Fluorescence quenched quinone methide based activity probes – a cautionary tale†

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A carbamate linked quenching group coupled with a pro-quinone methide reactive core provides an effective tool for studying enzyme function without problems associated with background fluorescence from unreacted probe. However, the relatively slow fragmentation of the carbamate linkage in such a strategy may cause problems of loss of signal or a decoupling of enzyme activity and labelling.

Introduction

Plant serine hydrolases are an important class of enzyme, involved in an array of physiological processes including senescence and programmed cell death, xylogenesis, tissue differentiation, the infection of plant cells, pathogenesis in virus-infected plants and germination.[1] One particular group of serine hydrolases are the carboxylesterases (CXE) involved in the bioactivation and metabolism of many crop protection agents.[2] As a result, understanding the differences in expression levels and function between carboxylesterases from crop and weed species is of significant importance for the agrochemical industries. In this report we describe our effort to generate and evaluate an intrinsically non-fluorescent covalent labelling probe for carboxylesterase enzymes that on activation becomes fluorescent.[3] Such a directed approach allows for the unambiguous identification of active carboxylesterases which are encoded by a superfamily of genes, mostly of unknown function.[4]

In recent years the use of chemical probes to explore protein function has become a particularly attractive and popular strategy.[5] Such probes can be crudely categorised as either responsive or affinity/activity based. Responsive probes commonly provide a simple measurable output, usually optical, in relation to protein (enzyme) function. In most cases this is achieved by a change in chemical structure, either the binding of an analyte or the rupture of a chemical bond, causing a change in fluorescence. Whilst in many cases this is ratiometric, a more favourable situation, providing greater sensitivity, is produced with an "off–on" (dark–light) switch. Whilst such probes provide information about the function of a protein they do not facilitate the identification of the target protein in a complex proteome. More recently the concept of activity based protein profiling (ABPP) has been developed by Cravatt, Bogyo and others.[6–11] In this approach the protein(s) of interest are covalently labelled according to a specific reactivity mode. The labelling moiety is additionally conjugated with various tags that facilitate the detection and isolation of the labelled protein. However, whilst this approach identifies the activity it does not necessarily provide any indication as to the level of activity. It is therefore desirable to combine the approaches and use the structural change in the probe that occurs on labelling to trigger a change in fluorescence to provide a compound that not only reports the activity of an enzyme but also facilitates its identification from a complex proteome. Such activity probes were first described by Bogyo who exploited the selective cleavage of acyloxymethyl ketones by cysteine dependent proteases in which enzyme action caused loss of a FRET based quenching group leading to a "switch on" of fluorescence.[12,13] Whilst elegant and very effective, the extension of this approach to other enzyme classes requires the complete redesign of probe structure. An attractive solution to this challenge is to utilise a common reactive core and reporting element that can be decorated with a variety of different enzyme activatable groups. For this, reflecting their long history as mechanism based covalent inhibitors, we were attracted to the use of *para*-hydroxybenzyl halides and related derivatives. Whilst this has been exploited, by a number of researchers, to develop activity based probes exploring a range of enzyme classes including glycosidases, phosphatases and various proteases, in the vast majority of these cases the leaving group used to generate the quinone methide reactive electrophiles are halogens which are not easily coupled to a change in fluorescence.[14–19] Although, simple *para*-hydroxybenzylic esters can be activated in a similar fashion, in earlier studies exploring quinone methide based inactivation of acid phosphatases, the use of the corresponding benzylic acetate was shown to be ineffective in labelling or inactivating the enzyme.[14,20] We therefore speculated that a carbamate or carbonate linker to the quencher group would afford sufficiently rapid fragmentation to provide efficient labelling of the target protein.[21] Herein, we describe the synthesis and evaluation of such a pro-fluorescent esterase probe in which the fluorescence is quenched by a proximal nitro aromatic group. This probe undergoes selective activation by the plant serine hydrolase *Arabidopsis thaliana* carboxylesterase 12 (*At*CXE12) leading to covalent modification of the enzyme, Fig. 1. Studies using more complex protein mixtures reveal that such probes need to be used with caution as product release from the enzyme

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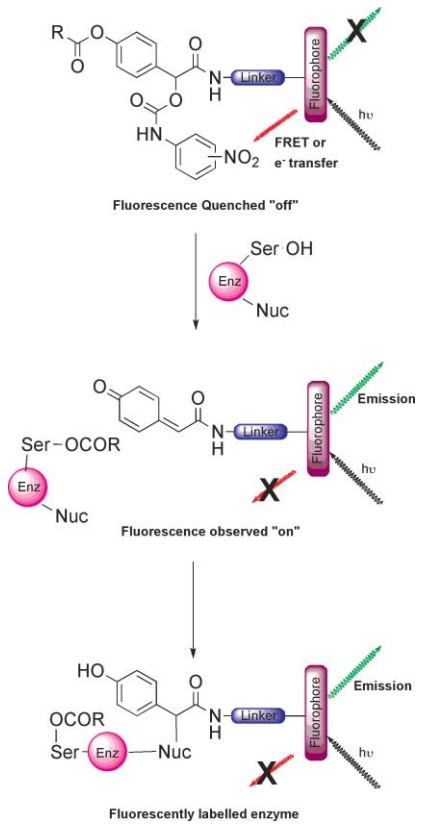


Fig. 1 Fluorescence quenched quinone methide protein probes.

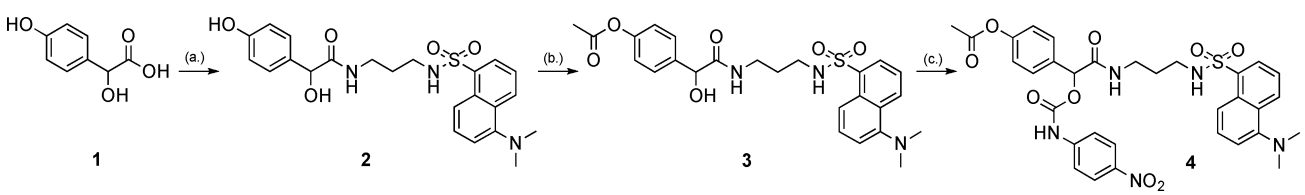
active site appears to occur at a competitive rate to covalent modification.

Results

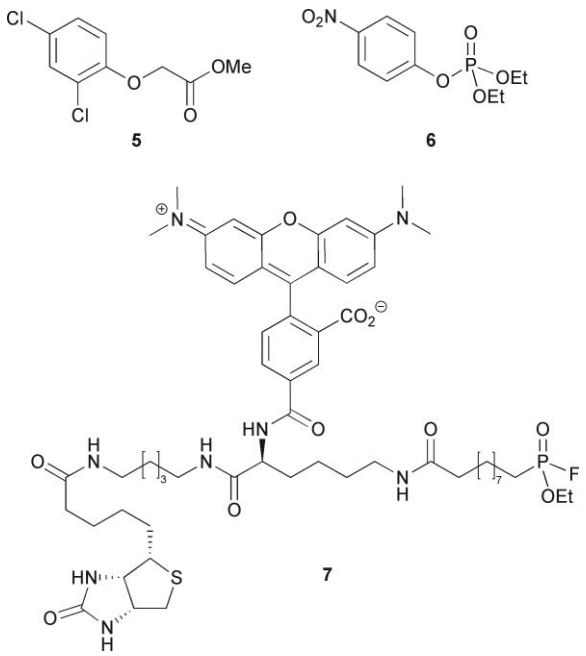
Synthesis started from 4-hydroxymandelic acid **1** by first coupling the fluorescent label, Scheme 1. In order to facilitate purification and handling through subsequent synthetic steps we opted to use the structurally simple dansyl group combined with a short propyldiamine spacer. Initial attempts to couple this directly using classical carbodiimide chemistry were not efficient and we ultimately used a straightforward sequence involving acid catalysed esterification which concomitantly afforded the benzylic ether, condensation with 1,3-diaminopropane, sulfonamide formation with dansyl chloride and then ether hydrolysis using aqueous 1 M HCl. The final steps of the synthesis required addition of a reactive head group and the fluorophore quencher. For this proof of principal study we opted to use a simple acetate and nitro-substituted aromatic as these are known to provide effective quenching.[22] Consequently, utilising the differences in pK_a (phenol pK_a ~ 10; benzylic alcohol pK_a ~ 15) the more acidic phenol group was acylated using acetyl chloride and DIPEA at 0 °C for 1 h followed by addition of the quencher group utilising *p*-nitrophenyl isocyanate and triethylamine.

