6.24 (dd, 1 H, $J$ = 4.5 Hz, $J$ = 1.5 Hz), 7.08 (dd, 1 H, $J$ = 4.5 Hz, $J$ = 4.5 Hz), 7.95 (d, 1 H, $J$ = 6.0 Hz), 8.19 (d, 1 H, $J$ = 12.0 Hz), 8.77 (s, 1 H); mass spectrum, $m/z$ 316 $(M + H)^+$; IR (KBr) 3420 (OH), 1720 (C=O) $cm^{-1}$.

**In Vitro Antibacterial Activity.** The in vitro antibacterial activity of the new compounds was tested in a side-by-side comparison with ciprofloxacin (1e) and determined by conventional agar dilution procedures. The organisms were grown overnight in brain-heart infusion (BHI) broth (Difco 0037-01-6) at 36 °C. Twofold dilutions of the stock solution (2000 μg/mL) of the test compound were made in BHI agar to obtain the test concentration ranging from 200 to 0.005 μg/mL. The plate was inoculated with approximately $10^4$ organisms. It was then incubated at 36 °C for 18 h. The minimum inhibitory concentrations (MIC, μg/mL) were the lowest concentrations of the test compounds that yielded no visible growth on the plate.

**Acknowledgment.** We thank Dwight Hardy and the staff of the Microbiological Team for their in vitro testing and Cyndy Davis for typing the manuscript.

# Analogues of 1,5-Bis(4-amidinophenoxy)pentane (Pentamidine) in the Treatment of Experimental *Pneumocystis carinii* Pneumonia

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A series of 33 analogues of the anti-*Pneumocystis carinii* drug 1,5-bis(4-amidinophenoxy)pentane (pentamidine) was synthesized for screening against a rat model of *P. carinii* pneumonia (PCP). Twenty-five of the compounds showed efficacy against PCP when compared to a saline-treated control group. Two compounds, 1,4-bis(4-amidinophenoxy)butane (butamidine, 6) and 1,3-bis(4-amidino-2-methoxyphenoxy)propane (DAMP, 16), were statistically more effective than the parent drug in treating PCP in the rat model of infection. In addition to their activity against PCP, the compounds were also evaluated for antitrypsin activity, ability to inhibit thymidylate synthetase, affinity for DNA, and toxicity. No correlation was observed between the tested molecular interactions of the diamidines and their effectiveness against PCP.

An aromatic diamidino compound, pentamidine, was discovered as early as 1957 to be an effective agent for the treatment of *P. carinii* pneumonia (PCP).[1] Since then the drug has seen continued use for the treatment of PCP despite an extensive list of adverse reactions that include nephrotoxicity, hepatotoxicity, hypotension, and sterile abscesses at the injection site.[2,3] However, pentamidine was a distant second to the relatively nontoxic diaminopyrimidinesulfonamide combinations for the treatment of PCP.[4] This preference changed drastically with the clinical upsurge of cases of PCP caused by the acquired immune deficiency syndrome (AIDS) and the observation that trimethoprim-sulfamethoxazole caused a high frequency of adverse reactions in patients with AIDS-related PCP.[5,6] This unfortunate circumstance, combined with the finding that PCP is the leading cause of morbidity and mortality in AIDS patients,[7,8] has caused an increased dependency on the use of pentamidine in treatment of AIDS-related PCP. Recent studies have shown that the toxicity of pentamidine can be greatly reduced and drug efficacy increased by aerosol administration.[9,10] Despite these findings there is still an urgent need for a safe and effective drug that can be given either by oral or by parenteral administration for treatment of PCP associated with AIDS.

There is published record of only a handful of pentamidine-related compounds as having been tested against PCP. The screening of large numbers of drugs against PCP has been limited due to the lack of a dependable in vitro assay system. Therefore, the evaluation of anti-*P. carinii* drugs has depended on a somewhat cumbersome and expensive model utilizing immunosuppressed animals. The model involves the administration of corticosteroids to rats for a period of 6-8 weeks, resulting in the spontaneous induction of PCP.[11-14] A number of drug studies have shown that the rat model of PCP is an effective predictor of drug efficacy in humans.[11,12] An early report demonstrated that a diamidine derivative, hydroxystilbamidine, showed some activity against *P. carinii* in the rat model of the disease.[11] Two recent studies demonstrated that several dicationic molecules with structures related

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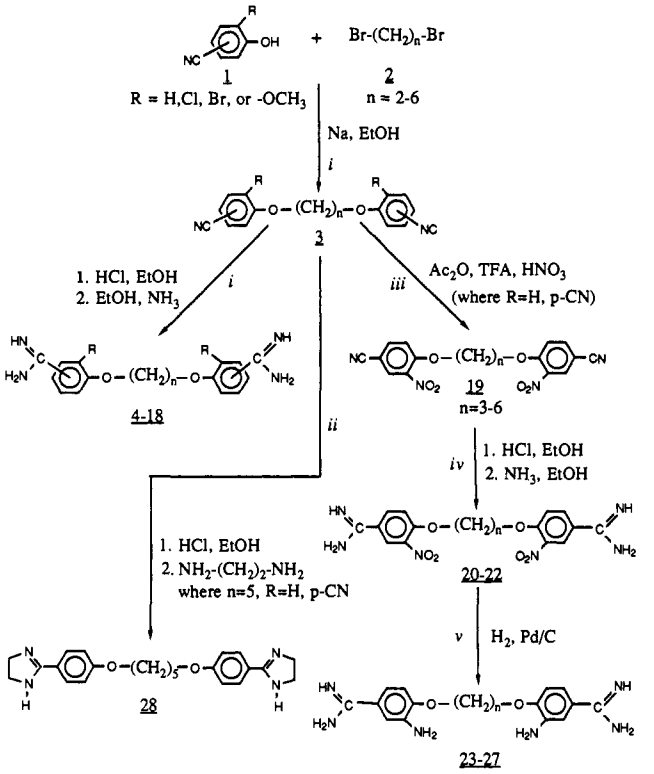
**Scheme I**

