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Anion binding to a cationic europium(III) probe enables the first real-time assay of heparan sulfotransferase activity†

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Sulfotransferases constitute a ubiquitous class of enzymes which are poorly understood due to the lack of a convenient tool for screening their activity. These enzymes use the anion PAPS (adenosine-3'-phosphate-5'-phosphosulfate) as a donor for a broad range of acceptor substrates, including carbohydrates, producing sulfated compounds and PAP (adenosine-3',5'-diphosphate) as a side product. We present a europium(III)-based probe that binds reversibly to both PAPS and PAP, producing a larger luminescence enhancement with the latter anion. We exploit this greater emission enhancement with PAP to demonstrate the first direct real-time assay of a heparan sulfate sulfotransferase using a multi-well plate format. The selective response of our probe towards PAP over structurally similar nucleoside phosphate anions, and over other anions, is investigated and discussed. This work opens the possibility of investigating more fully the roles played by this enzyme class in health and disease, including operationally simple inhibitor screening.

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Introduction

The past two decades have seen a multitude of studies aimed at fundamental understanding of lanthanide complexes and at their exploitation as luminescent sensors and probes.^{1–7} When suitably ligated and irradiated with light in the UV or visible range, certain lanthanide(III) ions undergo an electronic excitation; relaxation of this excited state leads to luminescence.⁸ Such lanthanide complexes possess a range of qualities that make them particularly interesting and useful for sensing and imaging purposes, including their typically large pseudo-Stokes shifts, narrow emission linewidths and long emission lifetimes (millisecond range for europium and terbium).^{9–12} Anion responsive lanthanide complexes may be designed by introducing one or more vacant coordination sites at the metal centre; these are occupied by water molecules in aqueous solution which deactivate the Ln(III) excited state through vibration of their O–H bonds. Thus, coordination of a target anion at the metal centre displaces one or more water molecules, leading to an extension of the excited state lifetime and increase of the luminescence. Careful design of the ligating group(s) can give rise to remarkable selectivity amongst

anions, allowing sensors to be created that signal anion binding through modulation of emission intensity, lifetime and spectral form.^{1,6} In particular, it has proved possible to discriminate between very similar phosphate-containing biomolecules in complex aqueous solutions, an area where we have been active.^{13–17}

Heparan sulfotransferases mediate the attachment of a sulfate group to an atom (usually oxygen, though sometimes nitrogen) in the heparanosan/heparan sulfate polysaccharide (Scheme 1). They exist in numerous isoforms with varying substrate preferences.¹⁸ The sulfated products of this reaction play important roles in cell communication¹⁹ and also feature in various pathologies including those of cancer,²⁰ Alzheimer's²¹ and the mucopolysaccharidoses.²² Heparan sulfotransferases belong to the wider group of sulfotransferases found across nature that use the universal sulfate donor compound PAPS (adenosine-3'-phosphate-5'-phosphosulfate),²³ and produce PAP (adenosine-3',5'-diphosphate) as a by-product (Scheme 1).

Very few methods have been disclosed for monitoring directly the activity of carbohydrate sulfotransferases. Those that have require radio-labelled substrates^{24–26} or specialized, expensive equipment and acceptor substrates.²⁷ The majority of published assays examine endpoints making the accurate determination of enzyme kinetics difficult and time consuming. Very few are adaptable to high throughput format, accounting for the small numbers of reports of inhibitors of these enzymes.^{24,28} Notably, a recent study by Eyers and co-workers demonstrated that sulfotransferases inhibitors can be identified in specialised high-throughput screens.²⁷ This work

