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Helenanolide-Type Sesquiterpene Lactones—III. Rates and Stereochemistry in the Reaction of Helenalin and Related Helenanolides with Sulfhydryl Containing Biomolecules*

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Abstract—The reactivity of the two potential Michael addition sites of the helenanolide-type sesquiterpene lactone helenalin towards the physiological thiols glutathione (GSH) and cysteine (cys) in aqueous solution was investigated by ^{1}H NMR spectroscopic experiments. In the presence of one molar equivalent of GSH, the reaction was shown to occur with high regio- and stereoselectivity at the β-position of C-2 in the cyclopentenone ring. Addition to the exocyclic methylene group at the lactone ring was found to occur in the presence of GSH in molar ratios over 1:1, but proceeded at a rate 10 times smaller than at C-2 leading to the 2β,13(11β)-bis-glutathionyl adduct. In contrast, addition of free cys highly favoured the exocyclic methylene group. Addition of GSH to the cyclopentenone of 11α,13-dihydrohelenalin (plenolin) showed the same characteristics as observed with helenalin while 2α-acetoxy-2,3-dihydro-4β*H*-helenalin (chamissonolide) did not form an adduct when incubated with an equimolar amount of GSH. Explanations for the observed differences in reactivity of the two potential reaction sites based on MO computations are given and implications for the biological activity of this type of sesquiterpene lactones are discussed. © 1997 Elsevier Science Ltd.

Introduction

Sesquiterpene lactones (STL) of the 10α-methylpseudoguaianolide (= helenanolide)-type are secondary plant metabolites isolated from numerous genera of the Compositae.[1] Many of them are known to possess a broad variety of biological and pharmacological activities.[2]

STL are known to bind covalently to sulfhydryl groups of biological molecules by Michael addition of their α,β-unsaturated carbonyl structures, which are common structural features of most STL. Thiol groups of enzymes are considered to be especially susceptible to this reaction, and STL have been demonstrated to inhibit a variety of important sulfhydryl-bearing enzymes, such as phosphofructokinase,[3] glycogen synthase,[4] inosine monophospate dehydrogenase,[5,6] and others.[6]

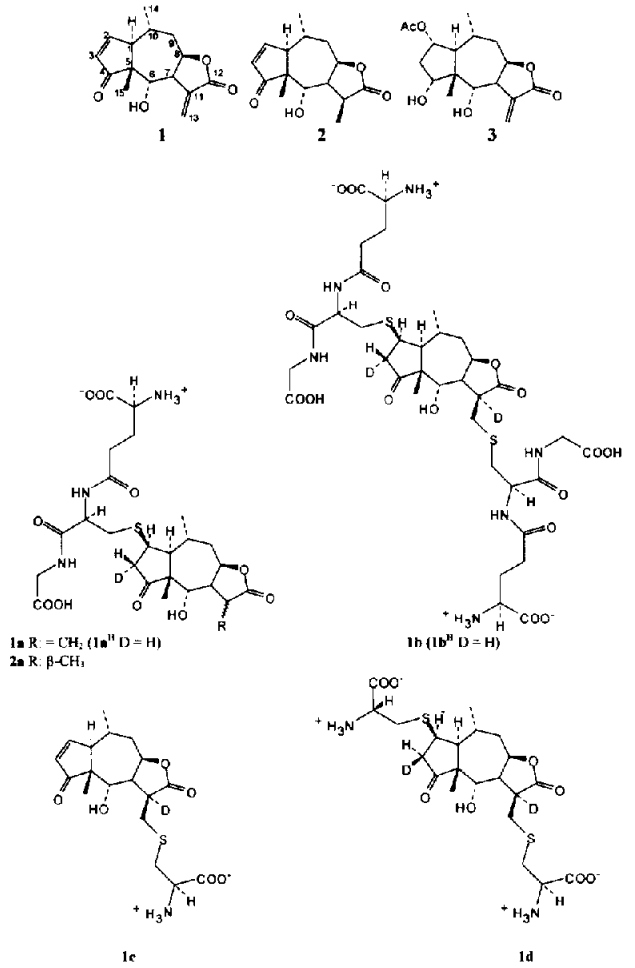
Among the compounds of the helenanolide group, especially helenalin **1** and its derivatives have received considerable attention in pharmacological research due to their potent antineoplastic and anti-inflammatory activity.[5–11] The intense activity of **1**, a constituent of *Helenium* species[1] and also of the medicinally used flowerheads of *Arnica montana* and further *Arnica* species,[1,12] has been explained by the fact that it possesses two reactive structure elements, an α,β-unsaturated cyclopentenone ring as well as the exocyclic methylene group in conjugation with the lactone carbonyl.[5–11,13]

Considering the generally accepted mechanism of action, the question arises, whether the reaction of the different potentially alkylating structure elements is of a certain specificity, so that some selectivity might exist concerning the sulfhydryl containing target structures. Several earlier reports have shown that helenalin and related compounds react with sulfhydryl reagents such as cysteine (cys) and glutathione (Gly-Cys-γGlu, = GSH).[5,6,14–16]

GSH is an extraordinarily important physiological peptide that is involved in numerous essential processes within all living cells.[17] It has been shown that some STL affect intracellular GSH levels[18–20] and thus, some of their bioactivities could be caused by interference with intracellular GSH balance, which would seriously affect cell function. On the other hand, spontaneous reaction of STL with GSH might, to some extent, protect other sulfhydryl containing structures within the cell and thus decrease the rate of enzyme inhibition. However, although some data on reaction rates are available in the literature,[5,6] little attention has been paid to reaction products, stereochemical aspects, and possible selectivity of the different potential reaction sites.

