An OregonGreen488-labelled d-amino acid for visualizing peptidoglycan by super-resolution STED nanoscopy

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METHOD

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INTRODUCTION

The peptidoglycan (PG) layer is a continuous elastic mesh that surrounds the cytoplasmic membrane of bacteria [1]. It is a single layer thick in Gram-negative strains, but has a multi-layered structure in Gram-positive strains. Structures of the PG from the Gram-positive strains Staphylococcus aureus and Bacillus subtilis, obtained using atomic force microscopy, indicate that it is a non-homogeneous porous mesh that is tens of nanometres thick [2]. This mesh defines the shape of the bacterium and provides mechanical strength against the internal turgor pressure of the cell [3].

The PG layer is continually synthesized and remodelled so that bacterial cells can grow and subsequently divide (reviewed in [4]). These processes require the co-ordinated activities of PG hydrolases (lytic transglycosylases, amidases, endopeptidases and carboxypeptidases) and PG synthases (glycosyltransferases, transpeptidases and bifunctional enzymes) [5]. The former open up the PG layer, whilst the latter insert a disaccharide of N-acetylglucosamine and N-acetylmuramic acid containing a short stem peptide. The disaccharide is polymerized into glycan strands and the stem peptides are cross-linked to create the layer. Maintaining the integrity of the PG layer is essential for cell viability, as cells lyse when it is compromised, for example, through the inhibition of PG synthases by ß-lactam antibiotics [6, 7].

Fluorescent d-amino acids (FDAAs) are molecular probes that are widely used to label the PG layer. They are integrated by D,D- and L,D-transpeptidases, which exchange free d-amino acids and FDAAs into position 4 (Escherichia coli) or 5 (B. subtilis) of the stem peptide [8]. Once integrated, they can be visualized by fluorescence microscopy. The VanNieuwenhze laboratory has developed more than 10 different types of FDAAs [9, 10], as well as rotor-fluorogenic versions (RFDAAs) [11]. This collection includes fluorophores that span the entire visible spectrum, providing options for multi-colour labelling and imaging of the PG by sequential addition of different FDAAs. The collection of FDAAs has been used to study the morphology and growth of the PG, to correlate sites...
of PG synthesis with specific enzymes, to study PG turnover, and to determine the efficacy of antibiotics in perturbing PG synthesis (reviewed in [12]).

In recent years, super-resolution nanoscopy has emerged as a powerful technique for studying PG biosynthesis at the nanoscale. Two commonly used techniques are 3D structured illumination microscopy (3D-SIM) and stimulated emission depletion (STED) (reviewed in [13–15]). 3D-SIM can be used to reveal the dynamic and time-dependent processes, but only offers modest spatial resolution compared to other methods. STED imaging generally yields a higher spatial resolution but is typically limited to fixed samples, and is therefore not useful for studying dynamic processes. These methods have been used to probe the relative organization of fluorescently labelled proteins involved in cell division and PG biosynthesis [16–18]. Unfortunately, most FDAAs are not well suited for STED nanoscopy as the attached fluorophores do not possess the requisite photophysical properties. Only Atto488DA (green-shifted), Cy₅-DA and TADA (both yellow to orange shifted) could potentially be used [10], but they are not commercially available and have therefore not yet been extensively tested.

Herein we describe the synthesis and characterization of an OregonGreen488-labelled d-amino acid (OGDA). The OregonGreen488 fluorophore has an excitation maximum of 501 nm and an emission maximum of 526 nm. It was chosen because it has a high extinction coefficient and fluorescence quantum yield, making it one of the best performing dyes for STED nanoscopy [19]. A fluorescein-labelled n-amino acid (FLDA) was also synthesized and used for comparison to the STED imaging with OGDA. The fluorescein fluorophore has an excitation maximum of 494 nm and emission maximum of 512 nm. It is more suitable for confocal fluorescence microscopy as it lacks the proper photostability characteristics for STED nanoscopy.

THEORY AND IMPLEMENTATION

Synthesis of two novel FDAAs

Two novel FDAAs were synthesized using a suitably protected d-alanine analogue, namely, N⁴-Boc- d-2,3-diaminopropionic acid, reacting with N-hydroxysuccinimide esters of either fluorescein (as a mixture of positional isomers) or OregonGreen488 (Scheme S1, available in the online version of this article). The coupling was performed under basic conditions using previously developed experimental procedures [10, 20] followed by deprotection of the tert-butoxycarbonyl group with acid and finally purification on a short reversed-phase C18 column. The fluorescein-labelled n-alanine is hereafter referred to as FLDA, and the OregonGreen488-labelled d-alanine is referred to as OGDA.