Pleasingly, comparison of the absorption profiles for probe **4** and the parent diol **2**, which lacks the quenching group, revealed that whilst both absorb at a maximum of 335 nm the emission profiles are distinctly different (Fig. 2A, black line). Whereas **2** exhibits a fluorescence quantum yield of 89% (red line), analysis of the emission spectrum for **4** reveals a clear quenching effect resulting in a quantum yield of fluorescence of only 8% (blue line). By comparison with the absorption spectrum for simple carbamates of *p*-nitroanilide the lack of an emission band for **2** below 400 nm suggests that the mechanism for this quenching involves electron transfer not FRET. Moreover, confirmation that ester hydrolysis would be accompanied by fragmentation and initiation of fluorescence was achieved by simple chemical hydrolysis of **4** utilising 1 M KOH in 0.1 M Tris buffer (3 ml, pH 7.4) at room temperature (Fig. 2B). Spectrophotometric measurements (excitation: 333 nm; emission: 400–600 nm) clearly revealed, that at room temperature, **4** undergoes very rapid (<5 min) hydrolysis leading to a 6-fold increase in intensity relative to the parent probe. Prolonged reaction times resulted in a slow decrease in fluorescence signal due to base mediated decomposition of **2**.

Having established that **4** was capable of chemical activation attention then moved to explore its utility as a probe for enzyme activity. As a model system we opted to explore the use of a recombinant carboxylesterase, *At*CXE12, originally isolated from *Arabidopsis thaliana* as the principal enzyme responsible for the hydrolytic bioactivation of methyl 2,4-dichlorophenoxyacetate **5** a pro-herbicide commonly used in the control of dicotyledonous weeds.[23] Previous work in the Edwards group has demonstrated that *At*CXE12 is a classic serine hydrolase and as such is sensitive to inhibition by the organophosphate insecticide paraoxon **6** and a biotinylated probe **7** bearing a fluorophosphonate reactive group.[2] Incubation of **4** (10 µl, 10 mM in acetone) in 0.1 M Tris buffer (3 ml, pH 7.4) with purified recombinant *At*CXE12 (50 µl, 0.8 mg ml^{-1}) at 37 °C revealed a similar increase in fluorescence to that determined chemically, albeit requiring 3 h to reach complete conversion (Fig. 2C). Importantly, the probe proved resistant to hydrolysis both in the absence of enzyme (cyan and blue line), or in the presence of enzyme denatured by heating. These results clearly demonstrate that **4** is a substrate for serine hydrolase *At*CXE12 and that the fluorescence increase is due solely to the enzyme action on the probe.



Scheme 1 *Reagents and conditions*: (a.) (i). c. H_2SO_4, MeOH, reflux, 72%; (ii). 1,3-diaminopropane, r.t., 84%; (iii). dansyl chloride, Et_3N, THF, –78 °C–r.t., 85%; (iv) aq. HCl (1 M), reflux, 4 h, 73%; (b.) AcCl, DIPEA, MeCN, 0 °C–r.t., 57%; (c.) 4-nitrophenyl isocyanate, Et_3N, $CHCl_3$, r.t., 45%.



Having confirmed that **4** could be bioactivated by *At*CXE12, we then sought to establish whether it was labelling the protein. Consequently, following incubation of the enzyme with **4** for 1.5 h the protein fraction was isolated and analysed by SDS-PAGE gel with visualisation using both Coomassie staining (Fig. 3A) and UV light (Fig. 3B). In the latter, the observation of a fluorescently labelled polypeptide (lane 2) demonstrated that not only does *At*CXE12 turn over **4**, but also that this is accompanied by covalent labelling of the enzyme. Moreover, when *At*CXE12 was denatured or inhibited (lane 3 and 4) no fluorescent bands were observed, clearly demonstrating that this labelling is a result of enzyme activity. Repeating this experiment at a range of probe concentrations (Fig. 3C, 3D), clearly showed dose dependent fluorescent labelling ranging from 10 mM (lane 2) to 0.01 mM (lane 5), with the latter lower limit being only just detectable. From this experiment, it was determined that 0.1 mM (lane 4) was the ideal probe concentration for future work. Interestingly, staining for protein (Coomassie blue) (Fig. 3C) revealed that, when using higher concentrations of probe (lanes 2 & 3), the labelled polypeptides migrated with a noticeably higher molecular weight implying multiple protein modifications had occurred.

ESMS analysis of the labelled *At*CXE12 confirmed such an occurrence. Following deconvolution to neutral mass, peaks corresponding to addition of successive units of 440 ±2 Da to the parent protein, consistent with addition of the probe fragment **9**, could be observed (Fig. 4B). MALDI time-of-flight mass spectrometric analysis following tryptic digest[24] of the modified enzyme provided further insight into this covalent modification of *At*CXE12. Using a portion of the same sample used to prepare the gel described above (Fig. 3A lane 2) strong signals for both modified and unmodified peptide fragments, covering 50% of the protein sequence were observed, (Fig. 4D). These peptides incorporate the N-terminus of the protein, confirming the absence of methionine, a common feature for recombinant proteins expressed in *E. coli*.[25] This analysis revealed that the protein had been modified in multiple positions with these modifications being clustered into two distinct regions when overlaid onto the protein sequence (Fig. 5A, modified tryptic peptides underlined in blue). Although four of these tryptic peptides contain multiple potential sites for electrophilic modification, the *m/z* values suggest that these have only undergone a single modification. Whilst the fragment comprising residues (12–30) contains two serines and one cysteine MALDI time-of-flight MS/MS analysis indicated that, although labelling at the two Ser residues cannot be excluded, alkylation of the more nucleophilic thiol of Cys-25 was a more likely occurrence.[26] However, the observation of labelling of the other tryptic peptides which lack a Cys residue suggest that this is not a pre-requisite for covalent modification. In particular, MS/MS analysis of the tryptic fragment HADFSR (amino acid residues 166–171) containing a single nucleophilic serine residue revealed a modification of +440 Da consistent with capture of the quinone methide electrophile by the serine hydroxyl group. Using a homology model, built using MOE®,[27] derived from the plant carboxylesterase *Ae*CXE1 crystallised from kiwi fruit (*Actinidia eriantha*) with 33% sequence identity to *At*CXE12 (Fig. 5A grey boxes),[28] and aligning the putative active site residues which are highly conserved between the *At*CXE family (Fig. 5A green boxes),[29] revealed that the nucleophilic residues, lying in the regions underlined in blue were outside the predicted active site (Fig. 5A, 5B). To explore this, simple docking experiments were performed in MOE®. In this **4**, which had all hydrogens added and was energy minimized using Hamiltonian-Force Field-MMFF94x, was docked with the *At*CXE12 model using the default MOE settings (scoring function was London dG with a placement of Triangle matcher, 30 conformers of **4** were retained and rescored with Alpha HB) (Fig. 5C, 5D). Consistent with the observed labelling the results suggested that the probe targeting domain (acetate head group) was buried in the protein with the latent electrophilic carbon of the quinone methide being exposed to the relatively unstructured N-terminal region of the protein as indicated by the MS analysis of the tryptic digest.

