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Evaluation of Sulfatase-Directed Quinone Methide Traps for Proteomics

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Abstract

Sulfatases hydrolytically cleave sulfate esters through a unique catalytic aldehyde, which is introduced by a posttranslational oxidation. To profile active sulfatases in health and disease, activity-based proteomic tools are needed. Herein, quinone methide (QM) traps directed against sulfatases are evaluated as activity-based proteomic probes (ABPPs). Starting from a *p*-fluoromethylphenyl sulfate scaffold, enzymatically generated QM traps can inactivate bacterial aryl sulfatases from *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and human steroid sulfatase. However, multiple enzyme-generated QMs form, diffuse, and non-specifically label purified enzyme. In complex proteomes, QM labelling is sulfatase-dependent but also non-specific. Thus, fluoromethylphenyl sulfates are poor ABPPs for sulfatases.

Keywords

Quinone methide; Enzyme inhibitor; Suicide inhibitor; Sulfatase probe; Activity-based proteomics

1. Introduction

The goal of activity-based proteomics (ABP) is to isolate and characterize specific enzyme classes within the context of the proteome. ABP targets active enzyme classes by using activity-based proteomic probes (ABPPs), which are most commonly equipped with broadly active mechanism-based inhibitors that can elicit turnover-dependent capture. In doing so, important information on catalytically active enzyme members is provided by ABPPs. Powerful ABPPs are available for several types of enzymes, including cysteine proteases and serine hydrolases; however, many other enzyme classes await the development of ABP tools.¹

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Supplementary Material

Supporting information is available online.

The biochemical characterization of sulfatases would benefit from ABP tools. Type I sulfatases (EC 3.1.5.6) are a large class of enzymes found in prokaryotes and eukaryotes that cleave sulfate esters by a unique hydrolytic mechanism dependent on the catalytic aldehyde, formylglycine (FGly).² FGly is installed post-translationally in eukaryotic sulfatases from encoded cysteine residues by formylglycine generating enzyme (FGE also known as SUMF1); while, in prokaryotes FGly can originate from either cysteine or serine residues by SUMF1 (activates Cys-type sulfatases), AtsB (activates Ser-type sulfatases), and potentially other activating enzymes.^{2–4} Because sulfatases require activation, protein abundance does not necessarily reflect biological activity. Multiple sulfatase deficiency (MSD) is a fatal human genetic disorder characterized by normal sulfatase protein production but inadequate FGly activation by FGE.² To date, eight different human disorders are known to be caused by the deficiency of single sulfatase genes.⁵ The human aryl sulfatases (ARS) D-K have been identified, but have unknown substrates and biological functions.^{2,3} Furthermore, sulfatases are increasingly found in pathological settings such as bacterial infection, cancer, and inflammation.^{2,6} ABP tools can help to better define the roles of active sulfatases in these processes.

We have been interested in developing ABP tools for the selective detection and isolation of sulfatases within complex mixtures using quinone methide (QM) traps (Fig. 1).⁷ QM traps are considered promising general ABPs for hydrolytic enzymes.^{8,9} The QM trap concept involves *in situ* generation of a reactive QM intermediate that is dependent on enzymatic turnover of a suicide inhibitor. Typically, QMs are the product of hydrolytically liberated fluoromethylphenolates, which then spontaneously fragment by fluoride elimination. Importantly, the enzymatically-generated QM features an electrophilic carbon atom poised to covalently capture a nucleophile via Michaeladdition. In an ideal ABPP, the QM will capture a properly disposed active site residue conserved in the targeted enzyme class, resulting in turnover-dependent inactivation and specific protein labelling. Herein, we evaluate the utility of sulfatase-directed QM traps as ABPPs for sulfatases. As shown in Figure 1, our QM traps are based on fluoromethylphenyl sulfate substrates. By containing the masked fluoromethyl within the minimal phenyl sulfate motif, these traps are designed to have broad-ranged reactivity against sulfatases, which are known to be generally tolerant of small aryl sulfate substrates.²

