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Helenanolide Type Sesquiterpene Lactones. Part 5:† The Role of Glutathione Addition Under Physiological Conditions

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Abstract—Sesquiterpene lactones (STLs) are known to exert most of their numerous biological activities through inhibition of enzymes and other functional proteins by forming covalent bonds with free cysteine residues in these macromolecules. The question arises how these drugs can alkylate such vital target structures instead of being quickly deactivated by reaction with the cysteine group of glutathione (GSH) which is present in high concentrations in all cells. We have measured in this study the pH dependent kinetics of GSH addition to the cyclopentenone and α-methylene-γ-lactone group of helenanolide type sesquiterpene lactones using UV–spectrophotometry. The reaction with GSH at physiological pH proceeds very quickly but is reversible so that a fraction of STL molecules will always be available for reaction with protein targets. In agreement with these chemical data, helenalin-mono- and -bis-glutathionyl adducts were demonstrated to inhibit the nuclear transcription factor NF-κB at concentrations similar to the free sesquiterpene lactone.

Introduction

Sesquiterpene lactones (STLs) have received considerable attention because of their numerous biological activities.[1] These compounds exert a strong anti-inflammatory and antineoplastic activity and may therefore represent lead structures for the development of therapeutic agents. However, many STLs also cause acute or chronic toxicity. Most of the observed biological effects have been attributed to these compounds' capability to deactivate enzymes and other essential proteins by formation of covalent bonds with free cysteine sulfhydryl groups in such polypeptides.[1] This mechanism is based on a Michael type addition of α,β-unsaturated carbonyl structures which are a common structural feature of many STLs (for overview on STLs structure see refs 2 and 3).

It has been demonstrated, that STLs such as helenalin, exert their anti-inflammatory activity by inhibiting the nuclear transcription factor NF-κB.[4–6] This protein is essential in activating the transcription of genes that encode important inflammatory cytokines such as interleukins 1, 2, 6 and 8, as well as enzymes such as cycloogygenase II and inducible nitric oxide synthase.[7] The observation that strong NF-κB inhibitory activity of STLs correlates with alkylant bifunctionality[6] and is probably directed selectively towards the p65 subunit,[5] has recently led to a proposed molecular mechanism for NF-κB deactivation by STLs. This model involves a cross linking of two essential cysteine residues in the DNA binding region of the p65 subunit by bifunctional alkylant STLs.[6] The question arose, however, whether and to what extent STL would be able to reach their macromolecular targets within a living cell, where high concentrations (0.5–10 mM) of the low molecular weight thiol gluathione (Gly-Cys-γ-Glu, GSH) are present.[8] This thiol should be able to deactivate STLs before they reach any vital protein targets. The stereochemistry and kinetics of the Michael addition of GSH to the reactive centers of the helenanolide helenalin (**1a**) and some of its derivatives have previously been studied by NMR spectroscopic analyses.[9] It was demonstrated that the cyclopentenone (CP) structure reacts more readily with GSH than the α-methylene-γ-lactone (ML).

Key words: Natural products; terpenes; biomimetic reactions; kinetics; sesquiterpene lactones; glutathione addition; Michael addition; nuclear transcription factor NF-κB.

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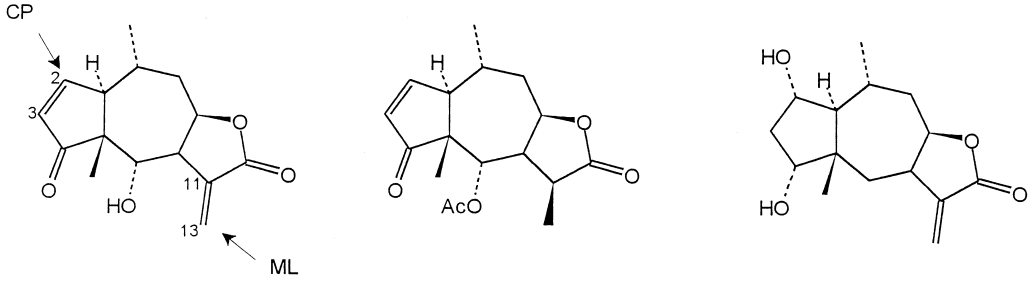
Due to the low rate of GSH addition to helenalin observed in the NMR experiments even at unphysiologically high STL and GSH concentrations, it was concluded that spontaneous reaction of STL with GSH in a living cell would possibly not lead to a significant deactivation of such drugs. However, these NMR experiments were not carried out under physiological conditions, but in unbuffered aqueous GSH solution at pH ≈3. It is, however, known that the reactivity of thiol compounds increases with pH due to formation of the thiolate anion,[10] so that we were interested in measuring the reaction rate at higher pH values which would allow for a more realistic estimation of the role of GSH addition to STLs in living cells.

