

Electronically tuned N-terminal glycine selective bioconjugation enables in vitro and in vivo species-specific *Staphylococcus aureus* targeting

Corresponding Author: Professor Vishal Rai

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors described an aldehyde-based chemical probe designed to conjugate with N-terminal glycine. However, this aromatic aldehyde exhibits excessive reactivity in live cells, especially when an electron-withdrawing group is attached, which compromises its selectivity. Due to this fundamental issue, the paper is not suitable for publication.

Reviewer #2

(Remarks to the Author)

The manuscript by Kumar et al. reports a chemical tool for modifying the N-terminal glycine using a newly developed Rapi-Gly-tag reagent. This technology is an advance over existing N-terminal gly modification methods as it uses chemical tuning of the aldehyde rather than H-bonding directed activation of the aldehyde. This allows the use of the reagent in conditions compatible with in vivo and cellular applications. The authors show that the proposed technology works on N-terminal Glycine-containing proteins, using Insulin as the model protein. Further, the authors validate the method using six-amino acid peptides. The technology was also used to selectively label the N-terminal glycine on a unique pentaglycine interpeptide bridge on the *S. aureus* peptidoglycan cell wall. As the pentaglycine interpeptide bridge is important for membrane integrity, the authors show that disrupting it affects the bacterial viability and biofilm formation. The antimicrobial effect was further tested using a *Drosophila* model and multiple mouse skin infection models.

Overall, this manuscript introduces a potentially valuable new chemical modification tool for targeting N-terminal glycine. The manuscript has a vast amount of data (with 9 main figures and over 160 supplemental figures) and the new labeling method is applied to many different systems both in vitro and in vivo. The work is generally executed thoroughly, and most of the conclusions are justified. The main issues with the work are the lack of key controls in places, lack of clear analysis of overall target selectivity and the presentation and discussion of the results. This manuscript has a large number of figures which do not focus on concisely presenting the key findings, making it not likely accessible to an average reader. The text is overly wordy and generally unclear in sections. The intro has many citations to other work which is mentioned in a way that is hard to follow because it lacks details. Many of those other citations are not needed. The authors need to concisely present the key prior art, how it works and why the new method is better. That is what is relevant for this paper. Also, the figures are overdone with too many diagrams and images that are not needed. This comes at the expense of showing the data in a way that a reader can follow without having to process so much extra noise. Regardless of the issues with the controls and presentation of the results, the work is potentially exciting and likely to be useful for many applications. This work fits well with the broad audience of Nature Communications and could be made suitable for publication after the authors address all the comments suggested below.

Major points:

1. This paper is an example of trying to do too much in one paper, combined with excessively complex and large figures and somewhat confusing text resulting in a paper which is hard to get through. The results seem quite strong and the method is good but this paper needs to be distilled down to something that an average reader could actually process.

2. The authors have chosen to use unusual methods to display simple data. For example, in figure 2c they use some kind of spider web like diagram when a simple bar graph would make more sense. Similarly, panels d-g should be bar or line graphs with error shown. For panel h there is no need for diagrams and images of proteins and eppendorff tubes with data shown using a cartoon speedometer. The data should simply be plotted with error bars included. Is there error for panel K?
3. In figure 3 there is no need to show the protein folds and panel c is just a cartoon of the labeling which is not needed. The data should be shown for this experiment – which is shown in panel b but could be just a single simple bar graph.
4. For figure 6 the interesting data is the time and concentration dependence which is in the supplemental figures. The overall intensity of labeling could be quantified and plotted to show the saturation of labeling with some representative images to show what the labeling looks like at a high magnification. The panel g is just another cartoon with no actual data.
5. A key experiment is missing to show how selective the labeling with the probe actually is. The authors need to treat cells with the alkyne tagged probe at the optimal concentration and time and then do click to add fluorophore and run the gel to show protein labeling. My guess is that there will be proteins that get labeled by the probe. Similarly, they do the pull down and mass spec but only look for the glycopeptide from the cell wall. The key question is what proteins are labeled? It would be interesting and important to use the probe for full chemoproteomics to identify any labeled proteins not just the Gly5 peptide. Reports show that the bacterial 30s ribosomal protein S3 also contains an N-terminal glycine after processing the formyl-methionine residue by methionine aminopeptidase. Do the authors find any labeling of the ribosomal protein?
6. A key negative control would be to make an alkyne probe with no substitution other than the aldehyde. This should not modify any N-terminal gly and will give an indication of the background labeling in cells and in vivo.
7. For figure 10b why was only one concentration of the probe used for E.coli compared to a full dose response in Staph?
8. For the mouse data in figure 9i – why is the signal from the fluorescent probe not co-localized with the bioluminescent signal? The fluorescent signal is much more diffuse. This experiment also needs a better negative control where either a non-active fluorescent probe is used or E. coli infection is used to control for non-specific probe uptake at the infection site (which could be due to inflammation).
9. There is no negative control for the functional studies on growth or biofilm formation. There needs to be a similar probe that does not label the N-gly5 peptide but that has the aryl-aldehyde and tag. It is impossible to tell if the compound is just toxic to the cells because of general aldehyde reactivity with proteins inside the cell. While E.coli is a decent control, it is gram negative so the probe may just not get inside those cells and cause cell death by modifying proteins.
10. The N-Gly specificity with proteins suggests that this platform shows >90% conversion of proteins with molecular weight between 5-15kDa, and labeling is significantly reduced for 2.8kDa melittin or 27kDa GFP. Can the authors comment on why it is not equally efficient at labeling N-glycine-containing proteins?
11. The authors propose that the rapi-Gly-tag platform is useful for live-cell tagging. For the flow cytometry data, the authors only measure the MFI of Cy3, which does not indicate whether the cells are live and intact. The authors should use a second dye, such as propidium iodide, to validate bacterial viability side-by-side across all tested concentrations, ensuring that the cells are intact.
12. For live-cell imaging, the authors should use a control dye, such as FM4-64 or Nile red for membrane labeling and Vancomycin-fluoresceine for cell wall labeling and validated that the probe is confined to the cell wall.
13. For the live cell imaging specificity data in figure 7 a much more elegant experiment which would take up much less space in the figures would be to mix cultures of all the bacteria and include a GFP labeled staph. Then image for GFP and show that the probe only labels the GFP positive cells in the culture.
14. In Supplementary Figure 138 (b), in flow cytometry data, why is the 5mM probe not showing any labeling and going to an untreated state? How did the authors measure the %labeled population? Why didn't the authors think that 2n would be highly toxic to the cells, causing cell death at 5 mM?
15. It is surprising that authors did not observe any reduction in N-Gly-modified cells in the presence of 1% SDS. Reports suggest 1% SDS completely disrupts the membrane. How is the labeled population quantified?
16. What is the concentration of 2n used in Figure 6(b)
17. Figure 7(a), E. coli merge panels do not match.
18. Why did the authors remove the lag phase part of the growth curves in Figure 10(a)? The authors tested the effect of 2n on bacterial viability and observed ~90% growth inhibition at 200 μ M. What is happening at the initial time points at lower concentrations? Is it interfering with the lag phase?
19. For 2u used in the FRET assay, the description is unclear. Since both panels, Cy3 and Cy5, label the same thing, what should the outcome be? The authors should discuss more about what they observed and the advantages of using such a probe.
20. Did the authors test the stability of 2t in a 20% sucrose solution for Drosophila experiments?
21. In Drosophila experiments (Figure 9d, CLSM panel 4), the S. aureus labeling appears more smeary than punctate. Also, a stain for the epithelial lining is missing. Not sure what exactly we are looking at. Authors should provide clearer images to help readers understand the data. Supplementary Figure 150: Cy5 labelling by 2t is giving much more background than pre-FITC labeling? Can the authors explain why there is more labeling with the Cy5? How did the author validate that the labeling is coming from live bacteria in that region? Instead of using the pre-FITC method, the authors should have used 2u for pre-labeling with one probe (FITC) and a second labeling after dissection to confirm that the signals originate only from bacteria, thereby reducing background.
22. Did the authors test whether 2n is bactericidal or bacteriostatic?
23. For the biofilm inhibition assay in Figure 10(d) and Supplementary Figure 154, it is surprising to see bacteria in 1X MIC in the SEM panels. MIC suggests no bacterial growth at the lowest concentration. Should Biofilm form at MIC or higher concentration, when there are no bacteria around? Looks like MIC is greater than what is reported here. The authors should show the growth inhibition curves for each concentration tested and test the biofilm inhibition at concentrations below the MIC for this assay or suggest the mechanism of biofilm inhibition.
24. In Figure 10(h), L-lys and Gly are indicated by the same symbol color, making it very difficult to differentiate between them.
25. Supplementary Figure 162 (b), do the authors have any comments on why they are observing increased collagen

density for 2n-treated wounds in comparison to uninfected wound healing?
26. The composition of the ointment used for healing is not mentioned in the protocol.

Minor points and corrections:

1. Line 161 should be changed to 4-CHO (2i), not 4-NO₂
2. Line 165, should be changed to 4-CN (2h), not 4-CHO
3. In Figure 2, the authors have not specified the insulin concentration used in each experiment in the figure legends.
4. Figure 2h, the authors have not mentioned the concentration of ADP and ATP
5. Supplementary Table 6 and Supplementary Figure 13, the reaction significantly dropped to 50% when 50-100 μM insulin was used. Do the authors have any reasons for this concentration-dependent reduction in reactivity? Was it due to insufficient 2j, or if insulin was aggregating at that concentration?
6. Figure 3, showing the reaction conditions, which is 20 μM; if it is a peptide concentration, it does not match the conditions mentioned in the Supplementary Table 15.
7. Supplementary Figure 17, panel c: should be 3k
8. Supplementary Figure 36, the converted peak should be labeled 3h
9. Supplementary Table 15, %conversion should be 5a not 7a
10. Supplementary Table 16, %conversion should be 5, not 7
11. Supplementary Figure 69: Mass is not clearly visible
12. In Figure 5c, the panels in the flow chart are not clear between the steps from Cu click to Cu free.
13. Figure 5, panel e, is not labeled, and line 355 should be Fig 5d.
14. There is no mention of microscopy data in the description shown in the last panel of Figure 5
15. Line 376, Fig 6c-e is incorrect, cell labeling should be Fig 6c-d
16. In Figure 6e, the flow cytometry data are not mentioned in the description.
17. Reversing the cellular bioconjugation with PLP data is interesting. However, did the authors normalize the PLP data with the viable bacteria
18. According to the description, the panels in Figure 8 are not easy to comprehend. Figure (a) should be divided into 3 separate panels with proper labeling for easy understanding.
19. Figure 9e, correct the panels (i), (ii), and (iii).
20. The supplementary figures are not in order. Sup Fig. 89 comes before sup Fig. 88.
21. In sup fig 153 what are the images of the mice in the top vs bottom images for each panel. It is not clear from the legend. I think the top is fluorescence and bottom is bioluminescence but that needs to be labeled.
22. Having a 162 supplemental figures is excessive. It seems like these need to be organized into actual figures vs. MS profiles to validate adducts. The adducts could be grouped into some kind of supplemental data set so that the actual supplement figures that are important for the main paper are easier to access.

Reviewer #3

(Remarks to the Author)

In the submitted manuscript, Rai and co-workers describe a new strategy to target the N-terminal glycine residue in peptides and proteins. This work represents an evolution of a previous technology reported by the same group; however, this does not diminish the significance of the present study, as the new protocol effectively enables applications ranging from in vitro systems to live cells and in vivo models. Using electronically tunable reagents, the method allows rapid and highly selective C–C bond formation at the N-terminal glycine while maintaining reactivity across diverse biomolecular environments. The resulting aminoalcohol bioconjugates were structurally validated by NMR, LC–MS, and single-crystal XRD, supported by DFT calculations and control experiments. The platform further enables the installation of bioorthogonal handles for versatile downstream functionalization and remains stable in complex biological media.

Importantly, this platform enables species-specific detection and targeting of *Staphylococcus aureus* by labeling the Gly5 signature in the bacterial cell wall, with demonstrated selectivity in co-cultures, live cells, *Drosophila melanogaster*, and murine infection models. The authors performed a thorough study and explored a wide range of applications to validate the technology, which is commendable. Technically, the work is solid and I have only a few minor comments.

The manuscript is very dense, and the use of large multi-panel figures sometimes makes it difficult to locate specific information.

The authors should revise the compound numbering on page 5 (first paragraph), as it does not match the numbering shown in Figure 2a.

The nitro substituent appears to favor C–C bond formation; however, the authors do not mention the potential toxicity concerns associated with this functional group, which are well known.

The DFT calculations should also include a comparison with the least-performing compounds in order to better rationalize the observed differences in reactivity.

Once these comments are addressed, the manuscript may be considered for publication.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)
Review report attached

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed all of my many concerns and have added some important new data with controls that look good. This work is now ready for publication. My only suggestion would be to include the controls in the main figures when possible since it seems like most of the negative controls ended up in the SI figures.

Reviewer #3

(Remarks to the Author)
In the revised manuscript, Rai and co-workers have addressed the comments raised by this referee very carefully, particularly those concerning the scientific aspects of the work. The DFT calculations have been significantly improved, and the simplified figures enhance the readability of the manuscript. Overall, I believe the authors have done an excellent job addressing all the questions and concerns raised by this referee. This work is now suitable to be accepted

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)
I have reviewed the revised version of this manuscript, focusing on the computational aspects, as in my initial evaluation. The authors have satisfactorily addressed the points raised in my previous report. The revisions have resolved the minor issues previously identified, and no further concerns remain. I therefore recommend the manuscript for publication in its current form.

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Point-by-point response

Reviewer 1

Reviewer 1: The authors described an aldehyde-based chemical probe designed to conjugate with N-terminal glycine. However, this aromatic aldehyde exhibits excessive reactivity in live cells, especially when an electron-withdrawing group is attached, which compromises its selectivity. Due to this fundamental issue, the paper is not suitable for publication.

