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Original article

Phenylthio-derivatives of α-methylene-γ-lactones as pro-drugs of cytotoxic agents§

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Abstract – A series of substituted phenylthio-derivatives of grosheimin (**1**), a natural cytotoxic guaianolide, were investigated with the aim of providing insight into their mechanism of action as cytotoxic agents against KB cell lines. Hydrolysis data, kinetics, in the presence and in the absence of H_2O_2, and the valuation of lipophilicity were correlated with cytotoxicity values and with Hammett-σ-values of substituents (R) at the thiophenol ring. These compounds behave as 'pro-drugs' which release the cytotoxic agent grosheimin by sulphur-oxidation promoted by H_2O_2 and subsequent retro-elimination which depends on the nature and position of the R substituent. © Elsevier, Paris

sesquiterpene lactones / pro-drugs / cytotoxicity

1. Introduction

Natural and semi-synthetic unsaturated-γ-lactones have been the subject of continuous research spanning nearly three decades because of their anti-inflammatory, antibacterial, anti-hyperlipidemic, antiallergic and especially because of their cytotoxic activity [1–4]. Sesquiterpene α-methylene-γ-lactones are alkylating agents and have been used as antitumour drugs in some cases [5]. The potential tumour-inhibiting ability of this class of sesquiterpene γ-lactones derives from a Michael-type interaction of the α,β-unsaturated moiety with nucleophilic endocellular components, such as sulphydril enzymes. The usefulness of most of these lactones is limited because of their toxicity which gives rise to indiscriminate reactions towards biological nucleophiles. Consequently, a number of efforts were made to achieve lower toxicity via lipophilicity modulation, or via chemical derivatization [6, 7].

Appropriate precursors of α-methylene-γ-lactones, able to release the cytotoxic agent selectively in tumour cells, can decrease the toxicity of this class of compounds. Our interest in such postulated precursors has been focused [8] on a series of phenylselenoderivatives of cytotoxic guaianolides with different functionalized sesquiterpene skeletons. Their cytotoxicity, evaluated in vitro against KB cells, showed a generalized increase of bioactivity (ID_{50}) compared to the parent lactone compound, thus supporting the hypothesis of in situ generation of the corresponding α-methylene-γ-lactones, probably via the formation of fast retro-syn-eliminating selenoxides [9, 10].

The activation of such masked α,β-unsaturated lactones by means of intracellular oxidation processes might occur preferentially in tumour cells, since some of them are deficient in catalase, peroxidase, superoxidodismutase and, therefore, more sensitive than normal cells to H_2O_2 and oxygen radical mediated cytotoxicity [11–15].

None of the predicted guaianolide selenoderivatives were active in vivo as antitumour agents in leukaemia screening by standard NCI protocols, at dose levels of 60–240 mg/kg (unpublished data).

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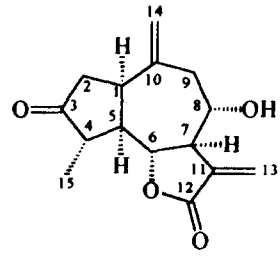
† Deceased March 26, 1994.

Table I. Chemical, physico-chemical and pharmacological data of compounds **1–18.**



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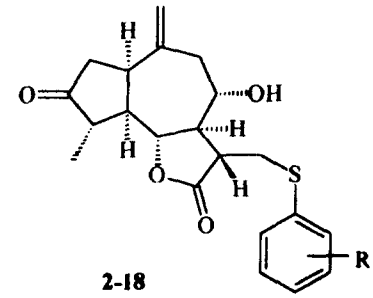

2-18

Compounds	Thiephenol type		R-substituents properties			In vitro KB cytotoxicity		Hydrolysis kinetics (I/h)		Hydrophilicity R_{RT}
	R-subsitutents	R-position	σ Hammett	Fσ Hammett	π hydrophob.	ID_{50} ($M \times 10^{-5}$)	% inhib. 5 μg/ml	lgki	$lgkiH_2O_2$	
1	-	-	-	-	-	0.56	-	-	-	1.0
2	2,3,5,6 tetra F	-	-	-	-	0.39	-	6.05	6.05	3.9
3	Me	p	- 0.17	- 0.05	+ 0.50	0.64	-	5.40	5.69	3.4
4	Me	m	- 0.07	- 0.05	+ 0.50	0.86	-	5.23	5.65	3.7
5	Me	o	-	- 0.05	+ 0.50	1.00	-	5.18	5.60	3.3
6	H	-	0.00	0.00	0.00	1.12	-	5.08	5.59	2.4
7	NHCOMe	p	0.00	+ 0.47	- 0.97	-	22.25	4.09	4.68	1.2
8	OMe	p	- 0.27	+ 0.41	- 0.02	-	19.66	4.12	4.53	2.2
9	OMe	m	+ 0.11	+ 0.41	- 0.02	-	19.09	4.08	4.40	2.9
10	OMe	o	-	+ 0.41	- 0.02	-	11.91	4.08	4.32	1.8
11	OH	p	- 0.37	+ 0.49	- 0.67	-	0.00	ki = 0	ki = 0	1.2
12	F	p	+ 0.06	+ 0.71	+ 0.13	-	0.00	ki = 0	ki = 0	2.3
13	Cl	p	+ 0.23	+ 0.70	+ 0.76	-	0.00	ki = 0	ki = 0	4.4
14	Cl	m	+ 0.37	+ 0.70	+ 0.76	-	0.00	ki = 0	ki = 0	4.6
15	Cl	o	-	+ 0.70	+ 0.76	-	0.00	ki = 0	ki = 0	3.2
16	Br	p	+ 0.23	+ 0.73	+ 0.94	-	0.00	ki = 0	ki = 0	5.0
17	Br	m	+ 0.39	+ 0.73	+ 0.94	-	0.00	ki = 0	ki = 0	5.5
18	Br	o	-	+ 0.73	+ 0.94	-	0.00	ki = 0	ki = 0	3.5

