Department of Organic Chemistry

Breaking The Wall: isolation and structure elucidation of the monomeric units of cell wall peptidoglycans from different bacterial strains

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Introduction

Peptidoglycan (PGN) is a major component of the bacterial cell-wall and is recognized by the immune system of higher organisms through innate immune receptors (1). The immunostimulatory activity of PGN is considered very interesting as an adjuvant in vaccines. Although polymeric PGN has too many side effects when used in vaccines for humans, fragments derived from PGN are also known to induce an effect on the immune system without toxic side effects. In this project, an enzymatic lysis is performed on PGN from *Staphylococcus aureus*, using different concentrations of Mutanolysin from *Steptomyces globisporus* specifically breaking β(1-4) interglycosidic bonds between NAG-NAG units. The fragments formed are separated by HPLC and characterized by NMR and MS. Furthermore, we have characterized Peptidoglycan Monomer (PGM) and Peptidoglycan Pentapeptide (PP) fragments, isolated from *Brevibacterium divaricatum* (2) and compared their NMR spectra to those of the fragments isolated from *Staphylococcus aureus*.

1. Enzymatic digestion

PGN lysis was performed at different Mutanolysin concentrations by addition of 100, 200 and 400 µL of stock solution. The digestion was performed at 37 °C for 24 h. The PGN fragments were separated from the insoluble fragments using centrifugation and membrane filtration.

2. Isolation: HPLC

The fragments were separated by reverse phase HPLC using a water-acetonitrile with linear gradient (0:100%).

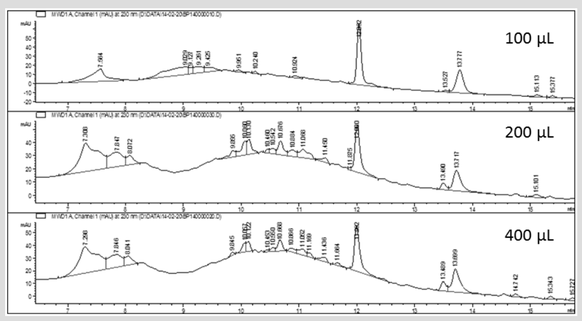


Fig 1: HPLC chromatograms for different concentrations of added enzyme in H2O/ACN

3. Structural characterisation: MS

Retention time (min)	Fragments (m/z)
7	186.15, 316.2, 387.15, 105.15
12	186.15, 316.20, 149.1, 142,2
13.5	184.2,214.20
14	316.2, 387.15, 654.3, 638.25, 637.20

Mainly smaller fragments were detected by LC-MS mixed with low amount of larger MW fragments.

Conclusion

- Full assignments of the NMR spectra for PP and PGM from *Brevibacterium divaricatum* have been achieved.
- Enzyme digest of PGN from *Staphylococcus aureus* resulted in small molecular weight fragments according to LC-MS. Characteristic patterns indicating amino acids, peptides and carbohydrate fragments can be seen in the NMR spectra.

4. PGN fragments from Brevibacterium divaricatum

All spectra recorded on:
- Bruker Avance II 700MHz
- 5mm TXI-Z ATMA probe head

Assignment procedure
- TOCSY: the whole amino acid spin system is identified in the fingerprint region starting from the NH resonance
- NOESY and HMBC: the relative positions of the amino acids is derived thus leading to sequential assignment

