volvement of intracellular Ca^{2+} in the hyperpolarization is shown by passing a current during the burst (Fig. 2C). A depolarizing current of 20 na approximately doubled the number of action potentials during the burst and caused a comparable absorbance increase. The hyperpolarization that followed was larger and of longer duration, and the onset of the next burst was delayed until the absorbance had almost returned to its baseline value. This result suggests that the onset of the burst is determined by the fall of the free intracellular Ca^{2+} concentration below some threshold value. When the cell was voltage-clamped after a burst to the average value of its resting potential, the absorbance declined below its baseline value at the time when the next burst was expected (the interval between bursts in R15 is normally quite regular). The next burst occurred immediately after the cell was released from voltage clamp; the frequency of action potentials was somewhat higher than normal, an effect that can be attributed to the internal Ca^{2+} concentration being lower than normal at the start of the burst.

Our experiments demonstrate that changes in intracellular Ca^{2+} play a fundamental role in bursting pacemaker activity. They suggest that the interval between bursts depends on the rate at which free intracellular Ca^{2+} is sequestered or transported out of the cytoplasm. They also suggest, however, that at least one other factor is involved in the generation of the burst. If activation of an outward current by intracellular Ca^{2+} were the only factor, the membrane potential would hyperpolarize during the burst as a result of the increase in intracellular Ca^{2+} concentration, and this is clearly not the case (Fig. 1). The delay in onset of the hyperpolarization until the end of the burst could be due either to inhibition of the Ca^{2+}-activated outward current or to antagonism by a maintained inward current during the burst. Experiments on analogous cells in the mollusk *Helix* (*19*) suggest that the second explanation is more probable, as they provide evidence for a voltage-dependent inward current, showing slow activation and inactivation, which is carried primarily by Ca^{2+} ions.

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14. The magnitude of the membrane potential oscillation associated with each burst was also reduced by treatment with low external Ca^{2+} or by saline solutions containing Mn^{2+} or Co^{2+}. However, at the concentrations used, these treatments did not completely abolish the absorbance or membrane potential changes.
15. The dye was calibrated at 16°C in the presence of 500 m*M* KCl and 2.5 m*M* $MgCl_2$, *p*H 7.3, to match intracellular conditions. Di Polo *et al.* (*11*) give the effective Ca-EGTA/EGTA dissociation constant at this *p*H and ionic strength as $1.5 \times 10^{-7} M$; the additional presence of 2.5 m*M* $MgCl_2$ is not expected to increase it significantly. The calibration was linear over the range studied (0 to $4.5 \times 10^{-7} M$ free Ca^{2+}, based on the dissociation constant above). Although we believe that our calibration gives a resonable estimate of changes in free intracellular Ca^{2+}, it should be realized that there are several potential sources of error in attempting to match in vitro conditions with those in the cell, so the values given can be only approximate.
    We should stress that our dye calibration is based on changes in *free* intracellular Ca^{2+}. The corresponding changes in concentration of the Ca-dye complex (which can be calculated from the molar extinction coefficient of the complex) are somewhat higher, but the presence of the dye at the concentration used in these experiments is unlikely to increase significantly the total intracellular buffering power toward Ca^{2+}. The increase in *total* intracellular Ca^{2+} during the burst may be many times higher than the increase in free Ca^{2+}, but physiologically the free Ca^{2+} level is expected to be the more important parameter.
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Sesquiterpene Antitumor Agents: Inhibitors of Cellular Metabolism

Abstract. *Helenalin and tenulin injected into CF_1 male mice bearing Ehrlich ascites tumors inhibit DNA synthesis and DNA polymerase enzymatic activity in the tumor cells. Helenalin inhibited protein synthesis. Both drugs increased the concentration of adenosine 3',5'-monophosphate, and interfered with glycolytic and mitochondrial energy processes. Cholesterol synthesis was also inhibited, resulting in lower serum cholesterol levels in tumor-bearing animals. Data obtained in vitro indicate that the cyclopentenone-bearing sesquiterpene lactone and related compounds do not alkylate purine bases of nucleic acids but rather undergo a Michael-type addition reaction with the sulfhydryl groups of reduced glutathione and* L-*cysteine. Thus, the inhibition of cellular enzyme activities and metabolism that has been observed with these drugs might be explained by the occurrence of a Michael-type reaction.*

