Structure–Activity Relationships among *In Vivo* Active Germacranolides

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Abstract □ Nine structurally related germacranolides from *Eupatorium semiserratum* and *Eriophyllum confertiflorum* were assayed in two standard tumor systems (PS and KB) to determine the structural features required for *in vivo* antileukemic activity. The moieties necessary for *in vivo* activity were found to be an α,β-unsaturated ester side chain adjacent to the γ-lactone and either a primary or secondary allylic alcohol or both.

Keyphrases □ Germacranolides—isolated from *Eupatorium semiserratum* and *Eriophyllum confertiflorum*, antineoplastic activity evaluated □ *Eupatorium semiserratum*—germacranolides isolated, antineoplastic activity evaluated □ *Eriophyllum confertiflorum*—germacranolides isolated, antineoplastic activity evaluated □ Antineoplastic activity—germacranolides isolated from *Eupatorium semiserratum* and *Eriophyllum confertiflorum* evaluated □ Structure–activity relationships—germacranolides isolated from *Eupatorium semiserratum* and *Eriophyllum confertiflorum* evaluated for antineoplastic activity

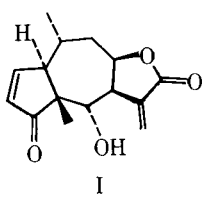
The discovery of antineoplastic activity in various sesquiterpene lactones derived from plant sources has stimulated considerable synthetic and biological interest in these compounds, and several studies relating structural features to cytotoxicity were reported recently (1–5). However, with the exception of the pseudoguianolide helenalin (I) (6, 7), few reports correlated structural requirements with *in vivo* activity.

The relatively large amounts of material necessary for *in vivo* testing (because of the high doses required to show activity) has imposed serious limitations on structure–activity studies of many compounds, especially natural products, which are available only in small quantities. Comparisons of compounds with diverse structural differences and functionalities that have potentially different modes of action can also complicate the interpretation of test results. Therefore, the study reported here concentrated on eriofertin (II), eriofertopin (V), and deacetyleupaserrin (VIII), germacranolides that are structurally similar and that have shown significant *in vivo* activity[1] against P-388 lymphocytic leukemia in the mouse (Table I).

The compounds are multifunctional. They contain an α-methylene butyrolactone ring, a functionality that has been shown to be necessary for cytotoxicity (1), and an unsaturated ester moiety adjacent to the lactone ring. Oxygenation in the form of either an OH or *O*-acyl group adjacent to the α-methylene butyrolactone moiety has been shown to increase the rate of cysteine addition to the butyrolactone ring (1) and may account, in part, for the significant *in vivo* activity. These compounds also contain primary and secondary allylic alcohols, which may serve as potential alkylation sites for a nucleophile on a biological macromolecule.

EXPERIMENTAL[2]

Deacetyleupaserrin (VIII), eupaserrin (IX), and acetyleupaserrin (X) were available from *Eupatorium semiserratum* DC (Asteraceae) as described previously (9).



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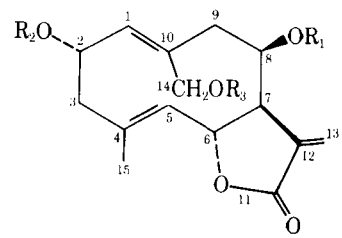
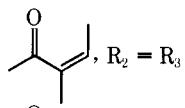
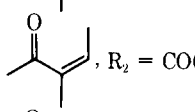
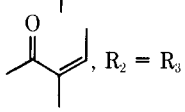
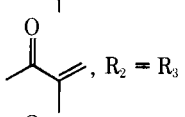
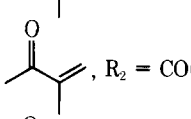


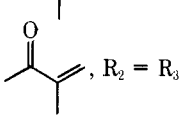
II: R_1 =  , $R_2 = R_3 = H$

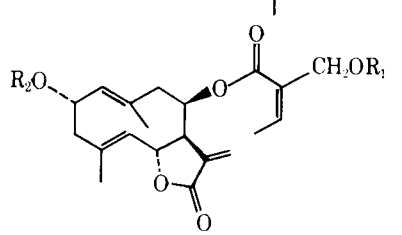
III: R_1 =  , $R_2 = COCH_3$, $R_3 = H$

IV: R_1 =  , $R_2 = R_3 = COCH_3$

V: R_1 =  , $R_2 = R_3 = H$

VI: R_1 =  , $R_2 = COCH_3$, $R_3 = H$

VII: R_1 =  , $R_2 = R_3 = COCH_3$



VIII: $R_1 = R_2 = H$
IX: $R_1 = COCH_3$, $R_2 = H$
X: $R_1 = R_2 = COCH_3$

[1] Cytotoxicity and *in vivo* activity were assayed under the auspices of the National Cancer Institute according to standard protocols (8). Compounds II–X show cytotoxic activity against KB cell culture in the range of $ED_{50} = 10–10^{-1}$ μg/ml.

