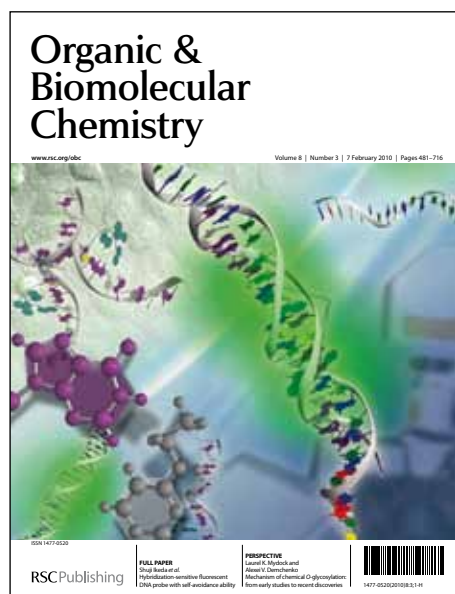


# Organic & Biomolecular Chemistry

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**Communication** View Article Online**Reactivity Assessment of Chalcones by a Kinetic Thiol Assay**Sabine Amslinger,<sup>a,\*</sup> Nafisah Al-Rifai,<sup>a,†</sup> Katrin Winter,<sup>a</sup> Kilian Wörmann,<sup>a</sup> Rebekka Scholz,<sup>a</sup> Paul Baumeister<sup>a</sup> and Martin Wild<sup>a</sup>

Received (in XXX, XXX) Xth XXXXXXXXXX 20XX, Accepted Xth XXXXXXXXXX 20XX

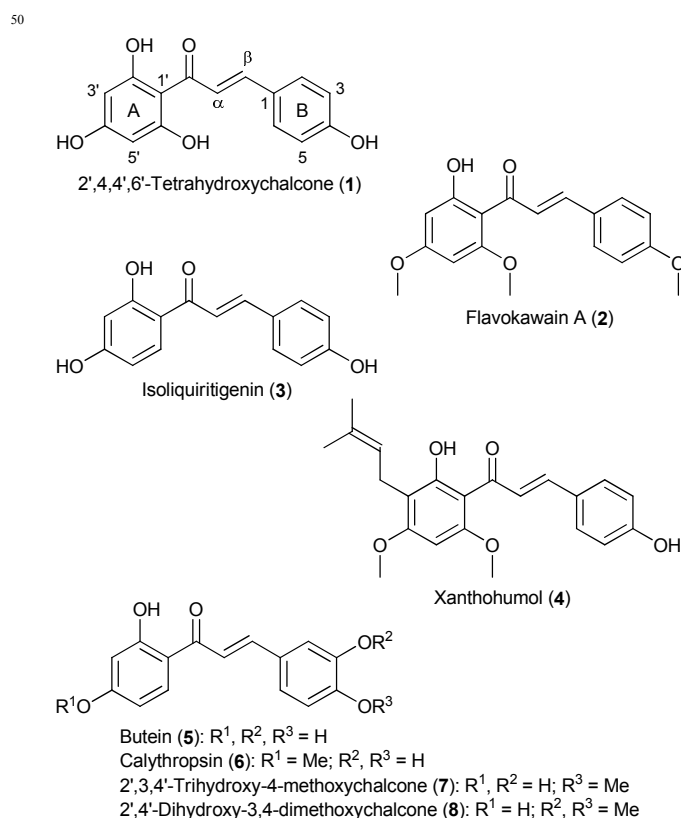
DOI: 10.1039/b000000x

The electrophilic nature of chalcones (1,3-diphenyl-prop-2-ene-1-ones) and many other  $\alpha,\beta$ -unsaturated carbonyl compounds is crucial for their biological activity, which is often based on thiol-mediated regulation processes. To better predict their biological activity a simple screening assay for the assessment of the second-order rate constants ( $k_2$ ) in thia-Michael additions was developed. Hence, a clear structure activity relationship of 16 differentially decorated hydroxy-alkoxychalcones upon the addition of cysteamine could be established. Moreover, amongst other naturally occurring  $\alpha,\beta$ -unsaturated carbonyl compounds  $k_2$  values for curcumin and cinnamaldehyde were gained while cinnamic acids or esters gave no or very slow reactions.