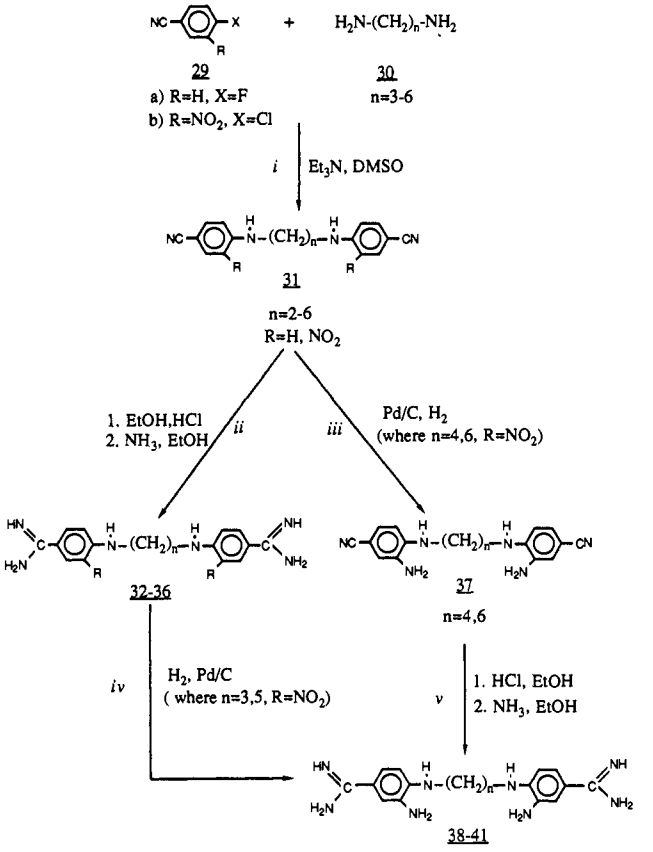



to pentamidine also possessed activity against rat PCP.[12,13] These studies, while showing promise for pentamidine type compounds against PCP, added few data to determine the structure–activity correlations or the mechanism of activity of this class of compounds against *P. carinii*.

This paper describes the synthesis and the anti-*P. carinii* activity of 33 analogues of pentamidine against the rat model of the disease. Also, we have determined the anti-protease activity, thymidylate synthetase inhibitory activity, and the DNA-binding ability of selected analogues to look for correlations between these activities and anti-*P. carinii* potency.

**Chemistry.** For the present study, all of the compounds were synthesized in our laboratory. The syntheses of compounds **5–12**, **14**, and **15** have been previously reported, and they were prepared according to the established route as outlined in Scheme I.[15,16] While the activity of compound **28** against *Trypanosoma rhodesiense* and *Plasmodium berghei* has been reported,[17] the synthesis has been detailed only in a final report in an Army contract.[18] The compound was synthesized by replacing ammonia with diaminoethane according to Scheme I, route ii. The dinitro analogues of pentamidine (**20–22**) were prepared by dinitration (Scheme I, route iii) of the corresponding dinitriles (**3**) followed by conversion of nitrile derivatives **19** to the respective amidines (Scheme I, route iv). Diamino derivatives **23–27** were prepared from the corresponding dinitro compounds **20–22** by hydrogenation with Pd/C (Scheme I, route v). The synthesis of $\alpha,\omega$-bis(4-amidinoanilino)alkanes **32–34** was accomplished by nucleophilic displacement reaction (Scheme II, route i) of 4-fluorobenzonitrile (**29a**) with the appropriate diaminoalkane (**30**) to give the corresponding dibenzonitrile derivative **31**. The nitriles were converted to amidines in the usual manner (Scheme II, route ii) to give the desired products **32–34**. Similarly, 4-chloro-3-nitrobenzonitrile (**29b**) was used as the starting reagent to give the desired $\alpha,\omega$-bis(4-amidino-2-nitroanilino)alkanes **35** and **36**. 1,3-Bis(4-amidino-2-aminoanilino)propane (**38**) and 1,5-bis(4-amidino-2-aminoanilino)pentane (**40**) were prepared by reduction (Scheme II, route iv) of the corresponding nitro derivatives **35** and **36** using Pd/C, while the four- and six-carbon analogues **39** and **41** were synthesized by first reducing (Scheme II, route iii) the desired $\alpha,\omega$-bis(4-cyano-2-nitroanilino)alkane **31** to the corresponding amine **37**. The nitriles were then converted to the amidine products (Scheme II, route v). Since the intermediate 1,2-bis(4-cyanoanilino)ethane was insoluble in any appropriate solvent, the synthesis of 1,2-bis(4-amidino-2-aminoanilino)ethane (**45**) was prepared according to the steps outlined in Scheme III. 4-Chloro-3-nitrobenzonitrile (**29**) was reacted with a 10-fold excess of ethylenediamine (**30**) at 25 °C (Scheme III, route i) to give monosubstituted product **42**. The nitro group was reduced, and the product was reacted with a second mole of **29** (Scheme III, route ii) to give 1-(2-amino-4-cyanoanilino)-2-(4-cyano-2-nitroanilino)ethane. The diamidine was prepared, followed by reduction of the second nitro group (Scheme III, route iii) to give **44**.

