2-Substituted 1,2-Dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-diones. A New Class of Antitumor Agent

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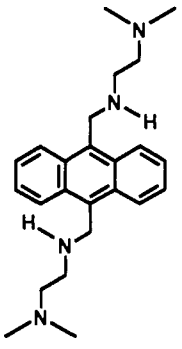
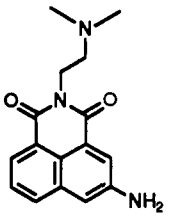
A new class of antitumor agents, having structural analogy to amonafide, but differing by the addition of a fourth ring in the nucleus, was synthesized conveniently from anthracene. Compounds with a variety of substituents, containing a basic nitrogen atom and located on the imide nitrogen, were prepared. Thirteen of 19 new compounds had greater growth inhibitory potency than amonafide in a panel of cultured murine and human tumor cells using the sulforhodamine B and MTT dye assays. The most active agents were similarly more toxic than amonafide to normal neonatal rat myocytes in vitro, but they had better chemotherapeutic indexes. From these compounds, the one with a 2-(dimethylamino)ethyl side chain (named azonafide) was chosen for further study. It showed high potency against a panel of cultured human colon cancer cells and it was active against ip P388 leukemia and subcutaneous B16 melanoma in mice. Preliminary structure–activity correlations suggest that the basicity of the side-chain nitrogen and the length of side chain are important determinants of antitumor potency in vitro. Steric hindrance and rigidity of the side chains might be other determinants.

In a previous study, we synthesized 9,10-disubstituted anthracenes related to bisantrene.[1] One of these compounds (1), having two [[2-(dimethylamino)ethyl]amino]-methyl side chains, showed potent growth inhibitory activity against human tumor cells in culture although it had only marginal activity against P388 leukemia in mice. Following the report that stiffening and flattening the side chains attached to polynuclear aromatic systems increased antitumor activity,[2] we sought to define a more rigid analogue of 1. The antitumor activity of amonafide



1



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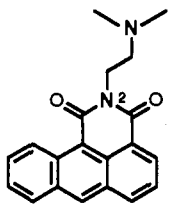

3

(nafidimide, 2)[3-5] suggested that modification of the side chain to incorporate the 1,3-piperidinedione functionality might achieve this affect. Addition of one such functionality to the more planar anthracene nucleus would produce 2-[2′-(dimethylamino)ethyl]-1,2-dihydro-3*H*-dibenz[*de,h*]-isoquinoline-1,3-dione (3). Although this structure has only one basic nitrogen atom in the side chain, it was known that, in compounds with three or more rings, only one such side chain is needed for potent antitumor activity.[6] On the basis of these factors, a study was undertaken on the synthesis and antitumor evaluation of 3 and a variety of analogues. In this article, analogues with variations in the side chain are described. A study on substituents in the nucleus is in progress and will be reported later.

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Chemistry

The synthesis of 3 and its analogues began with anthracene (4), which was converted into anthracene-1,9-dicarboxylic acid (6) by the known method involving condensation with oxalyl chloride in the presence of aluminum chloride to give tetracyclic dione 5, followed by oxidation with alkaline hydrogen peroxide (Scheme I).[7] Diacid 6 gave the corresponding anhydride 7 on heating in acetic anhydride. Compound 3 and its side-chain analogues 8–25 were obtained by heating diacid 6 or anhydride 7 with the appropriate amines in refluxing toluene. Analogue 8 with a 2-(methylamino)ethyl side chain was also prepared accidentally in an attempt to brominate the nucleus of 3. No bromo derivative could be isolated, but a small amount of 8 was obtained. It is apparently the result of N-demethylation. A more effective preparation of 8 was treatment of 6 with 2-(methylamino)ethylamine. The yields and physical properties of 3 and its analogues including ^{1}H NMR signals for the side chains are given in Table I. They all were converted into hydrochloride salts for antitumor testing.

Biology

All analogues were tested for cytotoxicity in a panel of cultured tumor cells. The sulforhodamine B (SRB) assay[8] was used for UACC375 human melanoma and OVCAR3 human ovarian cancer cells, which adhere to the plates. In this assay, the cells are fixed with trichloroacetic acid and then stained with the dye. The amount of protein-bound dye is measured spectrophotometrically. Choice of OVCAR is based on its resistance to a number of standard chemotherapeutic agents.[9] Cytotoxicity to the sensitive and multi-drug-resistant L1210 leukemia cells, which do not adhere to culture plates, was determined by the MTT tetrazolium dye assay.[10,11] This assay measures mitochondrial dehydrogenase activity that reduces MTT to a colored formazan compound, which after solubilization in DMSO absorbs light at 570 nm. As shown in Table II, 13 of the 19 new compounds were more active than

