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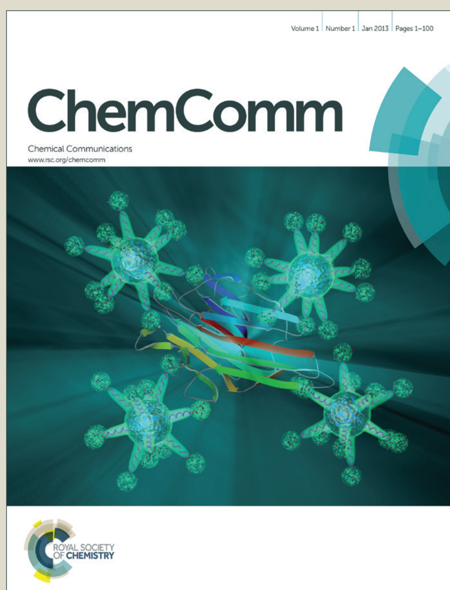

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A Phenacrylate Scaffold for Tunable Thiol Activation and Release

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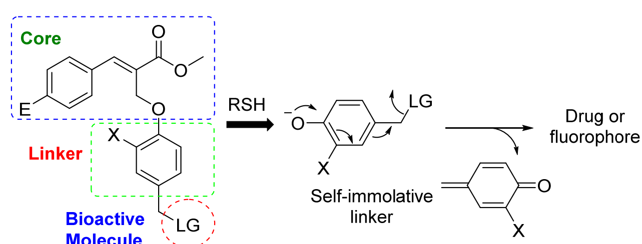
A thiol-selective 2-methyl-3-phenacrylate scaffold with spatiotemporal control over delivery of a cargo is reported. The half-lives of decomposition could be tuned from 30 min to 1 day and the scaffold's utility in thiol-inducible fluorophore release in cell-free as well as within cells is demonstrated.

Selective and covalent modification of thiols has numerous applications in biochemistry, imaging and medicine.[1-3] Thiols in proteins or peptides can be conjugated to a small molecule[4-5] or a macromolecule and is useful for interrogating cellular events, studying enzyme function and in drug discovery.[6-10] Cancers, for example, have elevated thiol levels in comparison with their normal paired tissue.[3, 11-13] Hence, this property can be exploited for directed delivery of drugs or fluorophores (for imaging) inside cells.[3, 11-12, 14] Here, a bioactive molecule or fluorophore is conjugated to a scaffold which is stable in buffer. Upon selective reaction with a thiol the active drug molecule or fluorophore is released.[15-16] The relative abundance of thiols in cancers might facilitate site-specific localization of the biomolecule. Among the methods available for thiol-mediated activation and release, the disulfide-based scaffold, which is cleaved by glutathione to initiate a rearrangement leading to drug or fluorophore release, is widely used for drug delivery and imaging.[2, 17-19] However, disulfides are susceptible to cleavage not just by glutathione but also by reductases[20] and this scaffold does not have any structural handle for controlling rate of release of the bioactive small molecule. Hence, this method might not be suitable for slow release of drugs, for example. Finn and coworkers reported an oxanorbornadiene-based methodology for thiol activation and release[21-22] where the rate of drug release could be tuned by structural modifications to the scaffold and is well suited for release of a fluorophore, possibly for imaging. For applications in drug release, however, this scaffold may have limited utility as the drug is still conjugated to a furan. Here, we report results of our design, synthesis and evaluation of a novel scaffold whose reaction rates with a biological thiol can be modulated by simple structural modifications.

