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Reactivity assessment of chalcones by a kinetic thiol assay†

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Sabine Amslinger,‡* Nafisah Al-Rifai,‡ Katrin Winter, Kilian Wörmann, Rebekka Scholz, Paul Baumeister and Martin Wild

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The electrophilic nature of chalcones (1,3-diphenylprop-2-en-1-ones) and many other α,β-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 addition of cysteamine could be established. Moreover, amongst other naturally occurring α,β-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-diphenylprop-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, bactericidal, antifungal, antimalarial, antileishmanial to chemoprotective and chemopreventive activity, but also cytotoxic and antiviral properties were found.[1] Their activity is mostly based either on the Michael acceptor activity of the α,β-unsaturated carbonyl system, or their radical scavenging or reductive potential which are often referred to as antioxidative behaviour.[2] The Michael acceptor activity is affected both by the decoration of the aromatic rings, and also, even more effectively, by an α-X-substitution of the double bond of the enone system.[3] 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.[1d] Nevertheless, there is no simple, efficient quantitative method to assess and compare the reactivity of different chalcones and other α,β-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 α-H-chalcones was assessed since this is the pattern found in almost all natural products of this class – the only exception being the α-hydroxychalcones.[4]

To be able to determine structure–activity relationships we picked mainly 2′,3,4,4′-tetrasubstituted chalcones, including the natural products butein (**5**) and calythropsin (**6**) (Fig. 1). This structural motif is less abundant in nature since it differs

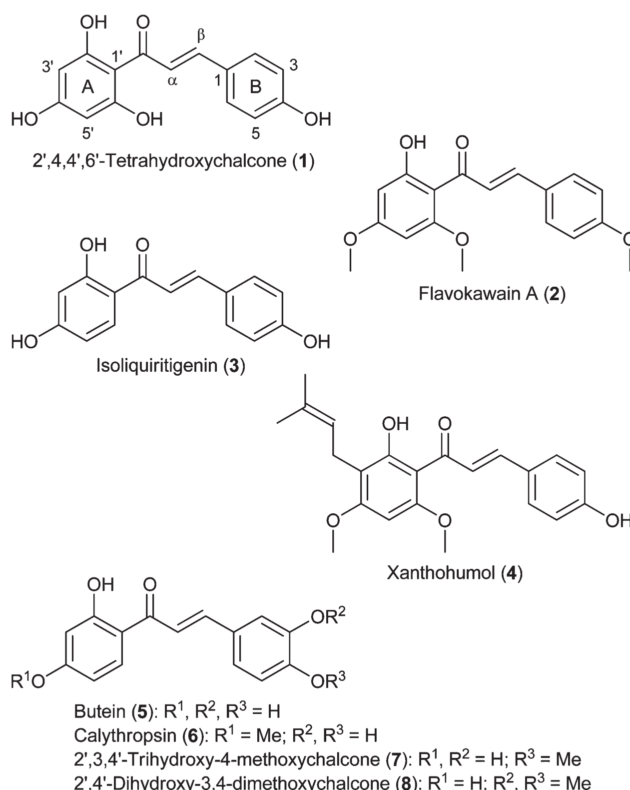

Fig. 1 Natural chalcones used in this study **2–8** and biosynthetic precursor **1**.

Institut für Organische Chemie, Universität Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany. E-mail: sabine.amslinger@chemie.uni-regensburg.de; Fax: +49 941 943-4121; Tel: +49 941 943-4650

†Electronic supplementary information (ESI) available: Experimental details, ^{1}H NMR, ^{13}C NMR spectra of **14–18**. CCDC 909409. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2ob27163j

‡These authors contributed equally to this work.