**Scheme II**




## Results and Discussion

Thirty-one analogues of pentamidine (Table I) were synthesized and screened for their ability to reduce the

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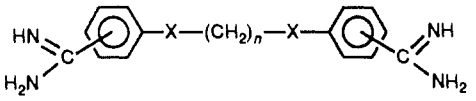
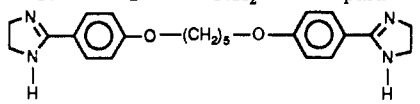
Table I. Structure and Chemical Data of Pentamidine Analogues



| no. | X | *n* | R | am | mp, °C | formula[a] |
|---|---|---|---|---|---|---|
| 4 | O | 2 | H | para | >300 | $C_{16}H_{18}N_4O_2 \cdot 2HCl \cdot 1.1H_2O$ |
| 5 (propamidine) | O | 3 | H | para | 144–145 dec | $C_{17}H_{20}N_4O_2 \cdot 2HCl \cdot 1.3H_2O$ |
| 6 (butamidine) | O | 4 | H | para | 267–270 | $C_{18}H_{22}N_4O_2 \cdot 2HCl \cdot 0.4H_2O$ |
| 7 (pentamidine) | O | 5 | H | para | 247 | $C_{19}H_{24}N_4O_2 \cdot 2HCl \cdot H_2O$ |
| 8 | O | 6 | H | para | 252 | $C_{20}H_{26}N_4O_2 \cdot 2HCl \cdot 2.5H_2O$ |
| 9 | O | 3 | H | meta | 300 | $C_{17}H_{20}N_4O_2 \cdot 2HCl \cdot 1.9H_2O$ |
| 10 | O | 4 | H | meta | 257 | $C_{18}H_{22}N_2O_2 \cdot 2HCl \cdot 1.8H_2O$ |
| 11 | O | 5 | H | meta | 133–134 | $C_{19}H_{24}N_4O_2 \cdot 2HCl$ |
| 12 | O | 6 | H | meta | 268 | $C_{20}H_{26}N_4O_2 \cdot 2HCl \cdot 0.3H_2O$ |
| 13 | O | 4 | Cl | para | >300 | $C_{18}H_{20}N_4O_2Cl_2 \cdot 2HCl$ |
| 14 | O | 5 | Cl | para | 247–248 | $C_{19}H_{22}N_4O_2Cl_2 \cdot 2HCl \cdot 1.1H_2O$ |
| 15 | O | 5 | Br | para | 254–255 | $C_{19}H_{22}N_4O_2Br_2 \cdot 2HCl \cdot H_2O$ |
| 16 (DAMP) | O | 3 | $OCH_3$ | para | 293 | $C_{19}H_{24}N_4O_4 \cdot 2HCl$ |
| 17 | O | 4 | $OCH_3$ | para | 297–299 | $C_{20}H_{26}N_4O_4 \cdot 2HCl \cdot 1.2H_2O$ |
| 18 | O | 5 | $OCH_3$ | para | 258–259 | $C_{21}H_{28}N_4O_4 \cdot 2HCl \cdot 2H_2O$ |
| 20 | O | 4 | $NO_2$ | para | 298 | $C_{18}H_{20}N_6O_6 \cdot 2HCl$ |
| 21 | O | 5 | $NO_2$ | para | 255 | $C_{19}H_{22}N_6O_6 \cdot 2HCl$ |
| 22 | O | 6 | $NO_2$ | para | 287–288 dec | $C_{20}H_{24}N_6O_6 \cdot 2HCl$ |
| 23 | O | 2 | $NH_2$ | para | >300 | $C_{16}H_{20}N_6O_2 \cdot 4HCl \cdot 0.4H_2O$ |
| 24 | O | 3 | $NH_2$ | para | 273 dec | $C_{17}H_{22}N_6O_2 \cdot 4HCl \cdot 1.9H_2O \cdot 0.6EtOH$ |
| 25 | O | 4 | $NH_2$ | para | 285 | $C_{18}H_{24}N_6O_2 \cdot 4HCl \cdot H_2O$ |
| 26 | O | 5 | $NH_2$ | para | 270 | $C_{19}H_{26}N_6O_2 \cdot 4HCl \cdot 0.6H_2O$ |
| 27 | O | 6 | $NH_2$ | para | 290 dec | $C_{20}H_{28}N_6O_2 \cdot 4HCl \cdot 0.5H_2O \cdot EtOH$ |
| 32 | N | 3 | H | para | 298–300 | $C_{17}H_{22}N_6 \cdot 2HCl$ |
| 33 | N | 4 | H | para | >300 | $C_{18}H_{24}N_6 \cdot 2HCl \cdot 0.5H_2O$ |
| 34 | N | 5 | H | para | 295 | $C_{19}H_{26}N_6 \cdot 2HCl \cdot 1.3H_2O$ |
| 35 | N | 3 | $NO_2$ | para | 295 dec | $C_{17}H_{20}N_8O_4 \cdot 2HCl \cdot H_2O$ |
| 36 | N | 5 | $NO_2$ | para | 293–294 | $C_{19}H_{24}N_8O_4 \cdot 2HCl \cdot 2H_2O \cdot 0.2EtOH$ |
| 38 | N | 3 | $NH_2$ | para | 229 | $C_{17}H_{24}N_8 \cdot 4HCl \cdot 1.1H_2O \cdot 0.3EtOH$ |
| 39 | N | 4 | $NH_2$ | para | 274–275 dec | $C_{18}H_{26}N_8 \cdot 4HCl$ |
| 40 | N | 5 | $NH_2$ | para | 300–303 | $C_{19}H_{28}N_8 \cdot 4HCl \cdot 2H_2O$ |
| 41 | N | 6 | $NH_2$ | para | 285 dec | $C_{20}H_{30}N_8 \cdot 4HCl \cdot 1.7H_2O$ |
| 44 | N | 2 | $NH_2$ | para | >300 | $C_{16}H_{22}N_8 \cdot 4HCl \cdot 1.6H_2O \cdot 0.4EtOH$ |
| 28 |  | | | | 147 | $C_{23}H_{28}N_4O_2 \cdot 2HCl \cdot 2.2H_2O$ |