In order to obtain such information, the reactions of helenalin **1**, 11α,13-dihydrohelenalin (plenolin) **2**, and 2α-acetoxy-2,3-dihydro-4β*H*-helenalin (chamissonolide) **3** with GSH and cys were studied by ^{1}H NMR spectroscopy. Reaction rates could be measured for the different reactive structure elements and stereo- and regioselectivities could be observed, which will be meaningful to further research on the biological activities of these compounds and other STL.

*The presented work was carried out for the major part at the Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana, U.S.A.



Structures of sesquiterpene lactones 1–3 and of the GSH- and cys adducts.

Results and Discussion

The rate diagrams obtained for three different concentrations of GSH exposed to 20 μmol mL^{-1} **1** in D_2O are depicted in Figure 1. Of the two potential reaction sites of helenalin, the cyclopentenone structure (reaction site C-2) was found to react with GSH considerably faster than the exocyclic methylene group (reaction site C-13). The reaction of **1** with GSH in equimolar amounts (20 μmol mL^{-1}) therefore yielded almost exclusively (>90%) a single adduct (**1a**). The structure of **1a** could be determined from the resulting ^{1}H NMR, COSY, and NOESY spectra (^{1}H NMR data see Table 1). Most surprisingly, the addition at C-2 occurred stereoselectively from the β-face of the helenalin molecule. The stereochemistry at C-2 and C-3 was deduced from the coupling constants of H-1, H-2, and H-3 in comparison with calculated values for force field-minimized computer models of the four possible diastereomers in the deuterated product (Table 2). The relatively small coupling of 6.4 Hz between H-2 and H-1 and the absence of a measurable coupling between H-2 and H-3 are only possible if the molecule possesses the relative configuration depicted in Table 2 A (i.e. if H-1 and H-2 are both α oriented and H-3 occupies the β-position while the deuteron at C-3 is in the α-position).

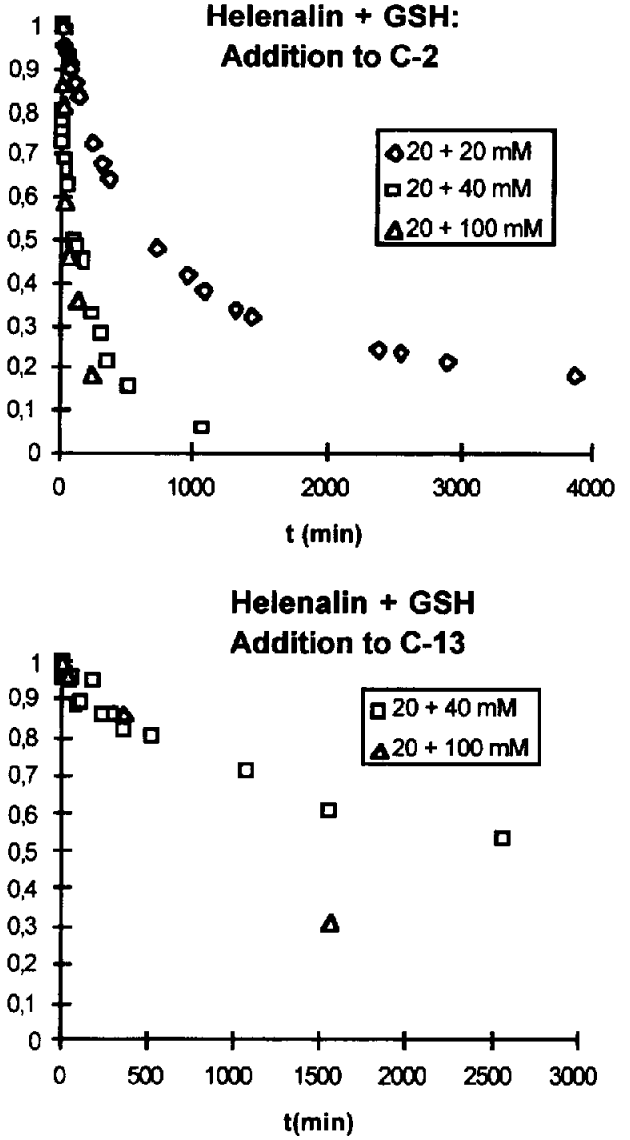


Figure 1. Reaction of **1** with three different concentrations of GSH.

In a recent communication, **1** was shown to be subjected to a fast exchange ($k \sim 3$–4×10^5 s^{-1} at 298 K, acetone-d_6) of two different twist-chair conformations, in one of which the pseudosymmetry axis C_2 passes through C-7 (TC7) and in the other one through C-10 (TC10). Both populations are present in a ratio of about 6:4 (TC7:TC10) in D_2O with an energy difference between both geometries of only about 0.97 kJ mol^{-1}.[21] The monoglutathionyl adduct **1a** was found to distinctly prefer the TC7 geometry. The coupling constants of H-9α and H-9β with H-10 of 10.7 and 2.3 Hz, respectively, are almost identical with those of 11α,13-dihydrohelenalin derivatives that were shown to adopt the TC7 conformation (compare $^3J_{9,10}$ with those of **1** (Table 1), and Schmidt[21]). Hence, the conformational equilibrium as observed with helenalin is shifted completely towards the TC7 conformation on addition to the Δ2,3-double bond. This finding corroborates our hypothesis that the stability of the different geometries within the cycloheptane ring of **1** is not only affected by hydrogenation of the Δ11,13-double bond and possible steric interac-

Table 1. ^{1}H NMR data of the GSH adducts of **1** and **2** (**1a**, **1b**, and **2a**) (400 MHz, D_2O;[a] data of **1** and **2** in D_2O are included for easier comparison)