2. Results and Discussion

A good ABPP must be able to specifically label a targeted enzyme class within a complex proteome. To develop ABPPs for sulfatases, we designed sulfatase-directed QM probes **1–4** and evaluated their ability to irreversibly inhibit and label sulfatases. Turnover-dependent capture of active site residues by irreversible inhibitors (known as mechanism-based inhibition, irreversible inhibition, or suicide inactivation) shuts down catalysis and is biochemically characterized by time- and concentration-dependent inhibition.¹⁰ Early work with QM traps based on difluoromethylphenyl sulfates (DFPS **1** and **2**, Fig. 1a) indicated that these compounds are not irreversible inhibitors of aryl sulfatases, as demonstrated with an aryl sulfatase from *Pseudomonas aeruginosa* (PARS⁷ and from *Klebsiella pneumoniae* (KARS) (see SI, Fig. S1). Although **1** and **2** are likely substrates for PARS and KARS, their utility as ABPPs is questionable, since the enzymatically-formed QM traps **5a** and **5b** do not effectively inactivate sulfatases. However, it is possible that QM adducts are formed by nucleophiles situated outside of the active site. For ABPPs, specific labelling of the target enzyme is paramount to active site inactivation. Thus, the scenario of a turnover-dependent QM being specifically captured outside of the active site is suitable in the context of a proteomics approach, provided it occurs before the QM diffuses out of the active site into a generally reactive space. As such, we next turned to creating a more reactive QM-trap for our sulfatase-directed ABPP.

We postulated that a more reactive sulfatase-directed QM trap would be afforded starting from a monofluoromethylphenyl sulfate (MFPS) scaffold (Fig. 1b). This scaffold offers several potential improvements to QM reactivity, specificity, and labelling over the masked difluoro analogs **1** and **2**. First, the monofluoro analogs cannot undergo side reactions that can complicate QM formation and inactivation kinetics.^{6b,11} Second, QMs generated from monofluoromethyl phenolates form and react faster than those generated from difluoromethyl phenolates.^{6b,12} Thus, MFPS containing structures like **3** and **4** should accelerate sulfatase capture, thereby increasing the likelihood of specificity, perhaps even labelling within the active site. Importantly, the MFPS probes still contain the key phenyl sulfate motif that should make them broadly accessible to the sulfatase enzyme class.² For a complete sulfatase-directed ABPP, the MFPS trapping scaffold was combined with a reporter group for detection in a proteomic mixture (e.g., fluorescein in **4**; see SI Scheme S1 for synthetic details).

To begin ABPP characterization, the broad-ranged biochemical activity of model MFPS probe **3** was tested against a panel of sulfatases. Four disparate sulfatase members were included: recombinantly expressed and purified Cys- and Sertype aryl sulfatases from bacteria (PARS⁷ and KARS¹³), as well as human aryl sulfatase G (ARSG¹⁴) and steroid sulfatase (STS¹⁵). Each has distinct features: the bacterial sulfatases are active against a broad range of aryl sulfate substrates under basic (pH of 8.9 for PARS) and neutral (pH 7.5 for KARS) conditions; STS is a membrane-bound sulfatase of the endoplasmic reticulum with neutral pH optimum, a high specificity for steroid sulfates, and a noted involvement in hormone-dependent cancers; and, ARSG is a newly discovered lysosomal enzyme, with acidic pH optimum and unknown biological substrate and function.^{2-4,6i,14} Sulfatase activity was determined by standard assay methods (see Experimental). Briefly, KARS, PARS and ARSG activities were monitored using a *para*-nitrocatechol sulfate (*p*NCS) colorimetric assay, and STS activity was determined using a radioactive dehydroepiandrosterone-3-sulfate (³DHEAS) assay.¹⁶ To test MFPS inhibition, enzymes were pre-incubated with model ABPP **3**, before being diluted into appropriate assay buffer to measure activity; final activities are reported as a percentage of the uninhibited control.

The dose-dependent inhibitory activity of MFPS probe **3** against the sulfatase panel at representative time points is shown in Figure 2. The inhibition potential was variable, with the strongest inactivation in shortest time periods being observed for the bacterial enzymes (i.e., roughly estimated 50 % inactivation of KARS and PARS after a 30 min pre-incubation with 0.8 mM and 5 mM **3** respectively), intermediate inactivation of STS (i.e., around 50 % after a 60 min pre-incubation at 15 mM **3**) and very little inactivation of ARSG. It is surprising that ARSG remained unaffected at all tested pre-incubation concentrations of **3** even after overnight incubation (see SI, Fig. S2), because the enzyme is known to accept aryl sulfate substrates, albeit with a preference for *ortho*-hydroxylated aryl sulfates.¹⁴ Possibly, the acidic pH conditions needed for ARSG activity affect QM trap formation (e.g., pH-dependent thiol additions to QM have been observed¹⁷). In addition to dose-dependence, KARS, PARS and STS also exhibited time-dependent inhibition, as exemplified for KARS in Figure 3a (also see SI, Fig. S2). Time-dependent inactivation indicates that the sulfatase active site can be shut down by the MFPS probes, an improvement over the original DFPS **1** and **2** probes that did not show any time-dependent inhibition (SI, Fig. S1).