Similar observations of multiple covalent modifications have previously been recorded for related quinone methide inhibitors.[16,17,30] Such multiple labelling suggests that release of either the quinone methide **8** or the unfragmented probe from the active site is occurring at a rate competitive with the labelling process.[31] Such behaviour would have significant implications for the identification of a specific protein from a complex mixture as found in a crude cell extract. In such a situation competition for the reactive probe might arise from either a non-specific binding protein or the competing reaction of an alternative nucleophile. Proteins undergoing such covalent labelling might be anticipated to bear reactive nucleophilic residues within their active site such as that found in the reactive cysteine dependent lambda class of glutathione transferases, a class of glutathione transferases (GST) involved in redox reactions such as thiol exchange.[32] In order to explore this possibility, mixtures of the lambda glutathione transferase isolated from wheat (*Ta*GSTL1),[33] bovine serum albumin (BSA), which is known to bind various drug like molecules in a non-specific fashion, and *At*CXE12, were mixed and, following incubation with **4** for 30 min, the protein fractions isolated and analysed by SDS-PAGE (Fig. 6). Importantly these three proteins have significantly different masses allowing easy identification in this analysis. In the absence of *At*CXE12 no fluorescence could be detected (lanes 6 and 7) indicating that activation of **4** is specific to esterase activity. However, although

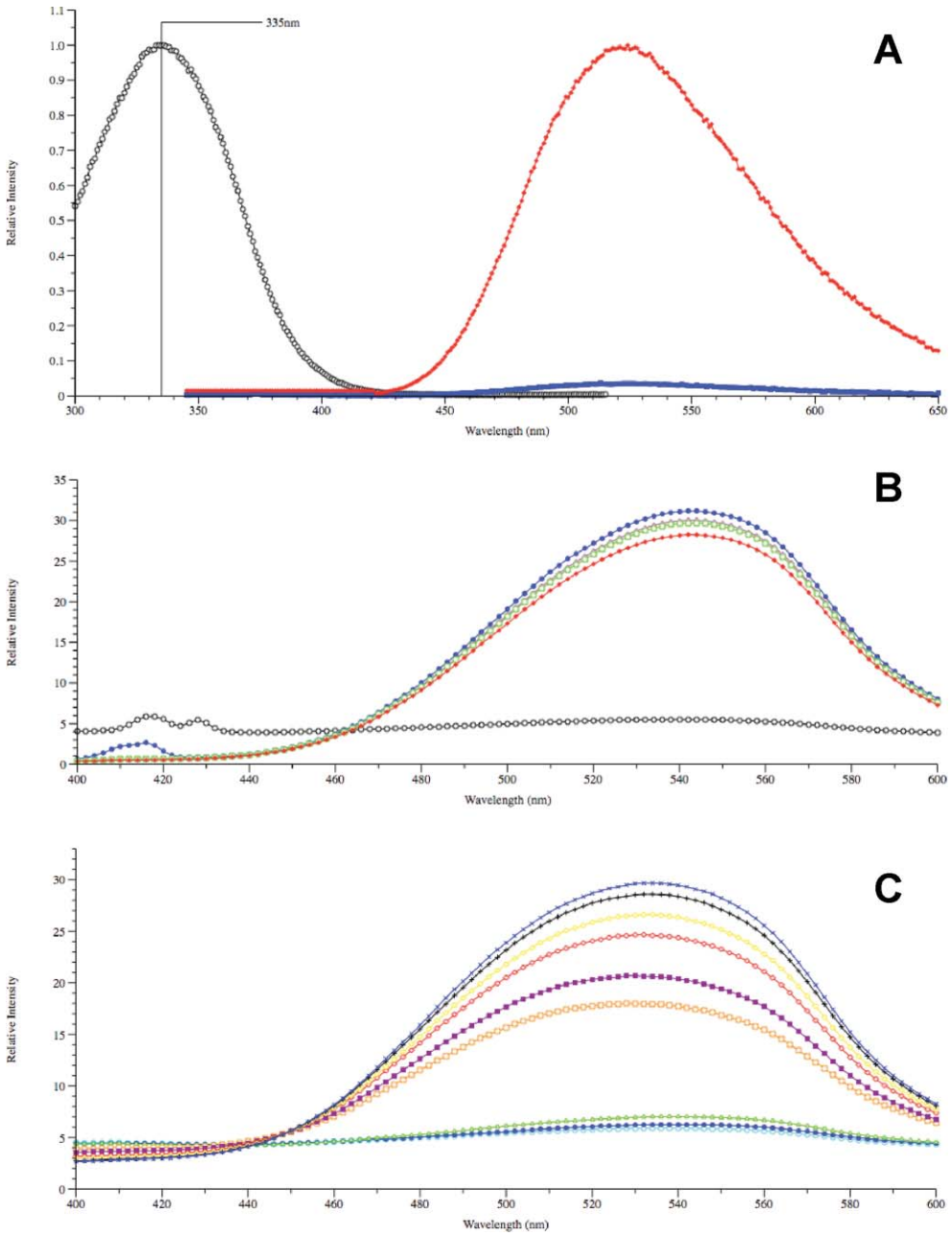


Fig. 2 Spectroscopic profiles: (**A**) Absorption (black) and emission (red) profile for the unquenched probe **2** and the absorption (black) and emission (blue) profile for the quenched probe **4**; (**B**) emission spectra for the hydrolysis of **4** with 1 M KOH (black – no base, blue – 30 s, pink – 5 min, green – 10 min, red – 30 min); (**C**) emission spectra for the incubation of **4** with *At*CXE12 at 37 °C (cyan – no *At*CXE12, blue – 10 min no *At*CXE12, green – 30 s, orange – 5 min, purple – 10 min, red – 30 min, yellow – 1 h, black – 2 h, blue – 3 h).

no labelling of BSA could be observed, when the reaction was undertaken in the presence of a combination of *Ta*GSTL1 and *At*CXE12 (lane 4) the band for *At*CXE12 exhibited considerably reduced fluorescence when compared with a sample of esterase enzyme alone, whilst the band for *Ta*GSTL1 was clearly labelled. Consistent with this observation ESMS analysis of the labelled band revealed that selective mono-labelling of the GST had occurred with a peak at 29266 Da (Fig. 7) corresponding to addition of one molecule of **9** to the parent protein. *Ta*GSTL1 contains a reactive cysteine (Cys-41) and the preferential reaction of such a residue reflects the greater nucleophilicity of the active site GST thiol residue when compared to the residues labelled in *At*CXE12. This selective labelling of *Ta*GSTL1 in preference to *At*CXE12 confirms that release of **8** or its immediate precursor into the solvent milieu from the esterase active site occurs more rapidly than labelling events. Reflecting this, although attempts to treat a crude plant extract with **4** resulted in the onset of fluorescence, SDS-PAGE analysis did not reveal any discretely labelled bands. This can be attributed to dispersion of the signal among several different protein targets or the preferential capture of the reactive electrophile by other cellular nucleophiles notably GSH through a classical xenobiotic response mechanism.[32]