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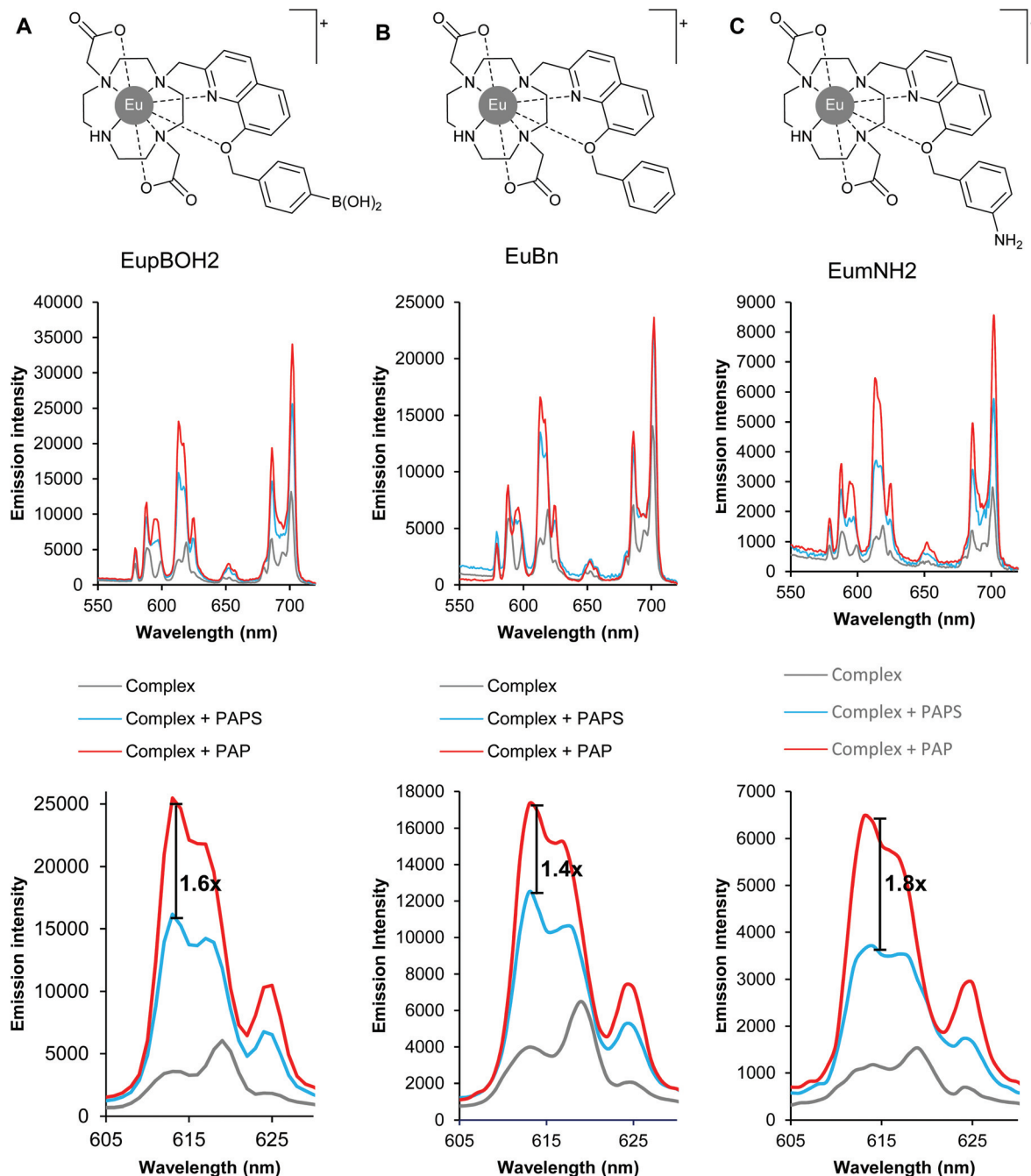