Response by authors: We respectfully disagree with the reviewer's conclusion that the aromatic aldehyde exhibits excessive reactivity in live cells. If that is a perception in a few segments of the scientific community, these results become even more critical. They unambiguously demonstrate that electronic tuning of the aldehyde scaffold significantly influences the balance between reactivity and selectivity, enabling exclusive labeling of the N-terminal glycine under biologically relevant conditions. We further demonstrate species-specific labeling of *S. aureus*. Again, no labeling was observed in other bacterial strains (Fig. 7a, Supplementary Fig. 126). Notably, we observe *S. aureus*-specific labeling even in co-culture with other bacterial strains and eukaryotic cells (Figs. 7b and 8a-c). Furthermore, global chemoproteomics with labeled *S. aureus* cells reveals no protein targets (Fig. R1, Supplementary Fig. 125), reconfirming the platform's selectivity. To further address the concern, we assessed the cytotoxicity of *rapi*-Gly-tag reagent in eukaryotic Vero cells and observed a selectivity index >11 relative to the MIC of *S. aureus* (Fig. 9c, Supplementary Table 19). Additionally, *in vivo* species-specific targeting of *S. aureus* in *Drosophila melanogaster* and mice models demonstrates the N-Gly exclusivity (Figs. 9d-e). Overall, the studies in this manuscript establish the selectivity with comprehensive data at molecular, cellular, and *in vivo* levels. We are confident that these investigations, including the additional experiments in the revised manuscript, will not leave any doubt about the selectivity of the *rapi*-Gly-tag platform.

Reviewer 2

Reviewer 2: The manuscript by Kumar et al. reports a chemical tool for modifying the N-terminal glycine using a newly developed Rapi-Gly-tag reagent. This technology is an advance over existing N-terminal gly modification methods as it uses chemical tuning of the aldehyde rather than H-bonding directed activation of the aldehyde. This allows the use of the reagent in conditions compatible with *in vivo* and cellular applications. The authors show that the proposed technology works on N-terminal Glycine- containing proteins, using Insulin as the model protein. Further, the authors validate the method using six-amino acid peptides. The technology was also used to selectively label the N-terminal glycine on a unique pentaglycine interpeptide bridge on the *S. aureus* peptidoglycan cell wall. As the pentaglycine interpeptide bridge is important for membrane integrity, the authors show that disrupting it affects the bacterial viability and biofilm formation. The antimicrobial effect was further tested using a *Drosophila* model and multiple mouse skin infection models.

Overall, this manuscript introduces a potentially valuable new chemical modification tool for targeting N-terminal glycine. The manuscript has a vast amount of data (with 9 main figures and over 160 supplemental figures) and the new labeling method is applied to many different systems both *in vitro* and *in vivo*. The work is generally executed thoroughly, and most of the conclusions are justified. The main issues with the work are the lack of key controls in places, lack of clear analysis of overall target selectivity and the presentation and discussion of the

results. This manuscript has a large number of figures which do not focus on concisely presenting the key findings, making it not likely accessible to an average reader. The text is overly wordy and generally unclear in sections. The intro has many citations to other work which is mentioned in a way that hard to follow because it lacks details. Many of those other citations are not needed. The authors need to concisely present the key prior art, how it works and why the new method is better. That is what is relevant for this paper. Also, the figures are overdone with too many diagrams and images that are not needed. This comes at the expense of showing the data in a way that a reader can follow without having to process so much extra noise. Regardless of the issues with the controls and presentation of the results, the work is potentially exciting and likely to be useful for many applications. This work fits well with the broad audience of Nature Communications and could be made suitable for publication after the authors address all the comments suggested below.

Response by authors: We sincerely thank the reviewer for the careful evaluation of the manuscript and for recognizing the potential significance of the *rapi*-Gly-tag platform. We are encouraged by the reviewer's positive assessment and appreciation for its broad utility in chemical biology. In particular, we are amazed by the depth of the assessment, which covers each segment of the manuscript and the supplementary information. We acknowledge that it is a very comprehensive manuscript and would have taken significant effort. Thank you very much for helping us improve the overall quality of the manuscript.

We appreciate the reviewer's constructive suggestions regarding the need for additional controls, improved selectivity analysis, and clearer presentation of the results. In response, we have substantially revised the manuscript to improve clarity, conciseness, and accessibility for a broad readership by performing the suggested experiments and incorporating required changes.

In the revised version, we have streamlined the Introduction to more clearly summarize the relevant prior art, the mechanistic basis of existing methods, and the conceptual advances introduced by *rapi*-Gly-tag technology. We have also carefully revised the figures and the overall organization of the manuscript to better emphasize the key findings and reduce additional visual complexity, as per the suggestions.

Importantly, we have incorporated additional control experiments and expanded selectivity analyses by performing chemoproteomics to address the reviewer's concerns regarding specificity and target selectivity. These additions further strengthen the mechanistic basis and biological relevance of the developed *rapi*-Gly-tag platform. We thank the reviewer again for the thoughtful and constructive comments, which have significantly improved the quality and presentation of the manuscript.

Major points

Reviewer 2, Comment 1: This paper is an example of trying to do too much in one paper, combined with excessively complex and large figures and somewhat confusing text resulting in a paper which is hard to get through. The results seem quite strong and the method is good but this paper needs to be distilled down to something that an average reader could actually process.

Response by authors: Thank you for this thoughtful and constructive feedback. We have revised the introduction and figures (2, 6, and 7) for the ease of general readers.

Reviewer 2, Comment 2: The authors have chosen to use unusual methods to display simple data. For example, in figure 2c they use some kind of spider web like diagram when a simple bar graph would make more sense. Similarly, panels d-g should be bar or line graphs with error shown. For panel h there is no need for diagrams and images of proteins and Eppendorf tubes with data shown using a cartoon speedometer. The data should simply be plotted with error bars included. Is there error for panel K?

Response by authors: Thank you for these suggestions regarding data presentation. To address these concerns, we have comprehensively revised Fig. 2 to improve readability and align it with conventional representations. In particular, the spider-web representation in Fig. 2c has been replaced with a bar graph. Likewise, panels d–g have been replotted using standard bar graphs with error bars to facilitate clearer comparison of the datasets. For panel h, we have removed the speedometer representation and plotted the data using a bar graph with appropriate error bars.

Regarding panel k, the bar graph with error bars now has been provided in the supplementary (Supplementary Fig.35), and the drop-line plot has been included in the main manuscript for clear representation within the available space.

Reviewer 2, Comment 3: In figure 3 there is no need to show the protein folds and panel c is just a cartoon of the labeling which is not needed. The data should be shown for this experiment – which is shown in panel b but could be just a single simple bar graph.

Response by authors: We thank the reviewer for raising this point. However, the feedback from a few readers indicated that it is important to show that the method operates independent of protein's structural diversity. The structure of proteins in Fig. 3 address this in a subtle manner. The data for extent of labeling is provided below the structure of each protein, and related MS data is provided in the supplementary information (section 3.7).

Reviewer 2, Comment 4: For figure 6 the interesting data is the time and concentration dependence which is in the supplemental figures. The overall intensity of labeling could be quantified and plotted to show the saturation of labeling with some representative images to show what the labeling looks like at a high magnification. The panel g is just another cartoon with no actual data.

Response by authors: Thank you for this suggestion. We agree that time- and concentration-dependent labeling experiments are important for initial method screening. In response, we have now incorporated the corresponding FACS-based quantitative labeling data into Figure 6 in panels b and c. The updated representation clearly illustrates labeling trends as a function of time and concentration. The panel g outlines the enrichment and analysis workflow for clarity to readers. The panel also contains the associated mass spectrometry data for the enriched peptide, which we believe is important for demonstrating the analytical validation.

Reviewer 2, Comment 5: A key experiment is missing to show how selective the labeling with the probe actually is. The authors need to treat cells with the alkyne tagged probe at the optimal concentration and time and then do click to add fluorophore and run the gel to show protein labeling. My guess is that there will be proteins that get labeled by the probe. Similarly, they do the pull down and mass spec but only look for the glycopeptide from the cell wall. The key question is what proteins are labeled? It would be interesting and important to use the probe for full chemoproteomics to identify any labeled proteins not just the Gly5 peptide. Reports show that the bacterial 30s ribosomal protein S3 also contains an N-terminal glycine

after processing the formyl-methionine residue by methionine aminopeptidase. Do the authors find any labeling of the ribosomal protein?

Response by authors: We thank the reviewer for raising this important and insightful point for discussion. We agree that evaluating potential off-target protein labeling is important for understanding the selectivity profile of the developed *rapi*-Gly-tag platform. To address this concern, we have performed additional experiments using the alkyne-tagged reagent (**2n**) under the optimal labeling conditions, followed by click conjugation with Cy3-azide and in-gel fluorescence analysis (Fig. R1a). These experiments revealed no observable protein modification in the cytosolic fractions; however, there was some fluorescent signal observed in total and insoluble fractions. We believe it would come from peptidoglycan fragments. The corresponding gel images have now been included in the Supplementary Information under the additional results section (Supplementary Fig. 125a).

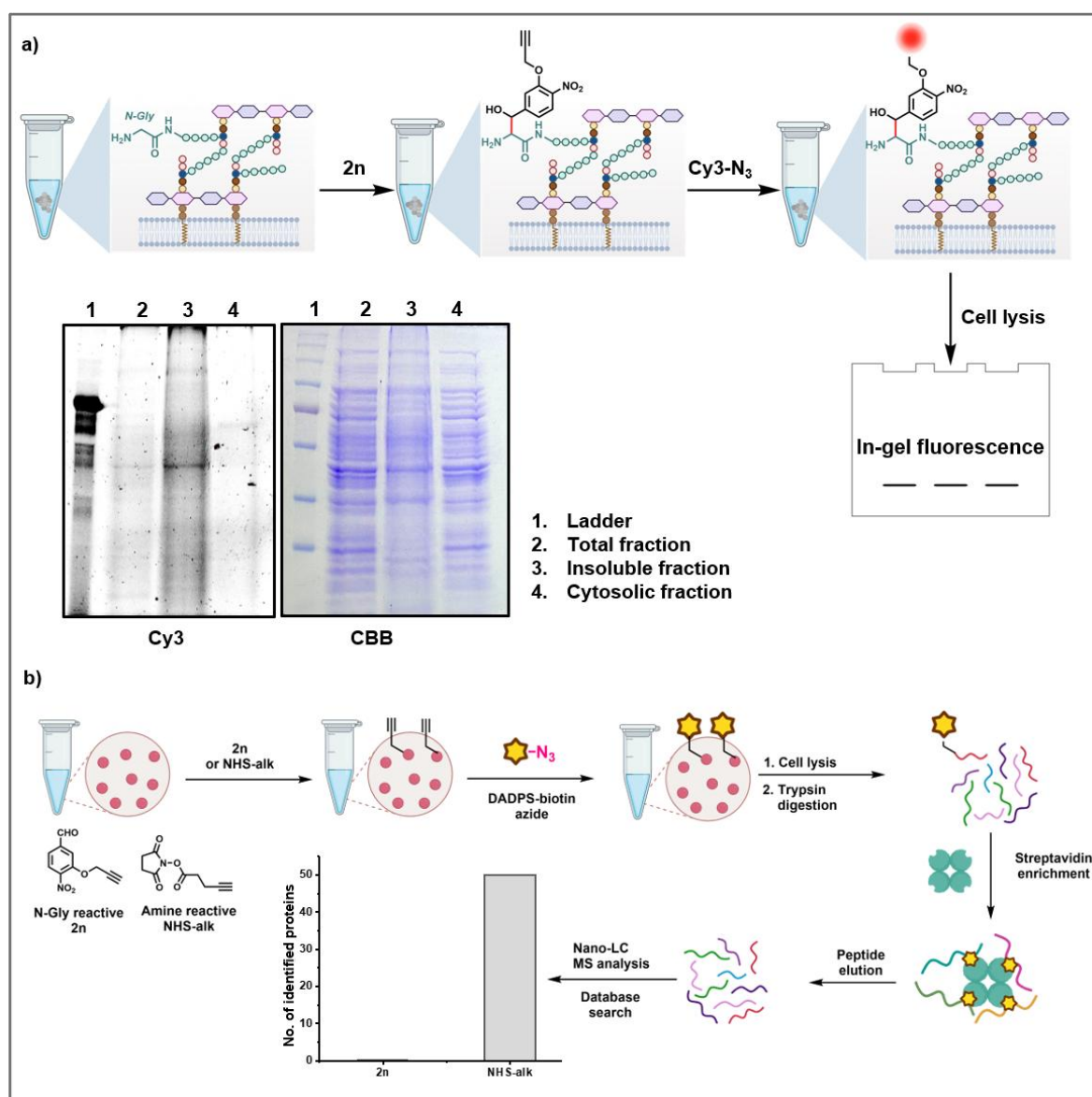


Fig. R1 | Selectivity of *rapi*-Gly-tag platform. a, in-gel fluorescence analysis of labeled cells with **2n** (2 mM, 2 h) followed by click conjugation of Cy3-azide. **b,** Global chemoproteomics analysis for identification of labeled proteins. Cells were incubated with **2n** (2 mM, 2 h) and NHS-alk (positive control, 200 μ M, 2 h) separately. Subsequently, click conjugation with DADPS-biotin azide. Next, cells were lysed, the proteome was trypsin-digested, labeled peptides were enriched, and analysed by nano-

LC Orbitrap. A detailed experimental procedure has been included in the supplementary information (section 2.21).