[2] Melting points were determined on a Mettler FP2 melting-point apparatus and are uncorrected. UV absorption spectra were determined on a Beckman model DK-2A recording spectrophotometer. IR spectra were determined on a Perkin-Elmer model 257 recording spectrophotometer. NMR spectra were determined on a JEOL PS-100 p FT NMR spectrometer interfaced to a Texas Instruments JEOL 980A computer, with tetramethylsilane as the internal standard. Mass spectra were determined on Hitachi Perkin-Elmer model RMU-6E and AEI model MS902 spectrometers at the University of Virginia. Values of $[\alpha]_D$ were determined on a Perkin-Elmer model 141 polarimeter.

Table I—Activity of II–X against P-388 Lymphocytic Leukemia in the Mouse

Compound	Dose, mg/kg	Weight Difference[a]	T/C, %[b]
II	40	−2.4	130
	20	−2.2	148
	10	−2.0	121
III	30	0.7	86
	15	1.2	93
	7.5	0.7	98
IV	40	1.7	126
	20	−1.1	109
	10	0.9	95
V	40	−3.6	Toxic
	20	−2.0	167
	10	−1.4	159
VI	30	−0.8	130
	15	0.2	120
	7.5	1.3	115
VII	30	−0.2	106
	15	−0.7	100
	7.5	−1.9	101
VIII	18	−2.1	150
	12	−0.2	155
	8.0	−0.4	160
IX	44	−2.4	150
	30	−2.3	145
	20	−2.1	122
X	30	−2.0	123
	15	−1.7	123
	7.5	−0.6	101

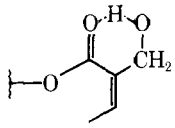
[a] The difference, in grams, between the weights of test and control animals. [b] The ratio (expressed as a percent) of the median survival time of the treated group of mice to the median survival time of the control group. A T/C ≥ 125% indicates activity in the National Institutes of Health protocol (8).

Eriofertopin (V) and 2-*O*-acetyleriofertopin (VI) were isolated from *Eriophyllum confertiflorum* Gray (Asteraceae)[3], and eriofertopin diacetate (VII) was prepared from eriofertopin as described previously (10). Eriofertin (II), 2-*O*-acetyleriofertin (III), and eriofertin diacetate (IV) were isolated from *E. confertiflorum* or prepared as described below. Compounds II–IV had all been reported previously (11), but complete spectral data for II and III were not given.

Isolation Procedure—Fractionation of a 95% ethanol (soxhlet) extract of *E. confertiflorum* (1 kg), guided by assays against human carcinoma of the nasopharynx cell culture (KB) and P-388 lymphocytic leukemia in the mouse (PS), showed the activity to be successively concentrated in the chloroform phase of a chloroform–water partition, the 10% aqueous methanol phase of a 10% aqueous methanol–*n*-hexane[4] partition, the 20% aqueous methanol phase of a 20% aqueous methanol–carbon tetrachloride partition, and the chloroform phase of a chloroform–40% aqueous methanol partition. Column chromatography of the final chloroform partition on silica gel 60[5] yielded two active fractions.

Eriofertin (II)—Extensive preparative TLC of the more polar fraction on silica gel 60 gave a more polar compound, eriofertopin (V), as described previously (10), and a less polar compound, eriofertin (II), as a brittle white foam, $[\alpha]_D^{21}$ +70° (c, 1.0 in $CHCl_3$); IR ($CHCl_3$): 3430, 1765, and 1725 cm^{-1}; NMR ($CDCl_3$): δ 1.70 (3H, s, 4-CH_3), 1.87 (3H, d, J = 1.2 Hz, 2′-CH_3), 1.97 (3H, d, J = 8 Hz, 3′-CH_3), 2.99 (1H, m, 7-H), 3.37 (2H, dd, J = 5.4, 14.4 Hz, 9-H_2), 3.76, 4.26 (2H, 2d, J = 12.7 Hz, 14-H_2), 4.83 (1H, dt, J = 5.8, 10 Hz, 2-H), 5.05 (3H, m, 1-H, 5-H, 6-H), 5.64, 6.33 (2H, 2d, J = 3.4 Hz, 13-H_2), 5.82 (1H, m, 8-H), and 6.13 (1H, dq, J = 1.2, 8 Hz, 3′-H) ppm; mass spectrum: *m/e* 363.1793 (M^+ + H, calc. for $C_{20}H_{27}O_6$, 363.1808).