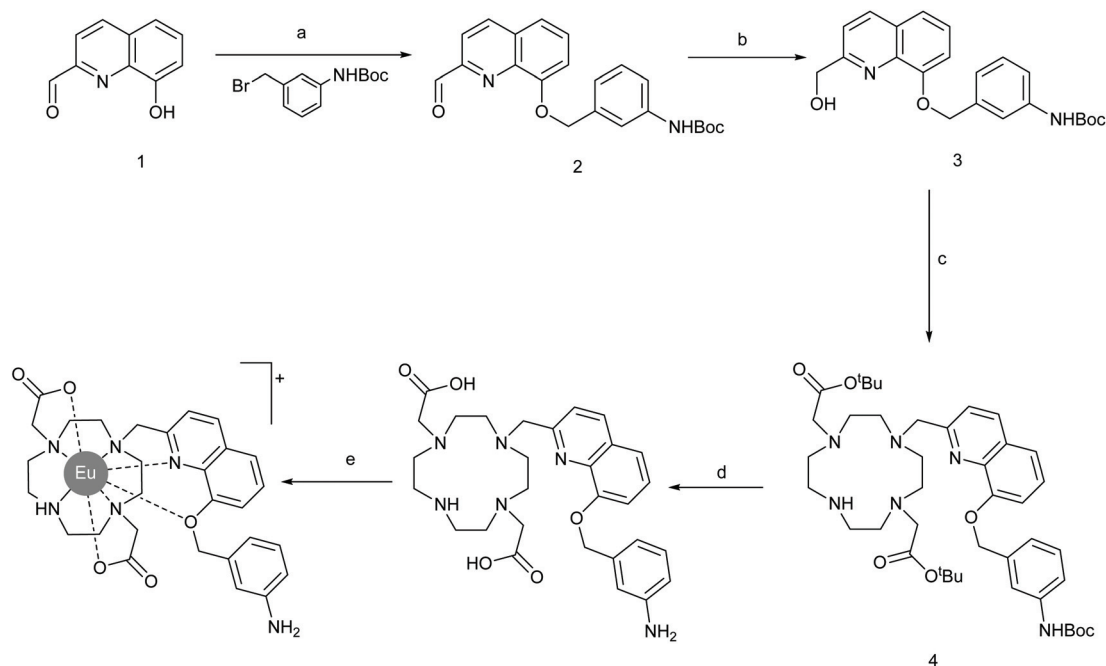
Fig. 1 Structures of cationic Eu(III) complexes and their emission responses alone and in the presence of PAPS and PAP. All three Eu(III) probes give greater emission enhancements upon binding PAP compared with PAPS (A–C); removal of the boronate group resulted in a loss of discrimination (B vs. A) while installing an amino group increased it (C vs. A, B). All experiments conducted using 5 μM Eu(III) complex, 250 μM anion in 50 mM TRIS at pH 7.4, rt.

identical to the unsubstituted complex **EuBn** and with a very similar extinction coefficient. The emission spectrum was also similar to **EuBn**, characterised by two components in the $\Delta J = 1$ (585–600 nm) emission band and three components in the $\Delta J = 2$ (605–630 nm) band. We suspected that the much lower quantum yield of **EumNH2** and its smaller emission lifetime in water and D_2O (Table 1, Table S1†) was caused by intramolecular photoinduced electron transfer (PET) from the

nitrogen lone pair on the aniline ring quenching the europium excited state.

Consistently, the emission from this complex was found to be highly pH-sensitive with much higher emission intensity observed at lower pH values (Fig. S6†). Further support for the involvement of the aniline nitrogen lone pair was the presence of an absorption band centred at 292 nm which is absent from the spectrum of the parent complex (compare Fig. S3 and S4†)





Scheme 2 Synthesis of **EumNH2**. Conditions: (a) K_2CO_3 , DMF, rt, 2 h, 88%; (b) NaBH_4 , MeOH, 0 °C to rt, 55%; (c) (i) MsCl , Et_3N , DCM, 0 °C to rt, (ii) DO2A *tert*-butyl ester, K_2CO_3 , MeCN, 60 °C, 18hr, 71% over two steps; (d) 3 : 1 DCM/TFA, 18 h, quant; (e) $\text{EuCl}_3 \cdot 6\text{H}_2\text{O}$, water, 60 °C, pH \geq 7.2, prep HPLC, 9%.

