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Tumor Inhibitors. 69. Structure–Cytotoxicity Relationships among the Sesquiterpene Lactones[1]

S. MORRIS KUPCHAN,* M. A. EAKIN, AND A. M. THOMAS[2]

Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901

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The presence of an α-methylene-γ-lactone is essential for significant cytotoxic activity among the sesquiterpene lactones. The biological activity is enhanced by the presence of certain additional α,β-unsaturated carbonyl functions. In addition, the activity of these compounds increases with increasing lipophilicity, but does not appear to correlate with reactivity toward cysteine. Those sesquiterpenes with demonstrated *in vivo* antitumor activity have common features which set them apart from the majority of the sesquiterpene lactones.

A number of significantly cytotoxic sesquiterpene lactones have been isolated during the course of a continuing search for antitumor agents from plant sources.[3] Despite the common occurrence of cytotoxic activity and, to a lesser extent, *in vivo* antitumor activity, this class of compounds has not been subjected to a systematic study of structure–activity relationships. The great structural diversity of these compounds (Chart I) precluded the determination of most of the free energy parameters required for a rigorous Hansch analysis. Other complicating factors, such as solubility difficulties in solvents used for the biological assay and biological variations in the responses, have been noted previously.[4] In spite of these limitations, we felt that structure–activity relationship studies of these compounds should reveal general relationships of value to any further biological study in the area of sesquiterpene lactones.

Using the data available at the time, Hartwell and Abbott[4] surveyed the structural variety of the cytotoxic sesquiterpene lactones and attempted a statistical treatment of the structure–activity problem. As they noted, the small number of examples available made it difficult to arrive at definitive conclusions. It was noted, however, that all the cytotoxic sesquiterpenes were lactones, of which all but one were α,β-unsaturated, and that the α,β-ethylenic linkage was exocyclic in every case.[4] At that time, this fact was deemed to be of questionable significance, in view of insufficient testing data on endocyclic analogs.

The cytotoxic sesquiterpene lactones characterized in these laboratories were isolated with the help of biological activity as a guide to fractionation at every stage.[3] Such a procedure, based on activity as a guide, would not be expected to have been selective for one type of compound. Thus, the repeated isolation of α-methylenelactones, and not the endocyclic analogs, from activity-directed fractionations was deemed significant. Furthermore, it has been shown that, in contrast to α-methylene-γ-lactones, which react rapidly with cysteine to form stable adducts, endocyclic α,β-unsaturated-γ-lactones react slowly with cysteine, to form unstable adducts.[5] These marked chemical differences, coupled with the fact that elephantopin-bis(cysteine) adduct, which contains an endocyclic $\Delta^{\alpha,\beta}$-lactone, is inactive[6] led us to believe that the endocyclic lactone does not contribute significantly to the cytotoxic activity of the parent compound, elephantopin. These observations lent support to the importance of α-methylene-γ-lactones for cytotoxic activity. That the occurrence of this functional grouping was intrinsically connected with cytotoxicity was also suggested by the results of cysteine addition studies,[6] which showed that cytotoxicity was related to sensitivity to thiol addition. It was postulated that ". . .the reactions of α-methylene-γ-lactones and other conjugated systems with biologically important sulfhydryl groups may play a significant role in the mechanisms by which these compounds exert their biological activities."[6]

A more extensive study of structure–cytotoxicity relationships was undertaken, in order to delineate further the structural requirements for biological activity.

Experimental Section

The sesquiterpene lactones were assayed for inhibitory activity *in vitro* against cells derived from human carcinoma of the naso-

(1) (a) Part 68: S. M. Kupchan, V. H. Davies, T. Fujita, M. R. Cox, and R. F. Bryan, *J. Amer. Chem. Soc.*, **93**, 4916 (1971). (b) This work was supported by grants from the National Cancer Institute (CA-11718) and the American Cancer Society (T-275).

(2) National Institutes of Health Postdoctoral Fellow, 1969–1971.

