Structure−Cytotoxicity Relationships of Some Helenanolide-Type Sesquiterpene Lactones

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This study deals with the cytotoxicity of helenanolide-type (10α-methylpseudoguaianolide) sesquiterpene lactones. We determined the influence of substitution patterns on the toxicity of 21 helenanolides to a cloned Ehrlich ascites tumor cell line, EN2. Within a series of helenalin esters, the acetate (**2**) and isobutyrate (**3**) were more toxic than helenalin itself (**1**). Esters with larger acyl groups (tiglate **4** and isovalerate **5**) exhibited a decreased toxicity compared with the parent alcohol (**1**). Similar relationships were observed between the 6,8-diastereomer of helenalin, mexicanin I (**6**) and its acetate (**7**) and isovalerate (**8**). In contrast, cytotoxicity within a series of 11α,13-dihydrohelenalin esters (**9**−**12**) was shown to be directly related to the size and lipophilicity of the ester side chain, dihydrohelenalin (**9**) being the least toxic compound in this group. Investigation of several 2,3-dihydrohelenalin derivatives (**13**−**21**) with 2α-hydroxy-4-oxo- and 2α,4α-dihydroxy- or -*O*-acyl-substituted cyclopentane rings (arnifolins and chamissonolides, respectively), for which no pharmacological data have been reported so far, revealed further interesting influences of the substitution pattern on cytotoxicity. The results may be interpreted in terms of lipophilicity and steric effects on the accessibility of the reactive sites considered responsible for biological activity.

Sesquiterpene lactones are natural products occurring in many plant families, but most widely distributed within the Compositae (Asteraceae). These compounds are known for their various biological activities, including cytotoxicity to tumor cells.[1] Alkylation of biological nucleophiles by α,β-unsaturated carbonyl structures in a Michael-type addition is considered to be the general mechanism of action. Covalent binding of sesquiterpene lactones to free sulfhydryl groups in proteins may interfere with the functions of these macromolecules[2−4]. Consequently, sesquiterpene lactones inhibit a large number of enzymes involved in key biological processes, namely DNA and RNA synthesis, protein synthesis, purine synthesis, glycolysis, citric acid cycle, and the mitochondrial electron transport chain.[3,5]

Although the exact cellular targets affected by sesquiterpene lactones are not known, it may be speculated that the cytotoxic effect is a consequence of alkylation of several enzyme sites. Then, the cytotoxicity may be directly related to the steric accessibility of the targets. Because the targets and the sesquiterpene lactones are both three-dimensional structures with their own specific environment, the Michael addition that takes place between these reactants will have a certain degree of specificity.

In many studies on the cytotoxic effects of helenanolide−sesquiterpene lactones, only little attention has been paid to possible effects of the variable molecular geometry on their activity. Differences in molecular conformation may affect both the steric accessibility of Michael addition sites and lipophilicity. It was, therefore, the aim of the present study to investigate the relationships between the cytotoxicity of 21 helenanolides and their structures, with special attention to the above-mentioned aspects. The compounds were divided into four groups, namely helenalins (**1**−**5**), mexicanins I (**6**−**8**), 11α,13-dihydrohelenalins (**9**−**12**), and 2,3-dihydrohelenalins (**13,14** arnifolins, **15**−**21** chamissonolides).

Results and Discussion

EN2 cells were incubated with each compound for 2 h and 72 h. The IC_{50} values obtained by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay are listed in Table 1. In agreement with previous findings,[1−6] compounds of the helenalin and mexicanin I series (**1**−**8**) were found to be the most toxic, probably due to their bifunctionality as alkylating agents. The isobutyryl ester **3** was equally toxic compared to the commonly used antitumor agent cisplatin. The presence of only one alkylation site still yielded considerable cytotoxicity [dihydrohelenalins (**9**−**12**), arnifolin **13**, and chamissonolides (**15**−**20**)]. Compounds **14** (11α,13-dihydroarnifolin) and **21** (11α,13-dihydrochamissonolide) lacking both the α-methylene-γ-lactone and the cyclopentenone structure were the least toxic.

