ChemBioChem

Research Article
doi.org/10.1002/cbic.202400037

Chemistry Europe
European Chemical Societies Publishing

www.chembiochem.org

Probing Peptidoglycan Synthesis in the Gut Commensal *Akkermansia Muciniphila* with Bioorthogonal Chemical Reporters

Tjerk J. Sminia,[a] Steven Aalvink,[b] Hanna de Jong,[c] Marcel H. Tempelaars,[d] Han Zuilhof,[a, e] Tjakko Abee,[d] Willem M. de Vos,[b, f] Hanne L. P. Tytgat[+],[b, g] and Tom Wennekes[+*][a, c]

Our gut microbiota directly influences human physiology in health and disease. The myriad of surface glycoconjugates in both the bacterial cell envelope and our gut cells dominate the microbiota-host interface and play a critical role in host response and microbiota homeostasis. Among these, peptidoglycan is the basic glycan polymer offering the cell rigidity and a basis on which many other glycoconjugates are anchored. To directly study peptidoglycan in gut commensals and obtain the molecular insight required to understand their functional activities we need effective techniques like chemical probes to label peptidoglycan in live bacteria. Here we report a chemically guided approach to study peptidoglycan in a key mucin-degrading gut microbiota member of the *Verrucomicrobia* phylum, *Akkermansia muciniphila*. Two novel non-toxic tetrazine click-compatible peptidoglycan probes with either a cyclopropene or isonitrile handle allowed for the detection and imaging of peptidoglycan synthesis in this intestinal species.

Introduction

Research of the past decade has uncovered the great diversity and abundance of the human gut microbiota and how these mainly anaerobic bacteria directly influence human physiology in health and disease. They protect against invading microbes, aid in degrading non-digestible dietary components, produce key nutrients and vitamins, and prime the immune system.[1] Mapping the key molecules of individual microbiota members that mediate microbiota-host interactions will be crucial to understand how this complex bacterial community controls human wellbeing by preserving mucosal homeostasis. Surface glycans and their glycoconjugates dominate the host-microbiota interface. Recent studies have highlighted the especially important role of glycoconjugates in the cell envelope of microbiota members on both mucosal homeostasis and dysbiosis.[2]

Acquiring a molecular understanding of bacterial glycoconjugate composition, activity and associated enzymes in their native setting is best achieved by a chemically guided approach[3] as the non-template driven assembly of glycoconjugates prevents the use of conventional molecular biology tagging methods. Microbiology increasingly uses the metabolic incorporation of chemical reporters for this purpose.[4] It allows for the direct imaging of cell envelope glycoconjugates, the modulation of their structures and subsequent study of their functional activities. For the application of this method on gut microbial ecosystems there is however a critical need for a molecular toolbox of validated bioorthogonal chemical reporters that work in live (an)aerobic microbiota members.

We aimed to develop a method for investigating the cell envelope of *Akkermansia muciniphila*, a key Gram-negative, oval-shaped, non-motile anaerobic bacterium in healthy human gut microbiota.[5] It colonizes the mucus layer of the gastrointestinal tract and specializes in the degradation of complex

[a] *T. J. Sminia, H. Zuilhof, Dr. T. Wennekes[+]*
Laboratory of Organic Chemistry, Wageningen University and Research, Wageningen, The Netherlands,
E-mail: t.wennekes@uu.nl

[b] *S. Aalvink, W. M. de Vos, H. L. P. Tytgat[+]*
Laboratory of Microbiology, Wageningen University and Research, Wageningen, The Netherlands

[c] *H. de Jong, Dr. T. Wennekes[+]*
Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands

[d] *M. H. Tempelaars, T. Abee*
Laboratory of Food Microbiology, Wageningen University and Research, Wageningen, The Netherlands

[e] *H. Zuilhof*
School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, P.R. China

[f] *W. M. de Vos*
Human Microbiome Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[g] *H. L. P. Tytgat[+]*
Current address: Nestlé Institute of Health Sciences, Nestlé Research, Lausanne, Switzerland

[+] *Dr. Tom Wennekes and Hanne L. P. Tytgat contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202400037

mucosal *O*-glycans. As a mucin-degrader, *A. muciniphila* is less dependent on the influx of dietary components, being able to use mucus as its sole carbon and nitrogen source,[5b] and the bacterium supports other microbiota members by providing access to mucus monosaccharides. This places it in a key position to shape the overall microbial composition. Due to its close proximity within the mucus layer to its human host *A. muciniphila* may also exert a relatively large, stimulating, effect on our immune system. *A. muciniphila* also stimulates host mucin production, increases mucus layer thickness and strengthens intestinal barrier function.[6] Its lowered intestinal colonization has been correlated with inflammatory bowel disease, obesity and various other diseases. Intervention studies in mice showed its potential to protect against high-fat-diet induced obesity and more recently, the safety and application of *A. muciniphila* as a therapeutic for human obesity and associated disorders was shown.[7]

To understand and modulate the interaction of the commensal *A. muciniphila* with its host and other gut microbiota members it is essential to have access to tailor-made small-molecule tools to study its basic physiology and function at the molecular level and especially its understudied cell envelope glycoconjugates. It was recently shown that the cell envelope of *A. muciniphila* contains peptidoglycan (PG) with immunostimulatory muropeptides.[8] This glycoconjugate plays a key role in cell division, cell envelope maintenance and through human PG recognition proteins also in microbiota-host interactions.[9] Given the important functions of PG and because *A. muciniphila* belongs to the deeply rooted Verrucomicrobia[5b] that are part of the PVC superphylum, of which members may lack PG,[10] we wanted to expand the current toolbox for PG analysis by designing new tools for its labeling and show its application in *A. muciniphila*.

Here, we report the development of two non-toxic D-Ala-dipetide-based PG probes, equipped with a cyclopropene or isonitrile chemical reporter, which were used to further demonstrate that *A. muciniphila* indeed synthesizes PG and show its location in the cell envelope periplasm (Figure 1). Both chemical probes and their labeling with a tetrazine click reaction proved non-toxic under anaerobic culturing conditions and allowed for PG detection via flow cytometry and confocal microscopy in live bacteria.