2-*O*-Acetyleriofertin (III)—Extensive preparative TLC of the less polar column fraction on silica gel 60 gave a more polar compound, 2-*O*-acetyleriofertopin (VI), as described previously (10), and a less polar compound, 2-*O*-acetyleriofertin (III), as an oil, $[\alpha]_D^{21}$ +45.4° (c, 1.0 in $CHCl_3$); IR ($CHCl_3$): 3500, 1765, 1733, and 1725 cm^{-1}; NMR ($CDCl_3$): δ 1.77 (3H, s, 4-CH_3), 1.86 (3H, d, J = 1.2 Hz, 2′-CH_3), 1.97 (3H, d, J = 8 Hz, 3′-CH_3), 2.08 (3H, s, 2-$OCOCH_3$), 2.97 (1H, m, 7-H), 3.19 (2H, dd, J = 5.6, 14.0 Hz, 9-H_2), 3.75, 4.40 (2H, 2d, J = 13.7 Hz, 14-H_2), 5.10 (3H, m, 1-H, 5-H, 6-H), 5.66, 6.33 (2H, 2d, J = 3.4 Hz, 13-H_2), 5.75 (1H, m, 2-H), 5.82 (1H, m, 8-H), and 6.13 (1H, dq, J = 1.2, 8 Hz, 3′-H) ppm; mass spectrum: *m/e* 405 (M^+ + H).



A

Eriofertin Diacetate (IV)—Eriofertin (5.0 mg) was treated with 0.25 ml of acetic anhydride and 0.5 ml of pyridine in 1.0 ml of benzene for 20 hr at room temperature, affording IV (2.2 mg) [identical with reported melting point, IR, NMR, and mass spectral data (11)].

RESULTS AND DISCUSSION

As can be seen in Table I, acetylation had a pronounced effect on the antileukemic activities of eriofertin (II), eriofertopin (V), and deacetyleupaserrin (VIII). Eupaserrin (IX), in which the primary allylic alcohol of the sarracinate moiety had been acetylated, was approximately as active as the diol but at a much higher dose. This activity could be a result of the more efficient transport of VIII compared to IX to the active site. When both alcohols of VIII were acetylated, the antileukemic activity was lost. This same effect was noted with both II and V.

2-*O*-Acetyleriofertin (III) and 2-*O*-acetyleriofertopin (VI), compounds in which the secondary allylic alcohol was acetylated, showed either a lack of or greatly diminished antileukemic activity. Acetylation of both alcohols in II and V, affording eriofertin diacetate (IV) and eriofertopin diacetate (VII), respectively, also resulted in greatly diminished *in vivo* activity. These results suggest that the primary allylic alcohol and, particularly, the secondary allylic alcohol contribute to the high *in vivo* activity of these compounds.

The ester moieties also exhibit some effect on the antileukemic activity, with the compounds containing methacrylate (V) and sarracinate (VIII) esters being somewhat more active than those with angelate (II) and acetylsarracinate (IX) esters. The reduced activity of II as compared to V may be a result of steric hindrance to the addition of a biological nucleophile by the larger β-methyl group of the angelate in II as compared to the smaller β-hydrogen of the methacrylate moiety in V. In the acetylsarracinate moiety of IX, the possibility of intramolecular hydrogen bonding such as Structure A in the sarracinate moiety of VIII, which might facilitate conjugate addition, has been eliminated, decreasing the rate of addition of a biological nucleophile to the acetylsarracinate group.

It is apparent that an α-methylene butyrolactone ring alone is not sufficient for *in vivo* antileukemic activity among the germacranolides discussed. An α,β-unsaturated ester side chain adjacent to the γ-lactone and a primary or secondary allylic alcohol appear to be vital for high *in vivo* activity. These and analogous functional groups can be found in most of the germacranolides and many of the other sesquiterpene lactones that have demonstrated significant *in vivo* activity.

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[3] Stems, leaves, and flowers were collected in California in April 1973. Dr. Robert E. Perdue, Jr., U.S. Department of Agriculture, Beltsville, Md., supplied the dried plant material in accordance with the program developed by the National Cancer Institute. Voucher specimen SPJ-3303 is on deposit at the Beltsville Agricultural Research Center of the U.S. Department of Agriculture, Beltsville, Md.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XXXV: Examination of Possible Pharmacokinetic Interaction between (*R*)-(+)- and (*S*)-(−)-Warfarin in Humans

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Received August 22, 1977, from the *Department of Pharmaceutics, State University of New York at Buffalo, Amherst, NY 14260, the ‡Department of Medicine, Santa Clara Medical Center, San Jose, CA 95128, and the §Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261. Accepted for publication September 20, 1977.