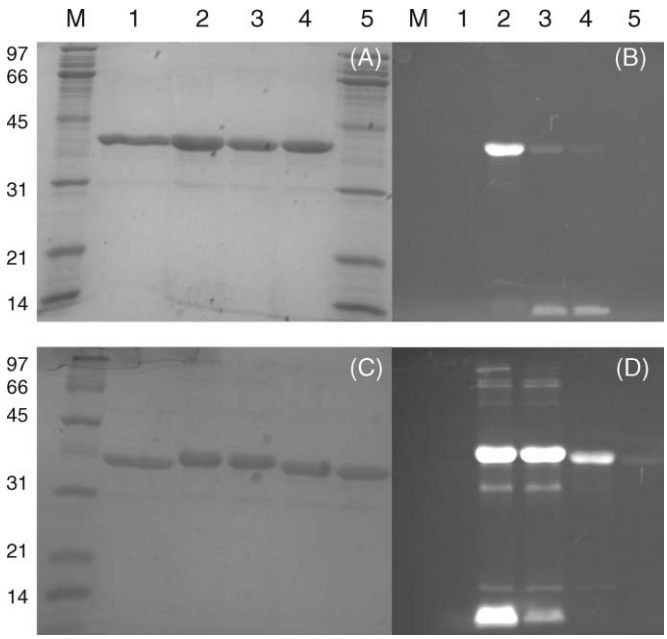


Fig. 3 SDS-PAGE analysis of *At*CXE12 following incubation with **4** and imaged with (A) Coomassie Blue or (B) fluorescence; M & 5. Low molecular weight marker (sizes in kDa as indicated); 1. *At*CXE12; 2. *At*CXE12 + **4**; 3. heat denatured *At*CXE12 + **4**; 4. paraoxon inhibited *At*CXE12 + **4**. SDS PAGE gel probing incubation of *At*CXE12 with varying concentrations of **4** visualised by staining with (C) Coomassie Blue or (D) fluorescence; M. low molecular weight marker (sizes in kDa as indicated); 1. *At*CXE12; 2. *At*CXE12 + **4** (10 mM); 3. *At*CXE12 + **4** (1 mM); 4. *At*CXE12 + **4** (0.1 mM); 5. *At*CXE12 + **4** (0.01 mM).

In conclusion, fluorophore tagged quinone methides continue to represent an attractive core to construct activity-based probes that can easily be modified with a variety of targeting groups. Moreover, the application of such probes can be considerably enhanced through the use of fluorescence quenching systems, coupled through a carbamate linker, which remove problems traditionally associated with background fluorescence of unreactive probe. However, the use of a carbamate linkage places limitations on the applications in proteome mining studies in that fragmentation and labelling may not be competitive with dissociation from the desired target protein resulting in multiple labelling events. Whilst multiple labelling is not necessarily a significant problem, in complex protein mixtures, loss of probe to the general media can result in labelling of multiple proteins and loss of signal or, more importantly, when a protein containing greater nucleophilic residues is present in the mixture, a decoupling of activity and labelling. In order to make the fluorescence quenched quinone methide activity based probe strategy viable it is necessary to utilise an effective nucleofuge that provides rapid fragmentation to generate the reactive electrophile before dissociation from the target enzyme can occur. In this respect, it is pertinent to note that even a number of early quinone methide based probes using fluoride as the leaving group demonstrated multiple labelling events,[17,30] suggesting that the use of even such simple quinone methide based probes in activity based protein profiling experiments must be undertaken with caution.

Experimental section

All air and/or moisture sensitive reactions were carried out under an argon atmosphere. Solvents were purified and dried following established protocols. Petrol refers to petroleum spirit boiling in the 40–60 °C range. Ether refers to diethyl ether. All commercially available reagents were used as received unless otherwise stated. Flash column chromatography was performed according to the method of Still *et al.* using 200–400 mesh silica gel.[34] Yields refer to isolated yields of products of greater than 95% purity as determined by ^{1}H + ^{13}C NMR spectroscopy. Melting points were determined using Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate) on a Perkin-Elmer FT-IR 1600 spectrometer. Unless otherwise stated ^{1}H NMR spectra were recorded in $CDCl_3$ on Varian Mercury-200, Varian VXR-400, Bruker Avance-400, Varian Inova-500, Varian VNMRS-700, and are reported as follows; chemical shift δ (ppm) (number of protons, multiplicity, coupling constant J (Hz), assignment). Residual protic solvent $CHCl_3$ ($\delta_H = 7.26$) was used as the internal reference. ^{13}C NMR spectra were recorded at 63 MHz or 126 MHz, using the central resonance of $CDCl_3$ ($\delta_C =$ 77.0 ppm) as the internal reference. All ^{13}C spectra were proton decoupled. Assignment of spectra was carried out using DEPT, COSY, HSQC, HMBC and NOESY experiments. High resolution accurate mass measurement was performed on a LTQFT mass spectrometer (ThermoFinnigan Corporation) using flow-injection electrospray ionisation. Intact proteins were analysed on a Q-ToF Premier mass spectrometer (Waters Corporation). A MassPrep desalting cartridge (Waters Corporation) was used to clean up the proteins, eluting at 70% MeCN containing 0.1% formic acid, prior to electrospray ionisation. The charge state distribution was deconvoluted to give the neutral mass of the analyte molecule using MaxEnt1 algorithm as part of MassLynx data processing software (Waters Corporation). Products of the tryptic digest were analysed on an Autoflex II ToF/ToF mass spectrometer (Bruker Daltonik GmBH) using MALDI ionisation. Here, 5 μg μl^{-1} of digested material dissolved in 50% MeCN and 50% aqueous solution containing 0.1% TFA was mixed 9 : 1 with matrix and spotted onto the MALDI target. The matrix used was 50 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 30% MeCN and 70% aqueous solution containing 0.1% TFA. All spectrofluorometric assays were performed on a JASCO FP-6200 fluorescence spectrometer.

Synthesis of probe 4

Methyl 2-(4-hydroxyphenyl)-2-methoxyethanoate. A solution of 4-hydroxymandelic acid **1** (5.0 g, 26.8 mmol) and concentrated sulfuric acid (0.5 ml) in anhydrous methanol (50 ml) was heated under reflux for 3 h. The reaction was then cooled to room temperature and quenched with sat. aq. $NaHCO_3$ (20 ml). The methanol was then removed under reduced pressure and the aqueous phase extracted with DCM (3 × 30 ml). The organic layers were combined, dried over $MgSO_4$, filtered, concentrated and dried *in vacuo* to afford the title ester as a white solid (3.8 g, 72%); R_f 0.6 (DCM–MeOH 9 : 1); m.p. 104–105 °C; ν_{max} (ATR) 3432–3186 (broad-OH), 1739 (C=O), 1596, 1513, 1446, 1265, 1224, 1192, 1087, 971, 907, 839, 815, 764 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 3.37 (3H, s, OCH_3), 3.72 (3H, s, CO_2CH_3), 4.71 (1H, s, ArC*H*), 5.60