Furthermore, we extended our experiments from Gly₅-directed proteomics to global chemoproteomics. In this perspective, we investigated the protein target(s) labeled with the *rapi*-Gly-tag reagent (**2n**, Fig. R1b). As a positive control, we used an amine-reactive N-hydroxysuccinimide derivative of an alkyne (NHS-alk) to validate the workflow. At first, we incubated *S. aureus* cells with alkyne-equipped reagents (**2n** or **NHS-alk**). Subsequently, we installed acid-cleavable biotin azide (DADPS biotin azide) via click chemistry. Further, we lysed the cells and performed trypsin digestion. Now, biotinylated peptides were enriched using streptavidin agarose beads. Finally, we subjected the enriched peptides to nano-LC-Orbitrap and analyzed the data with Proteome Discoverer. Importantly, our analyses confirmed that no protein, including the bacterial 30S ribosomal protein S3, is targeted by **2n** under the given conditions. On the other hand, 50 proteins were enriched and identified with NHS-alk reagent. These findings further support the probe's preferential reactivity toward the exposed pentaglycine motifs in the *S. aureus* cell wall. These investigations have been included in the revised manuscript (Page 14). The results have been included in the revised Supplementary Information (Supplementary Fig. 125b).

Reviewer 2, Comment 6: A key negative control would be to make an alkyne probe with no substitution other than the aldehyde. This should not modify any N-terminal gly and will give an indication of the background labeling in cells and in vivo.

Response by authors: We thank the reviewer for this valuable suggestion to include an unsubstituted aldehyde probe as a negative control to assess background labeling. To address this point, we synthesized the corresponding alkyne-tagged aryl aldehyde probe (**2v**) lacking the electron-withdrawing substituent (-NO₂) and evaluated its reactivity under identical experimental conditions. We first examined this control probe using insulin, a model N-terminal glycine-containing protein, and observed no modification, in contrast to 85% modification by nitro-containing reagent **2n** (Fig. R2a). Subsequently, we examined **2v**'s reactivity with *S. aureus* cells under the optimized reaction conditions and analysed them using confocal laser scanning microscopy (CLSM, Fig. R2c) and flow cytometry (Fig. R2d). Interestingly, no labeling was observed with **2v**, contrary to significant labeling by **2n**. These results further support our central hypothesis that electronic tuning of the aldehyde scaffold is critical for enabling efficient N-terminal glycine labeling. Moreover, the absence of detectable labeling with the unsubstituted control probe indicates that nonspecific background modification under cellular and biological conditions is minimal. The corresponding experimental data and discussion have now been included in the revised manuscript (page 14) and Supplementary Information (Supplementary Fig. 135).

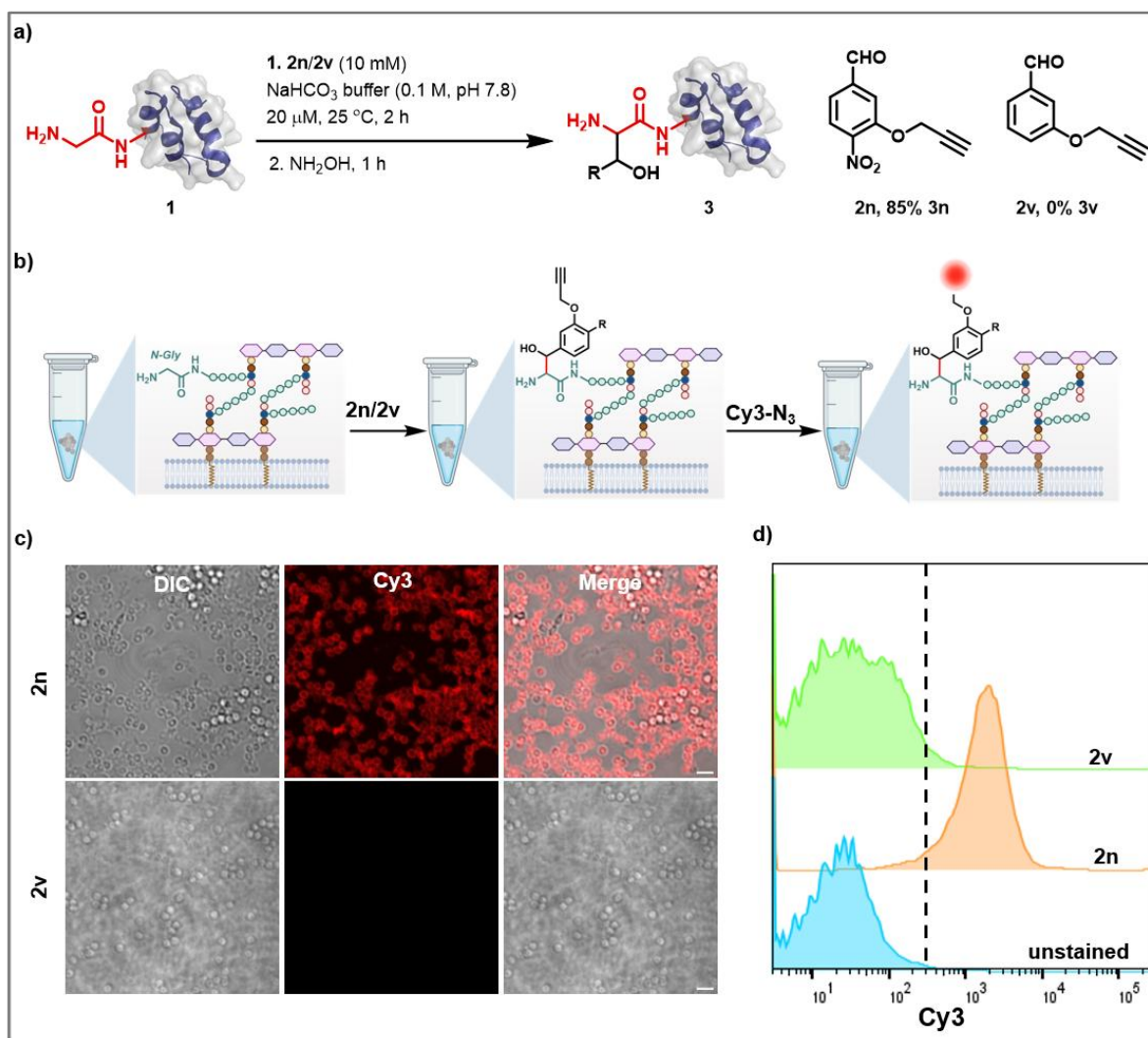


Fig. R2 | Negative control for selectivity of the *rapi*-Gly-tag platform. **a**, labeling of insulin with *rapi*-Gly-tag reagent **2n** and **2v** (control reagent). **b**, Workflow for labeling of *S. aureus* with **2n/2v** followed by click conjugation of Cy3-azide. **c**, CLSM analysis, **d**, flow cytometry analysis for labeling of *S. aureus* with **2n/2v**.

Reviewer 2, Comment 7: For figure 10b why was only one concentration of the probe used for *E. coli* compared to a full dose response in Staph?

Response by authors: The dose dependent experiments with *S. aureus* showed a significant growth inhibition at 200 μ M of **2n** (Fig 10a). Hence, we selected this concentration to assess the effect on *E. coli* (Figure 10b). These results confirm that *E. coli* remains unaffected at concentration of **2n** that is sufficient for significant inhibition of *S. aureus*. However it was expected, as the imaging-based investigations with *E. coli* using up to 2 mM **2n** already confirmed the absence of labeling (Fig. 7).

Reviewer 2, Comment 8: For the mouse data in figure 9i – why is the signal from the fluorescent probe not co-localized with the bioluminescent signal? The fluorescent signal is much more diffuse. This experiment also needs a better negative control where either a non-active fluorescent probe is used or *E. coli* infection is used to control for non-specific probe uptake at the infection site (which could be due to inflammation).

Response by authors: We thank the reviewer for this observation. The apparent differences in the distribution of fluorescence and bioluminescence signals may arise from the

fundamentally different imaging modalities and biological processes being monitored. The bioluminescent signal is generated exclusively by metabolically active *S. aureus* cells. In contrast, the fluorescent labeling occurs with Gly₅-containing bacterial cell wall and can remain associated with bacterial biomass (live or dead) at the infection site. Furthermore, fluorescence imaging is subject to photon scattering and tissue absorption, which can result in a broader and more diffuse signal distribution compared with the highly localized bioluminescent signal. Despite these differences, the fluorescence signal is consistently observed at the site of infection identified by bioluminescence. To address the concern, we performed a control experiment in which we treated it directly with DBCO-Cy5 without the **2t** exposure. Notably, we do not observe a fluorescent signal in this negative control.

To address the reviewer's concern regarding nonspecific probe accumulation caused by inflammation, we performed an additional control experiment using an *E. coli* infection model (Fig. R3). Here, mice infected with *E. coli* and treated with **2t** followed by DBCO-Cy5 in identical conditions, displayed no detectable fluorescence signal at the infection site. These results demonstrate that probe accumulation is not driven by nonspecific uptake into inflamed tissue but instead depends on the presence of *S. aureus*. The absence of signal in the *E. coli* control, therefore, provides strong evidence for the specificity of the *rapi*-Gly-tag platform *in vivo*. We have revised the manuscript text and incorporated the control experiments into the Supplementary Information (Supplementary Fig. 141).

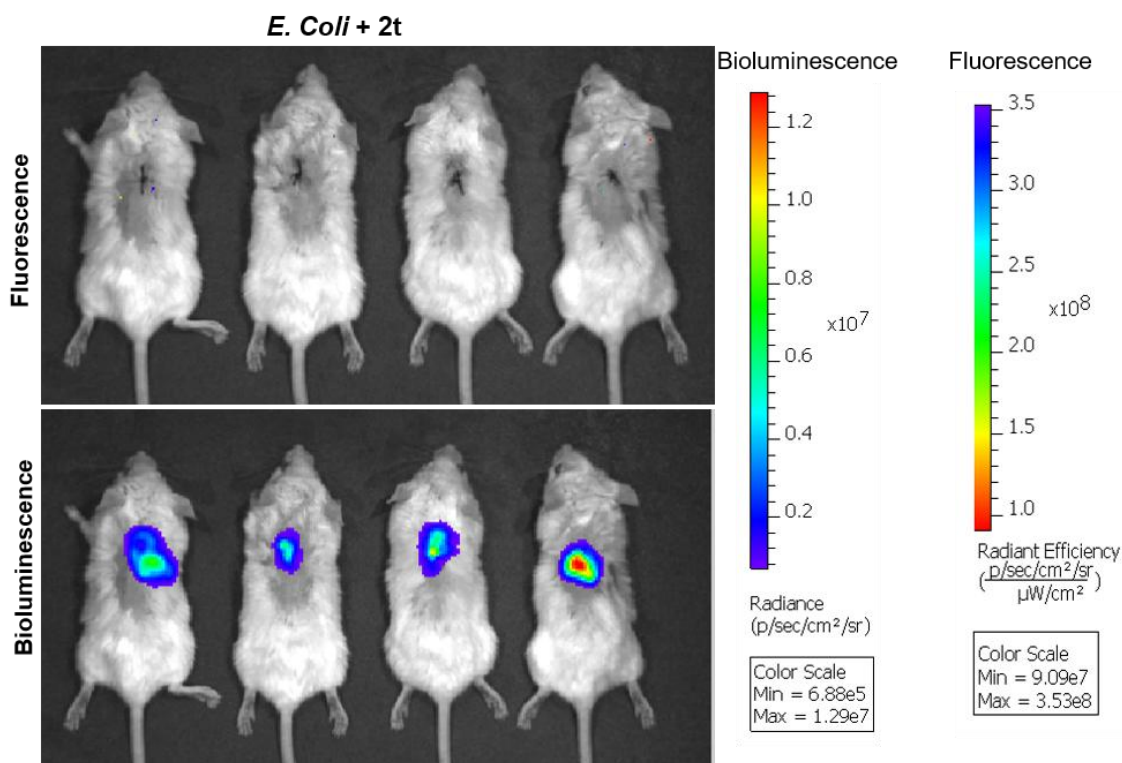


Fig. R3 | *E. Coli* control for selectivity of *rapi*-Gly-tag platform. *In vivo* imaging of *E. coli*-infected mice treated with **2t**. Imaging was performed for n = 4. The top images in the panel indicate fluorescence signals, and the bottom images indicate bioluminescence signals.

Reviewer 2, Comment 9: There is no negative control for the functional studies on growth or biofilm formation. There needs to be a similar probe that does not label the N-gly5 peptide but that has the aryl-aldehyde and tag. It is impossible to tell if the compound is just toxic to the cells because of general aldehyde reactivity with proteins inside the cell. While *E. coli* is a

decent control, it is gram negative so the probe may just not get inside those cells and cause cell death by modifying proteins.

Response by authors: We thank the reviewer for this critical point regarding the inclusion of an additional negative control for the functional growth and biofilm studies. To address the concern, we synthesized and used the negative control reagent **2v** (also see, response to comment 6). The reagent **2v** contains the aryl-aldehyde and alkyne tags but lacks the -NO₂ group required for N-Gly modification. We next evaluated the functional effects of **2v** in *S. aureus*. Growth inhibition assays were performed over a concentration range of 50–500 μ M (Fig. R4a). Notably, we did not observe growth inhibition even at 500 μ M, which is 2.5-fold higher than the concentration of **2n** (200 μ M), at which the latter exhibits complete growth inhibition. Furthermore, we investigated the effect of **2v** on biofilm formation using both crystal violet staining and SEM analysis (Fig. R4b-c). In contrast to probe **2n**, treatment with **2v** did not result in a significant reduction in biofilm formation up to 500 μ M. Together, these control experiments strongly support that the observed antibacterial and antibiofilm effects of probe **2n** are not due to nonspecific aldehyde-mediated cellular toxicity, but rather arise from selective targeting of the N-Gly₅ peptide in the *S. aureus* cell wall. These additional data and discussion have now been incorporated into the revised manuscript (page 21) and Supplementary Information (Supplementary Fig. 154).