H	δ (ppm)	**1** mult.	*J* (Hz)	δ (ppm)	**1a** mult.	*J* (Hz)	δ (ppm)	**1b** mult.	*J* (Hz)
1	2.964	ddd[dt]	11.5, 2 × ≈2	2.648	dd	6.4, 11.2	2.644	dd	6.3, 11.2
2	7.980	dd	6.1, 1.3	3.537[f]	d	6.5	3.531[f]	d	6.4
3	6.036	dd	5.9, 2.9	2.728[f]	s		2.723[f]	s	
6	4.326	d	1.7	4.114	d	2.3	4.103	s	
7	3.565	dddd[dq]	8.8, 3 × 1–2	3.511	dddd[dq]	8.1, 3 × 2.5	2.960[g]	d	6.4
8	5.099	ddd[dt]	2 × 8–9, 2.5,	4.970	ddd	7.8, 6.5, 1.3	4.864	dd [br t]	2 × 5–6
9α	1.741	ddd	15.4, 7.6, 2.5	1.779	ddd	15.7, 10.7, 1.6	1.702	dd	11.9, 15.1
9β	2.280	ddd	15.4, 8.0, 4.2	2.227	ddd	15.6, 6.4, 2.3	2.284	ddd	14.9, 5.2, 2.4
10	2.028	m		1.973	m		1.964	m	
11							(D)[g]		
13a	6.290	d	3.0	6.255	d	2.5	3.027	d	13.3
13b	5.935	d	3.0	5.941	d	2.3	2.754	d	13.2
CH_3-13									
CH_3-14	1.178	d	6.7	1.078	d	6.4	1.079	d	6.5
CH_3-15	0.849	s		0.799	s		0.846	s	
cys-βHa				3.024	dd	14.3, 4.6	3.02	–[e]	
							3.091	dd	14.1, 5.5
cys-βHb				2.822	dd	14.2, 9.9	2.820	dd	14.0, 10.1
							2.902	dd	14.1, 8.0
cys-αH				4.502	dd	9.9, 4.6	4.500	dd	9.8, 4.7
							4.591	dd	8.0, 5.5
gly-$αH_2$				3.875	–[b, c]		3.886	–[b, d]	
glu-$γH_2$				2.467	–[b, c]		2.468	–[b, d]	
glu-$βH_2$				2.092	–[b, c]		2.090	–[b, d]	
glu-αH				3.725	dd[t]	6.5	3.733	–[b, c]	

H	δ (ppm)	**2** mult.	*J* (Hz)	δ (ppm)	**2a** mult.	*J* (Hz)
1	3.008	ddd[dt]	11.3, 2 × ≈2	2.636	dd	6.3, 11.1
2	7.918	dd	5.9, 1.6	3.502	d	6.4
3	6.005	dd	5.8, 2.9	2.675	s	
6	4.211	s		4.064	s	
7	2.936	dd	10.7, 6.2	2.819	dd	10.8, 5.6
8	4.898	ddd[dt]	2 × 6, 1.5	4.825	dd[brt]	2 × 6
9α	1.682	ddd	15.5; 11.5; 1.5	1.684	dd	15.4, 11.5
9β	2.330	ddd	15.6, 6.6, 1.6	2.251	ddd	15.6, 6.6, 1.6
10	2.008	m		1.938	m	
11	3.261	dq	10.7, 3 × 7.3	3.184	dq	9.9, 3 × 7.4
13a						
13b						
CH_3-13	1.201	d	7.3	1.187	d	7.3
CH_3-14	1.122	d	6.7	1.058	d	6.4
CH_3-15	0.811	s		0.825	s	
cys-βHa				2.983	dd	14.2, 4.5
cys-βHb				2.783	dd	14.2, 9.9
cys-αH				4.471	dd	10.0, 4.6
gly-$αH_2$				3.835	–[b, c]	
glu-$γH_2$				2.442	–[b, c]	
glu-$βH_2$				2.053	–[b, c]	
glu-αH				3.670	dd[t]	6.4

[a]Five drops of acetone-d_6 were added for better solubility; [b]Signals not first order; [c] Intensity 2H; [d]Intensity 4H; [e]Overlapping signal, multiplicity not determined. [f]Signals for $\mathbf{1a}^H$ and $\mathbf{1b}^H$ (500.13 MHz): H-2: 3.55, dd [t], 2* 6–7; H-3a/b: non first-order AB part of ABX system at 2.71. [g]$\mathbf{1b}^H$: H7: 2.98, dd, 6.4, 9.5; H-11: 3.36, ddd [dt], 5.8, 2* 10.1; H-13a: 2.98, dd, 13.0, 6.0; H-13b: 2.78, dd, 12.8, 10.1.

tions of the resulting methyl group, but also by the hybridization of carbon atoms C-2 and C-3 in the cyclopentane ring of such helenanolides.[22]

When incubated with 2 and 5 mol equiv of GSH, slow formation of the 2β, 13(11β)-bis-glutathionyl adduct **1b** of helenalin was to be observed after initial formation of **1a**. Figure 2 shows the NMR spectra obtained during the reaction of **1** with 2 mol equiv of GSH.