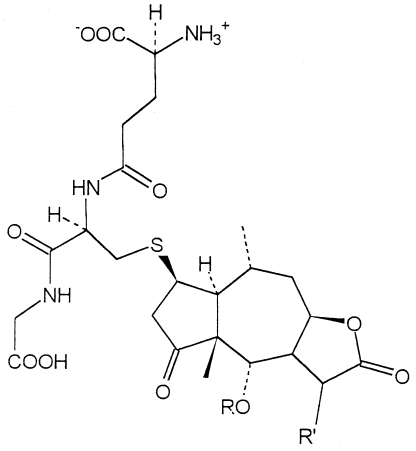
Thus, in the present study we investigated whether STLs are capable of reaching functional protein targets in living cells without being deactivated by reaction with GSH.

Results and Discussion

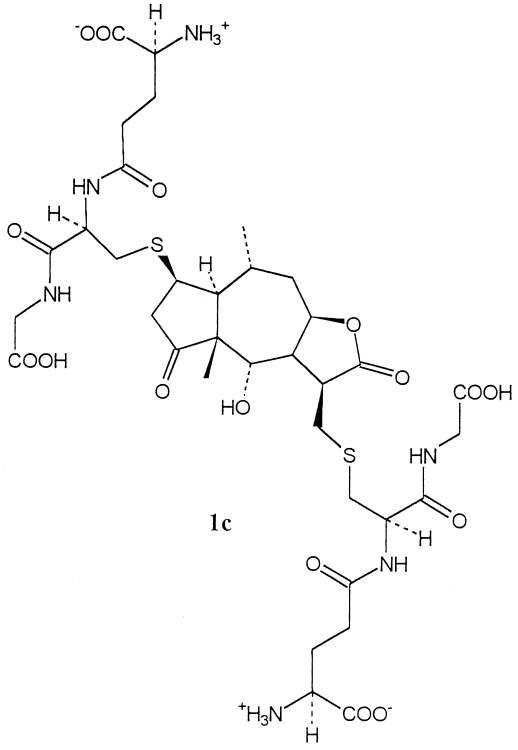
It has been demonstrated in a previous study that the reaction of the cyclopentenone (CP) and α-methylene-γ-lactone (ML) structure of helenalin (**1a**) with GSH follows different kinetics, the former being about 10 times more reactive than the latter.[9] Thus, when incubated with GSH at a molar ratio of 1:1, **1a** exclusively forms 2β-S-glutathionyl-2,3-dihydrohelenalin **1b** while formation of the bis adduct 2β,13-bis-*S*-glutathionyl-2,3, 11α,13-tetrahydrohelenalin **1c** requires higher amounts of GSH. These studies were carried out by ^{1}H NMR measurements at comparatively high concentrations (20 mM **1a** and 20–100 mM GSH) and in unbuffered aqueous (D_2O) solution, where the pH value is about 3.1, due to the acidity of GSH. In order to gain information on the formation and stability of STL–GSH adducts under physiological conditions, mechanistic studies were carried out in buffered solutions in a pH range between 5 and 8 and in a concentration range comparable with in vivo conditions (c(STL) = 10^{-4} M, c (GSH) = 10^{-4} and 5×10^{-4} M). To this end, UV spectrophotometric analysis was used. Due to the fact that the absorption maxima of the CP and ML chromophore overlap, **1a** possesses a broad UV absorption band (λ_{max} = 220 nm[11]), so that reaction kinetics at the different centres can not be monitored simultaneously by



1a **2a** **3**



	R	R'
1b	H	=CH_2
2b	Ac	β-CH_3



1c

measurement of UV absorption. We therefore chose as model compounds two helenalin derivatives, 11α,13-dihydrohelenalin acetate (**2a**) and 2-deacetyl-6-deoxy-chamissonolide (2α-hydroxy-2,3-dihydro-4β*H*-6-deoxy-helenalin) (**3**), each of which possesses only either the CP or the ML site, respectively ($\lambda_{max} = 230$ nm (**2a**) and 215 nm (**3**) in phosphate buffered H_2O at pH 8.0).