Incorporation of FDAAs into the peptidoglycan of Gram-positive bacteria

We tested whether three Gram-positive strains could incorporate FLDA and OGDA into their PG. Exponentially growing Streptococcus mutans, S. aureus and B. subtilis were labelled with 1 mM of either FLDA or OGDA for 1–5 min. This labelling time roughly corresponded to between 2% (S. mutans, S. aureus) and 20% (B. subtilis) of the cell cycle. Cells were then washed, fixed and imaged by confocal microscopy so that regions of incorporation into the PG layer could be visualized. We observed that both FLDA and OGDA were incorporated into the division site in nearly all cells (Fig. 1a, b). This observation was anticipated, as this is where PG synthesis is predominantly occurring [10, 20]. These data indicate that both FLDA and OGDA are efficiently incorporated into newly synthesized PG of Gram-positive bacteria and that they can be imaged by confocal microscopy.

We also imaged the same cells with super-resolution STED nanoscopy [21]. As anticipated, FLDA was challenging to image (Fig. 1a), because the photophysical properties of fluorescein are not optimal for super-resolution microscopy. In contrast, OGDA was relatively easy to image (Fig. 1b), as the OregonGreen488 fluorophore is highly resistant to bleaching and extremely photo-stable [19]. Quantification of fluorescence intensities from raw STED images indicated that OGDA was on average twice as bright as FLDA (Fig. 1c). To assess the resolution that could be obtained using OGDA, the STED images of S. mutans were further analysed. Here the minimal resolvable peak-to-peak distance of fluorescence maxima at the division site of OGDA-labelled cells was estimated to be approximately 85 nm (Fig. 2). It is anticipated that this number will vary from experiment to experiment. Nevertheless, it unequivocally shows that it is viable to use OGDA to image PG incorporation using super-resolution nanoscopy.

We carried out a virtual time lapse in S. mutans with OGDA and the yellow to orange shifted TADA [10]. This enabled us to capture the history of PG synthesis at the division site over time. In this experiment S. mutans cells were first pulse-labelled with OGDA, and then with TADA at intervals of 10 min (Fig. 3a). We observed that the OGDA was initially inserted into the PG at the division site, but was subsequently pushed away from the division site as new TADA-labelled PG was synthesized, resulting in a stripe

Impact Statement

Herein we describe the synthesis and characterization of a d-amino acid conjugated to the OregonGreen488 fluorophore (which we have named OGDA). We demonstrate that OGDA is incorporated into the peptidoglycan layer of Gram-positive, and some Gram-negative, bacteria. Since the OregonGreen488 fluorophore has suitable photophysical properties, OGDA can be imaged by super-resolution nanoscopy. OGDA is compatible with other fluorescent d-amino acids and will be a valuable resource for studying peptidoglycan biosynthesis, inhibition of peptidoglycan biosynthesis by antibiotics, and peptidoglycan turnover.
pattern (Fig. 3b). If cells were in a very late phase of division when the labelling was initiated, TADA was also incorporated into the division sites of the daughter cells (Fig. 3c).

As STED is a relatively complex microscopy approach and not yet accessible to many researchers, we also imaged cells by super-resolution structured illumination microscopy (SIM), a technique that is more broadly accessible (Fig. 3d). Finally, we also reversed the labelling sequence, with TADA first and OGDA last, to ensure that the order of the labelling did not influence the outcomes. As expected, we observed similar results, but with an inverted colour pattern (Fig. S1). Taken together, these data indicate that OGDA is compatible with TADA for super-resolution imaging using both STED and SIM [10].

**Incorporation of FDAAs into the peptidoglycan of Gram-negative bacteria**

It has previously been documented that most FDAAs do not work in Gram-negative bacteria because they cannot cross the outer membrane [11, 12]. This membrane acts as a permeability barrier to most chemical compounds, in particular those that are larger than 500–600 Da [22]. As OGDA is 498 Da, we tested whether it could be incorporated into the model Gram-negative bacterium *E. coli*.

