Antioxidant and Antiradical Activity of Hydroxy-Substituted 4-Thiaflavanes

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In memoriam Professor *Hanns Fischer*

The antioxidant activities of several hydroxy-substituted 4-thiaflavanes, compounds **1**–**3**, were determined by measuring their ability of inhibiting the autoxidation of styrene or cumene. On this basis, the role played by the number and position of OH groups and by the oxidation state of the S-atom was quantified and rationalized. With these data, it should be possible to optimize the structural features of these 'double-faced' antioxidants for structure–activity-relationship studies. A comparison between the kinetic data (k_{inh}) reported in this paper and the previously reported values of the antiradical activities (SC_{50}), measured by the DPPH$^{\cdot}$ bleaching method, for **1**–**3** is made (*Table*).

Introduction. – A tremendous effort is underway for investigating the properties of naturally occurring antioxidants because of their potential clinical relevance. Consumption with the diet of small molecules showing antioxidant activity is, indeed, a healthy habit, which contributes to keep under control the concentration of free radicals and other reactive oxygen species (ROS) in biological tissues. An abnormal increase of oxidative species has been related to several pathologies and to aging itself [1].

However, much less work has been committed to develop synthetic antioxidants designed to optimize the antioxidant activity, while satisfying other important criteria such as solubility, bioavailability, and lack of toxicity [2]. We have recently published the access to a new family of radical scavengers possessing the 4-thiaflavane skeleton (*Fig. 1*) [3][4]. When properly substituted with hydroxy (OH) groups, these heterocyclic derivatives are able to mimic the antiradical activity of either flavonoids bearing a catechol group on the *B*-ring (like catechin) or tocopherols (vitamin E), due to the presence of the thiachromane moiety (rings *A* and *C*). Hence, these compounds possess what we call a 'double-faced' antioxidant activity, matching the character of the two more-important families of natural antioxidants. This is an appealing property in consideration that natural antioxidants can efficiently transform a potentially dangerous ROS into a safe molecule by operating synergistically through a cascade of redox reactions [1][5]. As a matter of fact, selected hydroxy 4-thiaflavanes showed effective *in vitro* protection against DNA oxidative damage caused by either peroxy radicals or hydroxy radicals obtained by *Fenton* chemistry [6].

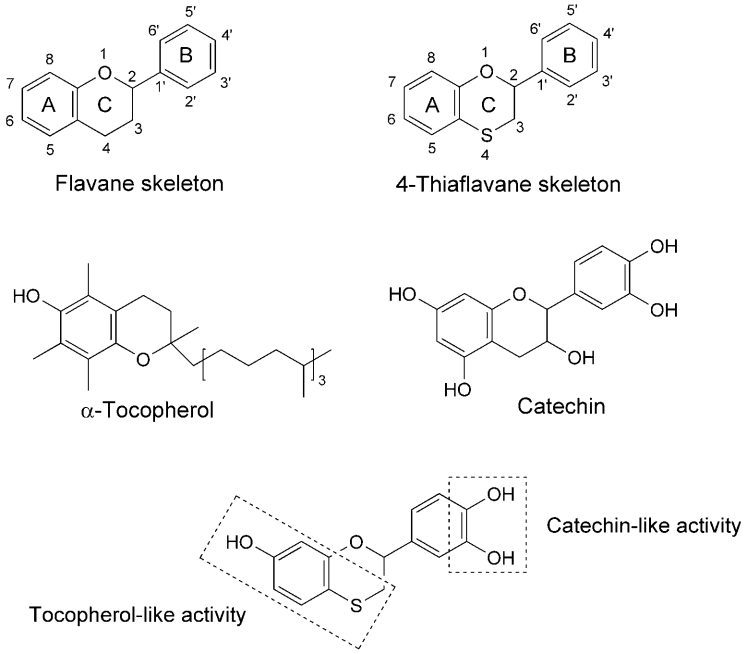


Fig. 1. *Flavane and 4-thiaflavan skeletons.* The latter shows 'double-faced' antioxidant activity.