Consistent with the NMR results,[9] the CP site of **2a** was found to be far more reactive towards GSH than the ML site of **3**. Figure 1A shows the progress of the reaction of 100 μM **2a** and **3**, in the presence of 500 μM GSH at pH 5 to 8 over the first 10 min. At physiological pH 7, **2a** quickly reacts with a half life ($t_{1/2}$) of about 6 min, while the concentration of **3** shows only a very small decrease.

The reaction rate of **2a** and **3** with GSH was found to be strongly pH dependent, higher pH leading to increased reaction rates. Linearization of the reaction data was possible equally well by a second-order and a (pseudo)first-order rate law. The rate constants and half-live data calculated from these measurements, together with those obtained by NMR spectroscopy at pH 3[9] are given in Table 1. Figure 1B shows a plot of the second-order rate constants versus pH. The rate constants exhibit an approximately exponential dependence on pH, which is in agreement with a base catalyzed reaction mechanism in which the thiol reacts in its deprotonated form (i.e. as the thiolate anion),[10] whose concentration increases with pH (pK_a of SH group in GSH = 9.1[12]).

Thus, the reaction of the CP moiety proceeds at a very high speed in the physiological pH range between 7 and 8 while the ML site reacts rather sluggishly. Taking into account, that the GSH concentration in this experiment represents only the lower boundary of that observed in living cells (between 0.5 and 10 mM[8]), it can be expected that the CP site will quickly and to a large extent be transformed into the GSH adducts once the STL enters a cell, while the ML site, due to its lower reactivity towards GSH may be capable of reaching protein cysteines before reacting with the tripeptide. Calculated $t_{1/2}$ values, for example, at c(STL) = 10^{-5} M(10 μM,

Table 1. Second-order (k_2, M^{-1} min^{-1}) and pseudo-first-order (k_1, min^{-1}) rate constants for the cyclopentenone (CP) and α-methylene-γ-lactone (ML) moiety of helenanolides at different pH values. Half lives $t_{1/2}$ (min) were calculated from the rate constants for c_0(SL) = 100 μM and c_0(GSH) = 500 μM

	CP				ML			
pH	k_2	k_1	$t_{1/2}(k_2)$	$t_{1/2}(k_1)$	k_2	k_1	$t_{1/2}(k_2)$	$t_{1/2}(k_1)$
8[a]	480	0.2167	3.06	3.20	77	0.0372	19.15	18.63
7[a]	216	0.1109	6.81	6.25	16	0.0078	93.3	88.87
6[a]	57	0.0286	25.79	24.24	1.75	0.0006	840	1155.25
5[a]	14	0.0068	105	101.93	n.d[c]	n.d.	—	—
3[b]	0.07	n.d.	2×10^4	–	0.009	—	1.6×10^5	—

[a] Determined with **2a** (CP) and **3** (ML) at c_0(STL) = 100 μM, c_0(GSH) = 500 μM by UV spectrophotometry.
[b] Determined with **1a** at c_0(STL) = 20 mM, c_0(GSH) = 100 mM by NMR spectroscopy.[9]
[c] n.d.: not determined.

