Synthesis of (Dialkylamino)alkyl-Disubstituted Pyrimido[5,6,1-*de*]acridines, a Novel Group of Anticancer Agents Active on a Multidrug Resistant Cell Line

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A series of pyrimidoacridine derivatives with two basic side chains, **7a–e**, was synthesized, as potential antitumor drugs, starting from 2-[2-(dimethylamino)ethyl]-6-chloropyrimido[5,6,1-*de*]acridine-1,3,7-trione (**6**) and a suitable (alkylamino)alkylamine. The products **6** and **7a–e** showed significant cytotoxic activity *in vitro* against L1210 leukemia. Compounds **7a,d** were 2 orders of magnitude more cytotoxic than ametantrone. All compounds were also examined for their activity on LoVo and resistant LoVo/Dx cell lines. Unlike ametantrone, the compounds have shown to be able to overcome the multidrug resistance. Compounds **7a,d**, the two most active *in vitro*, were tested *in vivo* against murine P388 leukemia showing good activity.

Introduction

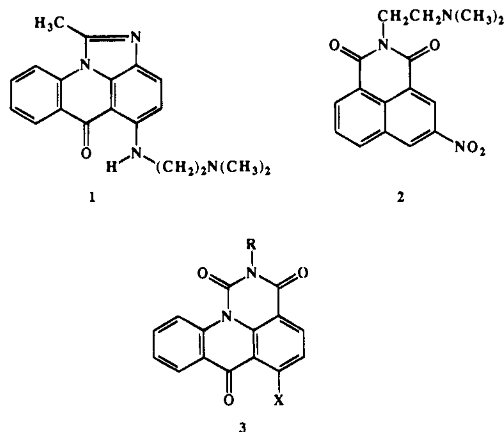
The noticeable results obtained in the field of antitumor drugs with the fully synthetic DNA-interacting agents of anthracenedione and acridine groups, such as mitoxantrone[1–5] and amsacrine[6,7] respectively, have raised in the last decade a great interest in the synthesis and structural modifications of these two classes of compounds. Anthrapyrazoles,[8,9] pyrazoloacridines,[10–12] benzothiopyranoindazoles,[13,14] acridinecarboxamides,[15,16] imidazoacridinones,[17,18] and triazoloacridinones[19] are the most representative examples of the research in this area, and some of them are currently undergoing clinical trials.

The main aim of the search for new structures within the mentioned groups was not only to develop novel synthetic compounds with antitumor activity but also to overcome two major undesirable properties of related anthracycline antibiotics: their cardiotoxicity (peroxidating activity) and the ability to induce resistance. Although the rationale for the design of nonperoxidating compounds has been recently proposed,[20–22] the clear concepts for the development of agents active on resistant tumor cells are still lacking. The synthesis of novel structural types of anthracenedione and acridine derivatives and analogs could be a way to find the desired leads.

All the above so far investigated compounds are characterized by the presence of a planar polycyclic chromophore, able to intercalate into DNA, and of, at least, one basic side chain which can increase DNA binding affinity and, in some cases, also solubility under physiological conditions.

A great effort has been devoted in the modification of the chromophore moiety and in determining its optimal characteristics. The results indicate that the planar portion of the molecule does not necessarily have to be a linear tricyclic system. Although some data indicate a positive relationship between DNA binding and antitumor activity,[23–25] it is normally accepted that DNA intercalation is a necessary, but not sufficient, condition for antitumor activity.[26] It has also been pointed out that DNA binding, while possibly improving intrinsic activity, can be even deleterious for distribution and penetration into the cells,[26,27] and on this basis, some classes of minimal DNA-intercalating agents have been obtained.[24,28] However, there are many aspects that must be considered for antitumor activity and among these the specificity of the interaction with DNA.[29] Some data have demonstrated that larger intercalators show more specific interactions.[30,31]

As a part of a research devoted to the investigation of new groups of antitumor-intercalating agents, recently we have reported[32,33] the synthesis and the antitumor activity of a series of *N*-(alkylamino)alkyl derivatives of pyrimido[5,6,1-*de*]acridine-1,3,7-triones **3** which can be seen as a combination in a unique structure of the acridone-type (**1**) and mitonafide[34] (**2**) chromophores. The biological activities of these compounds were not exciting, only one compound possessing borderline antitumor activity *in vivo*.



