Antitumor Agents. 4.[†,1] Cytotoxicity and *in Vivo* Activity of Helenalin Esters and Related Derivatives

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Sesquiterpene lactones possess certain structural features which appear to be essential for cytotoxic activity.[2-4] An investigation of the structure-cytotoxicity relationship for the linear pseudoguaianolide helenalin (1) revealed that the α-methylene-γ-lactone system was less important than the α,β-unsaturated ketonic moiety for the maintenance of a high level of cytotoxicity.[1] In this paper we have extended our investigation to consider esters of helenalin. It has been inferred that a parent molecule could be made more cytotoxic by increasing the lipophilic character and/or by adding groups (such as conjugated esters) which might act as alkylating agents.[4] As part of our continuing structure-cytotoxicity investigation of helenalin, we decided to study the effect of incorporating the above characteristics into the parent molecule through an ester side chain. Table I lists the esters and dimethylamine adducts of the esters which were synthesized and tested *in vitro* against cells derived from human epidermoid carcinoma of larynx (H.Ep.-2).[§] In addition, Table II lists six of the esters which were tested *in vivo* against the Ehrlich ascites carcinoma.

Cytotoxicity and Structure-Activity Relationships. A comparison of the relative C_{50} values for the cytotoxicity of the compounds listed in Table I illustrates effects on the parent molecule 1. Masking of the C_6-OH as in compound 2 results in decreased cytotoxicity. However, as the lipophilic character of the ester is increased, cytotoxicity is regained (compare compounds 2, 9, 10, and 11).

Additional alkylating groups appear to enhance cytotoxicity (compare haloacetates 3 and 4 to 2). However, cytotoxicity is enhanced significantly when a conjugated ester group is added to the parent molecule (compare compounds 6–8 and 13, 15 and 16 to parent molecule 1). The importance of conjugation in the ester moiety was obvious since destruction of the conjugated system resulted in a marked decrease in activity (compare compounds 7 with 9 and 13 with 14). An aromatic nucleus in conjugation with the ester carbonyl appears to be highly significant (compare compound 13 to parent molecule 1); moreover, the gradation of activity exhibited by compounds 12, 13, and 15 suggests that there is an optimum position for the aromatic nucleus. The significant level of cytotoxicity shown by compound 5 may be attributable to direct alkylation of biological nucleophiles but most likely is associated with facile elimination of HBr to generate compound 6.

Previously it was shown that the α,β-unsaturated ketonic moiety contributed most significantly to the cytotoxicity of helenalin (1). We have observed a similar requirement in the case of the dimethylamine adducts of the esters. The high levels of cytotoxicity exhibited by compounds 17–20 compared to the parent esters (compounds 7, 9, 13, and 14) indicated that the α-methylene-γ-lactone moiety made a minor contribution to cytotoxic activity. Moreover, a comparison of the activities of compounds 17 to 18 and 19 to 20 further verified the importance of conjugation in the ester moiety.

Activity against the Ehrlich Ascites Carcinoma. Six helenalin esters were screened at the maximum effective dose of helenalin (38.2 μmol/kg/day). The results listed in Table II indicate an obvious correlation between cytotoxicity and animal mortality. It is of interest to note that both 5-fluorouracil and helenalin are highly effective against the ascites tumor at the same concentration. Moreover, the fact that all survivors showed essentially 100% inhibition of the ascites tumor would tend to indicate that each compound can act as an effective inhibitor but at a lower dose level than helenalin.

Experimental Section

Biological Methods. The derivatives of 1 were assayed for inhibitory activity *in vitro* against cells derived from human epidermoid carcinoma of larynx (H.Ep.-2) according to a rapid microtiter method previously described.[3,5] For the present report the ED_{50} is expressed in micromolar concentration (C_{50}) in order to facilitate a comparison of the derivatives to 1 in terms of relative cytotoxicity. On this basis a compound listed in Table I exhibiting a relative $C_{50} < 1$ indicates that it is more toxic than 1.

The *in vivo* assay was carried out by testing the compounds *vs.* the Ehrlich ascites carcinoma in CF-1 male mice (initial wt 30–35 g) by minor modifications of a procedure described previously.[6] Both test and control animals received an intraperitoneal injection of 0.1 ml of ascitic fluid, collected from a suitable donor mouse which had borne the ascites carcinoma 6–7 days and diluted with saline to a cell concentration of 10% by volume. Each animal received a dose based on individual weight; *e.g.*, a 34-g test animal received 5 mg/kg in 0.17 ml of vehicle. Similarly a 34-g vehicle control animal received 0.17 ml of vehicle. For each assay the mice were divided into a non-vehicle control group, a vehicle control group, and several experimental groups of ten mice each. The six helenalin esters listed in Table II were assayed at the maximum effective dose established for 1.