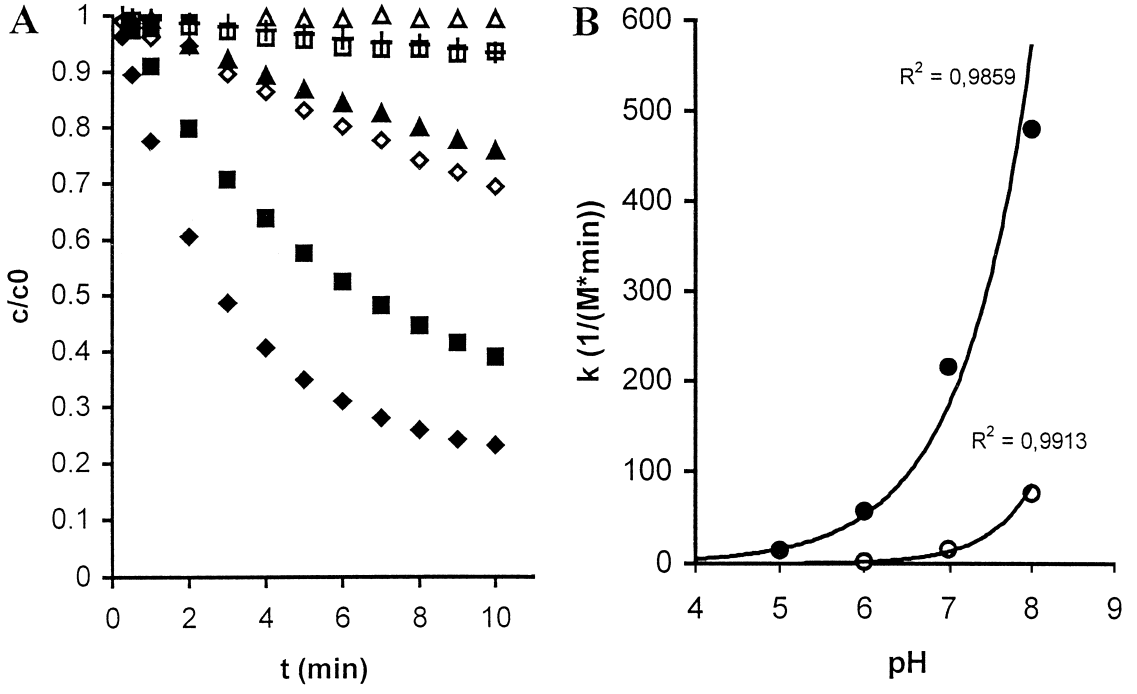


Figure 1. The CP site of helenanolides is far more reactive towards GSH than the ML site. A. Reaction of 100 μM **2a** (filled symbols) and **3** (open symbols) with 500 μM GSH at different pH values (unreacted fraction of STL plotted versus time): rhombic, pH 8; square, pH 7; triangle, pH 6; cross, pH 5, compound **2a**; compound **3** showed no measurable decrease of absorption at pH 5. B. Apparent second-order rate constants deduced from data shown in A, plotted versus pH (filled symbols: **2a**, open symbols: **3**). Exponential trendlines with their squared correlation coefficients are shown.

≈concentration at which **1a** leads to complete inhibition of NF-κB) and a GSH concentration of 5 mM are 0.6 min for CP and 8.7 min for ML. How then can STLs, especially those that possess only a CP group, effectively alkylate protein sulfhydryl groups leading to a biological effect, instead of immediately and completely being deactivated by reaction with GSH?

It has previously been demonstrated that addition of mercaptanes such as β-morpholinoethanethiol to a number of electrophiles (under non-physiological conditions) is reversible to a variable extent. This was shown by the different adduct yields at equimolar reactant ratio and by the extent of adduct decomposition in sodium hydroxide solutions.[13] In order to gain information on the reversibility of the addition of GSH to STLs under physiological conditions, kinetic studies were carried out at an equimolar ratio. When incubated with an equimolar amount of GSH (100 μM each), **2a** still showed considerable reactivity at higher pH values. In case of **3**, a very slow decrease in absorption could only be observed at pH 8, while at lower pH the reaction proceeded too slowly to be monitored at all. Quite conspicuously, however, the reaction of **2a** with 1 mol equivalent GSH reached equilibrium when about 63% of the STL were still unreacted (pH 8) indicating that the reverse reaction, i.e. hydrolysis of the adduct, was indeed taking place in the solution. The resulting equilibrium constant K (=c(Adduct)/[c(STL)*c(GSH)]=c(Adduct)/c(STL)^2) of 0.93 shows that the product is energetically about as stable as the educts. In order to study the reverse reaction, the GSH adduct of **2a**, 2β-S-glutathionyl-2,3,11α,13-tetrahydrohelenalin **2b**, was prepared (preparation and ^{1}H NMR data see Experimental) and its decomposition in 100 μM solution was monitored at different pH values. The resulting data are plotted together with those of the forward reaction at an equimolar ratio in Figure 2.

