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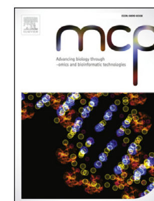

# Synthetic scale-up of a novel fluorescent probe and its biological evaluation for surface detection of *Staphylococcus aureus*

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This paper reports on the LGX fluorometric test for enzymatic MRSA/MSSA detection. It highlights the reasons rhodamines have been overlooked and also strategies to improve the synthesis of rhodamine-peptide conjugates. Evaluation of the LGX test for detection of MRSA/MSSA on surfaces is undertaken in the presence of potentially confounding *E. coli* and *S. epidermidis* for the first time.
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## 1. Introduction

The growth in the number of infections caused by antibiotic resistant pathogens has prompted healthcare agencies around the world to generate strategic plans [1]. These plans incorporate the restriction of antibiotic use to slow the emergence of resistant pathogens, as well as the development of new antibiotics, to which resistance will inevitably emerge. The former part of this strategy requires rapid, affordable diagnostics to determine the best clinical course of action [2] (see Fig. 1)

The principle drawbacks of PCR are the laboratory requirements and the expense of each test. This drawback is demonstrated in the UK insofar that PCR is not routinely used to detect problem pathogens such as MRSA. Bacterial culture is time-consuming (48–72 hrs) and as reported in the NHS NOW report specifically concerning MRSA, it is often the case that patients are discharged before the results of the test are known [3].

PCR techniques rely upon the enzymatic amplification of a gene until sufficient quantities can be detected. Bacterial culture relies upon the growth rate of a bacteria, augmented by the best media. An alternative approach involves the targeting of enzymes expressed by bacteria, which cleave between specific amino acid sequences [4].

To observe the actions of bacterially expressed enzymes and in common with other detection methods, a chromogenic response is arguably the most useful. This is evidenced by the recently reported rhodamine based fluorogenic probe (**1**). This probe, (incorporated into a test subsequently referred to as LGX) has shown good selectivity and sensitivity for both Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA) [5].

Rhodamine 110 (**2**) represents one of the most active fluorophores known, with a high extinction coefficient and a fluorescence quantum yield which is near unity. Rhodamine has been used as a marker in a wide variety of enyzmic activity studies (serine protease [6], esterase [7], caspase [8] and DT diaphorase [9]). Essentially the $NH_2$ groups of rhodamine 110 are conjugated to a polypeptide, which mimics the natural substrate of the enzyme in question. Upon cleavage of the polypeptide mimic, the rhodamine is released and in aqueous solution undergoes a conversion to a highly fluorescent zwitterionic form. The change in rhodamine's

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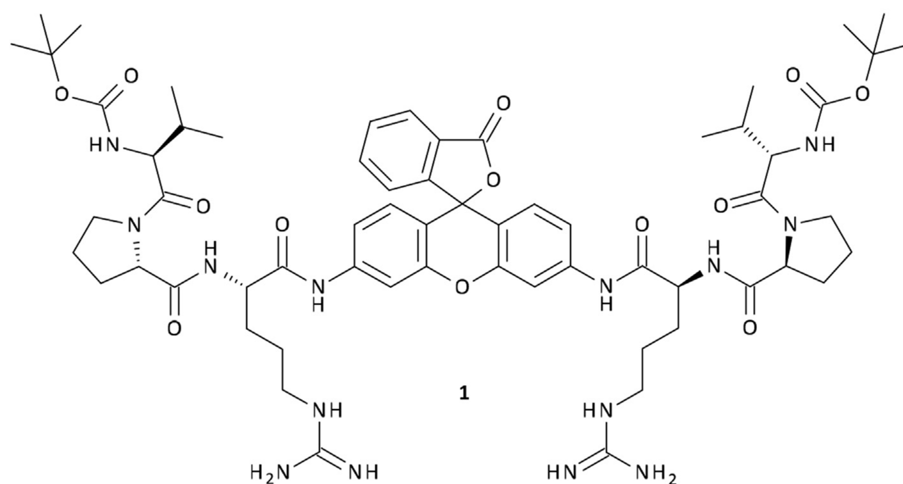


**Fig. 1.** Fluorogenic Probe (**1**) in LGX test.

fluorescence quantum yield from its unconjugated (lactone form), to conjugated (zwitterionic form) is considerable (Rho 110 $\phi_{lactone} = 0.006$ $\phi_{zwitterion} = 0.98$). This sharp increase in fluorescence is easily detectable by even handheld instruments and often just the naked eye.

