.62 mmol, 1.74 mL) of piperidine. The product was chromatographed on silica gel with petroleum ether–ethyl acetate (1:1 by volume) to get the pure product in the form of an oil 5. By adding ethereal hydrochloride to the ether solution of the free base, hydrochloride salt of 5 (1.04 g, 78%, mp 202 °C) was obtained: UV λ_{max} (ϵ) (MeOH) 218 (55 750), 238 (75 680), 320 (68 940) nm; IR 3300, 2940, 2680, 1595, 1495, 1385, 1275, 1160, 1050, 825, 745 cm^{-1}; ^{1}H-NMR δ 1.53 (m, 6 H, H$_c$, H$_d$, and H$_e$), 2.30 (m, 2 H, H$_j$), 2.56–2.67 (m, 4 H, H$_a$ and H$_b$), 3.45–3.70 (m, 4 H, H$_k$ and H$_m$); ^{13}C-NMR δ 20.21, 21.93, 22.50, 41.05, 53.18, 54.62; EIMS (m/z) 308 (M$^+$).

10-[3′-[(β-Hydroxyethyl)piperazino]propyl]phenoxazine (6). The procedure used for 2 is repeated with 1 g (3.86 mmol) of 1, 1.5 g of KI, 2.12 g of K_2CO_3, and 2 g (15.4 mmol, 1.9 mL) of (β-hydroxyethyl)piperazine. The free base was recrystallized in petroleum ether–ether mixture (7:3 by volume) to give 6 (1.16 g, 85%, mp 108 °C): UV λ_{max} (ϵ) (MeOH) 217 (55 115), 239 (72 955), 322 (66 225) nm; IR 3060, 2820, 1630, 1595, 1495, 1385, 1270, 1160, 1070, 980, 850, 810, 735 cm^{-1}; ^{1}H-NMR δ 1.74 (m, 2 H, H$_j$), 2.33–2.80 (m, 12 H, H$_a$ and H$_b$, H$_c$ and H$_d$, H$_e$ and H$_m$), 2.79 (s, 1 H, H$_g$, disappearing on D_2O exchange), 3.47–3.65 (m, 4 H, H$_k$ and H$_f$); ^{13}C-NMR δ 22.58, 41.72, 52.96, 53.28, 55.19, 57.77, 59.34; MS (m/z) 353 (M$^+$).

10-(3′-*N*-Pyrrolidinopropyl)phenoxazine (7). Procedure used for 2 was repeated with 1 g of 1, 1.5 g of KI, 2 g of K_2CO_3, and 1.1 g (15.5 mmol, 1.3 mL) of pyrrolidine. The product was purified by column chromatography, and the oil was converted into hydrochloride salt 7 (1.02 g, 80%, mp 158–159 °C): UV λ_{max} (ϵ) (MeOH) 217 (50 145), 239 (76 595), 319 (54 265) nm; IR 3300, 2660, 1590, 1490, 1375, 1270, 1130, 920, 820, 745 cm^{-1}; ^{1}H-NMR δ 2.01–2.17 (t, 4 H, H$_c$ and H$_d$, J = 13 Hz), 2.21 (m, 2 H, H$_j$), 3.06–3.14 (t, 4 H, H$_a$ and H$_b$), 3.60–3.67 (m, 4 H, H$_k$ and H$_m$); ^{13}C-NMR δ 22.25, 23.30, 40.90, 52.80, 53.63; MS (m/z) 294 (M$^+$).

10-(4′-Chlorobutyl)phenoxazine (8). Compound 8 (8.4 g, 80%, mp 46 °C) in the pure form was prepared following the procedure used for 1 with 7 g of phenoxazine (0.04 mol), 1.63 g of sodium amide, and 4.36 mL of 1-bromo-4-chlorobutane (0.04 mol): UV λ_{max} (ϵ) (MeOH) 212 (42 425), 238 (60 185), 320 (53 875) nm; IR 3060, 2980, 1630, 1590, 1495, 1380, 1280, 1130, 915, 840, 730 cm^{-1}; ^{1}H-NMR δ 1.75 (m, 4 H, H$_j$ and H$_m$), 3.38–3.50 (m, 4 H, H$_k$ and H$_n$); ^{13}C-NMR δ 22.60, 29.87, 43.27, 44.61; EIMS (m/z) 273 (M$^+$).