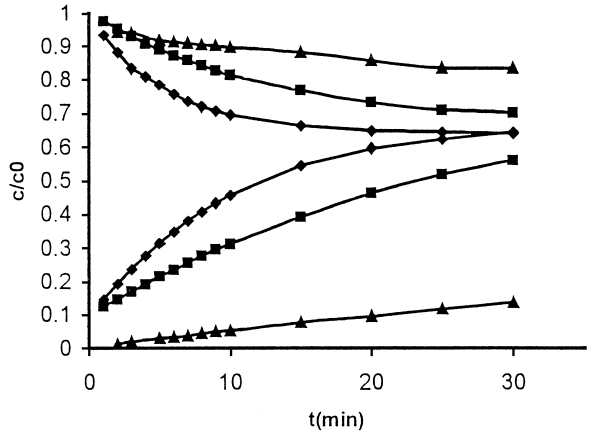
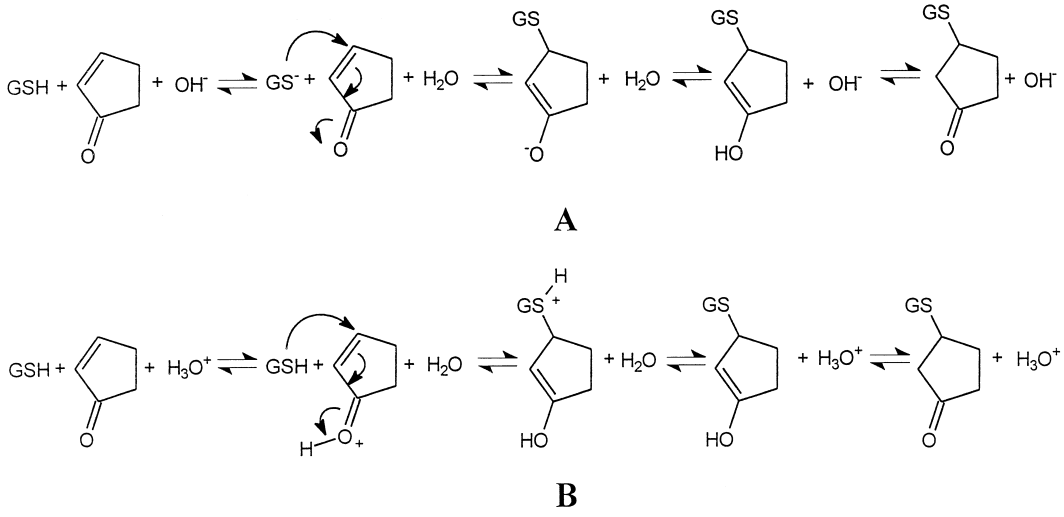


Figure 2. Reversibility of GSH addition to **2a**. Free STL concentrations for the reaction of 100 μM **2a** with 100 μM GSH (descending) and for decomposition of 100 μM **2b** (ascending) at pH 8 (rhombi), pH 7 (squares) and pH 6 (triangles).

Under the conditions of the above-mentioned NMR experiments at pH ≈3, the reaction proceeds to completeness (or at least to a much higher K value). Compared with the reaction at higher pH the reaction speed is extremely slow even at concentrations 200 times higher than applied here, which previously led us to the conclusion that GSH addition to STL should not play a significant role in a living cell.[9] The difference in the equilibrium constants observed in acidic and neutral or slightly basic solution indicates that the mechanism by which the reaction proceeds changes with pH. Scheme 1 shows the conceivable mechanisms of the reaction under prevalence of OH^- and H_3O^+, respectively. At low pH, where the speed of the reaction is extremely low, the equilibrium lies on the product side, i.e. the product is energetically more favourable in acidic solution than the educts. The overall equilibrium is determined by the



Scheme 1. Conceivable reaction mechanisms for addition of GSH to CP site of helenanolides under prevalence of OH^- (A) and H_3O^+ (B), respectively. A. At higher pH values (in the physiological range), the reaction proceeds very quickly but is reversible. The overall equilibrium (K≈1) is determined by the ratio of acidity of GSH's thiol group (forward reaction) and of the adduct's enol tautomer (reverse reaction). B. At low pH values (in unbuffered GSH solution), the speed of reaction is very low. The equilibrium lies on the product side ($K \gg 1$) since protonation at the thioether-sulfur (initiating reverse reaction) is less favourable as compared with protonation of the conjugated carbonyl oxygen (forward reaction).

proton affinity of the educt and the product. Thus, in agreement with the basicity of the educt's conjugated carbonyl oxygen being higher than that of the product's thioether-sulfur, the equilibrium lies on the product side. With increasing pH, the stability of the adduct decreases, so that at pH 8 it is about as stable as the educts leading to the observed equilibrium constant of about 1. Here, the overall equilibrium depends on the acidity of GSH's thiol group and of the product's enol tautomer, which appear to lie in a similar range. Here the reaction rate is much higher, indicating that the activation barrier of the base catalyzed mechanism is considerably lower than that of the acid catalyzed reaction.