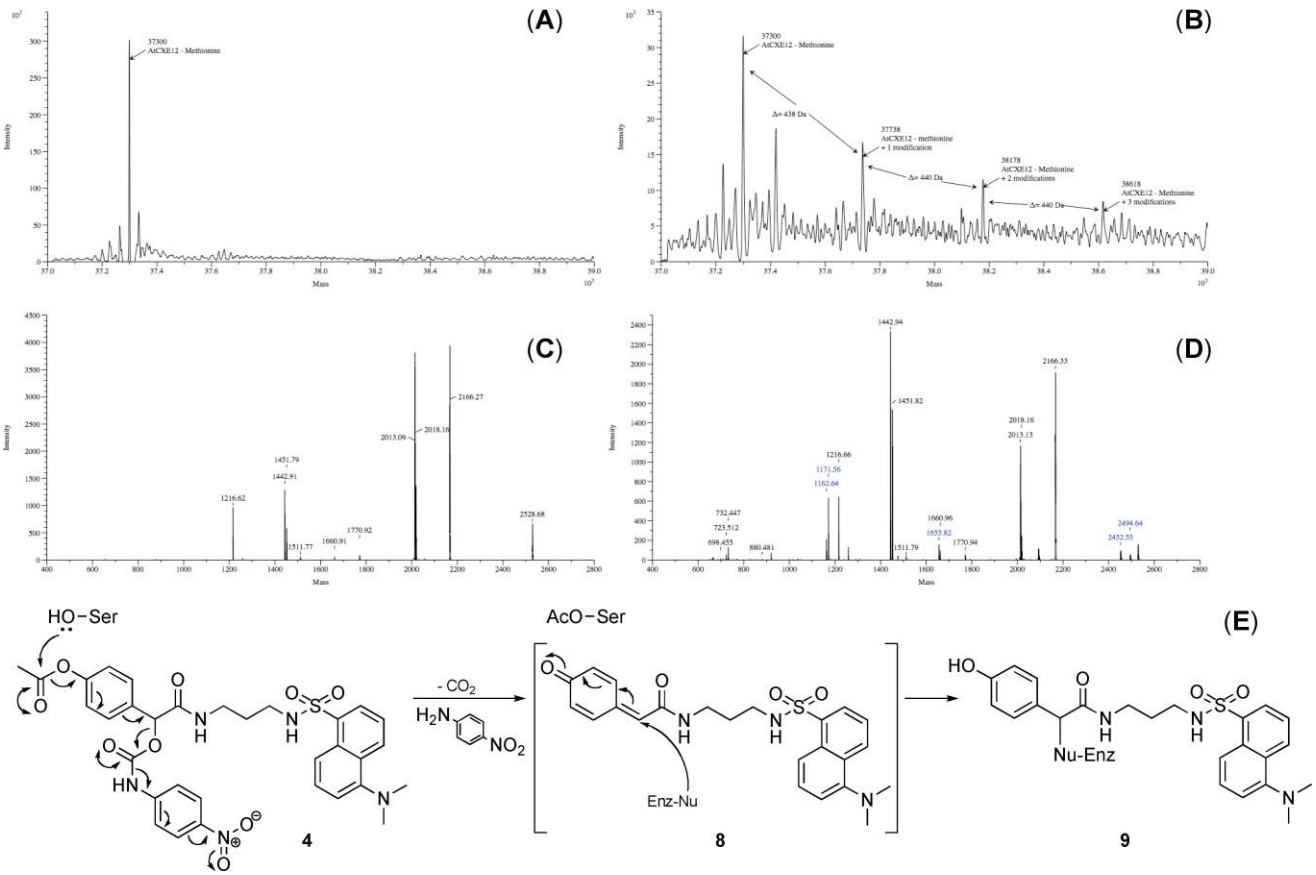


Fig. 4 (**A**) De-convoluted ES mass spectrum reporting the neutral mass of intact pure recombinant *At*CXE12; (**B**) de-convoluted ES mass spectrum reporting the neutral mass *At*CXE12 covalently modified with **9**; (**C**) MALDI MS analysis of unmodified *At*CXE12 following tryptic digest; (**D**). MALDI MS analysis of covalently modified *At*CXE12 following incubation with **4** and subsequent tryptic digest; (**E**). mechanism for the turnover of **4** by *At*CXE12 leading to quinone methide **8** and finally quenching with a nucleophile to give rise to covalently modified enzyme **9**.

(1H, br s, O*H*), 6.81 (2H, d, *J* 8, Ar-*H*), 7.30 (2H, d, *J* 8, Ar-*H*); δ_C (100 MHz, $CDCl_3$) 52.4 (O*C*H$_3$), 57.0 (CO_2CH$_3$), 82.1 (Ar*C*H), 115.6 (Ar-*C*), 127.8 (*ipso*-Ar-*C*), 128.8 (Ar-*C*), 156.5 (*ipso*-Ar-*C*), 171.7 (*C*=O); *m*/*z* (ES$^+$) 219 (MNa^+), 415 ($2MNa^+$); HRMS (ES$^+$) Found MNa^+, 219.06238 ($C_{10}H_{12}O_4Na$ requires 219.06278); Elemental Analysis [Found C, 61.16%; H, 6.08%; required for $C_{10}H_{12}O_4$ C, 61.22%; H, 6.16%].

***N*-(3-Aminopropyl)-2-(4-hydroxyphenyl)-2-methoxyethanamide.** A solution of *methyl 2-(4-hydroxyphenyl)-2-methoxyethanoate*, (1.2 g, 6.1 mmol) in 1,3-diaminopropane (4 ml) was stirred at room temperature for 28 h. The excess 1,3-diaminopropane was then removed *via* Kugelrohr distillation to give a brown solid which was washed with hot EtOAc to afford the title amide as a white powder (1.2 g, 84%); R_f 0.1 (DCM–MeOH 9 : 1); m.p. 154–156 °C; ν_{max} (ATR) 3364 (NH_2), 3340 (NH_2), 3108–2804 (broad-OH, NH), 1622 (C=O), 1564, 1542, 1484, 1434, 1379, 1340, 1310, 1266, 1152, 1046, 980, 817, 770 cm^{-1}; δ_H (400 MHz, D_2O) 1.78 (2H, quint, *J* 7, $CH_2CH_2CH_2$), 2.77 (2H, t, *J* 7, CH_2NH_2), 3.30 (2H, t, *J* 7, CH_2NH), 3.37 (3H, s, OC*H*$_3$), 4.63 (1H, s, ArC*H*), 6.70 (2H, d, *J* 8, Ar-*H*), 7.16 (2H, d, *J* 8, Ar-*H*); δ_C NMR (126 MHz, $CDCl_3$) 28.2 ($CH_2CH_2CH_2$), 36.1 (CH_2NH), 37.3 (CH_2NH_2), 56.8 (O*C*H$_3$), 83.3 (Ar*C*H), 118.1 (Ar-*C*), 124.0 (*ipso*-Ar-*C*), 129.2 (Ar-*C*), 163.9 (*ipso*-Ar-*C*), 174.4 (*C*=O); *m*/*z* (ES$^+$) 239 (MH^+), 261 (MNa^+), 277 (MK^+); HRMS (ES$^+$) Found MH^+, 239.13895 ($C_{12}H_{19}O_3N_2$ requires 239.13902).