The stereochemistry at C-11 of **1b** is affirmed by the coupling constant $J_{7,11}$ = 10 Hz in the spectrum of the product isolated after reaction in H_2O ($\mathbf{1b}^H$), which is very similar to the value in 11α,13-dihydrohelenalin and would be higher (ca. 13 Hz) in case of an 11β,13-dihydrohelenalin derivative.[21] Furthermore, nuclear Overhauser effects between H-13b and CH_3-15 as well as H-6 were observed in the NOESY spectrum, which could not occur if the side chain were α-oriented (distance H-13b ... CH_3-15 2.3 Å and H-13b ... H-6 2.2

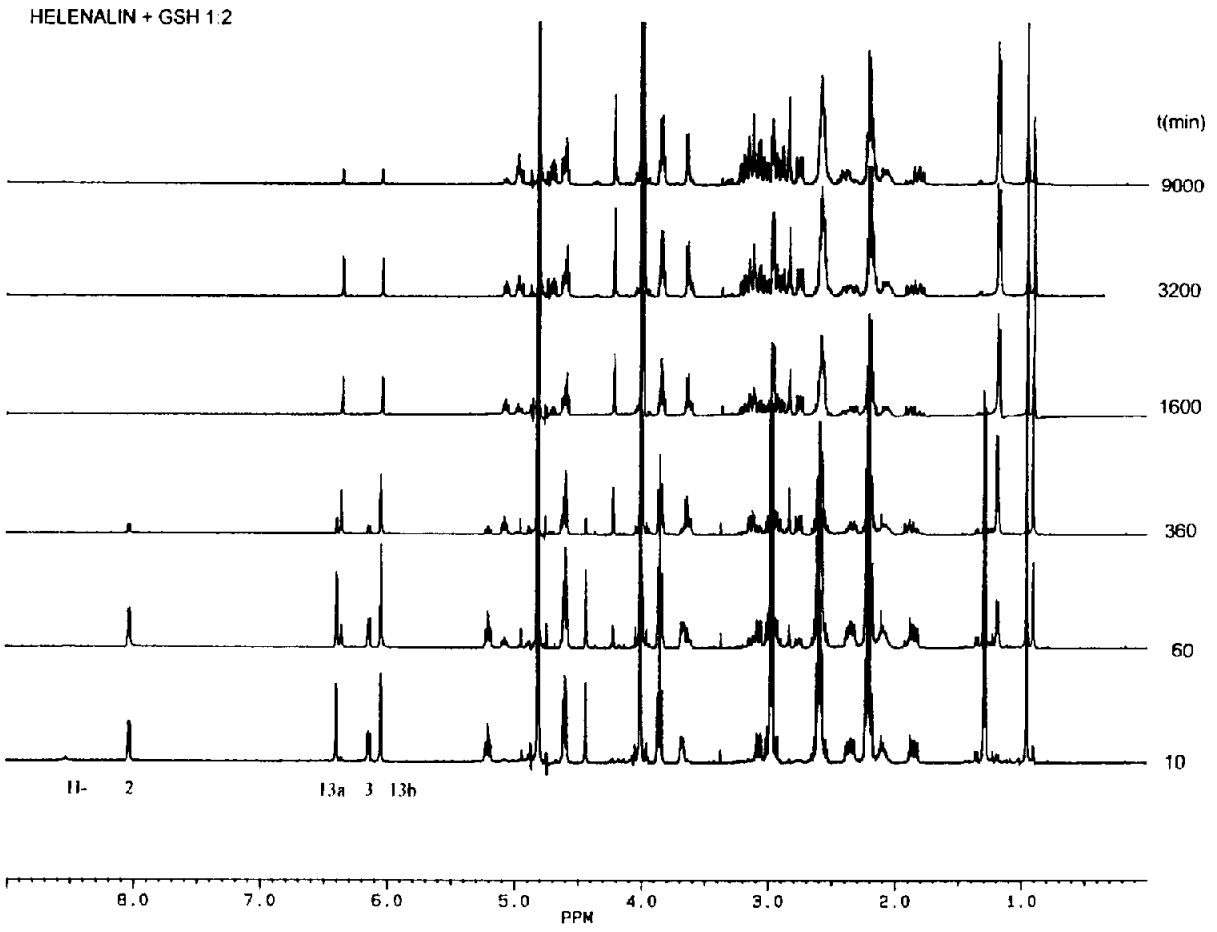


Figure 2. ^{1}H NMR spectra obtained during the reaction of **1** with 2 mol equiv of GSH.

Å for β-CH_2-S-R, both distances >4 Å for α-CH_2-S-R (AM1 models of the 11α- and 11β-configurated *t*-But-S-adducts)).

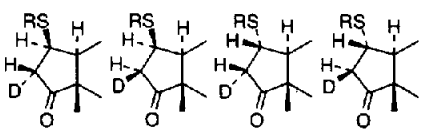
The approximate second-order rate constants for GSH addition obtained by linearization of the data sets (see Table 3) showed that the rate of addition to C-2 (average k_2 0.08 mol^{-1} min^{-1}) is about 10 times higher than for C-13 (average k_2 0.008 mol^{-1} min^{-1}).

These results are in agreement with earlier findings of Picman et al., who reported that helenalin yields only one monoadduct when incubated with an equimolar amount of GSH, without further characterization of the product.[14]

In contrast, other authors[15,16] reported that the olefinic proton signals of both reactive sites of **1**, H-2/H-3 and H-13a/b had disappeared from the ^{1}H NMR spectrum after 4 h incubation with an approximately *equimolar amount* of GSH (10 mg each = 3.8 µmol **1** and 3.3 µmol GSH). This, with respect to stoichiometry, is impossible since two molecules of GSH cannot be added to each molecule of helenalin if a ratio of only 1:1 molecules is present.