To investigate if the time-dependent inactivation of sulfatases by MFPS trap **3** (as clearly shown for KARS in Fig 3a) is caused by specific labelling, the stoichiometry of QM activation and QM-adduct formation was next gauged by ¹⁹F-NMR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). As shown in Figure 3b, real-time formation of the QM trap **5c** is visible in ¹⁹F-NMR experiments: in the NMR-spectrum, probe **3** has a chemical shift of $\delta = -180$ ppm; while the free fluoride

signal at $\delta = -120$ ppm emerges with QM formation, that is, as sulfate is cleaved and fluoride is eliminated.^{8a,b} Using low activity MFPS probe concentrations with KARS (140 μ M **3** and 70 nM KARS), slow QM activation kinetics were monitored over two weeks. Fluoride release ceased after 12 h due to complete enzyme inactivation (notably, under the same conditions, untreated enzyme remained active and probe **3** did not decompose). At complete inactivation, the integral ratio of ¹⁹F-NMR signals between **3**-bound fluorine and liberated fluoride indicates that approximately 20 % of probe **3** was converted to QM. Thus, nearly 400 molecules of probe must be turned over on average by each enzyme molecule before it is inactivated, when considering the 2000-fold molar excess of probe **3** over KARS. Although the 400:1 MFPS **3** to KARS inactivation ratio is surprisingly better than those previously reported with other hydrolases,^{8a,b} such a high ratio is consistent with non-specific labelling. Indeed, after treatment with **3**KARS multiply labelled with up to 7 QM-adducts is apparent by MALDI-ToF MS (Fig. 3c). PARS shows similar QM activation by ¹⁹F-NMR (SI, Fig. S3) and an average of 5 QM-enzyme adducts by MALDI-ToF MS after incubation with **3** (Fig. 3d). Thus, the data in Figure 3 suggest that inactivation of purified sulfatases by MFPS probes is consistent with non-specific protein labelling.

As a final test for ABPP utility, the MFPS probe **4** was applied to complex proteomic mixtures. Probe activation and selectivity was assessed using three model proteomes containing, (i) an active sulfatase, (ii) an inactive sulfatase, or (iii) no sulfatase. These proteomic samples consisted of crude lysates from *E. coli* DH5 α cells which expressed (i) recombinant KARS together with its post-translational activating enzyme AtsB,¹³ (ii) recombinant KARS alone, which will remain inactive without AtsB, and (iii) no sulfatase (null vector control). Notably, *E. coli* does not express endogenous sulfatases and shows no endogenous KARS activation activity.¹³ Proteomes were labelled with 1 mM of the fluorescent probe **4** for 60 minutes, separated by 2D-PAGE, and visualized by fluorescence scanning and then Coomassie staining (Fig. 4).

As shown in Figure 4, fluorescent labelling by **4** is turnover-dependent. The development of fluorescent spots is significant only in the presence of active KARS (Fig. 4a) and not in control proteomes (inactive KARS in Fig. 4b; and without sulfatases in Fig. 4c). However, most of the Coomassie stained proteins in the active KARS proteome also show up in the fluorescence scan of the gel (Figure 4a, left versus right panel), indicating that the probe labels most of the proteins in the sample population. Non-specific labelling of enzymes from a wide range of protein classes, including kinases, dehydrogenases, transaldolases, and a methyl transferase, was confirmed by trypsination and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of fluorescent gel spots (see SI, Fig. S4 and Table S1). Adding small-molecule nucleophiles (e.g., ethanolamine) to trap diffused activated QM species also resulted in decreased labelling of KARS, suggesting that even labelling of the QM-generating enzyme is non-specific (SI, Fig. S5). These nonspecific labelling results in complex proteomes support that QMs generated in a sulfatase-dependent manner diffuse and react indiscriminately.