10-[4′-(*N*-Diethylamino)butyl]phenoxazine (9). The procedure used for 2 was followed with 1 g (3.66 mmol) of 8, 1.5 g of KI, 2 g of K_2CO_3, and 1.07 g (14.63 mmol, 1.5 mL) of *N,N*-diethylamine to obtain 9. The oily product was chromatographed on the silica gel with CH_3OH–$CHCl_3$ (3:1), and the hydrochloride salt (0.76 g, 60%, mp 127 °C) was obtained in the pure form: UV λ_{max} (ϵ) (MeOH) 213 (58 885), 239 (90 590), 320 (67 920) nm; IR 3300, 2940, 1590, 1495, 1380, 925, 750 cm^{-1}; ^{1}H-NMR δ 1.33 (broad s, 6 H, H$_c$ and H$_d$), 1.66–1.91 (m, 4 H, H$_j$ and H$_m$), 3.05 (m, 6 H, H$_a$, H$_b$ and H$_n$), 3.50 (m, 2 H, H$_k$); ^{13}C-NMR δ 8.54, 21.02, 22.46, 43.05, 46.50, 51.26; MS (m/z) 310 (M$^+$).

10-[4′-[*N*-Bis(hydroxyethyl)amino]butyl]phenoxazine (10). Compound 10 as its hydrochloride salt (1.11 g, 80%, mp 115 °C) was obtained by following the procedure of 3 with 1 g (3.66 mmol) of 8, 1.5 g of KI, and 1.54 g (14.65 mmol, 1.4 mL) of *N,N*-diethanolamine followed by column chromatography: UV λ_{max} (ϵ) (MeOH) 210 (43 275), 238 (78 235), 321 (59 330) nm; IR 3280, 2850, 1630, 1590, 1490, 1375, 1270, 1135, 1020, 925, 845, 740 cm^{-1}; ^{1}H-NMR δ 1.70–1.98 (m, 4 H, H$_j$ and H$_m$), 3.35–3.57 (m, 10 H, H$_a$, H$_b$, H$_n$, H$_k$, H$_e$, and H$_f$), 3.95 (t, 4 H, H$_c$ and H$_d$, J = 7 Hz); ^{13}C-NMR δ 19.98, 21.10, 42.06, 52.92, 54.78, 54.96; EIMS (m/z) 342 (M$^+$).

10-(4′-*N*-Morpholinobutyl)phenoxazine (11). The procedure used for 4 was repeated with 1 g (3.66 mmol) of 8, 1.5 g of KI, 2 g of K_2CO_3, and 1.30 g (14.61 mmol, 1.3 mL) of morpholine. The product was recrystallized in ether–petroleum ether mixture (3:1) to give 11 (0.95 g, 80%, mp 89 °C): UV λ_{max} (ϵ) 213 (41 000), 239 (55 715), 321 (53 915) nm; IR 2960, 2810, 1630, 1495, 1380, 1295, 1130, 1070, 970, 870, 825, 745 cm^{-1}; ^{1}H-NMR δ 1.61–1.74 (m, 4 H, H$_j$ and H$_m$), 2.40–2.50 (m, 6 H, H$_a$, H$_b$, and H$_n$), 3.49 (m, 2 H, H$_k$), 3.50–3.78 (t, 4 H, H$_c$ and H$_d$, J = 12 Hz); ^{13}C-NMR δ 22.34, 23.50, 43.63, 53.67, 57.91, 66.97; EIMS (m/z) 324 (M$^+$). Anal. ($C_{20}H_{24}N_2O_2$) C, H, N.

10-(4′-*N*-Piperidinobutyl)phenoxazine (12). One gram (3.66 mmol) of 8, 1.5 g of KI, 2 g of K_2CO_3, 1.45 g (17.03 mmol, 1.5 mL) of piperidine were refluxed and processed according to the procedure used for 10. Purification by column chromatography afforded the free amine as a brown oil which was converted into hydrochloride salt 12 (1.18 g, 90%, mp 190 °C): UV λ_{max} (ϵ) 210 (46 575), 238 (69 390), 320 (53 455) nm; IR 3320, 2940, 1625, 1490, 1380, 1270, 1130, 1060, 955, 820, 730 cm^{-1}; ^{1}H-NMR δ 1.44–1.82 (m, 6 H, H$_c$, H$_d$, and H$_e$), 1.98–2.18 (m, 4 H, H$_j$ and H$_m$), 2.70–2.97 (m, 4 H, H$_a$ and H$_b$), 3.39–3.45 (m, 4 H, H$_k$ and H$_n$); ^{13}C-NMR

δ 20.96, 21.79, 22.48, 43.08, 52.91, 56.70; EIMS (*m/z*) 322 (M^+).