Page et al., in a more recent study reported on second-order rate constants for GSH addition to a number of STL. Their data for **1**, 2,3-dihydro-**1** and 11α,13-

Table 2. Theoretical $^3J_{H,H}$-coupling constants in the cyclopentanone ring of the four possible diastereomers of the C-2-SR adducts of **1** (AM1-models of the cys-adducts) and experimental values of compounds **1a**, **1b**, and **1d**



$^3J_{H,H}$	A	B	C	D	1a	1b	1c
1, 2	5.6	5.6	11.3	11.3	6.5	6.4	6.3
2, 3	1.3	8.4	9.2	8.9	<1	<1	7.6

Table 3. Second-order rate constants (k_2, mol^{-1} min^{-1}) for the addition of GSH to helenalin (**1**)

c_0(1:GSH)(mM)	Site	R^2	k_2	$t_{1/2}$	$t_{1/2(exp)}$
20:20	C-2	0.992	0.061	819	750
	C-13	–	–		
20:40	C-2	0.985	0.100	203	120
	C-13	0.989	0.007	2896	2800
20:100	C-2	0.973	0.071	103	60
	C-13	0.976	0.009	817	1100

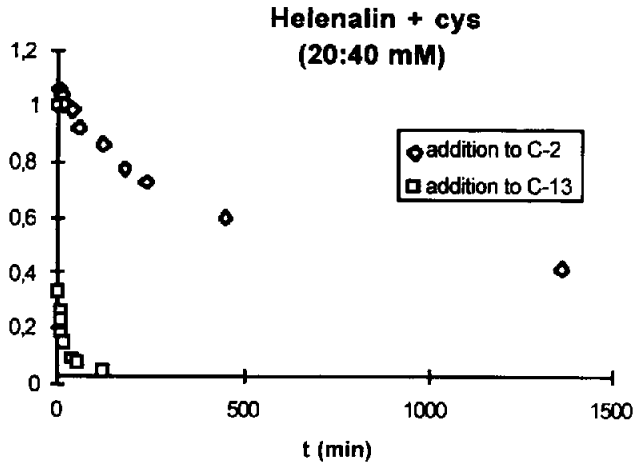


Figure 3. Reaction of **1** with 2 mol equiv of cys.

dihydro-**1** (plenolin), obtained by measurement of the decreasing GSH concentration in phosphate-buffered solution (pH 7.4), indicated that the reactivity of the exomethylene lactone should be somewhat higher than that of the cyclopentenone moiety.[5]

Regarding this discrepancy with the results obtained in the present study, the reaction of **1** with an equimolar amount of GSH was repeated in phosphate buffer pH 7.4. Formation of a single product was observed, which could be isolated and identified to be the helenalin-2β-mono-GSH adduct $\mathbf{1a^H}$ (^{1}H and ^{13}C NMR data see Tables 1 and 4, respectively) confirming that the above-mentioned regioselectivity of GSH addition also exists at physiological pH.

Moreover, the reactions of 11α,13-dihydrohelenalin **2** and of the 2,3-dihydrohelenalin derivative chamissonolide **3** with GSH were investigated. The reaction of **2** with GSH in equimolar ratio (20 mM) proceeded at a similar rate as observed for **1** ($k_2 \approx 0.04$ mol^{-1} min^{-1}) leading to a single adduct, **2a** (^{1}H NMR data see Table 1).

Table 4. ^{13}C NMR shifts (ppm) of the helenalin mono- and bis-GSH adducts $\mathbf{1a^H}$ and $\mathbf{1b^H}$ (125 MHz, D_2O, assignments of all protonated carbons confirmed by 2-D ^{1}H/^{13}C-shift correlation)

C	$\mathbf{1a^H}$	$\mathbf{1b^H}$	
1	49.78	49.90	
2	42.46	42.47	
3	47.33	47.07	
4	223.48	223.79	
5	56.25	54.78	
6	78.42	70.30	
7	49.18	49.82	
8	81.50	82.10	
9	38.19	37.79	
10	25.70	25.58	
11	138.38	45.38	
12	174.36[a]	180.64	
13	126.49	28.14	
14	20.10	19.71	
15	16.36	15.34	
CON	173.03[a]	173.00[b]	172.82[b]
CH-α	53.13	53.44	52.88
CH_2-β	34.18	33.88	33.72
gly			
COO	175.21[a]	175.15[b]	175.15[b]
CH_2-α	42.89	42.15	42.15
glu			
COO	174.38[a]	174.03[b]	174.17[b]
CH-α	54.60	54.21	54.21
CH_2-β	26.81	26.51	26.44
CH_2-γ	31.98	31.65	31.65
CON-δ	175.53[a]	175.19[b]	175.19[b]

[a,b]Assignments may be interchanged.

Incubation of **3** with an equimolar amount of GSH (20 mM), however, did not lead to any measurable formation of an adduct for a period of 20 h. Hence, the reactivity of C-2 and C-13 as observed with **1** was reproduced also with compounds **2** and **3**.

The reaction kinetics presented by Page et al.[5] for GSH addition, on the background of the results of the

Table 5. ^{1}H NMR data of the cys-adducts of **1** (**1c** and **1d**) (400 MHz, D_2O[a])

H	**1c**			**1d**		
	δ (ppm)	mult.	*J* (Hz)	δ (ppm)	mult.	*J* (Hz)
1	≈3.0	[b]		2.655	dd	11.1, 6.3
2	7.946	dd	6.1, 1.2	3.567	dd [br t]	2 × 6–7
3	6.032	dd	6.0, 3.0	2.712	d	7.6
6	4.237	s		4.107	s	
7	≈3.1	[b]		2.98	[b]	
8	4.945	ddd [dt]	2 × 6.1, 1.6	4.886	ddd [br t]	2 × 6–7, (<1)
9α	1.700	ddd	15.4, 11.7, 1.3	1.732	ddd	15.4,12.1, (<1)
9β	2.370	ddd	15.8, 5.9, 2.0	2.309	ddd	15.6, 6.6, 1.7
10	≈2.04	m[b]		≈2.04	m[b]	
13a	3.07	[b]		3.07	[b]	
13b	2.799	d	13.3	2.802	d	13.3
CH_3-14	1.147	d	6.7	1.130	d	6.6
CH_3-15	0.848	s		0.848	s	
cys-αH	3.930	dd	7.5, 4.7	3.882	dd	7.4, 4.6
				3.841	dd	7.4, 4.2
cys-βH_2	≈3.1	[b]		≈3.1	[b]	

[a]Five drops of acetone-d_6 were added for better solubility. [b]Overlapping signals, multiplicity not accessible, δ determined from centres of COSY cross peaks.

present study appear somewhat doubtful, all the more since it was not product formation that served as a probe for the reactions' progress.

