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Synthesis of novel muramic acid derivatives and their interaction with lysozyme: Action of lysozyme revisited

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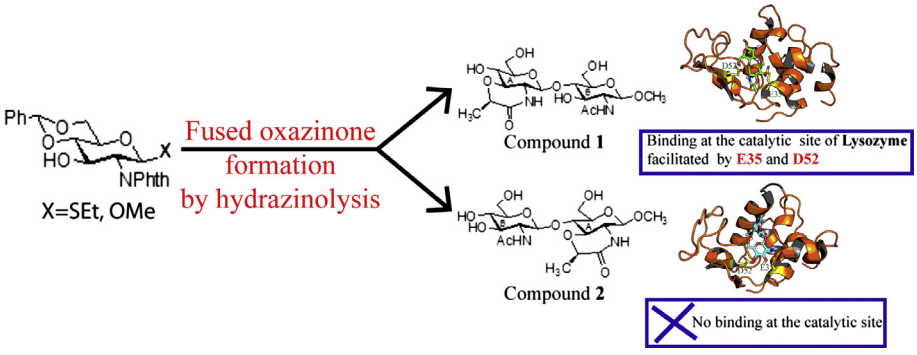
GRAPHICAL ABSTRACT



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Dedicated to Prof. Dr. Thomas Peters, University of Lübeck, Germany on the occasion of his 60th birthday.

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ABSTRACT

Hypothesis: The interaction of lysozyme with the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) unit of peptidoglycan (PGN) polymer of the bacterial cell wall is of immense importance to understand the mechanism of lysozyme on PGN.
Experiments: The synthesis of three novel NAM derivatives containing fused oxazinone ring to the NAM moiety has been achieved. The synthesized compounds were evaluated for their potential as a glycomimetic acceptor of lysozyme using different biophysical and computational methods such as ^{1}H NMR, STD NMR, DOSY and Molecular docking.
Findings: Novel modified muramic acid derivatives have been synthesized in excellent yield containing fused cyclooxazine ring embedded on the muramic acid moiety using a newly developed hydrazinolysis reaction condition. From various biophysical studies, it has been established that the compound containing endo modified muramic acid moiety (compound **1**) shows significant binding property for the lysozyme while the other isomer (compound **2**) did not bind to the lysozyme. The catalytic residues Glu35 and Asp52 were found to be in the close proximity for the active molecule which justifies the selectivity of this molecule in conjunction to lysozyme enzymatic activity.

1. Introduction

The peptidoglycan (PGN) layer in the plasma membrane of bacteria serves as a permeability barrier against most of the invading

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antimicrobial agents [1]. It provides the foundation for bacterial cell walls, designates a bacterium's structural integrity and anchors diverse protein complexes and extracellular organelles to the cell surface [2]. It exhibits substantial porosity and flexibility for the administration of essential nutrients, ions, and minerals for the survival of bacteria [3]. Because of its unique nature, peptidoglycan layer can be considered as the primary targets for many host defence antimicrobial drugs over the years [4]. It is worth mentioning that the thickness of peptidoglycan coating for Gram-positive bacteria is denser than that of Gram-negative bacteria [5].

The basic structure of peptidoglycan comprises a linear sugar backbone of alternating components of N-acetylmuramic acid (NAM) and, N-acetylglucosamine (NAG), which are connected by β-(1 → 4)-glycosidic bond [6]. Additionally, N-acetylmuramic acid residues are cross-linked to peptide chain of 3 to 5 amino acids such as D-alanine, L-alanine, D-glutamic acid etc depending upon the nature of bacterial strains [4].

Lysozyme being a host hydrolytic enzyme, degrades peptidoglycan layer and causes bacterial cell lysis [7,8]. It is a 14 kDa protein and presents in the major component of the innate immunity such as vertebrate, invertebrates, and plants [9]. Particularly, in vertebrates, lysozyme is known to be present in secretions of the tear, digestive tract, nasal, milk, and respiratory system [10].

The mechanism of action by which the lysozyme disintegrates the bacterial cell wall is the hydrolysis of the NAM-β-(1 → 4)-NAG glycosidic bond [11,12]. Mechanistically, from the crystal structure, it has been observed that the β-1,4 glycosidic (C—O) bond of NAM-NAG comes in close proximity to the two potentially catalytic residues, Glu35 and Asp52 of lysozyme, which leads to the cleavage of the glycosidic bond [7,12,13]. However, the controversial debate still persists over the last 50 years about the mechanistic aspects of cleavage i.e., whether the mechanism is mediated by S_N1 or/and S_N2 pathway [13,14]. The rationale of this debate is based on the problem of trapping of two intermediates ca. glycosyl-enzyme intermediate and cation intermediate, which are not possible to detect at the same time. Therefore, for better understanding the atomistic details of lysozyme enzyme mechanism it is of immense importance to know the details of the interaction between lysozyme and the carbohydrate components such as NAM and NAG units. In this regard, it can be noted that the bacteria can change the composition and perhaps the structure of their peptidoglycan, depending on the growth conditions, as well as response to their environmental surroundings [15].