A 2-methyl-3-phenacrylate-based scaffold was considered for tunable reactivity with thiols (Scheme 1). The core consists of a 2-methylene-3-arylacrylate functionality connected with a phenol-based self-immolative linker, which can be attached to a drug or fluorophore that is to be released. Michael addition[23] of a thiol produces an enol(ate), which can rearrange to produce a self-immolative phenolate; rearrangement of this intermediate would result in the release a leaving group, LG^-. α-Halo acetamides have been frequently used for labeling thiols in biomolecules[24-25] supporting a direct S_N2 attack on the ethereal carbon, which produces a phenolate and subsequent rearrangement can release the bioactive molecule LG^-. The carbonyl group is distant from the reaction centre but remains in conjugation while the aryl group communicates with the ether carbon and together these groups might help tuning of reaction rates. The pK_a of the phenol and hence leaving group ability of the linker can also be modulated by changing substituent X to attain the desired reaction rate and hence spatiotemporally controlled release



Scheme 1. Proposed 2-methyl-3-phenacrylate scaffold for tunable thiol activation. LG = leaving group

In order to synthesize this scaffold, bromide **2a** was prepared from the corresponding alcohol **1a** by a PBr_3-mediated bromination reaction (Table 1, entry 1).[26-27] The alcohol **1a** was in turn prepared by a Baylis-Hillman reaction of benzaldehyde and methyl acrylate.[28-29]

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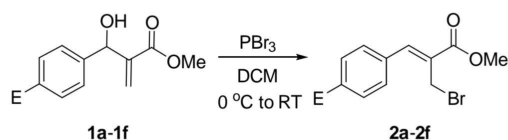
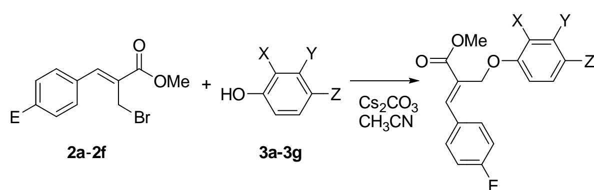
Next, **2a** was reacted with various phenols to produce the corresponding phenol ethers **4a-9a** in yields ranging from 75-95% (Table 2, entries 1-6).[30]

Table 1. Synthesis of bromides **2a-2f**



Entry	E	Reactant	Product	% Yield
1	H	**1a**	**2a**	79
2	NO_2	**1b**	**2b**	89
3	CN	**1c**	**2c**	71
4	Br	**1d**	**2d**	71
5	F	**1e**	**2e**	99
6	OMe	**1f**	**2f**	66

Table 2. Synthesis of **4a-9a, 8b-8f** and **10.**



Entry	E	RBr	X	Y	Z	ArOH	Prod
1	H	**2a**	H	H	Me	**3a**	**4a**
2	H	**2a**	Br	H	Me	**3b**	**5a**
3	H	**2a**	H	OMe	H	**3c**	**6a**
4	H	**2a**	H	H	CN	**3d**	**7a**
5	H	**2a**	NO_2	H	Me	**3e**	**8a**
6	H	**2a**	H	H	NO_2	**3f**	**9a**
7	NO_2	**2b**	NO_2	H	Me	**3e**	**8b**
8	CN	**2c**	NO_2	H	Me	**3e**	**8c**
9	Br	**2d**	NO_2	H	Me	**3e**	**8d**
10	F	**2e**	NO_2	H	Me	**3e**	**8e**
11	OMe	**2f**	NO_2	H	Me	**3e**	**8f**
12	CN	**2c**	NO_2	H	CH_2OH	**3g**	**8g**

In order to study if these compounds were capable of reacting with glutathione, **4a**-**9a** were reacted with 10 eq. GSH in pH 7.4 buffer at 37 °C and the amount of compound remaining was determined. A plot of % remaining versus pK_a[31] of the corresponding phenol leaving group shows a nearly linear relationship (Figure 1a) suggesting that reactivity with thiols could be tuned.

Due to ease of monitoring of the product **3e** formed during thiol-mediated activation, we chose **8a** for further studies. A time course for decomposition of **8a** (Figure 1b) in the presence of glutathione was conducted and a pseudo first order rate constant $\boldsymbol{k_x}$ for loss of **8a** was found as 0.072 h^{-1}, with a half-life of 9.6 h (Table 3). The formation of **3e** was monitored during the aforementioned reaction and a rate constant of 0.072 h^{-1} and with an excellent yield of **3e** (78%, Table 3).