An increase of incubation time from 2 h to 72 h resulted in higher cytotoxicity. The relative increase of toxicity within the compounds **1**-**8**, however, was

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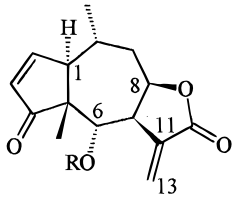
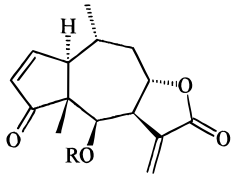
HELENALINS



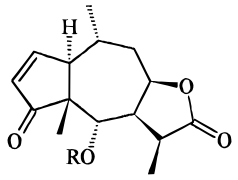
	R
1	H
2	Acetyl
3	Isobutyryl
4	Tigloyl
5	Isovaleryl

MEXICANINS I



	R
6	H
7	Acetyl
8	Isovaleryl

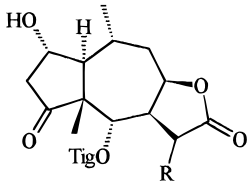
11α, 13 DIHYDROHELENALINS



	R
9	H
10	Acetyl
11	Tigloyl
12	Isovaleryl

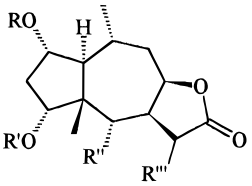
2,3 DIHYDROHELENALINS

- ARNIFOLINS



	R
13	$=CH_2$
14	β-CH_3

- CHAMISSONOLIDES



	R	R'	R''	R'''
15	H	H	H	$=CH_2$
16	H	H	OH	$=CH_2$
17	Acetyl	H	H	$=CH_2$
18	Acetyl	H	OH	$=CH_2$
19	H	H	OTigloyl	$=CH_2$
20	H	Tigloyl	OH	$=CH_2$
21	Acetyl	H	OH	β-CH_3

smaller than that of **9**−**21**; comparing 2-h to 72-h incubation, the IC_{50} values of **1**−**8** decreased about six-fold; those of **9**−**21**, about 13-fold. This may be related to the presence of two alkylating centers in compounds **1**−**8** exerting their cytotoxic effect faster.

Within the 11α,13-dihydrohelenalin group (**9**−**12**), cytotoxicity appeared to be directly proportional to the size of the ester group (i.e., lipophilicity). The isovalerate **12**, the most lipophilic compound, was the most toxic, and the alcohol **9** was the least toxic compound. Chromatographic behavior determined by HPLC and TLC showed relative retention time (rt_R) and the relative R_f (rR_f) values increasing with the presence of larger ester groups (Table 2). Higher lipophilicity determined by the HPLC and TLC systems may result in facilitated penetration through the lipophilic plasma membrane of the tumor cells, and subsequently, in higher cytotoxicity.

Interestingly, the simple relationship between ester size and cytotoxicity could not be found within the helenalin series (**1**−**5**), despite similar increases in lipophilic behavior (Table 2). Although the acetate **2** and the isobutyrate **3** were more toxic than helenalin **1**, decreased toxicity was observed in compounds with larger ester groups (tiglate **4** and isovalerate **5**). The same was found for the mexicanin I derivatives. The acetate **7** was more toxic and the isovalerate **8** less toxic than the parent alcohol **6**. These findings indicate that for compounds possessing an α-methylene-γ-lactone group a size optimum of an adjacent ester group exists. In compounds not bearing this alkylating center (**9**−**12**) an increase in lipophilicity causes the predicted increase in cytotoxicity. In the helenalin and mexicanin I derivatives with larger ester groups, the favorable effect of higher lipophilicity is obviously being outmatched by steric hindrance of the exocyclic methylene's approach towards a target structure by the bulky ester side chains. In 11α,13-dihydrohelenalin and its esters, cytotoxicity is caused by the cyclopentenone moiety, with the alkylation site at C-2. The distance of this center from the ester group at C-6 is considerably larger so that its approach to a target molecule should be less affected.