[a] Elemental analysis (C, H, N) within ±0.4% of the theoretical value.

severity of PCP in immunosuppressed rats as measured by histologic scoring of Grocott's methenamine silver (GMS) stained lung sections. Animals treated with the test compounds were compared to those treated with the parent drug (pentamidine) and a saline-treated control group. A typical experiment included six groups of animals (eight animals/group) consisting of four test groups, a pentamidine control group, and a saline control group. Since no statistical variation was observed in the histological lung scores of either control group from experiment to experiment, the test results were combined, and the results are reported in Table II. Each compound was tested at 10 mg/kg per day by iv injection unless low solubility or high toxicity necessitated a reduced test dose.

Twenty-five of the compounds tested (compounds 4 and 15 could not be tested due to insolubilities) produced a statistically significant reduction in lung PCP (regardless of the test dose). Only one compound (**44**) was inactive when tested at the optimal dose level of 10 mg/kg. The other compounds (**12, 13, 20,** and **35**) not exhibiting activity against PCP were tested at lower doses due to the lack of solubility. While 15 compounds proved statistically equal to pentamidine in reducing the extent of PCP, only two analogues (6 and 16) produced a mean histologic score that was significantly lower than that of the parent drug. The activity observed for 1,3-bis(4-amidino-2-methoxyphenoxy)propane (16) was especially noteworthy because this analogue produced a mean histologic score that was significantly lower than pentamidine at one-half the dosage level of the parent drug. Two other methoxy-substituted analogues (17 and 18) also showed good activity against PCP; however, compound 18 proved to be highly toxic at the standard dose of 10 mg/kg. Substitution of the amidino groups of pentamidine by imidazoline moieties gave a compound (**28**) that was equally as potent as pentamidine and caused no toxic side effects. The effectiveness of the imidazoline moiety in experimental PCP has been previously reported for imidocarb.[12] Dianilino analogues **32–36, 38–41,** and **44** were not appreciably more effective than the respective diphenoxy derivatives. However, several compounds in this series proved to be highly toxic. Likewise, substitution of the aromatic rings with nitro groups (compounds **20–22, 35,** and **36**) produced compounds that were highly toxic relative to the unsubstituted analogues. Amino substitution of the aromatic rings (compounds **23–27**) caused no significant change in activity or toxicity. Halogen substitution (compounds **13–15**) produced molecules of very low solubility that exhibited no activity against PCP at the highest soluble concentration.

Since little is known about the mechanism of action of pentamidine against PCP, we investigated the possibility that a correlation existed between one of the reported molecular interactions of amidine type molecules and their

Table II. Extent of Disease by Histologic Score[a]

| no.[b] | n | mean score | no. of animals per scoring group 0.5 | 1 | 2 | 3 | 4 | toxicity |
|---|---|---|---|---|---|---|---|---|
| saline | 72 | 3.2 | 1 | 2 | 10 | 25 | 34 | 0 |
| 4 | | not tested in animals—insoluble | | | | | | |
| 5 | 8 | 0.9[c] | 3 | 4 | 1 | 0 | 0 | + |
| 6 | 8 | 0.5[c,d] | 8 | 0 | 0 | 0 | 0 | 0 |
| 7 | 63 | 1.1[c] | 21 | 28 | 12 | 2 | 0 | ++ |
| 8 | 7 | 0.9[c] | 3 | 3 | 1 | 0 | 0 | +++ |
| 9 | 8 | 1.6[c,e] | 2 | 1 | 4 | 1 | 0 | 0 |
| 10 | 7 | 1.9[c,d] | 2 | 1 | 1 | 3 | 0 | 0 |
| 11 | 16 | 1.7[c,d] | 4 | 3 | 6 | 2 | 1 | + |
| 12 | 8 | 1.6[c] | 1 | 4 | 1 | 2 | 0 | + |
| 13[j] | 8 | 2.7[g] | 0 | 1 | 2 | 3 | 2 | 0 |
| 14[i] | 8 | 3.5[g] | 0 | 0 | 0 | 4 | 4 | 0 |
| 15 | | not tested in animals—insoluble | | | | | | |
| 16[h] | 8 | 0.6[c,e] | 7 | 1 | 0 | 0 | 0 | 0 |
| 17[h] | 8 | 0.9[c] | 3 | 4 | 1 | 0 | 0 | 0 |
| 18 | 14 | 1.6[c,d] | 2 | 4 | 7 | 1 | 0 | ++++ |
| 20[i] | 8 | 3.1[g] | 0 | 0 | 3 | 1 | 4 | ++++ |
| 21[i] | 8 | 2.0[c,g] | 0 | 2 | 4 | 2 | 0 | +++ |
| 22[j] | 6 | 0.8[c] | 4 | 1 | 1 | 0 | 0 | ++++ |
| 23 | 8 | 0.7[c] | 4 | 4 | 0 | 0 | 0 | 0 |
| 24 | 7 | 1.0[c] | 2 | 4 | 1 | 0 | 0 | 0 |
| 25 | 8 | 1.0[c] | 2 | 5 | 1 | 0 | 0 | 0 |
| 26 | 15 | 0.9[c] | 8 | 5 | 1 | 1 | 0 | + |
| 27[h] | 8 | 2.2[f,g] | 0 | 1 | 4 | 3 | 0 | +++ |
| 28 | 8 | 0.7[c] | 5 | 3 | 0 | 0 | 0 | 0 |
| 32 | 8 | 0.9[c] | 5 | 1 | 2 | 0 | 0 | + |
| 33 | 4 | 0.6[c] | 3 | 1 | 0 | 0 | 0 | ++++ |
| 34 | 7 | 1.6[c,e] | 1 | 2 | 3 | 1 | 0 | +++ |
| 35[h] | 8 | 3.7[g] | 0 | 0 | 0 | 2 | 6 | ++ |
| 36[h] | 7 | 1.9[c,d] | 1 | 3 | 0 | 2 | 1 | +++ |
| 38 | 7 | 1.2[c] | 1 | 4 | 2 | 0 | 0 | + |
| 39 | 8 | 1.6[c,e] | 0 | 4 | 3 | 1 | 0 | + |
| 40 | 8 | 1.2[c] | 3 | 2 | 3 | 0 | 0 | + |
| 41 | 7 | 1.5[c,e] | 3 | 1 | 2 | 0 | 1 | + |
| 44 | 8 | 2.6[g] | 0 | 0 | 4 | 3 | 1 | + |