In the reaction with cys, helenalin most surprisingly exhibited a completely different behaviour. In mixtures of **1** with cys (20 μmol mL^{-1} **1**, 20 and 40 μmol mL^{-1} cys), the exocyclic methylene protons H-13a/b disappeared much faster ($t_{1/2}$ < 5 min) than the signals of H-2 and H-3, indicating a dramatically increased reactivity of the C-13 reaction site towards the sulfhydryl group of the free amino acid (see Fig. 3).

Thus, the C-13-mono-cys-adduct **1c** (^{1}H NMR data see Table 5) was the main product in the reaction with 1 mol equivalent of cys.

Exposed to the double molar amount of cys, **1** yielded the bis-cys adduct **1d** (^{1}H NMR data see Table 5; $t_{1/2}$ for addition to C-13 < 5 min, for addition to C-2 ≈1000 min).

In analogy with the GSH adducts, the cys adducts adopt the TC7 geometry as indicated by their coupling constants in the H-9α and H-9β signals (Table 5). NOE-interactions between H-13b and H-6 as well as CH_3-15 once more proved the stereochemistry at C-11 of the C-13-cys adducts. Also, as observed with GSH, the addition of cys to C-2 occurs from the β-face of the molecule, as could be deduced from the coupling constants of H-1, H-2, and H-3 (Table 2). In contrast with the GSH reaction, however, the deuteron is added to C-3 from the β-direction in this case. The coupling constant between H-2 and H-3 is 7.6 Hz and thus, although this value is somewhat smaller than expected from the computer model, the cys-adduct must possess configuration B in Table 2.

In order to explain the observed regio- and stereoselectivities, a computational investigation of helenalin-thiol adducts was carried out utilizing the semi-empirical MO method AM1. It has to be noted that the GSH molecule is known to possess a high degree of conformational freedom in aqueous solution, the most stable conformer contributing only about 2% of the total population.[23] Hence, a vast number of energetically similar conformations may be expected to be present within the glutathionyl rests of the adducts.

Table 6. AM1 calculated enthalpies of reaction ($\delta\Delta H_f = \Delta H_f$(product)$-\sum\Delta H_f$(educts) (kJ mol^{-1})) of methylsulfide (Me-SH)-, *tert*-butylsulfide (*t*-But-SH) and cys adducts of cyclopentene-3-one (A), α-methylene-γ-butyrolactone (B) and the corresponding substructures within the helenalin molecule

	A	B	I	
			C-2β	C-13(11β)
Me-SH	−104.58	−98.34	−98.59	−80.40
t-But-SH	−99.51	−94.08	−94.28	−76.30
cys	−99.14	−128.42	−91.31	109.47
cys[a]	−93.91	−88.09	−91.90	−72.95

[a] cys in the α-amino-carboxylic acid form.

For the computer models, thiols with a smaller number of rotational degrees of freedom, methylsulfide (Me-SH) and *t*-butylsulfide (*t*-But-SH) were chosen.

The computed energy differences for addition of these mercaptanes to both reaction sites (models were calculated for cyclopentene-3-one and α-methylene-γ-butyrolactone and for helenalin with both, Me-SH and *t*-But-SH) were in agreement with the experimentally measured difference in reactivity between C-2 and C-13 towards GSH. As expected, the α,β-unsaturated ketone structure yields the thermodynamically more favourable product. The cyclopentenone adducts were calculated to be more favourable than the α-methylenebutyrolactone adducts by about 20 kJ mol^{-1} (Table 6).

The models for the C-2 adducts indicated that the 2α adducts should be energetically somewhat more favourable (6–8 kJ mol^{-1}) than the 2β configurated compounds actually formed. This stereoselective reaction at C-2 with both, cys and GSH, might be explained by a stereoelectronic effect arising from an interaction of the sp^3 orbital of the C-1–H-1 bond with the π-electron system of the Δ2,3-double bond. The slightly acidic H-1 is oriented perpendicular to the plane of the double bond and some of the electron density of the C–H bond will be distracted by the positive partial charge at C-2 so that the overall electron density on the α-side of the cyclopentenone ring is somewhat higher than on the β-side, which will facilitate the nucleophile's attack from the β-direction.

Since computer models for the C-13 adducts yielded more favourable energies (15–20 kJ mol^{-1}) for an 11α-oriented side chain, reaction at this site is likely to be determined by steric control. An approach of the sulfhydryl compound to the exomethylene site is far more probable from the α-direction (Fig. 4). The final addition of a proton or deuteron, which determines the stereochemistry at C-11 of the adduct for steric reasons, should also occur from the α-side.