Although still strongly active, the mexicanin I derivatives (**6**−**8**) were slightly, but significantly, less toxic than their stereoisomers of the helenalin group (**1**, **2**, **5**). These discrepancies may be caused by different stereochemistry and the resulting differences in the three-dimensional shape. The helenalin molecule has been shown to possess a rather high degree of conformational flexibility and is subject to a very fast conformational exchange at room temperature,[7,8] whereas mexicanin I, due to the 7,8-trans fused lactone ring, is a rather rigid molecule. This difference may affect the bioactivities. The possibility of reacting with sulfhydryl groups in different environments may increase with steric flexibility. Moreover, the solubility of helenalin is higher than mexicanin I in polar as well as in nonpolar solvents, which may facilitate the molecule in reaching its targets inside a living cell. Comparing mexicanin I ester **8** to the parent compound **6**, we found that toxicity was reduced about two-fold, similar to the corresponding pair, **5** and **1**. As stated above, hindered approach to a target molecule might be the reason for the decreased toxicity of the isovalerate.

The steric hindrance caused by the tigloyl group in the 2-deacetylchamissonolide series is clearly illustrated by the fact that 2-deacetyl-4-*O*-tigloylchamissonlide (**20**), in which the tigloyl rest is farther away from the alkylating center, was twice as toxic as the 6-*O*-tigloyl derivative **19**. Moreover, the unesterified parent alcohol, 2-deacetylchamissonolide (**16**), although expected to be less toxic than its esters because of its higher

Table 1. Mean IC_{50} (μM) Values ± the Standard Deviation ($n \geq 3$) of the *Arnica* Sesquiterpene Lactones as Measured by the MTT Assay[a]

compound	2 h	72 h
helenalin (**1**)	2.4 ± 0.49	0.39 ± 0.09
6-*O*-acetylhelenalin (**2**)	1.3 ± 0.12	0.22 ± 0.07
6-*O*-isobutyrylhelenalin (**3**)	1.3 ± 0.49	0.17 ± 0.02[b]
6-*O*-tigloylhelenalin (**4**)	5.3 ± 0.10[b]	1.00 ± 0.18[b]
6-*O*-isovalerylhelenalin (**5**)	3.2 ± 0.17	0.72 ± 0.08[b]
mexicanin I (**6**)	2.7 ± 0.67	0.56 ± 0.10[b]
6-*O*-acetylmexicanin I (**7**)	2.0 ± 0.24	0.36 ± 0.06[c]
6-*O*-isovalerylmexicanin I (**8**)	5.4 ± 1.4	0.99 ± 0.15[c]
11α,13-dihydrohelenalin (**9**)	128 ± 17	6.5 ± 2.2
6-*O*-acetyl dihydrohelenalin (**10**)	43 ± 5.6[d]	3.5 ± 0.70[d]
6-*O*-tigloyl dihydrohelenalin (**11**)	19 ± 1.9[d]	2.6 ± 0.61[d]
6-*O*-isovaleryl dihydrohelenalin (**12**)	20 ± 2.7[d]	1.7 ± 0.33[d]
arnifolin (**13**)	62 ± 7.4	4.0 ± 0.3
11α,13-dihydroarnifolin (**14**)	>100	76 ± 5.7
2-desacetyl-6-deoxychamissonolide (**15**)	n.d[e]	15.2 ± 1.1[f]
2-deacetylchamissonolide (**16**)	n.d	13.6 ± 0.5[g]
6-deoxychamissonolide (**17**)	102 ± 3.3	10.6 ± 1.7
Chamissonolide (**18**)	106 ± 17	7.6 ± 0.6
2-deacetyl-6-*O*-tigloylchamissonolide (**19**)	>100	20.7 ± 0.5[h]
2-deacetyl-4-*O*-tigloylchamissonolide (**20**)	116 ± 5.7	10.4 ± 1.6
11α,13-dihydrochamissonolide (**21**)	>100	>100
cisplatin	1.4 ± 0.10	0.18 ± 0.04

[a] Incubation times were 2 h or 72 h. [b] Significantly different from **1**. [c] Significantly different from **6**. [d] Significantly different from **9**. [e] n.d. stands for not determined. [f] Significantly different from **17**. [g] Significantly different from **18**. [h] Significantly different from **20**.