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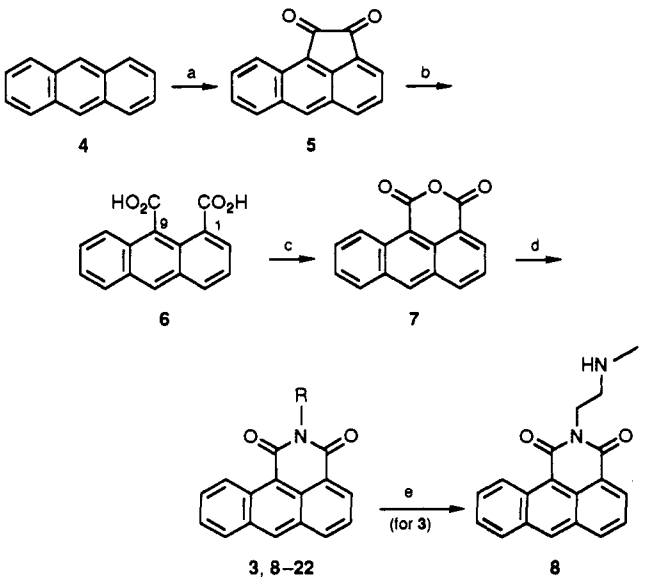
Scheme I[a]



[a] (a) ClCOCOCl, $AlCl_3$; (b) H_2O_2, NaOH; (c) Ac_2O, reflux; (d) RNH_2, toluene, reflux; (e) Br_2/AcOH.

amonafide (2) against all four tumor cell lines. The four most active compounds were 3, which has the same side chain as amonafide; 8 and 13, which have the 2-(methylamino)ethyl and 2-pyrrolidinoethyl side chains, respectively, also found in amonafide analogues that have significant antitumor activity in cell culture; and 10, which has the 2-[(2-hydroxyethyl)amino]ethyl side chain of mitoxantrone.[12] Other compounds more potent than amonafide in these assays included 9, 11, 14, 15, 16, 17, 18, 19, and 25, all of which bear side chains having strongly basic nitrogen atoms. None of the analogues with weakly basic side chains (20–24) had high potencies and pyridine-substituted analogues 20–22 were especially weak.

Because cardiotoxicity is a serious side effect of certain intercalating agents, including doxorubicin, daunorubicin, and mitoxantrone,[13] it was important to determine the cardiotoxicities of all new compounds relative to their toxicity to tumor cells. The cardiotoxicities of all of the analogues are compared with that of amonafide (2) in Table III. This comparison is based on toxicity to cultured neonatal rat heart myocytes, an assay that has been shown to correlate with human cardiotoxicity for anthracyclines and other antitumor agents.[13] It shows that amonafide has low cardiotoxicity relative to the analogues; however, in terms of the ratio of myocyte toxicity to tumor cell toxicity, at least seven compounds are better than amonafide. Analogues 8, 9, and 16 have significantly better therapeutic ratios than amonafide, at least in cell culture.

Compound 3 was tested in additional tumor models. It has been given the unofficial name "azonafide" to suggest its structural resemblance to amonafide. Table IV shows its activities in a panel of human colon carcinomas, using the MTT assay.[8] It had IC_{50} values in the 3–63 nM range. Azonafide also is active against P388 leukemia in mice. Table V shows a comparison of the relative increases in life span (ILS) given by azonafide and amonafide in the intraperitoneal assay. On a schedule of days 1, 5, and 9, both compounds were effective, with a slight advantage shown by amonafide (ILS 87.5% vs 79%) at the highest dose used (45 mg/kg). Azonafide gave a 90% ILS on a schedule of 5 mg/kg on days 1–5. In a more stringent assay, subcutaneous B16 melanoma in mice with the drug administered ip, azonafide gave a 74% reduction in tumor size on day 16, which was significantly greater than the 23% reduction shown by amonafide at twice the dose level. Humane considerations prevented continuing the experiment until the death of the mice.