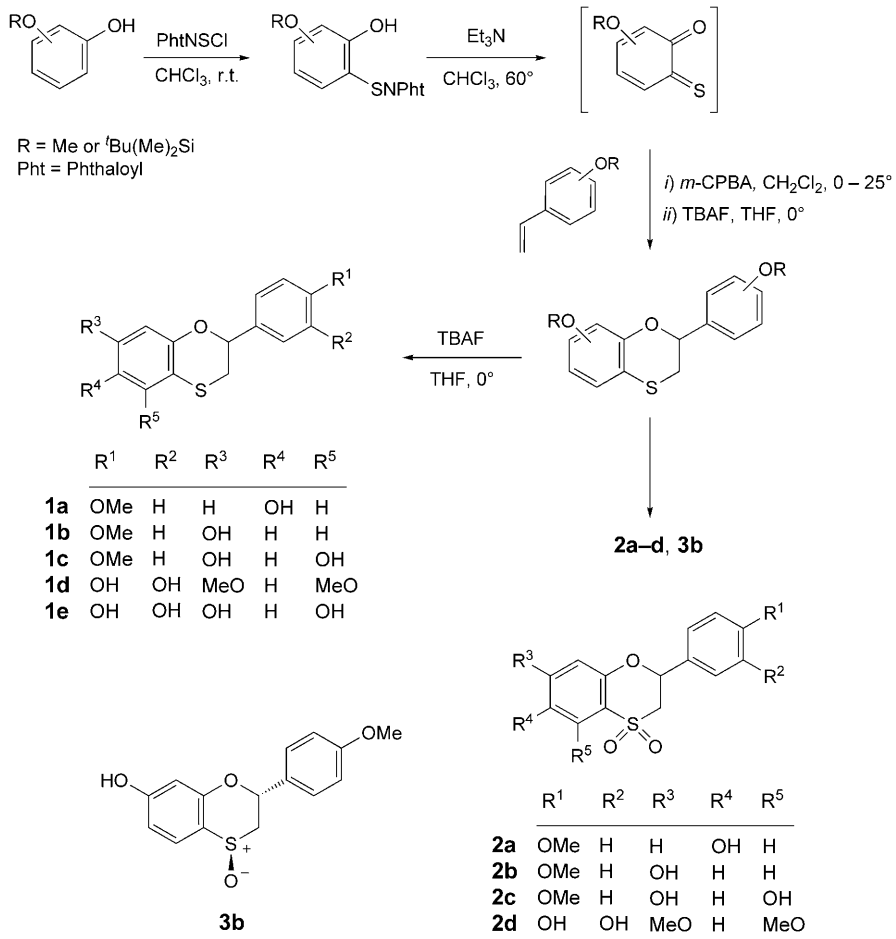
In order to have a rough estimate of the activity of these synthetic antioxidants, the antiradical activities of thiaflavanes were evaluated [4] by measuring their ability to quench the purple color of the 1,1-diphenyl-2-picrylhydrazyl (=1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazine) radical (DPPH$^{\bullet}$) in MeOH, expressed in terms of the SC_{50} index [7][8]. These simple measurements, commonly used for evaluating the total antioxidant activity of natural extracts, have experienced several criticisms. The most important one resides in the fact that DPPH$^{\bullet}$ bleaching is usually measured after a definite reaction time (*e.g.*, 20 min) [4], therefore this method is likely to reflect the stoichiometry rather than the rate of the inhibition process [9]. Moreover, it should also be stressed that there is little structural relationship between DPPH$^{\bullet}$ and the free radicals actually responsible for oxidative stress (essentially peroxy radicals; ROO$^{\bullet}$) [10].

In this paper, we report the determination of the inhibition rate constants k_{inh} for the reaction of several OH-substituted 4-thiaflavanes with ROO$^{\bullet}$ radicals, obtained by studying the inhibition of the autoxidation of styrene or cumene. The aim was to verify these compounds' actual antioxidant activities as a function of ring substitution, as well as depending on the oxidation state of the S-atom. A comparison between the k_{inh} and SC_{50} values for these compounds will be also discussed.