Thus, at physiological pH and high GSH-concentration as present in living cells (500 μM to 10 mM), GSH adducts will always be formed. From the experiments at equimolar concentration, however, it becomes clear that the reaction in the physiological pH range is essentially reversible so that the alkylating structure elements can be released from the GSH adducts allowing them to react with thiol groups of a target protein. The stability of such STL–protein adducts will be influenced by additional factors, such as the capability of the STL molecule to engage in further stabilizing interactions of hydrophobic and/or electrostatic/polar nature, with structure elements on the protein side.

In order to investigate whether these results from an in-vitro chemical study can be transferred to a biological system, we tested the NF-κB inhibitory activity of the isolated mono- and bis-glutathionyl adducts of helenalin (**1a**), 2β-*S*-glutathionyl-2,3-dihydrohelenalin (**1b**) and 2β,13-bis-*S*-glutathionyl-2,3,11α,13-tetrahydrohelenalin (**1c**) (for synthesis, purification and structural elucidation see ref 9). Under the assay conditions described previously,[4–6] both adducts were found to inhibit NF-κB DNA binding at concentrations only slightly higher than those required of helenalin itself. Nearly complete inhibition of specific NF-κB binding to the ^{32}P-labelled oligonucleotide was achieved with 10 μM **1a**, 20 μM **1b** and 50 μM **1c** (see Fig. 3). It is noteworthy that **1c** does not possess an alkylating structure element capable of reacting with the protein's SH-groups. However, this is considered an essential requirement for NF-κB inhibition by STL.[6] Its inhibitory activity can be explained by the instability of the thioether groups at physiological pH, i.e. the reversibility of GSH addition. This result clearly demonstrates how STLs are able to exert their biological activity in spite of the presence of GSH.