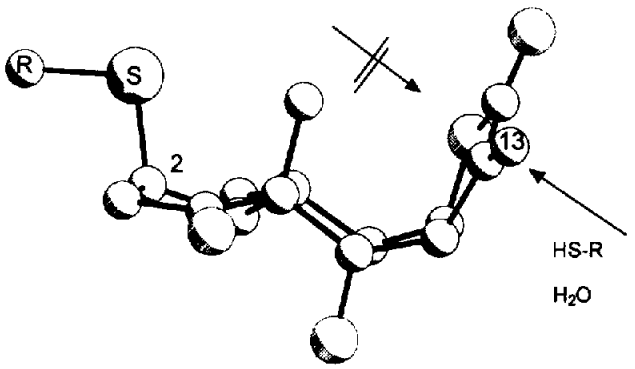


Figure 4. Addition to the exomethylene group occurs favourably from the α-side of the helenalin molecule (the figure shows a model of a C-2β-monoadduct). Proton donation by a solvent molecule determining the stereochemistry of the adduct is also more easily possible from this side leading to a C-11β configured product.

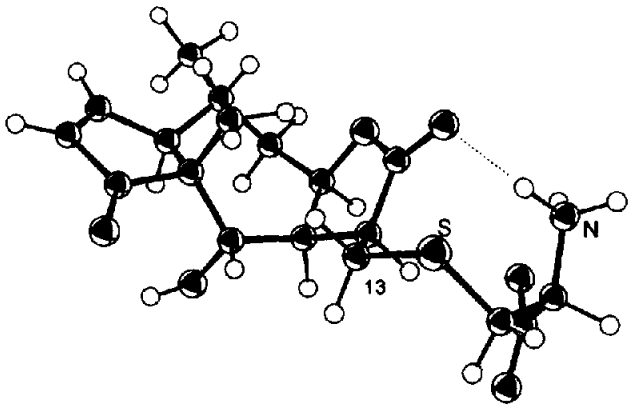


Figure 5. ORTEP plot of the AM1 minimized structure of **1c**. Dotted line: interaction between ammonium and lactone carbonyl.

As a possible reason for the dramatic increase in reactivity of the exomethylene group towards free cys, an electrostatic interaction of the ammonium group of cys with the lactone carbonyl (carbonyl and double bond in a cisoid orientation) of the STL might stabilize the transition state and product, while a similar interaction would not be likely to occur in case of the C-2 adduct. The most favourable conformer found for the C-13(11β)-cys-adduct of **1** is indeed characterized by a small distance between the ammonium group and the lactone carbonyl (N–H^+ ... O=C 2.16 Å, see Fig. 5). In contrast with the *t*-But-S- and Me-S-adducts, this C-13-cys adduct was calculated to be more favourable than the C-2β adduct by 18 kJ mol^{-1} (Table 6). This difference can be interpreted as a consequence of the zwitterionic nature of the amino acid, since models of the adducts with cys in the α-aminocarboxylic acid form, otherwise identical in geometry, yielded energy differences comparable with those of the Me-S- and *t*-But-S-adducts (i.e. the C-2 adduct is lower in energy by 19 kJ mol^{-1}). It may be concluded, that the reaction of cys with the exomethylene lactone occurs at such a highly increased rate due to the cisoid arrangement of the carbonyl and the exomethylene group, which allows an approach of the ammonium- and the sulfhydryl group at the same time. During the addition of cys to C-2 in the cyclopentenone, such a simultaneous approach is not possible because of the transoid orientation of the α,β-unsaturated structure and the higher distance between the oxygen atom and the reactive carbon.

The tested concentration range for GSH, 20–100 mM, is relatively high compared with physiological cellular GSH concentrations that lie in a range of 0.5–10 mM.[17,24] At the same time, the sesquiterpene lactone concentration of 20 mM is far above the concentrations necessary to obtain biological effects which usually lie in the micromolar range and below.[6–11] Since reaction rates, even in these unphysiologically high concentrations, are relatively low, chances for a spontaneous deactivation of the molecules by GSH addition within a living organism must be rather small. More specific reaction sites in enzymes may thus be reached by the unchanged helenalin molecule.

The reaction with cys, on the other hand, proceeded at a very high rate. However, free cys concentrations in blood plasma and cells are known to be very low, in a range of 0.01–0.1 mM,[24] which is only 1/200–1/2000 of the concentrations applied here, so that spontaneous deactivation of STL by free cys in a physiological environment would, if at all, proceed at a much lower rate.

The adverse behaviour of helenalin towards the SH groups of free cys and the tripeptide GSH shows that reactivity towards one particular model nucleophile cannot be extrapolated to predict biological activity in a living system since SH groups are present in a great variety of different environments within a living cell. This explains the observation made by Kupchan et al.[6] that no correlation between the rate of cys addition and bioactivity (in this case cytotoxicity) exists.

The findings presented here clearly demonstrate that the chemical environment of the target sulfhydryl plays a crucial role, whose importance should increase for reactions in more sterically demanding situations (e.g. on the surface or even in hydrophobic pockets of polypeptides). High flexibility as observed among helenalin and its esters[21] will certainly increase a molecule's success in reacting in such more complicated environments. The presence of positively charged or, possibly, hydrogen bond donating structure elements in the right steric position in relation to the SH group on the target side will strongly influence the rate of addition. The view of previous authors, that selectivity of individual sesquiterpene lactones towards particular receptor structures in enzymes exists (see Willuhn[13] and literature cited therein), is reinforced by these findings. Further research on the stereochemistry and reactivity of sesquiterpene lactones towards a wider variety of oligo- and polypeptide structures will possibly lead to further structural information on such selectivities, which should allow the optimization of the lead structures with respect to a desired biological effect. Investigations in this direction are in progress.

Experimental

Compounds

The sesquiterpene lactones were isolated from *Arnica* species as reported previously.[12] GSH and cys were used as purchased from Sigma Chemicals. The purity of all compounds was confirmed prior to the experiments by recording ^{1}H NMR spectra.