Results and Discussion. – *Synthesis.* The preparation of the 4-thiaflavanes **1a**–**e** was achieved by an inverse-electron-demand hetero-*Diels–Alder* reaction of *ortho*-thioquinones with styrenes [3][4][11]. The thioquinones were, in turn, obtained from the corresponding *ortho*-hydroxylated *N*-thiophthalimides following our original synthetic

procedure that foresees the use of the phthalimidesulfenyl chloride (PhtNSCl) as the key reagent (*Scheme*). Protection of the OH groups, on either the thioquinones and styrenes, as *tert*-butyl(dimethyl)silyl (TBDMS) ethers was used, deprotection with $Bu_4NF \cdot H_2O$ (TBAF) being the final step in all cases [3][4]. The sulfones **2a**–**d** and the sulfoxide **3b** were prepared by oxidation with the proper amount of 'metachloroperbenzoic acid' (=3-chlorobenzenecarboperoxoic acid; *m*-CPBA) of the *O*-silylated 4-thiaflavanes, followed by deprotection (*Scheme*) [4].

Scheme



	R^1	R^2	R^3	R^4	R^5
1a	OMe	H	H	OH	H
1b	OMe	H	OH	H	H
1c	OMe	H	OH	H	OH
1d	OH	OH	MeO	H	MeO
1e	OH	OH	OH	H	OH

	R^1	R^2	R^3	R^4	R^5
2a	OMe	H	H	OH	H
2b	OMe	H	OH	H	H
2c	OMe	H	OH	H	OH
2d	OH	OH	MeO	H	MeO

Kinetics. The determination of the rate constants k_{inh} for the reaction with peroxy radicals of the above derivatives was made by studying the inhibition of the thermally initiated autoxidation of either styrene or cumene [12], based on the processes described in *Eqns. 1–6*. Cumene was employed because its lower oxidizability improves the antioxidant behavior of a given compound, allowing one to differentiate more easily the antioxidant activities of only moderately effective inhibitors.

$$\text{Initiator} \xrightarrow{R_i} R^{\bullet} \quad (1)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + RH \xrightarrow{k_p} ROOH + R^{\bullet} \quad (3)$$

$$ROO^{\bullet} + ROO^{\bullet} \xrightarrow{2k_t} \text{products} \quad (4)$$

$$ROO^{\bullet} + ArOH \xrightarrow{k_{inh}} ROOH + ArO^{\bullet} \quad (5)$$

$$ROO^{\bullet} + ArO^{\bullet} \rightarrow \text{products} \quad (6)$$

The reaction was followed by monitoring the O_2 consumption during autoxidation with an automatic gas-absorption-recording apparatus, built in our laboratory [13], which uses as detector a commercial differential-pressure transducer. The reactions, initiated by the thermal decomposition of 2,2′-azobis(2,4-dimethylvaleronitrile) (=2,2′-azobis(2,4-dimethylpentanenitrile); AMVN), were carried out at 30° under controlled conditions in air-saturated styrene or cumene solution in the presence of each antioxidant. α-Tocopherol was used as reference chain-breaking inhibitor. The inhibition rate constants, k_{inh}, were determined by means of a kinetic treatment consisting in the measure of the initial rates of oxidation of the substrate both in the presence ($-d[O_2]/dt = R_{ox}$) and in the absence ($(-d[O_2]/dt)_0 = R_{ox,0}$) of a known amount of antioxidant (ArOH). The k_{inh} values were calculated from these data by means of *Eqn. 7* [13].

$$\frac{R_{ox,0}}{R_{ox}} - \frac{R_{ox}}{R_{ox,0}} = \frac{n\, k_{inh}[AH]_0}{\sqrt{2k_t R_i}} \quad (7)$$