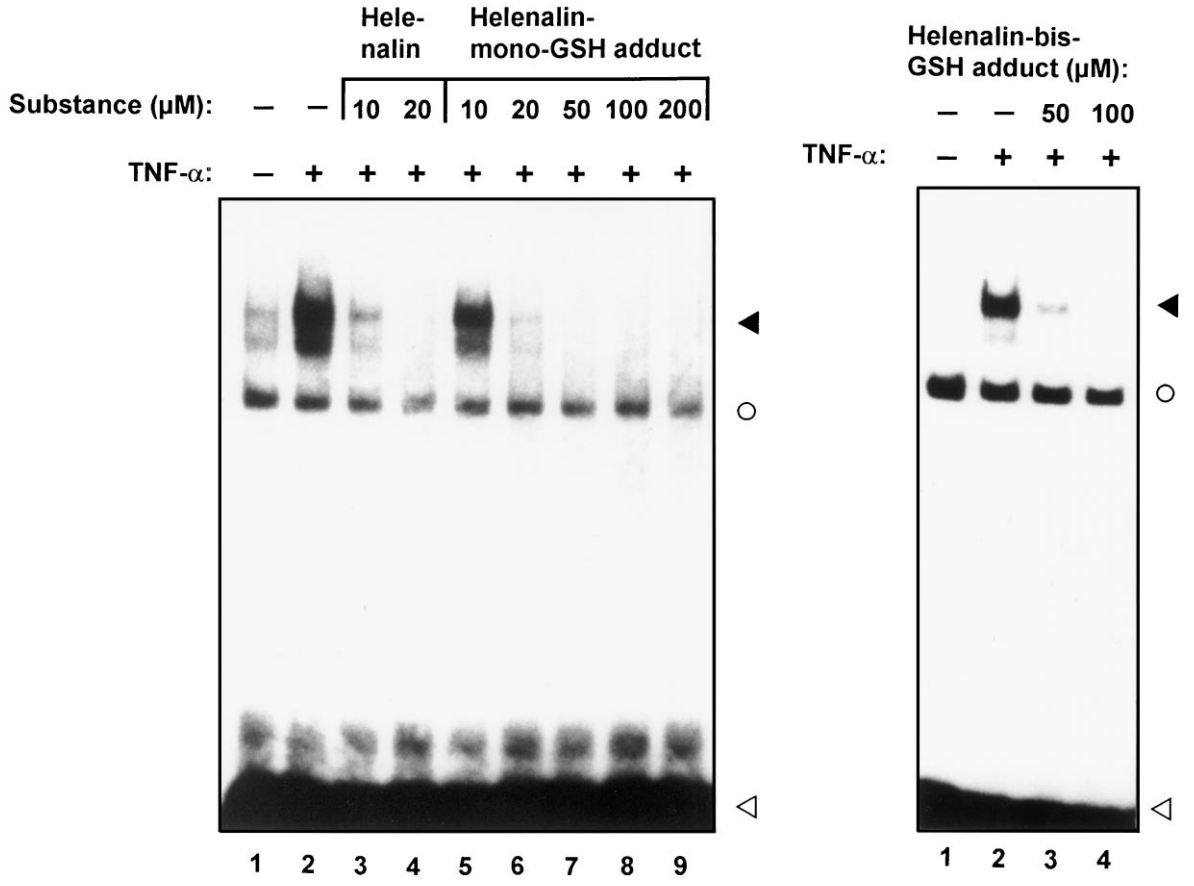


Figure 3. Inhibition of NF-κB DNA binding by helenalin (**1a**), and its mono- and bis-glutathionyl adducts (**1b** and **1c**, respectively). The slight shift in lane 3 (**1a**) and 6 (**1b**) in the left panel can be neglected, because untreated cells (lane 1) already show NF-κB activation to a small extent. Lane 1 shows unstimulated control cells. In lane 2, cells were treated with 200 U/mL TNF-α. In the other lanes, cells were pretreated for 1 h with the specified concentrations of the drugs before TNF-α stimulation. The filled arrowhead indicates the position of the NF-κB-DNA-complexes. The circle denotes a non-specific activity binding to the probe, the open arrowhead shows unbound oligonucleotide.

Conclusions

Under intracellular conditions, i.e. at neutral pH and high GSH concentration, a large fraction of STLs is transformed into the GSH adducts. The high reactivity of the CP site will lead to very fast and almost complete adduct formation, while the ML site is far less reactive and will react to a high extent only at much higher ratios of c(GSH)/c(STL). These, however, can be expected to occur inside certain cells. Reaction with free cysteine residues of macromolecules must therefore be preceded by hydrolysis of the GSH-adducts' thioether moieties, releasing the α,β-unsaturated carbonyl structures. This reaction was proven to occur in vitro at physiological pH. The NF-κB inhibitory activity of the helenalin-2,13-bis-GSH-adduct **1b** confirms that decomposition of the GSH adducts takes place also in a biological system. The present study thus demonstrates how STLs can overcome the cells' protection by high intracellular GSH concentrations to alkylate protein structures leading to their numerous biological effects. Possible involvement of glutathione *S*-transferase enzymes in adduct formation is currently under study, and first results indicate that the rate of addition is not influenced by a commercially available hepatic glutathione *S*-transferase.[14]

The observation that the decomposition of GSH adducts is negligibly slow at low pH may be of specific interest with respect to STLs' use as anti-inflammatory agents. When applied to acutely inflamed tissue which is usually acidotic, GSH adducts would be stable enough to prevent reaction of STLs with soluble proteins which is the prerequisite for STLs' most important side effect, sensitization leading to contact dermatitis. It has previously been demonstrated that amino acid adducts of alantolactone lack the sensitizing potency of the free STL.[15] It is, however, unclear at present whether such adducts themselves are capable of penetrating the cytoplasmic membrane or, whether only the fraction of STLs which is released from the adduct is able to enter the cell.

Further studies will have to show whether, for example, **1c** can serve as a therapeutic agent in treatment of acute inflammations of the skin without the sensitizing potential of free helenalin.

Experimental

Chemicals

The sesquiterpene lactones **1a** and **2a** were isolated from *Arnica* species (see literature cited in ref 16), **3** was obtained by hydrolysis of 6-deoxychamissonolide as described previously.[17] The glutathionyl adducts of **1a** were prepared as reported previously.[9] Glutathione was purchased from Sigma Chemicals. Buffers: 0.1 M KH_2PO_4 (A), 0.1 M NaOH (B) pH 8: 50 mL A + 46.7 mL B; pH 7: 50 mL A + 29.1 mL B; pH 6: 50 mL A + 5.6 mL B; pH 5: 14.8 mL 0.2M NaOAc + 35.2 mL 0.1 M AcOH.