Table 1 Photophysical parameters of the Eu(III) probes measured in water at 295 K For full data see Table S1†

	EupBOH2	EuBn	EumNH2
λ_{max} (nm)	322	322	322
ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	2900	2900	2500
Φ^a (%)	1.2	1.5	0.3
$\tau_{\text{H}_2\text{O}}^b$ (ms)	0.18	0.17	0.05
$\tau_{\text{D}_2\text{O}}^b$ (ms)	0.25	0.23	0.07
q^c	1.29	0.76	— ^d

^a Quantum yields were calculated relative to quinine sulfate standard ($\Phi_{\text{em}} = 0.58$); errors in quantum yields are $\pm 20\%$. ^b Lifetime experiments conducted using 50 μM probe, $\lambda_{\text{ex}} = 322$ nm, $\lambda_{\text{em}} = 620$ nm. ^c Hydration state, q , was estimated using literature methods (errors are $\pm 20\%$). ^d Very short emission lifetimes prevented accurate estimation of the hydration state.

and which disappears at low pH (Fig. S6†). We assign this band to an $n \rightarrow \pi^*$ transition. We attribute the emission enhancement and spectral form changes observed above pH 8 (Fig. S6†) to the coordination of hydroxide, but importantly the emission spectrum is stable in the pH range 6–8, rendering this compound potentially suitable for sulfotransferase assays.

The emission lifetime of the parent and boronic acid complexes in water were similar and extended in D_2O as expected (Table 1 and S1†), giving rise to an estimation³⁵ of the number of bound water molecules, q , of 1 within experimental error. We interpret these non-integer values as suggesting dynamic behaviour of the bound waters, including interaction with outer sphere molecules. The emission lifetime of complex **EumNH2** was much shorter, consistent with PET quenching

by the aniline nitrogen lone pair. Estimation of the q value for such complexes becomes difficult as the emission lifetime begins to overlap with the water exchange timescale.³⁵ It is nonetheless significant that the emission lifetime was longer in D_2O suggesting that the complex is hydrated in the absence of anions. When the overall emission intensity was measured in D_2O it was found to be 2.9 times higher than in H_2O , consistent with energy transfer to O–H vibrations being a significant non-radiative quenching pathway (Fig. S6†).

We were pleased to discover that, despite being less emissive, the aniline complex **EumNH2** outperformed both our initial boronic acid and unsubstituted benzene complex showing a 1.8-fold discrimination in emission between PAP and PAPS at 613 nm (Fig. 1C). Analysis of the anion binding titration data (Fig. S1†) indicated that **EumNH2** has a genuine preference for binding PAP over PAPS ($\log K_a = 4.0$ and 3.5, respectively), albeit with a modest 5-fold selectivity for the former anion.

We wished to confirm our hypothesis that the phosphate groups in PAPS and PAP coordinate to the Eu(III) ion displacing the bound water, and are thus responsible for the observed enhancement in emission and change in spectral form. First, we measured the emission response of **EumNH2** in the presence of a small range of anions and found that inorganic phosphate was unique in giving a significant increase in luminescence especially in the $\Delta J = 2$ band; other anions (sulfate, bicarbonate, nitrate, lactate and chloride) gave negligible changes in luminescence (Fig. S7†), indicating that these anions do not bind to **EumNH2**. Such high selectivity for phosphate was observed recently for structurally related Eu(III) receptors containing a bulky 8-(benzyloxy)quinoline pendant



arm that chelates the Eu(III) ion, leaving a single coordination site for the phosphate binding.²⁹

Other oxyanions that prefer a bidentate binding mode, including bicarbonate and lactate,³⁶ do not bind to the receptors due to the steric hindrance imposed by the ligand, which blocks the 'axial' coordination site. The emission spectral form changes observed for **EumNH2** in the presence of inorganic phosphate (Fig. S7†) matched that of PAP and PAPS, confirming that the phosphate-Eu(III) coordination is the primary interaction involved in the host-guest complexes involving PAP or PAPS.