As the high Sharpless retro-elimination reactivity of the selenoxide intermediates [16] could be responsible for the in vivo inactiveness of the tested seleno-guaianolides, we substituted the Se with a sulphur atom, which has a lower retro-elimination reactivity, by preparing a series of phenylthio derivatives (**2–18**) of the natural cytotoxic guaianolide grosheimin (**1**) *(table I).*

According to the mechanism of action reported in *figure 1*, the lower reactivity of sulphur to oxidation and to retro-elimination compared to those of selenium can provide a more highly selective process, particularly in the cytosol environment of cancer cells. Moreover, the change of the nature of the R substituents' position in the aromatic ring increases the selectivity of the potential pro-drugs by modulating their effectiveness on the process rate of generating the cytotoxic agent (**1**).

If substituent R is an electron-withdrawing group, it increases the electrophilicity of the sulphur atom thereby decreasing its ability to be oxidized in the first step of the sulfoxide intermediate, but also increasing the ability of the sulphinic (-enic) group to undergo retro-elimination and vice-versa.

The aim of this study was to provide new evidence supporting the mechanism of action of these postulated pro-drugs as 'masked α,β-unsaturated lactones'. Thus, we have now investigated the effect of both the substitution of Se/S and of the nature and the position of various R substituents at the thiophenol ring on the ability of these lactone precursors to act as selective cytotoxic agents.

The thiophenols added to the exomethylene group of the guaianolide grosheimin (**1**) have different substituted groups whose electron-withdrawing and polarization effects are measurable by the Hammett-σ-constants (position-dependent) and by the π-hydrophobic constants (position-independent) *(table I).*

Clearly, compounds **2** (because of the poly-substituted nature of the aromatic ring) and **5**, **10**, **15**, **18** (owing to

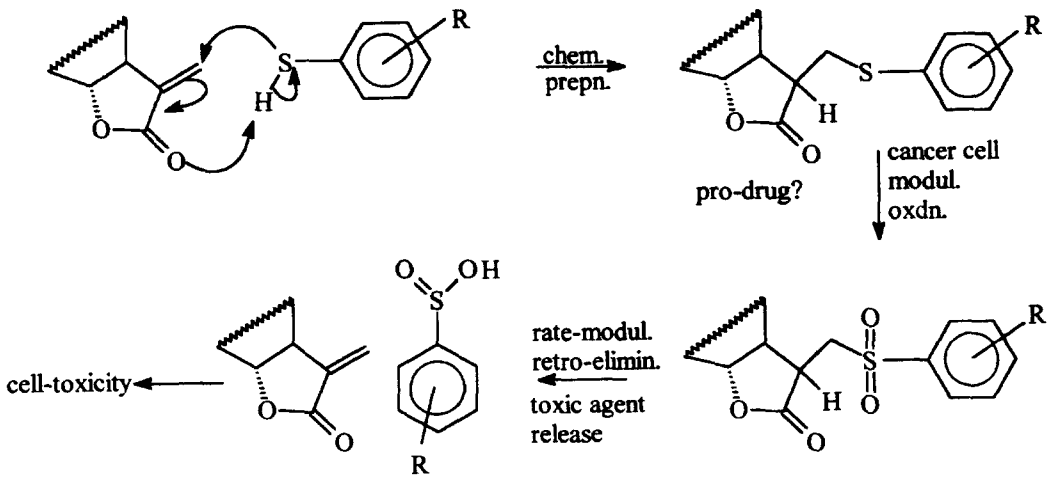

Figure 1. Preparation and proposed mechanism of action of compounds **2–18**.

the ortho position of their R substituent, which is clearly responsible for unquantifiable steric valuation) are not particularly suitable for such a rigorous valuation. However, they are useful to give further information on this series of products.