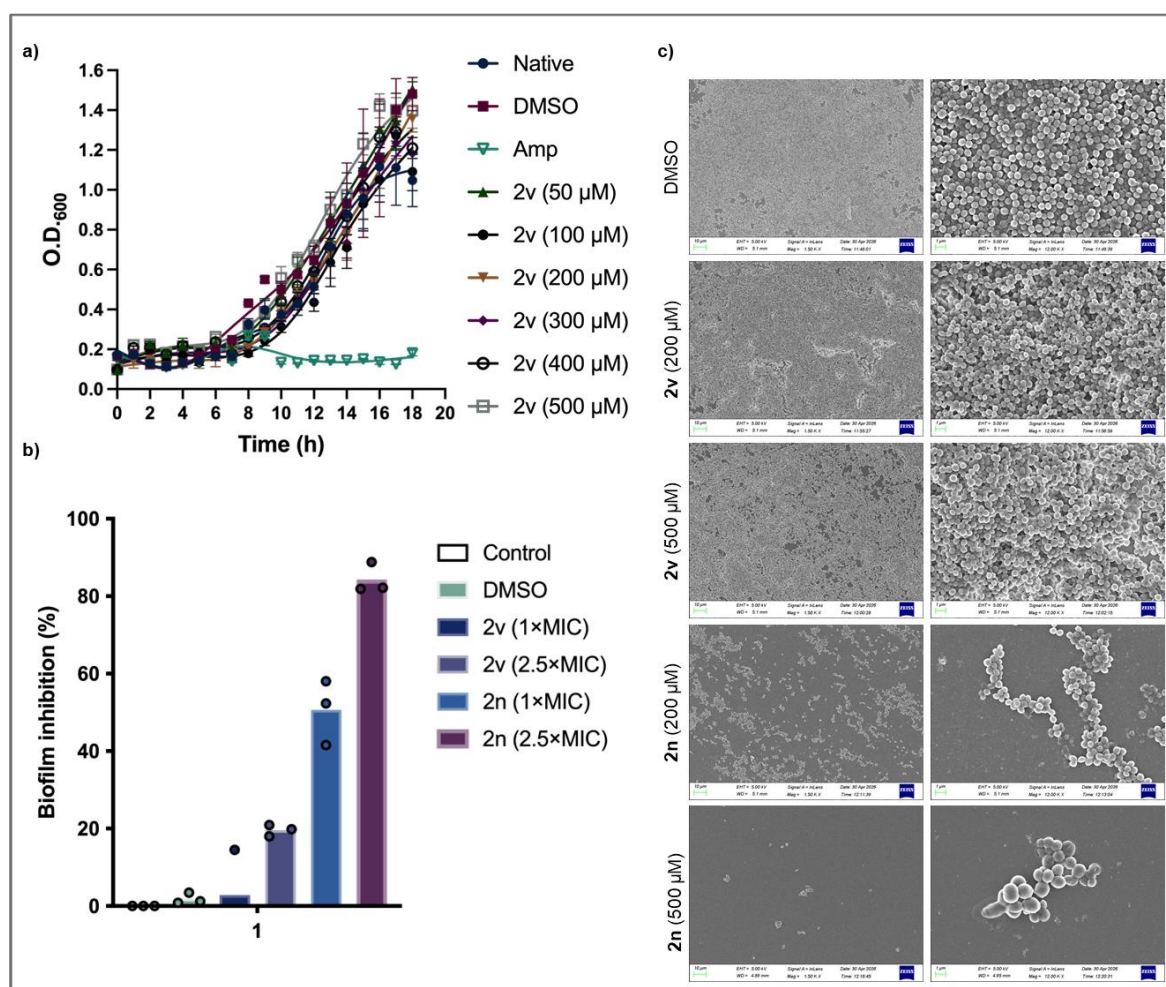


Fig. R4 | Functional studies of negative control reagent **2v**. **a**, Growth curves of *S. aureus* upon treatment with different concentrations of **2v** (50–500 μ M). **b**, **c**, Inhibition of *S. aureus* biofilm formation: Crystal violet assay and FE-SEM analysis showing reduction in biofilm formation when treated with

different concentrations of **2n/2v** at 1×MIC and 2.5×MIC. Scale bar, 10 μ m (left panel), 1 μ m (Right panel).

Reviewer 2, Comment 10: The N-Gly specificity with proteins suggests that this platform shows >90% conversion of proteins with molecular weight between 5-15kDa, and labeling is significantly reduced for 2.8kDa melittin or 27kDa GFP. Can the authors comment on why it is not equally efficient at labeling N-glycine-containing proteins?

Response by authors: We thank the reviewer for asking this insightful question. For simplicity to readers, a similar method has been used for all the proteins. However, the rate of reaction differs for structurally diverse proteins in a size-independent manner. These observations align with other protein bioconjugation methods. At the same time, the rationale behind these trends is not completely understood by the scientific community at this point.

Reviewer 2, Comment 11: The authors propose that the rapi-Gly-tag platform is useful for live-cell tagging. For the flow cytometry data, the authors only measure the MFI of Cy3, which does not indicate whether the cells are live and intact. The authors should use a second dye, such as propidium iodide, to validate bacterial viability side-by-side across all tested concentrations, ensuring that the cells are intact.

Response by authors: We thank the reviewer for this suggestion regarding the assessment of bacterial viability during the live-cell labeling experiments. To address the concern, we performed cellular viability analyses using propidium iodide (PI) staining. *S. aureus* cells treated with probe **2n** over a concentration range of 0.125–5 mM were incubated with PI (15 μ g/mL) for 15 min at 4 °C and subsequently analyzed by flow cytometry (Fig. R5).

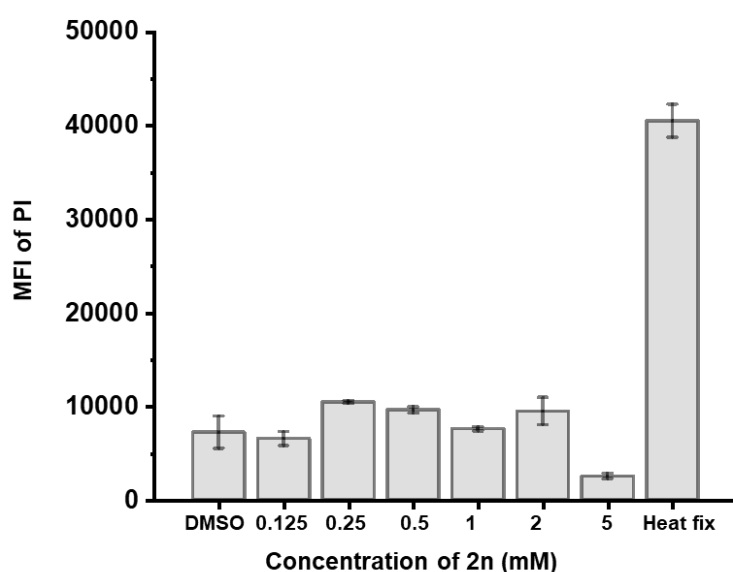


Fig. R5 | *S. aureus* viability analysis upon treatment with **2n**. Bacteria were incubated at different concentrations of **2n** (0.5-5 mM) for 2 h, followed by propidium iodide treatment (15 μ g/mL) for 15 min at 4 °C and subsequently analyzed by flow cytometry. MFI represents mean fluorescence intensity.

Notably, we did not observe significant PI uptake in **2n**-treated samples compared to the DMSO-treated control across all tested concentrations, indicating that the bacterial membranes remained largely intact under the labeling conditions employed. In contrast, heat-fixed *S. aureus* cells treated with PI showed strong fluorescence and were used as a positive control for membrane-compromised cells.

These results support the use of the rapi-Gly-tag platform for live-cell tagging. Under the given conditions across the tested concentrations, the cells remain live and intact. The corresponding flow cytometry data and analysis have now been included in the revised Supplementary Information (Supplementary Fig. 128). Also, the related text has been edited in the manuscript (page 16).

Reviewer 2, Comment 12: For live-cell imaging, the authors should use a control dye, such as FM4-64 or Nile red for membrane labeling and Vancomycin-fluoresceine for cell wall labeling and validated that the probe is confined to the cell wall.

Response by authors: We thank the reviewer for this point regarding the validation of probe localization in live-cell imaging experiments. In this perspective, we performed additional co-localization studies using established cell wall- and membrane-targeting dyes to evaluate whether the probe labeling is confined to the bacterial cell wall (Fig. R6).

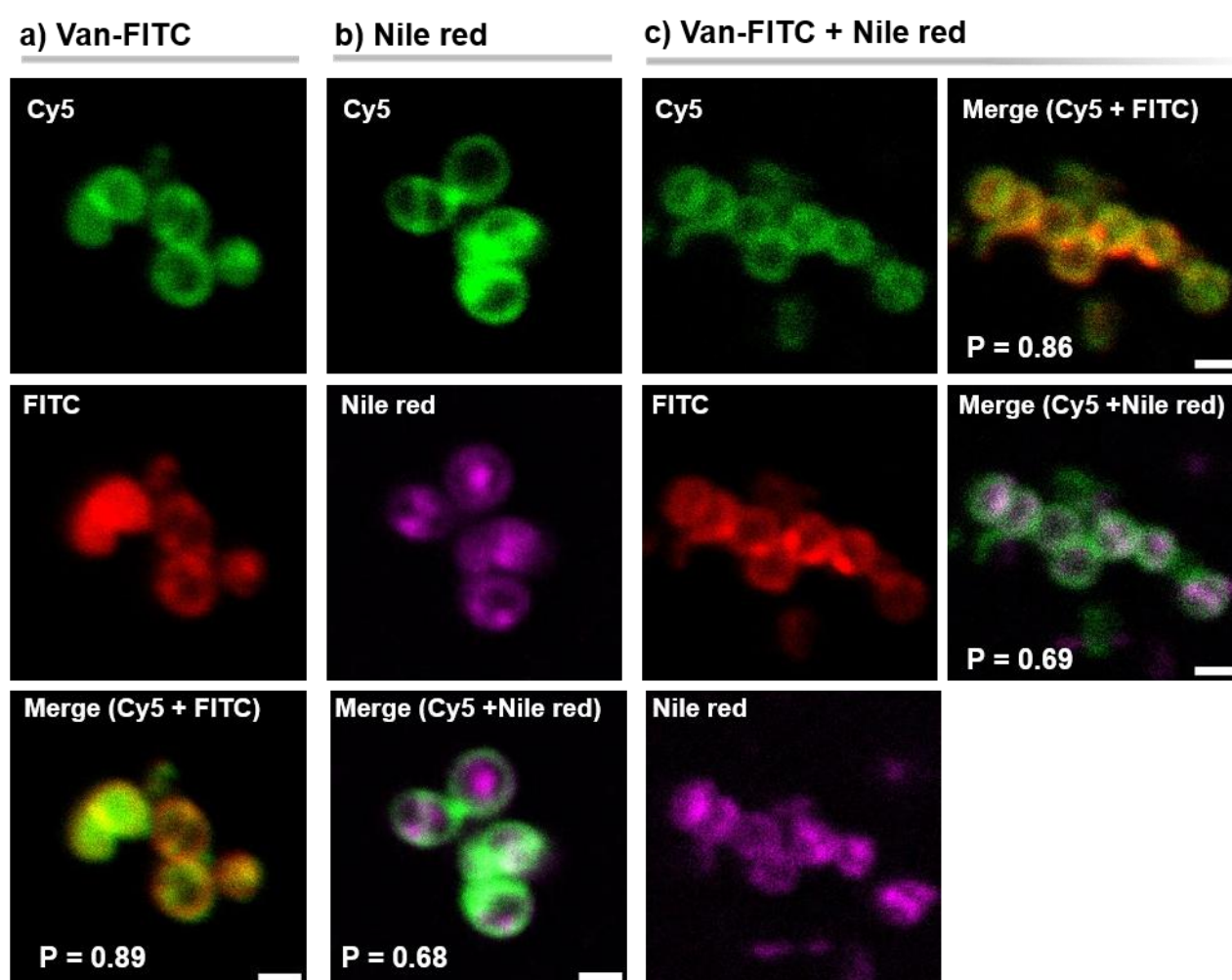


Fig. R6 | Co-localization study of *rapi*-Gly-tag labeling. co-localization of **2n**-led Cy5 labeling with **a**, cell wall-targeting Van-FITC labeling. **b**, membrane targeting Nile red labeling. **c**, co-localization of **2n**-led Cy5 labeling with both Van-FITC and Nile red. Scale bar, 1 μ m.

Following labeling of *S. aureus* with probe **2n**, we performed click conjugation using Cy5-azide instead of Cy3-azide, as the excitation/emission profile of Cy3 overlaps with Nile red staining. For cell wall staining, Cy5-labeled cells were incubated with Vancomycin-FITC (Van-FITC, 50 μ g/mL) for 30 min. For membrane staining, cells

were incubated with Nile red (20 µg/mL) for 10 min at 4 °C. As established earlier, the cell wall (20–40 nm) and membrane (2–5 nm) are separated only by 2–4 nm.^{1,2,3} Hence, confocal laser scanning microscopy (CLSM) experiments were performed under three separate conditions. In the first experiment, Cy5-labeled cells were co-stained only with Van-FITC. We observed substantial co-localization between the Cy5 and Van-FITC signals, with a Pearson correlation coefficient of 0.89, supporting predominant co-localization of the probes at the bacterial cell wall. In the second experiment, Cy5-labeled cells were stained with Nile red. In this case, we observed significantly lower co-localization between the Cy5 and membrane signals, with a Pearson correlation coefficient of 0.68, indicating limited association of the probe with the bacterial membrane. In the third experiment, Cy5-labeled cells were sequentially stained with both Van-FITC and Nile red with a Pearson correlation coefficient of 0.86 and 0.69, respectively. These analyses support the localization of probe **2n** in the cell wall. The corresponding CLSM images and analyses have now been included in the revised Supplementary Information (Supplementary Fig. 122). Also, the related text has been edited in the manuscript (page 12).

