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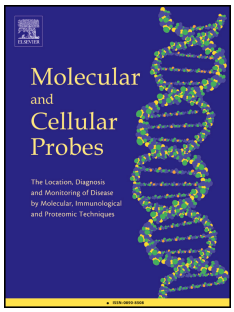

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Title: Synthetic scale-up of a novel fluorescent probe and its biological evaluation for surface detection of *Staphylococcus aureus*

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Abstract: 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.

Keywords: Rhodamine, Synthesis, Peptide, Fluorescence, Conjugate, Protease, Pathogen

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]

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 (48-72hrs) 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 *Staphylococcu aureus* (MRSA) and Methicillin Sensitive *Staphylococcus aureus* (MSSA).[5]

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

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], 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 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.

Figure 2. Rhodamine 110 (**2**)

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 $(CBz\text{-}Arg)_2$-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 525nm. 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 $(Boc\text{-}Val\text{-}Pro\text{-}Arg)_2$–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 are 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]

Figure 3. Asymmetric xanthone synthesis, as per A. Young-Hoon *et al.*

The initial step is an Ullmann condensation requiring high temperatures and long reaction times. Work-up in hot concentrated sulphuric acid induces ring closure, to produce the xanthone core. In our hands, this step resulted in the expected xanthone **3** in a consistent yield of 30% (Figure 4 (a)). NMR data confirmed this (See SI-1). Having functionalised the NH_2 of xanthone **3** with an appropriately protected amino acid e.g. N-Boc-Arginine$(Cbz)_2$ –OH to give **4**, the upper ring system could be introduced using a Grignard type approach as indicated in Figure 4.

Whilst the test reaction of phenyl magnesium bromide and unfunctionalised xanthone **6** successfully produced tertiary alcohol **7** (Figure 4 (b)), the Grignard reaction using acetal protected benzaldehyde on nitro amino xanthone **3** was unsuccessful in the production of **8**, further elaboration to **9** and **10** were therefore not possible (Figure 4(c)). Upon examination of the crude mixture, the expected signals were observed neither by NMR nor by MS, instead we obtained a complex and intractable mixture of deeply purple coloured products which could not be successfully isolated and characterised.

Figure 4. Proposed incorporation of C ring using Grignard followed by mild oxidation.

The reaction was tested a number of times with varying excesses of Grignard reagent without generating the product of interest (See Table SI-4). This result warranted further investigation, upon which the Bartoli indole synthesis was implicated. This synthesis detailed the reaction of vinyl Grignard reagents with aryl nitro compounds, demonstrating the reactivity of the aryl nitro group towards Grignard reagents.[15] It is possible that attack on the deactivated ketone group of the xanthone was slower than on the nitro group demonstrating the incompatibility of nitro arenes with Grignard reagents, aiding explanation of the observed results.

2.1.2 Samarium Iodide

During the investigation into new routes towards non-commercially available Rhodamine dye analogues, the reducing agent samarium iodide (SmI_2) was noticed. Attention was given to this due to its ability to produce γ-lactones via the reductive coupling of α, β- unsaturated esters with carbonyl compounds, and the broad scope provided by this ability. The process by which these systems were formed starts with a carbonyl such as a ketone or aldehyde, which is reduced to form a ketyl type radical. The unsaturated compound then acts as a radicalophile to form the first bond with the ketyl radical, cyclisation follows. As a variety of γ- lactones couple in this way, investigation into the production of these lactones from more complex substrates such as xanthone was carried out.[16]

Figure 5. (a) Reported cyclisation reaction on ideal system with SmI_2 (b) SmI_2 facilitated lactone formation (c) SmI_2 facilitated conjugated lactone formation

The reductive cyclisation test of cyclohexanone (**11**) shown in Figure 5(a) was carried out successfully to produce **12**, (See SI - 5) however, when applied to our xanthone (**6**) even after 20 hours at room temperature there had been no reaction or indication of **13**. The reaction was repeated under reflux conditions and the number of equivalents of SmI_2 increased from 2.5 to 5. This was to address the possibility of the commercially obtained SmI_2 solution being of lower concentration than that stated, which is widely mentioned in literature regarding its use.[13] Subsequent reactions involved freshly prepared SmI_2 solution produced from Imamoto's method, standardised under nitrogen against a known volumetric solution of ultra-pure iodine in THF (See Table SI - 6).[17,18]

Following standardisation of the freshly prepared SmI_2 solution, further reactions were performed: two at room temperature for 20 hours as before, using precisely 2.5 equivalents of SmI_2: one of which with, and one without the s-Butanol additive. This would compare with previous attempts so as to rule out the possibility of the reaction being unsuccessful due to inconsistent SmI_2 solution concentrations, or indeed the inclusion of the alcohol proton donor additive which is usually required for this type of reductive coupling.[19]

Figure 6. Excess SmI_2 reduces the xanthone in the presence of a hydrogen donor

In the presence of the s-Butanol, xanthene was produced in the reaction mixture (Figure 6), implying that the carbonyl was simply reduced down to a CH_2, but also indicates that xanthone is incompatible with the reductive coupling and thus unable to produce rhodamine analogues **13** – **16** (Figure 5(b) & (c)), conceivably due to stabilisation of the ketyl radical species formed in the first step.