Table 2. Chromatographic Behavior of the Helenanolides under Study using HPLC and TLC

compound	rt_R[a]	hR_f[b]	rR_f[a]
1	0.752	24	1.60
2	1.012	48	3.20
3	2.655	68	4.53
4	3.527	66	4.40
5	4.860	75	5.00
6	0.675	24	1.60
7	1.187	42	2.80
8	3.502	78	5.20
9	0.581	18	1.20
10	0.903	43	2.87
11	2.933	61	4.07
12	4.252	71	4.73
13	1.721	28	1.87
14	1.285	25	1.67
15	*d*	(24[e])	(0.52[e])
16	*f*	(18[e])	(0.39[e])
17	1.707	21 (60[e])	1.40
18	1.000	15 (46[e])	1.00
19	2.004	6	0.40
20	0.941	24	1.60
21	*g*	10	0.66

[a] Relative retention time compared to **18** as determined by HPLC. [b] $100 \times R_f$. [c] Relative R_f compared to **18**. [d] Not available. [e] TLC-system: toluene−EtOAc (20:80). [f] HPLC−rt_R **16** in another system (1.8 mL/min, 25% MeOH isocratic): 0.670. [g] UV not detectable.

polarity, was still more active than **19**. Interestingly, **19** and **20** showed divergent chromatographic behavior in TLC and HPLC[9] (Table 2). According to the HPLC-rt_R value, **19** was relatively lipophilic, while the hR_f value suggested a comparatively high polarity. Compound **20** showed the opposite behavior having a relatively low rt_R value, but a high hR_f. Consequently, based on these chromatography data, we cannot predict which of these two compounds is the most lipophilic, with respect to penetration through a biological membrane. However, the fact that **16** was somewhat less toxic than the 2-*O*-acetyl and the 4-*O*-tigloyl derivatives (**18**, and **20**, both with free hydroxyl functions at C-6) may very well be explained by its higher polarity (Table 2).

Within the tested group of 2,3-dihydrohelenalin derivatives (**13**−**21**), arnifolin (**13**) showed the strongest cytotoxic effect. It was found to be five times more toxic than 2-deacetyl-6-*O*-tigloylchamissonolide (**19**). It has been demonstrated that the two compounds adopt completely different molecular conformations.[10] Chamissonolide derivatives have been shown to possess a twist boat conformation,[9−11] whereas arnifolin adopts a twist chair conformation.[10] The steric hindrance of the exomethylene group by the bulky ester side chain would be stronger in a twist boat form, where the ester side chain is in an equatorial position coming closer to the molecule's sole alkylating center than in the twist chair conformer. This is illustrated by Figure 1, which shows molecular models of **13**, **19**, and **20** from two different angles. The five-fold difference in IC_{50} values between **13** and **19**, however, is not likely to be caused by this steric effect alone, because the cytotoxicity of **19** and its isomer **20** differed only by a factor 2. The hydroxyl at C-4 of **19** is replaced by a carbonyl function in **13**, and apart from possible steric effects, **13** might to some extent be transformed into 6-*O*-tigloylhelenalin (**4**) by metabolic dehydratation within a living cell, which would create an additional Michael addition site and enhance its cytotoxicity. Compound **14** has no Michael addition site. Therefore, an equivalent conversion of 11α,13-dihydroarnifolin **14** into **11** could explain the very low, but still measurable, cytotoxicity of **14**; however, it has been demonstrated that α,*β*-unsaturated ester groups have a low alkylating potency of their own.[12] Thus, it cannot be excluded that the tiglic ester moiety itself causes the weak cytotoxicity. As expected from its chemical structure, 6-deoxychamissonolide (**17**) was more lipophilic than chamissonolide (**18**) (Table 2). Yet, **17** turned out to be somewhat less toxic (Table 1). The same relationship was found between the corre-