10-[4′-[(β-Hydroxyethyl)piperazino]butyl]phenoxazine (13). The procedure used for 6 was repeated with 1 g (3.66 mmol) of 8, 1.5 g of KI, and 1.9 g (14.6 mmol, 1.8 mL) of (β-hydroxyethyl)piperazine. The oily residue was treated with 500 μL of ethyl acetate first and then with petroleum ether (20 mL), when white crystalline solid separated out. The solid was recrystallized to give pure 13 (1.21 g, 90%, mp 114 °C): UV λ_{max} (ε) (MeOH) 239 (71 535), 320 (57 655) nm; IR 3060, 2940, 2860, 1590, 1495, 1380, 1225, 1135, 1020, 935, 830, 740 cm^{-1}; ^{1}H-NMR δ 1.58 (broad s, 4 H, H_l and H_m), 2.36–2.51 (m, 12 H, H_a, H_b, H_c, H_d, H_e, and H_n), 3.42 (broad s, 3 H, H_k and H_g), 3.58–3.63 (t, 2 H, H_f, J = 7 Hz); ^{13}C-NMR δ 22.28, 23.72, 43.60, 53.11, 57.38, 57.96, 59.76; EIMS (*m/z*) 367 (M^+).

10-(4′-*N*-Pyrrolidinobutyl)phenoxazine (14). The experimental steps used for 2 were repeated, taking 1 g (3.66 mmol) of 8, 1.5 g of KI, 2 g of K_2CO_3, and 1.04 g (14.6 mmol, 1.22 mL) of pyrrolidine as reactants. The product was chromatographed on silica gel with $CHCl_3$–MeOH (1:1) to give the free amine as a brown oil. An ether solution of this oil was treated with ethereal hydrogen chloride to secure pure 14 (0.90 g, 71%, mp 170 °C) hydrochloride salt: UV λ_{max} (ε) (MeOH) 211 (51 780), 238 (75 210), 320 (64 735) nm; IR 3060, 2840, 1590, 1495, 1380, 1295, 1160, 1045, 915, 830, 740 cm^{-1}; ^{1}H-NMR δ 1.64–2.10 (m, 8 H, H_l, H_m, H_c, and H_d), 2.97–3.17 (m, 6 H, H_a, H_b, and H_n), 3.45–3.54 (m, 2 H, H_k); ^{13}C-NMR δ 22.47, 23.27, 43.14, 53.50, 54.91; EIMS (*m/z*) 308 (M^+).

10-(Chloroacetyl)phenoxazine (15). To a solution of 5 g (0.03 mol) of phenoxazine dissolved in 100 mL of anhydrous acetonitrile containing 10 mL of anhydrous ether was added dropwise 7 mL (9.926 g, 0.09 mol) of chloroacetyl chloride with constant stirring. The reaction mixture was stirred at room temperature for 5 h when white crystalline solid separated out. The crystals were filtered, washed several times with petroleum ether–ether mixture (9:1), and dried under high vacuum to get pure 15 (6.03 g, 85%, mp 143–144 °C): UV λ_{max} (ε) (MeOH) 218 (46 725), 249 (50 908), 287 (46 925) nm; IR 3070, 1675, 1580, 1480, 1410, 1350, 1260, 1115, 1040, 860, 750 cm^{-1}; ^{1}H-NMR δ 4.32 (s 2 H, H_l); ^{13}C-NMR δ 41.51, 170; EIMS (*m/z*) 259 (M^+).

10-[(*N*-Diethylamino)acetyl]phenoxazine (16). One gram (3.9 mmol) of 15 was dissolved in 150 mL of anhydrous acetonitrile, and 1.5 g of KI and 1.13 g (15.45 mmol, 1.6 mL) of *N*,*N*-diethylamine were added to it. The reaction mixture was refluxed for 1 h when substantial amount of the product was formed. The mixture was processed as in compound 2 and the oily product scratched with a spatula to get a white crystalline solid which was further recrystallized in ethyl acetate and petroleum ether mixture to get pure 16 (0.86 g, 75%, mp 39 °C): UV λ_{max} (ε) (MeOH) 220 (50 330), 246 (59 085), 287 (41 335) nm; IR 2800, 1685, 1580, 1480, 1320, 1210, 1150, 1060, 940, 860, 755, 670 cm^{-1}; ^{1}H-NMR δ 0.95 (t, 6 H, H_c and H_d, J = 7 Hz), 2.60 (q, 4 H, H_a and H_b), 3.55 (s, 2 H, H_l); ^{13}C-NMR δ 12.08, 47.04, 54.99, 169.84; MS (*m/z*) 296 (M^+).