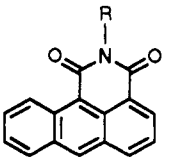
Structure–Activity Relationships

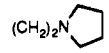
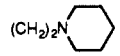
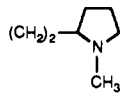
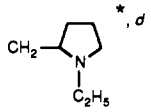
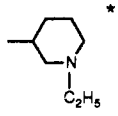
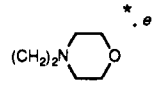
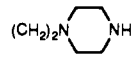
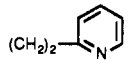
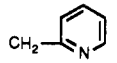
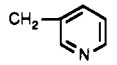
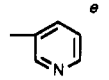
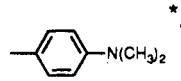
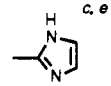
According to the data in Table II, two factors in the side-chain structure appear to be highly significant in determining the antitumor potencies of azonafide analogues: chain length and basicity of the nitrogen atom or atoms. The size of side-chain substituents might also help determine potency. Chain length probably is important in placing the protonated side-chain nitrogen in proximity to functional groups suitable for hydrogen bonding on the DNA double helix after the tetracyclic nucleus has intercalated. Bulky substituents can inhibit this binding mode. The probability of intercalation is based on analogy with amonafide and mitonafide, which are known intercalators.[14,15] This probability is supported by some DNA binding studies using 3 and calf thymus DNA (Experimental Section). The visible absorption maximum of 3 shifts from 443 to 457 mm in the presence of DNA. Furthermore, 3 increases the transition melt temperature of DNA by 15.2 °C, a value consistent with strong binding intercalators such as doxorubicin. Side chain length alterations affect cytotoxic potency. Thus, compounds 9 and 11, which have one more methylene group in the side chain, are less potent than the related compounds 3 and 10. A similar result was found for the propylene homologue of amonafide,[3] or 8-nitromitonafide.[16]

The basicity of the nitrogen determines its extent of protonation at physiological pH and this factor affects the ability of the molecule to pass through lipophilic membranes and bind to DNA. Azonafide, amonafide, and 9 have a pK_a of about 7.3, which means that about 67% of the molecules are protonated at the cell culture pH of 7.0. A low degree of protonation is desirable for cell penetration; however, DNA binding is favored by a high degree of protonation. Two-thirds protonation appears to be an effective compromise in view of the high potencies of azonafide and certain analogues. The poor antitumor activity of pyridine-containing analogues 20–23 and aniline derivative 24 possibly is a consequence of their low basicity. Compound 20 has a pK_a of 5.4, which indicates only about 2.5% protonation at pH 7.0. Imidazole derivative 24 should have adequate basicity for partial protonation. Its shorter side chain might be responsible for relatively low potency. The low potency of aziridine derivative 12 (96-fold less potent than 3) can also be explained by low basicity, as aziridines are less basic than other aliphatic amines by a $\Delta pK_a \approx 2$.

Ring size has a significant effect on potency. For example, 13, with a 5-membered ring, is almost 3 times as potent as 14, with a 6-membered ring. Furthermore, 5-membered-ring compounds 15 and 16 are more active than the isomeric 6-membered ring compound 17. In both cases, the 5-membered-ring compounds have better toxicity ratios than the 6-membered-ring analogues. Among the compounds with nonaromatic six-membered rings, piperidine analogue 14 is considerably more active than the corresponding morpholine (18) and piperazine (19) analogues. This effect might be attributed to the increased

Table I. Preparation and Properties of 2-Substituted 1,2-Dihydro-3*H*-dibenz[*de*,*h*]isoquinoline-1,3-diones[a]