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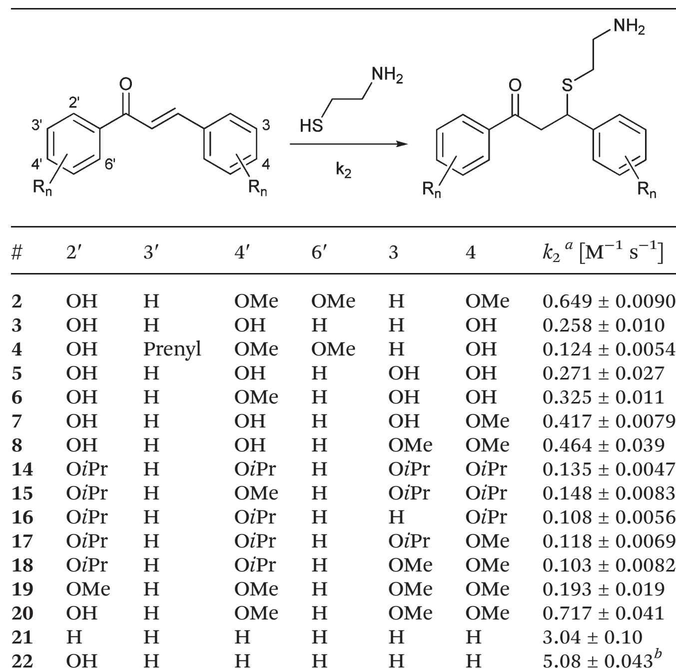
in two positions from its biosynthetic precursor 2′,4,4′,6′-tetrahydroxychalcone (**1**).

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**, **14–20** and **22** (see Table 1) used in this study were done by classical Claisen–Schmidt condensations of methoxylated or isopropyl protected acetophenones and benzaldehydes using $Ba(OH)_2 \cdot 8H_2O$ as the base in MeOH (Scheme 1).[5]

The deprotection of the corresponding isopropoxy ethers was performed with BCl_3 in CH_2Cl_2. 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.

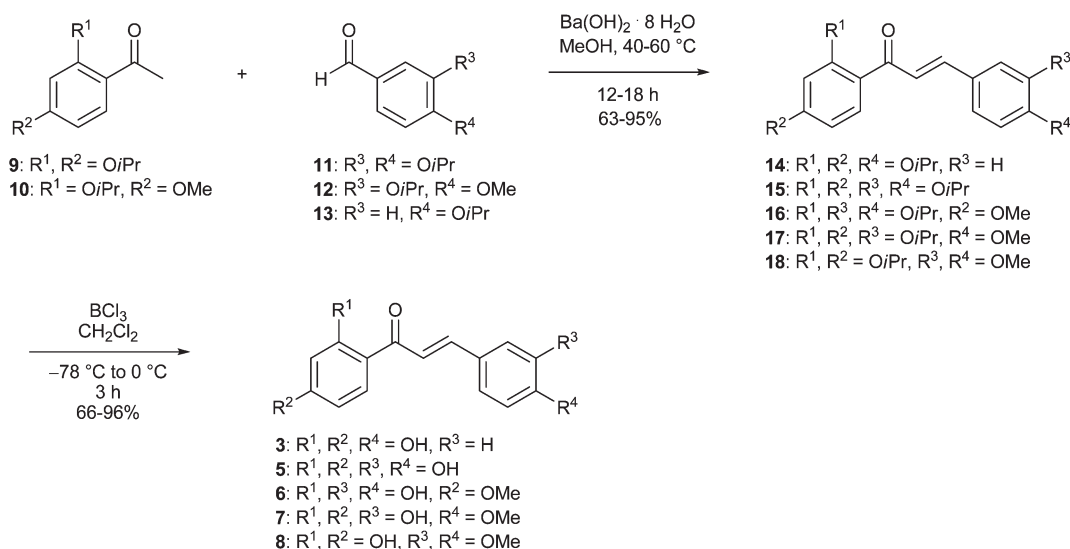
In order to be able to efficiently compare Michael acceptor activities, we decided to use a 96-well microtiter plate based screening system since it allows for a simple and quick handling together with rather small amounts of material needed to determine the k_2 values. Since we expected to see great 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 α-X-chalcones, where X can be different substituents like halogen or carbon-based residues.[3] 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,[6] 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 the 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

Table 1 k_2-Values of α-H-chalcones with cysteamine at 25 °C



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

[a] 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 concentration of 40 μM for chalcones and 12 to 500 fold cysteamine. [b] Done by stopped flow technique.



Scheme 1 Synthesis of hydroxylated and alkoxylated α-H-chalcones used in the kinetic studies involving isopropyl ethers.

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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[7] 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′-tetramethoxychalcone (**19**) showed a fast reaction with cysteamine (Fig. 2) and cysteine, intermediate reactivity with DTT and a slow reaction with 2-mercaptoethanol, as well as GSH.