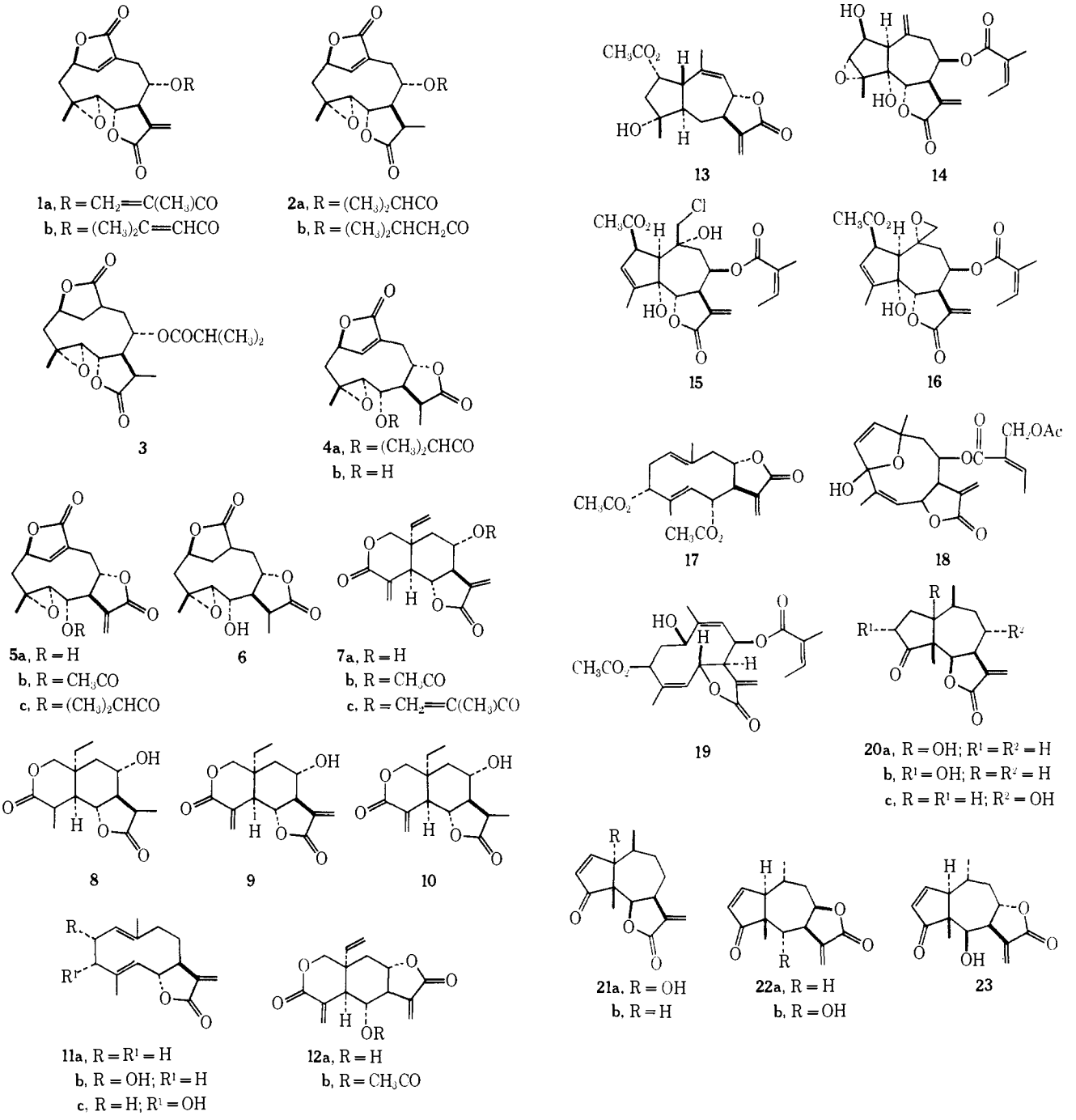
(3) S. M. Kupchan, *Trans. N. Y. Acad. Sci.*, **32**, 85 (1970).

(4) J. L. Hartwell and B. J. Abbott, *Advan. Pharmacol. Chemother.*, **7**, 117 (1969).

(5) S. M. Kupchan, T. J. Giacobbe, I. S. Krull, A. M. Thomas, M. A. Eakin, and D. C. Fessler, *J. Org. Chem.*, **35**, 3539 (1970).

(6) S. M. Kupchan, D. C. Fessler, M. A. Eakin, and T. J. Giacobbe, *Science*, **168**, 376 (1970).

CHART I
STRUCTURES OF THE CYTOTOXIC SESQUITERPENE LACTONES



pharynx (KB) carried in cell culture.[7] In order to minimize variations due to solvent effects on cytotoxicity, all of the compds were assayed using propylene glycol as the vehicle. It was recognized that, when only a few test results on one compd are available, an extraneous value will have an inordinate effect on the mean. The median value is less influenced by the extraneous number and so is more likely to represent the most probable value.[8] For this reason the ED_{50} of each compd has been defined as the median of the available testing data (usually either 3 or 4 tests per compd). If a compd was found to inhibit growth to 50% of the control growth at a dose of 4 μg/ml or less ($ED_{50} \leq 4$ μg/ml) it was deemed significantly cytotoxic. A compd is considered to be devoid of activity if the ED_{50} is greater than 100 μg/ml.