[a] Histologic scoring: 0.5 = <10 cysts found per two sections; 1 = scattered cysts, <10% of lung involved; 2 = scattered cysts, 10–25% lung involved or small foci of infection; 3 = scattered cysts, 25–50% of lung involved with some intense areas of infection; 4 = >50% of lung involved with many intense areas of focal infection. [b] All compounds were tested at 10 mg/kg unless otherwise indicated. [c] $P$ <0.001 when compared to saline controls. [d] $P$ <0.01 when compared to pentamidine. [e] $P$ <0.05 when compared to pentamidine. [f] $P$ <0.01 when compared to saline controls. [g] $P$ <0.001 when compared to pentamidine. [h] Tested at 5 mg/kg. [i] Tested at 2.5 mg/kg. [j] Tested at 1.25 mg/kg.

activity against PCP. Specifically, it has been shown that aromatic amidines can interfere with folic acid synthesis,[22] bind to RNA and DNA,[23] suppress RNA polymerase,[24] and are potent inhibitors of trypsin-like proteases.[15,16] Attempts to correlate certain in vitro activities of amidine analogues with their in vivo action against PCP are limited since the in vivo activity will be influenced by the dosing regimen and the metabolism and pharmacokinetics of the diamidines. However, it was felt that testing a wide variety of pentamidine analogues against PCP and comparing these results with the compounds' DNA binding affinity and enzyme (trypsin and thymidylate synthetase, TS) inhibitory potency could generate data that would, at least, suggest or eliminate a mechanism by which these compounds exert their anti-PCP effect.

Seventeen of the compounds were examined for their inhibition of both human (myeloid cells) and bacterial (*Lactobacillus casei*) thymidylate synthetase. The results

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Scheme III

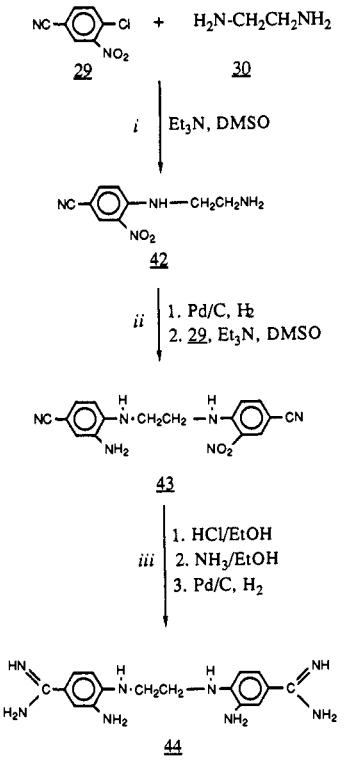


Table III. DNA Binding and Inhibition of Trypsin by Pentamidine Analogues

| no. | DNA binding:[a] $\Delta T_m$, °C | trypsin inhibition:[b] $K_i$, μM | no. | DNA binding:[a] $\Delta T_m$, °C | trypsin inhibition:[b] $K_i$, μM |
|---|---|---|---|---|---|
| 4 | 5.4 | 7.3 | 22 | NT[c] | NT[c] |
| 5 | 14.7 | 3.3 | 23 | 8.8 | 5.0 |
| 6 | 8.3 | 1.5 | 24 | 13.6 | 5.8 |
| 7 | 10.7 | 2.3 | 25 | 9.6 | 9.1 |
| 8 | 9.1 | 2.7 | 26 | 10.8 | 1.8 |
| 9 | 7.6 | 1.1 | 27 | 9.5 | 293.0 |
| 10 | 8.1 | 1.8 | 28 | 10.5 | >2000.0 |
| 11 | 6.8 | 3.8 | 32 | 11.6 | 1.1 |
| 12 | 7.3 | 4.0 | 33 | 10.3 | 1.6 |
| 13 | 8.9 | NT[d] | 34 | 11.3 | 1.4 |
| 14 | 10.5 | NT[d] | 35 | 12.8 | 4.3 |
| 15 | NT[e] | NT[e] | 36 | 12.7 | 8.0 |
| 16 | 16.3 | 1.3 | 38 | 11.0 | 10.3 |
| 17 | 10.2 | NT[d] | 39 | 10.5 | 11.1 |
| 18 | 12.0 | 1.3 | 40 | 11.0 | 4.8 |
| 20 | 8.5 | 5.4 | 41 | 8.3 | 5.3 |
| 21 | 9.7 | 1.0 | 44 | 9.1 | 12.7 |

[a] From ref 20. [b] From ref 15. [c] Not tested, insufficient amount of compound available. [d] Not tested. [e] Not tested, insoluble.

indicated that the compounds inhibited the enzymes only at high concentrations ($\sim 10^{-3}$ M). Furthermore, no structure–activity correlations or preference for either the human or bacterial enzyme were observed.