2.1.3 Directed metalation

As one of the better known routes to aryl containing structures, directed metalation was investigated in the context of rhodamine conjugate synthesis. The Wuts group explored the use of dialkyl hydrazides as directed lithiating agents and this showed promise.[20] Their work addressed the difficulty found when hydrolysing amide-type directed metalating agents and found that hydrazides were more favourable. Secondary butyllithium (s-BuLi) facilitated ortho-metalation of N',N'-dimethylbenzohydrazide (**17**) generated lithium species **18** (Figure 7(a)), followed by exposure to a number of standard electrophiles resulted in the free acid ortho addition product following oxidation. This is consistent with previously demonstrated amide chemistry, differing only by the mild oxidants required such as $CuCl_2$ or H_5IO_6.[20] More importantly, if the electrophile had aldehyde or ketone functionality it was noted that lactonisation often occurred spontaneously during workup and isolation, in the case of addition to cyclohexanone (**19**), leading to spirocyclic phthalide **20**. If this chemistry were compatible with xanthones, it could lead to a more reliable and cost effective route to rhodamine conjugates and potentially facilitate the generation of novel rhodamine analogues.

This route was initially tested on the substrate demonstrated in the literature (**19**) in order to confirm the viability of this reaction (See Figure 7(b) and supplementary information for data SI – 8). After confirmation, the same reaction conditions were applied to the simplest unfunctionalised xanthone core structure (**6**) with success (See Figure 7(c) and supplementary information for data SI – 9).

Figure 7. General scheme for direct metalation of xanthones

The hydrazide (**17**) shown in Figure 7. underwent ortho-metalation with s-BuLi at -78^{o}C followed by addition to **6**. Various reaction times and equivalents were assessed; each time the crude material was a complex mixture which defied purification after standard workup. However, acidification with concentrated HCl resulted in the slow formation of the spirophthalide which crystallised over night to form needle like white crystals which were washed with cold ether and isolated. Upon examination by ^{1}H NMR these appeared to be the expected product,

the previously unreported rhodamine core structure **21**. Apparently the acidification facilitated dehydrative cyclisation to form the upper spirophthalide ring, which allowed the compound to crystallise out of solution. This demonstrated the viability of the directed metalation approach for the production of further rhodamine analogues **22, 23** (Figure 7(c)), however the reaction proved intolerant of the amino/nitro arrangement (Figure 7(d)).

2.2 Improvement to standard peptide chemistry

2.2.1 Optimising coupling agents and conditions

The ability to scale-up the synthesis of rhodamine conjugates for affordable, real-world application had, at that point not been achieved, and we turned our attention back to the original coupling approaches to the rhodamine 110 core structure (**2**).

The problems associated with the coupling of arginine to rhodamine are a useful exemplar reaction to the coupling of any amino acid to rhodamine. In respect of the steric bulk, nucleophilicity and basic nature of the guanidinium side-chain moiety (leading to possible side-reactions) on the arginine, this combination represents the worst-case scenario for such a coupling.

Coupling Reagent	Isolated yield
EEDQ	-
PPh_3Cl	-
EDCI/Oxyma	5%
COMU	8%
HATU	25%

Table 1. Initial coupling of Boc-$(CBz)_2$Arginine to Rhodamine. Yields are the purified (Boc-$(CBz)_2$Arginine$)_2$-Rhodamine.

A variety of different coupling agents and protecting groups were used (Table 1) and it was possible to optimise the conditions to obtain an initial coupling yield of 25%.This was achieved when using the powerful coupling agent HATU, which has been shown to be effective in difficult couplings especially with sterically hindered amino acids.[21,22] Equally as important, the yield was consistent throughout several runs and represents a sound method for scaling the initial coupling.

2.2.2 Scale-up problems with deprotection of Cbz groups

The previously reported synthesis of (Boc-Val-Pro-Arg)$_2$–Rhodamine involves the direct attachment of an exhaustively CBz protected Boc-arginine with a particularly poor yield (17%), with subsequent amino acids being added prior to deprotection *via* hydrogenation. However, this approach is not amenable to scale-up and larger amounts of (Boc-Val-Pro-Arg(CBz)$_2$)$_2$–Rhodamine cannot be successfully deprotected either by using standard or non-standard hydrogenation techniques. This is evidenced in a report by Peakdale Molecular Ltd, which can be found in supporting information (SI - 10 and SI - 11). Indeed, the rate of hydrogenation of the central benzyl ester was shown to be greater for (Boc-Val-Pro-Arg(CBz)$_2$)$_2$–Rhodamine (**1**) than rate of loss of the Cbz groups (Figure 8).