no.	R	% yield	melt temp, °C	anal.	^{1}H NMR signals for the 2-substituent, δ[b]
3	$(CH_2)_2N(CH_3)_2$	77	126–128	C,H,N	2.3 (s, 6, NCH_3), 2.4 (t, 2, NCH_2), 4.0–4.25 (t, 2, $CONCH_2$)
8	$(CH_2)_2NHCH_3$	30	165–166	C,H,N	1.22 (s, 1, NH), 2.55 (s, 3, NCH_3), 3.06–3.11 (t, 2, NCH_2), 4.39–4.44 (t, 2, $CONCH_2$)
9	$(CH_2)_3N(CH_3)_2$	89	111–113	C,H,N	1.9–2.15 (quin, 2, CCH_2C), 2.3 (s, 6, NCH_3), 2.4–2.6 (t, 2, NCH_2), 4.2–4.4 (t, 2, $CONCH_2$)
10	$(CH_2)_2NH(CH_2)_2OH$[d]	16	160–162	C,H,N	2.57 (s, 1, NH), 2.77–2.81 (t, 2, NCH_2CH_2O), 2.96–3.02 (t, 2, NCH_2CH_2N), 3.18–3.33 (br s, 1, OH), 3.55–3.6 (t, 2, CH_2O), 4.31–4.37 (t, 2, $CONCH_2$)
11	$(CH_2)_3N(CH_2CH_2OH)_2$	79	139–141	C,H,N	1.8–2.1 (quin, 2, CCH_2C), 2.65–2.8 (m, 6, NCH_2), 3.0–3.3 (br, 2, OH), 3.68–3.78 (t, 4, CH_2OH),4.2–4.4 (t, 2, $CONCH_2$)
12	$(CH_2)_2N$[†]	10	139–141	C,H[f]	1.26–1.28 (d, 2, H above aziridine ring), 1.78–1.80 (d, 2, H below aziridine ring), 2.58–2.63 (t, 2, NCH_2), 4.45–4.50 (t, 2, $CONCH_2$)
13		92	162–164	C,H,N	1.65–1.95 (m, 4, CCH_2C), 2.5–3.0 (m, 6, NCH_2), 4.35–4.55 (t, 2, $CONCH_2$)
14		99	171–173	C,H,N	1.1–1.8 (m, 6, CCH_2C), 2.5–2.9 (m, 6, NCH_2), 4.35–4.55 (t, 2, $CONCH_2$)
15		86	119–122	C,H,N	1.8–2.15 (m, 4, CCH_2C), 2.2–2.4 (m, 2, NCH_2CH_2CH), 2.4–2.75 (s over m, 5, NCH_2 + NCH_3), 3.35–3.53 (m, 1, NCH), 4.27–4.35 (t, 2, $CONCH_2$)
16		99	128–130	H,N[g]	1.45–1.55 (t, 3, CH_3), 2.00–2.40 (m, 4, CCH_2C), 3.10–3.28 (m, 2, NCH_2CH_3), 3.70–3.95 (m, 3, NCH_2 endocyclic + NCH), 4.70–4.75 (t, 2, $CONCH_2$)
17		92	163–165	C,H,N	1.05–1.20 (t, 3, CH_3), 1.3–2.2 (m, 4, CCH_2C), 2.25–2.75 (m, 4, NCH_2 endocyclic), 2.9–3.1 (m, 2, NCH_2 exocyclic), 5.2–5.6 (m, 1, CONCH)
18		61	177–179	C,H,N	3.27–3.37 (m, 6, CH_2N), 3.53–3.63 (t, 4, CH_2O), 4.28–4.34 (t, 2, $CONCH_2$)
19		38	181–183	C,H[h]	1.94 (s, 1, NH), 2.75–2.83 (m, 6, H axial + NCH_2 exocyclic), 3.01–3.07 (m, 4, H equatorial), 4.40–4.42 (t, 2, $CONCH_2$)
20		80	167–169	C,H,N	3.27–3.34 (t, 2, CH_2-py), 4.63–4.69 (t, 2, $CONCH_2$), 7.14–7.17 (t, 1, H5′), 7.30–7.33 (d, 1, H3′), 7.57–7.65 [t (over t from anthracene), 1, H4′], 8.57–8.59 (d, 1, H6′)
21		95	230–232	C,H,N	5.65 (s, 2, CH_2), 7.13–7.18 (t, 1, H5′), 7.36–7.40 (d, 1, H3′), 7.57–7.63 (t, 1, H4′), 8.52–8.55 (d, 1, H6′)
22		48	211–213	C,H,N	6.45 (s, 2, CH_2), 7.23–7.28 (t, 1, H5′), 7.94–7.97 [d (over d from anthracene), 1, H4′], 8.51–8.53 (d, 1, H6′), 8.93 (s, 1, H2′)
23		19	290–291	C,H,N	7.53–7.55 (t, 1, H5′), 7.76–7.78 (d, 1, H4′), 8.678–8.683 [d (meta coupled), 1, H2′], 8.74–8.76 (d, 1, H6′)
24		82	332–334	C,H,N	3.18 (s, 6, NCH_3), 7.47–7.61 [d (over t from anthracene), 2, H3′ + H5′], 7.69–7.72 (d, 2, H2′ + H6′)
25		61	329–332	C,H[i]	7.25 (s, 2, H4′ + H5′)

[a] Analytical results were within 0.4% of theoretical values for all elements (C, H, N) except as shown in subsequent footnotes. In some examples, 1/4 mol of water (asterisk) or 1/2 mol of water (dagger) was added to reconcile calculated and found values. [b] The NMR solvent was $CDCl_3$ unless indicated otherwise. Signals for the anthracene nucleus were essentially the same for all compounds. They are given in the Experimental Section. [c] The yield is based on reacted starting material. Unreacted starting material was recovered by chromatography. [d] The NMR solvent was $CDCl_3$ + DMSO-d_6. [e] The NMR solvent was DMSO-d_6. [f] N: calcd, 8.32; found 7.67. [g] C: calcd, 76.14; found, 76.55. [h] N: calcd, 11.69; found, 11.27. [i] N: calcd, 13.42; found, 12.92.

hydrophilility of **18** and **19**. The open-chain analogues **10** and **11** are more potent than the corresponding 6-membered ring compounds **18** and **19** despite being more hydrophilic. The side chain **10** also conferred potency to 8-nitromitonafide.[16] In addition to their low basicity, the size and rigidity of pyridine-containing analogues **20–23** and dimethylanilino analogue **24** might contribute to their lack of potency. Even if they are protonated to a small extent, it may be difficult for them to assume conformations appropriate for hydrogen bonding to DNA.