It is possible that addition of PAP and PAPS also leads to suppression of PET quenching, contributing to the overall

enhancement in emission intensity.^{37,38} If blocking of PET, rather than water displacement, was the main mechanism for the emission response then we would expect similar emission enhancements on binding anions in H₂O and D₂O. In fact, we observed a 2.8-fold increase in overall intensity on binding PAP in H₂O but only 1.3-fold in D₂O; binding PAPS gave a 2.0-fold increase in H₂O but only 1.2-fold in D₂O (Fig. S6†). The smaller enhancements in emission in D₂O can be attributed to reduced quenching effect of O–D *versus* O–H oscillators. Additionally, suppression of PET by lowering the pH did not lead to changes in spectral form (Fig. S6†) unlike binding anions (Fig. 1, 2 and S7†). We thus conclude that the emission

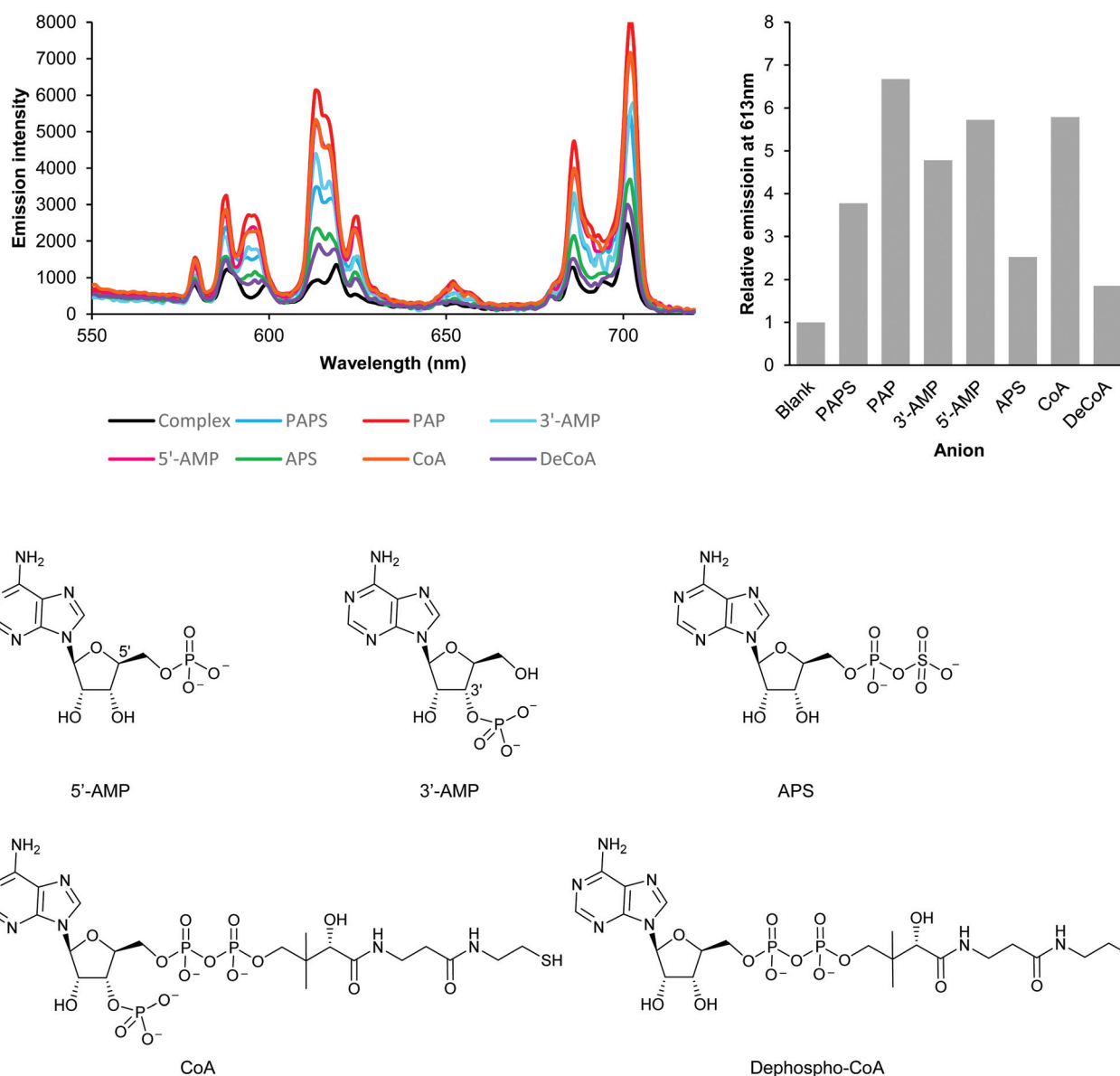


Fig. 2 Investigation of anion binding modes by analysis of changes in the emission intensity and spectral form of **EumNH2**. Emission enhancement occurs if the anion possesses either a 3'-phosphate (PAPS, 3'-AMP, CoA), a 5'-phosphate (5'-CoA) or both (PAP); the absence of both 3'- and 5'-phosphates (APS, dephospho-CoA) results in very limited emission enhancement. Experiments conducted using 5 μ M Eu(III) complex, 250 μ M anion in 50 mM TRIS at pH 7.4, 295 K. Very similar results were obtained for **EupBOH2** and **EuOBn** (see Fig. S8†).



the emission spectrum recorded. 1 μL of 10 mM anion solution in distilled water was added (final anion concentration 250 μM) and the emission recorded again. For inorganic anions (Fig. S5†) 1.6 μL of a 25 mM solution in HEPES was used, final concentration 1 mM.

Anion binding titrations

Anion binding titrations were carried out in 50 mM TRIS buffer at pH 7.4. Stock solutions of PAPS and PAP containing Eu(III) complex (5 μM) were made up at 0.1, 1 and 5 mM anion. The appropriate anion stock solution was added incrementally to 40 μL of Eu(III) complex (5 μM) and the emission spectrum was recorded after each addition. The ratio of emission bands 605–630 nm/585–600 nm ($\Delta J = 2/\Delta J = 1$) was plotted as a function of anion concentration. The data was analysed using a nonlinear least-squares curve fitting procedure, based on a 1 : 1 binding model described by the equation:

$$\text{FB} = \frac{\frac{1}{K_a} + [\text{A}] + [\text{Eu}] - \sqrt{\left(\frac{1}{K_a} + [\text{A}] + [\text{Eu}]\right)^2 + 4[\text{A}][\text{Eu}]}}{2[\text{Eu}]}$$

where FB is the fraction bound, calculated by $(I - I_0)/(I_1 - I_0)$ where I is the emission intensity at $[\text{A}]$, I_0 is the initial emission intensity, and I_1 is the final emission intensity. $[\text{A}]$ is the total concentration of anion in solution, $[\text{Eu}]$ is the total concentration of Eu(III) complex, K_a is the apparent binding constant.

Microplate-based sulfotransferase simulations

Varying ratios of a solution of PAPS and PAP containing a 5 μM Eu(III) complex in 50 mM TRIS at pH 7.4 were added to a 384-well plate, in triplicate, to a total well volume of 40 μL . The plate was incubated for 10 minutes prior to reading. Time-resolved emission intensities were recorded in the range 610–630 nm (integration time of 60–400 μs) with excitation at 292–366 nm. The mean of the triplicate intensity values was plotted against the percentage of PAP. Error bars indicate the standard error in the mean value.

Microplate-based sulfotransferase reactions

1.5 μL of 200 μM **EumNH2** was added to 58.5 μL of 20 μM GST-HS3ST1 and the mixture incubated at rt for 5 minutes. 6 \times 30 μL of a 5 μM solution of **EumNH2** in 50 mM TRIS pH 7.4 were placed in a 384-well plate and to these were added the mixture of **EumNH2** and GST-HS3ST1 (final concentrations both 5 μM). 3 μL of either 50 mM TRIS pH 7.4 or 100 mg mL^{-1} heparin were added to 3 wells each and the plate allowed to incubate at rt for 5 minutes. 1 μL of PAPS (10 mM in distilled water) was added to each well (final concentration 250 μM). The wells were scanned every 15 minutes for 3 hours then every hour for an additional 20 hours exciting at 292–364 nm and measuring emission at 620 ± 5 nm. Emission change was calculated by normalising to the emission at $t = 0$, averaging over replicates and subtracting the signal from wells with TRIS from the signal from wells with heparin.

Conflicts of interest

There are no conflicts to declare.

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