Figure 8. Diagram showing the hydrogenolysis of the lactone of (Boc-Val-Pro-Arg(CBz)$_2$)$_2$–Rhodamine (2**4**) and Lactone formation using DDQ and the reduced (Boc-Val-Pro-Arg)$_2$–Rhodamine derivative (**25**).

For the real world application of (Boc-Val-Pro-Arg)$_2$–Rhodamine (**1**) to the *in situ* detection of *S. aureus* in both clinical and non clinical environments, the final CBz deprotection step and its selectivity is critical and equally applicable to the development of other protease probes containing the CBz protecting group. This is because the hydrogenated lactone (**25**) is not fluorescent and so its liberation upon cleavage of the peptide side chain produces no fluorescence contrast change, rendering the probe ineffective.

Since all hydrogenation steps failed to selectively remove the CBz groups and leave the central lactone intact when scaled up, an alternative approach was adopted. If we cannot avoid the loss of the lactone, would it be possible to reform the required functionality after exhaustive hydrogenation (removing the Cbz groups and opening up the lactone to give **25**) through subsequent oxidation? The oxidant 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was selected for its oxidative coupling ability and a test reaction was set up to be monitored by proton NMR. Dissolution of the inactivated product in MeOD with an excess of DDQ allowed reaction progress to be monitored by proton NMR at 1 minute intervals.[23] We were delighted to observe the reappearance of the lactone functionality through disappearance of the signal for the benzylic proton in question. This method was then applied to a scaled up completion of the synthesis, using 8 equivalents of DDQ in MeOH, it was possible to reform the central lactone of the rhodamine core (Figure 8) to yield **1**.

The yield of this reaction is high (83%) and the correct structure was verified with NMR and HR MS (See SI - 12 & SI - 13). Details of the synthetic chemistry, including general experimental spectroscopic data are available in supplementary information.

2.3 Biological Testing For Surface Detection

2.3.1 Preparation of bacterial samples

Clinical isolates of MRSA, MSSA, *S. epidermidis* and *E. coli*, were revived from - 80°C and cultured overnight on Brain Heart Infusion (BHI) agar at 37°C, followed by two subcultures on nutrient agar. For each experiment, an inoculum of a respective bacterial species was cultured overnight in 100 mL nutrient broth under shaking conditions, and each bacterial sample was then washed with 1 x phosphate buffered saline (PBS) by centrifugation at 4000 rpm, and bacterial concentrations of 10^4, 10^3 and 10^2 colony forming units per ml (CFU mL^{-1}), were created by serial dilution of the bacteria in 1x PBS.

Co-cultures of varying ratios of MRSA/MSSA with *S. epidermidis* and *E. coli* were produced using the relative cell count percentages as detailed in the table below (Table 2).

Co-culture	**MRSA or MSSA**	***S. epidermidis***	***E.coli***
A	20 %	10%	70%
B	20 %	20%	60%
C	20 %	30%	50%
D	20 %	40%	40%
E	20 %	50%	30%
F	20 %	60%	20%
G	20 %	70%	10%

Table 2. Bacterial co-culture ratios designated A-G

Stainless steel coupons were washed, rinsed and placed in a glass petri dish followed by sterilisation using an autoclave. These were placed in an incubator overnight to ensure thorough drying of each plate. A bacterial inoculum of a single strain of MRSA or MSSA, in addition to *S. epidermidis* and *E. coli*, was cultured overnight in 100 mL nutrient broth. A 4 mL aliquot of each bacterial suspension was then cultured in 100 mL nutrient broth aerobically for 4 hours under shaking conditions. Each bacterial sample was harvested by centrifugation at 4000 rpm and washed with 1 x Phosphate buffered saline (PBS), and bacterial concentrations of 10^4, 10^3 and 10^2

CFU mL^{-1} were generated by serial dilution of bacteria in 1x PBS supplemented with 10 % heat inactivated fetal bovine serum (FBS).

A 50 μL aliquot of each bacterial sample was pipetted aseptically onto the steel coupons, and these were incubated aerobically overnight at 37°C. A cotton wool swab soaked in PBS was then used to remove the bacteria, and this was placed in an Eppendorf tube with 100 μL of sterile PBS, the tube was then vortexed to dislodge bacteria from the swab.

This was performed twice: once with using the LGX test (using a 100 μM LGX solution), and once using bacterial culture to determine cell viability. Swabbing was performed as described above in the experimental section. This process was repeated 3 times and the results shown in Figure 9 and 10 are averages with standard deviation error bars.