This equation allows the evaluation of k_{inh} even when the inhibition and termination rates (*Eqn. 4*) are comparable. The use of *Eqn. 7* requires knowledge of the initiation rate R_i, which was determined in preliminary experiments (see *Exper. Part*); the termination constant $2k_t$ for the self-combination of peroxy radicals was reported in the literature as 4.2×10^7 and 4.6×10^4 M^{-1} s^{-1} for styrylperoxy [12] and cumylperoxy radicals [14], respectively. The term n in *Eqn. 7* represents the stoichiometric coefficient, *i.e.*, the number of peroxy radicals trapped by each antioxidant molecule; it can be determined from *Eqn. 8* by measuring the length of the induction period τ during which the rate of O_2 consumption is strongly reduced. For classical chain-breaking antioxidants acting according to the processes described in *Eqns. 5* and *6*, a value of $n = 2$ is expected.

$$n = R_i\, \tau/[AH] \quad (8)$$

The experimental traces of O_2 consumption recorded during the oxidation of styrene, reported in *Figs. 2* and *3*, show that only antioxidants containing the catechol ring, *i.e.*, compounds **1d**, **2d**, and **1e**, give clearly distinct inhibition periods. When using the less easily oxidizable cumene (*Fig. 4*), the inhibition period is more clearly visible, even with less-reactive phenols, which allows one to determine n also for **1a**,

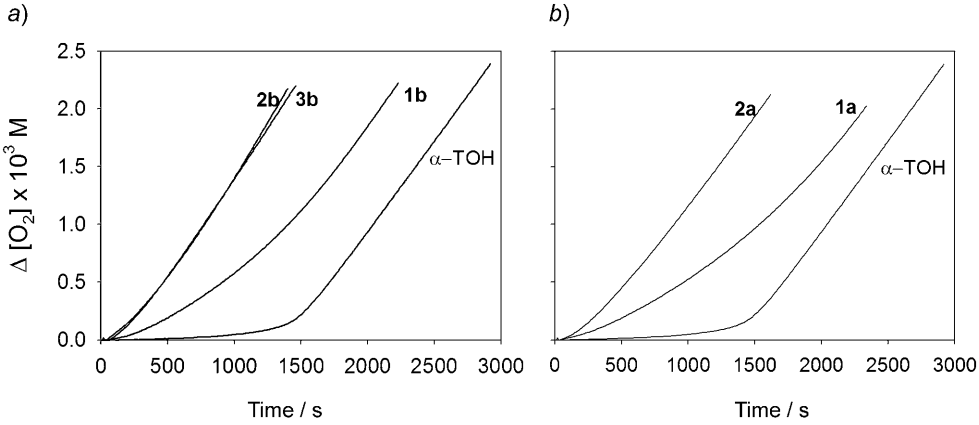


Fig. 2. *O_2-Consumption traces at 30° during AMVN* (5 mM)*-initiated autoxidation of styrene* (4.3M) *in chlorobenzene in the presence of* α*-tocopherol* (α-TOH; 5 μM) *and thiaflavanes* (5 μM) *containing* a) *the S-atom and* b) *the O-atom in* para *position with respect to the phenolic OH group of ring* A. AMVN = 2,2′-Azobis(2,4-dimethylvaleronitrile).

1b, **2a**, and **1c**. In the case of the thiaflavane **2c**, an *n* value of 2 was assumed since, even in cumene, no clear induction period could be observed.

The experimental values of the rate constant, k_{inh}, and the stoichiometric factor *n* measured for **1a**–**e**, **2a**–**d**, and **3b**, as well as the SC_{50} values previously published [4], are reported in the *Table*. An examination of the data shows that the presence of a catechol group provides a large contribution to the antioxidant efficacy of these compounds, independently on the nature of the thiachromane moiety, on the presence of OH substituents in this group, and also on the oxidation state of the S-atom. Actually,