We next tested if changing electronics on the aryl ring of this scaffold influenced rates of thiol activation and release. Bromides with electron withdrawing and electron donating groups attached to the aryl group were prepared (Table 1); these bromides were reacted with **3e** to give **8b**-**8f** in excellent yields (Table 2, entries 7-11). Glutathione-mediated decomposition of these compounds (see ESI) was conducted and rate constants ($\boldsymbol{k_x}$) for decomposition as well as formation of **3e** ($\boldsymbol{k_f}$) were determined (Table 3).

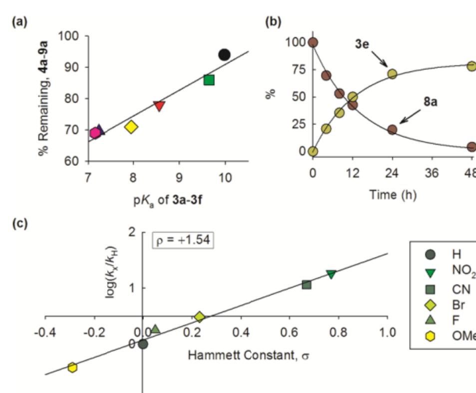


Figure 1. (a) A comparison of GSH-mediated decomposition of **4a**-**9a** monitored by HPLC analysis of reaction mixtures after 4 h and the reported p*K*as of the corresponding leaving groups, **3a**-**3f**; (b) Time courses of GSH-mediated decomposition of **8a** and formation of **3e** were monitored by HPLC analysis. Curve fitting gave rate constants for decomposition of **8a** and formation of **3e**; (c) Linear Free Energy Relationship Analysis of log($\boldsymbol{k_x}/\boldsymbol{k_H}$) obtained from rate constants for thiol-mediated decomposition of **8a**-**8f** (Table 3) and Hammett constants.

When **8c** was reacted with thiophenol in pH 7.4 buffer, we find **3h** (Figure 2) as the major product supporting direct displacement as the dominant pathway (ESI, Figure S2). The literature contains numerous examples of Michael attack by thiols suggesting the likelihood of this pathway.[1, 3, 5] If the rate determining step is conjugate addition of the thiol, the rate of decomposition of the compound must: (a) be significantly different from the rates of appearance of phenolate; and (b) not depend on the leaving group ability. However, due to nearly identical rates of decomposition ($\boldsymbol{k_x}$, Table 3) as well as release ($\boldsymbol{k_f}$, Table 3) and the dependence on the leaving group (Figure 1a), it appears that S_N2 (or possibly S_N2' followed by rapid thiol exchange) for displacement of the leaving group by GSH is more likely (ESI, Figure S3). Furthermore, our results are consistent with a previous report conducted with **2a** and sodium azide as the nucleophile, where the authors did not detect the Michael-attack intermediate.[27]

Table 3. Glutathione-mediated decomposition of **8a-8f** and release of **3e**.

Compd	E	σ[a]	k_X[b]	$t_{1/2}$, h[c]	k_f[d]	% Yield[e]
8a	H	0	0.072	9.6	0.077	78
8b	NO_2	0.77	1.32	0.5	1.44	88
8c	CN	0.67	0.83	0.8	1.20	79
8d	Br	0.23	0.22	3.15	0.20	73
8e	F	0.05	0.128	4.9	0.124	84
8f	OMe	–0.29	0.0274	25	0.030	79

[a]Hammett constant. [b]Pseudo first-order rate constant for decomposition expressed in h^{-1} was obtained by curve fitting ($R^2 \geq 0.995$, P-value <0.001, see ESI Table S1); [c]Half-life of decomposition of the compound; [d]First-order rate constant for formation of **3e** in h^{-1} was determined by curve fitting ($R^2 \geq 0.995$, P-value <0.001, see ESI Table S1); [e]Maximum yield of the phenol obtained during the decomposition.