2.3.2 Preparation of LGX

A 100 μM and 50 μM solution of LGX was prepared by dissolving fluorophore **1** in 2.5 % methanol followed by dilution in 1 x PBS. Solutions of Tris Base (0.05 M) and NaCl (0.1 M) in deionised H_2O were added to the solution until an optimum pH of 8.5 was achieved. The appropriate amount of human prothrombin in 1 x PBS was then added to produce final prothrombin concentrations of 83.6 nM and 41.8 nM, respectively. The resulting solution was termed "LGX". The concentrations of LGX, subsequently discussed, refer to the concentration of the active fluorophore **1**. The presence of coagulase positive bacteria was determined by addition of 100 μM LGX solution followed by5μL prothrombin. The fluorescence was then recorded (excitation 488 nm and emission 525 nm) at 15 minute intervals over a 2.5 hour time period using a benchtop fluorimeter (BMG FLUOstar OPTIMA).

3. Discussion

As can be observed, the majority of samples test positive for MRSA (denoted by a fluorescence reading exceeding 20,000 RFU). Figure 7 demonstrates the effects of co-culturing *E. coli* and *S. epidermidis* on the efficacy of detecting MRSA using the LGX system. The graph depicts the mean fluorescence response of 2 strains of MRSA tested on separate occasions.

Figure 9. Fluorescence response of LGX for the different co-cultures of MRSA (20% of 10^3 CFU/ml) over 2.5 hours. Results also include LGX (100uM), Negative Control (*E. coli* 10^3 CFU/ml) and Postive Control (MRSA 10^3 CFU/ml).

Figure 10. Fluorescence response for LGX for the different co-cultures of MSSA (20% of 10^3 CFU/ml) over 2.5 hours. Results also include LGX (100uM), Negative Control (*E. coli* 10^3 CFU/ml) and Postive Control (MSSA10^3 CFU/ml)

Over the 2.5 hour test period, fluorescence intensity increases gradually for all samples with the positive control (containing the coagulase positive MRSA strain alone) showing a much increased response. The varying ratios of *S. epidermidis* and *E. coli* however do not bear any conclusive effect on the efficacy of LGX as the fluorescence intensity is not affected by more *S. epidermidis* and less *E. coli* or *vice versa*. Also, the absence of *S. epidermidis* and *E. coli* also does not affect the potential of LGX to detect very small amounts of MRSA (at 20% of the original bacterial concentration), and so, this data suggests that the presence of other bacteria does not hamper the efficacy of LGX to detect MRSA.

However, it is noted that the presence of FBS in the experimental system may interfere with the fluorescence potential of LGX, and this requires further investigation.

Figure 8 demonstrates the effects of co culturing *E. coli* and *S. epidermidis* and MSSA (a coagulase positive organism) on the efficacy of detecting MSSA using the LGX system (ratios of MSSA/MRSA with contaminating organisms can be found in Table 2). As with MRSA (Figure 8), the data shows the average of 2 strains of MSSA tested on separate occasions. Over the 2.5 hour test period, fluorescence intensity remained constant, and the varying ratios of *S. epidermidis* and *E. coli* do not tend to bear a conclusive effect of the efficacy of LGX as the fluorescence intensity is not affected by more *S. epidermidis* and less *E. coli* or *vice versa*. Also, the absence of *S. epidermidis* and *E. coli* did tend to increase the potential of LGX to detect very small amounts of MRSA (at 20% of the original bacterial concentration) (Figure 7). This observation is not so

clear when considering MSSA (Figure 8). This result is interesting in the respect that previous experiments have shown that LGX is more sensitive at detecting MSSA when compared to testing for MSSA (fluorescence intensity peaks at lower time points). This may be indicative that this system, in a more "real life" scenario, may be more sensitive and selective in terms of MRSA detection over MSSA or that the expression of coagulase might be heightened in the MRSA strains. However, as with the previous two experiments, the presence of FBS again may also be a factor in the detection of fluorescence from LGX, this observation clearly requires further investigation.

Additionally, the swabbing experiment was repeated, and 20 μL of each bacterial ratio (for MRSA and MSSA) was plated onto nutrient agar, mannitol salt agar, MRSA agar and MacConkey agar, to confirm the ratios of bacteria were correct and also if this method of determining a "real life" situation in the absence of culturing bacteria together would make a viable method for testing the protocol. Bacterial cell counts for each sample showed an appropriate level of bacterial species in each co-culture set based upon the defining parameters of said culture grouping confirming that this method does not affect cell viability of one bacteria over any other.

The value of LGX in the detection of MRSA/MSSA without the need for culturing of the bacteria has been reported in the context of its use for analysing swabs from patients. In this paper, the authors report strategies for scale-up and show a significant increase in the achievable yield. For the first time reformation of a benzylic lactone after hydrogenolysis is reported for a rhodamine fluorophore and the synthetic organic significance is that the tripeptide substrate is unaffected by the use of DDQ, highlighting the practical value of this approach to an applied chemistry problem.