In order to get further insights on the exact site of lysozyme action on the peptidoglycan chain as well as to identify possible mimicking acceptor of lysozyme, here, we have synthesized a number of modified disaccharide and monosaccharide derivatives of the peptidoglycan chain containing NAM and NAG to study their interactions with lysozyme. In the synthesized compounds, the carboxylic group of the (*R*)-lactic acid present in the muramic acid moiety was coupled with the amine present in the C-2 position to make an oxazinone moiety fused with the sugar ring. Therefore, the 2-acetamido and the carboxylic group in the NAM moiety of the synthesized compounds are not available for any interaction with lysozyme and hence could impart some unexplored observations.

In the present work, we focused towards (i) the development of the synthetic strategies for the synthesis of disaccharide derivatives in which an oxazinone moiety is fused with ring carbons of the NAM; (ii) biophysical characterization of the mode of interactions between lysozyme and designed cyclic oligosaccharides. The key features of the synthetic strategy are: (a) preparation of lactate ether in the presence of *N*-phthalimido group in the substrate by controlling the reaction condition; (b) formation of the oxazinone ring during the removal of *N*-phthalimido group by hydrazinolysis. It is worth mentioning that exclusive formation of the fused oxazinone ring during the removal of phthalimido group using hydrazine hydrate has been observed for the first time.

The understanding of the interaction mechanism was revealed through a variety of Nuclear Magnetic Resonance (NMR) experiments in conjunction with molecular docking. This result will pave way for further development of glycomimetics for the lysozyme peptidoglycan interactions.

2. Materials and methods

2.1. Materials

The compounds were dissolved in sterilized Millipore water or phosphate buffer components of which were bought from Sigma-Aldrich Co. (St. Louis, Mo.). 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) and deuterium oxide (D_2O) were obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA).Commercially available anhydrous grade organic solvents stored over molecular sieves (Sigma-Aldrich Co., St. Louis, Mo.) are used in all reactions. *N*-iodosuccinimide (NIS), trimethylsilyl trifluoromethanesulfonate (TMSOTf) were purchased from Sigma-Aldrich Co.(St. Louis, Mo).

2.2. General methods for synthesis and identification

All reactions were monitored by thin layer chromatography over silica gel coated TLC plates. The spots on TLC were visualized under UV lamp and by warming the plates on a hot plate after sprayed with 10% H_2SO_4 in EtOH. Silica gel 230–400 mesh was used for column chromatography. NMR spectra were recorded on Bruker Avance 500 MHz using $CDCl_3$ as solvent and TMS as internal reference unless stated otherwise. Chemical shift value is expressed in δ ppm. The complete assignment of proton and carbon spectra was carried out by using a standard set of NMR experiments, e.g. ^{1}H NMR, ^{13}C NMR, ^{13}C DEPT 135, 2D COSY and 2D HSQC etc. MALDI-MS was recorded on a Bruker mass spectrometer. Optical rotations were recorded in a Jasco P-2000 spectrometer.

2.2.1. Synthesis and characterization of ethyl 4,6-O-benzylidene-3-O-[(R)-1-bezyloxycarbonylethyl]-2-deoxy-2-N-phthalimido-1-thio-β-d-glucopyranoside (6)

A solution of compound **4** (2 g, 4.53 mmol) and benzyl (*S*)-lactate triflate (**5**; 2.8 g, 8.97 mmol) in dry DMF (10 mL) was cooled to 0 °C. To the cooled reaction mixture was added NaH (50% oil coated, 550 mg, 11.45 mmol) and it was allowed to stir at room temperature for 3 h. The reaction was quenched with CH_3OH (1 mL), diluted with water (100 mL) and extracted with CH_2Cl_2 (100 mL). The organic layer was washed with satd. $NaHCO_3$ (50 mL), dried (Na_2SO_4) and concentrated. The crude product was purified over SiO_2 using hexane-EtOAc (6:1) as eluant to give pure compound **6** (2 g, 73%). Yellow oil; $[\alpha]_D^{25}$ + 26 (*c* 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.98–7.19 (m, 14 H, Ar-H), 5.69 (s, 1 H, PhC*H*), 5.55 (d, *J* = 10.5 Hz, 1 H, H-1), 4.90 (d, *J* = 12.0 Hz, 1 H, PhC*H*), 4.81 (d, *J* = 12.0 Hz, 1 H, PhC), 4.62–4.46 (m, 4 H, C*H*, H-4, H-6_{ab}), 3.93 (t, *J* = 10.0 Hz each, 1 H, H-3), 3.84 (t, *J* = 9.0 Hz each, 1 H, H-2), 3.81–3.76 (m, 1 H, H-5), 2.87–2.76 (m, 2 H, S$CH_2$$CH_3$), 1.44 (d, *J* = 6.5 Hz, 3 H, CCH_3), 1.34 (t, *J* = 7.4 Hz each, 3 H, SCH$_2$-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.6 (*C*O), 168.7, 167.7 (*C*O-Phth), 141.0–122.9 (Ar—C), 101.3 (PhCH), 83.2 (C-1), 81.8 (C-4), 76.5 (C-3), 75.5 (*C*H), 70.1 (C-5), 68.7 (PhCH_2), 66.1 (C-6), 54.3 (C-2), 23.9 (SCH_2CH$_3$), 18.9 (CCH_3), 14.9 (SC$H_2$$CH_3$); MALDI-MS: 626.1 $[M + Na]^+$; Anal. Calcd. for $C_{33}H_{33}NO_8S$ (603.68): C, 65.66; H, 5.51; found: C, 65.50; H, 5.70.