All of the compounds were found to bind to calf thymus DNA with $\Delta T_m$s ranging from 5.4 to 16.3 °C (Table III). One of the most effective compounds (**16**) against PCP proved to be the strongest DNA binding agent. However, the only other correlation that could be made about activity against PCP and DNA binding was that all of the molecules bound to DNA, and most of the analogues exhibited at least minimal activity against PCP.

The $K_i$ values for 25 compounds were determined against trypsin. All but two of the compounds were found to have potent activity against trypsin with $K_i$ values ranging from 1.1 to 12.7 μM. Only compound **28** had no detectable anti-trypsin activity. However, this compound

was found to be an effective agent in the treatment of PCP. This finding provides strong evidence that the antiprotease activity of diamidines is not germane to their potency against PCP.

In conclusion, the current studies clearly show that several pentamidine analogues are equally as effective as pentamidine in controlling PCP in the rat model of infection. Furthermore, two of the analogues were significantly more potent than pentamidine against PCP, and several compounds appeared less toxic than the parent drug. The study failed to determine the mechanism of action of the molecules against PCP, but did provide strong evidence to rule out the inhibition of proteases or TS as the mechanism of action. Further studies directed at the molecular biology of the organism and the pharmacokinetics and metabolic characteristics of pentamidine analogues will lead to a better understanding of how pentamidine-related compounds exert their anti-PCP effect. This, in turn, will allow for a more systematic design of future pentamidine analogues.

## Experimental Section

**Chemistry.** Melting points or decomposition points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. The $^1H$ NMR spectra were recorded on a Varian EM-390 90-MHz spectrometer. Infrared spectra were obtained on a Perkin-Elmer Model 1320 spectrophotometer. Because of their lack of unusual features, the IR and NMR spectral data were not included; however, they were obtained for all compounds and were consistent with the assigned structures. TLC analysis was performed on precoated TLC sheets (silica gel 60A F-254) from Whatman. HPLC analysis of the target compounds was performed on a Hewlett-Packard Model 1084B liquid chromatograph equipped with a Model 79841 A autoinjector and a Model 79850B reporting integrator. A Zorbax ODS 250 X 4.6 mm reverse-phase column with 5-$\mu$m particle size from Du Pont was used. The compounds were detected by a Hewlett-Packard Model 79875A variable-wavelength ultraviolet spectrophotometer. The method of the HPLC assay has been previously detailed.[19] Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Where analyses are indicated on the final products, results within ±0.40 of the theoretical value were considered acceptable. Inclusion of EtOH or $H_2O$ in the formula was confirmed by $^1H$ NMR spectra. Starting materials and reagents were purchased from Aldrich, Milwaukee, WI; Fisher Scientific, Atlanta, GA; and Fluka, Ronkonkoma, NY.

**General Procedure for the Synthesis of Compounds 4–18 (Scheme I, Route i).** The procedure adopted for the synthesis of **5** is described.

Small pieces of Na (1.2 g, 0.052 mol) were added slowly to a stirred solution of 25 mL of dry EtOH. After complete dissolution of the Na, a solution of 4-cyanophenol (**1**, 6 g, 0.050 mol) in 25 mL of dry EtOH was added to the stirred mixture followed by the dropwise addition of 1,3-dibromopropane (**2**, 5.1 g, 0.025 mol). The reaction mixture was stirred at reflux until TLC showed the reaction to be complete (2 days) and then it was cooled. The resulting precipitate was filtered, washed with copious amounts of water, and dried under vacuum to give 5.7 g (82%) of 1,3-bis(4-cyanophenoxy)propane (**3**) as a white solid. The product was used in the next step without further purification.

A mixture of 1,3-bis(4-cyanophenoxy)propane (3.5 g, 0.0126 mol) and 20 mL of dry EtOH in 377 mL of dry benzene was cooled to 0–5 °C and saturated with HCl gas. The stirring mixture was sealed and allowed to warm to room temperature. After 3 days an IR spectrum of an aliquot determined that all of the starting nitrile was converted to the imidate. The mixture was allowed to cool to room temperature, and 200 mL of ether was added. The resulting precipitate was filtered, washed with dry ether, and dried under nitrogen. The solid was dissolved in 252 mL of dry EtOH and 252 mL of ethanolic ammonia and heated to 50 °C for 6 h. The volume of the mixture was reduced by one-half, and ether was added to precipitate the product as a white solid. The solid was recrystallized once from 2 N HCl to give 4.2 g (82%) of **5** as fluffy, white crystals.

**Synthesis of 1,5-Bis(4-imidazolinophenoxy)pentane (28, Scheme I, Route ii).** The desired dinitrile **3** was prepared according to the preceding method.

A solution of 5.5 g (0.018 mol) of 1,5-bis(4-cyanophenoxy)-pentane in 539 mL of dry benzene and 29 mL of EtOH was saturated with HCl gas. The bis-imidate was isolated as described in the previous reaction and reacted for 3 h in 200 mL of refluxing MeOH with 4 g of ethylenediamine. The solvent was removed by evaporation and the residue recrystallized from 2 N HCl to give 7.4 g (81%) of **28**.

**General Procedure for the Synthesis of 20–22 (Scheme I, Routes iii and iv).** The procedure for the synthesis of **22** is described.

The desired dinitrile **3** was prepared according to the previously described method. To a solution of 1,6-bis(4-cyanophenoxy)-hexane (2.5 g, 0.0078 mol) in 30 mL of TFA was added dropwise a solution of 4.2 mL of 70% $HNO_3$ in 7 mL of $Ac_2O$. The mixture was heated at 45 °C for 24 h and poured into 500 mL of $H_2O$ and ice. The precipitate was filtered, dried, and recrystallized from TFA to give 2.7 g (84%) of dried dinitro product **19**.