Table II. Activity of 1,2-Dihydro-3*H*-dibenz[*de*,*h*]isoquinoline-1,3-diones against Tumor Cells in Culture[a]

no.	testing code, EL-	IC_{50}, nM				
		UACC375[b] melanoma	OVCAR3[c] ovarian	L1210 sensitive	L1210[d] resistant	avg[e]
2	amonafide	2031	2180	625	625	1365
3	1	71	57	7.0	7.0	36
8	22	59	44	10	20.6	33
9	11	245	462	54	82	211
10	66	67	81	18	41	52
11	12	217	163	76	142	150
12	68	2532	7911	791	791	3006
13	8	39	66	14.5	11.8	33
14	7	178	89	51	51	92
15	42	190	203	51	56	125
16	41	119	214	36	55	106
17	9	1650	1185	635	609	1020
18	40	511	639	512	512	544
19	48	463	1968	174	463	767
20	43	6443	3866	6443	15464	8054
21	39	12032	17380	26738	26740	20722
22	38	11363	8021	1872	2674	5983
23	44	2493	1939	544	554	1380
24	10	4975	16169	187	1791	4537
25	45	1622	2434	207	148	1103

[a] The murine leukemia experiments were based on continuous drug exposure using the MTT assay (Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 1988, *48*, 589–601.) Determination of cytotoxicity against AUCC375 and OVCAR 3 utilized the sulforhodamine B assay (Skehan, P.; Strong, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening, *J. Natl. Cancer Inst.* 1990, *82*, 1107–1112. [b] A human melanoma line obtained from the University of Arizona Cancer Center. [c] A human ovarian cancer cell line obtained from the NCI. This carcinoma was resistant to all standard anticancer drugs. [d] A murine leukemia cell line. The resistant strain is multiple drug resistant. [e] An average (mean) for IC_{50} values in the four tumor cell lines.

Table III. Cardiotoxicity of 1,2-Dihydro-3*H*-dibenz[*de*,*h*]isoquinoline-1,3-diones in Cell Culture[a]

no.	cardiotoxicity IC_{50} (nM)	toxicity ratio[b] myocyte/ tumor cell	no.	cardiotoxicity IC_{50} (nM)	toxicity ratio[b] myocyte/ tumor cell
2	48400	36	16	15200	143
3	1983	56	17	8760	8.3
8	7060	211	18	>25000	>50
9	11410	113	19	6713	8.8
10	943	18	20	>25000	>3
11	8910	60	21	>25000	>1
12	not determined		22	>25000	>4
13	920	28	23	>25000	>20
14	10400	13	24	37313	8
15	7080	57	25	not determined	

[a] Neonatal rate heart myocyte assay, 1 h exposure. Cytotoxicity is measured by the ATP/protein ratio compared with untreated controls. The IC_{50} is the 1-h drug concentration that reduces this ratio to 50% of that in untreated control myocytes (Dorr, R. T.; Bozak, K. A.; Shipp, N. G.; Hendrix, M.; Alberts, D. S.; Ahmann, F. *In Vitro* Rat Myocyte Cardiotoxicity Model for Antitumor Antibiotics Using Adenosine Triphosphate/protein Ratios. *Cancer Res.* 1988, *48*, 5222–5227). [b] The quotient of the IC_{50} in the myocytes divided by the mean IC_{50} in the four tumor cell lines (from Table II). This ratio has been used previously to compare anthracycline antitumor agents (Dorr, R. T.; Shipp, N. G.; Lee, K. M. Comparison of Cytotoxicity in Heart Cells and Tumor Cells Exposed to DNA Intercalating Agents *In Vitro*. *Anti-Cancer Drugs* 1991, *2*, 27–33.

Table IV. Activities of Azonafide (3) in Human Colon Carcinoma Tumors in Vitro

growth inhibitory activity	(IC_{50} in nM)[a]
Lo Vo	6
Colo 205	36
HT 29	63
HCT 15	8
Colo 480	3
mean (SD)	23(26)

[a] Continous drug exposure using the MTT assay (Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czervinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 1988, *48*, 589–601.)