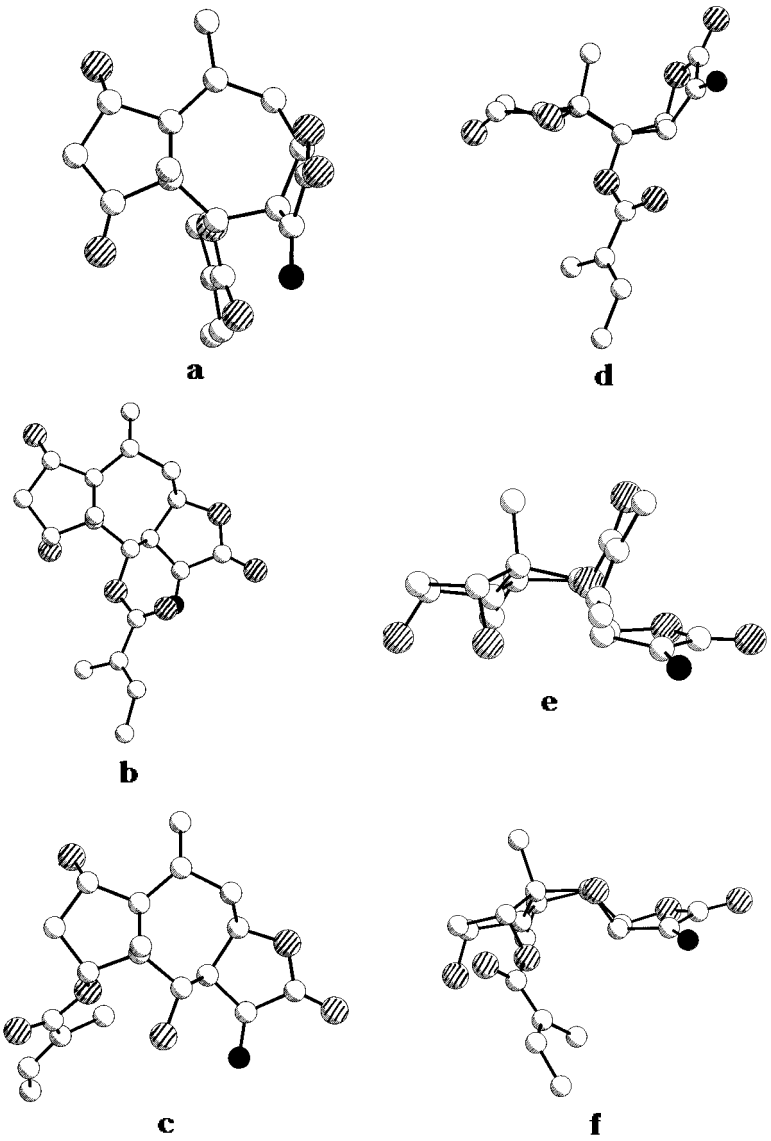


Figure 1. Molecular models of **13** (a, d), **19** (b, e), and **20** (c, f), viewed from two different angles (see Experimental Section). Oxygen atoms are hatched for clarity; α-methylene carbons involved in Michael addition are colored black. The lateral view of **19** (e) shows the possible hindrance of the exocyclic methylene by the bulky tigloyl ester side chain.

sponding 2-deacetyl derivatives **15** and **16**. This finding is in agreement with Kupchan *et al.*[13] who found enhanced activities for compounds bearing an oxygen function at C-6. We suppose that the oxygen at C-6 may form a hydrogen bond with amino acid residues of a target protein adjacent to the reactive cysteine. This possibly increases the reactivity to the Michael addition site and the toxic effect to some extent.

Although the target molecules affected by the sesquiterpene lactones in the cell line used in this study are not known, our findings clearly demonstrate that differences in substitution pattern and molecular geometry of sesquiterpene lactones should be considered in the interpretation of their biological activity. Results obtained in a particular cytotoxicity testing system depend on parameters, such as the cell line and the assay chosen to measure cytotoxicity. These parameters may account for differences between some of our results and data previously published for helenalin esters.[5,12,14,15] Recently, we have demonstrated for two sesquiterpene lactones each containing a Michael addition site (artemisitene, eupatoriopicrin) that the MTT and the classical clonogenic assay yielded comparable cytotoxicity data indicating actual cell death.[16] It is desirable to extend the present investigations to other cell line systems and to other classes of sesquiterpene lactones in order to draw more general conclusions.

Experimental Section

Sesquiterpene Lactones−Natural Products. Most of the compounds under study were isolated as natural products from different *Arnica* species as reported previously: helenalin (**1**) and 11α,13-dihydrohelenalin (**9**);[17] helenalin esters and 11α,13-dihydrohelenalin esters (**2**−**5** and **10**−**12**);[18] mexicanin I (**6**);[19] 6-deoxychamissonolide (**17**);[20] and arnifolin (**13**), 11α,13-dihydroarnifolin (**14**), chamissonolide (**18**), 2-deacetyl-6-*O*-tigloylchamissonolide (**19**), 2-deacetyl-4-*O*-tigloylchamissonolide (**20**), 11α,13-dihydrochamissonolide (**21**).[9]

Sesquiterpene Lactones−Semisynthetic Derivatives. Mexicanin I Acetate (7). Ten mg (38.2 μmol) of mexicanin I was refluxed for 3 h with 5 mL of a 10% solution of Ac_2O in pyridine. The solvent was evaporated *in vacuo* and the residue purified by vacuum liquid chromatography (VLC) on 15 g silica with hexane−EtOAc (6:4) to give 6 mg (19.7 μmol) mexicanin I acetate.

