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## Graphical abstract

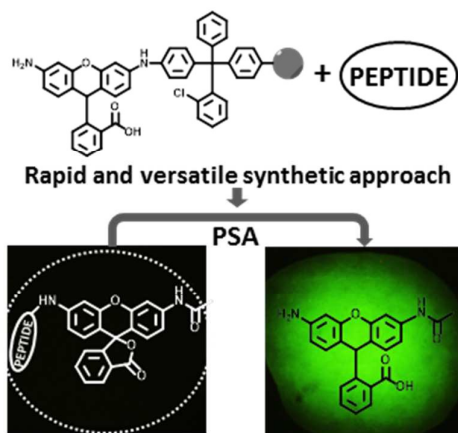
### Solid-phase synthesis of Rhodamine-110 fluorogenic substrates and their application in forensic analysis

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A novel synthesis demonstrates the rapid and efficient preparation of fluorogenic substrates containing Rhodamine-110 fluorophores for the detection of biological evidence.



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## Solid-phase synthesis of Rhodamine-110 fluorogenic substrates and their application in forensic analysis

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**A novel synthetic route for the rapid and efficient preparation of fluorogenic substrates utilizing Rhodamine-110 or similar fluorophores is reported. Applicability of the synthesized peptide substrate within a forensic casework context is also presented.**

Last year, over 10,000 exhibits were submitted to the Evidence Recovery Unit (ERU) of the Metropolitan Police Service. Body fluid stains such as blood, semen and saliva are often the most important forms of biological evidence encountered in forensic casework. Determining the identity of such deposits can be used to further a criminal investigation as it may provide information on both the nature of the offence and the genetic origin of the fluid (through DNA profiling).<sup>1</sup> Consequently, over 90% of these submitted exhibits required examination for the presence of biological fluids.

However, with meticulous visual examination by trained personnel being the most common method of detection, determining the presence and location of any fluid deposits may be very challenging, especially with small, or latent traces on dark backgrounds which may be easily overlooked.<sup>1</sup> Long wave UV or violet light sources may aid detection by exploiting the auto-fluorescent properties of some intra-fluidic molecules but their use is restricted to a select number of surface types.<sup>2</sup> The colorimetric presumptive assays used in order to identify the type of fluid present are also considered by many in the forensic community to be limited by issues of specificity,<sup>3</sup> safety,<sup>4</sup> and their detrimental effects on DNA recovery.<sup>5</sup> Developing a fast, dependable and specific method of fluid

detection would save time and money. Biosensors that simultaneously detect and identify body fluids could represent a cost and time efficient alternative to present day techniques. The development of fluorogenic peptide substrates specific to each body fluid type could improve body fluid search efficiency through the identification of fluid-specific proteases in situ via fluorescence emission.<sup>6,7</sup>

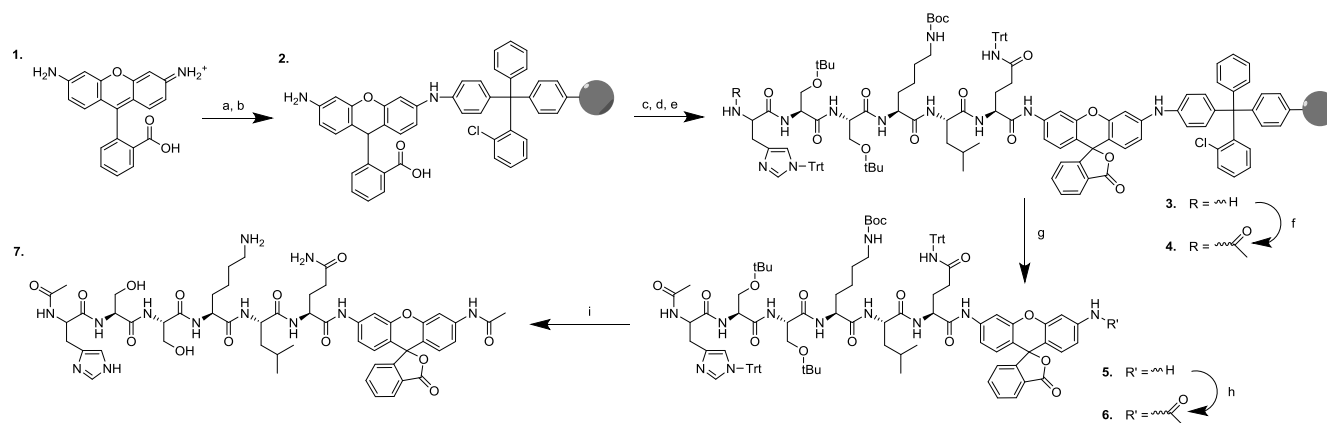
Rhodamine-110 (Rh-110) is a xanthene-based fluorescent dye containing two aromatic amine moieties, the symmetric or asymmetric modification of which may shift the existing equilibrium of the molecule from a highly fluorescent quinoid towards a colorless lactone formation, quenching fluorescence emission.<sup>8</sup> This has cemented Rhodamine-110 as a popular pro-fluorophore for use within many protease substrates via the direct attachment of specific amino acid chains (with a positive fluorescence signal achieved via the proteolytic separation of peptide and fluorophore in the presence of a target enzyme).<sup>8</sup> The physical and spectral characteristics of Rho-110 are considered highly desirable for biological fluid detection, as they display a high quantum yield and are relatively pH insensitive. Moreover, Rho-110 based substrates exhibit an almost non-existent emission upon peptide conjugation, preventing background fluorescence and ensuring high enzymatic assay sensitivities.<sup>8</sup>