Conclusions

The 2-substituted 1,2-dihydro-3*H*-dibenz[*de*,*h*]isoquinoline-1,3-diones provide a new class of antitumor agents that can be synthesized readily from anthracene. They have structural analogy to amonafide (2), but differ from it in being tetracyclic systems. More than half of these compounds were more potent than amonafide against murine and human cancer cells in vitro, and some of them, including the most potent compounds 3 and 8, had better chemotherapeutic indexes than amonafide. Among these compounds, 2-(methylamino)ethyl derivative 8 had the highest potency and lowest cardiotoxicity. The 2-(dimethylamino)ethyl derivative 3, named azonafide, was more active than amonafide against a panel of human colon cancer cells and nearly as effective, at lower doses, against leukemia in mice.

The impressive superiority of azonafide (3) and certain analogues to amonafide (2) in the in vitro assays probably reflects their greater intrinsic cytotoxicity to cancer cells. Loss of this advantage in the ip murine P388 leukemia assay could be caused by factors such as absorption into various tissues around the peritoneal cavity and to metabolism. Future studies will address this problem.

It is encouraging to find that cardiotoxicity and cytotoxicity to tumors can be separated substantially in azonafide analogues. The in vitro toxicity ratios vary from 8.3 to 211, with significant margins (>100-fold ratios) found for 8, 9, and 16.

One potential advantage of azonafide and its analogues reported above is that they lack the primary amino group of amonafide, which is subject to toxicity-enhancing N-acetylation in humans.[17] These results and expectations suggest that further analogues of azonafide should be prepared and screened against tumors. The synthesis of

Table V. Activities against Tumors in Mice

compd	dose (mg/kg)	schedule (days)	L1210 leukemia % ILS[a]	sc B16 melanoma % TGI[b]
amonafide (2)	30	1,5,9	45	23
	45	1,5,9	87.5	
azonafide (3)	15	1,5,9	25	74
	30	1,5,9	62.5	
	45	1,5,9	79	
	5	1–5	90	

[a] Conducted according to standard NCI protocol. The leukemia cells were given ip. Results are expressed as the percent increase in life span (ILS) = 100 × [(life span trated − life span controls)/life span controls]. [b] 10^6 B16 melanoma cells are injected subcutaneously into C57/VBL male mice. The compounds are given ip. Tumor growth is measured by calipers using the widest perpendicular widths of palpable subcutaneous tumor as the endpoint. These widths are converted to an estimated tumor mass according to the formula $L_{mm} \times W^2_{mm}/2$ = mg. The percent tumor growth inhibition (TGI) is calculated by the equation

$$\% \text{ TGI} = \frac{(\text{wt control tumor} - \text{wt tumor})}{\text{wt control tumor}} \times 100$$

analogues with a variety of substituents introduced at all of the open positions on the anthracene nucleus is in progress. Further studies on the antitumor activity, toxicity, and pharmacology of azonafide are in progress and will be published elsewhere.

Experimental Section

Melting points were recorded on a Mel-Temp melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker 250 WM spectrometer and absorptions are reported as downfield from Me_4Si. Elemental analyses were performed by Desert Analytics, Inc., Tucson, AZ.

General Procedure for the Preparation of 2-Substituted 1,2-Dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-diones (3, 8–18, 20–22). A suspension of 1 equiv of anthracene-1,9-dicarboxylic acid or its anhydride (especially in the case of 12) in toluene (3, 8–9, 12–14, 17) or a toluene–ethanol mixture (4:1) was treated with 1.1 equiv (1.5 in the case of 8 and 12) of the appropriate amine. The mixture was refluxed under nitrogen for a period from 1 to 18 h as monitored by TLC (chloroform–methanol 9:1, 8:2, or 7:3). The reaction mixture (usually a clear solution) was filtered from any tarry or insoluble material. The filtrate was evaporated in vacuum to dryness and the yellow residue was purified by chromatography on silica gel (neutral alumina in the case of 12) and/or crystallization. Solvents for column chromatography were toluene–methanol 8:2 (for 20) and chloroform–methanol 9:1 (3) and 8:2 (11) and for preparative thin-layer chromatography were chloroform–methanol 9:1 (15) and 8.5:1.5 (10), toluene–methanol 8.5:1.5 (8), 1.5% triethylamine in chloroform (18), and chloroform (12, 20–22). Crystallization solvents were hexanes (15, 16), toluene (3, 10, 11, 21, 22), and mixtures of hexanes and toluene 7:1 (for 8) and 1:1 or 2:1 (for the rest of the compounds). ^{1}H NMR ($CDCl_3$, TMS, δ in ppm) for compound 3: δ 2.41 (s, 6, NCH_3), 2.67–2.73 (t, 2, NCH_2), 4.33–4.39 (t, 2, $CONCH_2$), 7.49–7.56 (t, 1, H-9), 7.56–7.62 (t, 1, H-5), 7.69–7.75 (t, 1, H-10), 7.92–7.95 (d, 1, H-8), 8.14–8.17 (d, 1, H-4), 8.57 (s, 1, H-7), 8.57–8.60 (d, 1, H-6), 9.80–9.83 (d, 1, H-11). All the compounds listed in Table I have ^{1}H NMR signals similar to that of 3 except for the 2-substituents which are given in the table.