For the present report, ED_{50} is expressed also in micromolar concn. This avoids variation due to differences in mol wt, and is consequently more convenient for comparisons of cytotoxicities with structural variation. On this scale significant activity corresponds to a C_{50} of approx 15 μM or less, and a $C_{50} > 300$ μM implies complete lack of activity.

Second-order rate constants for the reactions of the sesquiterpene lactones with cysteine were detd spectrophotometrically with a Beckman DK-2A spectrophotometer equipped with a thermostated cell compartment. To a 10^{-4} M soln of L-cysteine in 0.067 M phosphate buffer (pH 7.4), prepd in a 1.00-cm quartz cell (vol 3.60 ml), was added a 10^{-2} M soln of lactone in THF (36 μl) and the resultant soln was mixed rapidly. After an appropriate reaction time, the SH content was measured by quenching the reaction with an excess of a THF soln of 2,2'-pyridine disulfide, which reacts with cysteine to give 2-thiopyridone (ϵ 7780 at 343 mμ).

The partition coefficients ($P = c_{octanol}/c_{H_2O}$) were detd essen-

(7) Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemotherapy Rep.*, **25**, 1 (1962).

(8) R. B. Dean and W. J. Dixon, *Anal. Chem.*, **23**, 636 (1951).

tially as described by Fujita, *et al.*,[9] using a Beckman DK-2A spectrophotometer. Typically a 0.1-mg sample was partitioned between 5 ml of H_2O and 1-octanol. The vol of 1-octanol was chosen so that the absorbance of the H_2O layer after partitioning had a value between 0.2 and 0.9 using a 1-cm cell. In a few cases, due to poor solubility in H_2O, the absorbances were measured for the 1-octanol phase. Adherence to Beer's law was detd for these materials in 1-octanol. The absorption max (210–230 mμ) for the α-methylene-γ-lactone chromophore was measured.

The biological activity, log $1/C_{50}$, was correlated with log P by a Burroughs B-5500 computer using a least-squares analysis program.

Results and Discussion

The structures of the cytotoxic sesquiterpene lactones are diverse in two respects: they show considerable variation in carbon skeletons, and contain a variety of combinations of functional groups (Chart I). The first problem was the determination of which functional groups contributed to cytotoxicity. As discussed above, preliminary evidence suggested that the α-methylene-γ-lactone was of major importance. It has been possible to demonstrate that cytotoxicity is critically dependent upon the presence of this functional group.

Many of the naturally occurring sesquiterpenes isolated in these laboratories were modified in the course of structural elucidation. Evaluation of the cytotoxic activities of the transformation products was often informative, since it gave a clear indication of just which functional groups contributed to cytotoxic activity. Selective modification of certain functional groups always resulted in complete loss of cytotoxicity, thus identifying the activity conferring functionalities. In this way the so-called "active functional groups" were revealed from amongst the wide variety present in the sesquiterpene lactones.

Elephantopin[10] (**1a**, C_{50} = 3.22 μ*M*) on reduction gave the inactive derivatives tetrahydroelephantopin[1] (**2a**) and hexahydroelephantopin[10] (**3**). The isomeric derivative dihydroelephantol isobutyrate[10] (**4a**) was also inactive. The hydrolysis product elephantol[10] (**5a**) retained some, but not significant, activity (C_{50} = 123.2 μ*M*), whereas dihydro- and tetrahydroelephantol[10] (**4b** and **6**, respectively) were inactive. Likewise the closely related compound elephantin[10] (**1b**, C_{50} = 2.51 μ*M*) also became inactive upon partial hydrogenation to tetrahydroelephantin (**2b**). Thus it appears that for these compounds only the α-methylene-γ-lactone and the conjugated ester side chain contribute to cytotoxicity. The endocyclic $\Delta^{\alpha,\beta}$-lactone appears not to confer cytotoxicity. That the unsaturated ester is less important than the α-methylene-γ-lactone is suggested by the high activity of elephantol acetate[10] (**5b**, C_{50} = 11.4 μ*M*) and elephantol isobutyrate (**5c**, C_{50} = 11.4 μ*M*).