However, all reported symmetric and asymmetrically labeled Rh-110 peptide substrates have so far been synthesized through solution chemistry, a task that is made problematic due to the poor nucleophilicity of the Rh-110 aromatic amines,<sup>9</sup> and often leads to inefficient peptide-fluorophore acylations. Final product yields are inevitably very low, as each intermediate must undergo substantial purification.<sup>10</sup> This makes the cost of Rh-110-based substrates high and since the

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**Fig. 1** Schematic of Rh-110 PSA substrate development: a) 2-ct resin (0.27mmol), DCM, DIPEA, 24h, b) MeOH, 10 min, c) Fmoc-Gln(Trt)-OH (3mmol), DMF/Pyridine, EDC (2.5mmol), 24h, x 2, d) 20% piperidine/DMF, 30 min, e) 6 x AA, Oxyma, DIC, DMF, 1hr, f) 10 x (CH<sub>3</sub>CO)<sub>2</sub>O, DIPEA, DMF, 1h, g) AcOH:TfE:DCM, 1h, h) CH<sub>3</sub>COCl, DIPEA, DMF, 48h, i) TFA:TIPS:TA:Phenol.

aim of the research is to produce a spray that could be used directly on forensic evidence, this cost would be unacceptable. We surmised that preparing large quantities of peptide substrates in a rapid and efficient manner could be achieved via the direct attachment of Rh-110 to a solid support. In this procedure (Fig. 1), it is envisioned that one of the aromatic amine groups of Rh-110 may be anchored to a functionalized solid support, keeping the remaining amine group available for the subsequent stepwise incorporation of amino acids by standard Fmoc-SPPS. This route offers advantages over typical solution-phase conjugation techniques by allowing the incubation of fluorophores with large molar excesses of any desired amino acid residue, which may be removed without extensive liquid-liquid extraction and chromatographic purification steps, thereby increasing overall product yield. Instead, excess amino acids, coupling reagents and other by-products may be removed by simple resin wash and filtration processes. Therefore, a fluorogenic substrate for the purpose of detecting seminal fluid within a forensic casework framework was constructed through the sequential coupling of immobilized Rh-110 to amino acids HSSKQLQ, a sequence previously reported by Denmeade *et al.*<sup>11</sup> as a proteolytic cleavage site of seminal fluid-specific protease prostate specific antigen (PSA).