The half-lives of decomposition of **8a**-**8f** were found to be 30 min–1 day (Table 3) suggesting a high degree of tunability of release of the free phenolate. The yields of **3e** during decomposition was >75% in a majority of the cases supporting the possibility of efficient release upon activation by a thiol (Table 3). Linear free energy relationship analysis (Figure 1c) of rates of decomposition of **8a**-**8f**

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showed a nearly linear relationship between Hammett constant σ and log(k_X/k_H). Linear regression analysis yielded a ρ-value of +1.54 (R = 0.9944) supporting a moderate but predictable electronic effect on thiol-mediated decomposition of phenacrylates (Figure 1c).

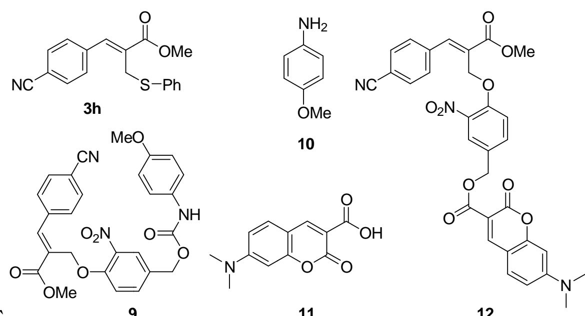

Figure 2. Compounds **3h**, **9**, **10**, **11** and **12**.

With these results in hand, we tested if the scaffold was selective towards thiols. When **8b** was reacted with amino acids that do not contain a free thiol, we found no evidence for decomposition after 4 h (see ESI, Figure S1); whereas this compound was nearly completely decomposed when this reaction was conducted with glutathione (Table 2) suggesting a high degree of selectivity towards activation by biological thiols (see ESI, Figure S1).

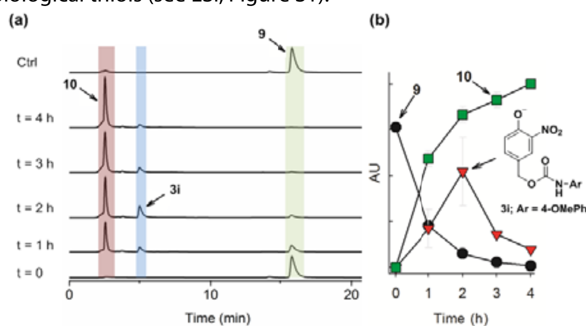

Figure 3. Representative HPLC traces for decomposition of **9** in the presence of glutathione (GSH, 10 eq.) to release of **10**. The formation of an intermediate was observed by HPLC analysis and MS analysis of this fraction suggested that this intermediate was **3i**. Ctrl is **9** incubated in buffer for 4 h. (b) Time course of the aforementioned decomposition of **9** in the presence of GSH.

In order to test the suitability of this scaffold for release of a bioactive molecule, 4-(hydroxymethyl)-2-nitrophenol[32-33] (**3g**, Table 2, entry 12) was prepared using a reported procedure. Reaction of **3g** with **2c** gave **8g** (Table 2, entry 12). This alcohol was further reacted with 4-nitrophenyl (4-methoxyphenyl)carbamate to give **9** (Figure 2). This compound upon exposure to a thiol should undergo decomposition to release the 2-nitrophenolate intermediate **3i**, which in turn would undergo decomposition to produce CO_2 and 4-anisidine **10** (Figure 2). HPLC analysis of the reaction mixture of **9** and GSH showed gradual decomposition of **9** during 4 h and concomitant formation of **10** (Figure 3a). In addition, the formation of an intermediate was observed (Figure 3b); mass spectrometry of this intermediate supported **3i** (ESI, Figure S4) thus providing further evidence for the proposed mechanism (ESI, Figure S3). In the absence of glutathione, we found **9** to be stable in buffer during this time period (Ctrl, Figure 3).