Results and Discussion

Our first goal was to develop chemical probes that would be able to detect and image PG synthesis in live anaerobic bacteria. As a constituent of bacterial cell envelopes that preserves cell rigidity and shape, PG forms an anchoring point for many cell wall-associated molecules and plays a key role in cell growth and division.[11] If present, as in the recent discovery for A. muciniphila, it forms a unique opportunity to get insight in the cell envelope dynamics of bacterial cells. PG consists of an alternating polymeric chain of β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) with a

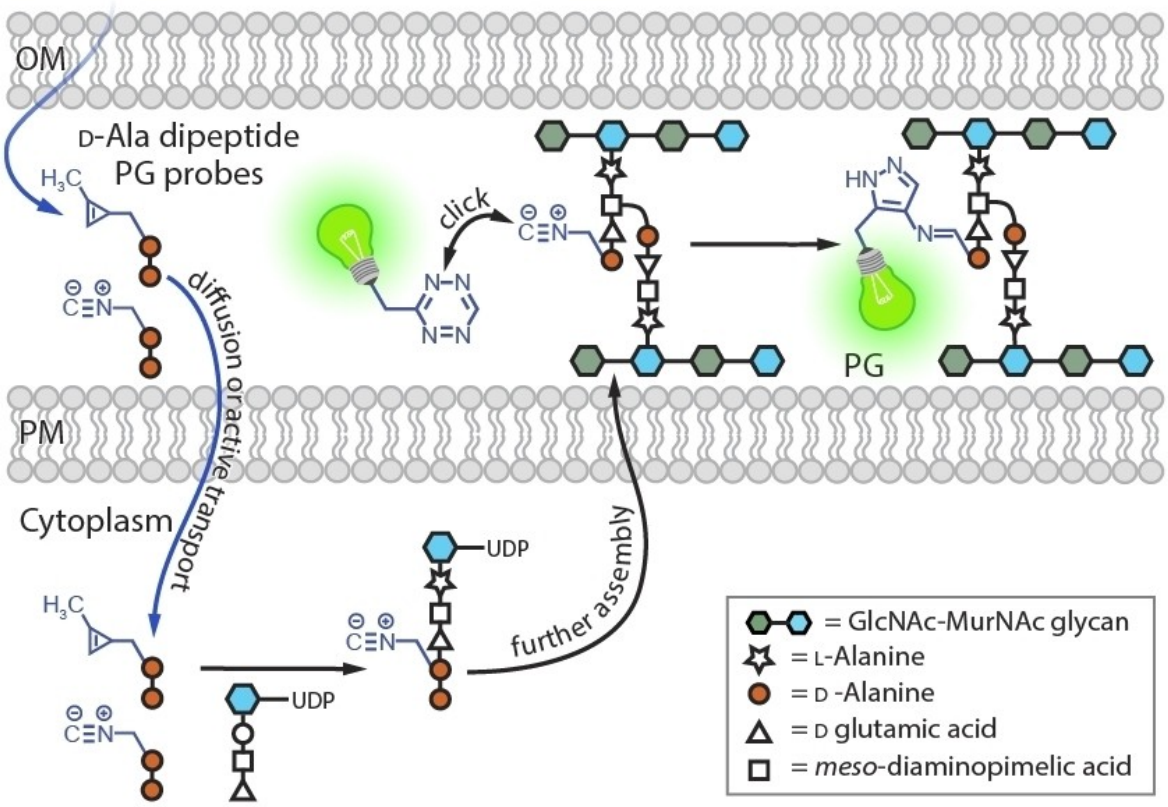


Figure 1. Overview of metabolic labeling with PG probes. OM: Outer membrane, PG: peptidoglycan, PM: plasma membrane, UDP: uridine diphosphate.

pentapeptide attached to the latter that enables crosslinking between chains. The glycan chain of PG can function as a target for metabolic labeling, but as the glucosamine core of this is not unique to bacteria this can limit their application.[4e,12] The so-called PG stem peptide contains two terminal D-Alanine residues that are of special interest, as these are unique to bacterial PG. In recent years, this fact has been exploited to study PG via metabolic incorporation of unnatural D-amino acid-based probes (Figure 1). Although some of these probes have a prepended bulky fluorophore, most probes rely on a secondary click reaction step between a chemical reporter group on the metabolically incorporated D-Ala-based probe and a complementary group attached to a fluorophore.[3h,13] This smaller size of the initial probe also better permits PG labeling of Gram-negative bacteria whose outer membrane make them less accessible to fluorophore-prepended bulky PG probes. Liechti and co-workers used this approach to prove the presence of PG in another PVC supercluster member, Chlamydia trachomatis, with an alkyne-containing D-Ala dipeptide probe.[13e] Their probe however relies on a copper-catalyzed azide-alkyne cycloaddition (CuAAC) in which the used copper is toxic to bacteria. In general, there is a lack of bioorthogonal PG probes that closely mimic D-alanine and even more crucially that have been validated for their efficacy in non-toxic labeling and detection of bacterial PG in vivo.

The [4 + 1] cycloaddition of isonitriles and tetrazines is a recent addition[14] to the bioorthogonal click chemistry toolbox. We envisioned that the sterically small isonitrile group would allow for a close mimic of D-alanine. This should only result in a minor perturbation of the PG macromolecular structure once incorporated and thereby limit an effect on the dynamics of PG production and degradation. Additionally, the inverse electron demand Diels Alder (invDA) reaction between strained alkenes and tetrazines is among the fastest bioorthogonal reactions currently known and allows for the use of relatively compact cyclopropene chemical reporters.[4f] Both these copper independent click reactions have been proven to be bioorthogonal and were already applied successfully in in vivo glycan imaging.[14b,15] We therefore designed a D-Ala-based probe with either an isonitrile or cyclopropene chemical reporter group. As modified single D-Ala amino acids are sometimes poorly incorporated into PG and are not selective in labeling PG formation and maturation dynamics,[13d,k] we chose to adapt the design reported by Liechti and co-workers based on a D-Ala dipeptide motif.[13e] By installing the bio-orthogonal isonitrile or cyclopropene chemical reporter group on the N-terminus of the D-Ala dipeptide we could target labeling to the crucial fourth position of the stem peptide[13e] that is actively involved in PG formation and maturation (Figure 1).

We started our synthesis route with the commercially available Fmoc-D-asparagine-OH (1) for both the cyclopropene and isonitrile probe (see Figure 2, detailed synthetic procedures in SI). A hypervalent iodine mediated Hofmann rearrangement under basic conditions efficiently produced an amine intermediate (2) in 99% yield.[16] After temporary Boc-protection of the obtained amine, a peptide coupling under standard carbodiimide coupling conditions with D-Ala-OCH3.HCl produced dipeptide precursor 4. The Boc-group was removed with trifluoroacetic acid (TFA), yielding the free amine dipeptide as a TFA salt intermediate (5). From this point onwards the synthesis of the cyclopropane (7) and isonitrile (10) dipeptide PG probe diverged. In the case of the cyclopropene-containing probe, the free amine was reacted with activated para-nitrophenylcarbonate cyclopropene resulting in compound 6 with a cyclopropene handle on the protected D-Ala dipeptide.[15a] The synthesis of this cyclopropene handle was reported by Prescher and co-workers and later adapted by us and others.[15,17] From the diverse family of cyclopropenes we chose a methyl-cyclopropene carbamate handle as the most suitable in terms of stability during probe synthesis and reactivity during the labeling.