Reviewer 2, Comment 13: For the live cell imaging specificity data in figure 7 a much more elegant experiment which would take up much less space in the figures would be to mix cultures of all the bacteria and include a GFP labeled staph. Then image for GFP and show that the probe only labels the GFP positive cells in the culture.

Response by authors: We thank the reviewer for this suggestion. To increase the biological complexity systematically, we moved from proteins to single strain of live cells. Subsequently, we extended our investigations to a co-culture of two cells (different combinations). While one had N-Gly₅ signature, the others were devoid of it. Even though this approach was more cumbersome than mix culture of all bacteria, it provided unambiguous shape differentiation in the imaging-based analysis. However, we do agree that Fig. 7 consumes a lot of space. To address this, we have retained representative images for one Gram-positive and one Gram-negative co-culture experiment in the revised manuscript. We transferred the remaining dataset to the Supplementary Information while giving appropriate citation in the figure caption.

Reviewer 2, Comment 14: In Supplementary Figure 138 (b), in flow cytometry data, why is the 5 mM probe not showing any labeling and going to an untreated state? How did the authors measure the %labeled population? Why didn't the authors think that **2n** would be highly toxic to the cells, causing cell death at 5 mM?

Response by authors: We thank the reviewer for carefully examining the flow cytometry data in Supplementary Figure 138b and for raising this important point. The reduced labeling observed at 5 mM concentration of probe **2n** is primarily attributed to solubility limitations. The reagent **2n** at 5 mM led to noticeable precipitation in the reaction mixture. This reduces the effective concentration of the probe. Consequently, the labeling efficiency at 5 mM was lower than that observed at 2 mM, where the reagent remained fully soluble under the experimental conditions.

To further address this concern, we directly assessed the possibility of probe-induced toxicity at 5 mM of **2n**. Here, we performed bacterial viability studies using propidium iodide (PI) staining (also see, response to Comment 11). Notably, even after treatment with 5 mM **2n**, we

did not observe significant PI uptake compared to the DMSO-treated control, indicating that the cell death is less likely to be responsible for lower labeling.

The percentage of labeled cells was quantified by flow cytometry using fluorescence gating relative to the untreated control population. Cells exhibiting fluorescence intensity above the background threshold established from untreated samples were considered labeled.

Reviewer 2, Comment 15: It is surprising that authors did not observe any reduction in N-Gly-modified cells in the presence of 1% SDS. Reports suggest 1% SDS completely disrupts the membrane. How is the labeled population quantified?

Response by authors: We thank the reviewer for raising this point. We must apologise as there was an error in reporting the SDS concentration. The experiment was performed with 0.1% SDS, and not 1%. In fact, the SDS was limited to 0.1% to avoid the compromise of cell integrity. The manuscript and corresponding figure caption have now been corrected. The labeled population was quantified from the gated fluorescent-positive cell population obtained from flow cytometry analysis relative to the untreated control. The gating strategy was kept consistent across all samples to determine the percentage of labeled cells.

Reviewer 2, Comment 16: What is the concentration of 2n used in Figure 6(b) 17. Figure 7(a), *E. coli* merge panels do not match.

Response by authors: The concentration of 2n used in Figure 6(b) was 2 mM. We have now clarified this in the revised manuscript and figure caption. We also apologize for the error in the merged panels for *E. coli* in Figure 7(a). The incorrect merge images have now been corrected in the revised main manuscript (Fig. 7) and in the Supplementary Information (Supplementary Fig. 126).

Reviewer 2, Comment 17: Why did the authors remove the lag phase part of the growth curves in Figure 10(a)? The authors tested the effect of 2n on bacterial viability and observed ~90% growth inhibition at 200 μ M. What is happening at the initial time points at lower concentrations? Is it interfering with the lag phase?

Response by authors: We thank the reviewer for raising this point. In the earlier version, we did not focus on the lag phase because our primary objective was to evaluate overall growth inhibition caused by 2n. However, based on the reviewer's suggestion, we have now repeated the experiment while covering the lag phase. From the revised growth curves (Fig R7), we do not observe any significant interference with the lag phase at lower concentrations of 2n.

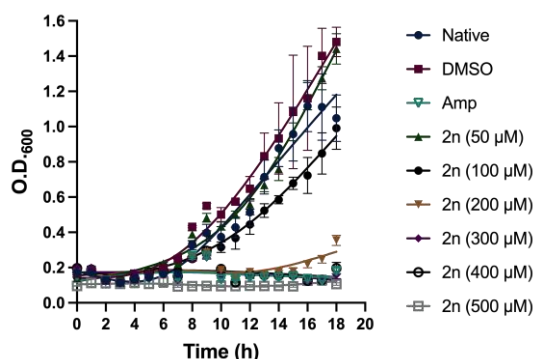


Fig. R7 | Complete Growth curves data, including lag phase, for *S. aureus* upon treatment with different concentrations of 2n (50-500 μ M).

Reviewer 2, Comment 18: For 2u used in the FRET assay, the description is unclear. Since both panels, Cy3 and Cy5, label the same thing, what should the outcome be? The authors should discuss more about what they observed and the advantages of using such a probe.

Response by authors: We thank the reviewer for this comment. We have now clarified the description of **2u** and expanded the discussion regarding the FRET and the advantages of this dual labeling strategy in the revised manuscript (page 16).

FRET has established itself as a powerful technique while offering complementarity compared to single-fluorophore-based methods. Its diverse advantages include significantly improved spatial resolution. For a representative example, see the highlighted sections in the Fig. R8 below (extracted from Extended data Fig. 1, main manuscript).

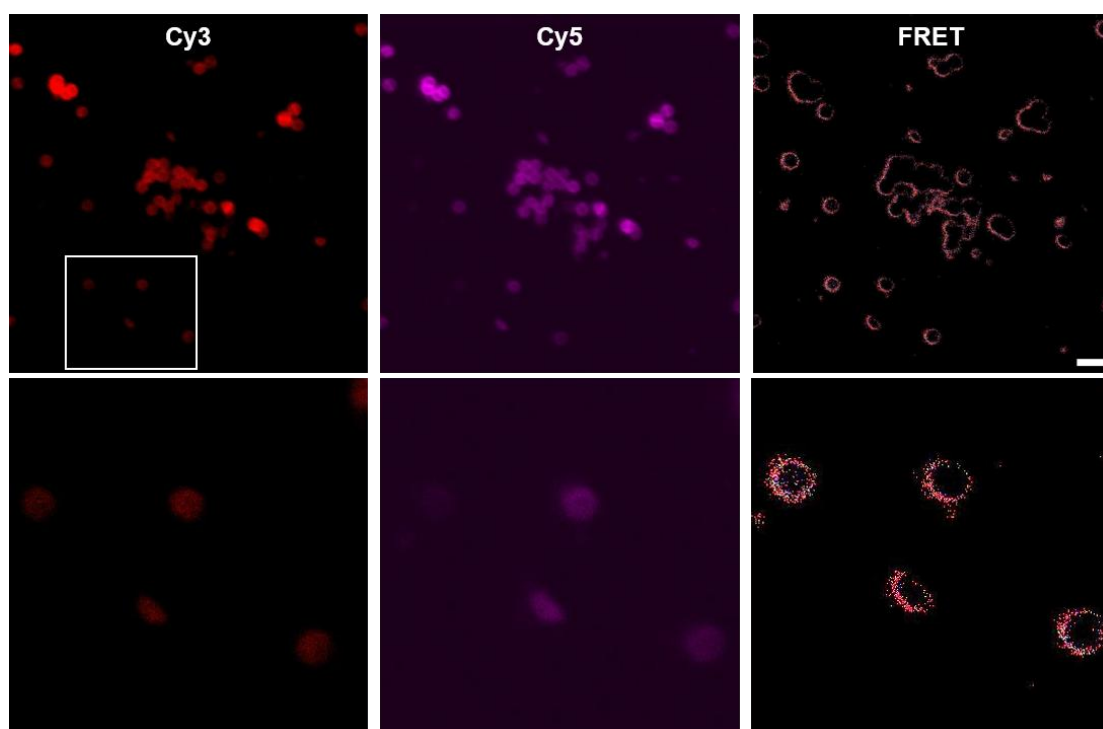


Fig. R8 | FRET analysis using the Cy3 (donor) / Cy5 (acceptor) pair. Scale bar, 2 μ m. The lower panel is enlarged from the highlighted section in the upper panel.

Reviewer 2, Comment 19: Did the authors test the stability of 2t in a 20% sucrose solution for *Drosophila* experiments?

Response by authors: We thank the reviewer for raising this point. To evaluate the stability of **2t** under the experimental conditions used for the *Drosophila* studies, we performed LC-MS analysis of **2t** (200 μ M) in 20% sucrose solution at 0 h and after 2 h incubation. The LC-MS results indicated that **2t** remained stable over this time period, with no detectable decomposition (Fig. R9). These results support the suitability of **2t** for the *Drosophila* feeding experiments performed in this study.

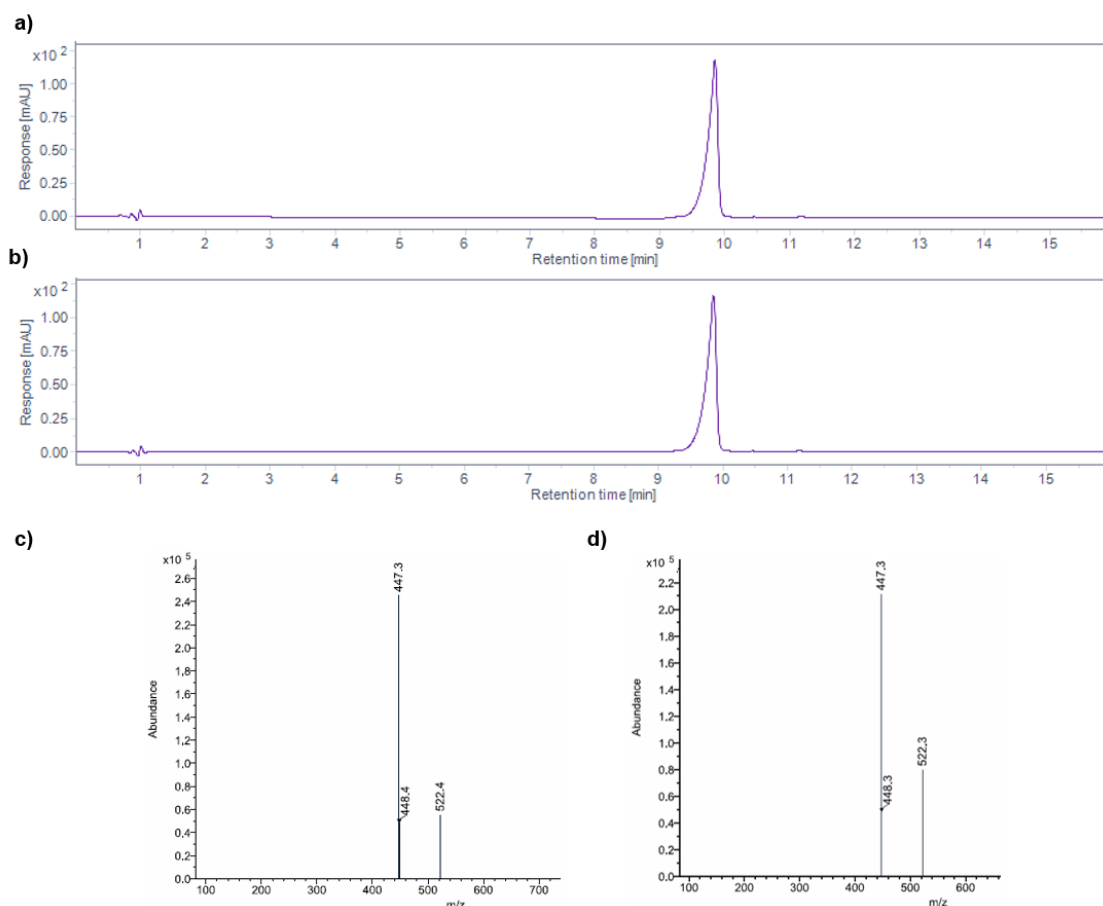


Fig. R9 | Stability of 2t in 20% sucrose. a, LC chromatogram of 2t in 20 % sucrose at 0 h. **b,** LC chromatogram of 2t in 20 % sucrose after 2 h of incubation. **c,** MS spectrum of the corresponding LC peak at 9-10 min for stability at 0 h, $[M+H]^+$ and $[M+2K-2H]^+$. **d,** MS spectrum of the corresponding LC peak at 9-10 min for stability at 2 h, $[M+H]^+$ and $[M+2K-2H]^+$.

Reviewer 2, Comment 20: In *Drosophila* experiments (Figure 9d, CLSM panel 4), the *S. aureus* labeling appears more smeary than punctate. Also, a stain for the epithelial lining is missing. Not sure what exactly we are looking at. Authors should provide clearer images to help readers understand the data. Supplementary Figure 150: Cy5 labelling by 2t is giving much more background than pre-FITC labeling? Can the authors explain why there is more labeling with the Cy5? How did the author validate that the labeling is coming from live bacteria in that region? Instead of using the pre-FITC method, the authors should have used 2u for pre-labeling with one probe (FITC) and a second labeling after dissection to confirm that the signals originate only from bacteria, thereby reducing background.