Helenalin (**1**), a sesquiterpene lactone, has been shown to be a potent antitumor agent in the Walker 256 ascites carcinosarcoma and Ehrlich ascites tumor, and marginally active in the P-388 lymphocytic leukemia screen (*1–3*). Its cytotoxic activity has been delineated in the HEp-2 cell line. Studies of the relation between the structure and activity of this compound have established that one of the structural requirements for significant cytotoxic antitumor activity is that the $O{=}C{-}C{=}CH_2$ moiety be part of an ester or part of a ketone or lactone (*4, 5*). Kupchan has demonstrated that the α-methylene-γ-lactone system can act as the alkylating center for cytotoxic antitumor lactones, for example, elephantopin, vernolepin, and euparotin acetate. A Michael-like reaction between biological nucleophiles, such as L-cysteine (*6*) or sulfhydryl-containing enzymes, for example, phosphofructokinase (*7*) and glycogen synthetase (*8*), was proposed. The $O{=}C{-}C{=}CH_2$ system in a ketone unit, for example, the β-unsubstituted cyclopentenone moiety, might act as an essential alkylating center, particularly since the α-methylene-γ-lactone was observed to be less important than the cyclopentenone

moiety for the maintenance of a high level of cytotoxicity. Furthermore, recent studies have established that compounds containing no α-methylene-γ-lactonic moiety, such as plenolin (**2**) (*9*) and tenulin (**3**) (*10*) as well as cyclopentanone (**4**) (*11*), possess significant antitumor activity (*12*).

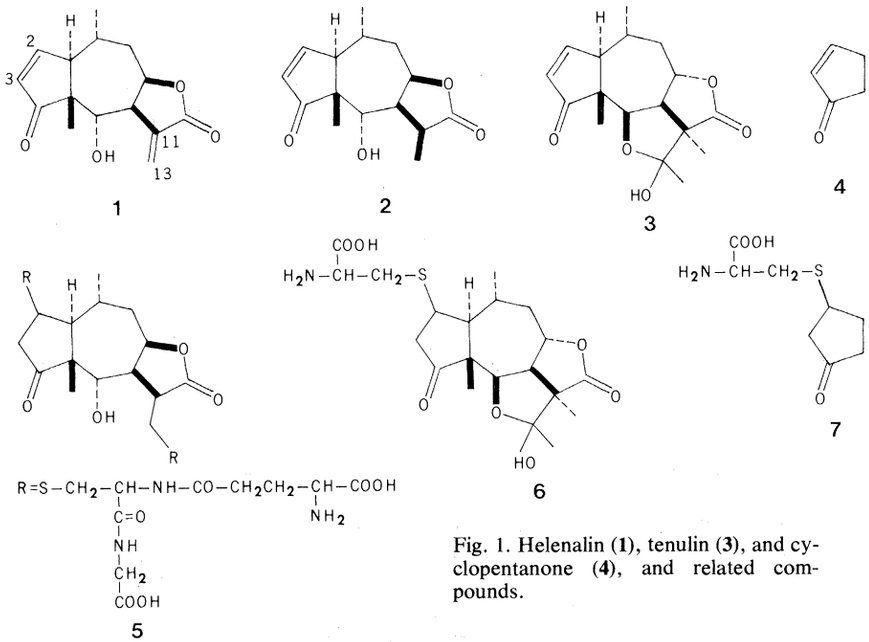

Fig. 1. Helenalin (**1**), tenulin (**3**), and cyclopentanone (**4**), and related compounds.