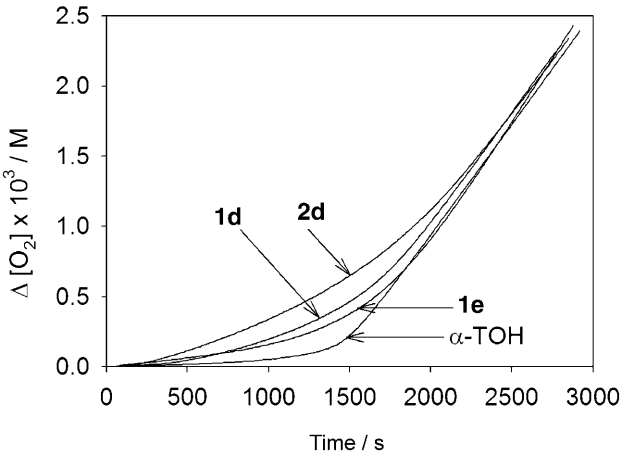


Fig. 3. *O_2 Consumption at 30° during the AMVN* (5 mM)*-initiated autoxidation of styrene* (4.3M) *in chlorobenzene in the presence of thiaflavanes containing the catechol ring* (**1d**, **1e**, **2d**) *and* α*-tocopherol* (5 μM)

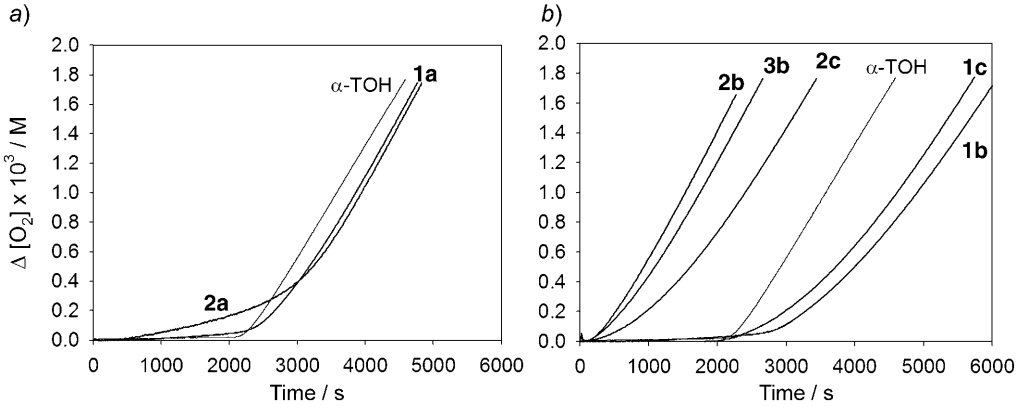


Fig. 4. *O_2 Consumption at 30° during AMVN* (5 mM) *initiated autoxidation of cumene* (7.1M) *in the presence of the investigated thiaflavanes* (5 μM) *containing* a) *the O-atom and* b) *the S-atom in* para *position with respect to the phenolic OH group of ring* A

Table. *Antioxidant and Antiradical Parameters for* **1**, **2**, *and* **3b**

Entry	Compound	k_{inh} [M^{-1} s^{-1}][a])	n[a])	SC_{50} [μM][b])
1	**1a**	$(1.7\pm0.3)\times10^5$	2.2[e])	23
2	**2a**	$(1.3\pm0.2)\times10^4$[c])	2.4[e])	210
3	**1b**	$(1.2\pm0.2)\times10^5$	2.9[e])	18
4	**2b**	$<10^3$	n.d.[d])	>300
5	**3b**	$<10^3$	n.d.	>300
6	**1c**	$(3.9\pm0.8)\times10^5$	2.8[e])	12
7	**2c**	$(2.3\pm0.5)\times10^3$[c])	2[e])	>300
8	**1d**	$(5.5\pm1.1)\times10^5$	1.7	16
9	**2d**	$(2.6\pm0.5)\times10^5$	1.8	15
10	**1e**	$(6.8\pm1.3)\times10^5$	2.1	8
11	Catechol	$(5.3\pm0.5)\times10^5$	1.9	
12	α-Tocopherol	3.2×10^6[f])	2	

[a]) Measured in styrene; mean of three determinations. The error in n is ±0.2. [b]) Antioxidant concentration causing the fading of 50% absorbance of 100 μM DPPH·, 20 min after mixing [4]. [c]) Obtained in cumene. [d]) Not determined. [e]) Assumed value (see text). [f]) Data from [15].