***N*-(3-(5-(Dimethylamino)naphthalene-1-sulfonamido)propyl)-2-(4-hydroxyphenyl)-2-methoxyethanamide.** Triethylamine (320 µl, 1.2 mmol) and dansyl chloride (311 mg, 1.2 mmol) in THF (5 ml) were added to a suspension of *N-(3-aminopropyl)-2-(4-hydroxyphenyl)-2-methoxyethanamide*, (250 mg, 1.1 mmol) in THF (20 ml) which had been cooled to −78 °C. The resulting yellow suspension was allowed to warm gradually to room temperature and then stirred for 18 h. The reaction was quenched with sat. aq. NH_4Cl (20 ml) and extracted with EtOAc (2 × 20 ml). The organic layers were combined, dried over $MgSO_4$, filtered, concentrated and dried *in vacuo*. Flash chromatography (n-hexane–EtOAc 1 : 1) afforded the title sulfonamide as a green oil (402 mg, 85%); R_f 0.5 (EtOAc); ν_{max} (ATR) 3424–3112 (broad-OH, NH), 2942, 2874, 2830, 2792, 1654 (C=O), 1652, 1590, 1536, 1516, 1454, 1311 (SO_2), 1226, 1139 (SO_2), 1091, 910, 787, 726, 620 cm^{-1}; δ_H (400 MHz, $CDCl_3$) 1.48 (2H, quint, *J* 6, $CH_2CH_2CH_2$), 2.76 (2H, t, *J* 6, CH_2NHSO_2), 2.87 (6H, s, N(C*H*$_3$)$_2$), 3.15 (2H, t, *J* 6, CH_2NHCO), 3.21 (3H, s, OC*H*$_3$), 4.44 (1H, s, ArC*H*), 5.86 (1H, t, *J* 6, N*H*), 6.54 (2H, d, *J* 8, Ar-*H*), 7.07 (2H, d, *J* 8, Ar-*H*), 7.13 (1H, d, *J* 8, Ar-*H*), 7.29 (1H, t, *J* 6, N*H*), 7.46 (2H, m, Ar-*H*), 8.06 (1H, d, *J* 8, Ar-*H*), 8.20 (1H, d, *J* 8, Ar-*H*), 8.44 (1H, d, *J* 8, Ar-*H*), 8.83 (1H, br s, O*H*); δ_C NMR (126 MHz, $CDCl_3$) 29.6 (CH_2), 35.6 (CH_2),

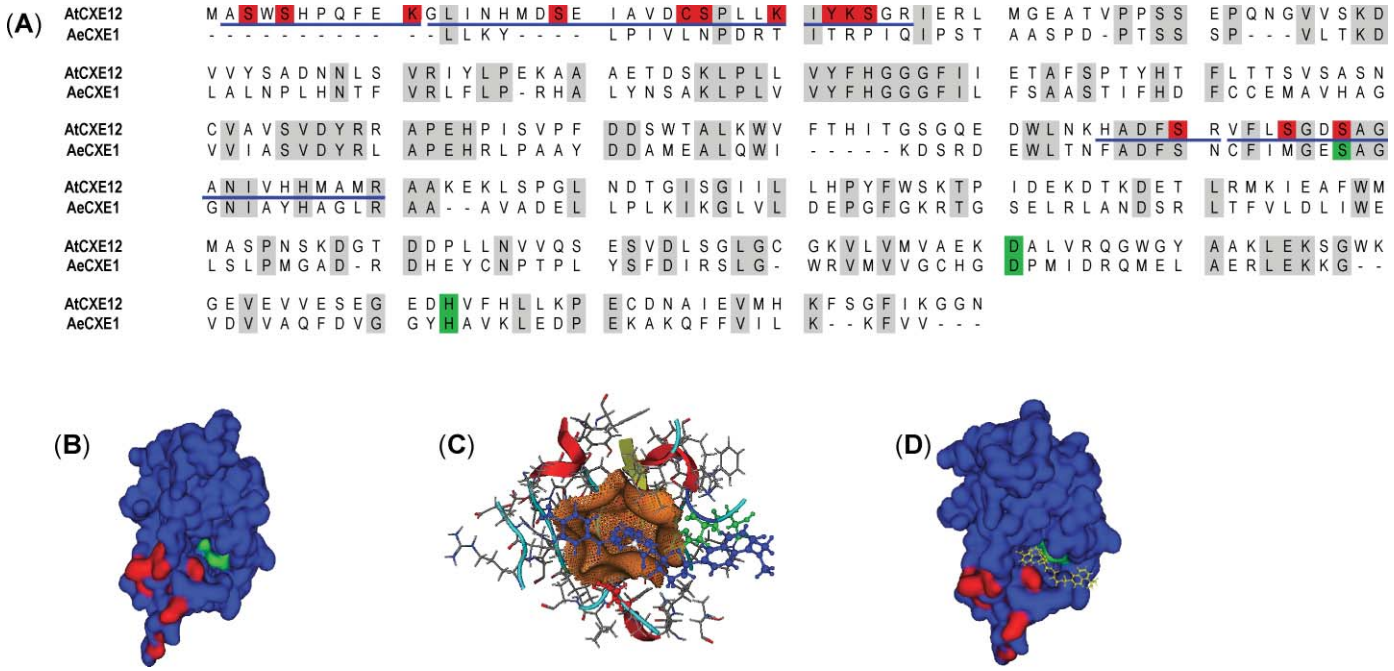


Fig. 5 (**A**) Protein sequences of *At*CXE12 and *Ae*CXE1 showing conserved residues (grey); active site residues (green), sites of potential covalent modification (red); and peptides modified by probe as identified by MALDI analysis following tryptic digestion (blue underlining); (**B**) *At*CXE12 showing Gaussian surface contacts with active site residues (green) and potential modified sites (red); (**C**) docked conformation of **4** (blue) in the putative active site of *At*CXE12 utilising MOE-Dock®; **(D)** docked conformation of **4** (orange) in the putative active site of *At*CXE12 utilising MOE-Dock® showing Gaussian contacts for the active site residues (green) and potential modified sites (red).

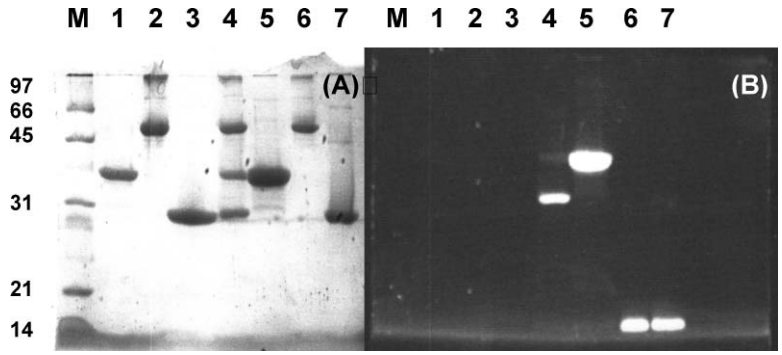


Fig. 6 SDS-PAGE gel analysis of *At*CXE12, BSA and *Ta*GSTL1 following incubation with **4**. M. Molecular weight markers (sizes in kDa as shown); 1. pure *At*CXE12; 2. BSA; 3. *Ta*GSTL1; 4. *At*CXE12, BSA, *Ta*GSTL1 + **4**; 5. *At*CXE12 + **4**; 6. BSA + **4**; 7. *Ta*GSTL1 + **4**. (**A**) Gel visualised by protein staining; (**B**) probe labelling visualised by fluorescence.

39.9 (CH_2), 45.4 (N(CH_3)$_2$), 56.5 (OCH_3), 83.0 (Ar-*C*H), 115.3 (Ar-*C*), 115.6 (Ar-*C*), 119.1 (*ipso*-Ar-*C*), 122.1 (Ar-*C*), 123.2 (Ar-*C*), 127.5 (Ar-*C*), 128.4 (Ar-*C*), 128.5 (Ar-*C*), 129.2 (Ar-*C*), 129.5 (Ar-*C*), 135.1 (*ipso*-Ar-*C*), 151.6 (*ipso*-Ar-*C*), 156.8 (*ipso*-Ar-*C*), 172.6 (*C*=O); *m/z* (ES$^+$) 494 (MNa$^+$); HRMS (ES$^+$) Found MNa$^+$, 494.17159 ($C_{24}H_{29}O_5N_3Na^{32}S$ requires 494.17201).