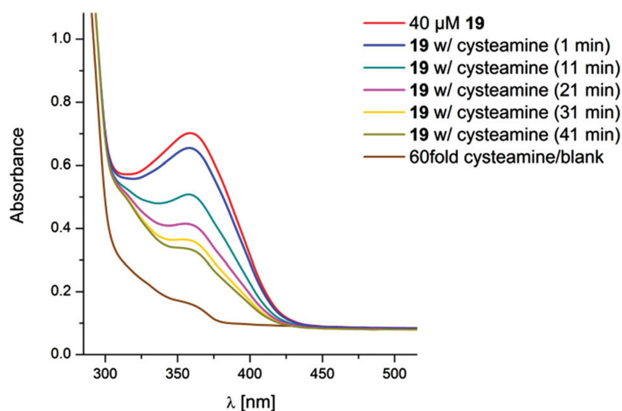
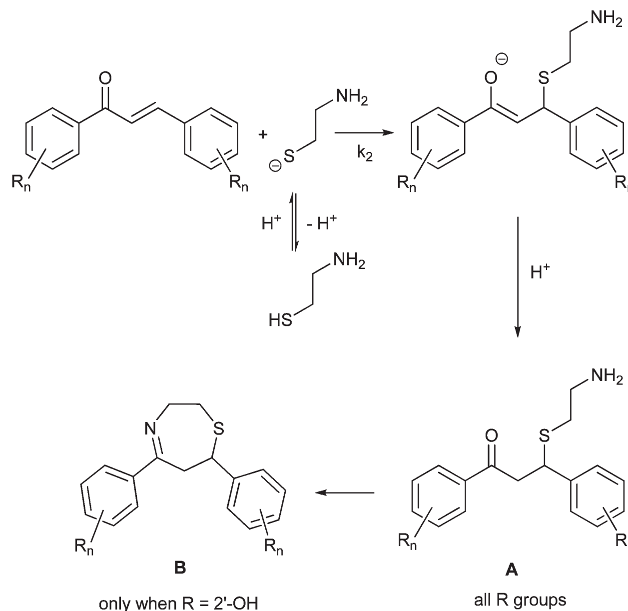


Fig. 2 UV spectra of 2′,3,4,4′-tetramethoxychalcone (**19**) without and with cysteamine at 25 °C (in a plastic microtiter plate with a PCR foil).

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

Assuming that there is always a sufficient amount of thiolate present, which concentration is directly proportional to



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

the thiol concentration, one can use the following equations to calculate the second order rate constant k_2:

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

under pseudo-first-order conditions:

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

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

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

k_{obs} values were gained from the time-dependent decay of the absorbance (A_t) of the αβ (chalcone) with thiol (cysteamine) by fitting the data of individual experiments to the first order exponential eqn (5):

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

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 α-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[8] that a 2′-OH group is essential for the reactivity of the chalcones.[9] 2′-Hydroxychalcone (**22**) is the most active compound with a k_2 value of 5.08 M^{-1} s^{-1} for its reaction with cysteamine.

There is about 1.7 fold increase in activity 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

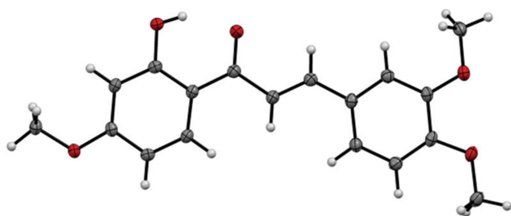
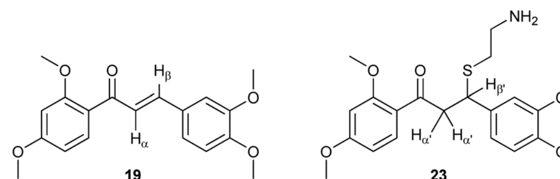


Fig. 3 X-ray structure of 2′-hydroxy-3,4,4′-trimethoxychalcone (**20**).