Since the presence of a second basic side chain could increase not only the binding to DNA, through additional interactions, but also decisively modify the physicochemical properties and solubility of these compounds, we decided to synthesize a new series of pyrimidoacridones bearing an additional [(alkylamino)-alkyl]amino side chain attached in position 6 of the chromophore.

Chemistry. In a similar way to that previously used,[33] the reaction of 1-chloro-9,10-dihydro-9-oxo-4-

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Scheme 1

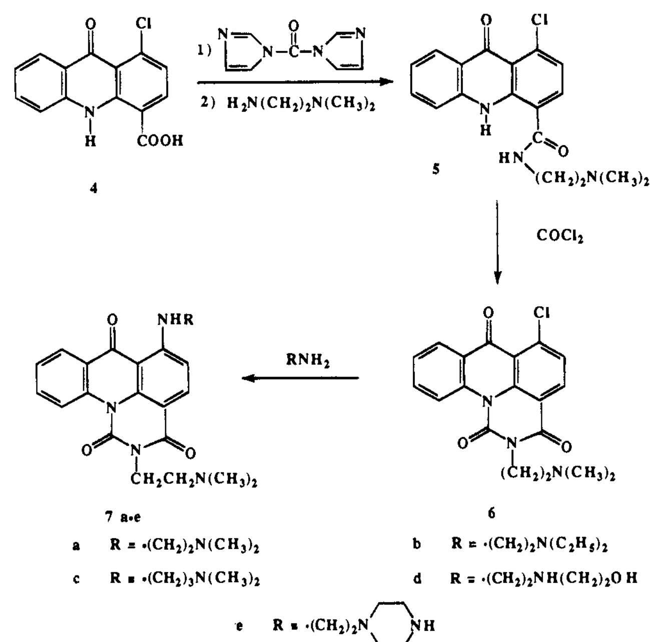

Table 1. *In Vitro* Cytotoxic Activity of Compounds **6** and **7a–e** in Comparison with Ametantrone (Am) on L1210 Murine Leukemia and Human Colon Adenocarcinoma Cell Lines Sensitive (LoVo) and Doxorubicin Resistant (LoVo/Dx)

	EC_{50}[a]			
compd	L1210	LoVo	LoVo/Dx	RI[b]
Am	0.35 ± 0.08	0.64 ± 0.13	36.8 ± 4.8	57.5
6	0.50 ± 0.06	1.2 ± 0.3	1.4 ± 0.3	1.2
7a	0.0022 ± 0.0006	0.022 ± 0.005	0.029 ± 0.003	1.3
7b	0.052 ± 0.01	0.052 ± 0.001	0.1 ± 0.02	1.9
7c	0.063 ± 0.01	0.19 ± 0.01	0.16 ± 0.01	0.8
7d	0.0045 ± 0.001	0.03 ± 0.01	0.23 ± 0.02	7.7
7e	0.21 ± 0.08	0.85 ± 0.04	1.0 ± 0.1	1.2

[a] Inhibiting concentration (μM) of 50% cellular growth ± SEM. [b] Resistance index: EC_{50} of LoVo/Dx cell line/EC_{50} of LoVo cell line. All compounds were tested as dihydrochlorides, except **6** which was monohydrochloride.

acridinecarboxylic acid (**4**),[35-37] with 1,1′-carbonyldiimidazole and *N,N*-dimethylethylenediamine, afforded *N*-[2-(dimethylamino)ethyl]-9,10-dihydro-1-chloro-9-oxo-4-acridinecarboxamide (**5**), which was cyclized with phosgene to the tetracyclic derivative **6**. From this, by substitution with a suitable (alkylamino)alkylamine, we obtained the disubstituted derivatives **7a–e** in satisfactory yields. In consideration of their low solubility in water, the compounds **6** and **7a–e** were converted into hydrochlorides by bubbling dry hydrogen chloride in a chloroform solution of the free bases. The structures were established on the basis of spectral data and elemental analysis.

Biological Results and Discussion

The results of cytotoxic activity of compounds **6** and **7a–e** against murine leukemia (L1210) and human colon adenocarcinoma sensitive (LoVo) and doxorubicin resistant (LoVo/Dx) cell lines are presented in Table 1. The data of the *in vivo* activity against murine P388 leukemia of compounds **7a,d** are shown in Table 2. All the derivatives were tested as hydrochlorides in order to overcome the low water solubility of free bases. Ametantrone was used as positive control in the *in vitro* testing.