A mixture of 2.6 g (0.006 mol) of dinitrile **19** ($n$ = 6), 300 mL of $CHCl_3$, and 150 mL of MeOH was cooled and saturated with HCl gas and reacted for 24 h as previously described. The diimidate was isolated by precipitation with ether and reacted at 40–60 °C with 150 mL of ethanolic ammonia and 150 mL of dry ethanol. The product was isolated by ether precipitation and filtration followed by recrystallization from 2 N HCl to give 1.1 g (35%) of **22**.

**General Procedure for the Synthesis of 23–27 (Scheme I, Route v).** The procedure adopted for the synthesis of **25** is described.

A mixture of 1.2 g (0.0024 mol) of **20** ($n$ = 4), 280 mg of 10% Pd/C, 56 mL of 95% EtOH, and 0.84 mL of 10 N HCl was hydrogenated at room temperature and 60 psi on a Parr hydrogenator for 6 h. The catalyst was filtered and washed with $H_2O$ and the combined filtrates were evaporated under vacuo. The residue was recrystallized from 2 N HCl to give 0.9 g (70.6%) of **25**.

**General Procedure for the Synthesis of 32–36 (Scheme II, Routes i and ii).** The procedure adopted for the synthesis of **33** and **35** is described.

**Compound 33.** A mixture of 4-fluorobenzonitrile (**29**, 11 g, 0.090 mol), 1,4-diaminobutane (**30**, 2 g, 0.023 mol), 14 mL of triethylamine, and 58 mL of dry DMSO was heated at 150 °C with stirring for 3 h. The mixture was poured into 1 L of iced water and the precipitate was collected by filtration. The solid was recrystallized from DMSO/$H_2O$ (8:1), filtered, and washed with water to give 3.2 g (48%) of dried product **31** ($n$ = 4, R = H).

A solution of 3.2 g (0.011 mol) of dinitrile **31** ($n$ = 4, R = H), in 300 mL of dry dioxane and 130 mL of dry MeOH was saturated with HCl gas. The sealed mixture was allowed to stir at room temperature until the IR indicated the absence of the dinitrile. The reaction required 21 days for completion during which time a second saturation with HCl gas was performed. The diimidate was converted to the corresponding amidine in the usual manner with EtOH (84 mL) and ethanolic ammonia (84 mL). The product was filtered, washed with ether, and recrystallized from $H_2O$ to give 1.9 g (43%) of **33**.

**Compound 35.** $p$-Chlorobenzonitrile (25 g, 0.18 mol) was added slowly to a cooled (0 °C) mixture of 37 g (0.36 mol) of $KNO_3$ and 150 mL of $H_2SO_4$. The mixture was stirred for 3 h at 3–5 °C and poured into 500 mL of iced water, and the precipitate was filtered. The solid was washed with water until neutral and recrystallized from EtOH/$H_2O$ (1:1) to give 30 g (88%) of **29a**.

A mixture of 5.5 g (0.03 mol) of 4-chloro-3-nitrobenzonitrile (**29a**), 1.1 g (0.015 mol) of diaminopropane, 10 mL of triethylamine, and 50 mL of DMSO was heated (120 °C) with stirring for 3 h. A solid was collected by filtration, washed with water, and recrystallized from DMSO/$H_2O$ (2:1) to give 4.5 g (41%) of **31** (R = $NO_2$, $n$ = 3).

A solution of 4.5 g of **31** (R = $NO_2$, $n$ = 3), 350 mL of dry dioxane, 400 mL of EtOAc, and 150 mL of dry MeOH was saturated with HCl gas, sealed, and allowed to stir for 7 days at room temperature. A solid was precipitated with ether, washed with ether, dried under $N_2$, and collected by filtration. The solid

material was dissolved in 200 mL of dry EtOH and 200 mL of ethanolic ammonia and heated to 60 °C for 24 h. After cooling, the product was precipitated with ether, filtered, and recrystallized from EtOH/HCl (5:1) to give 3.5 g (58%) of **35**.

**General Procedure for the Synthesis of 38 and 40 (Scheme II, Route iv).** The procedure adopted for the synthesis of **38** is described.

A mixture of 2.3 g (0.006 mol) of **35**, 1.0 g of Pd/C (10%), 1 mL of 10 N HCl, and 100 mL of 75% EtOH was hydrogenated for 3 h at 60 psi on a Parr hydrogenator. The catalyst was filtered, and the solvents were removed by evaporation. The residue was recrystallized from water to give 0.6 g (20%) of **38**.

**General Procedure for the Synthesis of 39 and 41 (Scheme II, Routes iii and v).** The procedure adopted for the synthesis of **39** is described.

A mixture of 3.0 g (0.008 mol) of **31**, 2 g of Pd/C (10%), 300 mL of TFA, and 25 mL of 10 N HCl was hydrogenated at 60 psi until 3 equiv of $H_2$ was consumed. The catalyst was removed by filtration, and the solvents were evaporated to give 2.4 g (94%) of **37** ($n = 4$). The product was used in the following reaction without further purification.

A mixture of 2.4 g of **37** ($n = 4$), 500 mL of dry dioxane, and 500 mL of dry MeOH was saturated with HCl gas at 5 °C, sealed and allowed to stir for 20 days at room temperature. The diimidate was precipitated with ether, collected by filtration under $N_2$, and dried under vacuum.

A solution of the diimidate in 200 mL of dry EtOH and 200 mL of ethanolic ammonia was heated and stirred at 60 °C for 3 days. The mixture was cooled and the product was collected by filtration. Recrystallization of the crude product from 2 N HCl gave 1 g (27%) of **39**.