The co-culturing evaluation demonstrates the potential application of LGX to surface detection of MRSA/MSSA in the 'real world' scenario. Significant fluorescence was still detectable despite the presence of contaminating bacterial strains (*E. coli* and *S. epidermidis*).

Author Contributions

LB and AS Carried out chemical synthesis of LGX/Derivatives and analysis of spectroscopic data

LM-R and MF designed and conducted microbiological experiments with LGX

ALG advised on the synthetic strategy, identified the DDQ dehydrogenation step and wrote the manuscript with LB.

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Supplementary Material

Synthetic experimental data is included along with tables referencing the strategies employed for the improved synthesis of LGX and its analogues. These are referenced in the manuscript as figures/tables SI-1 to SI-13

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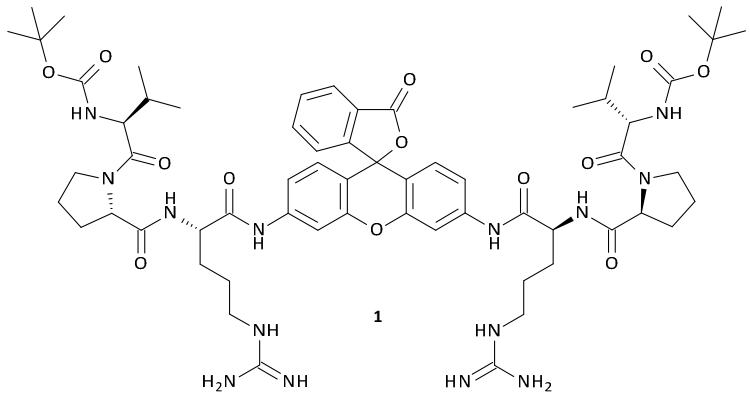

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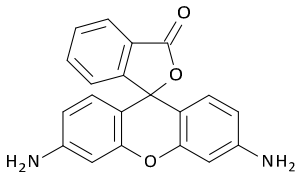

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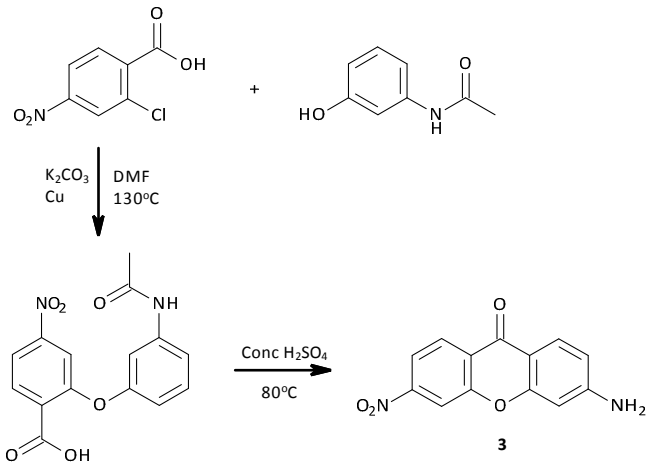

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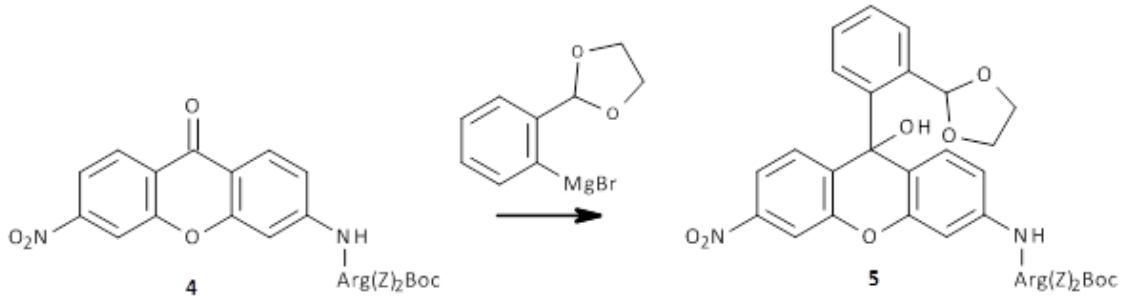

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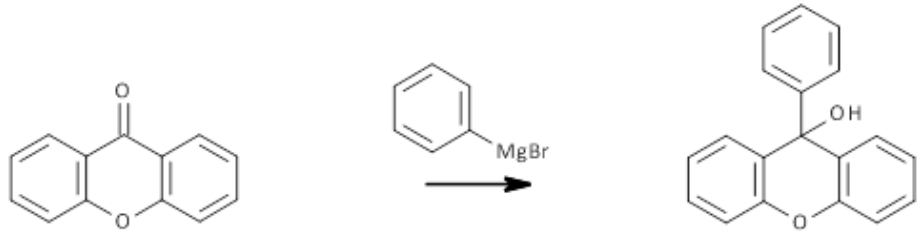