Our studies with QM-traps based on general masked monofluoromethylphenyl sulfates are consistent with sulfatase-generated QMs that quickly diffuse and indiscriminately label nucleophiles, making them poor ABPPs for sulfatases. During the course of our studies, efforts toward developing STS-directed QM-traps were put forth, which reinforce our findings.^{8e,f} Recombinantly over-expressed STS can be labelled with a biotinylated MFPS probe similar to **4** although non-specific labelling was apparent in crude mammalian lysates.^{8e} Apparently, STS can also evade QM-based inactivation by a masked difluorophenyl sulfate derivative of estrone sulfate (as with compounds **1** and **2** against PARS and KARS), whereas the corresponding monofluoromethyl analogue will irreversibly inactivate STS (as with **3** against PARS, KARS and STS).^{8f} Although the estrone sulfate-

masked QMs were not applied to proteomic endeavours, their corresponding STS-generated QMs showed diffusive behaviour and high QM to STS inactivation ratios.^{8f} It is interesting that the highly substrate-like scaffold of these estrone-based QM traps did not improve the specific labelling of STS. Specific capture of protein tyrosine phosphatases (PTPs) was recently achieved by making short phosphotyrosine peptide substrate analogues armed with a masked phospho-tyrosine QM.¹⁸ Although, flanking amino acid character has also been used to hone the specificity of protease-dependent quinole imine capture,⁹ the PTP example is the first to show specific capture of a quinone methide in the context of a complex proteome.¹⁸ Indeed, previous efforts using simple fluoromethylphenyl phosphates against PTPs resulted in a large degree of non-specific protein labelling,¹⁸ reflecting the nonspecific nature of many other hydrolytically generated QMs from masked fluoromethyl phenolates,⁸ including our own against sulfatases.

3. Conclusion

Sulfatases are key players in disease⁶ making ABPPs important for providing information about their catalytic integrity in the underlying pathobiochemical processes. As such, we examined sulfatase-directed QM traps based on masked fluoromethylphenyl sulfates as ABPPs for sulfatases. New sulfatase-directed probes based on MFPSs (**3** and **4**) were synthesized and found to have superior irreversible inactivation properties against aryl sulfatases in comparison to previously designed DFPSs (**1** and **2**).⁷ MFPS probes were active against aryl sulfatases operating at neutral and basic pH, including bacterial PARS and KARS and human STS; however, they were not active against the human lysosomal enzyme ARSG. Despite good inactivation and reasonable reactivity toward the enzyme class, further biochemical characterization of the MFPS probes with purified enzyme demonstrated that sulfatase inactivation occurs only after multiple QM traps are enzymatically generated. This leads to multiple enzyme labelling events, presumably through non-specific QM capture. The activity of MFPS probe **4** in complex proteomes confirmed that the sulfatase-directed QM traps required enzymatic activation, but lead to non-specific labelling of many different enzyme classes. Unfortunately, such behaviour does not meet the stringent requirements for an ABPP, which must be able to decisively report on the activity of a specific enzyme class by turnover-dependent labelling in the context of the greater proteome.^{1,9}

4. Experimental

4.1. Sulfatase Protein Expression

Literature procedures were followed for the preparation of: purified recombinant KARS (1.4 mg/mL in 50 mM Tris, pH 7.4, 250 mM NaCl, TBS);¹³ enriched KARS (0.7 mg/mL in TBS);¹⁹ purified recombinant PARS (3 mg/mL in 100 mM Tris, pH 7.5 with 20 % glycerol);⁷ and purified ARSG (17 µg/mL) from HT1080 ARSG-His cells.¹⁴ STS was expressed using a stable Tet-on HT1080 cell line.¹⁵ Cells were cultured in 10% fetal calf serum containing Dulbecco's modified Eagle's medium (Gibco) at 37 °C with 5 % CO₂. The cells were transiently transfected in 6-wells with 4 µg of the bidirectional, tet-responsive pBI-STS+FGE-HA expression plasmid¹⁵ coding for STS and FGE using Lipofectamine LTX (Invitrogen) as recommended. After transfection for 5 h protein expression was induced for 24 h by replacing the normal medium with medium containing 2 µg/mL doxycycline (Fisher Scientific). Cells were harvested by trypsinization and cell pellets were frozen at -20 °C. STS Pellets were resuspended in 150 µL TBS, pH 8.0, containing proteinase inhibitor cocktail 1:100 (Sigma), and lysed by 3×10 s sonication at 4 °C. Total protein concentration was 5.2 mg/mL. All protein concentrations were determined using Coomassie Plus Bradford reagent (Pierce).