A similar specificity in the "active functional groups" was found in derivatives of vernolepin[11] (**7a**, C_{50} = 6.52 μ*M*). While hexahydrovernolepin[11] (**8**) was inactive, dihydrovernolepin[12] (**9**, C_{50} = 6.85 μ*M*) was as active as the parent compound, thus implicating the α-methylenelactone groups as "active functionalities." Partial hydrogenation of **7a** gave the markedly less active derivative tetrahydrovernolepin[11] (**10**, C_{50} = 64.3 μ*M*) in which the α-methylene-γ-lactone had been saturated.[13]

This approach revealed 3 types of functional groups which impart some degree of cytotoxic activity in this series of compounds. The most common, and the only one which by itself confers significant cytotoxicity, is the α-methylene-γ-lactone grouping. All but one of the 50 known cytotoxic sesquiterpenes contain this grouping. There are 25 compounds which contain only this group as a potential "active functionality"; of these 20 are significantly active. No monofunctional sesquiterpene containing only an α,β-unsaturated ester or cyclopentenone has, to date, shown significant activity. As discussed below, however, the presence of these groups in α-methylene-γ-lactones does appear to *enhance* cytotoxicity.

The cytotoxicity of the α-methylene-γ-lactones studied varies from C_{50} = 0.35 μ*M* to C_{50} = 127 μ*M*. Clearly, other structural parameters influence the activity of these compounds. Since it appeared that the cytotoxicity was dependent upon the ability of the compounds to react with thiols,[6] it was thought that the rate of thiol addition might influence cytotoxicity. Rates of reaction of the sesquiterpene lactones with cysteine (Table I) were determined at pH 7.4 and 25°. Examination of these rates revealed a definite structural dependence.

The rate of addition to those compounds devoid of OH or *O*-acyl groups adjacent to the α-CH_2 on the γ-lactone ring was consistently in the order of 250 l./mole min^{-1}. A neighboring OH or *O*-acyl group produced a marked enhancement in the rate of cysteine addition. In these cases k_2 varied from 720 to 15,000 l./mole min^{-1}. This rate enhancement presumably arises from a neighboring group facilitation of the addition of S^- anion, or proton transfer at some intermediate stage in the addition. The reactivity did not appear to be affected by whether the compounds possessed cis or trans lactone ring junctions.

Attempts to find a direct correlation between the rate of cysteine addition and cytotoxicity were unsuccessful. Even when compounds of similar structure were compared, the cytotoxicity appeared to be randomly distributed when plotted as a function of reactivity. This lack of correlation may mean that the differences in reactivity have an insignificant influence upon cytotoxicity, relative to other structural parameters, or it may simply mean that cysteine is a poor model for the biological thiols affected by the cytotoxic action of these compounds.

Correlations between lipophilic character, which is a measure of the fraction of the dose administered reaching its receptor compartment[14] as measured by the partition coefficient in 1-octanol–H_2O, and various biological activities have proven very meaningful.[15-17]

(9) T. Fujita, J. Iwasa, and C. Hansch, *J. Amer. Chem. Soc.*, **86**, 5175 (1964).

(10) S. M. Kupchan, Y. Aynehchi, J. M. Cassady, H. K. Schnoes, and A. L. Burlingame, *J. Org. Chem.*, **34**, 3867 (1969).

(11) S. M. Kupchan, R. J. Hemingway, D. Werner, and A. Karim, *J. Org. Chem.*, **34**, 3903 (1969).

(12) S. M. Kupchan, T. J. Giacobbe, and I. S. Krull, *Tetrahedron Lett.*, 2859 (1970).

(13) This suggests that the α-methylene-δ-lactone is not as effective as the α-methylene-γ-lactone in imparting cytotoxicity, and, indeed, no monofunctional α-methylene-δ-lactones have yet shown significant cytotoxicity.

(14) J. M. Van Rossum, *Advan. Drug Res.*, **3**, 189 (1966).

(15) A. Lee, C. Hansch, and C. Church, *J. Med. Chem.*, **12**, 766 (1969).

(16) C. Hansch, *Accounts Chem. Res.*, **2**, 232 (1969).

(17) C. Hansch, *Annu. Rep. Med. Chem.*, **1967**, 348 (1968).