(Fig. 3) of the fairly flat 2′-hydroxy compound **20** (dihedral angle = 11.38°) compared to tetramethoxy analog **19**, which displays a dihedral angle of 26.88° between the two benzene rings.[10]

This influence of the 2′-OH group led to a k_2 value of 0.717 M^{-1} s^{-1} for hydroxyl compound **20**, whereas the 2′-methoxy compound **19** gave a k_2 value of 0.193 M^{-1} s^{-1}. Furthermore, since the double bond of the α,β-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 M^{-1} s^{-1}, **7** and **6**, gave k_2 values of 0.417 M^{-1} s^{-1} and 0.325 M^{-1} s^{-1}, respectively. On the other hand when regioisomers flavokawain A (**2**) and **20** are compared, which both carry three methoxy groups, this effect was not observed. Here, the steric hindrance 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 M^{-1} 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 2 fold, which could be explained by steric factors that lead to differences in the conjugation of the π-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 on the carbonyl group. This was recently shown for α,β-unsaturated aldehydes.[11]



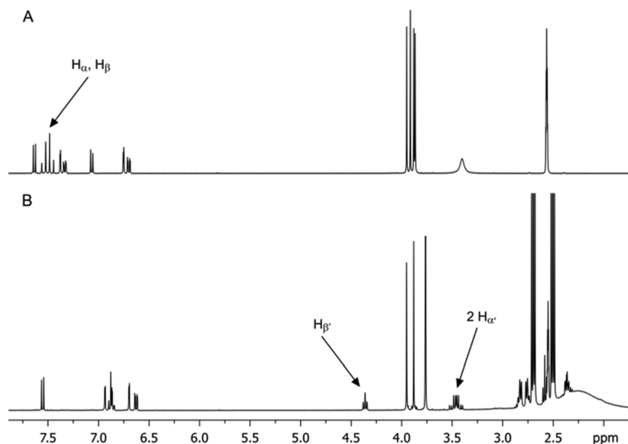


Fig. 4 ^{1}H NMR spectra of 2′,3,4,4′-tetramethoxychalcone (**19**) in DMSO-d_6. (A) **19** only, (B) **19** with 12 fold cysteamine after 5 min.

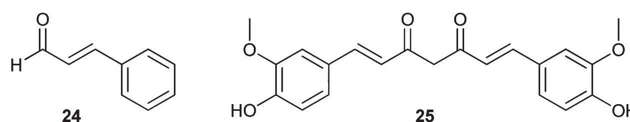


Fig. 5 Further active α,β-unsaturated carbonyl compounds.

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 12 fold cysteamine including an internal standard (Fig. 4). Fig. 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 α,β-unsaturated carbonyl compounds in our kinetic measurements, namely cinnamaldehyde (**24**), curcumin (**25**, Fig. 5), cinnamic acid methyl ester (**26**), cinnamic acid ethyl ester (**27**), 2′-hydroxycinnamic 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 **24** and **25** displayed sufficient reactivities so that their k_2 values could be determined. (Table 2)

Notably, the α,β-unsaturated aromatic aldehyde **24** demonstrates good reactivity with a k_2 value of 0.636 M^{-1} s^{-1} which is very close to flavokawain A (**2**). Curcumin (**25**), which gave a k_2 value of 0.0621 M^{-1} s^{-1}, is less reactive by a factor of about 10.

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Table 2 Kinetic studies of α,β-unsaturated carbonyl compounds with cysteamine at 25 °C

#	Compound	k_2 [a] [M^{-1} s^{-1}]
24	Cinnamaldehyde	0.636 ± 0.019
25	Curcumin	0.0660 ± 0.0079
26	Cinnamic acid methyl ester	<0.001 [b]
27	Cinnamic acid ethyl ester	<0.001 [b]
28	2′-Hydroxycinnamic acid	n.r. [c]
29	Cinnamic acid	n.r. [c]
30	Caffeic acid phenethyl ester (CAPE)	<0.001 [b]
31	Chlorogenic acid	<0.001 [b]
32	Caffeic acid	n.r. [c]
33	3-Hydroxycoumarin	<0.001 [b]
34	Kaempferol	n.r. [c]
35	Quercetin	n.r. [c]

[a] 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 concentration of 40 μM for α,β-unsaturated carbonyl compounds and 12 to 500 fold cysteamine. [b] Reactions were not complete after 63 h with 500 fold cysteamine. [c] No reaction was found within 63 h with 500 fold cysteamine.

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The MS experiments revealed the expected products including bisadducts in the cases for **24** and **25**. Additionally, aromatic α,β-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.

Acids **28**, **29**, **32**, and flavonols **34** and **35** were not reactive in our assay (all results were verified by MS studies). Attempts to determine k_2 for phenylvinylketone (1-phenylprop-2-en-1-one) failed since the absorption maximum of this compound lies beyond 300 nm and therefore our UV-VIS detection-based method cannot be used.