In case of the isonitrile PG probe, the free amine (**5**) was reacted with the mixed acetic formic anhydride. Different methods for the formation of a formamide are known,[18] but the procedure using the mixed acetic formic anhydride proved the most reproducible in providing formamide **8**. Other procedures such as formic acid in presence of dicyclohexylcarbodiimide (DCC) resulted in byproduct formation while other methods with methyl formate or catalysis with boric acid gave low to no product formation. With formamide **8** in hand we next assessed the standard method for formamide dehydration with $POCl_3$. Although complete consumption of **8** was observed and traces of the isonitrile could be detected, this method was not successful in producing the isonitrile. A milder dehydration method using Burgess reagent and refluxing DCM did however succeed in producing the protected isonitrile probe (**9**) in acceptable yield.[19] Next, Fmoc deprotection in both PG dipeptide probe precursors (**6** and **9**) provided our target PG probes **7** and **10** (Figure 2). The methyl ester was left in place, as we expected that the remaining methyl ester on the C-terminal D-Ala amino acid would be hydrolyzed by nonspecific bacterial esterases, as these dipeptides are believed to selectively label PG via a cytoplasmic route.[13e] Additionally, the C-terminal D-ala is also not directly involved in the chemistry of metabolic incorporation. The increased lipophilicity the ester imparts on the probes might facilitate the initial passive uptake of the dipeptide probe **4** and **6**.

The aim of our bioorthogonal PG probes was to image PG synthesis in live bacteria, but the incorporation of our unnatural D-Ala dipeptides **7** and **10** might have detrimental effects on bacterial growth, viability and physiology. Therefore, we evaluated their toxicity. As a benchmark, we first assessed toxicity of the probes in *Escherichia coli*, which did not reveal any detrimental effects of the chemical probes on specific growth rate (Figure 3A). Similarly, in *A. muciniphila* no significant growth defect was observed after addition of our isonitrile and cyclopropene probes. In contrast, addition of the copper-dependent alkyne probe of Liechti *et al.*, which was used as a benchmark, resulted in a significant growth defect in *A. muciniphila* cultures (Figure 3B).[13e] The cause of this growth defect with the alkyne dipetide probe is not yet understood, but one other structural difference between our probes and the benchmark probe is the presence of the methyl ester.

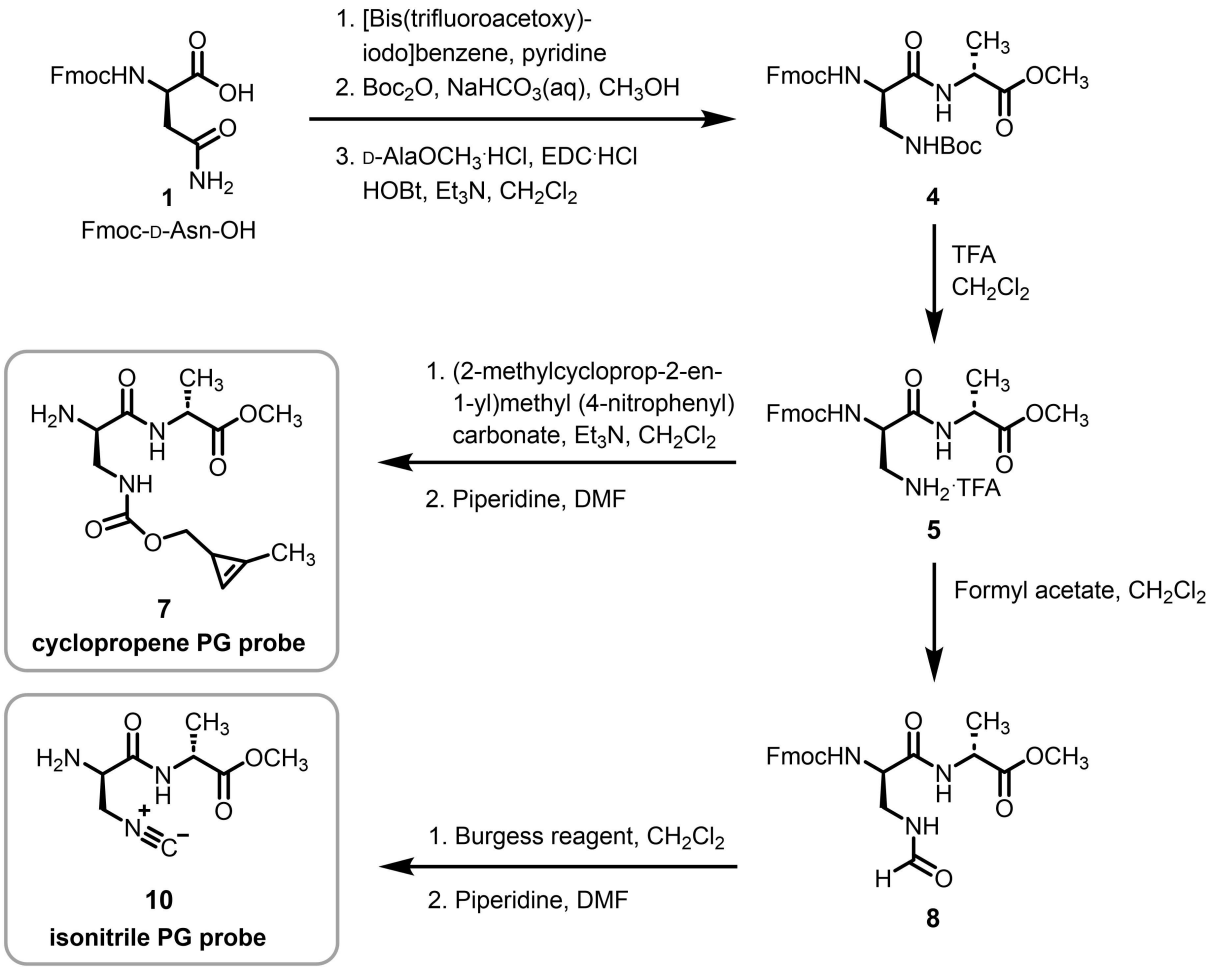


Figure 2. Synthesis of cyclopropene- and isonitrile-containing PG probes **7** and **10** (details on synthesis route are located in SI).

Next, we evaluated the combined toxicity of metabolic PG probe incorporation and the subsequent click reaction with fluorescent reporter groups for our probes in comparison to the alkyne PG probe with associated CuAAC click reaction. The viability of the bacteria was assessed after the click reaction by determining colony-forming units on CP-mucin agar for *A. muciniphila* and LB agar for *E. coli* (Figure 3C and D). This clearly revealed the expected cytotoxicity of the copper-dependent alkyne PG dipeptide probe and subsequent CuAAC reaction. In stark contrast with the alkyne probe, both *E. coli* and *A. muciniphila* showed sustained viability when subjected to our novel cyclopropane (**7**) and isonitrile (**10**) PG probes in combination with the bioorthogonal invDA or [4+1] cycloaddition reaction.