Chalcones (1,3-diphenyl prop-2-en-1-ones) are natural products from the class of plant polyphenols and the biochemical precursors of cyclic flavonoids. They display anti-inflammatory, antioxidative, anti-mitotic, bacteriacidal, antifungal, antimalarial, antileishmanial to chemoprotective and chemopreventive activity, but also cytotoxic and antiviral properties were found.<sup>1</sup> Their activity is mostly based either on the Michael acceptor activity of the  $\alpha,\beta$ -unsaturated carbonyl system, or their radical scavenging or reductive potential which are often referred to as antioxidative behaviour.<sup>2</sup> The Michael acceptor activity is affected both by the decoration of the aromatic rings, and also, even more effectively, by an  $\alpha$ -X-substitution of the double bond of the enone system.<sup>3</sup> Despite the fact that Michael acceptors are a neglected class of compounds in drug development their unique capability to address certain cysteine residues qualifies them as valuable tools to modulate biological activity.<sup>1d</sup> Nevertheless, there is no simple, efficient quantitative method to assess and compare the reactivity of different chalcones and other  $\alpha,\beta$ -unsaturated carbonyl compounds to be able to identify suitable electrophiles. Therefore, we developed a facile screening assay to determine the second-order rate constants ( $k_2$ ) of chalcones in thia-Michael additions. As a starting point, the reactivity of the  $\alpha$ -H-chalcones was assessed since this is the pattern found in almost all natural products of this class – the only exception being the  $\alpha$ -hydroxy-chalcones.<sup>4</sup>

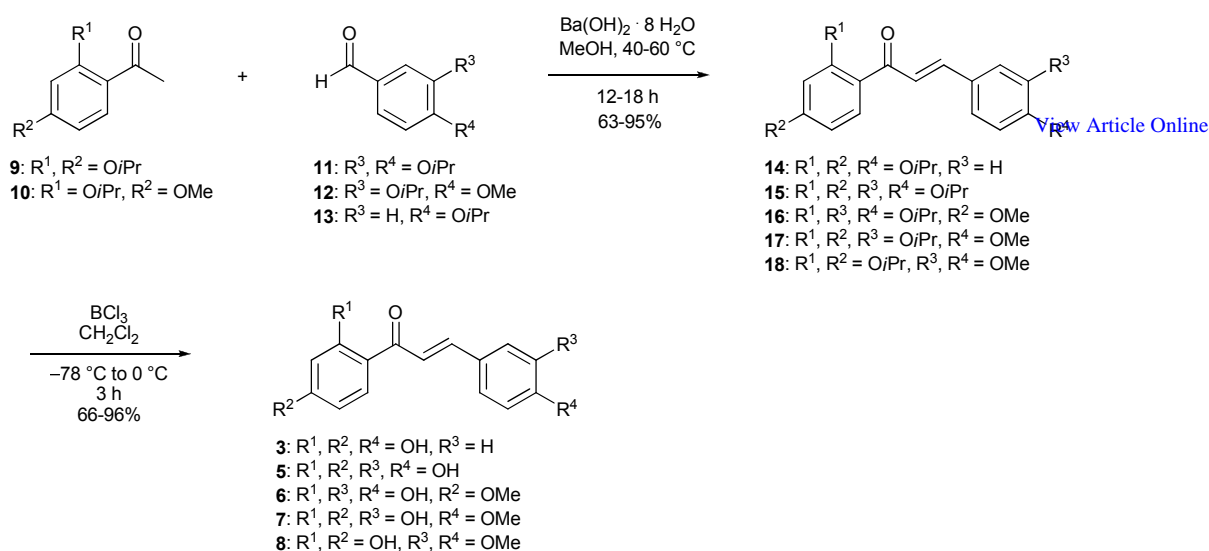
To be able to determine structure activity relationships we picked mainly 2',3,4,4'-tetrasubstituted chalcones, including the natural products butein (5) and calythrpsin (6).