2. Chemistry

Phenylthio derivatives **2–18** were obtained by reaction of grosheimin **1** with the appropriate thiophenol at room temperature with stirring under nitrogen atmosphere. Main details of the compounds' synthesis and characterization are reported in *tables II* and *III*. The reaction of grosheimin with the appropriate thiophenols generated compounds (**2–18**) with a new chiral centre. The ^{1}H-NMR signals showed the presence of only one stereoisomer. Unfortunately, the resolution at 200 MHz was not enough to assign the correct configuration. According to the results previously obtained for the grosheimin phenylseleno derivatives [8], the presence of the H_{11}-α stereoisomer can be hypothesized.

Hydrolysis kinetics were carried out at pH 7.4 and 37 °C in the absence and in the presence of a stoichiometric excess of H_2O_2 (k_i and $k_iH_2O_2$ values are reported in *table I, figure 2, plot 3* and *figure 3, plot 2*).

The measurement of the lipophilic-hydrophilic properties of all the compounds were carried out by means of an original HPLC reverse-phase method [17] on the relative retention time values (RRt) (*table I* and *figure 2, plot 4*). This physico-chemical characteristic is important in the pharmacokinetic phase of cell-membrane permeation by cytotoxic agents, especially in the case of the in vitro test in cell cultures.

3. Pharmacology

The cytostatic activity of the compounds **1–18** was tested in vitro against a cell line of human nasopharinx carcinoma (KB). The 18 tested compounds can be divided into three pharmacological groups: inactive (**11–18**), moderately active (**7–10**) with low cytostatic effect at a concentration of 5 μg/mL medium (corresponding to values greater than 10^{-5} M) and active (**1–6**) compounds
(*table I* and *figure 2, plot 1*).

4. Results and discussion

Results of in vitro tests, hydrolysis kinetics in the absence and in the presence of H_2O_2 and measurements of the lipophilic-hydrophilic properties of all compounds were correlated both qualitatively (*figure 2*) and, in part, quantitatively (*figure 3*). These correlations provided consistent suggestions on the role of both the nature and the position of the R-substituents at the thiophenol ring in the behaviour of these 'masked lactones' which can act as pro-drugs according to the postulated mechanism of action. Preliminary results of this study are summarized in *table I* and *figure 2* in which compounds **2–18** are plotted in order of increasing cytotoxicity (*figure 2, plot 1*) and correlated (i) with the respective values of the Hammett 'field effect' of the R substituent (Fσ) (*figure 2, plot 2*), (ii) with the values of the kinetic constants of

Table II. Compound synthesis and characterization.

Comp.	Thiophenol (Thi) added	Reaction time (hr)	Yield (% mol.)	m.p.* °C	MS (M/e)	UV (λmax, nm, EtOH)
2	2,3,5,6-tetraF-thi	2	38	175-78	441.1 (19.8 %, M^+, ^{32}S), 443.0 (2.1 %, M^+, ^{34}S)	272 (ε = 5010), 206 (ε = 13650)
3	p-CH_3-Thi	0.5	78	164-66	385.9 (0.9 %, M^+, ^{32}S)	256 (ε = 7420), 204 (ε = 14550)
4	m-CH_3-Thi	1	88	172-74	385.9 (2.5 %, M^+, ^{32}S)	255 (ε = 8370), 207 (ε = 18240)
5	o-CH_3-Thi	2	77	172-73	386.1 (36.7 %, M^+, ^{32}S), 387.9 (2.9 %, M^+, ^{34}S)	251 (ε = 3580), 207 (ε = 15510)
6	Thi	3	63	188-90	372.3 (57.1 %, M^+, ^{32}S), 374.3 (8.3 %, M^+, ^{34}S)	254 (ε = 5160), 205 (ε = 12280)
7	p-NHCO-CH_3-Thi	0.3	80	205-07	429.0 (33.6 %, M^+, ^{32}S), 431.1 (3.9 %, M^+, ^{34}S)	273 (ε = 21080), 205 (ε = 23930)
8	p-OCH_3-Thi	0.75	98	168-70	402.1 (6.2 %, M^+, ^{32}S), 404.0 (1.3 %, M^+, ^{34}S)	257 (ε = 13870), 229 (ε = 13330)
9	m-OCH_3-Thi	2	85	150-52	402.0 (5.1 %, M^+, ^{32}S)	286 (ε = 2452), 255 (ε = 6100), 217 (ε = 14680), 204 (ε = 17280)
10	o-OCH_3-Thi	0.25	98	216-18	402.2 (5.6 %, M^+, ^{32}S), 404.1 (0.9 %, M^+, ^{34}S)	286 (ε = 3230), 253 (ε = 4770), 205 (ε = 17890)
11	p-OH-Thi	2	74	134-36	388.2 (11.3 %, M^+, ^{32}S), 390.0 (2.7 %, M^+, ^{34}S)	
12	p-F-Thi	2	42	159-62	390.2 (28.7 %, M^+, ^{32}S), 392.1 (3 %, M^+, ^{34}S)	286 (ε = 16860)
13	p-Cl-Thi	0.6	97	180-82	406.5 (41.8 %, M^+, ^{32}S), 408.4 (9.6 %, M^+, ^{34}S)	260 (ε = 14240), 205 (ε = 19190)
14	m-Cl-Thi	0.25	93	177-79	406.4 (13.7 %, M^+, ^{32}S), 408.5 (2.8 %, M^+, ^{34}S)	258 (ε = 10010), 212 (ε = 18530), 205 (ε = 19140)
15	o-Cl-Thi	0.1	84	178-80	405.0 (36.4 %, M^+-1, ^{32}S), 407.2 (3.9 %, M^+-1, ^{34}S)	255 (ε = 5220), 204 (ε = 16700)
16	p-Br-Thi	0.15	87	212-14	451.0 (19.8 %, M^+, ^{32}S), 453.1 (2.1 %, M^+, ^{34}S)	263 (ε = 16800), 204 (ε = 23160)
17	m-Br-Thi	1	93	158-60	451.1 (31.0 %, M^+, ^{32}S), 453.2 (2.8 %, M^+, ^{34}S)	259 (ε = 10640), 206 (ε = 23450)
18	o-Br-Thi	1	87	188-91	451.1 (29.3 %, M^+, ^{32}S), 453.0 (1.8 %, M^+, ^{34}S)	255 (ε = 9630), 207 (ε = 26850)