10-(*N*-Morpholinoacetyl)phenoxazine (17). The same procedure used for 16 was employed with 1 g (3.86 mmol) of 15, 1.5 g of KI, and 1.35 g (16 mmol, 1.4 mL) of morpholine. The solid 17 was recrystallized in a mixture of ethyl acetate, petroleum ether, and ether, and the free base was converted into the hydrochloride salt (1.07 g, 80%, mp 130 °C) using ethereal hydrogen chloride: UV λ_{max} (ε) 213 (37 285), 246 (61 530), 287 (54 085) nm; IR 2980, 2860, 1690, 1485, 1355, 1270, 1120, 1070, 900, 855, 760, 640 cm^{-1}; ^{1}H-NMR δ 2.40–2.60 (t, 4 H, H_a and H_b, J = 12 Hz), 3.35 (s, 2 H, H_l), 3.50–3.70 (t, 4 H, H_c and H_d); ^{13}C-NMR δ 52.41, 57.01, 63.23, 163.40; MS (*m/z*) 310 (M^+).

10-(*N*-Piperidinoacetyl)phenoxazine (18). The method employed for 17 was used with 1 g (3.86 mmol) of 15, 1.5 g of KI, and 1.31 g (15.40 mmol, 1.52 mL) of piperidine to get 18 (0.95 g, 80%, mp 110–111 °C): UV λ_{max} (ε) (MeOH) 218 (39 275), 246 (55 525), 287 (44 350) nm; IR 2960, 1670, 1580, 1480, 1370, 1260, 1120, 940, 855, 765, 655 cm^{-1}; ^{1}H-NMR δ 1.51 (broad s, 6 H, H_c, H_d, and H_e), 2.44 (m, 4 H, H_a and H_b), 3.34 (s, 2 H, H_l); ^{13}C-NMR δ 23.92, 25.93, 54.15, 60.80, 168.92; EIMS (*m/z*) 308 (M^+). Anal. ($C_{19}H_{20}N_2O_2$) C, H, N.

10-[[(β-Hydroxyethyl)piperazino]acetyl]phenoxazine (19). The procedure used for 17 was repeated with 1 g (3.86 mmol) of 15, 1.5 g of KI, and 2 g (15.4 mmol, 1.9 mL) of (β-hydroxyethyl)piperazine. Recrystallization of the white solid yielded 19 (1.17 g, 86%, mp 70–71 °C): UV λ_{max} (ε) (MeOH) 213 (38 295), 246 (66 405), 287 (63 815) nm; IR 3200, 2940, 1685, 1480, 1265, 1160, 945, 855, 765, 640 cm^{-1}; ^{1}H-NMR δ 2.48 (m, 10 H, H_a, H_b, H_c, H_d, and H_e), 2.70 (s, 1 H, H_g, disappearing on D_2O exchange), 3.39 (s, 2 H, H_l), 3.60 (t, 2 H, H_f, J = 7 Hz); ^{13}C-NMR δ 52.70, 52.90, 57.70, 59.23, 59.80, 168.43; EIMS (*m/z*) 353 (M^+).

10-(*N*-Pyrrolidinoacetyl)phenoxazine (20). The experimental procedure used for 17 was employed with 1 g (3.86 mmol) of 15, 1.5 g of KI, and 1.1 g (15.4 mmol, 1.3 mL) of pyrrolidine. Purification by recrystallization afforded 1.02 g (80%, mp 96–98 °C) of 20: UV λ_{max} (ε) (MeOH) 214 (40 440), 240 (58 345), 286 (39 050) nm; IR 2980, 2820, 1695, 1480, 1340, 1270, 1100, 1040, 985, 855, 755, 640 cm^{-1}; ^{1}H-NMR δ 1.77 (t, 4 H, H_c and H_d, J = 7 Hz), 2.64 (t, 4 H, H_a and H_b), 3.51 (s, 2 H, H_l); ^{13}C-NMR δ 23.73, 53.83, 57.24, 168.92; EIMS (*m/z*) 294 (M^+).