Table 2. Antitumor Activity of Selected Compounds **7a,d** against P388 Murine Leukemia

compd	dose (mg/kg)	%T/C[a]	tox[b]
7a	0.19	166	0/6
	0.39	222	0/6
	0.78	222	0/6
	1.56	100	1/6
	3.12	70	6/6
7d	0.19	189	0/6
	0.39	244	0/6
	0.78	233	0/6
	1.56	167	0/6
	3.12	70	6/6

[a] %T/C = ratio of medium survival time of the treated to the control mice expressed as a percentage. %T/C ≥ 125 are considered indicative of significant activity. [b] Number of toxic deaths/total number of mice 7 days after the day 5 injection as measure of drug toxicity.

These studies are a part of our research program concerning the search for new structural types of synthetic anthracenedione and acridine analogs as lead compounds for the development of antitumor agents active on multidrug resistant tumors. The novel nonlinear tetracyclic pyrimidoacridone derivatives obtained fulfill these expectations.

The studied compounds exhibited some structure–activity relationships similar to those of known reference classical linear anthracenedione and acridine derivatives, but also some important differences have been noted.

The pyrimidoacridone derivatives showed high cytotoxic activity against L1210 leukemia; however, compounds **7a–e** with two basic side chains were much more active than compounds bearing only one chain such as 6-chloro derivative **6** and similar pyrimidoacridones previously reported.[33] This relation parallels the results reported for the classical linear cyclic compounds. The cytotoxic potency of **7a,d** was about 2 orders of magnitude higher than that of ametantrone. The similarities with linear compounds concerned also the side chain structures: two methylenes between the nitrogen atoms and not too bulky substituents at the terminal nitrogen (compounds **7a,d**) appeared to be optimal. Compound **7c**, bearing three methylene groups separating the nitrogen atoms, as well as compounds **7b,e**, with the terminal nitrogen atom substituted by bulky groups, were less active.

The essential novelty in biological behavior of the developed nonlinear compounds as compared to the linear reference ametantrone was their high activity against doxorubicin resistant LoVo/Dx cells, which constitute a multidrug resistant cell line.[38-40] As generally also in this case, sensitive LoVo cells are somewhat less susceptible than L1210 ones to the action of drugs. However, pyrimidoacridones **7a–d** still were much more active than ametantrone. But a drastic difference was noted with the resistant LoVo/Dx cells. The cytotoxicity of ametantrone against these cells dropped dramatically, while the susceptibility of LoVo/Dx to all compounds **6** and **7a–e** was practically unchanged as compared to normal LoVo cells, **7d** only having partial diminution, as clearly indicated by the resistance index (RI) values. This finding shows that this is a peculiarity of the examined compounds to overcome the multidrug resistance of the LoVo/Dx cell line, the higher RI value of **7d** being perhaps due to the hydrophilic character of the side chain in position 6.

Some parallelism can be noted between our results with pyrimidoacridones and those obtained by Krapcho et al. with azaametantrones.[38] The best results in overcoming the multidrug resistance of the LoVo/Dx cell line are obtained with tertiary amino side chains, particularly with a terminal (dimethylamino)ethyl residue and two methylenes between the nitrogen atoms, in strict correlation with compound **7a**, while one of the worse results is with the (hydroxyethyl)amino residue, in strict correlation with compound **7d**. The above data indicate that both side chain structures and the type of cyclic nucleus are of importance for the ability of the compounds to overcome the multidrug resistance. It seems that the pyrimidoacridone nucleus is favorable for this effect at least as much as the azaanthracenedione one.

Compounds **7a,d** were shown to exhibit also antileukemic activity *in vivo* against murine leukemia P388. The *in vivo* activity, with maximum $T/C = 244\%$ for **7d** and 222% for **7a**, much exceeds the value of 125%, considered indicative of significant activity.

It could be concluded that the developed nonlinear tetracyclic pyrimidoacridone derivatives constitute, on one side, a novel structural group of antitumor agents and, on the other side, a group of lead compounds that can overcome the multidrug resistance of the LoVo/Dx cell line.

At this stage of our investigation, the mechanism by which pyrimidoacridones act on the resistant LoVo/Dx cells is as yet unclear. However, due to the fact that the multidrug resistance of these cells is primarily due to the overexpression of P-glycoprotein,[40] it could be expected that the developed compounds are not substrates of this multidrug transporter. Anyway, further drug development modification studies, as well as fundamental studies aimed at the identification of structural factors and mechanisms by which pyrimidoacridones are active against multidrug resistant tumor cells, are underway.