* White crystals, from CH_2Cl_2 for **11** and from MeOH for all the other compounds.

hydrolysis, $\lg k_i$, at pH 7.4 and 37 °C in the absence of H_2O_2 and in the presence of H_2O_2 (*figure 2, plot 3*) and (iii) with the lipophilicity (*figure 2, plot 4*). These qualitative correlations clearly show a parallelism between the trend of the data of cytotoxicity, that of Fσ values of R substituents, and that of the two series of $\lg k_i$ values for all the three groups of: active (**2–6**), moderately active (**7–10**) and inactive (**11–18**) compounds.

The R-substituent at the thiophenol ring of the molecule may play a basic role, mainly because of its own 'electric field effect' quantified by the Hammett Fσ values [18, 19]. A dominant 'field effect' (Fσ) of the R substituent would affect the sulphur charge density in the phase of the proposed mechanism.

Further insights arise from the data of the hydrolysis kinetics of the tested compounds in the absence and in the presence of H_2O_2 (*figure 2, plot 3* and *table I*). These hydrolysis processes are the different simplified model-conditions of a normal and an oxidant environment for the 'masked lactones' inside hypothetical normal and tumour cells.

The rates of hydrolysis, followed by HPLC dosage of grosheimin (**1**), demonstrated first that the cytotoxic agent is actually released during the hydrolysis reaction of the active compounds (**2–10**). Second, the rates of hydrolysis were, as expected, of zero order in all cases. Third, and most important, the kinetic constants increase in the presence of H_2O_2 (*figure 2, plot 3* and *table I*) for all the active and moderately active compounds. This demonstrates the role of H_2O_2 as a promoter of the hydrolysis reaction rate, that is of 'grosheimin-release', and supports the basic hypothesis of the postulated mechanism. The only exception is compound **2** which will be discussed later.

Moreover, in the course of the hydrolysis reactions, the presence of the ultimate product of oxidation of the aromatic leaving group, as the totally oxidized R substituted benzen-sulfonate, was demonstrated by direct TLC

Table III. Main ^{1}H-NMR spectroscopy data of compounds 1–18.