This structural motif is less abundant in nature since it differs in two positions from its biosynthetic precursor 2',4,4',6'-tetrahydroxy chalcone (1).



**Figure 1** Natural chalcones used in this study 2-8 and biosynthetic precursor 1.

Additionally, we included isoliquiritigenin (3), flavokawain A (2) and the hops secondary metabolite xanthohumol (4) together with compounds 7 and 8. The syntheses of the chalcones 2, 3, 5-8 and 14-22 (see Table 1) used in this study were done by classical Claisen-Schmidt condensations of methoxylated or isopropyl protected acetophenones and benzaldehydes using  $Ba(OH)_2 \cdot 8 H_2O$  as the base in MeOH.<sup>5</sup>



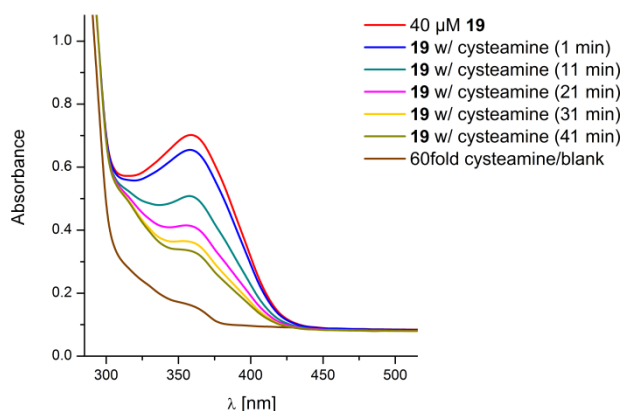
**Scheme 1** Synthesis of hydroxylated and alkoxyated  $\alpha$ -H-chalcones used in the kinetic studies involving isopropyl ethers.

The deprotection of the corresponding isopropoxy ethers was performed with BCl<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub>. The 2'-OH compounds flavokawain A (**2**), **20** and **22** without further hydroxyl groups could be directly prepared without an isopropoxy protection in the 2'-OH position. The final purification was merely done by column chromatography and recrystallization.

In order to be able to efficiently compare Michael acceptor activities, we decided to use a 96-well microtiter plate based screening system since it allows for a simple and quick handling together with rather small amounts of material needed to determine the  $k_2$  values. Since we expected to see great differences in reactivity, we needed to establish a kinetic assay system which could be used to measure both very fast and very slow reactions. And, thus, allowing for a differentiation between diverse substitution patterns on the aromatic rings of the chalcones. But also, even more importantly, one wants to be able to compare a wider range of compounds and thus discriminate between individual  $\alpha$ -X-chalcones, where X can be different substituents like halogen or carbon-based residues.<sup>3</sup> Such a task involves the optimization of pH, solvent mixtures, mixing procedures, avoiding thiol oxidation and easy and reproducible handling of big sample amounts. In our initial solvent screening we included buffer systems with a pH of 7.4 (physiological conditions) together with solvents such as acetone, acetonitrile,<sup>6</sup> EtOH, MeOH, and DMSO. Depending on the solvent used we could find large differences in the solubility and stability of the chalcones (data not shown). EtOH together with 100 mM TRIS-HCl pH 7.4 gave overall very good results. But, when we started longer experiments evaporation of solvent from individual wells in the microtiter plate reader impaired the results. Two measures could solve this problem: using ethylene glycol instead of EtOH due to its high boiling point and covering the 96-well plate with an optical clear PCR foil. The addition of 2 mM EDTA to the buffer was essential to avoid thiol oxidation<sup>7</sup> and superior to any attempts using a protective argon atmosphere. To determine the second-order rate constants ( $k_2$ ), pseudo-first order kinetic conditions were used to measure the exponential decay of

chalcones when a thiol is added.