Experimental Section

Melting points were determined on a Büchi 510 apparatus and are uncorrected. Thin layer chromatography (TLC) was accomplished on plates precoated with silica gel 60 F-254 (Merck). The IR spectra were recorded on a Perkin Elmer 297 spectrophotometer. The ^{1}H NMR spectra were recorded on a Varian VXR 300 instrument. Chemical shifts are reported as δ values (ppm) downfield from internal tetramethylsilane. The following NMR abbreviations are used, b (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), ar (aromatic proton), ex (exchangeable by addition of deuterium oxide), d ex (exchangeable by addition of deuterium oxide, but with difficulty). Quartets that are transformed into triplets by addition of deuterium oxide are labeled with an asterisk. All the elemental analyses were performed on a elemental analyzer (Model 1106) by Carlo Erba Strumentazione for C, H, and N and are within ±0.4% of the theoretical values.

Biological Studies. *In Vitro* Cytotoxic Evaluations: L1210 Murine Leukemia. Murine L1210 leukemia cells (RPMI) were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin G (100 000 units/L), and streptomycin (100 mg/L) in a controlled (air−5% CO_2) humidified atmosphere at 37 °C. The cells were transplanted every 2−3 days. The L1210 cells in logarithmic growth were suspended in the growth medium to give a final density of 0.05×10^6 cells/mL. The evaluation was performed as described previously for HeLa S_3 cells.[41] The cytotoxic activities (EC_{50} values) of the compounds were defined as their *in vitro* concentrations causing 50% inhibition of cell growth after 48 h exposure to the drug, as measured by the protein content of the cells. Each experiment was run at least five times, and results are presented as average values ± standard error of the mean (SEM).

Human Colon Adenocarcinoma LoVo and LoVo/Dx. Human colon adenocarcinoma sensitive cell line (LoVo) and doxorubicin resistant subline (LoVo/Dx) were kindly provided by Dr. Maria Grandi (Farmitalia Carlo Erba, Nerviano, Italy) through the courtesy of Dr. Giuliani. The cells were grown as a monolayer culture in Ham's F-12 medium supplemented with 10% fetal calf serum, penicillin G (100 000 units/L), and streptomycin (100 mg/L), 1% of a 200 mM L-glutamine solution, and 1% of a MEM vitamin solution 100X at 37 °C in a controlled humidifed atmosphere of 5% CO_2−95% air. The cells were transplanted as previously described.[39] The LoVo and LoVo/Dx cells in logarithmic growth were suspended in the growth medium to give a final density of 0.02×10^6 cells/mL. The evaluation was performed as described previously for HeLa S_3 cells.[41] The cytotoxic activities (EC_{50} values) of the compounds were defined as their *in vitro* concentrations causing 50% inhibition of cell growth after 72 h exposure to the drug, as measured by the protein content of the cells. Each experiment was run at least five times, and results are presented as average values ± standard error of the mean (SEM).

***In Vivo* Biological Evaluation: P388 Murine Leukemia.** Murine P388 leukemia was obtained from the Institute of Immunology and Experimental Therapy of Polish Academy of Sciences and injected ip in DBA/2 mice according to standard protocols from the National Cancer Institute.[42] For test purpose, mice [first-generation hybrid (BALB/c × DBA/2) F_1] were given 10^6 P388 cells ip on day 0. Twenty-four hours after tumor implantation, solutions of compounds in physiological silane were administered ip daily for 5 consecutive days. The treated group consisted of 6 and the control of 12 animals. The mean survival time (MST) of the treated (T) and control (C) groups was determined, and the percent of T/C was calculated by using the following formula: $\%T/C = [(\text{MST treated})/(\text{MST control})] \times 100$.