TABLE I

RATE OF CYSTEINE ADDITION, LOG *P*, AND CYTOTOXICITY OF SESQUITERPENE LACTONES

Compd	Ref	$k_{2(cys)}$ (l. M^{-1} min^{-1})	log *P*	ED_{50}[a]	C_{50}[b]	Obsvd log $(1/C_{50})$[c]	Calculated log $(1/C_{50})$[c]Δ Eq 1	Eq 2	Eq 3	Eq 4
Vernomenin (**12a**)	*d*	2,500 NL	0.86 − 1	35.0	127.0	3.89	4.66 −0.77			0.40 −0.51
Vernomenin acetate (**12b**)	*d*	1,500 NL	0.25	8.00	26.2	4.60	4.94 −0.34			4.80 −0.20
Vernolepin (**7a**)	*d*	15,000 NL	0.31	1.80	6.52	5.19	4.99 0.20			4.86 0.33
Vernolepin acetate (**7b**)	*d*	15,000 NL	0.79	2.70	8.49	5.07	5.34 −0.27			5.35 −0.28
Vernolepin methacrylate (**7c**)	*e*		1.56	0.42	1.22	5.92	5.90 −0.01			6.14 −0.23
Gaillardin (**13**)	*f*	280 ± 70	1.09	2.30	7.52	5.12	5.57 −0.45	5.19 −0.07		
Eupatundin (**14**)	*g*	2,000 NL	1.69	0.47	1.25	5.90	6.01 −0.11			6.27 −0.37
Eupachlorin acetate (**15**)	*g*	720 ± 75	1.42	0.16	0.35	6.46	5.81 0.65			5.99 0.47
Euparotin acetate (**16**)	*g*	3,200 ± 500	0.76	0.22	0.53	6.28	5.32 0.96			5.32 0.96
Costunolide (**11a**)	*h*	250 ± 65	1.70	0.57	2.46	5.61	6.02 −0.41	5.54 0.07		
Tamaulipin A (**11b**)	*i*	230 ± 40	1.23	1.26	5.08	5.30	5.67 −0.37	5.27 0.03		
Tamaulipin B (**11c**)	*j*	270 ± 65	1.40	2.60	10.5	4.98	5.80 −0.82	5.37 −0.39		
Chammissonin diacetate (**17**)	*k*	1,450 ± 250	1.14	2.13	6.12	5.21	5.60 −0.39	5.22 −0.01		
Liatrin (**18**)	*l*	2,600 ± 445	1.19	1.62	3.93	5.41	5.64 −0.23			5.76 −0.35
Eupacunin (**19**)	*m*	1,300 ± 220	1.16	0.84	2.07	5.68	5.62 0.06			5.73 −0.05
Elephantol (**5a**)	*n*	3,500 ± 265	0.36 − 1	36.0	123.2	3.91	4.28 −0.37	4.19 −0.28		
Elephantopin (**1a**)	*n*	2,600 ± 275	0.69	1.16	3.22	5.49	5.27 0.22			5.25 0.24
Elephantin (**1b**)	*n*	2,730 ± 400	1.05	0.94	2.51	5.61	5.54 0.07			5.62 −0.01
Coronopilin (**20a**)	*o*	270 ± 22	0.83	1.45	5.49	5.26	5.37 −0.11	5.04 0.22		
3-Hydroxydamsin (**20b**)	*p*	225 ± 40	0.66	2.65	10.00	5.00	5.25 −0.25	4.94 0.06		
Desacetylconfertiflorin (**20c**)	*q*	900 NL	0.23	2.30	8.71	5.06	4.93 0.13	4.69 0.37		
Parthenin (**21a**)	*r*	257 ± 33	0.77	0.34	1.30	5.89	5.33 0.56		5.91 −0.02	
Ambrosin (**21b**)	*r*	205 ± 30	1.03	0.45	1.83	5.74	5.52 0.22		5.88 −0.14	
Aromaticin (**22a**)	*s*	285 ± 70	1.17	0.34	1.38	5.86	5.63 0.23		5.87 −0.01	
Mexicanin I (**23**)	*t*	417 ± 54	0.36	0.33	1.26	5.90	5.03 0.88		5.95 −0.05	
Helenalin (**22b**)	*u*	1,375 ± 135	0.87	0.20	0.76	6.12	5.40 0.72		5.90 0.22	