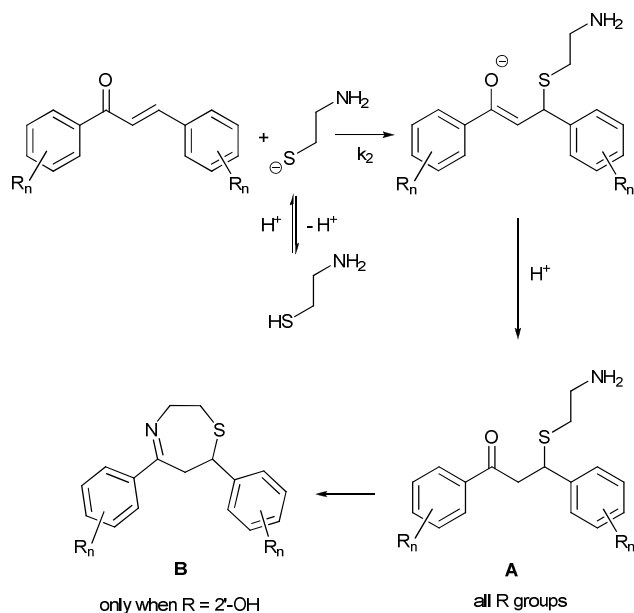
First, appropriate wavelengths had to be established based on LC-MS studies. At these wavelengths just the decay of the UV-bands of the corresponding starting materials occurred without an interference with product formation. Second, appropriate thiol concentrations to measure only the thia-Michael addition were needed. Especially, when free phenolic hydroxyl groups were present sufficient amounts of thiols had to be used so that the deprotonation of the phenol did not interfere. In an initial thiol screen we included cysteamine, cysteine, dithiothreitol (DTT), 2-mercaptoethanol, and glutathione (GSH). With different buffer and solvent mixtures we found that 2',3,4,4'-tetramethoxy-chalcone (**19**) showed a fast reaction with cysteamine and cysteine, intermediate reactivity with DTT and a slow reaction with 2-mercaptoethanol, as well as GSH.



**Figure 2** UV spectra of 2',3,4,4'-tetramethoxychalcone (**19**) without and with cysteamine at 25 °C (in plastic microtiter plate with PCR foil).

Cysteine was ruled out since it gave a yellow coloured addition product, which made it hard to find a suitable wavelength for the  $k_2$  determination. And, with a  $pK_a$  of 8.3 cysteamine has just the right reactivity as found in many surface thiols of proteins and is therefore a perfect model thiol for a reactivity screening. Additionally, since cysteamine is an aliphatic thiol it does not

absorb in the UV range of interest (Figure 2). Depending on the chalcone tested, the excess of thiol solution ranged from 12 to 500fold. A reaction sequence for the thia-Michael addition of cysteamine to chalcones is given in Scheme 2.



**Scheme 2** Reactions of chalcones with cysteamine relevant for kinetic studies.

Assuming there is always a sufficient amount of thiolate present, which concentration is directly proportional to the thiol concentration, one can use the following equations to calculate the second order rate constant  $k_2$ :

$$-\frac{d[\alpha\beta]}{dt} = k_2[\alpha\beta][Thiol] \quad (1)$$

under pseudo-first order conditions:

$$[Thiol] \gg [\alpha\beta] \rightarrow [Thiol] = const. = [Thiol]_0 \quad (2)$$

$$(2) \text{ in } (1): -\frac{d[\alpha\beta]}{dt} = k_{obs}[\alpha\beta] \quad (3)$$

$$\text{with } k_{obs} = k_2[Thiol]_0 \rightarrow k_2 = \frac{k_{obs}}{[Thiol]_0} \quad (4)$$