Table 1. Effects of helenalin and tenulin on the metabolism of Ehrlich ascites cells in CF_1 male mice. Six animals were used in each experiment. Results are expressed as means ± standard deviation. Data for the incorporation of ^{14}C- or ^{3}H-labeled thymidine, uridine, and leucine are expressed as disintegrations per minute (dpm) per milligram of DNA, RNA, and protein, respectively. Free activity of deoxyribonuclease, ribonuclease, and cathepsin activity is based on the micrograms of DNA, RNA, or protein hydrolyzed per minute per milligram of protein of Ehrlich ascites fluid. Cyclic AMP concentration was determined as picomoles per 10^6 cells. Phosphofructokinase and hexokinase activities were computed as optical density changes (of reduced nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide phosphate, respectively) at 340 nm per minute per milligram of protein. Serum cholesterol concentration was expressed as milligrams per 100 ml. HMG-CoA reductase activity is computed as disintegrations of ^{14}C-labeled cholesterol per minute per milligram of protein. Respiration studies were calculated as microliters of O_2 per hour per milligram of protein.

Experiment	Control (0.05 percent Tween-80)	Percentage of control	
		Helenalin (0.125 mg/day)	Tenulin (0.25 mg/kg)
	In vivo		
Incorporation of ^{14}C or ^{3}H			
[^{14}C]-Thymidine into DNA (dpm/mg of DNA)	100 ± 21	12 ± 2*	9 ± 2*
[^{3}H]-Uridine into RNA (dpm/mg of RNA)	100 ± 27	105 ± 51	
[^{14}C]-Leucine into protein (dpm/mg of protein)	100 ± 17	65 ± 14*	110 ± 16
Free activity of			
Deoxyribonuclease	100 ± 8	25 ± 8*	29 ± 15*
Ribonuclease	100 ± 16	32 ± 13*	40 ± 25*
Cathepsin	100 ± 32	19 ± 17*	22 ± 8*
Cyclic AMP concentration	100 ± 49	292 ± 148*	236 ± 92*
Enzyme activity			
Phosphofructokinase	100 ± 39	55 ± 16*	
Hexokinase activity	100 ± 32	17 ± 2*	
Serum cholesterol concentration	100 ± 6	61 ± 9*	
	In vitro		
HMG-CoA reductase activity	100 ± 24	63 ± 3*	36 ± 8*
Respiration studies			
Basal (state 4)	100 ± 3	72 ± 8*	52 ± 6*
ATP stimulated (state 3)	100 ± 4	60 ± 8*	64 ± 5*

*$P \leq .001$ in Student's *t*-test.

The exact mechanism of inhibition by helenalin (**1**) and tenulin (**3**) on cellular metabolism has not been explored. Compounds **1** and **3** (Fig. 1) were administered intraperitoneally to CF_1 male mice bearing Ehrlich ascites tumors on days 5, 6, and 7. On day 8, the animals were killed 1 hour after the administration of [^{14}C]-thymidine, [^{14}C]leucine, or [^{3}H]uridine, and the ascites fluid was collected. The incorporation of [^{14}C]thymidine into DNA (*13*) was significantly inhibited (Table 1) by helenalin (88 percent) and tenulin (91 percent). The incorporation of [^{3}H]uridine into RNA (*14*) was unaltered by helenalin administration. The incorporation of [^{14}C]leucine into protein (*15*) was inhibited by helenalin (35 percent), whereas tenulin had little effect. Studies on DNA metabolism and gene activity have demonstrated that compounds **1** and **3** inhibit nuclear DNA polymerase activity and the phosphorylation of histones and nonhistones (*1*, *2*). The catabolism of these macromolecules was not accelerated by compounds **1** and **3**. In fact, both compounds inhibited the hydrolytic activity of deoxyribonuclease (*16*), ribonuclease, and cathepsin (*p*H 5.0) (*17*). Helenalin and tenulin caused a significant increase in the concentrations of adenosine 3′,5′-monophosphate (cyclic AMP) (*18*) in Ehrlich ascites cells. Increased intracellular concentrations of cyclic AMP are associated with a reversal of tumor morphology (*19*), a reduction of cellular proliferation, and an enhancement of cellular differentiation (*20*). Kupchan and coworkers have indicated that α-methylene-γ-lactones inhibit respiratory glycolytic enzymes (*7*, *8*). Thus, the effects of compound **1** on the phosphofructokinase (E.C. 2.7.1.11) (*21*) and hexokinase (E.C. 2.7.1.1) (*22*) activity of 8-day Ehrlich ascites cell were determined. Helenalin treatment resulted in 45 percent inhibition of phosphofructokinase activity and 83 percent inhibition of hexokinase. The aerobic respiration of Ehrlich ascites cells in the presence of either helenalin (0.2 mg) or tenulin (0.2 mg) was examined, with succinate being used as the substrate. Both compounds inhibited basal respiration significantly, as well as adenosine triphosphate (ATP)–stimulated respiration (*23*). Further examination of these mice showed that the hypercholesteremic state (*24*) of tumor-bearing animals was reduced by 39 percent by helenalin. Compounds **1**, **2**, **3**, and **4** caused significant inhibition of the activity of β-hydroxy-β-methyl glutaryl-coenzyme A (HMG-CoA) reductase [^{14}C-labeled acetate incorporation into cholesterol (*12*)], with tenulin (**3**) having superior inhibitor