The efficacy of both our novel, non-toxic dipeptide probes (**7** and **10**) to detect and image PG synthesis was first successfully confirmed in *E. coli* using flow cytometry (FCM) and confocal imaging experiments (Figure 4). This initial result highlights the potential wider application of our probes for PG imaging and proved that the presence of the methyl ester does not prevent metabolic incorporation of the D-Ala dipeptide analogs. The probes do have the potential to intramolecularly cyclize to a non-metabolizable diketopiperazine derivative via the methyl ester, however, if it occurred as a side reaction, it did not hinder the application of the probes in our hands.

We next assessed PG presence in the cell envelope periplasm of *A. muciniphila.* Anaerobic cultures were incubated in the presence of either probe **7** or **10** for 1 h prior to sampling. The invDA or [4+1] cycloaddition with the fluorophore-appended click reagents, here a Cy3 tetrazine dyes was used, proceeded fast, with minimal background and did not affect the morphology of *A. muciniphila* (Figure S1). Confocal microscopy images of *A. muciniphila* probed (Figure 5 and Figure S2) with the non-toxic PG dipeptide probes showed clear fluorescence and provide compelling additional evidence for PG synthesis in this intestinal species. In a comparative FCM experiment in *A. muciniphila.*, probe **7** favorably compared to the established alkyne-based PG probe of Liechti and co-workers, rendering less background signal and thus offering a non-toxic alternative to the that Cu-dependent probe (Figure S3 and S4). The compatibility of these non-toxic probes and subsequent click reactions with bacterial growth opens perspectives towards the further use of these probes to monitor PG dynamics *in vivo* for this and other gut microbiota members. Another advantage of these new PG probes is their orthogon-

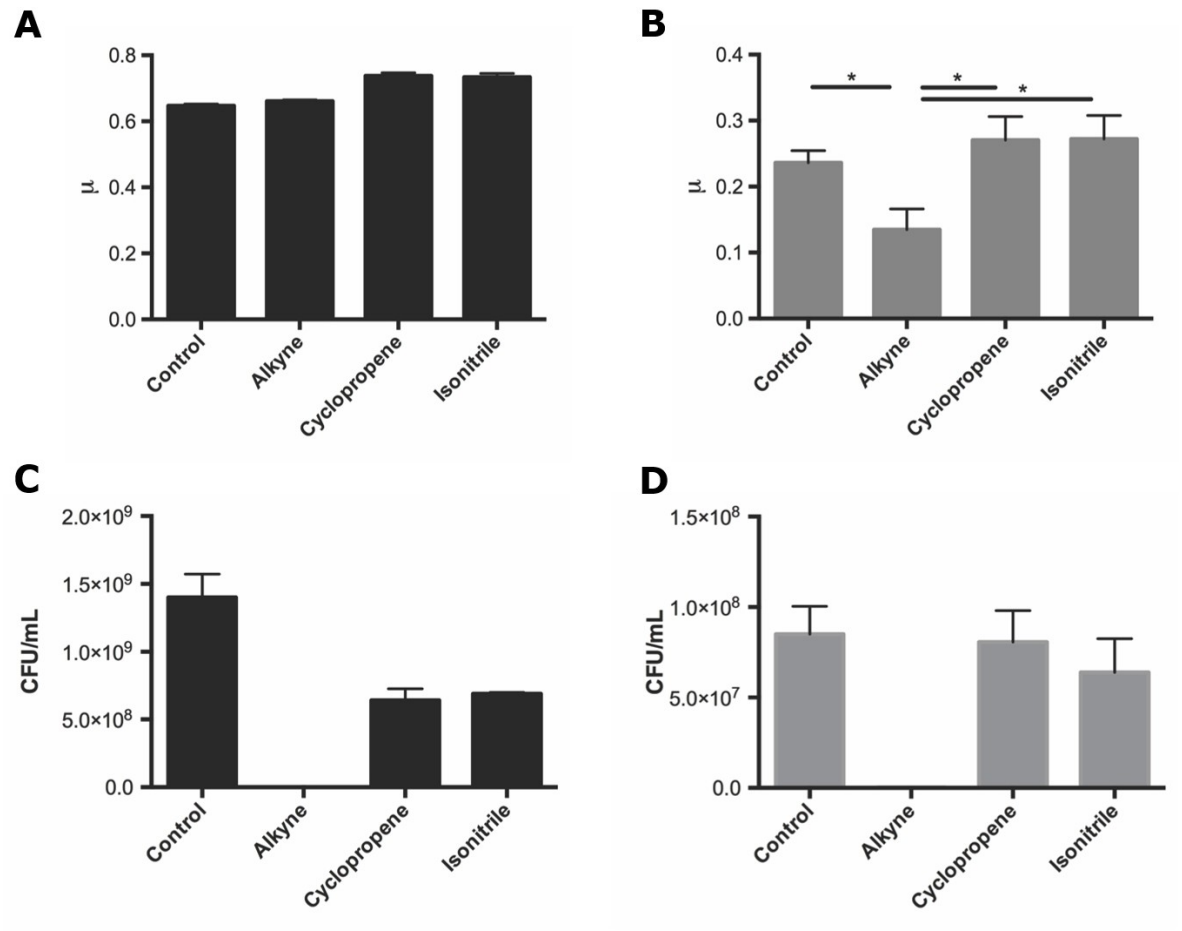


Figure 3. Specific growth rate (μ) of *E. coli* (A) and *A. muciniphila* (B) in the presence of 50 μM PG dipeptide probes (data represents mean of triplicates, error bars depict SEM). Colony-Forming Units (CFU) of *E. coli* (C) and *A. muciniphila* (D) after incubation with PG probes and their click reaction (CuAAC with azide for Alkyne PG probe and invDA or [4 + 1] with tetrazine for cyclopropene (**7**) or isonitrile (**10**) probes).

ality with other types of bioorthogonal chemistries such as the strain-promoted azide-alkyne cycloaddition (SPAAC).

Conclusions

The newly developed non-toxic cyclopropane (**7**) and isonitrile (**10**) D-Ala dipeptide PG probes, allowed us to successfully perform cell envelope engineering experiments in a live human gut microbiota member, namely the anaerobic mucin degrader, *A. muciniphila*. This provided a quick and general method to show the presence of PG in *A. muciniphila*. Peptidoglycan is positioned at the microbiota-host interface and is a not yet well understood mediator of the complex interactions tied to human well-being. Future elucidation of basic aspects of *A. muciniphila* molecular physiology with these probes will aid in further developing therapeutic opportunities for this key human gut microbiota member.