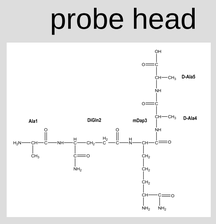


Fig 2: Structure of PP

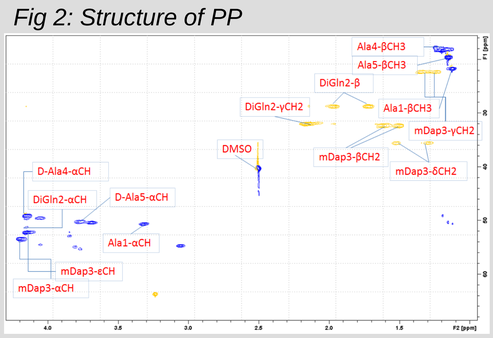


Fig 3: HSQC of PP in DMSO

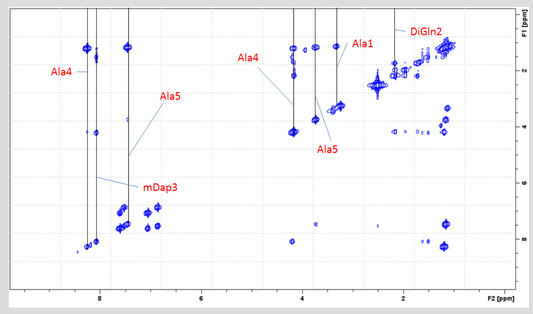


Fig 4: TOCSY of PP in DMSO. Vertical lines designate amino acid spin systems.

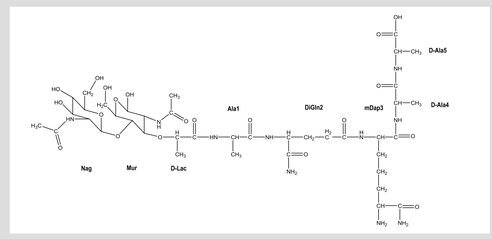


Fig 5: Structure of PGM

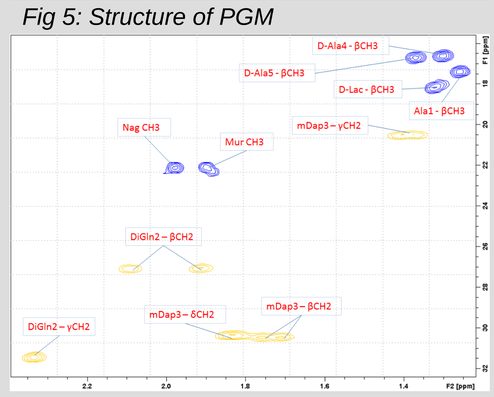
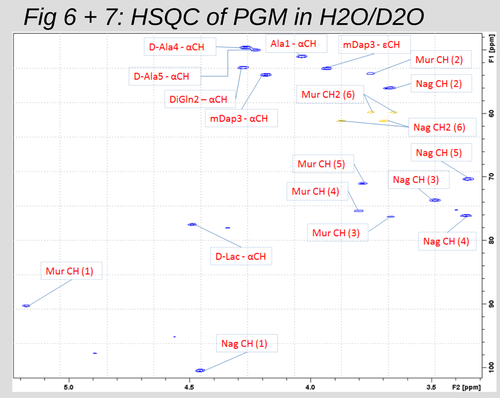


Fig 6 + 7: HSQC of PGM in H2O/D2O



5. Structural characterization: NMR

Identified spectral regions in TOCSY
- Aliphatic region
- Carbohydrate region
- Peptide region

The amide chemical shift distribution of oligopeptides such as PP or PGM is larger than that of the fragments. This pattern is rather characteristic of smaller amino acids or sugar-peptide fragments.