10-(Trifluoroacetyl)phenoxazine (21). To a solution of 200 mg (1.10 mmol) of phenoxazine in 10 mL anhydrous chloroform and 4 mL anhydrous ether was added 500 μL of (0.74 g, 3.54 mmol) trifluoroacetic anhydride and stirred at room temperature for 8 h. The formation of the product was monitored by TLC, extracted with chloroform and evaporated. The residue was subjected for column chromatography which afforded pure compound 21 (213 mg, 70%, mp 90 °C): UV λ_{max} (ε values not determined) (MeOH) 212, 238, 252 nm; IR 3375, 1695, 1580, 1480, 1390, 1290, 1170, 1030, 965, 850, 800, 760, 670 cm^{-1}; ^{13}C-NMR δ >200 ppm; EIMS (*m/z*) 279 (M^+).

Evaluation of N-Substituted Phenoxazines for Their Anti-MDR Activity. Cell Lines and Cell Cultures. A cloned line of human colon adenocarcinoma, GC_3/Cl[31], which is intrinsically resistant to VCR (~4-fold relative to KB-3-1), was routinely grown at 37 °C in antibiotic-free RPMI-1640 medium supplemented with 2 mM glutamine and 10% FBS (Hyclone Laboratories, Inc., Logan, UT) in a humidified atmosphere of 5% CO_2 and 95% air. Human epidermoid carcinoma KB-3-1 cells and a colchicine-selected MDR variant, $KBCh^R$-8-5, were obtained from Dr. M. Gottesman. $KBCh^R$-8-5 was cross-resistant to VCR (45-fold) and VLB (6.3-fold) and was grown in monolayer culture at 37 °C in DMEM with 10% FBS and L-glutamine in a humidified atmosphere of 10% CO_2 in air. The resistance of the $KBCh^R$-8-5 cells was maintained by culturing them with colchicine (10 ng/mL).

Accumulation Studies. 2 mL of cell suspensions (2×10^6 cells) were plated in 35 × 10 mm style "easy grip" culture dishes (Becton Dickinson Co., Lincoln Park, NJ). Cells were allowed to attach to plastic overnight at 37 °C. Medium was aspirated, and cells were washed with (2 × 2 mL) physiologic Tris (PT) buffer. Monolayers were incubated at room temperature for 10 min in PT buffer prior to aspiration and adding 1 mL of serum-free RPMI-1640 Hepes buffer (10.4 g RPMI-1640 medium in 1 L of 25 mM Hepes, pH 7.4) containing 70.4 nm [^{3}H]VCR (sp act. 7.1 Ci/mmol) or 49.5 nm [^{3}H]VLB (sp act. 10.1 Ci/mmol) with or without compounds 1–21 (100 μM) or VRP dissolved in H_2O and DMSO (final culture concentration <0.1% DMSO). After 2 h of incubation at room temperature, medium was rapidly aspirated to terminate drug accumulation, and monolayers were washed four times with ice-cold PBS (g/L: NaCl 8.0; $Na_2HPO_4 \cdot 12H_2O$, 2.9; KCl 0.2; KH_2PO_4, 0.2) and drained. To each dish, 1 mL of trypsin–EDTA (0.05% trypsin, 0.53 mM EDTA) was added. After 1 min, monolayers were pipetted to give a uniform suspension of cells, and radioactivity in 0.75 mL was determined by scintillation counting. Cell number per dish was determined on 200 μL of suspension using the method of Butler,[32] and amounts of intracellular VCR or VLB were determined (Table I).

Effect of N-Substituted Phenoxazines on Cell Survival. $KBCh^R$-8-5 cells were plated in triplicate at a density of 1000 cells per well and GC_3 at 3000 cells per well in Falcon 6-well flat-bottom tissue culture plates (Becton Dickinson Co., Lincoln Park, NJ). After 24 h, incubation medium was replaced with 3 mL of fresh medium containing compounds 1–4 or 10–14 or 18 at concentrations ranging from 1–100 μM (final culture concentration, 0.1% DMSO), and cells were incubated at 37 °C for a further 7 days. The medium was aspirated, and cells were washed once with 2 mL of 0.9% saline and dried overnight. Colonies were stained with 1 mL of 0.1% crystal violet followed by washing twice with distilled water and were counted using an automated ARTEK Model 880 colony counter. The IC_{50} values were determined from

concentration-percent cell-survival curves and were defined as the concentrations of phenoxazines required for 50% reduction in colonies compared to controls (Table II).