$k_{obs}$  values were gained from the time-dependent decay of the absorbance ( $A_t$ ) of the  $\alpha\beta$  (chalcone) with *Thiol* (cysteamine) by fitting the data of individual experiments to the first order exponential equation (5):

$$A_t = A_0 e^{-k_{obs}t} + C \quad \text{with } A_0 = A_t([\alpha\beta]_0) \quad (5)$$

The final  $k_2$  values were determined by plotting individual  $k_{obs}$  values against the corresponding different thiol concentrations. The results of the kinetic studies for  $\alpha$ -H-chalcones are given in Table 1. From the kinetic data for  $k_2$  a clear structure-activity relationship can be established. We could show as known from the literature<sup>8</sup> that a 2'-OH group is essential for the reactivity of the chalcones.<sup>9</sup> 2'-hydroxychalcone (**22**) is the most active

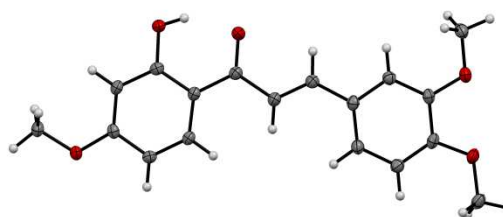
compound with a  $k_2$  value of  $5.08 \text{ M}^{-1}\text{s}^{-1}$  for its reaction with cysteamine.

**Table 1**  $k_2$ -values of  $\alpha$ -H-chalcones with cysteamine at 25 °C. [View Article Online](#)

#	2'	3'	4'	6'	3	4	$k_2$ [ $\text{M}^{-1}\text{s}^{-1}$ ] <sup>a</sup>
2	OH	H	OMe	OMe	H	OMe	$0.649 \pm 0.0090$
3	OH	H	OH	H	H	OH	$0.258 \pm 0.010$
4	OH	prenyl	OMe	OMe	H	OH	$0.124 \pm 0.0054$
5	OH	H	OH	H	OH	OH	$0.271 \pm 0.027$
6	OH	H	OMe	H	OH	OH	$0.325 \pm 0.011$
7	OH	H	OH	H	OH	OMe	$0.417 \pm 0.0079$
8	OH	H	OH	H	OMe	OMe	$0.464 \pm 0.039$
14	O <i>i</i> Pr	H	O <i>i</i> Pr	H	O <i>i</i> Pr	O <i>i</i> Pr	$0.135 \pm 0.0047$
15	O <i>i</i> Pr	H	OMe	H	O <i>i</i> Pr	O <i>i</i> Pr	$0.148 \pm 0.0083$
16	O <i>i</i> Pr	H	O <i>i</i> Pr	H	H	O <i>i</i> Pr	$0.108 \pm 0.0056$
17	O <i>i</i> Pr	H	O <i>i</i> Pr	H	O <i>i</i> Pr	OMe	$0.118 \pm 0.0069$
18	O <i>i</i> Pr	H	O <i>i</i> Pr	H	OMe	OMe	$0.103 \pm 0.0082$
19	OMe	H	OMe	H	OMe	OMe	$0.193 \pm 0.019$
20	OH	H	OMe	H	OMe	OMe	$0.717 \pm 0.041$
21	H	H	H	H	H	H	$3.04 \pm 0.10$
22	OH	H	H	H	H	H	$5.08 \pm 0.043^b$

<sup>a</sup> Reactions were carried out in 100 mM TRIS-HCl pH 7.4, 2 mM EDTA/ethylene glycol 20:80 under pseudo-first order conditions at a concentrations of 40  $\mu\text{M}$  for chalcones and 12 to 500fold cysteamine; <sup>b</sup> done by stopped flow technique.