Compound	H_6	H_8	H_{13}	$H_{13'}$	H_{14}	$H_{14'}$	H_{15}	Arom.	Others
1	4.00	3.92	6.31	6.38	5.11	5.87	1.17	-	-
	dd (10,10)	m	d (2)	d (2)	s	s	d (8)		
2	3.87	3.60	3.56	3.38	4.82	5.08	1.20	7.06	
	dd (9,9)	m	dd (4,13)	dd (4,13)	s	s	d (7)	tt (8,15)	
3	3.89	3.67	3.58	3.37	4.73	5.02	1.22	7.04-7.10	2.30 arom-Me
	dd (10,10)	m	dd (4,14)	dd (4,14)	s	s	d (7)		s
4	3.90	3.68	3.64	3.40	4.74	5.02	1.22	7.35-7.0	2.33 arom-Me
	dd (8,8)	m	dd (4,14)	dd (4,14)	s	s	d (7)		s
5	3.90	3.64	3.62	3.38	4.75	5.02	1.23	7.5-7.1	2.42 arom-Me
	dd (9,9)	m	dd (4,12)	dd (4,12)	s	s	d (7)		s
6	3.90	3.70	3.64	3.43	4.74	5.03	1.23	7.5-7.2	-
	dd (9,9)	m	dd (4,14)	dd (4,14)	s	s	d (8)		
7	3.98	3.67	3.51		4.69	5.04	1.22	7.6-7.4	2.13 acetyl-Me
	dd (9,9)	m	d (4)		s	s	d (8)		s
8	3.95	3.40	3.58	3.30	4.80	5.10	1.20	6.7-7.6	3.60-OMe
	dd (9,9)	m	dd (4,14)	dd (4,14)	s	s	d (7)		s
9	3.92	3.72	3.68	3.42	4.75	5.03	1.25	6.7-7.7	3.82-OMe
	dd (9,9)	m	dd (4, 14)	dd (4,14)	s	s	d (8)		s
10	3.82	3.53	3.40	3.38	4.59	4.93	1.10	6.7-7.5	3.88-OMe
	dd (8,8)	m	d (4)	d (4)	s	s	d (7)		s
11	3.84	3.57	3.42	3.25	4.65	4.97	1.22	6.6-7.5	-
	dd (9,9)	m	dd (4,13)	dd (4,13)	s	s	d (7)		
12	3.87	3.63	3.66	3.47	4.75	5.02	1.23	6.8-7.5	-
	dd (10, 10)	m	dd (4,13)	dd (4,13)	s	s	d(7)		
13	3.93	3.74	3.61	3.45	4.76	5.04	1.27	7.2-7.4	-
	dd (9,9)	ddd (5, 8, 13)	dd (4, 14)	dd (4, 14)	s	s	d (7)		
14	3.92	3.72	3.62	3.50	4.73	5.02	1.23	7.1-7.5	-
	dd (9,9)	ddd (4,9,14)	dd (4,11)	dd (4,11)	s	s	d (7)		
15	3.93	3.72	3.63	3.50	4.77	5.03	1.22	7.1-7.6	-
	dd (9,9)	ddd (5,12,12)	dd (4, 13)	dd (4,13)	s	s	d (7)		
16	3.98	3.68	3.67	3.54	4.71	5.25	1.22	7.3-7.5	-
	dd (9,9)	ddd (5, 11,11)	d (4)	d (4)	s	s	d (7)		
17	3.92	3.75	3.52	3.58	4.72	5.03	1.23	7.1-7.7	-
	dd (9,9)	m	d (4)	d (4)	s	s	d (7)		
18	3.95	3.70	3.50	3.65	4.74	5.04	1.25	7.1-7.7	-
	dd (9,9)	m	dd (4,14)	dd (4,14)	s	s	d (7)		

δ-values in ppm.
Coupling constants in Hz are in parentheses.
Solvent: $CDCl_3$.

comparison with the corresponding products of H_2O_2 oxidation obtained from commercial substituted thiophenols [20]. As further confirmation of our expectations, biologically inactive compounds (**11–18**) did not undergo the hydrolysis process in either of the experimental conditions.

Hydrolysis reactions carried out in the absence of H_2O_2 confirmed, by means of the test of free sulphydril groups [21] on compounds **2–10**, that the secondary product of the reaction was actually the substituted thiophenol in the reduced state. This was confirmed by TLC comparison with the respective commercial products used in the preparation of all the phenylthio derivatives.

Hydrolysis kinetics show that, as expected, the R substituent at the thiophenol moiety of the compounds affects the rate of reaction in the presence of H_2O_2. The strongly deactivating electron attracting groups, such as F, Cl and Br, may prevent sulfur oxidation and consequently further retroelimination. Conversely, the moderate electron-attracting substituents, such as OMe and NHCOMe, are sufficient for a moderate rate of S-oxidation and they may also assist consequent retroelimination. In the absence of S-oxidation they are not opposed to retroelimination, and this process proceeds more slowly.

Finally, weak electron-donating groups, such as Me or the indifferent H-atom, help or permit a higher rate of