***N*-(3-(5-(Dimethylamino)naphthalene-1-sulfonamido)propyl)-2-hydroxy-2-(4-hydroxyphenyl)ethanamide, 2.** A solution of *N-(3-(5-(dimethylamino)naphthalene-1-sulfonamido)propyl)-2-(4-hydroxyphenyl)-2-methoxyethanamide*, (2.0 g, 4.2 mmol) in aq. hydrochloric acid (1 M, 50 ml) was heated under reflux for 4 h. The reaction mixture was then cooled to room temperature, quenched with aq. $NaHCO_3$ and extracted with EtOAc (3 × 50 ml). The organic layers were combined, dried over $MgSO_4$, filtered, concentrated and dried *in vacuo*. Flash chromatography (EtOAc) afforded **2** as a yellow solid (1.4 g, 73%); R_f 0.4 (EtOAc–MeOH 95 : 5); m.p. 90–91 °C; ν_{max} (ATR) 3464–3042 (broad-OH, NH), 1650 (C=O), 1618, 1586, 1506, 1458, 1402, 1307 (SO_2), 1234, 1204, 1138 (SO_2), 1064, 832, 788, 621 cm^{-1}; δ_H (500 MHz, $(CD_3)_2CO$) 1.54 (2H, quint, *J* 6, $CH_2CH_2CH_2$), 2.86 (8H, m, CH_2NHCO + N(CH_3)$_2$), 3.19 (2H, dt, *J* 6, 3, CH_2NHSO_2), 4.90 (1H, s, ArC*H*), 5.07 (1H, s, O*H*), 6.74 (2H, d, *J* 8, Ar-*H*), 6.92 (1H, t, *J* 6, N*H*CO), 7.19 (2H, d, *J* 8, Ar-*H*), 7.24 (1H, d, *J* 7, Ar-*H*), 7.46 (1H, br t, *J* 3, N*H*SO_2), 7.59 (2H, m, Ar-*H*), 8.17 (1H, d, *J* 8, Ar-*H*), 8.34 (1H, s, ArO*H*), 8.38 (1H, d, *J* 9, Ar-*H*), 8.44 (1H, d, *J* 9, Ar-*H*); δ_C (126 MHz, $(CD_3)_2CO$) 28.6 ($CH_2CH_2CH_2$), 35.6 (CH_2NHCO), 40.1 (CH_2NHSO_2),

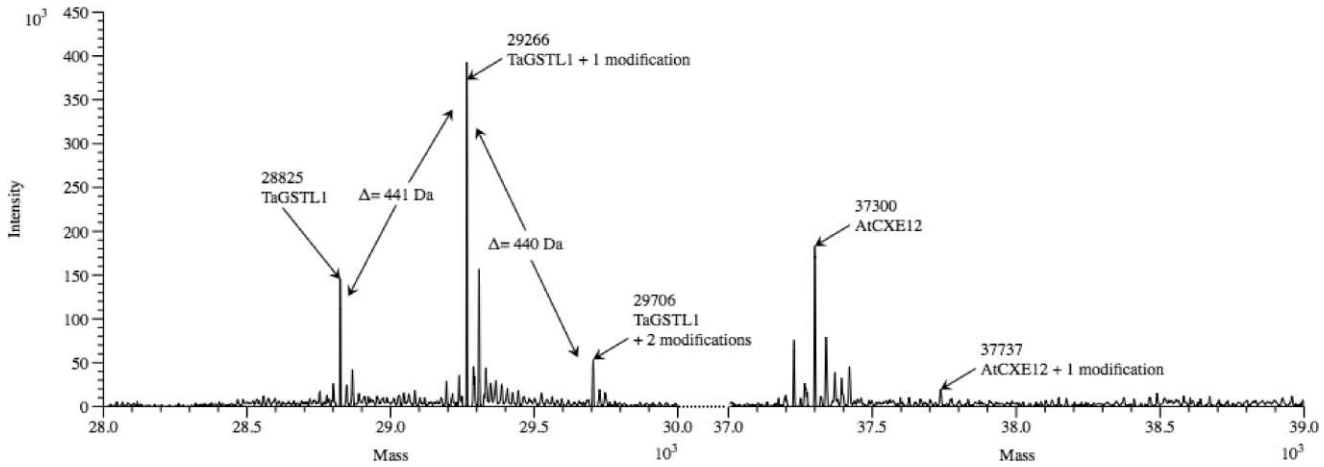


Fig. 7 De-convoluted ES mass spectrometric analysis reporting the neutral masses of a mixture of *At*CXE12, *Ta*GSTL1 and **4**. No significant peaks were observed outside the mass ranges shown.

45.0 (N(CH_3)$_2$), 73.8 (ArCH), 115.1 (Ar-C), 115.5 (Ar-C), 119.7 (Ar-C), 123.6 (Ar-C), 128.13 (Ar-C), 128.10 (Ar-C), 128.9 (Ar-C), 129.9 (Ar-C), 130.1 (*ipso*-Ar-C), 132.3 (*ipso*-Ar-C), 136.8 (*ipso*-Ar-C), 152.1 (*ipso*-Ar-C), 157.1 (*ipso*-Ar-C), 173.4 (C=O); m/z (ES$^+$) 458 (MH$^+$), 480 (MNa$^+$), 915 (2MH$^+$), 937 (2MNa$^+$); HRMS (ES$^+$) Found MH$^+$, 458.17456 ($C_{23}H_{28}O_5N_3{}^{32}S$ requires 458.17442).

4-(2-(3-(5-(Dimethylamino)naphthalene-1-sulfonamido)propylamino)-1-hydroxy-2-oxoethyl)phenyl ethanoate, 3. *N*,*N*′-Diisopropylethylamine (76 µl, 0.4 mmol) followed by acetyl chloride (31 µl, 0.4 mmol) were added to a solution of *N*-(*3*-(*5*-(*dimethylamino*)*naphthalene-1-sulfonamido*)*propyl*)-*2-hydroxy-2*-(*4-hydroxyphenyl*)*ethanamide*, **2** (200 mg, 0.4 mmol) in dry acetonitrile (5 ml) which had been cooled in an ice bath. The resulting yellow reaction mixture was allowed to stir in the ice-bath for 1 h. The solvent was then removed under reduced pressure and flash chromatography (DCM–EtOAc 1 : 1) afforded **3** as a yellow oil (124 mg, 57%). R_f 0.4 (DCM–EtOAc 1 : 1); ν_{max} (thin film) 3270–3432 (broad-NH), 1806, 1773 (C=O), 1650 (C=O), 1604, 1554, 1484, 1376 (SO_2), 1206, 1166, 1104 (SO_2), 950, 864, 786, 670, 632 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 1.48 (2H, quint, J 6, $CH_2CH_2CH_2$), 2.29 (3H, s, CH_3CO), 2.79 (2H, q, J 6, CH_2NHSO_2), 2.87 (6H, s, N(CH_3)$_2$), 3.20 (2H, m, CH_2NHCO), 4.05 (1H, d, J 3, OH), 4.86 (1H, d, J 3, ArCH), 5.76 (1H, t, J 6, NHSO$_2$), 6.74 (1H, t, J 6, NHCO), 6.98 (2H, d, J 9, Ar-H), 7.16 (1H, d, J 8, Ar-H), 7.30 (2H, d, J 9, Ar-H), 7.50 (2H, m, Ar-H), 8.14 (1H, d, J 8, Ar-H), 8.27 (1H, d, J 9, Ar-H), 8.53 (1H, d, J 9, Ar-H); δ_C (126 MHz, $CDCl_3$) 21.3 (CH_3CO), 29.6 ($CH_2CH_2CH_2$), 36.1 (CH_2NHCO), 40.5 (CH_2NHSO_2), 45.5 (N(CH_3)$_2$), 73.4 (ArCH), 115.4 (Ar-C), 119.0 (Ar-C), 122.0 (Ar-C), 123.3 (Ar-C), 127.9 (Ar-C), 128.5 (Ar-C), 129.3 (Ar-C), 129.6 (*ipso*-Ar-C), 130.0 (*ipso*-Ar-C), 130.4 (*ipso*-Ar-C), 135.1 (*ipso*-Ar-C), 137.2 (*ipso*-Ar-C), 150.7 (*ipso*-Ar-C), 152.0 (*ipso*-Ar-C), 169.8 (CH_3C=O), 173.1 (NHC=O); m/z (ES$^+$) 500 (MH$^+$), 522 (MNa$^+$), 1021 (2MNa$^+$); HRMS (ES$^+$) Found MNa$^+$, 522.16793 ($C_{25}H_{29}O_6N_3Na^{32}S$ requires 522.16693).