This is about 1.7fold increase in activity of compared to its parent compound chalcone (**21**), lacking the 2'-OH group. The 2'-OH group acts in two ways: 1) it activates the carbonyl group through the intramolecular H-bond and 2) stabilizes the conjugation in the system as shown by the x-ray structure (Figure 3) of the fairly flat 2'-hydroxy compound **20** (dihedral angle =  $11.38^\circ$ ) compared to tetramethoxy analog **19**, which displays a dihedral angle of  $26.88^\circ$  between the two benzene rings.<sup>10</sup>

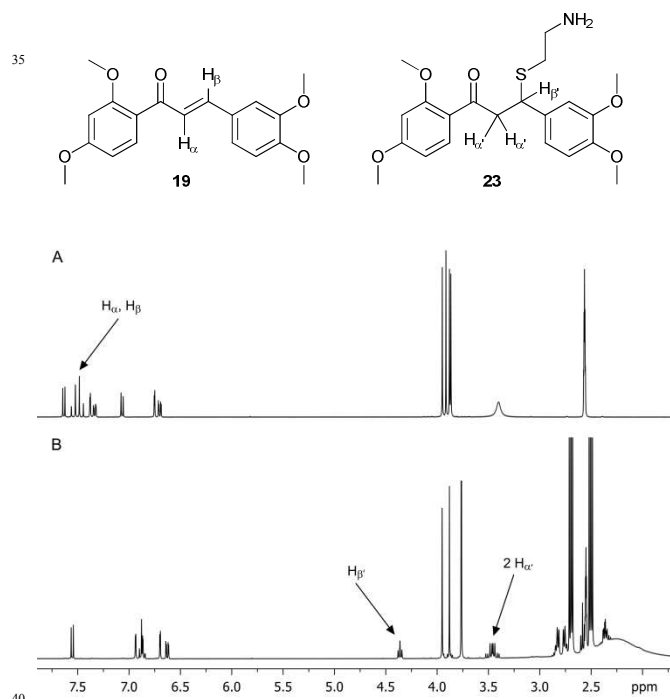


**Figure 3** X-ray structure of 2'-hydroxy-3,4,4'-trimethoxychalcone (**20**).

This influence of the 2'-OH group lead to a  $k_2$  value of  $0.717 \text{ M}^{-1}\text{s}^{-1}$  for hydroxyl compound **20**, whereas the 2'-methoxy compound **19** gave a  $k_2$  value of  $0.193 \text{ M}^{-1}\text{s}^{-1}$ . Furthermore, since the double bond of the  $\alpha,\beta$ -unsaturated carbonyl system can be referred to as a push-pull double bond, it is not surprising that the replacement of the more electron donating hydroxy substituent by a methoxy residue in the B-ring restores more reactivity compared to a similar exchange in the A-ring. Thus,

monomethoxy derivatives of tetrahydroxy compound butein (5) with a  $k_2$  value of  $0.271 \text{ M}^{-1}\text{s}^{-1}$ , 7 and 6, gave  $k_2$  values of  $0.417 \text{ M}^{-1}\text{s}^{-1}$  and  $0.325 \text{ M}^{-1}\text{s}^{-1}$ , respectively. On the other hand when regioisomers flavakawain A (2) and 20 are compared, which carry both three methoxy groups, this effect was not observed. Here, the steric hinderance of the 6'-methoxy group in 2 may contribute to less conjugation and therefore a loss in reactivity relative to 20. Xanthohumol (4) with a very electron-rich and sterically demanding A-ring is the least reactive compound in our 2'-OH chalcone series with a  $k_2$  value of  $0.124 \text{ M}^{-1}\text{s}^{-1}$ . A comparison of butein (5) with its 3-deshydroxy derivative isoliquiritigenin (3) which have unexpectedly nearly the same  $k_2$  values points to a particular big influence of the substituent in the 4-position of the B-ring. That is important to note for potential variations in the substitution patterns of future compounds. The isopropoxy compounds 14-18 behaved mostly similar to tetramethoxychalcone 19 being overall tetraalkoxy compounds. But, the overall reactivity varies in a range of 2fold, which could be explained by steric factors that lead to differences in the conjugation of the  $\pi$ -system.