Chemical Methods. Unless otherwise specified melting points were determined on a Thomas-Hoover melting point apparatus and are corrected. Ir spectra were determined in $CHCl_3$ with a Perkin-Elmer 257 grating ir spectrophotometer. Nmr spectra were measured in $CDCl_3$ with a Jeolco C-60 HL spectrometer (TMS) or with a Varian HA-100 spectrometer[#] (TMS), and chemical shifts were reported in δ (ppm) units: s, singlet; d, doublet; t, triplet; and m, multiplet; and *J* values in hertz. Silica gel for column chromatography refers to Baker AR No. 3405. Florisil for column chromatography refers to Floridin Co. Florisil 100–200 mesh. Silica gel for tlc refers to Merck silica gel G or GF_{254} developed with $CHCl_3$–acetone (85:15) or $CHCl_3$–EtOAC–Et_2NH (85:15:2) and visualized with 50% H_2SO_4 or uv light. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.

General Method for Ester Synthesis. The esters listed in Table I were synthesized by reaction of 1 with an acid chloride. Acid chlorides that were not commercially available were synthesized by refluxing the appropriate acid with $SOCl_2$. Typically, 300 mg of 1, 20% excess of acid chloride, in 10 ml of dry benzene was refluxed in the absence of moisture until the reaction was complete as determined by tlc. The benzene solution was washed with 5% $NaHCO_3$ and saturated aqueous NaCl, dried (Na_2SO_4), and concentrated *in vacuo* to an oil. If the product could not be isolated by crystallization from the crude oil, purification was accomplished by column chromatography on Florisil or silica gel or by preparative tlc.

Helenalin β-Bromopropionate (5). The title compound was synthesized as indicated above. However, upon completion of the re-

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[§]*In vitro* assay was carried out by E.-S. Huang.

[#]The 100-MHz nmr spectra were obtained by courtesy of J. Younger, Research Triangle Institute, Chemistry and Life Sciences Division, N. C.

Table I. Cytotoxicity of Helenalin Esters and Related Derivatives

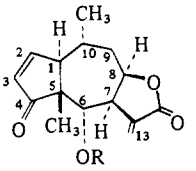
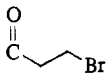
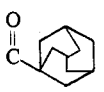
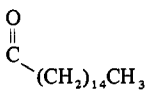
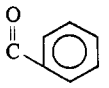
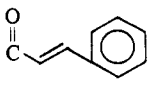
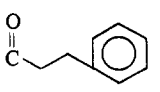
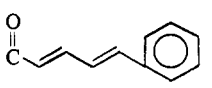
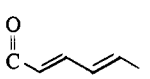
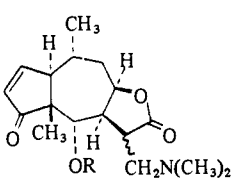
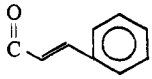
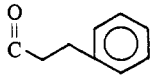
No.	R	Formula	Analyses[a,b]	Mp, °C	Recrystn solvent	Rel C_{50}[c] $\left(\frac{C_{50}\text{ ester}}{C_{50}\text{ helenalin}}\right)$ (H.Ep.-2)
						
1	H	$C_{15}H_{18}O_4$		170–172[d]	Benzene	1.00
2		$C_{17}H_{20}O_5$		180–180.5[e]	CH_2Cl_2–Et_2O	2.42
3		$C_{17}H_{19}O_5Br$	C, H, Br	170–172.5	Ip_2O–$CHCl_3$	1.40
4		$C_{17}H_{19}O_5I$	C, H	*f*		1.61
5		$C_{18}H_{21}O_5Br$	*g*	*h*		0.56
6		$C_{18}H_{20}O_5$	C, H	163–164	EtOH	0.65
7		$C_{20}H_{24}O_5$	C, H	151–152	EtOH	0.55
8		$C_{20}H_{24}O_5$	C, H	*f*		0.58
9		$C_{20}H_{26}O_5$	C, H	*h*		1.39
10		$C_{26}H_{32}O_5$	C, H	237–240 dec	EtOH	0.57
11		$C_{31}H_{48}O_5$	C, H	*h*		0.47
12		$C_{22}H_{22}O_5$	C, H	*f*		0.84
13		$C_{24}H_{24}O_5$	C, H	*f*		0.19
14		$C_{24}H_{26}O_5$	C, H	141–144	EtOH	0.49
15		$C_{26}H_{26}O_5$	C, H	*f*		0.39
16		$C_{21}H_{24}O_5$	*i*	*f*		0.50
						