[a] Median value in μg/ml. [b] Half-maximal effective dose in μmoles/l. [c] Log of reciprocal half-maximal effective dose in moles/l. [d] See ref 11. [e] S. M. Kupchan and R. J. Hemingway, unpublished results. [f] S. M. Kupchan, J. M. Cassady, J. E. Kelsey, H. K. Schnoes, D. H. Smith, and A. L. Burlingame, *J. Amer. Chem. Soc.*, **88**, 5292 (1966); T. A. Dullforce, G. A. Sim, D. N. J. White, J. E. Kelsey, and S. M. Kupchan, *Tetrahedron Lett.*, 973 (1969). [g] S. M. Kupchan, J. E. Kelsey, M. Maruyama, J. M. Cassady, J. C. Hemingway, and J. R. Knox, *J. Org. Chem.*, **34**, 3876 (1969). [h] See ref 18. [i] N. H. Fischer, T. J. Mabry, and H. B. Kagon, *Tetrahedron*, **24**, 4091 (1968). [j] N. H. Fischer and T. J. Mabry, *Chem. Commun.*, 1235 (1967). [k] T. A. Geissman, R. J. Turley, and S. Marayama, *J. Org. Chem.*, **31**, 2269 (1966). [l] See ref 1a. [m] S. M. Kupchan, M. Maruyama, R. J. Hemingway, J. C. Hemingway, S. Shibuya, T. Fujita, P. D. Cradwick, A. D. U. Hardy, and G. A. Sim, *J. Amer. Chem. Soc.*, **93**, 4914 (1971). [n] See ref 10. [o] W. Herz and G. Hogenauer, *J. Org. Chem.*, **26**, 5011 (1961). [p] H. E. Miller and T. J. Mabry, *ibid.*, **32**, 2929 (1967). [q] N. H. Fischer and T. J. Mabry, *Tetrahedron*, **23**, 2529 (1967). [r] W. Herz, H. Watanabe, M. Miyazaki, and Y. Kishida, *J. Amer. Chem. Soc.*, **84**, 2601 (1962). [s] J. Romo and P. Joseph-Nathan, *Tetrahedron*, **20**, 79 (1964). [t] E. Dominguez and J. Romo, *ibid.*, **19**, 1415 (1963). [u] W. Herz, A. Romo Di Vivar J., Romo, and N. Viswanathan, *J. Amer. Chem. Soc.*, **85**, 19 (1963).

Indeed this approach yielded results of considerable interest in our studies of the sesquiterpene lactones. Correlation of log $1/C_{50}$ with log *P* for all 26 compounds (eq 1) was not significant. Equation 1, however, does

$$\log (1/C_{50}) = 0.741(\log P) + 4.758 \quad (1)$$
$$n = 26,\ r = 0.660,\ s = 0.461$$

indicate that, despite the structural variety of the compounds included in the treatment, there is a definite relationship between lipophilic character and cytotoxicity.

Examination of the data (Table I) used to derive eq 1 was quite revealing. Of the 9 compounds containing only an α-methylene-γ-lactone as a potential "active functional group," 8 are less cytotoxic than predicted by eq 1. Of the 17 potentially difunctional compounds 11 are more cytotoxic than costunolide[18] (**11**), the most cytotoxic monofunctional sesquiterpene lactone studied. These observations suggested treatment of the monofunctional compounds as a class by themselves.

The results of the correlation of log $(1/C_{50})$ with log *P* for these 9 compounds are given by eq 2. It is

$$\log (1/C_{50}) = 0.580(\log P) + 4.557 \quad (2)$$
$$n = 9,\ r = 0.870,\ s = 0.218$$

satisfying that about 75% of the variation in cytotoxicity, for these monofunctional compounds, is explained by variation in log *P*, especially considering the variety of structures present. This result does indicate that other parameters are influencing the cytotoxic activity. Even for these simplest sesquiter-

(18) A. S. Rao, G. R. Kelkar, and S. C. Bhattacharyya, *Tetrahedron*, **9**, 275 (1960).

TABLE II

In Vivo ACTIVE SESQUITERPENE LACTONES[a]