Helenalin-2β-mono-glutathionyl adduct (1a^H). Helenalin (10.5 mg, 40 μmol) was dissolved in five drops of acetone, and 1 mL of phosphate buffer pH 7.4 was added. After degassing the sample (ultrasonic) for 30 min, 6.1 mg (20 μmol) of GSH were added. The progress of the reaction was monitored by TLC on Cellulose F254 plates with the upper phase of a mixture of *n*-butanol:H_2O:acetic acid (5:4:1) as mobile phase. Detection: UV 254 nm and 0.25% ninhydrin in acetone.

Formation of one single product was observed. (GSH: $hR_f = 10$, UV: −; ninhydrin: violet; helenalin: $hR_f > 90$, UV: +; ninhydrin: −; Product: hR_f 32; UV: +; ninhydrin: brown). When no further increase of the product spot was observed, another 20 µmol of GSH were added and the reaction continued for another day. After this, only a very small amount of unchanged helenalin could be detected. The reaction product was then purified using a column of 5 g Sephadex LH20 in H_2O, which yielded 4 mg of the pure compound in addition to 16 mg slightly impure product. For ^{13}C NMR data see Table 4.

Helenalin-2β,13(11β)-bis-glutathionyl adduct (1b^H). Helenalin (10.5 mg, 40 µmol) was dissolved as above. After addition of 49 mg (160 µmol) GSH, the mixture was allowed to stand at room temperature for three days and the progress of the reaction monitored by TLC (see above). When no detectable amounts of either unchanged helenalin or the monoadduct **1a^H** were left, the mixture was separated as mentioned above to give 17 mg of pure **1b^H** (TLC $hR_f = 3$). For ^{13}C NMR data see Table 4.

NMR spectroscopy

^1H NMR, COSY, and NOESY spectra were recorded on a Bruker AM400 NMR spectrometer at 400.13 MHz. For the COSY and NOESY spectra, the standard pulse programs COSY.AU and NOESY.AU as provided by Bruker were used. All pulse angles were 90° and the mixing time for the NOESY experiments was 1000 ± 20 ms.

The spectra of **1a^H** and **1b^H** after isolation were recorded on a Bruker DRX 500 NMR spectrometer at 500.13 MHz (^1H) and 125.77 MHz (^{13}C). 2-D $^1H/^{13}C$ shift correlated spectra were recorded on the same spectrometer using the pulse program INVBTP. Shift values are expressed relative to (external) TMS (δ(HDO) = 4.720 ppm). Solvents of a deuteration grade of 99.8% D were utilized in all cases.

Reactions monitored by ^1H NMR spectroscopy

For each reaction, 10 µmol STL were dissolved in five drops of acetone-d_6 (**1**) or five drops of each, acetone-d_6/methanol-d_4 (**2** and **3**) and diluted with D_2O to a volume of 0.5 mL in NMR tubes to give the specified concentration (c_0 = 20 µmol mL^{-1} = 20 mM). After adjusting the NMR spectrometer to the individual sample, the tube was removed and quickly mixed with the specified amounts of GSH or cys. The first spectrum was usually taken 5 min after the beginning of the reaction.

Measurement of reaction rates

The NMR signals in spectra recorded at different times during a reaction were integrated and reaction diagrams obtained by plotting the intensity of the decreasing signals of the starting materials (H-13a/b and/or H-2) versus time (Fig. 1).

In case of the reaction of **1** and **2** with GSH, the data were found to be in good agreement with second-order kinetics. For equimolar ratio, a good linear relationship between $1/c$ and time was obtained (linear regression was carried out using Microsoft Excel. For data for **1** see Table 3, data for **2**: R^2 0.965, k_2 = 0.039). For the reaction of **1** with 2 and 5 equiv of GSH, $1/(c_0(\text{GSH}) - c_0(\mathbf{1})) \times \ln((c_0(\text{GSH}) \times c_t\mathbf{1})/(c_0\mathbf{1} \times c_t(\text{GSH})))$ was plotted versus time.[25] Parameters derived from these graphs (R^2 values for the linear regression and second-order rate constants) are given in Table 3. The increasing deviation of the data from linearity for the reaction with 2 and 5 mol equiv illustrates that the reaction in these concentrations does not exactly follow second-order kinetics.

The data for addition of cys to C-13 could not be linearized by application of a second-order rate law. This reaction proceeded at an extremely high rate with $t_{1/2} < 5$ min. The best fit for a linear relationship (20:40 mM (**1**:cys)) was found for a reaction order of 2.5 (R^2 = 0.9997, $k_{2.5}$ = 0.58 mol$^{-1.5}$ min^{-1}).[25]

In the reaction of cys with C-2, however, the data could be linearized by plotting according to second order, which led to a k_2 value of 0.071 mol^{-1} min^{-1} (R^2 = 0.979) that shows that the reaction rate is very similar to the value for GSH addition at this reactive site.

Molecular models

Computer models were generated with the molecular modelling package Hyperchem, Release 4. As the starting geometry for the adducts of **1**, a model of the TC7 geometry as described in a previous communication[21] was used.

As a first step, MM+ optimized models of the educts were combined to a preliminary model of the product, which was minimized again. Molecular dynamics simulations were carried out at high simulation temperatures (generally 3 ps at T = 600 K) and different geometries resulting from this procedure were re-minimized in order to obtain a minimum energy structure for the products. The resulting geometries were then minimized using the semi-empirical MO method AM1, as implemented with Hyperchem. All models computed by this method were minimized using the Polak–Ribiere algorithm to an RMS gradient smaller than 0.01. Calculation of ^1H-coupling constants (Table 4) was carried out with the program PCMODEL after transformation of the Hyperchem files into MOPAC-format.

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