the thiaflavanes **1d** (two MeO groups), **1e** (two OH groups), and **2d** (sulfone), which all contain a catechol *B*-ring, have inhibition rate constants ranging from 2.6×10^5 to 6.8×10^5 M^{-1} s^{-1}, values very similar to that of catechol itself (5.3×10^5 M^{-1} s^{-1}). When taking into account that α-tocopherol, the best natural chain-breaking antioxidant, has a k_{inh} value of 3.2×10^6 M^{-1} s^{-1} [15], and that 2,6-di(*tert*-butyl)-4-methylphenol (BHT) and 2,6-di(*tert*-butyl)-4-methoxyphenol (BHA), two common synthetic antioxidants, have k_{inh} values of 1.0×10^4 M^{-1} s^{-1} and 1.1×10^5 M^{-1} s^{-1} [15], respectively, compounds **1d**, **1e**, and **2d** can be considered as antioxidants characterized by a medium-to-good inhibiting activity.

Another series of moderately good antioxidants are the thiaflavanes **1a**–**c** containing sulfur in its lower oxidation state, with k_{inh} values of 1.2×10^5 to 3.9×10^5 M^{-1} s^{-1}.

Among the mono-hydroxy compounds **1a** and **1b**, the latter one, where the OH group can conjugate with the endocyclic S-atom, is slightly less efficient than **1a** having an O-atom in conjugated position. This is in line with previous data on the activity of synthetic thia-tocopherol reported by *Ingold* and co-workers [16], and with very recent data obtained by us on the antioxidant activity of acyclic thio-substituted phenols [17]. These data contradict the claim that conjugation with bivalent sulfur should be superior to that with oxygen in stabilizing a phenoxy radical [18], and the widespread belief that substitution of oxygen with sulfur, or even better, with selenium or tellurium, should be a valuable means for the preparation of more-efficient antioxidants [18][19]. It should, however, be pointed out that the present experiments, *i.e.*, thermally initiated oxidations, provide a measure of the chain-breaking activity of antioxidants, while spontaneous oxidations, proceeding more slowly, might be inhibited efficiently also by sulfur and other chalcogen-containing phenols due to their ability to behave as preventive antioxidants by decomposing hydroperoxides to alcohols.

The introduction of an additional OH group on ring *A*, as in **1c**, increases the antioxidant activity due to a 'tocopherol-like' mechanism, with a k_{inh} value almost as good as that measured for those compounds possessing a catechol *B*-ring. This increase is likely due to electronic effects, since the second OH group on C(5) is expected to be H-bonded to the nearby S-atom, therefore being not available for abstraction by ROO$^{\bullet}$ radicals. However, semi-empirical [4] as well as *ab initio* [20] calculations, carried out on **1c** and **1d**, seem to suggest that the bond-dissociation enthalpy (BDE) for C(5)–OH is lower than that for C(7)–OH. A dedicated study is ongoing to confirm this anomalous and unexpected *ortho* effect.

Oxidation of the bivalent sulfur on the *C*-ring to higher oxidation states is critical for the 'tocopherol-like' behavior of thiaflavanes. In fact, the sulfones **2b**, **c** and the sulfoxide **3b** did not show significant retarding effects on either styrene or cumene oxidation. This result can be rationalized in consideration of the electron-withdrawing effect [15] exerted by the sulfone or the sulfoxide, which strongly depletes the ability of the phenolic OH to react with peroxy radicals (*Eqn. 5*).

Oxidation of sulfides to sulfones, on the other hand, affects also the activity of catechol-containing thiaflavanes, although only slightly (see data for **1d** and **2d**). This result indicates that any structural modification of antioxidants, even on positions not directly involved in the interaction with the attacking peroxy radicals, must be taken into account if fine-tuning of the antioxidant activity is desired.