The here described novel approach, using tetrazine-reactive D-Ala dipeptides add to the bacterial metabolic labeling molecular toolbox. It allowed for the live labeling and imaging of PG in its native cell envelope setting and offers an interesting and powerful new chemically guided approach to functionally analyze the presence and role of peptidoglycan in key microbiota members. The application in bacteria with an aerobic and anaerobic lifestyle illustrates the potential for the *in-situ* tracking of the PG in all gut bacteria.

Materials and Methods

General considerations, materials and chemical synthesis. Detailed experimental procedures used to synthesize both chemical probes **7** and **10** are located in the supporting information. An overview of the synthesis of the known alkyne dipeptide probe is presented in the supporting information (Scheme S1). All moisture sensitive reactions were conducted under an argon atmosphere, using oven-dried glassware and allowed to cool in a desiccator over anhydrous KOH, unless otherwise stated. Solvents were either purified over aluminum oxide under argon using a Pure Solv 400 solvent purification system (Innovative Technology, Amesbury, USA) (CH_2Cl_2, Et_2O, toluene, and THF) or dried over 3 Å molecular sieves (MeCN, CH_3OH, Pyridine). Commercially acquired chemicals were used without further purification unless stated otherwise. Burgess reagent was purchased from VWR. AF488-Azide and methyl-tetrazine-Sulfo-Cy3 were purchased from Jena Bioscience. All chemicals used for biological experiments were dissolved in DMSO (1 mM stock solution). Analytical TLC was performed using plates of silica gel (Merck 60 F-254 on aluminum) and visualised

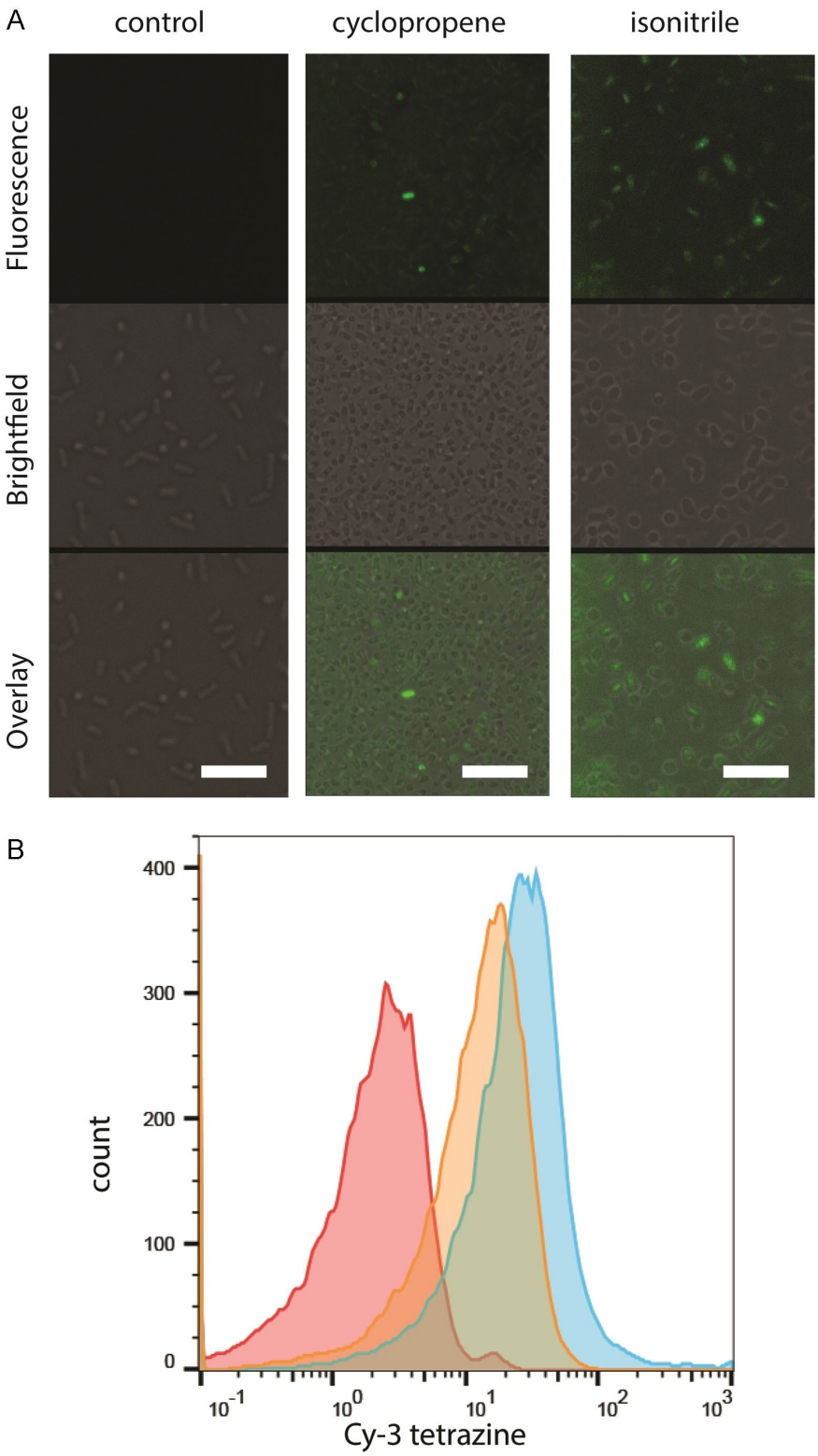


Figure 4. A. Confocal microscopy images of *E. coli* with control, cyclopropane **7**, and isonitrile **10** dipeptide PG probe (50 μM for 2 h). A click reaction with Cy-3 tetrazine dye was used for visualization. Scale bar = 5 μm. B. Flow cytometry analysis of control (red), isonitrile **10** (orange), and cyclopropane **7** (blue) PG dipeptide probes (50 μM for 2 h) in *E. coli*.

using UV light (254 nm), ninhydrin, or $KMnO_4$. Silica gel 60 (70–230 mesh) was used for flash chromatography.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 spectrometer (observation of ^{1}H nucleus 400 MHz, and of ^{13}C nucleus 100 MHz). Chemical shifts are reported in parts per million (ppm), calibrated on the residual peak of the solvent, whose values