activity. Previous data from ultraviolet analysis has indicated that helenalin and tenulin do not bind or intercalate with deoxyguanosine 5′-monophosphate, deoxyguanosine 5′-triphosphate, or DNA. Data obtained from nuclear magnetic resonance (NMR) studies confirm that there is no alkylation of the N-7 position of guanosine by helenalin or tenulin (*1*). Since many of the above cellular enzymes, for example, hexokinase and DNA polymerase, contain free SH groups, further effort was made to determine whether thiol groups were the sites of drug action.

Helenalin (**1**; 10 mg) was homogenized with reduced glutathione (10 mg) in deuterated water. After the mixture was allowed to stand at room temperature for 4 hours, the NMR spectrum (XL-100 Fourier transformed) of the supernatant revealed the complete disappearance of the characteristic signals for olefinic protons in the lactone (two H-13) as well as the ketone (H-2 and H-3). This indicated that reduced glutathione not only reacted with the expected α-methylene grouping of the γ-lactone, but also with the double bond of the cyclopentenone ring which would theoretically result in structure **5**. A similar result was also obtained when helenalin reacted with cysteine.

To test further this type of reaction with a cyclopentenone-bearing sesquiterpene lactone, equimolar quantities of L-cysteine hydrochloride (58 mg) and tenulin (**3**; 100 mg) were allowed to react in a mixture of ethanol and water (3 : 7 by volume; total volume, 1 ml) for 7 days, whereupon a thin-layer chromatogram (1-butanol, acetic acid, water; 3 : 1 : 1 by volume) of the reaction mixture showed, in addition to tenulin, cysteine, and cystine, a major product with an R_F value higher than that of cysteine and cystine but lower than that of tenulin. The major product was observed by thin-layer chromatography as a ninhydrin-positive spot, indicating an amino acid character. Identical results were obtained when this reaction was carried out in aqueous phosphate buffer at *p*H 7.4. The major reaction product which separated from the buffered reaction mixture was isolated as a white solid, melting point 231°C (decomposed), and analyzed for $C_{20}H_{29}NO_7S$. The infrared spectrum of the product was suggestive of its structural character (**6**); the product exhibited strong absorption at 1775 cm^{-1}, characteristic of the γ-lactone unit, but it lacked the 1705 cm^{-1} cyclopentenone absorption characteristic of tenulin. In addition, the sharp 3380 cm^{-1} hydroxyl absorption in tenulin was replaced in the adduct by a broadened absorption (3200 to 2400 cm^{-1}) characteristic of an amino acid residue. Furthermore, infrared signals at 1610 and 1580 cm^{-1} in the spectrum of cysteine hydrochloride are present in the reaction product (1600 cm^{-1}) but entirely absent in tenulin. The cyclopentanone absorption of the adduct **6** was seen at 1730 cm^{-1}.