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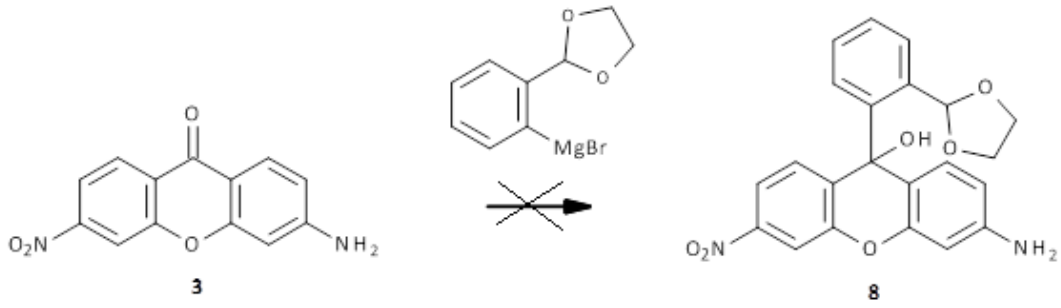

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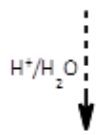

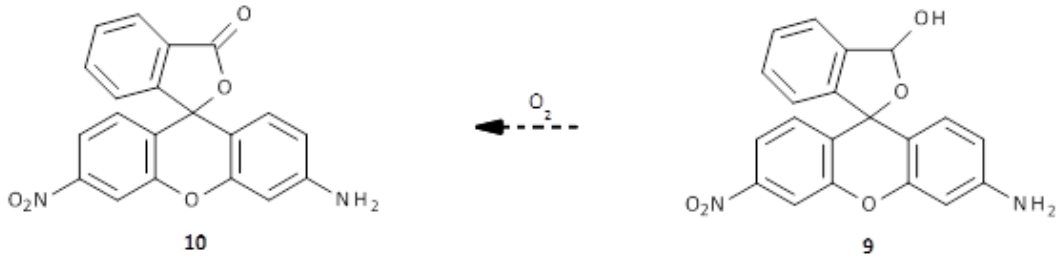

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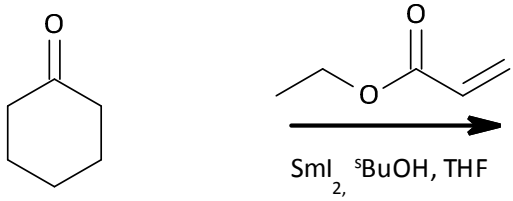

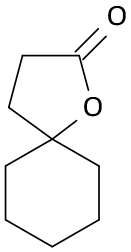

11 **12**

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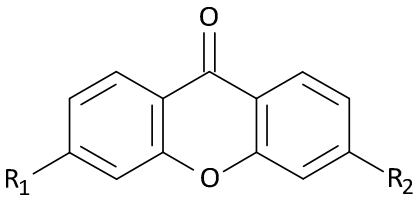

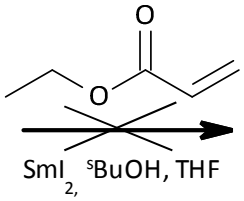

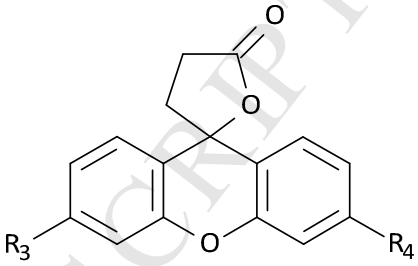

3, 6 **13, 14**

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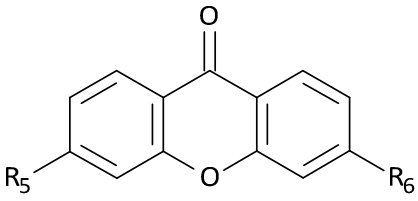
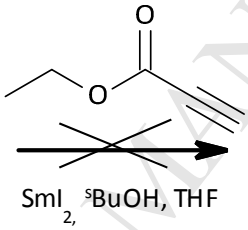
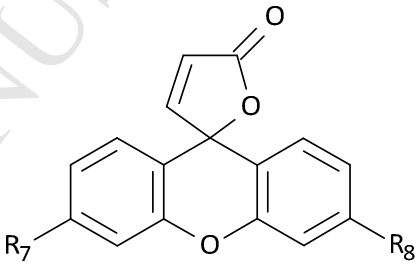
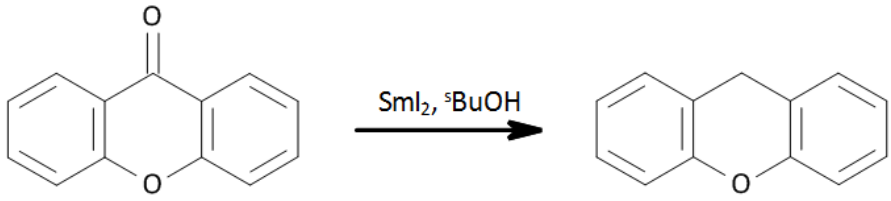