Preparation of 2-(2′-*N*-Piperazinoethyl)-1,2-dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-dione (19). A suspension of 248 mg (1 mmol) of anthracene-1,9-dicarboxylic acid anhydride in 30 mL of toluene was treated with a solution of 155 mg (1.2 mmol) of 1-(2-aminoethyl)piperazine in 5 mL of absolute ethanol. After refluxing for 16 h the solvent was evaporated and the residue (286 mg) was chromatographed on a silica gel column using chloroform–methanol 7:3 and then on a silica gel plate in a mixture of chloroform–methanol–triethylamine 9:1:0.2 to give a yellow solid that was digested with boiling toluene. The insoluble material (100 mg) was filtered and rejected. The filtrate upon evaporation gave 135 mg (38%) of 19, which crystallizes from hexane–toluene 1:1 (cf. Table I). Anal. ($C_{22}H_{21}N_3O_2$) C, H; N: calcd, 11.69; found, 11.27.

Preparation of 2-(3′-Pyridyl)-1,2-dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-dione (23). A suspension of 496 mg (2 mmol) of anthracene-1,9-dicarboxylic acid anhydride in 40 mL of toluene was treated with a solution of 621 mg (6.6 mmol) of 3-aminopyridine in 10 mL of absolute ethanol. After refluxing for 64 h the reaction mixture was cooled to room temperature and the yellow precipitate (459 mg) of unreacted anthracene 1,9-dicarboxylic acid anhydride was filtered. The filtrate was evaporated to dryness and the residue was purified by preparative thin-layer chromatography on silica gel in a mixture of toluene–methanol 8:2 to give 9 mg (19% based on reacted material) of 23, which was crystallized from hexanes–toluene 1:1 (cf. Table I). Anal. ($C_{21}H_{12}N_3O_3$) C, H, N.

In an attempt to overcome the limitation in yield of 23 caused by insolubility of the anhydride, the preparation was repeated with DMF as solvent. A mixture of 100 mg (0.4 mmol) of anthracene-1,9-dicarboxylic acid anhydride and 350 mg (3.7 mmol) of 3-aminopyridine in *N,N*-dimethylformamide was heated under reflux for 48 h, cooled, and concentrated to dryness. The residue was purified by preparative TLC on silica gel with toluene–methanol 8:2 as solvent. This procedure gave 26 mg (20%) of 23.

Preparation of 2-[4′-(Dimethylamino)phenyl]-1,2-dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-dione (24). A mixture of 300 mg (1.21 mmol) of anthracene-1,9-dicarboxylic acid anhydride and 494 mg (3.63 mmol) of *N,N*-dimethyl-*p*-phenylenediamine in 50 mL of absolute ethanol was heated under reflux for 24 h. Twenty milliliters of dry toluene was added and the mixture was refluxed for another 72 h. After cooling to room temperature the yellow solid was filtered and digested with boiling dioxane. The insoluble material (110 mg), unreacted anthracene-1,9-dicarboxylic acid anhydride, was filtered. The filtrate upon evaporation gave 230 mg (82% based on reacted anhydride) of 24, which crystallizes from dioxane and has the physical properties listed in Table I. Anal. ($C_{24}H_{18}N_2O_2 \cdot {}^1/_4H_2O$) C, H, N.

Preparation of 2-(2′-Imidazolyl)-1,2-dihydro-3*H*-dibenz[*de,h*]isoquinoline-1,3-dione (25). A suspension of 248 mg (1 mmol) of anthracene-1,9-dicarboxylic acid anhydride in 50 mL of absolute ethanol was treated with a solution of 249 (3 mmol) of 2-aminoimidazole in 5 mL water. The mixture was heated under reflux for 15 h. The insoluble yellow precipitate was filtered, washed with water, and dried in air to give 190 mg (61%) of 25 which was crystallized from *N,N*-dimethylformamide (cf. Table I). Anal. ($C_{19}H_{11}N_3O_2$) C, H; N: calcd, 13.42; found 12.92.

Microculture Tetrazolium Assay. This assay is based on reductive cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium (MTT) bromide to a colored formazan compound as an indicator of cell viability.[10] Tumor cells were plated at 50 000/well onto 96-well microtiter plates (Costar, Cambridge, MA). On day 2, drugs dissolved initially in DMSO (J.T. Baker, analytical grade) and then diluted serially with phosphate-buffered saline (pH 7.4) were added at concentrations of 10^1–10^{-5} μg/mL in half-log gradations. Final concentrations of DMSO did not exceed 0.1%. The plates were incubated at 37 °C with 5% CO_2, 95% air, and 100% relative humidity for 6 days.