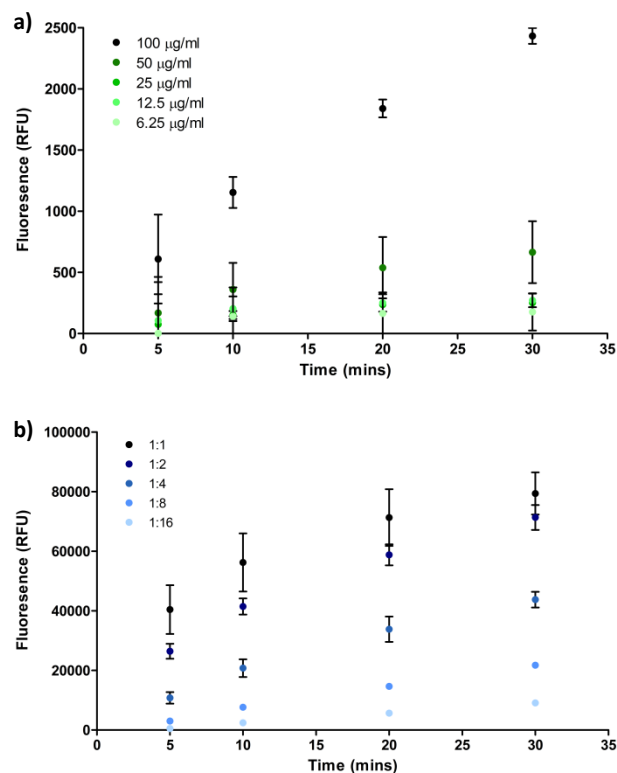
Rh-110 was successfully anchored to the 2-chlorotriptyl chloride resin (known to react with aromatic amines in the presence of

a base such as pyridine or *N,N*-Diisopropylethylamine (DIPEA))<sup>12</sup> in the first step towards substrate preparation. Upon reaction, the resin exhibited a deep red coloration that was retained after extensive washing, indicating successful dye incorporation. Cleavage of a small portion of the acid-labile resin, followed by ESI-MS analysis confirmed the presence of free Rh-110 (Fig. S1, ESI).

With a number of workers previously reporting the unsuccessful conjugation of bulky peptides to Rh-110 within solution,<sup>13</sup> product **3** was instead assembled via the stepwise addition of single amino acids in a series of individual reactions. Multiple large excesses of Fmoc-protected glutamine were first incubated with Rh-110-attached resin **2** in order to ensure complete coupling (which was confirmed via ESI-MS analysis after 'soft' cleavage of a small portion of resin (Fig. S2, ESI)), with successive amino acids incorporated through standard SPPS deprotection-wash-coupling-wash cycling to form a PSA-specific peptide backbone. Acetylation of the histidine N-terminus was performed to prevent substrate degradation from environmental aminopeptidase activity.<sup>14</sup>

As previously mentioned, many substrates utilizing Rh-110 fluorophores are bis-substituted, having been synthesized through the symmetric conjugation of identical peptides to both aromatic amines in order to quench fluorescence. However, in such substrates, a two-step enzymatic hydrolysis is required in order to cleave both peptide chains before a full

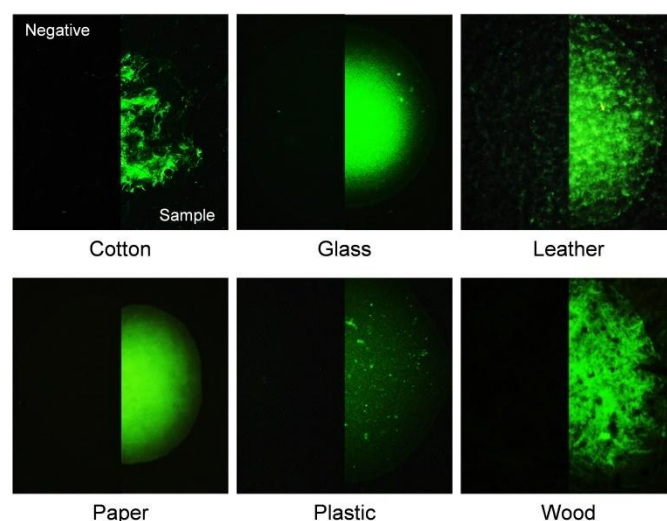
fluorescence signal is restored, consequently limiting the dynamic linear range of the assay.<sup>15</sup> As a result, the majority of fluorogenic Rh-110 peptide substrates now produced are mono-substituted, with secondary aromatic amines 'capped' through amide, carbamate or urea modifications.<sup>8</sup> This not only permits the assay to be completed within a single hydrolysis step, but also produces the same complete emission quenching effects as bis-peptide modification.<sup>16</sup> Therefore, a 'soft' cleavage of the resin was performed under mild acidic conditions to generate **5** from the solid support, allowing all tert-butyloxycarbonyl and trityl amino acid side-chain protecting groups to remain intact (preventing accidental amino acid side-chain modification) during the capping of the remaining Rh-110 aromatic amine by acetylation. Liquid-liquid extraction was then performed to separate aqueous-reactive acetyl chloride from organic-soluble products, which were subsequently treated with TFA and precipitated in ether to remove acid-labile side-chain protecting groups. RP-HPLC and high-resolution mass spectrometry were used to confirm the purity and identity of final substrate product **7** respectively (Fig. S3 and S4, ESI).