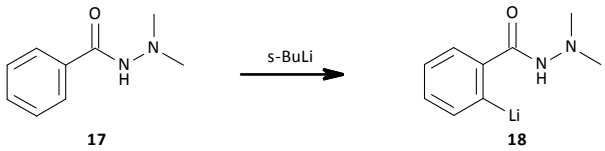
3, 6 **15, 16**

$R_1 = NO_2$, $R_2 = NH_2$ (**3**), $R_1 = R_2 = H$ (**6**)
$R_3 = NO_2$, $R_4 = NH_2$ (**13**), $R_3 = R_4 = H$ (**14**)
$R_5 = NO_2$, $R_6 = NH_2$ (**15**), $R_5 = R_6 =$ (**16**)



6 Xanthene

(a)



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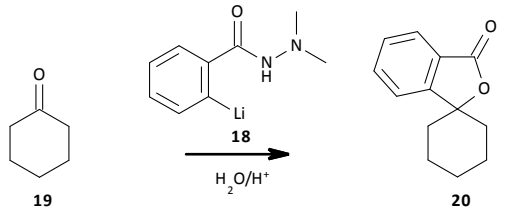

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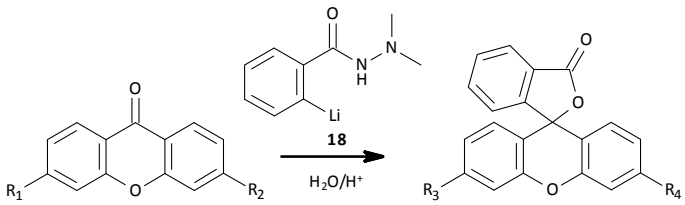

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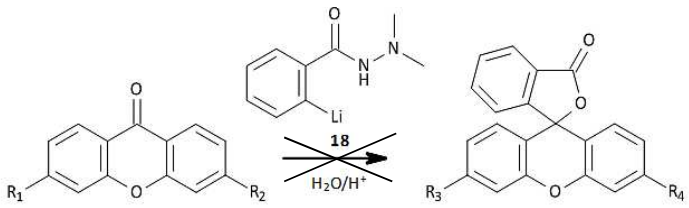

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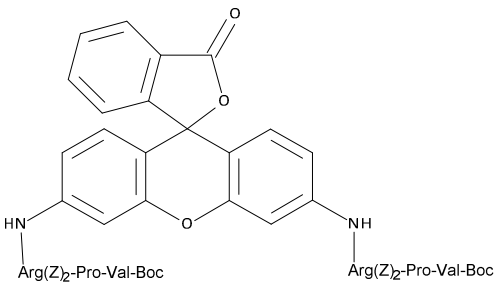

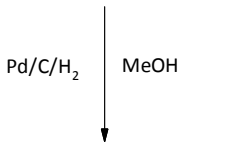

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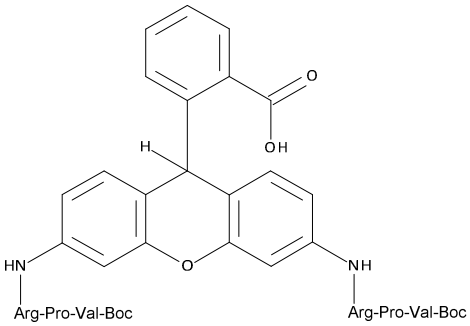

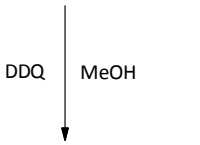

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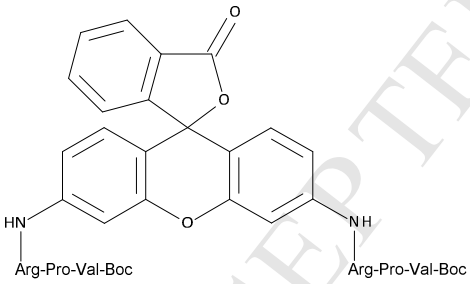