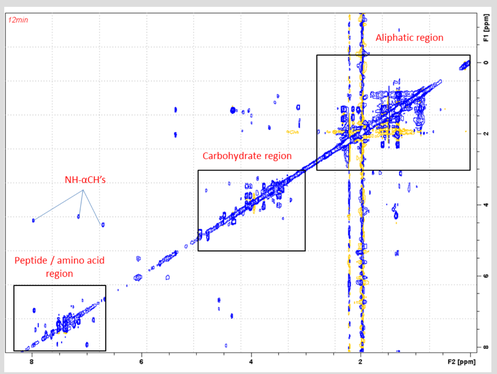


Fig 9: TOCSY of fragment isolated at 12 min retention time

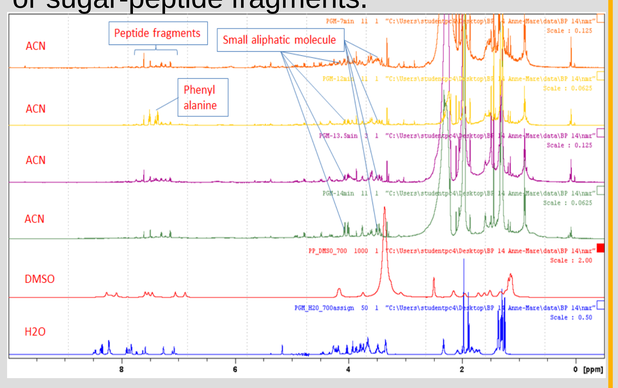


Fig 10: 1H NMR overlay of the fragments, PP and the PGM

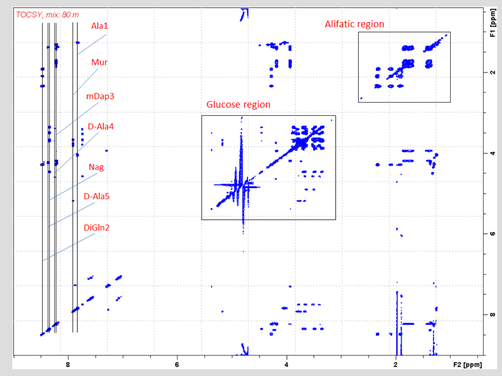


Fig 8: TOCSY of PGM with vertical lines indicating the amino acid spin systems

Safety in the NMR lab

- **Magnetic safety:**

A magnetic field surrounds the magnet in all directions. This field is invisible and does not get blocked by walls, floors or ceilings: hence the need to post warning signs. There are two regions around the NMR spectrometer: the inner and the outer zone.

The inner zone: from the magnetic center to the 1mT line, objects may suddenly be drawn towards the magnetic center. Under no circumstances should heavy ferromagnetic objects be located or moved within this zone; small steel objects cannot be allowed to lie on the floor near the magnet.

The outer zone: from the 1mT line to the 0.3mT line, the magnetic field may erase information stored on magnetic strips or discs. Pressurized gas cylinders made of steel should be located well beyond the outer zone and should be fixed to the wall.

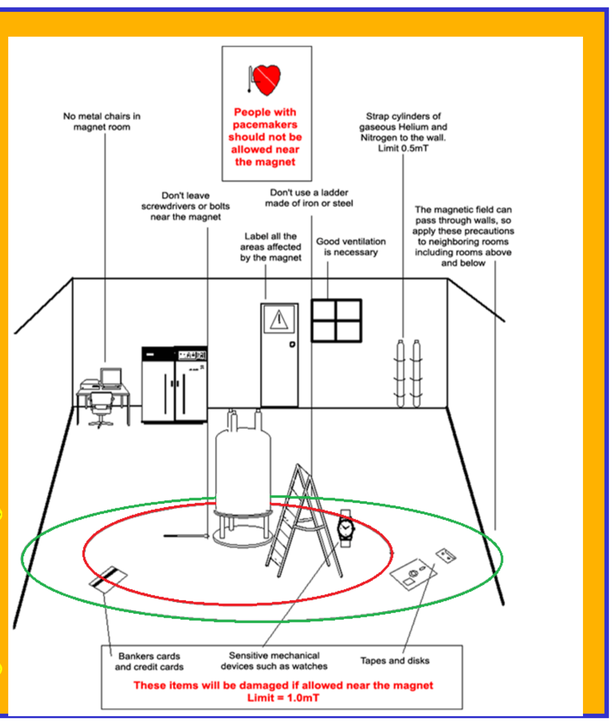
People fitted with surgical implants that contain ferromagnetic materials should not be allowed near the magnet.

- **Cryogenic safety:**

Liquid helium and nitrogen are required to keep the superconducting magnet at a very low temperature. Direct contact with these liquids can cause frostbite. Therefore safety goggles, gloves and a long sleeved shirt should always be worn when handling cryogens and passengers should never accompany cryogens in an elevator. Because of the volatility of the cryogens the room should be adequate ventilated, provided with an oxygen meter and it has to be checked whether the gases can escape from the magnet.

- **General laboratory safety:**

While performing sample preparations in the lab, it is obliged to wear a lab coat and safety goggles at all times. Used chemicals should be treated as mentioned on the recipients.



References
1. E. Girardin and D. J. Philpott, The role of peptidoglycan recognition in innate immunity
2. D. Keglevic et al., (1974) , isolation and study of the composition of a peptidoglycan complex excreted by the biotin-requiring mutant of Brevibacterium divaricatum
3. E. Butler, Bruker BioSpin Avance beginners, English version User Guide, version 004 (2008) 11:14

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