Effect of N-Substituted Phenoxazines on in Vitro Cytotoxicity of VLB and VCR. Cells were treated with graded concentrations of VCR and VLB in the absence or presence of nontoxic concentrations (Table III) of compounds 1, 3, 4, 11, or 18. The plates were then transferred to a CO_2 incubator and, after further incubation for 7 days at 37 °C, colonies were enumerated as described.

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Synthesis and Biological Activity of Ketomethylene Pseudopeptide Analogues as Thrombin Inhibitors[1]

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Ketomethylene pseudopeptide analogues Aa-Pro-Argψ($COCH_2$)Gly-pip, 1, where Aa are D- or L-amino acids (Dpa, β,β-diphenylalanine; αNal, α-naphthylalanine; βNal, β-naphthylalanine; Fgl, fluorenylglycine) with highly lipophilic side chains and ψ($COCH_2$) is a ketomethylene pseudopeptide bond, have been synthesized through a modified Dakin-West reaction under very mild conditions with a high yield using tripeptide 4 with a labile functional group directly on the side chain. Their enzymatic assay of thrombin inhibition has been carried out. The structure-activity relationship study indicated that a lipophilic side chain on the amino acid in the P_3 position is very important for binding to the apolar site of thrombin. Compound 1a with D-Dpa at the P_3 position has a K_i of 0.2 μM and it doubles thrombin clotting time at only 3 times higher concentration. These values are about 7 times better than those of the corresponding D-Phe analogues. Furthermore, 1a shows poor inhibitory activity against plasmin, factor Xa, urokinase, and kallikrein. Preliminary in vivo testing (3-4-kg rabbit as the animal model) shows no observable side effect (change of blood pressure and accumulation of blood platelet in lungs) at a dose of 1 mg/kg.

Interest in the design of synthetic inhibitors of serine proteases and especially of thrombin has grown enormously during the last few years. One type of such inhibitors is the ketomethylene pseudopeptide analogues, in which the -NH- group of the scissile P_1-P_1' bond, corresponding to the natural substrate, has been replaced by a methylene group. This type of peptide inhibitor has several advantages. Firstly, the ketomethylene bond is resistant to enzymatic degradation. Secondly, the keto group can possibly form a tetrahedral semi-ketal with the active site serine hydroxyl. Thirdly, the amino acid sequence on the carbonyl side of the ketomethylene bond can add binding affinity to the inhibitor.

The peptide sequence D-Phe-Pro-Arg, imitating the natural substrate of thrombin, has been widely used as a basis for inhibitors and substrates of thrombin.[2-6] The ketomethylene peptide analogue D-Phe-Pro-Argψ-($COCH_2$)Gly-pip, synthesized by Szelke and Jones,[6] showed good inhibitory activity toward thrombin.[7]

In the course of our studies on thrombin peptide inhibitors, it is found that a lipophilic side chain on the amino acid in the P_3 position is very important for binding to the apolar site of thrombin. We here report the synthesis and antithrombin effect of inhibitors with the structure shown in formula 1 (Scheme I), where D-Phe in the sequence D-Phe-Pro-Arg has been replaced by some unnatural, aromatic amino acids.

To introduce the ketomethylene moiety into the compound, Szelke and Jones[6] used a Dakin-West reaction[8] with N^{α}-formyl-protected amino acid as the starting material. This method gave us a very poor yield (1%). The benzene ring of an N^{α}-benzoyl amino acid activatives the oxazolone intermediate. We obtained a good yield (58%) by this method, but the very harsh conditions (HCl/$AcOH/H_2O$, 120 °C, 20 h)[9] needed to remove the benzoyl protecting group gave a very poor yield in this step. These hydrolysis conditions will limit the use of this reaction in the case of amino acids with labile functional groups on the side chains. The trifluoromethyl ketone analogue of

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(1) Abbreviations: Aa, α-amino acid; Dpa, β,β-diphenylalanine; αNal, α-naphthylalanine; βNal, β-naphthylalanine; Fgl, fluorenylglycine; ψ($COCH_2$), ketomethylene pseudopeptide bond; MMS, monomethyl succinate; Py, pyridine; TEA, triethylamine; pip, piperidine; Mtr, (4-methoxy-2,3,6-trimethylphenyl)sulfonyl; HOSu, *N*-hydroxysuccinimide; DCC, 1,3-dicyclohexylcarbodiimine; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

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