4-(2-(3-(5-(Dimethylamino)naphthalene-1-sulfonamido)propylamino)-1-(4-nitrophenylcarbamoyloxy)-2-oxoethyl)phenyl ethanoate, 4. 4-Nitrophenyl isocyanate (12 mg, 0.07 mmol) and triethylamine (cat) were added to a yellow solution of *4*-(*2*-(*3*-(*5*-(*dimethylamino*)*naphthalene-1-sulfonamido*)*propylamino*)-*1-hydroxy-2-oxoethyl*)*phenyl ethanoate*, **3** (40 mg, 0.08 mmol) in chloroform (2 ml). The reaction mixture was stirred at room temperature for 18 h, then washed with sat. aq. NH_4Cl (1 ml), dried and concentrated under reduced pressure. Flash chromatography (DCM, DCM–MeOH 98 : 2) afforded **4** as a yellow oil (21 mg, 45%); R_f 0.2 (CH_2Cl_2); ν_{max} (ATR) 3432–3156 (broad-NH), 2952, 2940, 2860, 1741 (C=O), 1663 (C=O), 1596, 1552, 1505, 1411, 1329 (SO_2), 1304, 1195 (SO_2), 1140, 1047, 850, 788, 749, 622 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 1.66 (2H, quint, J 5, $CH_2CH_2CH_2$), 2.29 (3H, s, CH_3CO), 2.90 (6H, s, N(CH_3)$_2$), 2.97 (2H, q, J 5, CH_2NHSO_2), 3.46 (2H, m, CH_2NHCO), 5.65 (1H, t, J 5, NHSO$_2$), 6.10 (1H, s, ArCH), 7.07 (2H, d, J 8, Ar-H), 7.20 (1H, d, J 8, Ar-H), 7.51 (6H, m, Ar-H), 7.73 (1H, t, J 5, NHCO), 8.05 (2H, d, J 8, Ar-H), 8.18 (1H, d, J 8, Ar-H), 8.22 (1H, d, J 9, Ar-H), 8.58 (1H, d, J 9, Ar-H), 8.60 (1H, s, NHCOO); δ_C (126 MHz, $CDCl_3$) 21.3 (CH_3CO), 28.5 ($CH_2CH_2CH_2$), 37.4 (CH_2NHSO_2), 42.1 (CH_2NHCO), 45.5 (N(CH_3)$_2$), 75.9 (ArCH), 115.6 (Ar-C), 118.0 (Ar-C), 122.1 (Ar-C), 123.3 (Ar-C), 125.1 (Ar-C), 128.7 (Ar-C), 128.7 (Ar-C), 129.0 (Ar-C), 129.4 (Ar-C), 129.5 (*ipso*-Ar-C), 130.1 (*ipso*-Ar-C), 131.3 (*ipso*-Ar-C), 133.5 (Ar-C), 133.7 (*ipso*-Ar-C), 143.1 (Ar-C), 144.1 (Ar-C), 151.1 (Ar-C), 151.2 (Ar-C), 168.5 (C=O), 168.5 (C=O), 169.6 (NHC=O); m/z (ES$^+$) 664 (MH$^+$), 686 (MNa$^+$); HRMS (ES$^+$) Found MH$^+$, 664.20734 ($C_{32}H_{34}O_9N_5{}^{32}S$ requires 664.20718).

Biological experimental procedures

Cloning, transformation and expression of *At*CXE12 and TaGSTL1. *At*CXE12[2] was sub-cloned into the pET-STRP3 vector[25] for expression with an N-terminal *Strep* tag and expressed in *E. coli* Rosetta2(DE3) under standard conditions (1 mM IPTG, 12 h, 37 °C). TaGSTL1 (Cla 30)[33] was amplified from wheat cDNA, cloned into pET-STRP3 and expressed in *E. coli* Tuner(DE3) containing the pRARE plasmid (Novagen) from log phase cultures with 0.2 mM IPTG for 4 h at 30 °C. In both cases cells were lysed by sonication in HBS buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.6) and the clarified supernatant was then purified by *Strep*-tactin affinity chromatography.[25]

Spectrophotometric assays

Assay utilising 1 M KOH. Tris buffer (0.1 M, 1 ml, pH 7.4) was treated with 10 µl **4** (10 mM in acetone) and vortexed, then diluted to 3 ml with buffer. The background fluorescence was then observed (excitation: 333 nm; emission 400–600 nm). Subsequent addition of 400 µl of 1 M KOH and monitoring of the emission profile at room temperature over 30 min revealed a sharp increase in fluorescence.

Assay utilising recombinant *At*CXE12. Tris buffer (0.1 M, 1 ml, pH 7.4) was treated with 10 µl **4** (10 mM in acetone) and vortexed, then diluted to 3 ml with buffer. The background fluorescence was then monitored (excitation: 333 nm; emission 400–600 nm). Incubation of the sample at 37 °C for 10 min and subsequent measurement revealed no increase in background fluorescence due to hydrolysis in the buffer. As a result, 50 µl of *At*CXE12 in STRP buffer was added, the reaction incubated at 37 °C and the fluorescence measured at 5, 10, 30, 60, 120, 180 min.

Electrophoretic analysis of probe reactivity. *At*CXE12 (50 µl, 0.86 mg ml^{-1}), **4** (10 µl, 0.1 mM in acetone) and Tris buffer (0.1 M, 10 µl, pH 7.4) were incubated for 1.5 h at 37 °C. When denatured enzyme was employed, 50 µl of *At*CXE12 was pre-heated at 90 °C for 15 min prior to addition of **4** and when inhibited enzyme was used, paraoxon (10 µl, 10 mM in acetone) was pre-incubated with *At*CXE12 prior to addition of **4**. To investigate the dependence of labelling on probe concentration, *At*CXE12 (50 µl, 0.86 mg ml^{-1}), varying concentrations of **4** (10 µl of 10 mM, 1 mM, 0.1 mM and 0.01 mM) and Tris buffer (0.1 M, 10 µl, pH 7.4) were incubated for 1.5 h at 37 °C. For investigating probe reactivity in protein mixtures, *At*CXE12 (100 µl, 0.86 mg ml^{-1}), *Ta*GSTL1 (5 µl, 3 mg ml^{-1}), BSA (5 µl, 1 mg ml^{-1}), **4** (10 µl, 0.1 mM) and Tris buffer (0.1 M, 10 µl, pH 7.4) were incubated for 30 min at 37 °C. Where necessary, samples were concentrated by precipitation with 4 vol acetone and resuspended in a small volume of buffer. Samples were analysed by SDS-PAGE using 12% acrylamide gels, after addition of 1 vol 2 × SDS-PAGE loading buffer and denaturation at 90 °C for 10 min. Gels were visualized by fluorescence on a Bio-Rad Molecular Imager Gel Doc XR system, and for protein content by staining with colloidal Coomassie Brilliant Blue G250.

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