^{1}H NMR (200 MHz, $CDCl_3$): δ 7.58 (1H, dd, J = 2, 6 Hz, H-2), 6.28 (1H, d, J = 4 Hz, H-13a), 6.12 (1H, dd, J = 3, 6 Hz, H-3), 5.94 (1H, d, J = 5 Hz, H-6), 5.69 (1H, d, J= 3 Hz, H-13b), 4.80 (1H, ddd, J = 12, 9, 3 Hz, H-8), 3.26 (1H, m, H-7), 2.77 (1H, ddd, J = 2, 2, 11 Hz, H-1), 2.56 (1H, ddd, J = 13, 5, 3 Hz, H-9b), 2.15 (1H, m, H-10), 2.07 (3H, s, CH_3 Ac), 1.36 (1H, ddd, J = 13, 12, 10 Hz, H-9a), 1.25 (3H, d, J = 7 Hz, CH_3-14), 1.22 (3H, s, CH_3-15).

Mexicanin I Isovalerate (8). Fifty-one mg (0.195 mmol) of mexicanin I, 25 mg (0.245 mmol) of isovaleric acid, 43 mg (0.208 mmol) of dicyclohexylcarbodiimide, and 3 mg (24.6 μmol) of 4-(dimethylamino)pyridine were dissolved in 13 mL of dry CH_2Cl_2 and stirred overnight. The reaction was quenched by addition of 25 mL of aqueous NaCl solution. The aqueous layer was extracted with 2 × 20 mL of CH_2Cl_2, the CH_2Cl_2 layers were combined and evaporated to dryness. The residue was subjected to VLC on 20 g of silica with hexane−EtOAc (6:4) to yield 18 mg (51.7 μmol) of mexicanin I isovalerate. ^{1}H NMR (250 MHz, $CDCl_3$): 7.58 (1H, dd, J = 2, 6 Hz, H-2), 6.28 (1H, d, J = 4 Hz, H-13a), 6.11 (1H, dd, J = 3, 6 Hz, H-3), 5.97 (1H, d, J = 5 Hz, H-6), 4.81 (1H, ddd, J = 12, 9, 3 Hz, H-8), 3.26 (1H, m, H-7), 2.78 (1H, ddd, J = 2, 2, 11 Hz, H-1), 2.57 (1H, ddd, J = 13, 5, 3 Hz, H-9b), 2.3−2.0 (4H, m, H-10, CH ival, CH_2 ival), 1.43 (1H, ddd, J = 13, 12, 10 Hz, H-9a), 1.26 (3H, d, J = 7 Hz, CH_3-14), 1.22 (3H, s, CH_3-15), 0.962 [6H, $(CH_3)_2$ ival].

2-Deacetyl-6-deoxychamissonolide (15). Ten mg (32.5 μmol) of 6-deoxychamissonolide (**17**) was dissolved in 5 mL of a 1% aqueous solution of NaOH and stirred at 60 °C for 2 h. After neutralization with dilute HCl, the aqueous solution was extracted with 3 × 20 mL CH_2Cl_2, and the organic layer was evaporated to dryness yielding 8 mg (30.1 μmol) of pure 2-deacetyl-6-deoxychamissonolide. ^{1}H-NMR findings were identical to those reported.[21]

2-Deacetylchamissonolide (16). Fifteen mg (46.3 μmol) of chamissonolide (**18**) was dissolved in 5 mL of a 1% aqueous solution of NaOH and stirred at 60 °C for 2 h. After neutralization with dilute HCl the aqueous solution was extracted with 3 × 20 mL CH_2Cl_2, and the organic layers were evaporated to dryness. The residue was subjected to column chromatography on 10 g of silica with cyclohexane−EtOAc (6:4) to yield 5 mg (17.7 μmol) of pure 2-deacetylchamissonolide. ^{1}H-NMR findings were identical to those reported.[21]

For all compounds, purity was confirmed by ^{1}H-NMR spectroscopy. The purity of all compounds was confirmed by HPLC and GC to be >95%.