After the 6-day exposure period, 50 μL of a 2 mg/mL MTT solution was added to each of the wells, and the plates were incubated an additional 4 h. The medium was then aspirated and the formazan product was solubilized by DMSO (100 μL/well). The intensity of the color, which is proportional to viable cell numbers, was quantitated by absorbance at 570 nm on an automated microculture plate reader (Biomek 1000, Beckman Instruments). Test results were calibrated in percent control absorbance from untreated tumor cells. Each drug concentration was tested in six wells, and the IC_{50} values were averaged. The results are given in Tables II and IV.

Sulforhodamine B Assay. This assay is based on the spectrophotometric determination of sulforhodamine B, a pink aminoxanthine dye, bound to cellular protein.[8] The plating of tumor cells, addition of drugs, and incubation was the same as described in the MTT assay. After the 8-day exposure period, the medium was aspirated and phosphate-buffered saline (PBS)

was added. The cells were fixed by gently layering 50 μL of 10% trichloroacetic acid (TCA) on top of the growth medium in each well. The cultures were incubated at 4 °C for 1 h and then washed several times with tap water. Plates were air-dried and background optical densities were measured in wells incubated with growth medium without cells. TCA-fixed cells were strained for 30 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid, then the SRB was removed, and the cultures were quickly rinsed four times with 1% acetic acid. After the cultures were dried in air, the bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5) for 5 min on a shaker. The OD at 564 nm was read on an automated microculture plate reader (Biomek 1000, Beckman Instruments). Protein content was determined by references to a calibration curve constructed with bovine serum albumin used as a standard. Each drug concentration was tested in six wells and the IC_{50} values were averaged. The results are given in Tables II and IV.

Antitumor Assays in Mice. The assay for P388 leukemia in mice was conducted as specified in the standard NCI protocol.[18] Freshly harvested tumor cells (10^6 cells) were injected ip into 10 adult DBA/2J male mice on day 0 and amonafide (2) or azonafide (3) was given ip on days 1, 5, and 9. The control group of 10 mice was given 10^6 tumor cells ip and injected with saline on the scheduled days. Results are expressed as the percent increase in life span (ILS) = 100 × (life span treated − life span controls)/life span controls, using median values for the groups of 10 mice.

In the B16 melanoma assay, 10^6 B16 cells were injected subcutaneously in the flank of C57/8L male mice (25–30 g) on day 0. Azonafide at 10 mg/kg was injected intraperitoneally on days 1, 5, and 9. The mean tumor size was calculated from bidimensional tumor measurements according to the equation mg tumor = $(L_{mm} \times W^2_{mm})/2$, and the inhibition of tumor growth was calculated by the equation

$$\% \text{ inhibition} = \left(\frac{\text{mean tumor size of controls} - \text{mean tumor size of treated}}{\text{mean tumor size of controls}}\right) \times 100$$

The experiment was terminated when the tumors in controls became so large as to severely debilitate the mice.

Effect of DNA on the Visible Absorption of 3. A solution of calf thymus DNA (244 μM in base pairs) in water containing 0.1 M NaCl, 0.1 M HEPES, and 0.001 M EDTA (pH 7.0) was treated with increasing amounts of 3 (from 10 to 162 μM) in the same buffer. The visible absorption maximum of 3 shift from 443 mm to a final value of 457 nm.

Transition Melt Temperatures. The buffer for these experiments was ion-exchanged water containing 0.01 M NaH_2PO_4 and 0.001 M EDTA with the pH tuned to 7.0 with NaOH solution. DNA solution was made by dissolving calf thymus DNA in buffer and adjusting the final concentration to about 5×10^{-5} M. This solution was made freshly before each measurement. An appropriate amount of 3 in the same buffer was added to give a ratio of 5:1 for DNA base pairs to 3. With buffer in the reference cuvette, the sample cuvette was heated from 25 to 100–105 °C at 0.8 °C/min, using a Perkin-Elmer Lambda 3A spectrophotometer with heated cell and temperature programmer and a PE R100A recorder.

pK_a Determinations. Solutions were prepared by dissolving 0.165 mmol of each compound as the hydrochloride in sufficient 0.982 N HCl to give 1 mequiv of acid beyond protonation of the amino group. These solutions were stirred and titrated with 0.926 N NaOH while the pH was measured in a Sargent-Welch Model IP pH meter. pH data were graphed and pK_a values were determined from points on the curve where the excess HCl was neutralized and 0.5 equiv of base per amino group had been added.

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