Synthesis: *N*-[2-(Dimethylamino)ethyl]-1-chloro-9,10-dihydro-9-oxo-4-acridinecarboxamide (5). 1-Chloro-9,10-dihydro-9-oxo-4-acridinecarboxylic acid (0.55 g, 2 mmol) and 1,1′-carbonyldiimidazole (0.65 g, 4 mmol) were suspended in DMF (10 mL); the mixture was stirred at room temperature with exclusion of moisture until all solids had dissolved. To the resulting solution cooled at 0 °C was added *N,N*-dimethylethylenediamine (10 mmol) in CH_2Cl_2 (10 mL) dropwise. The mixture was stirred for 30 min at room temperature and then diluted with $CHCl_3$ (30 mL) and extracted with 1 N aqueous Na_2CO_3 (20 mL); the organic layer was washed with water and evaporated to yield a crude oil which was purified by column chromatography on silica gel using $CHCl_3/C_6H_6/CH_3OH$ (3:3:4). The residue obtained by evaporation of fractions containing **5** was further purified by stirring in ether. Filtration of the mixture gave the pure amide **5** (0.60 g, 87%): mp 185−7 °C; IR (Nujol) 3380 cm^{-1} (NH); ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 6 H, 2 CH_3), 2.70 (t, 2 H, 2-C-CH_2), 3.62 (q*, 2 H, 2-CH_2), 7.18−7.33 (m, 3 H, 3 ar) 7.53 (b s, 1 H, amidic NH, ex), 8.00 (d, 1 H, ar), 8.38 (d, 1 H, ar), 8.62 (d, 1 H, ar), 12.42 (b s, 1 H, 10-H, ex). Anal. ($C_{18}H_{18}ClN_3O_2$) C,H,N.

6-Chloro-2-[2-(dimethylamino)ethyl]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (6). To a solution of the amide **5** (1.3 g, 3.78 mmol) in $CHCl_3$ (20 mL) and anhydrous triethylamine (4 mL) was added $COCl_2$ (20% in toluene, 9.75 mL, 19.5 mmol) in $CHCl_3$ (20 mL) dropwise under stirring at 0 °C. The resulting mixture was stirred for 30 min at room temperature and then extracted with 1 N NaOH (20 mL), washed with water (2 × 10 mL), dried on anhydrous Na_2SO_4, and evaporated to give a residue which was purified by column chromatography on silica gel using $CHCl_3/CH_3OH$ (19:1). The residue obtained by evaporation of fractions containing **6** was further purified by stirring in ether. Filtration of the mixture gave pure **6** (0.69 g, 49%): mp 132−4 °C; hydrochloride mp 288−90 °C; ^{1}H NMR ($CDCl_3$) δ 2.39 (s, 6 H, 2 CH_3), 2.75 (t, 2 H, 2-C-CH_2), 4.35 (t, 2 H, 2-CH_2), 7.49 (t, 1 H, 9-H, ar), 7.58 (d, 1 H, 5-H, ar), 7.74 (m, 1 H, 10-H, ar), 8.33 (m, 1 H, 11-H,

ar), 8.42 (d, 1 H, 4-H, ar), 8.52 (d, 1 H, 8-H, ar). Anal. ($C_{19}H_{16}ClN_3O_3$) C,H,N.

General Procedure for the Preparation of Compounds 7a–e. A solution of the 6-chloro derivative **6** (0.5 g, 1.35 mmol) and the suitable amine (4 mmol) in $CHCl_3$ (20 mL) was stirred at room temperature for 4–7 days and then evaporated. The residue was chromatographed on a silica gel column eluted as described below.

2-[2-(Dimethylamino)ethyl]-6-[[2-(dimethylamino)ethyl]amino]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (7a). The chromatography with $CHCl_3/CH_3OH$ (17:3) gave a residue which was further purified by stirring in ether. Filtration of the mixture gave pure **7a** (0.3 g, 55%): mp 181–3 °C; dihydrochloride mp 268–70 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 12 H, 4 CH_3), 2.67 (t, 4 H, 2-C-CH_2, 6-N-C-CH_2), 3.45 (q*, 2 H, 6-N-CH_2), 4.30 (t, 2 H, 2-CH_2), 6.68 (d, 1 H, 5-H, ar), 7.45 (t, 1 H, 9-H, ar), 7.70 (m, 1 H, 10-H, ar), 8.25 (d, 1 H, 4-H, ar), 8.43 (m, 1 H, 11-H, ar), 8.73 (d, 1 H, 8-H, ar), 10.95 (t, 1 H, NH, d ex). Anal. ($C_{23}H_{27}N_5O_3$) C,H,N.