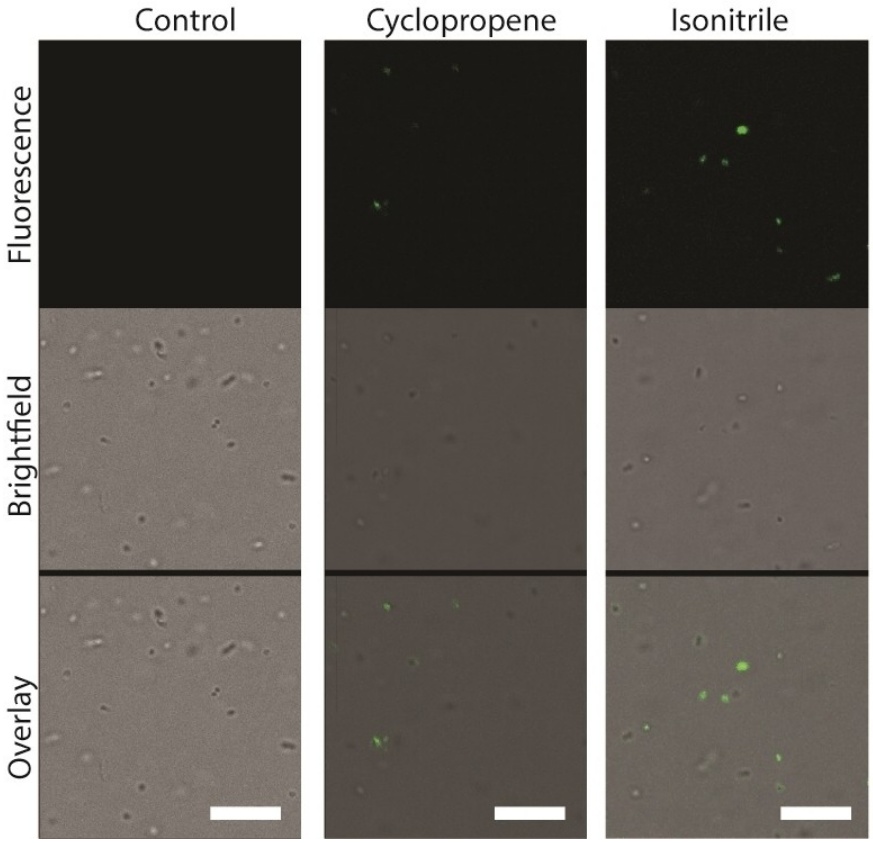


Figure 5. Confocal microscopy images of *A. muciniphila* after Cy-3 tetrazine labeling with and without addition of the PG dipeptide probes **7** or **10** at 50 μM for 2 h. Scale bar = 5 μm.

are referred to tetramethylsilane (TMS, δTMS = 0), as the internal standard. Coupling constants (*J*) are reported in Hertz (Hz), rounded to the nearest 0.5 Hz. Signal assignment was made from unambiguous chemical shifts and COSY, HSQC, HMBC, and DEPT. Electrospray ionization (ESI) mass analyses were performed on a Finnigan LXQ, while high-resolution ESI mass analyses were recorded on a Thermo Scientific Q Exactive High-Resolution mass spectrometer. Infra-red analyses were performed on a Bruker FT-IR spectrometer.

Biological Procedures

Bacterial strains and growth conditions. *Escherichia coli* K12 MG1655 was grown in Lysogeny Broth (LB) at 37 °C with aeration. *Akkermansia muciniphila* MucT (ATTC BAA-835)[5b] was cultivated anaerobically at 37 °C in CP medium supplemented with mucin[5b] or in CP-PT synthetic medium as described earlier.[20] Specific Growth Rate (SGR, μ) was calculated by measuring OD600 over a two hour growth interval during exponential growth of the respective bacteria. Colony forming units (CFUs) were determined by plating the *E. coli* on LB agar plates and *A. muciniphila* on CP-mucin agar plates.

Application of bioorthogonal probes. PG probes were filter-sterilized before application in microbiological experiments. The probes were added in a 50 μM final concentration to early exponential bacterial cultures (OD600 = 0.3). The cultures were incubated for 1–2 hours in the presence of the probes. After growth in the presence of the probes, cells were harvested and washed twice with PBS. Cells were resuspended in click buffer (CuAAC: 100 μM $CuSO_4$, 100 mM ascorbic acid, 5 μL of 1 mM AF488-Azide dye dissolved in PBS; tetrazine: 5 μL of 1 mM methyl-tetrazine-Sulfo-Cy3 dye in PBS). Cells were incubated for 1 hour at room temperature in the dark and then washed three times with PBS. Supernatant was resuspended in PBS prior to confocal or FCM analysis.

Confocal microscopy. Ibidi μ-slide eight well glass bottom slides were used for confocal microscopy. Solutions for CuAAC labelling were freshly prepared with the following concentrations: $CuSO_4$ (100 mM) and Ascorbic acid (5 mM). Live imaging was performed on a confocal laser scanning microscope (Leica TCS SP8X system). Alexa Fluor 488 was excited using an argon laser (488 nm), and fluorescence emission was detected from 500 to 540 nm. Cy-3 was excited using a white light continuum laser. Images were captured using a 633 water-immersion objective with a numeric aperture of 1.2 and a pinhole set to 1 Airy unit. Images were processed by ImageJ or LAS AF Lite.

Flow cytometry. Analysis of isonitrile PG probe 10 labelled cells was performed using a BD FACSaria III Cell Sorter fitted with BD FACSDIVA 6.1 software. Single cell populations were localised using FCS and SSC (488-nm 20 mW laser), gating pulse Area over pulse Width. Furthermore, Syto-9 (488-nm, 502 LP, 530/30 nm) was used to counterstain and distinguish cells from background. The Cy-3 tetrazine labelled PG isonitrile probe was detected using a 561-nm (50 mW) laser including a 582 nm band pass filter with a bandwidth of 15 nm. Data analysis was performed using FlowJo X (Treestar, USA).

Supplementary Material

An overview of all chemical procedures and characterization of the used peptidoglycan probes can be found in supplementary information.

Acknowledgements

The authors thank the Netherlands Foundation for Scientific Research (NWO) for funding via a VENI (722.011.006) and VIDI (723.014.005) grant of TW. Work at the Laboratory of Microbiology was supported by the Soehngen Institute of Anaerobic Microbiology (SIAM Gravity Grant of NWO, 024.002.002) and NWO Spinoza award of WMdV. We gratefully acknowledge Jan Willem Borst for confocal microscopy support (Laboratory of Biochemistry, Wageningen University). Yvette Luijkx, Connie de Kock and Jona Merx are acknowledged for synthesis support. Geert-Jan Boons is acknowledged for fruitful discussions. The authors declare no conflict of interest.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: *Akkermansia muciniphila* · biosynthesis · bioorthogonal labeling · click chemistry · chemical probes · microbiota · Peptidoglycan