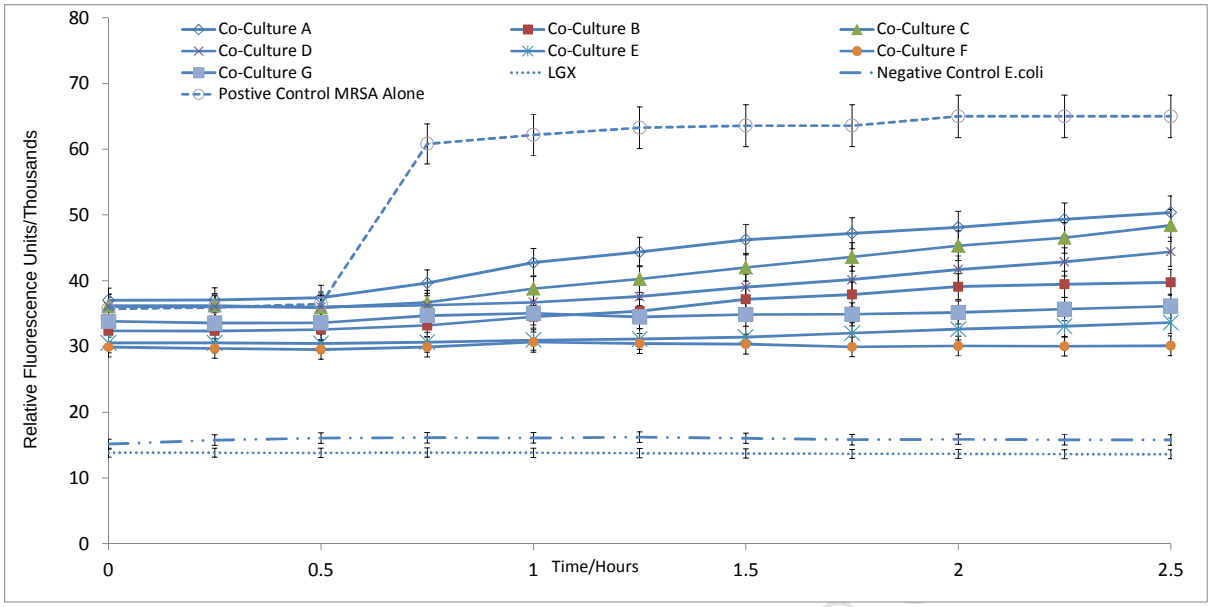

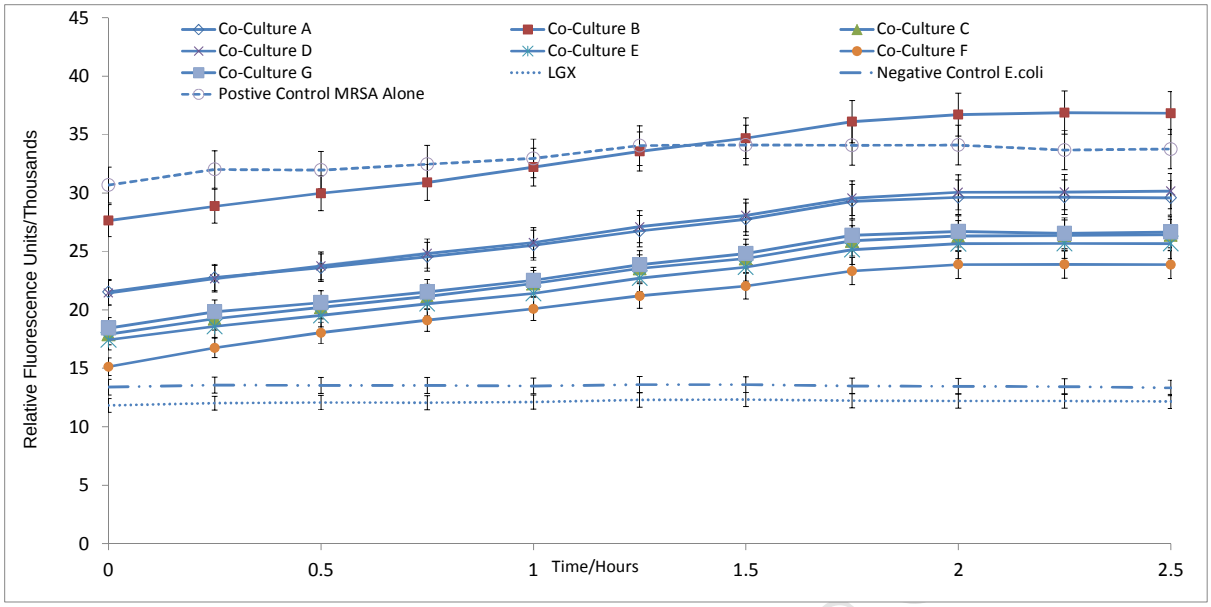

Highlights

- Scale-up of a rapid, simple fluorescence test for MRSA/MSSA is reported for the first time.
- MRSA/MSSA was detectable in our test despite the presence of contaminating bacterial strains (*E. coli* and *S. epidermidis*)
- The co-culturing evaluation demonstrates the potential application of LGX to surface detection of MRSA/MSSA in the 'real world' scenario.