**Fig. 2.** Confocal and STED images of a *S. mutans* cell labelled with OGDA. The fluorescence intensity was measured by drawing a line over the OGDA signal along the long axis of the cell. The graph shows the OGDA fluorescence intensity trace over the septum (yellow dotted line on the STED image). The peak-to-peak distance was ~85 nm. Scale bar, 500 nm.
In this series of experiments, cells were labelled with 1 mM of OGDA for 5 min, which corresponds to less than 20% of the cell cycle. Cells were then washed, fixed and imaged by confocal microscopy and STED so that regions of incorporation into the PG layer could be visualized. Although we observed fluorescence at the division site of some cells (Fig. 4a), the vast majority of cells were not labelled (data not shown). We also tested whether a strain lacking the glycosyltransferase WaaG (ΔwaaG) was more permeable to OGDA. This strain is a 'deep rough' lipopolysaccharide mutant that is considered 'leaky', as it is hyper-sensitive to antibiotics such as novobiocin (612 Da), erythromycin (733 Da), rifampicin (823 Da) and vancomycin (1449 Da) [23]. However, we did not observe increased incorporation of OGDA (data not shown). We did, however, observe incorporation of OGDA in Zymomonas mobilis, a Gram-negative bacterium that is used for industrial production of ethanol (Fig. 4b).

Here we observed that >95% of all cells were labelled at the division site after 5 min with 1 mM OGDA. Even though this labelling time corresponded to less than 5% of the cell cycle, many of the cells were also labelled in the perimeter of the membranes (Fig. S2).

CONCLUSION

Herein we describe the synthesis of two new FDAAs in the green spectrum: OregonGreen488–FDAA (OGDA) and fluorescein–FDAA (FLDA). Like FDAAs that have been described previously, they are integrated into the PG layer and can be visualized by fluorescence microscopy [9, 10]. They can therefore be used as molecular probes for localizing sites of peptidoglycan biosynthesis, for determining how antibiotics inhibit peptidoglycan biosynthesis and for studying peptidoglycan turnover.
Notably, OGDA is suitable for super-resolution STED nanoscopy as the OregonGreen488 fluorophore is resistant to bleaching and extremely photo-stable [19]. We used OGDA and STED to image sites of PG insertion at a resolution of ~85 nm. Moreover, we demonstrated that OGDA could be used in parallel with the orange-shifted TADA for dual-colour STED imaging. In principle, OGDA should also be compatible with the orange-shifted Cy3B ADA for STED imaging [10]. The availability of OGDA and STED to study PG insertion will help researchers to more accurately pinpoint sites of PG insertion in time and space, and determine whether given PG synthesizing proteins co-localize at these sites. This may help to address a number of open questions in bacterial cell wall synthesis.

Although OGDA worked in all in Gram-positive strains tested, it may have limited used in Gram-negative bacteria. We noted that it was incorporated into the PG layer of Z. mobilis, but that it was not efficiently incorporated into the PG layer of the E. coli strains we tested. This is most likely because the molecular weight of OGDA (498 Da) is close to the molecular weight cut-off for porins in the outer membrane in E. coli (500–600 Da) [22]. This problem has been noted for most FDAAs [11, 12].

**METHODS**

**General**

NHS–fluorescein (5/6-carboxyfluorescein succinimidyl ester), mixed isomer, OregonGreen488 carboxylic acid, succinimidyl ester, 6-isomer and Nα-Boc-α-2,3-diaminopropionic acid were purchased from Thermo Fisher Scientific (Kandel, Germany). Dry acetonitrile (ACN) was purchased from Honeywell Research Chemicals (Bucharest, Romania), and dry N,N-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Acros Organics (Morris Plains, NJ, USA). N,N-diisopropylethylamine (DIPEA) was purchased from Sigma-Aldrich (Geel, Belgium). All reagents were used as received. A nitrogen flow was used for reactions requiring inert atmosphere. Purification was performed on Sep-Pak C18 Plus short cartridges, 55–105 µm particle size (Waters, Milford, MA, USA). MQ water was obtained from the Elga Purelab Ultra Genetic Water Purification System (High Wycombe, UK). One-dimensional 1H and 13C, and 2D multiplicity-edited 1H,13C-HSQC nuclear magnetic resonance (NMR) spectra for the characterization of isolated compounds were recorded in MeOH-d4 at 298 K on a Bruker AVANCE III 700 MHz equipped with a 5 mm Z-Gradient Cryoprobe (1H/13C/15N), a Bruker AVANCE III 600 MHz spectrometer equipped with a 5 mm TXI inverse Z-Gradient 1H/D-31P/13C and a Bruker AVANCE III HD 400 MHz spectrometer equipped with a 5 mm BFB/H/D 5.0 Z probe. The NMR chemical shifts (δ) are reported in p.p.m. and referenced to residual methanol δH 3.31, internally to the MeOD-d4 solvent peak, δD 49.00, or externally to fluorobenzene, δD -115.42, in MeOD [24]. J coupling constants are reported in hertz (Hz). High-resolution mass spectra (HRMS) were recorded on Bruker Daltonics microOTOF or microTOFQ spectrometers (Bremen, Germany) using electrospray ionization (ESI) in the positive mode. Samples for mass spectrometry (MS) were prepared using a solution of acetone and H2O in a 1 : 1 ratio.