[1] a) M. Arumugam, J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, D. R. Mende, G. R. Fernandes, J. Tap, T. Bruls, J. M. Batto, M. Bertalan, N. Borruel, F. Casellas, L. Fernandez, L. Gautier, T. Hansen, M. Hattori, T. Hayashi, M. Kleerebezem, K. Kurokawa, M. Leclerc, F. Levenez, C. Manichanh, H. B. Nielsen, T. Nielsen, N. Pons, J. Poulain, J. Qin, T. Sicheritz-Ponten, S. Tims, D. Torrents, E. Ugarte, E. G. Zoetendal, J. Wang, F. Guarner, O. Pedersen, W. M. de Vos, S. Brunak, J. Dore, M. Antolin, F. Artiguenave, H. M. Blottiere, M. Almeida, C. Brechot, C. Cara, C. Chervaux, A. Cultrone, C. Delorme, G. Denariaz, R. Dervyn, K. U. Foerstner, C. Friss, M. van de Guchte, E. Guedon, F. Haimet, W. Huber, J. van Hylckama-Vlieg, A. Jamet, C. Juste, G. Kaci, J. Knol, O. Lakhdari, S. Layec, K. Le Roux, E. Maguin, A. Merieux, R. Melo Minardi, C. M'Rini, J. Muller, R. Oozeer, J. Parkhill, P. Renault, M. Rescigno, N. Sanchez, S. Sunagawa, A. Torrejon, K. Turner, G. Vandemeulebrouck, E. Varela, Y. Winogradsky, G. Zeller, J. Weissenbach, S. D. Ehrlich, P. Bork, *Nature* **2011**, *473*, 174–180; b) J. Qin, R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J. M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, P. Bork, S. D. Ehrlich, J. Wang, *Nature* **2010**, *464*, 59–65; c) *Nature* **2012**, *486*, 207–214.

[2] H. L. Tytgat, W. M. de Vos, *Trends Microbiol.* **2016**, *24*, 853–861.

[3] a) H. de Jong, M. J. Moure, J. E. M. Hartman, G. P. Bosman, J. Y. Ong, B. W. Bardoel, G. J. Boons, M. Wosten, T. Wennekes, *ChemBioChem.* **2022**, *23*, e202200340; b) H. de Jong, M. Wosten, T. Wennekes, *Glycobiology* **2022**, *32*, 11–22; c) Y. Luijkx, A. J. Henselijn, G. P. Bosman, D. A. T. Cramer, K. Giesbers, E. M. van 't Veld, G. J. Boons, A. J. R. Heck, K. R. Reiding, K. Strijbis, T. Wennekes, *Molecules* **2022**, *27*; d) Y. Luijkx, S. Jongkees, K. Strijbis, T. Wennekes, *Org. Biomol. Chem.* **2021**, *19*, 2968–2977; e) X. Meng, G. J. Boons, M. Wosten, T. Wennekes, *Angew. Chem.* **2021**, *60*, 24811–24816; f) S. van Kasteren, D. E. Rozen, *ISME Commun* **2023**, *3*, 9; g) Q. Zheng, P. V. Chang, *Isr. J. Chem.* **2023**, *63*; h) W. Wang, Q. Yang, Y. Du, X. Zhou, X. Du, Q. Wu, L. Lin, Y. Song, F. Li, C. Yang, W. Tan, *Angew. Chem.* **2020**, *59*, 2628–2633.

[4] a) E. L. Clark, M. Emmadi, K. L. Krupp, A. R. Podilapu, J. D. Helble, S. S. Kulkarni, D. H. Dube, *ACS Chem. Biol.* **2016**, *11*, 3365–3373; b) H. N. Foley, J. A. Stewart, H. W. Kavunja, S. R. Rundell, B. M. Swarts, *Angew. Chem.* **2016**, *55*, 2053–2057; c) J. E. Hudak, D. Alvarez, A. Skelly, U. H. von Andrian, D. L. Kasper, *Nat Microbiol* **2017**, *2*, 17099; d) N. Koppel, E. P. Balskus, *Cell Chem. Biol.* **2016**, *23*, 18–30; e) H. Liang, K. E. DeMeester, C. W. Hou, M. A. Parent, J. L. Caplan, C. L. Grimes, *Nat. Commun.* **2017**, *8*, 15015; f) T. J. Sminia, H. Zuilhof, T. Wennekes, *Carbohydr. Res.* **2016**, *435*, 121–141; g) N. Banahene, H. W. Kavunja, B. M. Swarts, *Chem. Rev.* **2022**, *122*, 3336–3413.

[5] a) C. Belzer, W. M. de Vos, *ISME J.* **2012**, *6*, 1449–1458; b) M. Derrien, E. E. Vaughan, C. M. Plugge, W. M. de Vos, *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 1469–1476.

[6] a) M. Derrien, C. Belzer, W. M. de Vos, *Microb. Pathog.* **2016**; b) M. Derrien, P. Van Baarlen, G. Hooiveld, E. Norin, M. Muller, W. M. de Vos, *Front. Microbiol.* **2011**, *2*, 166; c) C. W. Png, S. K. Linden, K. S. Gilshenan, E. G. Zoetendal, C. S. McSweeney, L. I. Sly, M. A. McGuckin, T. H. Florin, *Am. J. Gastroenterol.* **2010**, *105*, 2420–2428; d) J. Reunanen, V. Kainulainen, L. Huuskonen, N. Ottman, C. Belzer, H. Huhtinen, W. M. de Vos, R. Satokari, *Appl. Environ. Microbiol.* **2015**, *81*, 3655–3662; e) N. R. Shin, J. C. Lee, H. Y. Lee, M. S. Kim, T. W. Whon, M. S. Lee, J. W. Bae, *Gut* **2014**, *63*, 727–735.

[7] a) A. Everard, C. Belzer, L. Geurts, J. P. Ouwerkerk, C. Druart, L. B. Bindels, Y. Guiot, M. Derrien, G. G. Muccioli, N. M. Delzenne, W. M. de Vos, P. D. Cani, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 9066–9071; b) H. Plovier, A. Everard, C. Druart, C. Depommier, M. Van Hul, L. Geurts, J. Chilloux, N. Ottman, T. Duparc, L. Lichtenstein, A. Myridakis, N. M. Delzenne, J. Klievink, A. Bhattacharjee, K. C. van der Ark, S. Aalvink, L. O. Martinez, M. E. Dumas, D. Maiter, A. Loumaye, M. P. Hermans, J. P. Thissen, C. Belzer, W. M. de Vos, P. D. Cani, *Nat. Med.* **2016**.

[8] P. Garcia-Vello, H. L. P. Tytgat, J. Gray, J. Elzinga, F. Di Lorenzo, J. Biboy, D. Vollmer, C. De Castro, W. Vollmer, W. M. de Vos, A. Molinaro, *Glycobiology* **2022**, *32*, 712–719.

[9] J. Royet, D. Gupta, R. Dziarski, *Nature reviews. Immunology* **2011**, *11*, 837–851.

[10] D. P. Devos, *Trends Microbiol.* **2014**, *22*, 14–20.

[11] a) A. J. Egan, R. M. Cleverley, K. Peters, R. J. Lewis, W. Vollmer, *FEBS J.* **2016**; b) A. Typas, M. Banzhaf, C. A. Gross, W. Vollmer, *Nat. Rev. Microbiol.* **2012**, *10*, 123–136; c) W. Vollmer, S. J. Seligman, *Trends Microbiol.* **2010**, *18*, 59–66.