^{1}H NMR studies with different α,β-unsaturated natural products have been used to successfully group these into reversible and irreversible thiol sinks.[11] Under the conditions used (DMSO-d_6/dilution with $CDCl_3$) 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 α,β-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-κB, 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.[12] Moreover, metabolic transformations, such as the hydroxylation of the aromatic rings of the α,β-unsaturated carbonyl compounds, could change their reactivity and therefore overall biological activity.[13] Other α,β-unsaturated carbonyl compounds such as the flavonoles kaempferol (**34**) and quercetin (**35**) show significant biological activities such as cancer prevention,[14] 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.

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Notes and references

1 For selected reviews see: (*a*) M. L. Go, X. Wu and X. L. Liu, *Curr. Med. Chem.*, 2005, **12**, 483–499; (*b*) Z. Nowakowska, *Eur. J. Med. Chem.*, 2007, **42**, 125–137; (*c*) C. Kontogiorgis, M. Mantzanidou and D. Hadjipavlou-Litina, *Mini-Rev. Med. Chem.*, 2008, **8**, 1224–1242; (*d*) A.-M. Katsori and D. Hadjipavlou-Litina, *Expert Opin. Ther. Pat.*, 2011, **21**, 1575–1596.
2 S. Amslinger, *ChemMedChem*, 2010, **5**, 351–356.
3 Kinetic data for a series of alpha-X-chalcones based on our thiol assay will be published elsewhere.
4 (*a*) D. G. Roux and D. Ferreira, *Phytochemistry*, 1974, **13**, 2039–2048; (*b*) W. Greenaway and F. R. Whatley, *Phytochemistry*, 1990, **29**, 2551–2554; (*c*) R. Metuno, F. Ngandeu, A. T. Tchinda, B. Ngameni, G. D. W. F. Kapche, P. C. Djemgou, B. T. Ngadjui, M. Bezabih and B. M. Abegaz, *Biochem. Syst. Ecol.*, 2008, **36**, 148–152.
5 S. Sogawa, Y. Nihro, H. Ueda, A. Izumi, T. Miki, H. Matsumoto and T. Satoh, *J. Med. Chem.*, 1993, **36**, 3904–3909.

6 A kinetic measurement performed of **19** in a 1 : 1 mixture of MeCN/100 mM TRIS-HCl pH 7.4 (A. T. Dinkova-Kostova, M. A. Massiah, R. E. Bozak, R. J. Hicks and P. Talalay, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 3404–3409) gave a very fast reaction with GSH.

7 G. A. Bagiyan, I. K. Koroleva, N. V. Soroka and A. V. Ufimtsev, *Russ. Chem. Bull., Int. Ed.*, 2003, **52**, 1135–1141.

8 (*a*) F. Jin, X. Y. Jin, Y. L. Jin, D. W. Sohn, S.-A. Kim, D. H. Sohn, Y. C. Kim and H. S. Kim, *Arch. Pharmacal Res.*, 2007, **30**, 1359–1367; (*b*) A. T. Dinkova-Kostova, C. Abeygunawardana and P. Talalay, *J. Med. Chem.*, 1998, **41**, 5287–5296.

9 In some other aromatic α,β-unsaturated carbonyl compounds, *ortho*-hydroxyl groups in general proved to increase biological activity (A. T. Dinkova-Kostova and P. Talalay, *Carcinogenesis*, 1999, **20**, 911–914).

10 J. H. v. Tonder, T. J. Muller and B. C. B. Bezuidenhoudt, *Acta Crystallogr., Sect. E: Struct. Rep. Online*, 2010, **66**, o1798–o1799.

11 C. Avonto, O. Taglialatela-Scafati, F. Pollastro, A. Minassi, V. Di Marzo, L. De Petrocellis and G. Appendino, *Angew. Chem., Int. Ed.*, 2011, **50**, 467–471.

12 J. Heilmann, M. R. Wasescha and T. J. Schmidt, *Bioorg. Med. Chem.*, 2001, **9**, 2189–2194.

13 Y. Kohno, S. Kitamura, S. Sanoh, K. Sugihara, N. Fujimoto and S. Ohta, *Drug Metab. Dispos.*, 2005, **33**, 1115–1123.

14 H. S. Aiyer, A. M. Warri, D. R. Woode, L. Hilakivi-Clarke and R. Clarke, *J. Agric. Food Chem.*, 2012, **60**, 5693–5708.

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