**Fig. 2** Fluorescence response of final substrate **7** to a) concentrations of purified PSA protein and b) seminal fluid dilutions over time.

Spectrofluorometry was employed to observe the emission response of **7** quantitatively towards its intended enzymatic target. Whilst the final goal of this substrate is to identify the

presence of whole semen, it is important to confirm that a fluorescence response is only elicited as a result of PSA hydrolysis and not from interaction with any other molecule within the seminal fluid matrix. Therefore in addition to dilutions of seminal fluid, substrate **7** was incubated with varying concentrations of purified PSA protein. Immediate fluorescence signal increases upon the addition of both PSA (Fig. 2a) and seminal fluid (Fig. 2b) successfully demonstrated cleavage between Rh-110-Ac and the attached glutamine residue, consequently establishing the potential use of this substrate as a semen-specific biosensor. Responses generated by seminal fluid were found to be much higher compared to that of tested purified protein concentrations, which was expected considering the significantly larger levels of PSA usually contained within whole semen (approximately 0.5-3.0 mg/ml).<sup>17</sup> As it is important to determine the proteolytic specificity of fluorogenic substrates prior their application (especially within the context of forensic casework, in which a false-positive signal may represent an incorrect evidential identification), final substrate **7** was also incubated with identical concentrations of trypsin, proteinase K and aminopeptidase M as a measure of PSA selectivity. In all cases the substrate did not exhibit a positive response, thereby demonstrating a high specificity towards seminal fluid, as well as an inherent resistance to protease enzymes present within other body fluids or the surrounding environment (Fig. S5, ESI).



**Fig. 3** Detection of semen across six surfaces by substrate **7**. Seminal fluid-only negative controls are provided on the left side of each image.

With one of the advantages of this assay over current forensic testing techniques being the ability to simultaneously locate and identify fluid stains in situ via fluorescence, it is pertinent to establish that a positive substrate emission will not be affected by the physical nature of the evidential surface on which a body fluid is deposited. Substrate **7** was therefore



applied to small volumes of semen deposited on six surfaces routinely examined within criminal investigation, with subsequent signal responses observed by fluorescence microscopy. Upon reagent application, all deposits were successfully visualised, with surface material displaying little effect on resulting substrate emission (Fig. 3). Similar visualization was also achieved via the use of a hand-held Crime-lite® portable excitation source. As an additional method of observing reagent specificity, substrate **7** was also incubated with volumes of blood and saliva in situ. No signal was observed upon application (Fig. S6, ESI).

The identification of seminal fluid by PSA detection is currently achieved through absorption of a reconstituted suspect stain onto an immunochromatographic testing strip.<sup>18</sup> However, this technique is not only unable to locate the position of stains upon an evidential item but furthermore, consumes the fluid during the testing process, preventing further DNA analysis. An in situ PSA detection assay that allows downstream genetic profiling would consequently be considered advantageous. Therefore, substrate **7** was applied to a volume of seminal fluid deposited on a glass slide, with the resultant mixture being recovered by use of a cotton swab. Samples then underwent standard DNA extraction, quantification, amplification and capillary electrophoresis according to forensic protocols. Comparisons were then made to an untreated but identically processed reference sample to examine the effects of substrate application on genetic recovery. Differentiation of samples was not observed at any stage of the profiling process with the DNA concentrations of reagent-applied fluid and reference sample calculated at 0.66 and 0.61 ng/μl respectively. A full STR profile from both samples was also obtained (Fig. S7, ESI).

In summary, we have described a versatile detailed a synthetic route for the simple, rapid and inexpensive preparation of Rh-110-based fluorogenic substrates. Moreover, a substrate produced by this method has already shown great potential as a replacement for currently employed forensic body fluid testing techniques, This substrate allowed the specific detection of seminal fluid traces both within solution and in situ, without compromising subsequent genetic profiling processes. Future work will centre on the use of this method to produce fluorogenic substrates towards other biological fluids and latent fingermarks (something that has not currently been achieved). However, with fluorogenic substrates routinely employed in a variety of industrial and biomedical applications, the disclosed synthetic protocol is also likely to have a much larger implication beyond the field of forensic science.

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