**Synthesis of fluorescently labelled α-amino acid**

Fluorescent dye was allowed to attain room temperature (RT) before the vial was opened. To a flame-dried 5 ml flask succinimidyl ester dye derivative (5 mg, 1 eq) and Nα-Boc-α-2,3-diaminopropionic acid (1.4 eq) were added under nitrogen flow. Dry DMF (0.2 ml) and freshly distilled DIPEA (2.5 µl) were then added and the reaction was left under stirring for 2 h while being monitored by HRMS. Thereafter, the reaction mixture was co-evaporated under vacuum with dry toluene (40 °C).

Removal of the Boc protecting group was achieved by treatment with DCM/TFA (1 : 1, 2 ml) for 30 min at RT. The crude, concentrated under reduced pressure, was redissolved in MQ water (0.5 ml) and few drops of ACN and passed through a Sep-Pak C18 Plus short cartridge that had been pre-equilibrated with MQ-water (10 ml) and ACN (10 ml), using in sequence MQ water (5 ml), water/ACN (9 : 1, 5 ml), water/ACN (7 : 3, 5 ml) and ACN (5 ml). Fractions collected were checked by MS, pooled and freeze-dried twice to yield an orange/red powder.

**Analytical data**

Fluorescein α-amino acid (FDAA)

(R)-2-amino-3-(3′,6′-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-carboxamido) propanoic acid and (R)-2-amino-3-(3′,6′-dihydroxy-3-oxo-
3H-spiro[isobenzofuran-1,9’-xanthene]-6-carboxamido propanoic acid

1H NMR (700 MHz, MeOD, 298 K): δ 8.52 (dd, 1H, J 1.6 Hz, J 0.7 Hz, I 1), 8.23 (dd, 1H, J 8.0 Hz, J 1.6 Hz, I 11), 8.18 (dd, 1H, J 8.1 Hz, J 1.5 Hz, I 2), 8.12 (dd, 1H, J 8.1 Hz, J 0.7 Hz, I 2), 7.71 (dd, 1H, J 1.5 Hz, J 0.7 Hz, I 2), 7.34 (dd, 1H, J 8.0 Hz, J 0.7 Hz, I 1), 6.77–6.69 (4H, Ar), 6.61–6.57 (2H, Ar), 4.00 (1H, Hα, I 1), 3.90 (1H, Hβ, I 11/2), 3.88 (1H, Hα, I 11/2), 3.87 (1H, Hβ, I 11), 3.77 (1H, Hα, I 12), 3.75 (1H, Hβ, I 12). 13C NMR (175 MHz, MeOD, 298 K) selected data: δ 172.9, 172.8, 171.9, 171.8, 170.8, 170.7 (6×C=O), 156.0, 141.2 (2×quat), 137.8, 135.3, 131.7, 131.5, 128.0, 127.7, 127.0, 126.3, 116.3, 112.8, 104.6, 58.1, 58.0 (2×C), 43.1, 42.9 (2×C). ESI-HRMS: [M-H]− m/z calculated for C16H15N2O8F2 497.0802, found 497.0787. Yield: 81 %. See Fig. S3.

Images

Gated STED (gSTED) images were acquired on a Leica TCS SP8 STED 3× system in a similar way to that described by Söderström et al. [18] using a HC PL Apo 100× oil immersion objective with NA 1.40. OregonGreen488 was excited using a white laser operated at 488 nm. A STED laser line operated at 592 nm was used as a depletion laser. A detection time delay of 1.3 ms was applied. The total depletion laser intensity was in the order of 40 MW cm−2 for all STED imaging. The final pixel size was 24 nm and the scanning speed was 600 Hz. The pinhole size was set to 0.9 AU.

SIM images were acquired using a Zeiss ELYRA PS1 equipped with a pcO.edge sCMOS camera and 100×1.46 NA plan Apo oil immersion objective. The final pixel size in SIM images was 25 nm. Individual images were acquired using an acquisition time of 500 ms−1 image (a total of 25 images were acquired per SIM image reconstruction) and subsequently reconstructed from the raw data using ZEN2012 software [26].
The authors declare that there are no conflicts of interest.

**Author contributions**

B. S. and D. O. D. conceived the study. B. S. and A. R. performed the experiments. All authors analysed the data and wrote the manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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