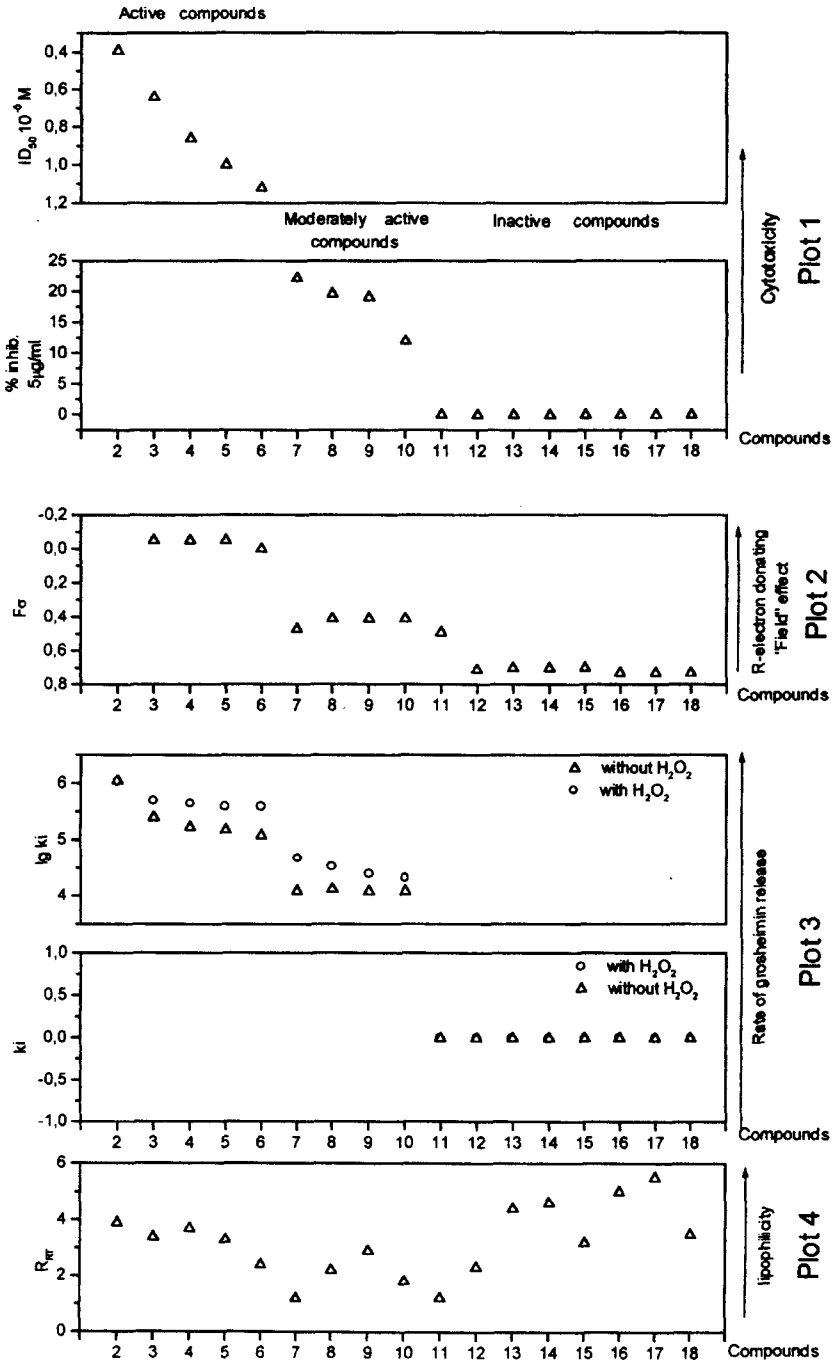

Figure 2. See text.

sulphur oxidation because they are not opposed to the consequent fast step of retroelimination which can proceed at a good rate although not activated by the previous S-oxidation.

In all cases, the sulphur-oxidation step is important in the total process of 'grosheimin-release' by hydrolysis as evidenced by all the reactive and unreactive phenylthio derivatives.