2β-*S*-Glutathionyl-2,3,11α,13-tetrahydrohelenalin acetate (2b). To a solution of 6.1 mg (20 μmol) of **2a** in 0.5 mL ethanol, 12.3 mg (40 μmol) reduced glutathione in 0.5 mL H_2O were added and the progress of the reaction monitored by TLC as reported previously;[9] product $R_f = 0.53$.

When no free **2a** could be detected anymore, the mixture was chromatographed on 10 g Sephadex LH-20 with H_2O as eluent and the fractions containing the pure adduct (TLC control) were combined and freeze dried.

^{1}H NMR (500 MHz, D_2O, external TMS): H-1: 2.68 (dd, 6.3, 11.4); H-2: 3.48 (*); H-3a + 3b: 2.71 (*); H-6: 5.10 (s); H-7: 2.79 (dd, 7.3, 10.1); H-8: 4.76 (br dd(t), 6–7); H-9α; 1.68 (ddd, (≈1, 11.8, 15.3); H-9β: 2.24 (ddd, 2.2, 6.6, 15.8); H-10 1.96 (m); H-11: 3.19 (dq, 7.3 (q), 10.1); CH_3-13: 1.22 (d, 7.3); CH_3-14: 1.01(d, 6.6); CH_3-15: 0.87 (s); cys: α-H: 4.43 (dd, 10.1, 4.6); β-H_2: 2.96 (dd, 4.6, 14.5), 2.77 (dd, 10.1, 14.5); gly: α-H_2: 3.78 (m); glu: α-H: 3.65 (t, 6.5); β-H_2: 2.01 (m); γ-H_2: 2.40 (m). *non-first-order spin system with $^3J_{2,3a} \approx 0$ Hz, $^3J_{2,3b} \approx 8$ Hz, $^2J_{3a,3b} \approx 16$ Hz; $^3J_{2,1} = 6.3$ Hz; $\delta\nu_0(3a,3b) \approx 1$ Hz.

UV–spectrophotometric measurements and data analysis

All reactions were carried out in buffered aqueous solution in 1 cm quartz cuvettes in a Beckman DB-G UV–vis spectrophotometer. Reactions of **2a** were monitored at $\lambda = 230$ nm, those of **3** at $\lambda = 215$ nm. A solution of the same concentration of GSH in the buffer was used for compensation. Of each reactant, a stock solution (EtOH (**2a** and **3**), H_2O (GSH and **2b**)) was prepared so that addition of 10 μL to 2.0 mL of buffer yielded the specified final concentration. Stock solutions of GSH were prepared freshly each day to avoid a decrease of concentration due to formation of glutathione disulfide. After recording the initial absorption of the STL solution A_0(STL), GSH was added, quickly mixed and the decrease in absorption monitored (increase in decomposition studies of **2b**). The resulting absorption data were processed with Microsoft Excel 97. Absolute concentrations at time t were calculated by multiplying A_t(STL)/A_0(STL) with the initial STL concentration c_0(STL). GSH concentrations then are $c_t(GSH) = c_0(GSH) - (c_0(STL) - c_t(STL))$. Second-order rate constants (k_2) were obtained from the reaction of 100 μM STL with 500 μM GSH (Table 1) by plotting $\ln\{(c_0(GSH)\cdot c_t(STL))/(c_t(GSH)\cdot c_0(STL))\}\cdot\{1/(c_0(GSH) - c_0(STL))\}$ versus time. The slope coefficients of the resulting linear functions represent k_2. Pseudo-first-order rate constants k_1 (min^{-1}, Table 1) were obtained by plotting ln c_t(STL) versus time, where k_1 is given by the slope of the resulting linear functions.

Half lives ($t_{1/2}$, Table 1) were calculated from the rate constants according to $t_{1/2} = \ln 2/k_1$ and $t_{1/2} = \ln [(0.5\cdot c_0(STL)\cdot c_0(GSH))/(c_0(STL)\cdot(c_0(GSH) - 0.5\cdot c_0(STL))] \cdot 1/(c_0(STL) - c_0(GSH)\cdot k_2)$.

NF-κB assay

All assay conditions were essentially identical with those reported previously.[4–6] Experiments were repeated three times with identical results.

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