Response by authors: We thank the reviewer for this suggestion. In response, we have repeated the *Drosophila* imaging experiments and included staining of the epithelial lining using DLG as a membrane marker to provide a clearer tissue context (Fig. R10). In the revised CLSM images, the epithelial linings are now clearly visible, enabling better visualization of *S. aureus* localization. Importantly, the Cy5 signal now appears as distinct punctate staining rather than smeary fluorescence, supporting specific bacterial labeling in the tissue environment. The updated data have been incorporated into the revised manuscript (Fig. 9d) and Supplementary Information (Supplementary Fig. 135).

Regarding Supplementary Figure 150, the higher background in the Cy5 channel is not a true representation of relative intensities. If we look closely into the entire z-stack series, the Cy5 labeling intensity and distribution are comparable to those in the pre-FITC labeling. For clarity, we have now included the complete stack images in the revised Supplementary Information (Fig. R11, Supplementary Fig. 137).

However, we agree with the reviewer that using **2u** with a pre-labeled probe can provide unambiguous information. Hence, we performed an experiment using **2u** for both pre-labeling and post-dissection validation of bacterial signals. Specifically, DBCO-Cy5 was first mixed with **2u** in a 1:1 ratio to generate a pre-conjugated Cy5–**2u** probe, which was then administered to *Drosophila* larvae for *in vivo* labeling. Following larval dissection, Cy3 labeling was performed. The resulting CLSM images show substantial overlap between the Cy5 and Cy3 signals, demonstrating strong colocalization of the two independently introduced fluorophores (Fig. R12). These results confirm that the observed fluorescence signals originate predominantly from *S. aureus* labeled by **2u**, and not from nonspecific background. The additional control experiment and corresponding images have now been included in the revised Supplementary Information (Supplementary Fig. 138) to provide further validation of *S. aureus*-specific labeling.

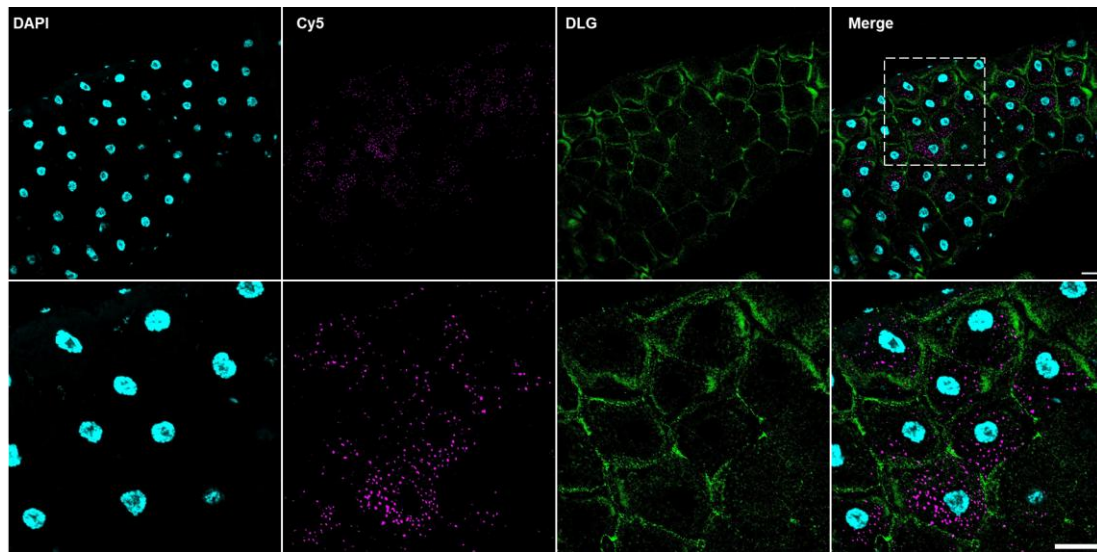


Fig. R10 | Specific detection of *S. aureus* in *Drosophila* gut: 2nd instar larvae were fed with either *S. aureus*, followed by treatment with **2t** (250 μ M, 2 h). The larval guts were dissected and subsequently treated with DBCO-Cy5. Next, immunofluorescence staining was performed for epithelial membrane staining (DLG marker). CLSM images show specific labeling of *S. aureus* within the mid-gut region of *Drosophila* larvae, its characteristic location. Scale bar, 20 μ m.

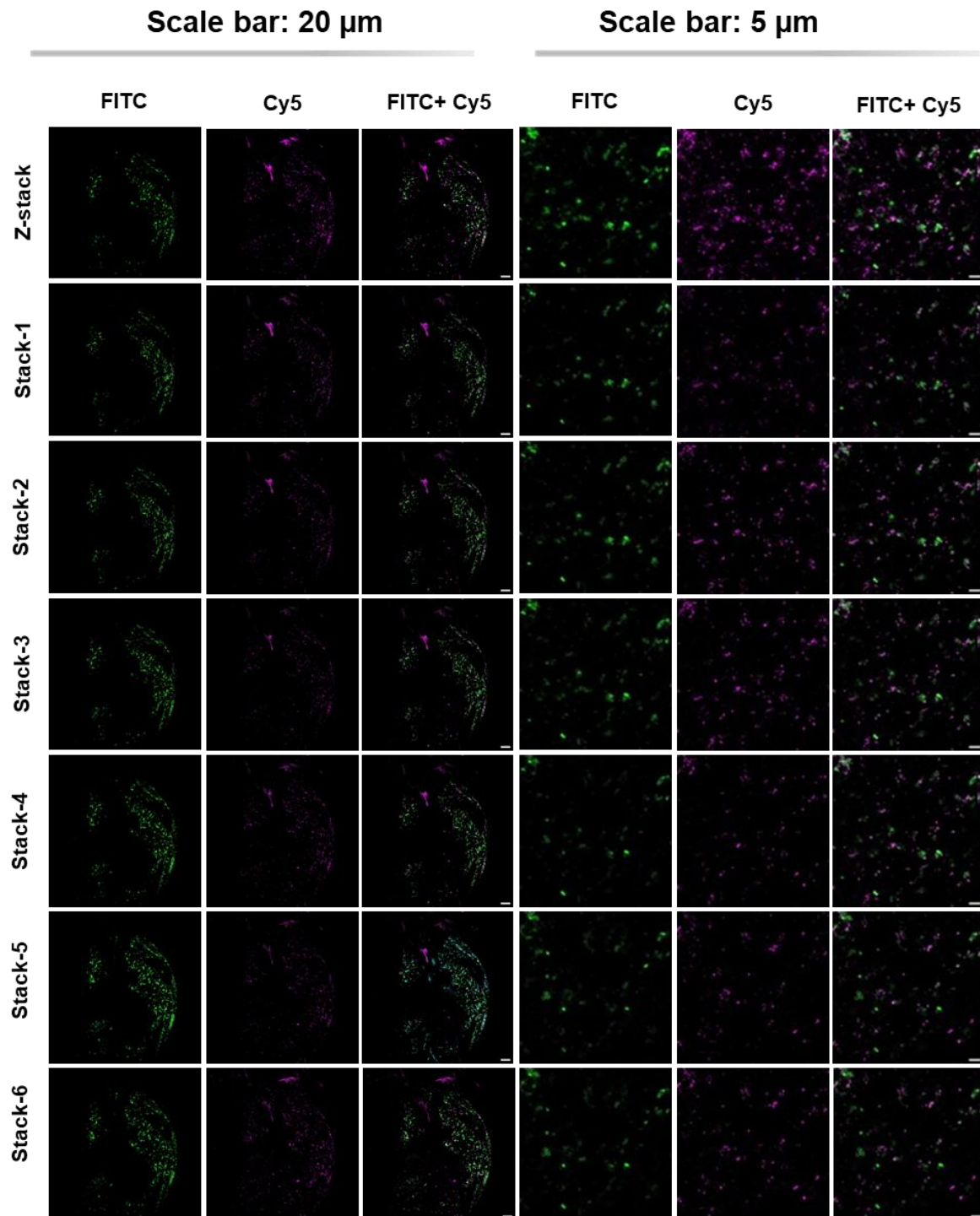


Fig. R11 | Stack analysis of pre- (FITC) and post-(Cy5) infection labeling of *S. aureus* in *Drosophila*.

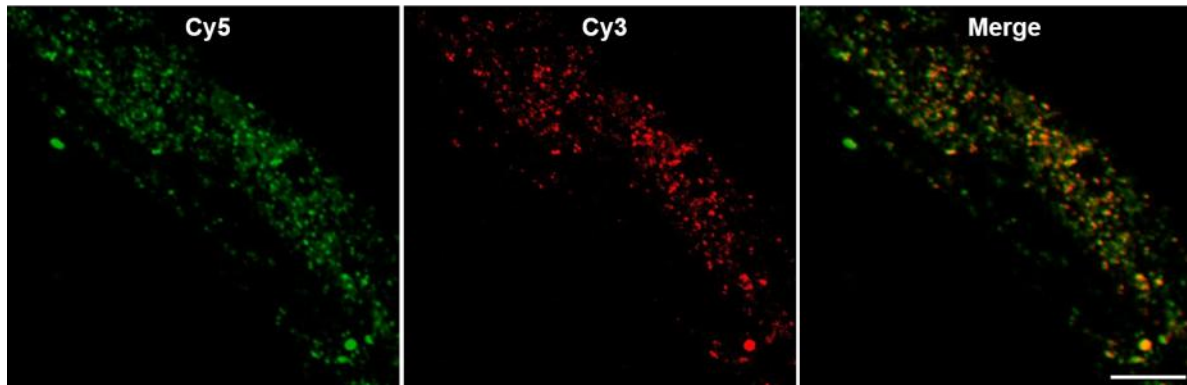


Fig. R12| Sequential dual labeling of *S. aureus* with **2u**. At first, infected larvae were fed with pre-conjugated 2u-Cy5, and Cy3 was conjugated post-dissection. Scale bar, 20 μ m.

Reviewer 2, Comment 21: Did the authors test whether **2n** is bactericidal or bacteriostatic?

Response by authors: We thank the reviewer for this question. To determine whether **2n** exhibits bacteriostatic or bactericidal activity, we performed a time-kill assay against *S. aureus* at different concentrations of **2n** and compared the results with vancomycin controls (Fig. R13). The results demonstrate that **2n** shows concentration-dependent bactericidal activity. At higher concentrations (5 $\times$ MIC), **2n** caused a complete loss of detectable bacterial colonies within 24 h, whereas intermediate concentrations produced partial but significant reductions in CFU counts. In contrast, lower concentrations showed only transient growth inhibition. These findings indicate that **2n** is not merely bacteriostatic but also exerts bactericidal effects in a concentration-dependent manner. The corresponding time-kill data have now been included in the revised Supplementary Information (Supplementary Fig. 142).

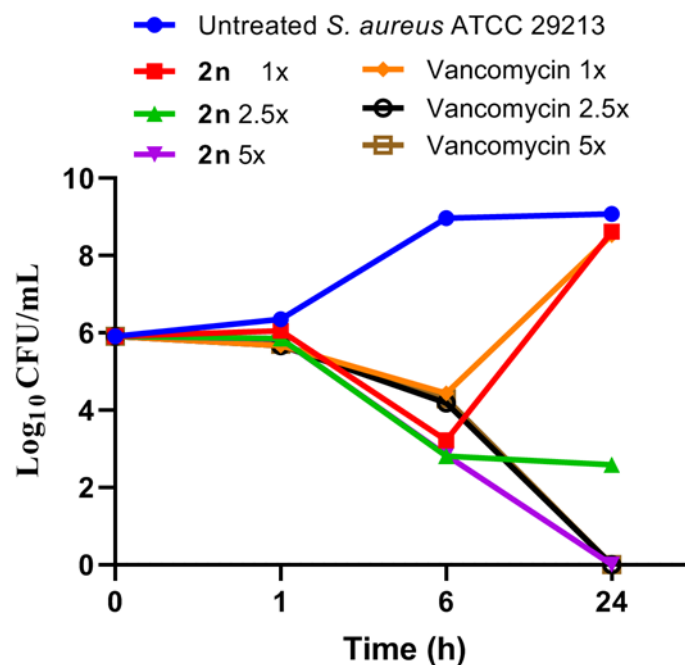


Fig. R13 | Time kill assay against *S. aureus* upon treatment of **2n** and vancomycin (positive control).

Reviewer 2, Comment 22: For the biofilm inhibition assay in Figure 10(d) and Supplementary Figure 154, it is surprising to see bacteria in 1X MIC in the SEM panels. MIC suggests no bacterial growth at the lowest concentration. Should Biofilm form at MIC or higher concentration, when there are no bacteria around? Looks like MIC is greater than what is reported here. The authors should show the growth inhibition curves for each concentration tested and test the biofilm inhibition at concentrations below the MIC for this assay or suggest the mechanism of biofilm inhibition.

Response by authors: We thank the reviewer for this comment. We would like to clarify that the MIC (minimum inhibitory concentration) is defined as the *lowest concentration at which no visible bacterial growth is observed*. Thus, MIC determination is based on macroscopic or optical assessment of planktonic bacteria and does not necessarily indicate complete elimination of all bacterial cells at the microscopic level. On the other hand, the sensitivity of SEM imaging makes it capable of detecting residual surface-associated bacteria, or partially damaged bacterial populations that may not contribute to visible growth in MIC assays. Therefore, the observation of bacterial structures in SEM images at 1× MIC is not uncommon and does not contradict the MIC determination. Moreover, in biofilm-related studies,⁴ it is common practice to evaluate antibiofilm effects at concentrations equal to or higher than the MIC. It is primarily because biofilm-associated bacteria exhibit greater tolerance and surface persistence compared to planktonic cells. Accordingly, higher concentrations are required for substantial disruption or complete reduction of biofilm architecture at the microscopic level.