Next, in order to test the suitability of this scaffold for activation and release of a fluorophore, a coupling reaction of *N*,*N*-dimethylaminocoumaric acid (**11**), a fluorophore was carried out with **3g** to produce **12**.[34-35] The ester **12** is weakly fluorescent in aqueous buffer while **11** is highly fluorescent under these conditions.[36] When reacted with a thiol, decomposition of **12** should generate a phenolate, which would self-immolate to generate **11** leading to a significant increase in fluorescence intensity (Scheme 1). We studied the reaction of **12** with 2-mercaptoethanol in methanolic pH 7.4 buffer and we found a gradual increase in fluorescence during 5 h (Figure 4a) attributable to the formation of **11** (Figure 4a, inset). The yield of **11** under these conditions was estimated as 76% (calculated from a calibration curve for **11**, see ESI, Figure S5) suggesting the potential for temporally controlled and efficient release.

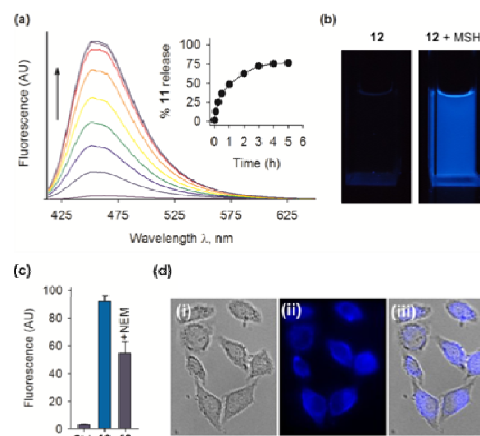

Figure 4. (a) Decomposition of **12** in the presence of 2-mercaptoethanol (MSH, 25 eq.) to release of **11** was monitored by fluorescence measurements over several hours. Inset: time course of **11** generated during this experiment was estimated based on a calibration curve for authentic **11** (see ESI, Figure S5). (b) Solutions of **12** incubated in pH 7.4 alone and in the presence of MSH (25 eq.) after 8 h. (c) Human cervical cancer HeLa cells were incubated with DMSO (Ctrl) and **12** (10 μM); + NEM indicates cells were treated with *N*-ethylmaleimide (NEM, 100 μM) for 30 min followed by addition of **12** (10 μM). Data presented is for fluorescence measured after 2 h. (d) Live cell images of HeLa cells incubated with **12** (10 μM) for 2 h at 37 °C: (i) brightfield, (ii) fluorescence and (iii) overlay.

Under similar conditions, no significant increase in fluorescence was observed in the absence of a thiol (Figure 4b) or in the presence of esterase (see ESI) supporting the thiol-selectivity of the trigger.

A cell viability assay using human cervical cancer HeLa cells showed that the IC_{50} of this compound was >25 μM suggesting that this scaffold is itself not significantly toxic (see ESI, Figure S6). Treatment of Hela cells with **12** and subsequent fluorescence measurement showed enhanced fluorescence attributable to the formation of **11** (Figure 4c). HeLa cells were incubated with *N*-ethylmaleimide (NEM), a quencher of free thiols for 30 min followed by addition of **12**. Here, we find significant decrease in fluorescence indicating thiol activation as a mechanism for fluorophore release in cells (Figure 4c). A similar experiment conducted using **12** in serum starved HeLa cells showed a dose-dependent increase in fluorescence supporting activation by thiols within cells to release the fluorophore (see ESI, Figure S7).

Finally, HeLa cells were treated with **12** and fluorescence microscopy revealed increased fluorescence corresponding to **11**

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supporting localization of this fluorophore within cells (Figure 4d). Together our data suggests that this scaffold is capable of permeating cells to deliver a bioactive cargo such as a fluorophore.

In summary, we report a novel scaffold that is suitable for thiol-mediated activation and release of a drug or fluorophore with a high degree of thiol-selectivity and half-lives ranging from 30 min to 1 day.[37-38] The most common methodology available i.e. the disulfide-based method has no structural handle for rate control. It is anticipated that the method presented here will be useful for selective thiol modification, thiol-mediated drug release as well as cellular imaging.[25]

Notes and references

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