This paper considers the previous synthetic literature for rhodamine 110 derivatives and reports on new exploratory approaches to the tailoring of this important fluorophore. Furthermore it addresses why rhodamine conjugates have not been more widely applied to pathogen detection *via* bacterial enzymatic action.

The most recent review of rhodamine 110 details the methodology used for the synthesis of rhodamine derivatives, which is essentially unchanged since the 1980's.

Rhodamine 110 is reacted with a protected amino acid, which is first activated with a coupling agent. Following deprotection and subsequent activation, further amino acids are added in a routine fashion, however the yields are often poor and the costs of the initial rhodamine are high (£100/g). In addition, there is no mention of racemisation in any of the seminal work, on which that review is based [10].

Recent work by the authors, reports the synthesis of a novel (Boc-Val-Pro-Arg)$_2$–Rhodamine (**1**), however the authors report considerable difficulty with which Arginine could be attached to rhodamine, which is at odds with the comparative ease of this achievement by Mangel et al., almost 33 years ago [11]. In reference to the original literature dealing with biologically relevant rhodamine conjugates, we observe multiple discrepancies between the compounds reported and the fluorescence data before and after enzymatic cleavage. There is no reported yield for the cathepsin C activated rhodamine [12] or caspase conjugates [8], yet these are reportedly known in various reviews. The synthesis of the serine protease activated rhodamine by Mangel et al. is also highly improbable in its simplicity and purification *via* centrifuge [11]. The only characterisation undertaken is TLC and low resolution MS. The elemental analysis deviates from the empirical formula, but water and HCl are inexplicably added to bring the observed values within tolerance. The absence of HPLC/NMR facilities when the work was done in 1982 means that it is unlikely the compound was specifically synthesised. The primary literature reports the (CBz-Arg)$_2$-Rhodamine to be a pink powder, yet the Sinclair group reported a white solid after HPLC purification. The biological testing of the serine protease activated rhodamine as then reported bears out the fact that whatever was synthesised is impure. The UV-Vis spectra indicates there is still fluorescence of the rhodamine conjugate at 525 nm. If the fluorescence quantum yield alone is three orders of magnitude lower for a Bis-NH$_2$ conjugated rhodamine than for the free fluorophore, this fluorescence should not be visible, yet it is shown to be fluorescent in the original paper from 1982. The conclusion has to be that a mixture of rhodamine, mono Arg-Rhodamine and bis Arg-Rhodamine was in fact made. Thus the contrast between conjugated and unconjugated rhodamine is comparatively small and significant amounts of protease are required to achieve this. As a consequence, any attempt to achieve a high sensitivity e.g. for the detection of pM concentrations of an enzyme expressed by a pathogen would be met with failure.

As indicated in the previous section rhodamine conjugates have been somewhat overlooked as the fluorescent component for practical enzymatic tests and there is a need to develop synthetic methodology to tune the rhodamine structure efficiently.

Previously reported synthesis has proven capricious with initial coupling reactions of amino acids with rhodamine being of very low yield and this makes them less amenable to scale-up. We discuss the different approaches and show an improvement in the overall yield of a recently reported rhodamine conjugate (Boc-Val-Pro-Arg)$_2$–Rhodamine (**1**), which has shown remarkable selectivity and sensitivity for MRSA and MSSA as reported by the authors [5].

## 2. Results

### 2.1. The xanthone approach

#### 2.1.1. Grignard

The main problem with functionalising the NH$_2$ groups on rhodamine (see Fig. 2) is the intrinsic lack of nucleophilicity of these, due to resonance and other effects [13]. To overcome this problem the approach was taken to reduce some of the possible conjugation through the rhodamine *via* removal of the top lactone aromatic. The aim was to achieve a better amino acid coupling yield and introduce the lactone ring at a later stage. The method followed that of A. Young-Hoon et al. whom originally developed the procedure to produce library of rosamines for combinatorial synthesis. These conditions were adapted to produce the initial required nitro amino xanthone (**3**) for further modification towards a number of novel unsymmetrical rhodamines for further study [14].

The initial step is an Ullmann condensation requiring high temperatures and long reaction times (see Fig. 3). Work-up in hot concentrated sulphuric acid induces ring closure, to produce the

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