17		$C_{22}H_{31}O_5N$	C, H, N	196–197	MeOH	0.65

Table I *(Continued)*

No.	R	Formula	Analyses[a,b]	Mp, °C	Recrystn solvent	Rel C_{50}[c] $\left(\frac{C_{50}\text{ ester}}{C_{50}\text{ helenalin}}\right)$ (H.Ep.-2)
18		$C_{22}H_{33}O_5N$	C, H, N	163.5–164.5	EtOH	1.96
19		$C_{26}H_{31}O_5N$	C, H[j,k]	191–192	EtOAc	0.46
20		$C_{26}H_{33}O_5N$	C, H[j,l]	146–146.5	EtOH	1.68

[a]Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. [b]All compounds gave satisfactory nmr and ir data. In all cases H-6 is a sharp s in the range of 4.45–5.57 as compared to broad s at 5.66 for 1 in the nmr spectrum. [c]Half-maximal effective dose. The C_{50} value of helenalin is 0.393 μmol/l. [d]R. Adams and W. Herz, *J. Amer. Chem. Soc.*, 71, 2546 (1949), reported mp 169–172° (benzene). [e]R. Adams and W. Herz, *J. Amer. Chem. Soc.*, 71, 2546 (1949), reported mp 179.5–180.5°. [f]Freeze-dried solids. [g]This compound was shown to be tlc homogeneous and had a molecular ion peak at *m/e* 396.0574 corresponding to $C_{18}H_{21}O_5Br$. [h]Oil. [i]This compound was shown to be tlc homogeneous and had *m/e* 356 (M^+). [j]Insufficient sample for N analysis. [k]*M/e* 437.2191 ($C_{26}H_{31}O_5N$). [l]*m/e* 439.2352 ($C_{26}H_{33}O_5N$).

Table II. Results of Screening Test *vs.* the Ehrlich Ascites Carcinoma[a]

Compd	Dose, μmol/kg/day	Mortality C	Mortality T	Volume T/C, ml	Volume SDT ± ml	Ascitocrit T/C	Ascitocrit SDT ± ml	Av TPCV, T as % of C
1[b]	38.2	2/10	1/10	0.16/6.83	0.31	0/0.228	0	0
1[b]	53.5	2/10	6/10	0/6.83	0	0/0.228	0	0
1[b]	68.8	2/10	4/10	0.13/6.83	0.32	0.005/0.228	0.01	0.04
2[c]	38.2	1/9	2/10	0/4.64	0	0/0.226	0	0
7[c]	38.2	1/9	7/10	0/4.64	0	0/0.226	0	0
9[c]	38.2	1/9	4/10	0/4.64	0	0/0.226	0	0
10[c]	38.2	2/10	9/10	0/5.94	0	0/0.277	0	0
13[c]	38.2	2/10	10/10					
14[c]	38.2	2/10	8/10	0/5.94	0	0/0.277	0	0
FU[c,d]	38.2	1/10	1/10	1.8/6.62	0.71	0.145/0.254	0.056	15.5

[a]T = treated group, C = vehicle control group, TPCV = average total packed cell volume of tumor cells on final day of assay, SD = standard deviation of TPCV of treated group. The average SD of the control group was ±1.27 ml and the average ascitocrit was SD ± 0.053. [b]Vehicle was 0.9% NaCl–DMSO (90:10). [c]Vehicle was 0.9% NaCl–DMSO (20:80). [d]FU = 5-fluorouracil.

action, the solvent was removed *in vacuo* and the crude oil was chromatographed on silica gel.**

Helenalin Acrylate (6). A solution of 1 (200 mg) and acrylic anhydride (0.5 ml) in 5 ml of dry pyridine was allowed to stand 5 hr at room temperature. Excess pyridine was removed *in vacuo* to yield an oil. The product was isolated by column chromatography on Florisil.

Helenalin Dimethylamine Adducts. Compounds 17–20 were synthesized by a procedure described previously.[1]

Helenalin (1). Helenalin (1) was isolated by column chromatography on silica gel of the crude extract of plant material *Helenium autumnale* L.

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References

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**Washing with cold 0.05% $NaHCO_3$ or chromatography of the crude oil on Florisil resulted in facile elimination of HBr to generate compound 6.

Syntheses of Analgesics. 34.[1] Synthesis of 3-Hydroxy-*N*-cyclopropylmethyl-9-azamorphinan (Studies on the Syntheses of Heterocyclic Compounds. 509[2])

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We have reported the synthesis of the 3-hydroxy-9-azamorphinan and N-substituted compounds,[3] some of which showed an analgesic activity, and especially 3-hydroxy-*N*-phenethyl- (**1**) and 3-hydroxy-*N*-cyclopropylmethyl-9-azamorphinans (**2**) were found to have a potent analgesic effect. The modified synthesis and pharmacological activity of **2** were investigated successively. Herein we wish to report these results.

9-Azamorphinan was synthesized by an eight-step procedure from 2-(3-methoxyphenyl)cyclohexanone (**3**) by