4.2. Inhibition Assays with QM-Inhibitor 3

4.2.1. Nitrocatechol Sulfate (pNCS) Activity Assay—Turnover of *p*NCS by sulfatases to *p*-nitrocatechol (pNC) is measured by absorbance at 515 nm after stopping the enzyme reactions with 0.33 M NaOH. Absorption measurements were made in flat transparent 96-well plates (Greiner) using an infinite M200 microplate reader (TECAN). All measurements were performed in triplicate and repeated twice.

KARS (1 μ M, in 50 mM Tris, pH 7.5, 250 mM NaCl) or PARS (1 μ M, in 50 mM Tris, pH 8.5) were incubated for various times with different concentrations of inhibitor **3** in 10 μ L assay volume at room temperature. After pre-incubation, the sulfatase activity assay was initiated by adding *p*NCS to a final volume of 310 μ L with a final assay composition of 35 nM sulfatase, 8 mM *p*NCS and 8 mM Tris (at pH 7.5 or pH 8.5, for KARS and PARS, respectively). The enzyme reaction was allowed to proceed for 10 min at room temperature before quenching by addition of 600 μ L 1 M NaOH. For ARSG the assay was conducted as follows: ARSG (20 nM, in 0.5 M NaOAc, pH 5.6) was incubated with **3** for 3 h and for 18 h at 37 °C. Sulfatase activity assay was then initiated by adding *p*NCS to a final concentration of 8 mM in 0.5 M NaOAc, pH 5.6, with a total volume of 150 μ L, and the enzyme was allowed to react for 60 min at 37 °C. The reaction was stopped by addition of 150 μ L 1 M NaOH. See Fig. 2Fig 3a, and SI, Fig. S2 for time- and concentration-dependent inhibition studies with all sulfatases.

4.2.2. Steroidsulfatase (STS) Activity Assay—STS lysate (5 μ L) was pre-incubated with inhibitor for 0, 1, 2 and 4 h at 37 °C with different concentrations of **3** in 50 mM Tris, pH 7.4 (final volume of 47.5 μ L). STS activity was determined using 3 H-Dehydroepiandrosterone-3-sulfate (3 DHEAS) as substrate.^{15,16} The reaction was started by adding DHEAS to a final concentration of 5 μ M containing 25000 cpm 3 H-DHEAS (kindly supplied by Bernhard Schmidt, Institut für Biochemie II, Universität Göttingen) in a final volume of 50 μ L. After 10 min of incubation at 37 °C the reaction was stopped by addition of 25 μ L 1 M NaOH. Radioactivity of the product 3 H-DHEA was detected by liquid scintillation counting as previously described.¹⁵ All measurements were done in triplicate and repeated twice.

4.2.3. 19 F-NMR Real-Time Experiments of Sulfate Cleavage of Probe 3—All experiments were performed in standard glass NMR tubes at 37 °C on a Bruker Avance 600. KARS (70 nM) was measured with compound **3** (140 μ M) in D₂O containing 10 mM Tris, pH 7.5 over 14 d. PARS (70 μ M) and **3** (5 mM) in D₂O containing 100 mM Tris, pH 8.5 were measured for 12 h.

4.2.4. MALDI-ToF Analysis of Probe 3-Labelled KARS and PARS—MALDI-ToF MS measurements were performed on an ultrafleXtreme mass spectrometer (Bruker Daltonik). The spectra were acquired in the linear mode. 5,000 single spectra were summarized with a 1 kHz smartbeam-II laser for each sample. Spectral processing (smoothing and baseline subtraction) was done in flexAnalysis (Bruker Daltonik). Purified KARS and PARS (20 μ L) were labelled with 5 mM **3** for 1 and 12 hours, respectively, at room temperature. For MALDI-ToF analysis: 2 μ L of the labelling mixture was mixed with 2 μ L of 2 % TFA and 2 μ L of matrix solution (7.6 mg 2,5-dihydroxyacetophenone dissolved in 375 μ L EtOH and 125 μ L of a solution containing 18 mg/mL aqueous diammonium hydrogen citrate solution). The protein-matrix mixture (0.5 μ L) was spotted onto a ground steel target for MALDI-ToF analysis.