That an addition reaction between cysteine and tenulin had occurred with destruction of the enone grouping is further supported by ultraviolet studies (Coleman-Hitachi 124). To $2.6 \times 10^{-4}M$ cysteine in 0.03*M* phosphate buffer (*p*H 7.2), prepared in a 1-cm quartz cell (volume 3.60 ml) was added 40 μl of $9.8 \times 10^{-3}M$ tenulin in phosphate buffer (*p*H 7.2). The resultant solution was stirred rapidly and the tenulin cyclopentenone absorbance at 225 nm was measured as a function of time. At the end of 4 days this absorbance had decreased by 30 percent of its original value. In a blank determination run as described above but without cysteine, the 225-nm absorbance fell by only 8 percent after 4 days. The formation of adduct **6** is indicated by these data. The small decrease in absorbance observed in the blank after 4 days is not unexpected because the slow addition of an oxygen nucleophile to an enone system has been observed for other sesquiterpene ketones (*25*).

Further direct evidence for the ability of cyclopentenone to form an adduct with cysteine was obtained by treatment of an equimolar amount of cyclopentenone with cysteine in deuterated water at room temperature. After 10 minutes, the NMR spectrum of the reaction mixture indicated the complete disappearance of the olefinic protons. The adduct (**7**), which was isolated as a white powder, melting point 168° to 170°C (decomposed), was analyzed for $C_8H_{13}NO_3S$ by evaporation of the solvent followed by recrystallization from 80 percent hot ethanol. Compound **7** showed a positive ninhydrin test, a strong infrared absorption band at 1742 cm^{-1} (cyclopentanone C=O), and exhibited NMR (D_2O) signals at δ 3.12 (2H, *d*, J = 8 hertz, $SC\underline{H}_2CH$–COOH) and 3.93 (1H, *t*, J = 8 hertz, $SCH_2C\underline{H}$–COOH).

The rapid reaction of cyclopentenone with glutathione in deuterated water was also observed. However, no reaction between cyclopentenone and histidine was seen after the reaction was allowed to stand overnight at room temperature, suggesting that the alkylation favors the sulfhydryl groups bearing enzymes or proteins rather than the imidazole functional group.

Although the evidence that thiol groups of cellular enzymes are the sites of alkylation by β-unsubstituted cyclopentenone and α-methylene-γ-lactones is not conclusive, and since our analyses were conducted in vitro so that we cannot exclude the possibility of metabolites of the drug binding the DNA, certainly the feasibility of this reaction is high and the theory is attractive.

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26. Although compounds **2** and **3** still possess an α-methyl-γ-lactonic moiety which might be regarded as a reactive functional group, our previous work has shown that compounds such as 2,3,11,13-tetrahydrohelenalin (*5*), which contains this moiety, lack significant cytotoxicity in vitro.
27. This investigation was supported by grants from the National Cancer Institute (CA-12360) and the American Cancer Society (CH-19) to K.-H.L. and (IN 15-P) to I.H.H. This report is part 20 in the series "Antitumor Agents"; for part 19 see K.-H. Lee, Y. Imakura, D. Sims, A. T. McPhail, K. D. Onan, *J. Chem. Soc. Commun.* (1976), p. 341.

28 September 1976

Photoreceptor Outer Segments: Accelerated Membrane Renewal in Rods After Exposure to Light

Abstract. *The rate of rod outer segment renewal in* Rana pipiens *tadpoles under constant light and under diurnal conditions of 12 or 2 hours light per day is significantly increased compared to that in animals in darkness. Furthermore, during 24 hours in light after 6 days in darkness the rate of renewal is three to four times that in darkness. In* Xenopus laevis *tadpoles the rate of renewal is more than five times greater during the first 8 hours of a normal diurnal cycle than during the following 16 hours. These observations demonstrate that bursts of renewal activity occur as a response to light, and suggest that a normal pattern of light alternating with darkness plays a fundamental role in the regulation of rod outer segment turnover.*

The rod outer segment (ROS) turnover process involves production and assembly of new membrane disks at the base coupled with shedding of groups of older disks at the distal tip where they are phagocytized and degraded by the pigment epithelium (*1–3*). Evidence for this concept comes from autoradiographic studies of eyes labeled with tritiated amino acids. In such studies a discrete radioactive band is formed at the base of the ROS and is gradually displaced toward the tip; eventually the label appears within phagosomes of the pigment epithelium. Shedding occurs intermittently, and light plays a major role in initiating the process (*4*). However, at a given temperature the addition of new disks is believed to proceed at a nearly constant rate that is modified only slightly by light (*2, 3*).