Compd	Ref	Tumor[b] system	Dose, mg/kg	Survivors	Animal wt differences, g ($T - C$)	Tumor evaluation,[c] T/C	$T/C \times 100$[c]
Elephantopin (**1a**)	*d*	WM	100	3/4	−21	900/4000	22
			50	4/4	−18	1000/4000	25
			25	3/4	+1	3900/4000	97
		PS	60	6/6	−1.1	12.5/10.0	125
			40	6/6	−1.1	14.0/10.0	140
			20	6/6	−2.4	14.0/10.0	140
Elephantin (**1b**)	*d*	WM	100	4/4	−17	600/5000	12
			50	4/4	−3	2700/5000	54
			25	4/4	+1	3000/5000	60
Eupachlorin acetate (**15**)	*e*	WM	400	3/4	−20	2100/5000	42
			300	3/4	−12	1900/5000	38
			200	4/4	−15	4400/5000	88
Euparotin acetate (**16**)	*e*	WM	100	2/4	−4	1800/7800	Toxic
			75	4/4	−10	1800/7800	23
			55	4/4	−5	9100/12800	71
Vernolepin (**7a**)	*f*	WM	14	2/6	−14	700/5200	Toxic
			12	5/6	−10	1700/5200	32
			10	6/6	−12	2400/5200	46
Liatrin (**18**)	*g*	PS	18	4/6	+2.5	12.0/9.5	126
			8	6/6	−1.4	15.0/9.5	157
			5	6/6	−1.5	15.0/9.5	157
Eupacunin (**19**)	*h*	WM	110	1/4	−8	500/5300	Toxic
			100	4/4	−3	2100/5300	39
			90	3/4	−2	1600/5300	30
		PS	60.0	6/6	−2.2	13.5/10.0	135
			40.0	6/6	−1.0	11.0/10.0	110
			27.0	6/6	−1.2	11.5/10.0	115

[a] Data are taken from the indicated references. [b] WM = Walker 256 im carcinosarcoma in rats, PS = P388 lymphocytic leukemia in mice. [c] For WM, wt of tumor in mg; a reduction in tumor weight to 42% or less is considered active. For PS, median survival time in days; an increase in survival time to 125% or greater is considered active. [d] See ref 10. [e] See footnote *g*, Table I. [f] See ref 11. [g] See ref 1a. [h] See footnote *m*, Table I.

pene lactones, the structures are diverse enough that steric factors, and perhaps electronic factors, undoubtedly vary quite widely. Such variations could quite easily explain the remaining variation in log $(1/C_{50})$.

The potentially "difunctional" sesquiterpene lactones were more complex, in that in addition to the α-methylene-γ-lactone, they contained a second functionality that varied significantly in nature. It was noticed, however, that the 5 compounds containing a conjugated cyclopentenone as the second functionality all had about the same cytotoxicity, even though they varied appreciably in lipophilicity. Correlation of the data for these compounds did reveal that the cytotoxicity was not dependent upon log P (eq 3). Due to the small

$$\log (1/C_{50}) = 0.095(\log P) + 5.982 \quad (3)$$
$$n = 5,\ r = -0.214,\ s = 0.120$$

number of compounds studied in this group, and to the small range of cytotoxicity, this result might not be extendable to all compounds containing conjugated cyclopentenones as the second functionality. However, for the present case, these 5 compounds are unique in that they show no dependence upon log P for cytotoxicity.

The 12 remaining sesquiterpenes contained as the second functionality either an α,β-unsaturated side chain ester or an α-methylene-δ-lactone. Although the correlation of log $(1/C_{50})$ with log P for these compounds (eq 4) was not as good as for the monofunctional compounds, it was significant. The poorer correlation for this group of compounds, as opposed to the monofunctional ones, can be readily explained by the greater structural variety. In addition to a variety of esters and lactones as the second active functionalities, some of them also contain epoxides, chlorohydrins, and other groups. This wide variety of functional groups may easily increase the effects of other parameters which can also influence cytotoxic activity.

$$\log (1/C_{50}) = 1.021(\log P) + 4.545 \quad (4)$$
$$n = 12,\ r = 0.801,\ s = 0.408$$

These correlations show that the presence of a conjugated ester, cyclopentenone, or α-methylene-δ-lactone in addition to the α-methylene-γ-lactone, increases cytotoxicity more than can be explained by changes in lipophilicity. On the basis of the partition studies, sesquiterpene lactones may be divided into 3 classes; monofunctional, those with a conjugated cyclopentenone as the second functionality, and those with either a conjugated side chain ester or α-methylene-δ-lactone as the second functionality.

The results reported above indicate that an increase in cytotoxicity accompanies increased lipophilicity among the sesquiterpene lactones. In terms of *in vivo* antitumor activity, however, this observation may not be helpful. Of the sesquiterpene lactones studied to date only 7 have shown *in vivo* activity (Table II). For these 7 compounds log $(1/C_{50})$ varies from 5.19 to 6.46, and, perhaps coincidentally, log P varies from 0.31 to 1.42. The moderate cytotoxicity

and lipophilic character of these 7 compounds seems to rule out the possibility of achieving enhanced *in vivo* activity by increasing lipophilicity and cytotoxicity. Perhaps more importantly, all 7 of these compounds contain, in addition to the α-methylene-γ-lactone, either an α-methylene-δ-lactone or a conjugated side chain ester as the second functionality, and an OH or *O*-acyl group adjacent to the CH_2 of the γ-lactone grouping. Probably as a result of this, these 7 compounds all show increased rates of cysteine addition, an observation that may only be of secondary importance.