6-[[2-(Diethylamino)ethyl]amino]-2-[2-(dimethylamino)ethyl]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (7b). The chromatography with $CHCl_3/CH_3OH$ (9:1) gave a residue which was further purified by stirring in ether. Filtration of the mixture gave pure **7b** (0.26 g, 43%): mp 122–4 °C; dihydrochloride mp 253–4 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 6 H, 2 C-CH_3), 2.38 (s, 6 H, 2 N-CH_3), 2.66 (q, 4 H, 2 ethyl CH_2), 2.70 (t, 2 H, 2-C-CH_2), 2.82 (t, 2 H, 6-N-C-CH_2), 3.44 (q*, 2 H, 6-N-CH_2), 4.30 (t, 2 H, 2-CH_2), 6.70 (d, 1 H, 5-H, ar), 7.44 (t, 1 H, 9-H, ar), 7.68 (m, 1 H, 10-H, ar), 8.23 (d, 1 H, 4-H, ar), 8.41 (m, 1 H, 11-H, ar), 8.71 (d, 1 H, 8-H, ar), 10.92 (t, 1 H, NH, d ex). Anal. ($C_{25}H_{31}N_5O_3$) C,H,N.

2-[2-(Dimethylamino)ethyl]-6-[[3-(dimethylamino)propyl]amino]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (7c). The chromatography with $CHCl_3/CH_3OH$ (7:3) gave a residue which was further purified by stirring in ether. Filtration of the mixture gave pure **7c** (0.36 g, 62%): mp 173–5 °C; dihydrochloride mp 279–80 °C dec; ^{1}H NMR ($CDCl_3$) δ 1.93 (quintet, 2 H, 6-N-C-CH_2), 2.27 (s, 6 H, 2 6-chain CH_3), 2.35 (s, 6 H, 2 2-chain CH_3), 2.44 (t, 2 H, 6-N-C-C-CH_2), 2.68 (t, 2 H, 2-C-CH_2), 3.44 (q*, 2 H, 6-N-CH_2), 4.30 (t, 2 H, 2-CH_2), 6.75 (d, 1 H, 5-H, ar), 7.44 (t, 1 H, 9-H, ar), 7.68 (m, 1 H, 10-H, ar), 8.23 (d, 1 H, 4-H, ar), 8.39 (m, 1 H, 11-H, ar), 8.71 (d, 1 H, 8-H, ar), 10.90 (t, 1 H, NH, d, ex). Anal. ($C_{24}H_{29}N_5O_3$) C,H,N.

2-[2-(Dimethylamino)ethyl]-6-[[2-[(2-hydroxyethyl)amino]ethyl]amino]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (7d). The chromatography with CH_3OH/EtOAc (8:2) gave a residue which was further purified by stirring in ether. Filtration of the mixture gave pure **7d** (0.195 g, 33%): mp 174–6 °C; dihydrochloride mp 274–5 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 6 H, 2 CH_3), 2.68 (t, 2 H, 2-C-CH_2), 2.91 (t, 2 H, N-CH_2-C-O), 3.08 (t, 2 H, 6-N-C-CH_2), 3.45 (q*, 2 H, 6-N-CH_2), 3.73 (t, 2 H, CH_2-O), 4.30 (t, 2 H, 2-CH_2), 6.65 (d, 1 H, 5-H, ar), 7.43 (t, 1 H, 9-H, ar), 7.68 (m, 1 H, 10-H, ar), 8.22 (d, 1 H, 4-H, ar), 8.40 (m, 1 H, 11-H, ar), 8.73 (d, 1 H, 8-H, ar), 11.18 (t, 1 H, 6-NH, d ex). Anal. ($C_{23}H_{27}N_5O_4$) C,H,N.

2-[2-(Dimethylamino)ethyl]-6-[[2-(1-piperazinyl)ethyl]amino]pyrimido[5,6,1-*de*]acridine-1,3,7-trione (7e). The flash chromatography with $CHCl_3/CH_3OH/32\%$ NH_3 (7:3:0.1) gave a residue which was further purified by stirring in ether. Filtration of the mixture gave pure **7e** (0.33 g, 53%): mp 105–7 °C; dihydrochloride mp 270–2 °C dec; ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 6 H, 2 CH_3), 2.55 (m, 2 H, 2-C-CH_2), 2.70 (m, 6 H, 3 CH_2), 2.91 (m, 2 H, CH_2), 3.42 (m, 4 H, 2 CH_2), 4.30 (t, 2 H, 2-CH_2), 6.98 (d, 1 H, 5-H, ar), 7.43 (t, 1 H, 9-H, ar), 7.68 (t, 1 H, 10-H, ar), 8.20 (d, 1 H, 4-H, ar), 8.25 (d, 1 H, 11-H, ar), 8.57 (d, 1 H, 8-H, ar). Anal. ($C_{25}H_{30}N_6O_3$) C,H,N.

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