Reviewer 2, Comment 23: In Figure 10(h), L-lys and Gly are indicated by the same symbol color, making it very difficult to differentiate between them.

Response by authors: We thank the reviewer for pointing this out. We agree that the use of similar shades of symbol colors for L-Lys and Gly in Fig. 10h is difficult to distinguish visually. To address this, we have updated the symbol color to clearly differentiate L-Lys from Gly in the revised version (Fig. 10h).

Reviewer 2, Comment 24: Supplementary Figure 162 (b), do the authors have any comments on why they are observing increased collagen density for 2n-treated wounds in comparison to uninfected wound healing?

Response by authors: We thank the reviewer for this observation of high collagen density in the treated group (*S. aureus* + 2n) with respect to the uninfected control group (*No S. aureus* + vehicle). At first, we thought that it might be due to the difference in tissue sections. However, a careful repeat of the experiments gave us a similar result. A plausible explanation for this observation could be that *S. aureus* infection can induce tissue damage and inflammatory responses, and subsequent antibacterial treatment may trigger compensatory wound-repair processes involving fibroblast activation and increased collagen deposition to restore tissue integrity.^{5 6} As a result, collagen accumulation in the treated infected wounds can exceed the levels observed in the uninfected healing control. Notably, the collagen fibers in the 2n-treated group appear more organized and continuous compared to the infected untreated group. However, we acknowledge that this interpretation remains a plausible explanation. Identifying the exact molecular basis underlying the enhanced collagen deposition would require a separate set of dedicated studies, including detailed analysis of inflammatory signaling pathways, fibroblast activity, and extracellular matrix remodeling, which are beyond the scope of an already extensive manuscript.

Reviewer 2, Comment 25: The composition of the ointment used for healing is not mentioned in the protocol.

Response by authors: We thank the reviewer for pointing this out. The topical formulation used in the wound-healing study consisted of 2% (w/w) **2n** in petroleum jelly, which served as the vehicle. We have now clarified the composition of the ointment formulation in the revised Supplementary Information and Experimental Section.

Minor points and corrections:

Response by authors: We sincerely thank the reviewer for the careful reading of the manuscript and for pointing out typographical errors, labeling inconsistencies, and figure clarity issues. We have carefully revised the manuscript and Supplementary Information accordingly. Detailed responses to each point are provided below.

Reviewer 2, Comment 1: Line 161 should be changed to 4-CHO (**2i**), not 4-NO₂

Response by authors: The error has been corrected from -NO₂ (**2i**) to -CHO (**2i**).

Reviewer 2, Comment 2: Line 165, should be changed to 4-CN (**2h**), not 4-CHO

Response by authors: The error has been corrected from -CHO (**2h**) to -CHO (**2i**).

Reviewer 2, Comment 3: In Figure 2, the authors have not specified the insulin concentration used in each experiment in the figure legends.

Response by authors: We thank the reviewer for highlighting this point. We have now included the insulin concentration (1, 20 μM) in the figure 2 legends.

Reviewer 2, Comment 4: Figure 2h, the authors have not mentioned the concentration of ADP and ATP

Response by authors: We thank the reviewer for highlighting this point. We have now specified the concentrations of biological additives used, including ADP (5 mM), ATP (5 mM), 10 % human serum, and DMEM cellular media, in the Figure 2 legends.

Reviewer 2, Comment 5: Supplementary Table 6 and Supplementary Figure 13, the reaction significantly dropped to 50% when 50-100μM insulin was used. Do the authors have any reasons for this concentration-dependent reduction in reactivity? Was it due to insufficient **2j**, or if insulin was aggregating at that concentration?

Response by authors: We thank the reviewer for this observation. In these experiments, the total proportional nanomoles of insulin and **2j** were kept constant, while only the solution volume was varied to achieve different insulin concentrations. Therefore, the reduced conversion observed at higher insulin concentrations is unlikely to arise from insufficient availability of **2j**. It is likely due to the well-established concentration-dependent self-assembly of insulin, which could reduce the accessibility of the reactive N-terminal site, thereby lowering the labeling efficiency. Such concentration-dependent outcomes are common in protein bioconjugation.

Reviewer 2, Comment 6: Figure 3, showing the reaction conditions, which is 20 μM; if it is a peptide concentration, it does not match the conditions mentioned in the Supplementary Table 15.

Response by authors: The peptide concentration in Figure 3 is 500 μM, as incorrectly stated in the figure. We have now updated Figure 3 to the correct peptide concentration.

Reviewer 2, Comment 7: Supplementary Figure 17, panel c: should be 3k

Response by authors: Supplementary Figure 17 has now been corrected to 3k in the revised Supplementary Information.

Reviewer 2, Comment 8: Supplementary Figure 36, the converted peak should be labeled 3h

Response by authors: The deconvoluted peak has now been correctly labeled as **3h** (Supplementary Figure 36) in the revised Supplementary Information.

Reviewer 2, Comment 9: Supplementary Table 15, %conversion should be 5a not 7a

Response by authors: We thank the reviewer for this typographical error. Table 15 has now been corrected accordingly.

Reviewer 2, Comment 10: Supplementary Table 16, %conversion should be 5, not 7

Response by authors: Table 16 in the Supplementary Information has now been corrected accordingly.

Reviewer 2, Comment 11: Supplementary Figure 69: Mass is not clearly visible

Response by authors: Supplementary Figure 69 has been updated for more clarity.

Reviewer 2, Comment 12: In Figure 5c, the panels in the flow chart are not clear between the steps from Cu click to Cu free.

Response by authors: We have now clarified in the figure caption that the bioconjugate generated from reagent **2n** was utilized for the Cu-catalyzed click reaction (Cu-click), whereas the bioconjugate generated from reagent **2p** was utilized for the Cu-free click reaction.

Reviewer 2, Comment 13: Figure 5, panel e, is not labeled, and line 355 should be Fig 5d.

Response by authors: There is no panel e in Fig.5. Now we have corrected the citation to Fig. 5d.

Reviewer 2, Comment 14: There is no mention of microscopy data in the description shown in the last panel of Figure 5

Response by authors: We have now added a description of the microscopy data in Fig. 5 for insulin bioactivity.

Reviewer 2, Comment 15: Line 376, Fig 6c-e is incorrect, cell labeling should be Fig 6c-d

Response by authors: The figure citation for cell labeling has been corrected to reflect the updated Fig. 6.

Reviewer 2, Comment 16: In Figure 6e, the flow cytometry data are not mentioned in the description.

Response by authors: The figure description has been modified in accordance with the updated Fig. 6.

Reviewer 2, Comment 17: Reversing the cellular bioconjugation with PLP data is interesting. However, did the authors normalize the PLP data with the viable bacteria.

Response by authors: We thank the reviewer for this comment. The PLP-mediated reversal experiment was performed in sodium bicarbonate buffer (200 μ L, 0.1 M, pH 7.8, 0.5 M NaCl) rather than in LB growth medium. We validated that the bacterial growth does not happen under these non-nutritive conditions within the given incubation period. The data has now been provided below for reviewer's reference (Fig. R14).

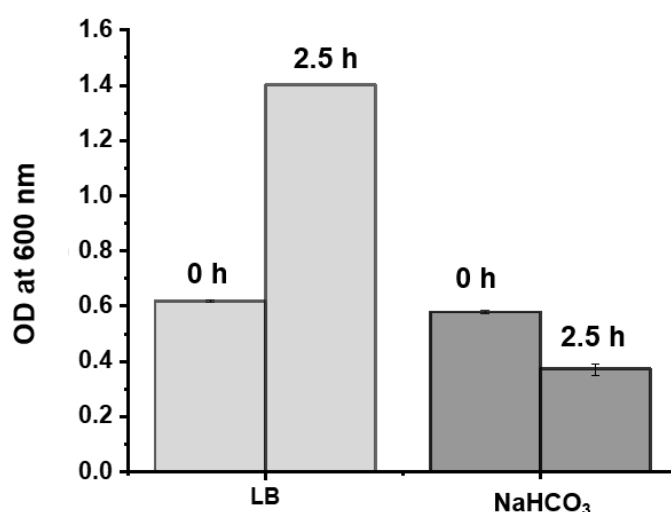


Fig. R14 | *S. aureus* growth in LB media and NaHCO₃ buffer at 0 and 2.5 h.

Reviewer 2, Comment 18: According to the description, the panels in Figure 8 are not easy to comprehend. Figure (a) should be divided into 3 separate panels with proper labeling for easy understanding.

Response by authors: Figure 8a has now been updated as Extended data Fig. 1. Now we have added the details for dual- and triple-probe installations to aid readers' understanding.

Reviewer 2, Comment 19: Figure 9e, correct the panels (i), (ii), and (iii).

Response by authors: In the revised manuscript, the Fig. 9e panels have been corrected.

Reviewer 2, Comment 20: The supplementary figures are not in order. Sup Fig. 89 comes before sup Fig. 88.

Response by authors: Supplementary Figs. 88 and 89 have now been rearranged into the proper sequential order in the revised Supplementary Information.

Reviewer 2, Comment 21: In sup fig 153 what are the images of the mice in the top vs bottom images for each panel. It is not clear from the legend. I think the top is fluorescence and bottom is bioluminescence but that needs to be labeled.

Response by authors: We have now clarified the caption of the revised Supplementary Figure 141 by explicitly stating that the top images in each panel represent fluorescence imaging, while the bottom images represent bioluminescence imaging.

Reviewer 2, Comment 22: Having a 162 supplemental figures is excessive. It seems like these need to be organized into actual figures vs. MS profiles to validate adducts. The adducts could be grouped into some kind of supplemental data set so that the actual supplement figures that are important for the main paper are easier to access.

Response by authors: We thank the reviewer for this suggestion. We have substantially revised the Supplementary Information and removed 20 figures (MS data from replicates). This reduces the overall length of main supplementary information and makes it easier to navigate. The replicates of MS data have now been compiled into a separate supplementary file.

Reviewer 3

Reviewer 3: In the submitted manuscript, Rai and co-workers describe a new strategy to target the N-terminal glycine residue in peptides and proteins. This work represents an evolution of a previous technology reported by the same group; however, this does not diminish the significance of the present study, as the new protocol effectively enables applications ranging from in vitro systems to live cells and in vivo models. Using electronically tunable reagents, the method allows rapid and highly selective C–C bond formation at the N-terminal glycine while maintaining reactivity across diverse biomolecular environments. The resulting aminoalcohol bioconjugates were structurally validated by NMR, LC–MS, and single-crystal XRD, supported by DFT calculations and control experiments. The platform further enables the installation of bioorthogonal handles for versatile downstream functionalization and remains stable in complex biological media.

Importantly, this platform enables species-specific detection and targeting of *Staphylococcus aureus* by labeling the Gly5 signature in the bacterial cell wall, with demonstrated selectivity in co-cultures, live cells, *Drosophila melanogaster*, and murine infection models. The authors performed a thorough study and explored a wide range of applications to validate the technology, which is commendable. Technically, the work is solid and I have only a few minor comments.

Response by authors: We sincerely thank the reviewer for the positive evaluation of our work and for recognizing both the conceptual advancement and broad applicability of the platform. We are particularly encouraged by the reviewer's appreciation of the strategy for selective N-terminal glycine targeting, the extensive validation across biomolecular and biological systems, and the demonstrated applicability toward *S. aureus* detection and targeting in complex in vivo settings. We also appreciate the reviewer's recognition of the thorough experimental and mechanistic investigations supporting this study. We have carefully considered the minor comments provided below and have revised the manuscript accordingly.

Reviewer 3, Comment 1: The manuscript is very dense, and the use of large multi-panel figures sometimes makes it difficult to locate specific information.

Response by authors: We thank the reviewer for this feedback. To address the concern, we have revised figures 6 and 7 substantially. Additionally, we have edited figures 2, 3, 4, 8, and 9. Further, Supplementary Information sections have also been revised to improve clarity and readability.

Reviewer 3, Comment 2: The authors should revise the compound numbering on page 5 (first paragraph), as it does not match the numbering shown in Figure 2a.

Response by authors: We thank the reviewer for pointing this out. We have carefully revised the compound numbering in the first paragraph on page 5 to ensure that it is fully consistent with the numbering scheme shown in Figure 2a. Particularly, the errors have been corrected from -NO₂ (2i) to -NO₂ (2j) and from -CHO (2h) to -CHO (2i), and these changes have now been updated in the revised manuscript.

Reviewer 3, Comment 3: The nitro substituent appears to favor C–C bond formation; however, the authors do not mention the potential toxicity concerns associated with this functional group, which are well known.

Response by authors: We thank the reviewer for raising this concern. The bacterial labeling, mammalian cell compatibility, and mouse wound-healing experiments, did not indicate toxicity or adverse tissue effects. For the latter, the treated groups showed improved wound-healing outcomes compared with infected, untreated controls. To address this point, we assessed cytotoxicity of *rapi*-Gly-tag reagent in eukaryotic Vero cells and observed a selectivity index >11 relative to the MIC of *S. aureus* (Fig. 9c, Supplementary Table 19).

Reviewer 3, Comment 4: The DFT calculations should also include a comparison with the least-performing compounds in order to better rationalize the observed differences in reactivity. Once these comments are addressed, the manuscript may be considered for publication.

Response by authors: We thank the reviewer for the suggestion. Based on the suggestion, we have carried out detailed DFT calculations. We calculated the free energy profile for the reaction of 4-methoxybenzaldehyde with **8a** using DFT methods (Fig R15). The **10-IN1** proceeds via water-assisted proton transfer to form the unstable intermediates **10-IN2**, with a transition-state barrier of 35.3 kcal/mol, which was 7.7 kcal/mol higher than that of **8a-IN2** (27.6 kcal/mol). Next, the intermolecular C-C bond-forming step gives rise to the stable intermediates **8a-IN3** via the transition state TS2, with a barrier of 23.8 kcal/mol. These computational results clearly indicate that the pathway is significantly more feasible with **2j** (4-nitrobenzaldehyde) as a substrate than with 4-methoxybenzaldehyde (**2b**). These results are in good agreement with the experimental observation and now been included in the revised Supplementary Information (Supplementary Fig. 99).