Reference Compound. Cisplatin (*cis*-dichlorodiammineplatinum II, Aldrich, Milwaukee, WI) was used as a reference compound. It was dissolved in H_2O (1 mg/mL) and stored at −80 °C for no longer than 1 month.

Chromatographic Assessment of Lipophilicity. Chromatographic behavior was used to estimate the order of lipophilicity of the sesquiterpene lactones under study. It was tested by HPLC retention times (t_R) and TLC hR_f (= 100 × R_f) values.

HPLC system: Hypersil ODS (RP 18) 5mm, 125 × 4.6 mm, flow 1.80 mL/min, isocratic elution with 52.5% MeOH−H_2O; detection at 220 nm; the relative retention times (rt_R) were based on that of chamissonolide **18** (t_{RCham}) using the formula $rt_R = t_{RX} - t_0/t_{RCham} - t_0$.

TLC system: Silica 60F_{254} (Merck); toluene−EtOAc 60:40 (v/v); the relative R_f (rR_f) values were based on that of **18** (Rf_{Cham}): $rR_f = R_{fX}/R_{fCham}$.

Cytotoxicity Testing. The murine EN2, a cloned Ehrlich ascites tumor cell line, was grown in suspension culture in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) plus 0.2 mg/mL streptomycin and 200 IU/mL penicillin G. The cell line was cultured at 37 °C in a shaking incubator. The doubling time of the cells was about 12 h. Exponentially growing cells were used for all experiments. In all experiments over 95% of the cells excluded trypan blue.

The principle of the cytotoxicity method is the reduction of the soluble MTT into a blue−purple formazan product, mainly by mitochondrial reductase activity inside living cells.[22] The number of cells was found to be proportional to the extent of formazan production. The test compounds were made as a 10-mM stock in 96% EtOH, except mexicanin I and its esters, which were dissolved in 100% DMSO. As a control, helenalin was dissolved in either EtOH or DMSO, which did not lead to differences in cytotoxicity.

The compounds were pipetted into 96-wells microtiter plates (Nunc, Roskilde, Denmark). Equal volumes of cell suspensions containing 500 cells and the compounds were mixed in the wells. The cells were incubated with the compounds for either 2 h or 72 h. After 2 h of exposure at 37 °C in a humidified incubator with 5% CO_2, the test compounds were washed away in three steps, always leaving the cells behind in 50 μL of medium. After each washing step, the plates were centrifuged for 10 min at 20 °C, 195*g*. The cells were further incubated for 72 h at 37 °C in a humidified incubator with 5% CO_2. Then, 20 μL of a 5-mg/mL stock solution of MTT (Sigma, St Louis, Mo) in phosphate-buffered saline (PBS: 1.6 mM KH_2PO_4, 6.5 mM $Na_2HPO_4 \cdot 12H_2O$, 0.137 mM NaCl, 2.7 mM KCl, pH 7.4) was added, and the plates were incubated for another 3 h 45 min. The medium was removed after centrifugation (15 min, 195*g*, 20 °C) leaving the insoluble formazan product behind, which was subsequently dissolved in 200 μL of 100% DMSO. After mixing, the absorbance (A) was measured at 550 nm using a spectrophotometer (Titertek Multiscan, Flow Laboratories, Irvine, Scotland). Cell growth was calculated using the formula:

$$\frac{[A_{550}\,(\text{treated cells}) - A_{550}\,(\text{culture medium})]100}{[A_{550}\,(\text{control cells}) - A_{550}\,(\text{culture medium})]}$$

A computer program (Graphpad) was used to calculate the test compound concentration resulting in 50% growth inhibition (IC_{50}), a parameter for cytotoxicity.

Statistics. The independent *T*-test was used for statistics, which is part of the SYSTAT software.

Computer Models. The depicted computer models of compounds **13**, **19**, and **20** represent the conformations recently determined by X-ray crystallographic analyses.[10] In Figure 1 they are depicted from two different viewing angles. For a−c the carbon atoms C-6 and C-10 were aligned with a hypothetical *y*-axis in the paper plane, while C-6 and C-10 were aligned with the corresponding *z*-axis in pictures d−f.

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