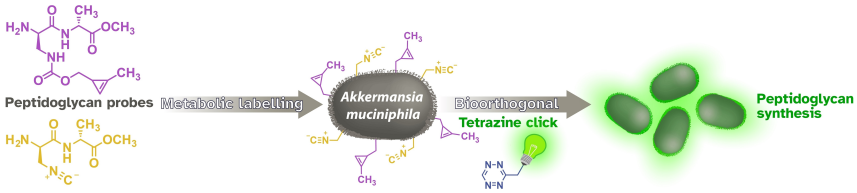
[12] a) K. E. DeMeester, H. Liang, M. R. Jensen, Z. S. Jones, E. A. D'Ambrosio, S. L. Scinto, J. Zhou, C. L. Grimes, *J. Am. Chem. Soc.* **2018**, *140*, 9458–9465; b) Y. Xu, V. M. Hernandez-Rocamora, J. H. Lorent, R. Cox, X. Wang, X. Bao, M. Stel, G. Vos, R. M. van den Bos, R. J. Pieters, J. Gray, W. Vollmer, E. Breukink, *iScience* **2022**, *25*, 104753; c) A. R. Brown, K. A. Wodzanowski, C. C. Santiago, S. N. Hyland, J. L. Follmar, P. Asare-Okai, C. L. Grimes, *ACS Chem. Biol.* **2021**, *16*, 1908–1916.

[13] a) R. T. Gale, E. D. Brown, *Curr. Opin. Microbiol.* **2015**, *27*, 69–77; b) O. Kocaoglu, E. E. Carlson, *Nat. Chem. Biol.* **2016**, *12*, 472–478; c) E. Kuru, H. V. Hughes, P. J. Brown, E. Hall, S. Tekkam, F. Cava, M. A. de Pedro, Y. V. Brun, M. S. VanNieuwenhze, *Angew. Chem.* **2012**, *51*, 12519–12523; d) E. Kuru, S. Tekkam, E. Hall, Y. V. Brun, M. S. Van Nieuwenhze, *Nat. Protoc.* **2015**, *10*, 33–52; e) G. W. Liechti, E. Kuru, E. Hall, A. Kalinda, Y. V. Brun, M. VanNieuwenhze, A. T. Maurelli, *Nature* **2014**, *506*, 507–510; f) S. E. Pidgeon, J. M. Fura, W. Leon, M. Birabaharan, D. Vezenov, M. M. Pires, *Angew. Chem.* **2015**, *54*, 6158–6162; g) S. E. Pidgeon, M. M. Pires, *Chem. Commun.* **2015**, *51*, 10330–10333; h) A. R. Sherratt, M. Chigrinova, D. A. MacKenzie, N. K. Rastogi, M. T. Ouattara, A. T. Pezacki, J. P. Pezacki, *Bioconjugate Chem.* **2016**, *27*, 1222–1226; i) P. Shieh, M. S. Siegrist, A. J. Cullen, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 5456–5461; j) M. S. Siegrist, B. M. Swarts, D. M. Fox, S. A. Lim, C. R. Bertozzi, *FEMS Microbiol. Rev.* **2015**, *39*, 184–202; k) M. S. Siegrist, S. Whiteside, J. C. Jewett, A. Aditham, F. Cava, C. R. Bertozzi, *ACS Chem. Biol.* **2013**, *8*, 500–

505; l) J. T. Ngo, S. R. Adams, T. J. Deerinck, D. Boassa, F. Rodriguez-Rivera, S. F. Palida, C. R. Bertozzi, M. H. Ellisman, R. Y. Tsien, *Nat. Chem. Biol.* **2016**, *12*, 459–465.
[14] a) H. Stockmann, A. A. Neves, S. Stairs, K. M. Brindle, F. J. Leeper, *Org. Biomol. Chem.* **2011**, *9*, 7303–7305; b) S. Stairs, A. A. Neves, H. Stockmann, Y. A. Wainman, H. Ireland-Zecchini, K. M. Brindle, F. J. Leeper, *ChemBioChem* **2013**, *14*, 1063–1067.
[15] a) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638–18643; b) J. Yang, J. Seckute, C. M. Cole, N. K. Devaraj, *Angew. Chem.* **2012**, *51*, 7476–7479.
[16] Y. H. Lau, D. R. Spring, *Synlett* **2011**, 1917–1919.
[17] a) D. M. Patterson, K. A. Jones, J. A. Prescher, *Mol. BioSyst.* **2014**, *10*, 1693–1697; b) A. K. Spate, V. F. Schart, J. Hafner, A. Niederwieser, T. U. Mayer, V. Wittmann, *Beilstein J. Org. Chem.* **2014**, *10*, 2235–2242.
[18] a) N. R. Aguiam, V. I. Castro, A. I. F. Ribeiro, R. D. V. Fernandes, C. M. Carvalho, S. P. G. Costa, S. M. M. A. Pereira-Lima, *Tetrahedron* **2013**, *69*, 9161–9165; b) T. B. Nguyen, J. Sorres, M. Q. Tran, L. Ermolenko, A. Al-Mourabit, *Org. Lett.* **2012**, *14*, 3202–3205.
[19] S. M. Creedon, H. K. Crowley, D. G. McCarthy, *J Chem Soc Perk T 1* **1998**, 1015–1017.
[20] H. Plovier, A. Everard, C. Druart, C. Depommier, M. Van Hul, L. Geurts, J. Chilloux, N. Ottman, T. Duparc, L. Lichtenstein, A. Myridakis, N. M. Delzenne, J. Klievink, A. Bhattacharjee, K. C. van der Ark, S. Aalvink, L. O. Martinez, M. E. Dumas, D. Maiter, A. Loumaye, M. P. Hermans, J. P. Thissen, C. Belzer, W. M. de Vos, P. D. Cani, *Nat. Med.* **2017**, *23*, 107–113.

Manuscript received: January 16, 2024
Revised manuscript received: April 29, 2024
Accepted manuscript online: April 30, 2024
Version of record online: ■■, ■■

RESEARCH ARTICLE



We here present cyclopropene and isonitrile functionalized analogs of D-Ala dipeptides, essential for peptidoglycan biosynthesis, as new members of the bacterial metabolic labeling toolkit. These non-toxic, tetrazine click-compatible probes allowed us to detect and image peptidoglycan in live *Akkermansia muciniphila* bacteria. Their effectiveness in this anaerobic bacterium and also aerobic bacteria (*E. coli*) shows their potential for *in situ* study of peptidoglycan.

*T. J. Sminia, S. Aalvink, H. de Jong, M. H. Tempelaars, H. Zuilhof, T. Abee, W. M. de Vos, H. L. P. Tytgat, Dr. T. Wennekes**

1 – 10

Probing Peptidoglycan Synthesis in the Gut Commensal *Akkermansia Muciniphila* with Bioorthogonal Chemical Reporters