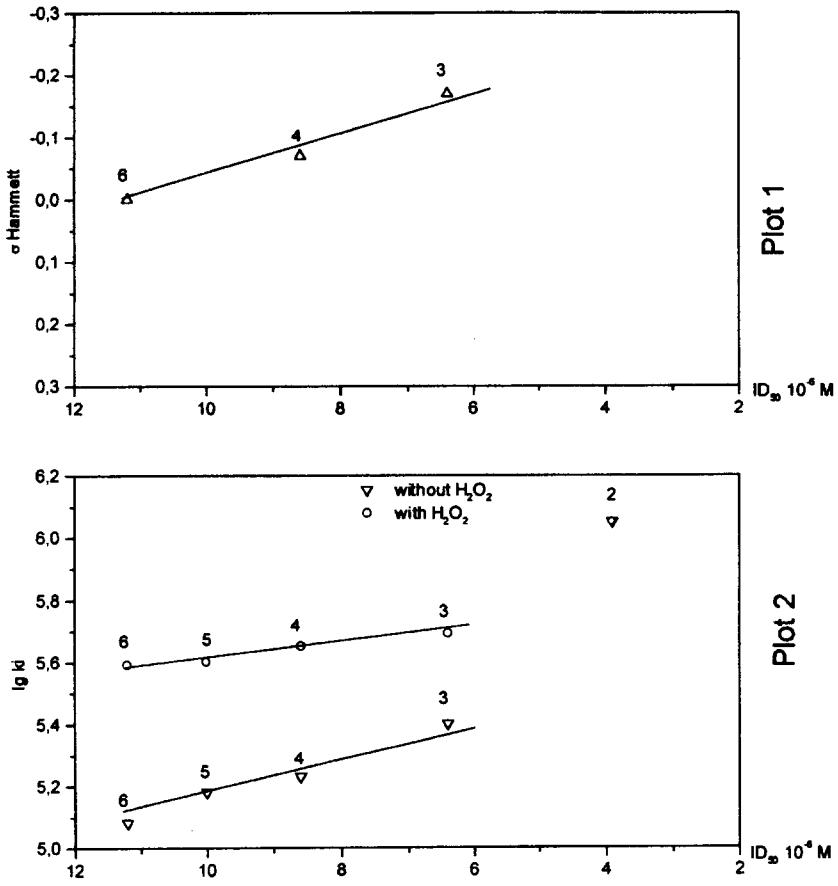

Figure 3. See text.

Compounds **11** and **2** showed a particular behaviour. Compound **11** (R = OH) was totally inactive as a cytotoxic agent and it was also inert to hydrolysis, even in the presence of H_2O_2 despite its positive, but moderately high, Fσ value (*figure 2, plot 2* and *table II*). Probably the OH group is deprotonated at pH = 7.4 so the retroelimination is prevented by a strong negative mesomeric effect ($\sigma = -0.52$; $\sigma^- = -0.81$) [18].

Compound **2** (R = 2,3,5,6-tetra F) resulted in the most active cytotoxic agent and it is surprising that it is even more active than natural grosheimin **1**. This result might be explained by a possible better ability of **2** to penetrate into the cells in comparison to **1**. The behaviour of **2** in the reactions of hydrolysis was surprising too. Under the experimental conditions, compound **2** was the fastest 'releaser' of grosheimin, and the two k_i values, in the absence and in the presence of H_2O_2, were identical (*figure 2, plot 3* and *table II*). This shows its absolute insensitivity towards the oxidant agent. Probably the very high electron-attracting effect of the tetrafluoro benzene ring (Hammett-σ-constants not available) promotes efficaciously and rapidly a simple base-assisted retroelimination process without the need for any preliminary oxidative activation at the sulfur centre (*figure 4*). Furthermore, this oxidation is improbable for the same reason.

The last question to be elucidated is whether, and how, the characteristics of lipophilicity/hydrophilicity of the compounds can affect the in vitro cytotoxicity by modulating pharmacokinetic phases of action and, if so, what specific role they would play. To this end, on the basis of our previous results obtained for the same series of products [17], we chose to utilize R_{RT} ratio values which are based on HPLC retention factors. The data reported in *figure 2, plot 4*, show that the differences in activity cannot be explained by differences in lipophilicity because no elements of parallelism in trend can be found between lipophilicity evaluation and bioactivity. These findings indicate that the lipophilic character of these substances seems to be less significant for cytotoxicity

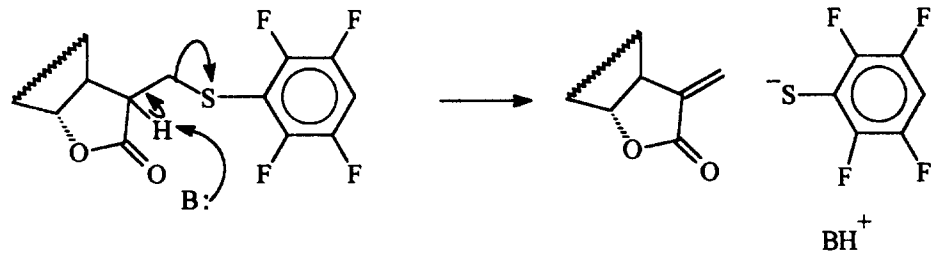

Figure 4. Base-assisted retroelimination process of compound **2**.

than the primary and secondary prerequisites of their chemical features.

In light of the above results, an attempt was made to find a quantitative correlation between bioactivity and molecular physico-chemical properties in the groups of active phenylthio derivatives **2–6**. ID_{50} values were plotted versus: (i) Hammett-constant values of the substituents; and (ii) hydrolysis kinetic k_i and $k_iH_2O_2$ constant values (*figure 3, plots 1, 2*). Sufficient linear correlation appears between ID_{50} and σ values for H (**6**), m-Me (**4**) and p-Me (**3**) substituents ($r = 0.988$) and good linearities were obtained by plotting ID_{50} versus $\lg k_i$ ($r = 0.992$) and $\lg k_iH_2O_2$ ($r = 0.983$). The anomaly of compound **2** in this trend may derive from the above-mentioned presumed difference in its mechanism of action. These linear correlations confirm our expectations and reinforce the hypothetical mechanism of action of these cytotoxic agents.