**Procedure for the Synthesis of 44 (Scheme III).** A mixture of 10 g (0.054 mol) of **29**, 50 mL (0.45 mol) of ethylenediamine, 8 mL of triethylamine, and 25 mL of DMSO was stirred at room temperature for 30 h, poured over 50 mL of iced water, and filtered. The precipitate was washed with water (3 × 50 mL), ether, and *n*-hexane and recrystallized from EtOAc to give 12 g (98%) of **42**.

A suspension of **42** (6 g, 0.026 mol), 2 g of Pd/C (10%), and 250 mL of EtOH (absolute) was hydrogenated at 60 psi for 3 h. The catalyst was filtered, and the solvents were removed under vacuum to give a viscous oil. The oil was dissolved in EtOAc and filtered, and the filtrate was dried with $MgSO_4$. Filtration of the drying agent and removal of the solvent gave 3.5 g (69%) of the reduced product. The product was used in the following reaction without further purification.

A solution of 2.0 g (0.011 mol) of **29**, 2.9 g (0.016 mol) of the reduced product of **42**, 4 mL of ethylenediamine, and 15 mL of DMSO was stirred at room temperature for 5 h. The mixture was poured into 150 mL of iced water and filtered, and the solid was washed with water (3 × 150 mL). The crude product was recrystallized from water/DMSO (2:1) to give 3.1 g (87%) of **43**.

A mixture of 2.0 g (0.006 mol) of **43**, 150 mL of dioxane, and 70 mL of dry MeOH was saturated with HCl gas, sealed, and stirred at room temperature for 48 h. A solid was precipitated with ether, filtered under nitrogen, and washed with copious amounts of ether. The dry solid was dissolved in 150 mL of dry EtOH and ethanolic ammonia and stirred at 60 °C for 18 h. After cooling, a solid was collected by filtration and recrystallized from 2 N HCl to give 2.1 g of the diamidine product.

A mixture of 1.3 g (0.0028 mol) of the diamidine of **43**, 0.5 g of Pd/C (10%), and 400 mL of EtOH (50%) was hydrogenated at 60 psi for 6 h on a Parr hydrogenator. The catalyst was removed by filtration, 1 mL of 10% HCl was added to the filtrate, and the solvent removed by evaporation. The residue was recrystallized from water to give 0.4 g (27%) of **44**.

**Animal Protocol.** The induction and treatment of PCP was carried out with only minor alterations to published methods:[11,13] Male Sprague–Dawley rats, barrier raised, not certified virus-free, and weighing 150–200 g each, were obtained from Hilltop Laboratories (Scottsdale, PA). Immediately upon arrival the animals were caged individually and were begun on a low protein (8%) diet (ICN Biomedicals, Cincinnati, OH), and on drinking water containing tetracycline (0.5 mg/mL) and dexamethasone (1.0 μg/mL). This regimen was continued for the next 8 weeks. The fluid intake was monitored daily and the animals were weighed weekly. At the beginning of the sixth week, animals were divided into groups of eight or more, and the test compounds were administered for 14 days by single daily iv injection. Generally, the daily dose was 10 mg/kg of body weight and was dissolved in 0.4 mL of saline. Smaller doses were given in a few cases where the compounds were of low solubility or were toxic at the higher concentration. Saline- and pentamidine-treated groups were included as controls.

**Assessment of the Activity of the Drugs against PCP.** Animals were sacrificed at the end of the eighth week by chloroform inhalation. The right lung was inflated in situ with 10% formalin and fixed for histologic examination. The lung tissue was sectioned in the long axis and exposed to the GMS stain, which selectively identified the walls of the *P. carinii* cysts. Stained sections were coded and then scored blindly by two examiners. The scoring system was as follows: 0.5, less than 10 cysts found per 2 sections examined; 1, scattered cysts with less than 10% of lung tissue involved; 2, scattered cysts and a few focally intense areas of involvement amounting to 10–25% of lung parenchyma; 3, scattered cysts and numerous focal areas of dense involvement totaling 25–50% of lung tissue; 4, cysts found throughout the specimen and numerous very intense focal areas of involvement occupying greater than 50% of lung section.

**Evaluation of Toxicity.** Liver, spleen, and kidneys were examined for pathologic changes by light microscopy. The health and general well-being of the animals were observed and recorded on a daily basis. Toxicity of the test compounds was evaluated at 10 mg/kg or the highest dose tested by the following criteria: 0 = no local, clinical or histologic toxicity; 1+ = animals survived without observable distress but there was some local toxicity at the injection site; 2+ = most animals survived, some with severe distress and marked local toxicity, and some systemic toxicity and histopathologic abnormalities were also observed; 3+ = multiple and severe toxic effects, but death of less than 50% of the animals with single or multiple doses; 4+ = at least 50% of the animals died.

**DNA-Binding Affinity.** The DNA binding of pentamidine and its analogues was measured, at low ionic strength, by determining the change in midpoint ($\Delta T_m$) of the thermal denaturation curve of sonicated calf thymus DNA at a 1:10 drug to base ratio. The magnitude of the $\Delta T_m$ is approximately proportional to the binding constant of the compound under these conditions. The procedure used has been described in detail.[20]

**Trypsin Inhibition Activity.** The inhibition constants ($K_i$ values) for the compounds against trypsin were determined graphically from an amidase assay. All tests were carried out at 37 °C and at a pH of 8.1. Details of the assay have been previously described.[15]

**Thymidylate Synthetase Assay.** Inhibitory activities of the compounds against both human (myeloid cells) and bacterial (*L. casei*) enzymes were determined according to a published method.[21]

**Statistical Studies.** Student's *t* test was used to calculate the *p* values of each test group when compared to the saline-treated and pentamidine-treated groups. The statistical analysis was carried out with the Statview 512+ software package (Brainpower, Inc., Calabasas, CA) on a Macintosh II computer.

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