4.2.5. Proteome Labelling with 4—Model proteome (50 μ g) was incubated with 1 mM probe **4** for 60 min, in 10 μ L buffer (50 mM Tris, pH 7.5, 250 mM NaCl), at room

temperature. The sample was diluted with 330 μ L DeStreak Rehydration Solution (GE Healthcare) containing 0.5 % IPG Buffer, pH 3–10 (GE Healthcare) and submitted to isoelectric focusing on Immobiline DryStrip gels pH 3–10 NL, 18 cm (GE Healthcare). An Ettan IPGphor (GE Healthcare) with the following program was used for focusing: 1. step, 10 V for 1 h; 2. step, 30 V for 12 h; 3. step 500 V for 1 h; 4. gradient, 1000 V for 8 h; 5. gradient, 8000 V for 3 h; 6. step, 8000 V for 2 h. Second dimension separation was achieved with a 10 % polyacrylamide gel, visualization of the fluorescence signal was done using a Fujifilm LAS 3000 with an excitation of $\lambda_{\text{ex}} = 460$ nm and detection using a FL-Y515 filter.

4.3. Synthesis of Sulfatase-Directed QM Probes

See supporting information (SI) online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Capture schemes for sulfatase-directed QM traps starting from masked fluoromethylphenyl sulfates, including: a) difluoromethylphenyl sulfates (DFPSs **1** and **2**), and b) monofluoromethylphenyl sulfates (MFPS probes **3** and **4**, see Fig. 2 and SI for detailed chemical structures). Specific sulfatase capture requires sulfatase-catalyzed sulfate cleavage and fluoride elimination to release a reactive QM (**5a–d**), which can then covalently trap a nucleophile, ideally a conserved sulfatase active site residue.

Figure 2.

Dose-dependent inhibition of KARS, PARS, STS and ARSG after pre-incubation with and without **3** (time is indicated below enzyme name. Bars represent the percentage of activity relative to control; with S.D., n = 3. (White: control, no inhibitor; grey: 1 mM **3**; black: 10 mM **3**).

Figure 3.

Evaluation of monofluoro-QM trap **3**. a) Time- and concentration-dependent inhibition of KARS by **3**. b) Real-time generation of QM intermediate **5c** as monitored by ^{19}F -NMR via the generation of free fluoride ($\delta = -120$ ppm) after KARS-catalyzed sulfate cleavage of **3** ($\delta = -180$ ppm). Conditions: 140 μM **3**, 370 nM KARS; spectral recording times as indicated; integrals were determined to add up to one. c) and d) Formation of multiple QM adducts, as shown by MALDI-TOF MS-spectral overlay of sulfatases c) KARS and d) PARS with spectra for untreated enzyme shown in black and for enzyme treated with **3** in grey. Conditions: labelling with 5 mM **3** (KARS for 12 h, PARS for 1 h). Untreated enzyme

(black line) shows expected masses: KARS with and without N-terminal signal peptide, $[M+H]^+_{KARS} = 65$ and 63 kDa, corresponding to inactive and active enzyme respectively; PARS, $[M+H]^+_{PARS} = 61$ kDa. Shifted and broadened distributions in molecular weight after treatment with **3** demonstrates multiple QM adducts labelling (each QM adduct adds 280 Da). The active KARS peak disappears with QM probe treatment (shift is 2 kDa), which corresponds to up to 7 QM labels (although the inactive KARS complicates the discernment of a true quantitative average). PARS shows an average of 5 QM adducts (shift 1.5 kDa). See SI for protein sequence details.

Figure 4. Labelling of model proteomic lysates using fluorescent MFPS probe **4** and 2D-PAGE. Model proteomes comprised of *E. coli* lysates containing: a) active KARS, b) inactive KARS, c) no sulfatase were incubated for 60 min with 1 mM probe **4**. Panels show 2D-gel images by fluorescence scanning (left) and Coomassie staining (right). Fluorescent labeling only occurs with active KARS; however, it is non-specific. Black frames indicate KARS, as verified by LC-MS/MS analysis (see SI).