An analysis of the parameter *n*, the number of peroxy radicals quenched by each antioxidant molecule, shows that 4-thiaflavanes behave as classical chain-breaking inhibitors. Some exceptions are worth of further comment: it appears in *Fig. 4*, *b* that thiaflavanes in which a phenolic OH group is conjugating with the S-atom (**1b** and **1c**) show, at the end of the strongly inhibited period corresponding to $n=2$, an O_2-consumption rate slightly lower than expected. This small effect, visible only when using cumene as oxidizable substrate, is likely due to some residual antioxidant activity of the oxidized products. Also, compound **1e** showed a surprising behavior, by inhibiting styrene autoxidation for a time lapse similar to that of **1d**, despite the fact that the former compound contains both 'tocopherol-like' and catechol moieties. Thus, an inhibition time twice as large should be expected.

Finally, to compare antioxidant-activity parameters obtained by different techniques, the rate constants of inhibition, k_{inh} (this work), are plotted in *Fig. 5* against the corresponding SC_{50} values determined previously [4]. It is evident that the examined 4-thiaflavanes can be divided in two groups, depending on whether k_{inh} is smaller or larger than 10^5 M^{-1} s^{-1}. Correspondingly, the SC_{50} values are much larger than 25, and lower or equal to 25, respectively, without any linear dependence on the kinetics rate constants.

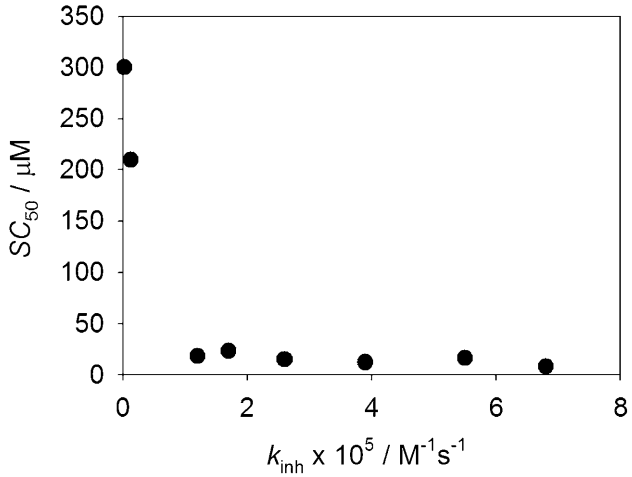


Fig. 5. *Plot of* k_{inh} vs. SC_{50} *for* **1**–**3**

To rationalize this observation, it should be taken into account that the DPPH· test consists in measuring, in alcoholic solution (MeOH), the concentration of antioxidant causing a 50% fading of the absorbance of 100 μM DPPH· after 20 min. As evidenced by *Berset* and co-workers [7], with good antioxidants such as ascorbic acid or δ-tocopherol, the reaction quickly reaches a steady state, and the SC_{50} value only reflects the stoichiometry of inhibition, that is the number of radicals trapped by each molecule of antioxidant. On the contrary, with less-efficient antioxidants, the SC_{50} value accounts both for the stoichiometry and the kinetics of inhibition, thus providing an estimate of the reactivity of the tested compound.

In the present case, with highly effective antioxidants able to scavenge two radicals per molecule, the SC_{50} value is expected to be equal to 25 μM, being the initial amount of DPPH· 100 μM. Therefore, the determined SC_{50} values of 23 and 18 μM for **1a** and **1b**, respecitvely, seem to correspond to the stoichiometric factors of these antioxidants ($n = 2.2$ and 2.9, resp.; see the *Table*). In the case of the three catechol derivatives **1d**, **1e**, and **2d**, the anomalously low SC_{50} values (16, 15, and 8 μM, resp.) are a clear indication that n is greater than 2. This result is likely due to the intervention of secondary reactions that regenerate the catechol structures from the *ortho*-quinones initially formed in the trapping of two DPPH· radicals. Actually, it has been very recently reported that protocatechuic acid esters (=3,4-dihydroxybenzoates) are able to trap in alcoholic solutions *ca.* 5 equiv. of DPPH· through a complex mechanism, implying reduction of the *ortho*-quinone intermediate by nucleophilic addition of an alcohol

molecule, with formation of new catechol derivatives that, in turn, may scavenge additional DPPH· radicals [21][22]. This secondary reaction seems to be unimportant in non-alcoholic solvents, where n is actually close to 2 (see the *Table*). A similar explanation might be given also for the anomalously low SC_{50} value of 12 μM for **1c**, although more-detailed studies are requested.