5. Conclusion

In conclusion, the present study contributes to the understanding of the mechanism of action of substituted phenylthio derivatives of α,β-unsaturated cytotoxic lactones which seem to behave as 'masked lactones', thus probably acting as pro-drugs in bioactivity. They release, in vitro, the cytotoxic agent by sulphur-oxidative promotion and by subsequent retroelimination of the thiosulphenic moiety.

These results also focus on the effect of both the nature and the position of R substituents at the thiophenol ring upon the chemical and physico-chemical properties providing correlations with cytotoxic activity.

6. Experimental protocols

6.1. Chemistry

Melting points were determined with a Kofler hot stage instrument and are uncorrected. ^{1}H-NMR spectra were recorded with a Bruker Spectrospin 200 MHz (TMS as an internal standard). Mass spectra were carried out with a spectrometer MAT-311-A, AEI-MS-902. UV spectra were obtained with a spectrophotometer Perkin Elmer 5515. HPLC apparatus consisted of a Perkin Elmer 410-LC Pump, Detector Perkin Elmer LC90J. Spectrophotometric UV, Integrator Trivector TRIO. Column Chromatography was performed on Merck Silicagel 60, TLC on DC Alufolien kieselgel F_{254} (Merck) analytical, detectors = H_2SO_4 20%, UV lamp (λ = 254 nm) and Stratochrom SIF-254 (Carlo Erba) preparative. TLC eluent was CH_2Cl_2/MeOH = 90/10. Elemental analyses (C, H, N, S for compound **7** and C, H, S for all the others) were carried out with Carlo Erba Elemental Analyzer model 1106 and were within ± 0.4% of theoretical values. Grosheimin **1** was extracted according to [6], other reagents were purchased from Carlo Erba (Italy) and Aldrich (Italy).

6.2. General procedure for phenylthio derivatives **2–18** *(table I)*

Grosheimin **1** (200 mg) was dissolved in MeOH containing triethylamine at room temperature and the appropriate thiophenol (stoichiometric excess) was added under N_2 and stirring. For compounds **2**, **7** and **11**, the reaction mixture was evaporated in vacuo and the crude residue chromatographed on preparative TLC. For the other phenylthio derivatives, the reaction mixture was acidified with aq. HCl 1 N, then the addition of cold water caused the precipitation of the crude product. It was filtered, washed with H_2O/MeOH:1/1 and dried.

6.3. K_i values of hydrolysis of compounds **2–18**

A quantity corresponding to 150 μmol of each compound (**2–18**) was dissolved in MeOH (1 mL, RS-HPLC) and 9 mL phosphate buffer pH 7.4 (H_2O RS-HPLC) was added at 37 °C, under stirring. Then, 20 μL reaction solution was directly injected in the HPLC pump. HPLC

conditions: Column Viosil C-18 (10 μ) l = 25 cm, ϕ = 4 mm, 29.000 t.p., mobile phase: MeOH/H_2O = 68/32 isocratic, flux = 0.8 mL/min, attenuation = 0.5, R_t (Grosh.) = 120 (± 5) s.

From the integral values (I) of Grosheimin peak versus time, for the compounds **2–10**, k_i values of the resulting zero-order straight line were obtained. d(I Grosh.)/dt = k_i.

6.4. $K_iH_2O_2$ values of hydrolysis of compounds **2–18**

These values were measured according to the same procedure used for the k_i values determination, after addition of H_2O_2 35% m/v (40 mL ca. 460 mmol) to the phenylthioderivatives solutions.

6.5. In vitro cytostatic activity

The previously described method [22] was followed. Minimal Eagle's Medium (MEM) with non-essential aminoacids and 10% newborn calf serum was used. A total of 10^5 KB cells, an established human tumour line, were incubated at 37 °C in Leighton tubes. After 24 h, the cells were attached to the glass and the medium was changed with MEM containing the compounds to be tested. The compounds were dissolved immediately before use in sterile dimethylsulfoxide (DMSO). First the percentage of growth inhibition at 5 μg/mL was evaluated. For the most active compounds, the ID_{50} (defined as the molar drug concentration required to inhibit cell growth by 50%) was determined. Further dilutions were carried out with culture medium to the required concentration. For each compound, at least five concentration levels were used. Each compound was tested on at least two separate occasions. Incubation was carried out at 37 °C for 72 h, the time interval in which exponential growth occurs. Cell growth was estimated by counting the cells which were detached from the glass surface with trypsin. The cytostatic activity was evaluated as percentage of growth inhibition in the treated tubes in respect to the controls. The significance of the results was evaluated by the Student's *t*-test ($P < 0.01$). The ID_{50} values were determined by linear regression analysis. We have chosen an ID_{50} value of about 10^{-5} M as our upper-limit criterion for a significant and promising level of activity in order to reduce the number of false positives [23]. Pharmacological data are reported in *table I*.

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