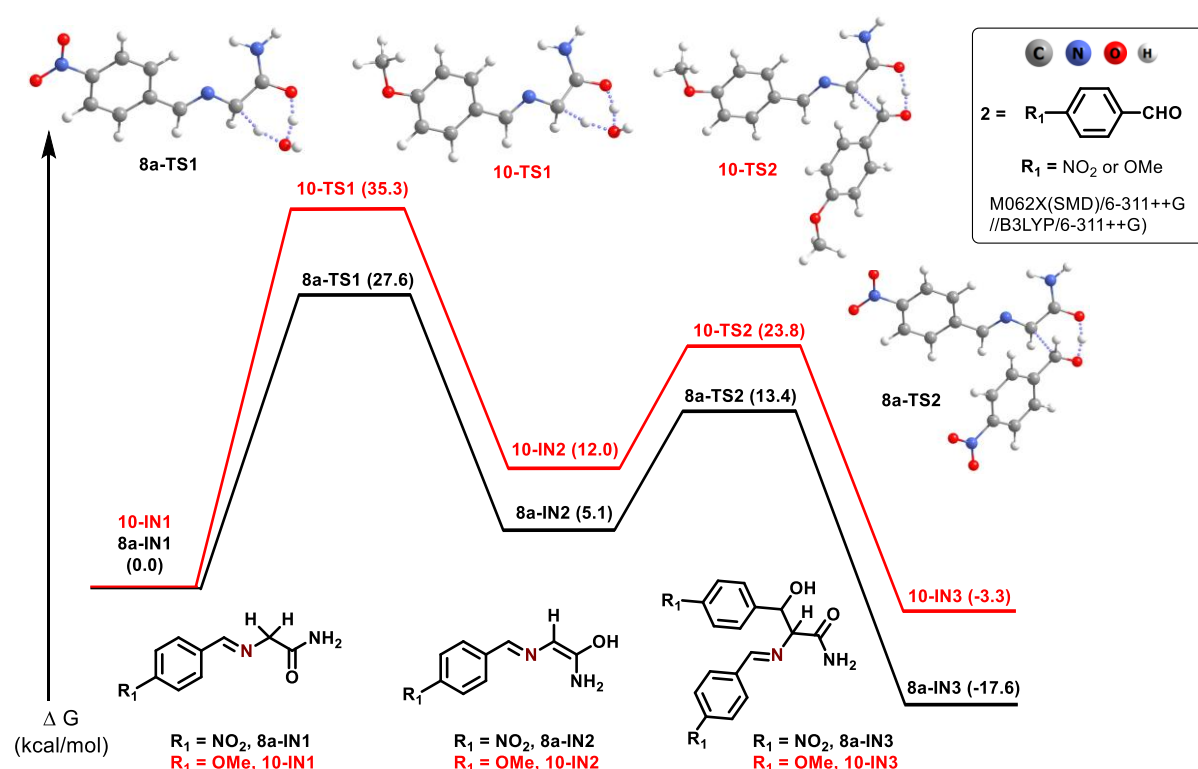


Fig. R15 | Comparative free energy profile for 4-nitrobenzaldehyde (**2j**) and 4-methoxybenzaldehyde (**2b**). Mechanistic insights by DFT calculation using M062X/6-311++G(d,p)//B3LYP/6-311++G(d,p) method. Non-essential hydrogen atoms are omitted for clarity.

Reviewer 4

Reviewer 4: I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response by authors: We thank the reviewer and co-reviewer for their careful evaluation of our manuscript and for their constructive comments and suggestions.

Reviewer 5

Reviewer 5, Comment 1: In this paper, the authors report a method for selectively tagging N-terminal glycine proteins in aqueous media using different bioorthogonal handles that enable late-stage modification. They further demonstrate that this strategy can be applied to selectively target *Staphylococcus aureus* in vivo. I found the manuscript scientifically compelling and impactful; however, the topic lies outside my primary area of expertise. I was asked specifically to evaluate only the computational component of the work, and the report below focuses exclusively on that aspect.

In Section 3.3, the authors investigate the mechanism of formation of a 1,2-aminoalcohol from the rapiGly Tag reagent 2j and Gly or Ala. The mechanistic pathway proposed is chemically reasonable and aligns well with mechanisms previously reported for analogous transformations. In addition, the detection of the proposed intermediates by HRMS offers valuable experimental support for the sequence of steps described.

From a computational standpoint, the authors employ the M06-2X/6-311++G(d,p)//B3LYP/6-311++G(d,p) level of theory. Although this cannot be considered state-of-the-art—more modern and robust approaches are now available—M06-2X nevertheless remains a sensible choice for capturing general mechanistic features. The results presented appear internally consistent and adequate for the purposes of the study.

Response by authors: We sincerely thank the reviewer for carefully evaluating the computational aspects of our work and for the positive assessment of the mechanistic study. We also appreciate the recognition that the experimentally detected HRMS intermediates provide meaningful support for the proposed mechanistic sequence. All the suggestions and comments have been addressed, and the corresponding changes have been incorporated in the revised manuscript. These changes have significantly improved the quality of this work, and we thank the reviewer for their input.

Reviewer 5, Comment 2: I recommend revising the statement on page 10, lines 285–286 (“All the energies were estimated in the presence of water using an explicit solvent model”) as well as the caption of Figure 4 (page 9, line 268), where it is stated that an explicit solvent model was used, as the calculations do not employ a fully explicit solvent model. As indicated in the Supporting Information, the authors combined a single explicit water molecule with the SMD continuum solvent model (SCRF), which corresponds to a hybrid cluster–continuum approach, not a fully explicit solvation model.

Response by authors: We thank the reviewer for pointing this out. We have corrected the errors as suggested.

Reviewer 5, Comment 3: Regarding stereochemical aspects, the calculated structure IN3 corresponds to the anti diastereomer of the 1,2-aminoalcohol product, in agreement with the configuration observed in the X-ray structure. Experimentally, however, the reaction yields a mixture of anti and syn diastereomers, as indicated by the NMR spectra. Was the syn diastereomer also computed and how does its energy compare to that of the anti isomer? A similar comment applies to the enol tautomer IN2: the authors depict the Z isomer, presumably because it is lower in energy than the E isomer. Maybe a brief comment in the Supporting Information would suffice.

Response by authors: We thank the reviewer for the constructive comments and supporting the manuscript. To address the point, the calculations for the syn and anti-diastereomer of the intermediate **IN3** and the E-Z isomers of **IN2** has now been included in the revised Supporting Information (Supplementary Figs. 100 and 101).

As the reviewer anticipated, the calculations indicate that the anti-diastereomer intermediate **8a-IN3** is more stable than its syn-diastereomer **8a'-IN3** (1.8 kcal/mol, Fig. R16). Similarly, anti-diastereomer intermediate **8b-IN3** is more stable than its syn-diastereomer **8b'-IN3** (3.3 kcal/mol).

We noticed that the enol tautomer **IN2** was depicted incorrectly. It has now been corrected in Fig. 4 of the revised version. The E-isomers (**8a-IN2** and **8b-IN2**) are more stable than the Z-isomers (**8a'-IN2** and **8b'-IN2**; 0.3 and 2.3 kcal/mol, respectively; Fig. R17).

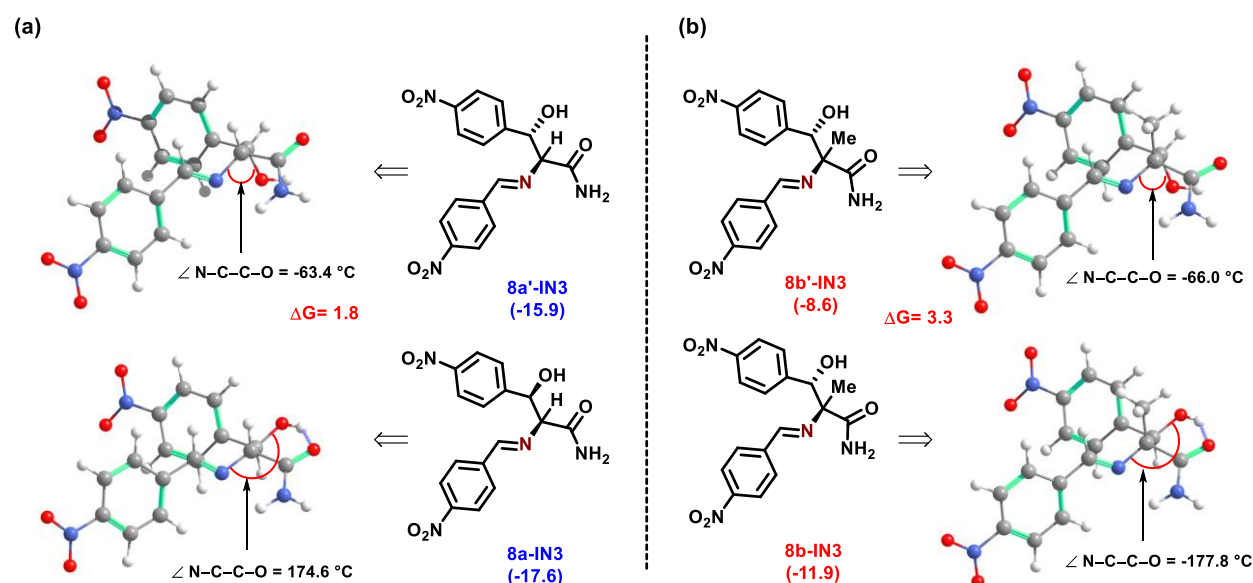


Fig. R16 | The conformation of syn- and anti-isomers structures for the intermediates (A) **8a-IN3**, and **8a'-IN3**; (B) **8b-IN3** and **8b'-IN3**.

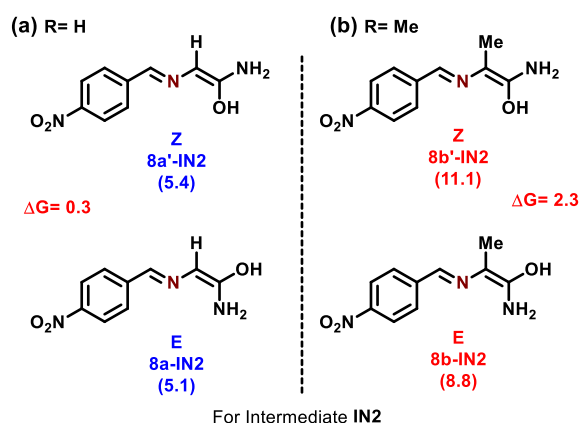


Fig. R17 | The calculated intermediate **IN2** ($R = -H, -Me$) for the *E*- and *Z*-isomeric forms.

Reviewer 5, Comment 4: Overall, the calculations convincingly support the conclusions and claims made in the manuscript, and no additional evidence appears necessary. The data analysis and interpretation are sound. While a few minor points would benefit from clarification, none undermine the validity of the study or require significant revision. The methodology is appropriate and meets the expected standards of the field, and the level of detail provided is sufficient to ensure reproducibility.

Response by authors: We thank the reviewer for supporting the computational investigations and for critical insights.

References

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REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have addressed all of my many concerns and have added some important new data with controls that look good. This work is now ready for publication. My only suggestion would be to include the controls in the main figures when possible, since it seems like most of the negative controls ended up in the SI figures.

Response by authors: We sincerely thank the reviewer for the positive evaluation of our work and for recognizing our efforts in addressing the previous concerns. We appreciate the reviewer's suggestion regarding the placement of the negative controls. To address this point, we have added the key negative control data to the main figures 6, 8, and 9.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, Rai and co-workers have addressed the comments raised by this referee carefully, particularly those concerning the scientific aspects of the work. The DFT calculations have been significantly improved, and the simplified figures enhance the readability of the manuscript. Overall, I believe the authors have done an excellent job addressing all the questions and concerns raised by this referee. This work is now suitable to be accepted.

Response by authors: We thank the reviewer for the positive comments about the manuscript.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response by authors: We thank the reviewer and co-reviewer for their careful evaluation of our manuscript and for their constructive comments and suggestions.

Reviewer #5 (Remarks to the Author):

I have reviewed the revised version of this manuscript, focusing on the computational aspects, as in my initial evaluation. The authors have satisfactorily addressed the points raised in my

previous report. The revisions have resolved the minor issues previously identified, and no further concerns remain. I therefore recommend the manuscript for publication in its current form.

Response by authors: We thank the reviewer for the positive comments.

In this paper, the authors report a method for selectively tagging N-terminal glycine proteins in aqueous media using different bioorthogonal handles that enable late-stage modification. They further demonstrate that this strategy can be applied to selectively target *Staphylococcus aureus* in vivo. I found the manuscript scientifically compelling and impactful; however, the topic lies outside my primary area of expertise. I was asked specifically to evaluate only the computational component of the work, and the report below focuses exclusively on that aspect.

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From a computational standpoint, the authors employ the M06-2X/6-311++G(d,p)//B3LYP/6-311++G(d,p) level of theory. Although this cannot be considered state-of-the-art—more modern and robust approaches are now available—M06-2X nevertheless remains a sensible choice for capturing general mechanistic features. The results presented appear internally consistent and adequate for the purposes of the study.

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Overall, the calculations convincingly support the conclusions and claims made in the manuscript, and no additional evidence appears necessary. The data analysis and interpretation are sound. While a few minor points would benefit from clarification, none undermine the validity of the study or require significant revision. The methodology is appropriate and meets the expected standards of the field, and the level of detail provided is sufficient to ensure reproducibility.