Of the compounds studied, only one more, vernolepin acetate, shares the structural features noted above for the 7 *in vivo* actives and it has not been available in sufficient quantity to allow *in vivo* testing. All of the other sesquiterpene lactones share some but not all of these properties, so it may be that the presence of the indicated structural features may be essential for *in vivo* activity among sesquiterpene lactones.

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Structure and Tumor-Promoting Activity of Anthralin (1,8-Dihydroxy-9-anthrone) and Related Compounds

ALVIN SEGAL, CHARLINE KATZ, AND BENJAMIN L. VAN DUUREN*

Laboratory of Organic Chemistry and Carcinogenesis, Institute of Environmental Medicine, New York University Medical Center, New York, New York 10016

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The compound anthralin, which is used for the treatment of skin diseases, is shown to exist normally in the semiquinone form, *i.e.*, 1,8-dihydroxy-9-anthrone. In Me_2CO or MeOH the compound spontaneously dimerizes and oxidizes to 1,8-dihydroxyanthraquinone. Anthralin dimer has the structure 1,6,7,12,18,21-hexahydroxy-5,12:6,11-di-*o*-benzo[*a*,*e*]cyclooctene. Anthralin is a potent tumor-promoting agent in 2-stage carcinogenesis. Its transformation products and other related compounds were inactive in this assay.

Anthralin (dithranol) has been used for many years for the treatment of psoriasis and related skin diseases.[1] This compound is of interest also because of its reported tumor-promoting activity in 2-stage carcinogenesis[2] on mouse skin and the possible occurrence of compounds of this type in tobacco tars. Anthralin is active in the sebaceous gland suppression test[3] which was at one time thought to be indicative of carcinogenic activity. It is mutagenic in yeast[4] and has been shown by absorption spectroscopic studies to bind to deoxyribonucleic acid.[5] Clinical experience suggests that the compound is irritating to human skin at high doses.[1]

While repeating the mouse skin experiment, we discovered that the accepted tautomeric structure for anthralin[6,7] is incorrect and, furthermore, that anthralin undergoes spontaneous dimerization and oxidation in Me_2CO; acetone is the solvent of choice for chronic mouse skin bioassays with this and many other compounds. Because of our finding that anthralin degrades in acetone solution which was also the solvent used in the earlier study,[2] it was important to reexamine the tumor-promoting activity with freshly prepared solutions of pure anthralin as well as that of the transformation products, *i.e.*, anthralin dimer and 1,8-dihydroxyanthraquinone. The present report describes the clarification of the structure of anthralin, a study of its transformation products, and the bioassay of anthralin and related compounds for tumor-promoting activity on mouse skin.

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Experimental Section

Animals.—Female ICR/Ha Swiss mice (A. R. Schmidt-Millerton Co., Millerton, N. Y.) were used for this experiment. They were vaccinated against ectromelia and started on test at age 7 weeks. Mice were housed on sterile wood chips (Absorb Dri, Fisher and Son, Bound Brook, N. J.), 10 to a cage, fed Purina Laboratory chow and H_2O *ad libitum*, and weighed regularly. The animal rooms were maintained at 22–24°.

Bioassay Procedure.—The backs of the mice were clipped free from hair the day before the initial treatment and then as needed for the duration of the experiment. The solns were all applied by micropipette in the interscapular region; a single treatment with 20 μg of 7,12-dimethylbenz[*a*]anthracene in 0.1 ml of Me_2CO was followed 2 weeks later by 3-times weekly application of anthralin or related compds in 0.1 ml of acetone. The dosages and duration of the experiments are given under Results below. The dosage of anthralin used was based on that used in the earlier study.[2] This is also the maximum tolerable dose. Higher doses were toxic and caused severe skin damage in mice. Animals were observed regularly and the tumors recorded; tumors greater than 1 mm in diameter were counted and charted regularly. Only tumors which persisted for 30 days or more were counted in the cumulative totals. The results presented below are based on these chartings. Animals bearing tumors that appeared grossly to be carcinomas were killed approximately 2 months after the tumors were clinically classified as malignant. All animals were autopsied at death and representative tumors and any gross abnormalities were excised, fixed in unbuffered 4% formalin, blocked in paraffin, stained with hematoxylin and eosin, and confirmed histologically. Included in the experimental protocol were control groups that received promoters alone, sol-