Abstract □ The elimination kinetics and anticoagulant effect produced by single 1.5-mg/kg doses of (*R*)-(+)-, (*S*)-(−)-, and racemic warfarin were determined in 10 healthy men. The results obtained in experiments with the individual enantiomers were used to predict the elimination kinetics and anticoagulant effect of racemic warfarin, assuming that there is no interaction between the two enantiomers. These predictions were compared to experimental results, and no significant differences were observed. This finding suggests that there are no pronounced pharmacokinetic or pharmacodynamic interactions between single large doses of (*R*)-(+)- and (*S*)-(−)-warfarin in humans.

Keyphrases □ Warfarin—(*R*)-(+)- and (*S*)-(−)-forms, elimination kinetics and anticoagulant effect compared, humans □ Elimination kinetics—(*R*)-(+)- and (*S*)-(−)-warfarin compared, humans □ Anticoagulant effect—(*R*)-(+)- and (*S*)-(−)-warfarin compared, humans □ Pharmacokinetics—(*R*)-(+)- and (*S*)-(−)-warfarin compared, humans □ Enantiomers—(*R*)-(+)- and (*S*)-(−)-warfarin, elimination kinetics and anticoagulant effect compared, humans

The anticoagulant warfarin is used therapeutically in the racemic form. The constituents of the racemic mixture, (*R*)-(+)- and (*S*)-(−)-warfarin, differ in pharmacokinetic characteristics, anticoagulant potency, and metabolic fate (1–9). The plasma warfarin concentrations of rats after intravenous administration of a dose of racemic warfarin are essentially equal to the sum of the concentrations observed after separate administration of half of the dose of each individual enantiomer, suggesting that one enantiomer does not measurably affect the pharmacokinetics of the other (8). On the other hand, Chan *et al.* (3) observed that the metabolic pattern of single doses of racemic warfarin in humans is not equivalent to the sum of the patterns obtained from administration of the individual enantiomers, suggesting an interaction between the two enantiomers (3).

To examine the possible pharmacokinetic and pharmacodynamic implications of the apparent metabolic interaction in humans, the kinetics of elimination and anticoagulant effect of a single dose of racemic warfarin were compared with the kinetics predicted on the basis of results obtained by separate administration of the individual enantiomers.

EXPERIMENTAL

Ten healthy men, 21–51 years old, received single oral doses of (*R*)-(+)-, (*S*)(−)-, and racemic warfarin, 1.5 mg/kg, at intervals of at least 1 month. Daily blood samples were obtained for 9 days, and the warfarin concentration and the prothrombin complex activity were determined in plasma. Details of these procedures were described previously (9).

The apparent volume of distribution, V_d, the elimination rate constant, k_{el}, and the total body clearance, *TC*, were determined (10), and the synthesis rate of prothrombin complex activity, R_{syn}, was calculated (11). The pharmacokinetic and pharmacodynamic data obtained following separate administration of the two enantiomers were used to make quantitative predictions of pharmacokinetic and pharmacodynamic patterns of single doses of racemic warfarin (6, 8).

RESULTS AND DISCUSSION

The total body clearances of (*R*)-(+)- and (*S*)-(−)-warfarin were 1.30 ± 0.47 and 2.08 ± 0.70 ml/hr/kg (mean ± *SD*), respectively. The individual data are reported elsewhere (10). The experimentally determined and predicted individual values of k_{el}, V_d, and *TC* for racemic warfarin are presented in Table I. There were no statistically significant differences between the experimental and predicted values; the correlation between

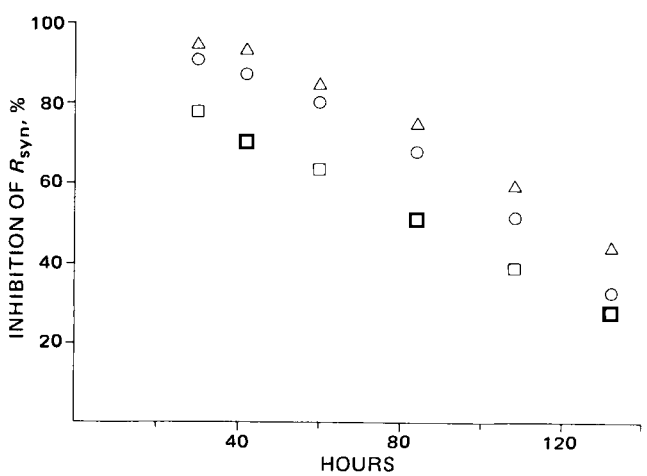

Figure 1—*Time course of inhibition of prothrombin complex activity synthesis rate, R_{syn}, produced by a single oral 1.5-mg/kg dose of* (S)-(−)-*warfarin* (△), *racemic warfarin* (○), *and* (R)-(+)-*warfarin* (□). *Average of data from 10 subjects.*