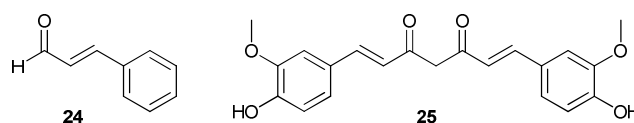
In order to prove the product formation as being claimed to be Michael adducts we performed LC-MS studies (data not shown) after our kinetic experiments directly from the unpurified reaction mixtures, since purification yielded no clean addition products. This fact is possibly caused as a result of a reversible reaction with cysteamine on silica gel. In the mass spectrometric analyses we could find either the expected cysteamine adduct A only or, additionally, the seven membered 1,4-thiazepine intramolecular condensation product B (Scheme 2) which is formed by the nucleophilic attack of the amine to the carbonyl group. This was recently shown for  $\alpha,\beta$ -unsaturated aldehydes.<sup>11</sup>



**Figure 4**  $^1\text{H}$  NMR spectra of 2',3,4,4'-tetramethoxychalcone (19) in DMSO- $d_6$ . A) 19 only, B) 19 with 12fold cysteamine after 5 min.

The initial addition product A was found in all cases whereas the cyclized product B was only found when a 2'-OH group was present (3, 4, 6, 7, 8, 20, and 22; with the exception of butein (5), and flavokawain A (2) where no B could be proven).

We also did NMR experiments to prove the formation of the adduct independently. Here, we used a solution of 19 in DMSO- $d_6$  and then added 12fold cysteamine using an internal standard (Figure 4). Figure 4B clearly shows the formation of one addition product with cysteamine. To further test our kinetic assay and put it into perspective we included some more  $\alpha,\beta$ -unsaturated carbonyl compounds in our kinetic measurements, namely cinnamaldehyde (24), curcumin (25), cinnamic acid methyl ester (26), cinnamic acid ethyl ester (27), 2'-hydroxy cinnamic acid (28), cinnamic acid (29), caffeic acid phenethyl ester (CAPE) (30), chlorogenic acid (31), caffeic acid (32), 3-hydroxycoumarin (33), kaempferol (34), and quercetin (35). Only cinnamic aldehyde and curcumin displayed sufficient reactivities so that their  $k_2$  values could be determined.



**Figure 5** Further active of  $\alpha,\beta$ -unsaturated carbonyl compounds.

Notably, the  $\alpha,\beta$ -unsaturated aromatic aldehyde 24 demonstrates good reactivity with a  $k_2$  value of  $0.636 \text{ M}^{-1}\text{s}^{-1}$  which is very close to flavokawain A. Curcumin (25), which gave a  $k_2$  value of  $0.0621 \text{ M}^{-1}\text{s}^{-1}$  is less reactive by a factor of about 10. The MS experiments revealed the expected products including bisadducts in the cases for 24 and 25. Additionally, aromatic  $\alpha,\beta$ -unsaturated esters 26, 27, 30, 31, and 33 gave very slow reactions that could be enhanced at higher pH values (8-10), but  $k_2$  values were not determined.

**Table 2** Kinetic studies of  $\alpha,\beta$ -unsaturated carbonyl compounds with cysteamine at  $25^\circ\text{C}$ .

#	compound	$k_2 [\text{M}^{-1}\text{s}^{-1}]^a$
24	Cinnamaldehyde	$0.636 \pm 0.019$
25	Curcumin	$0.0660 \pm 0.0079$
26	Cinnamic acid methyl ester	$< 0.001^b$
27	Cinnamic acid ethyl ester	$< 0.001^b$
28	2'-Hydroxy cinnamic acid	n. r. <sup>c</sup>
29	Cinnamic acid	n. r. <sup>c</sup>
30	Caffeic acid phenethyl ester (CAPE)	$< 0.001^b$
31	Chlorogenic acid	$< 0.001^b$
32	Caffeic acid	n. r. <sup>c</sup>
33	3-Hydroxycoumarin	$< 0.001^b$
34	Kaempferol	n. r. <sup>c</sup>
35	Quercetin	n. r. <sup>c</sup>

<sup>a</sup> Reactions were carried out in 100 mM TRIS-HCl pH 7.4, 2 mM EDTA/ethylene glycol 20:80 under pseudo-first order conditions at a concentrations of  $40 \mu\text{M}$  for  $\alpha,\beta$ -unsaturated carbonyl compounds and 12 to 500fold cysteamine; <sup>b</sup> reactions were not complete after 63 h with 500fold cysteamine; <sup>c</sup> no reaction was found within 63 h with 500fold cysteamine.