The last group of 4-thiaflavanes to consider comprises the sulfones **2a**–**c** and the sulfoxide **3b**. Disappointingly, these compounds were found to be too unreactive towards both DPPH· and peroxy radicals to allow any correlation between experimental data. In fact, for three of them, the SC_{50} values are out of the measurable range, the only exception being **2a**, and for two of them, the k_{inh} values were too small to be determined. Evidently, these thiaflavanes are very poor antioxidants.

Conclusions. – The antioxidant potential of OH-substituted 4-thiaflavanes were evaluated by measuring their activity in inhibiting the thermally initiated autoxidation of styrene and cumene. From the values of the rate constants k_{inh} for the reaction of these compounds with peroxy radicals, it is concluded that all compounds containing a catechol *B*-ring are good antioxidants, independent on the substitution pattern at the chromane moiety. In the absence of the catechol ring, substitution of OH group(s) at the *A*-ring confers a still satisfactory antioxidant activity to thiaflavanes, unless the S-atom is in its higher-oxidized states. Actually, in sulfoxides and sulfones, this property is almost completely lost.

On the basis of these results, we expect that a significant improvement of the antioxidant activity of such molecules can be achieved by selecting those with the OH group *para* to the 1,4-oxathiin O-atom (*i.e.*, on C(6)) and by inserting alkyl substituents on the *A*-ring to mimic more closely α-tocopherol (vitamin E).

A comparison between k_{inh} and the previously reported SC_{50} values of the antiradical activity measured by DPPH· bleaching reveals that the two series of data correlate only very roughly. This is due to the fact that the measure of k_{inh} essentially depends on the kinetics of the inhibition reaction, while that of SC_{50} provides values reflecting both the kinetics and the stoichiometry of the reaction with DPPH·. In the case of catechol antioxidants and presumably other polyphenols, additional complications arise from the observation that, in alcoholic solutions, they trap more than two DPPH· radicals per molecule.

Therefore, tests based on DPPH· bleaching can be useful in preliminary screening of a large number of potentially active compounds. However, to avoid misleading conclusions, the results obtained should be handled with care, keeping in mind that DPPH· has different properties and reactivities from those of biologically relevant radicals. When more-accurate structure–activity relationships are requested, procedures involving the determination of the kinetic rate constants for the reaction between peroxy radicals and antioxidants should be adopted.

Experimental Part

Compounds **1**, **2**, and **3b** were prepared as reported elsewhere [4]. The rate constants k_{inh} for the reaction of the title compounds with peroxy radicals were measured by following the autoxidation of either styrene or cumene at 303 K using as initiator 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). The reac-

tions were performed in a O_2-uptake apparatus built in our laboratories and based on a *Validyne-DP15* differential-pressure transducer, which has been previously described in detail [13]. The entire apparatus was immersed in a thermostated bath to ensure a const. temp. within $\pm 0.1°$.

In a typical experiment, an air-saturated chlorobenzene solution containing the oxidizable substrate and the antioxidant was equilibrated with the reference soln. containing an excess of α-tocopherol (1–10 mM) in the same solvent at 30°. After equilibration, a concentrated chlorobenzene soln. of AMVN was injected in both the reference and the sample flasks, and the O_2 consumption in the sample was measured (after calibration of the apparatus) from the differential pressure recorded with time between the two channels. This instrumental setting allowed us to have the N_2 production and the O_2 consumption derived from the azo-initiator decomposition already subtracted from the measured reaction rates. The antioxidant concentration was kept constant for all measurements (5.0 μM) to compare more easily their behavior. Initiation rates, R_i, were determined for each condition in preliminary experiments by the inhibitor method using α-tocopherol as reference antioxidant: $R_i = 2[\alpha\text{-tocopherol}]/\tau$ [12]. Induction-period lengths (τ) were determined by the intersection between the regression lines to the inhibited and the uninhibited traces.

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