We obtained evidence that rods exposed to light after long-term dark adaptation responded with massive shedding followed by rapid restoration of ROS length (*4, 5*). Tadpoles exposed to light for 24 hours after 7 days of dark adaptation showed a rapid decline (within 2 hours) of visual pigment concentration and ROS length which was paralleled by a great increase in the number of phagosomes within the pigment epithelium. During the following 22 hours, however, visual pigment concentration and ROS length were restored to control levels. We estimated that restoration of ROS length in this experiment would have required a three- to fourfold increase in the rate of renewal. The present study was initiated to examine the effects of light on ROS renewal by autoradiography.

To obtain baseline data on the effects of different lighting conditions on renewal, *Rana pipiens* tadpoles (*6*) each received an intraperitoneal injection of 25 μc of [4, 5-^{3}H] L-leucine (New England Nuclear, specific activity 5.0 c/mmole). During the week before and the first 24 hours after injection, animals were kept at room temperature (22° to 24°C) and were exposed to light for approximately 12 hours daily. Twenty-four hours after injection some of the eyes were fixed for autoradiography (*7*). The remaining tadpoles were maintained in incubators for 12 additional days at 23° ± 0.5°C in constant light (*8*), constant darkness, or in cyclic conditions of 12 or 2 hours of light per day. Samples of eyes from each group were fixed 7 and 13 days after injection. From autoradiographs, measurements were made from the base of the outer segment to the scleral edge of the radioactive band (*9*).

Displacement of the radioactive band was consistently greater in light than in darkness both 7 and 13 days after injection (Fig. 1A), which indicates that cyclic light of low intensity and short daily duration significantly stimulates ROS renewal. The average rate of displacement was increased by 54 percent from a low of 0.63 μm/day in darkness to 0.97 μm/day in constant light. At these rates band displacement was respectively 9.1 ± 0.12 μm (mean ± standard error) and 13.1 ± 0.47 μm by the end of the experiment (Fig. 1A). However, the magnitude of the effect of light on renewal was not proportional to the duration of light exposure each day. Thus, 2 hours of light per day was sufficient to produce 44 percent of the effect observed in constant light after 12 days as opposed to the 8 percent expected if the light effect were precisely additive. In addition, the rate of band displacement was higher in all treatments during the first half of the experiment than during the second half. For example, among animals receiving 12 hours light per day the rate of band displacement was 1 μm per day between days 1 and 7 and 0.63 μm/day thereafter (see Fig. 1B) yielding an overall average renewal rate of 0.83 μm/day (*10*).

To measure the effects of light on band displacement in animals previously dark-adapted for 6 days, some of the tadpoles in the previous experiment were exposed to light on day 7 and kept for one to four additional days after fixation. The results confirmed our previous suspicion that light exposure after long-term dark adaptation results in a great increase in ROS renewal. During 24 hours in constant light after 6 days in darkness the radioactive band advanced 2.6 μm, which corresponds to a rate four times that in darkness and more than two and a half times that in constant light. Likewise, during the first 24 hours on a diurnal cycle of 12 hours of light and 12 hours of darkness after 6 days in darkness, the radioactive band advanced 2.0 μm (Fig. 1B), which corresponds to a rate three times that in darkness and two times that in constant light. The high rate of renewal measured during the first 24 hours in this group was not sustained, however. Instead, it declined during the second through fourth days after light exposure to a level comparable to that of control animals receiving 12 hours of light per day (Fig. 1B).

These results indicate that a burst of ROS renewal occurs on exposure to light, and suggest that under normal diurnal conditions there is a daily fluctuation in the rate of renewal. Because of the relatively low renewal rate in *R. pipiens* tadpoles, however, it is difficult to measure