Acids 28, 29, 32, and flavonols 34, and 35 were not reactive in our assay (all results were verified by MS studies) (Table 2). Attempts to determine  $k_2$  for phenylvinylketone (1-phenyl-2-



propen-1-one) failed since the absorption maxima of this compound lies beyond 300 nm and therefore our UV-VIS detection-based method cannot be used.

<sup>5</sup> <sup>1</sup>H NMR studies with different  $\alpha,\beta$ -unsaturated natural products have been used to successfully group these into reversible and irreversible thiol sinks.<sup>11</sup> Under the conditions used (DMSO-*d*<sub>6</sub>/dilution with CDCl<sub>3</sub>) there is no need for a chromophore and important structural information is gained when more than one reactive site could be addressed in one molecule. On the other hand, to quantitatively compare libraries of compounds like chalcones where only small structural changes are introduced, our method allows for a very precise assessment of the reactivity of each single compound. Thus, our 96-well-microtiter plate assay can facilitate the evaluation of many and very important classes of molecules particularly aromatic  $\alpha,\beta$ -unsaturated carbonyl compounds like the polyphenols. Nevertheless, there are limitations to use the reactivity in thia-Michael additions for a prediction of biological activity. When surface cysteines need to be addressed as for example in the activation process of Nrf2 via the Keap1-Nrf2 pathway or in the inhibition of NF- $\kappa$ B, reactivity rather than accessibility determines whether an S-alkylation of their highly reactive sulfhydryl groups takes place. Very strong electrophiles could certainly lead to unspecific reactions with less reactive thiol groups, but they may be neutralized by the cellular electrophile trap glutathione (GSH), whose cysteine residue displays only moderate activity. This fact can be responsible for a reduced activity of certain potent electrophiles which therefore show less unspecific toxicity.<sup>12</sup> Moreover, metabolic transformations, such as the hydroxylation of the aromatic rings of the  $\alpha,\beta$ -unsaturated carbonyl compounds could change their reactivity and therefore overall biological activity.<sup>13</sup> Other  $\alpha,\beta$ -unsaturated carbonyl compounds such as the flavonoles kaempferol (**34**) and quercetin (**35**) show significant biological activities such as cancer prevention,<sup>14</sup> but have not proven to be electrophiles. **34** and **35** are considered as antioxidants because of their free phenolic hydroxyl groups that can act as reductants or induce ROS-mediated processes. Moreover, their structure itself could form non-covalent interactions and thus initiate biological activity apart from alkylation and redox reactions.

In summary, we have developed a new, simple kinetic assay whose solvent system allows the inclusion of compounds with quite different reactivities in Michael additions of thiols. We could show a structure-activity relationship within 16 chalcones and could compare them with the well studied curcumin. This assay can be used to better understand biological activities of Michael acceptors and therefore to help to overcome their poor standing in drug development.

## Acknowledgements

We thank Karl Amslinger for developing an Excel sheet for easy data processing, Sandra Schlee (Institut für Biophysik und Physikalische Biochemie, Universität Regensburg) for expert help with stopped-flow experiments, the Mass Spectrometry Unit (Universität Regensburg) for LC-MS measurements, the Fonds der Chemischen Industrie for funding (Liebig scholarship for

S.A.) and the DAAD for a PhD scholarship for N.A.

## Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental  
details, <sup>1</sup>H NMR, <sup>13</sup>C NMR spectra of **14-18**. CCDC 909409 (**21**). See  
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‡ These authors contributed equally to this work.
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