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ORIGINAL ARTICLE

Comparing naturally occurring glycosylated forms of proline rich antibacterial peptide, Drosocin

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Abstract Antimicrobial peptides (AMPs) are key players of innate immunity. Amongst various classes of AMPs, proline rich AMPs from insects enjoy special attention with few members of this class bearing *O*-glycosylation as post-translational modification. Drosocin, a 19 amino acid glycosylated AMP is a member of proline rich class, synthesized in the haemolymph of *Drosophila melanogaster* upon bacterial challenge. We report herein the chemical synthesis of drosocin carrying disaccharide (β-Gal(1 → 3)α-GalNAc) and comparison of its structural and functional properties with another naturally occurring monoglycosylated form of drosocin *i.e.* α-GalNAc-drosocin as well as with non-glycosylated drosocin. The disaccharide containing drosocin exhibited lower potency compared to monoglycosylated drosocin against all the tested Gram negative bacteria, suggesting the role of the distal sugar or increase in the sugar chain length on the activity. Circular dichroism studies failed to demonstrate the differential effect of sugars on the overall peptide conformation. Haemolytic and cytotoxic properties of drosocin were not altered due to an increase in the sugar chain length. In addition, we have also evaluated the effect of differentially glycosylated drosocins on two pro-inflammatory cytokines secreted by murine macrophages or LPS stimulated macrophages. All the drosocin forms tested, neither could stimulate the secretion of TNF-α and IL-6 nor could modulate LPS-induced levels of TNF-α and IL-6 in murine macrophages. This study provides insights about naturally occurring two different glycosylated forms of drosocin.

Keywords Antimicrobial peptides · Proline rich · Drosocin · T-antigen · Immunomodulation

Introduction

The universal presence of invading microorganisms commands the need for continuous protection of multicellular organisms. The innate immunity takes care of the initial interactions between the host and the microbes. It does not depend on exact antigen recognition, but instead recognizes common structural characteristics. Antimicrobial peptides (AMPs) are one such inducible effector molecules of innate immune mechanism [1]. AMPs are the most important elements of insects' defense against harmful bacteria and fungi. The insect-derived proline rich AMPs include apidaecin [2], a non-glycosylated AMP and *O*-glycosylated AMPs like drosocin [3], pyrrhocoricin [4] and formaecins [5], carrying a carbohydrate moiety at a conserved threonine residue [6].

Detailed understanding of carbohydrate protein/peptide crosstalk is difficult because of natural availability of glycoproteins/glycopeptides in low concentration, only and inherent heterogeneity of glycosylation. Proline rich antibacterial glycopeptides turn out to be an attractive model for studying the role of glycosylation in peptides' function. Earlier we have reported the effect of different monosaccharides, disaccharides and their interglycosidic linkages on the various properties of two proline rich AMPs, drosocin and formaecin [7, 8]. A number of glycoproteins have also been studied to elucidate the effect of the sugar chain length on their

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structure and function [9]. For example, in case of family 1 carbohydrate binding module (CBM) increase in glycan length increases the thermolysin resistance of CBM conferring various beneficial properties to the enzyme targeting cellulose for biomass degradation [10]. Contrary to this, in case of glycosylated antifreeze peptides and glycoproteins (AFGPs), the distal sugar was not essential for the activity. AFGPs with monosaccharide (αGalNAc-) can display thermal hysteresis activity [11]. Effect of the increase in glycan side chain or degree of glycosylation on protein stability has also been established [12].

Drosocin, a 19 amino-acid long peptide is active against Gram negative bacteria, carrying either a monosaccharide (α-GalNAc) or a disaccharide (β-Gal(1 → 3)α-GalNAc) on the threonine residue. Both these glycosylated forms of drosocin were found to be secreted by *Drosophila* on bacterial challenge [3]. Importance of glycosylation on its antibacterial activity has been previously discussed. Bulet and co-workers [13] showed the antibacterial activity pattern of disaccharide containing drosocin and its non-glycosylated form against multiple strains of bacteria but comparison of activity of differently glycosylated drosocins bearing monosaccharide and disaccharide with non-glycosylated drosocin was only studied against *E. coli* D22. For detailed comparative analysis of antibacterial activity of naturally occurring glycosylated forms of drosocin against various strains of *E. coli*, *Salmonella* and *Klebsiella*, we have synthesized drosocins carrying monosaccharide and disaccharide in the laboratory. Their antibacterial activities were also compared with that of non-glycosylated drosocin. Secondary structures of the peptides were characterized by circular dichroism (CD) spectrometry. Cytotoxic effects of monosaccharide and disaccharide containing drosocins and non-glycosylated drosocin on mammalian cells were analyzed by haemolytic and MTT assays. Various antimicrobial peptides are known to regulate innate and adaptive immune responses in the host. Cathelicidins like LL-37, are known to act as effector molecules of the innate immune system and control the release of pro-inflammatory mediators like TNF-α from LPS-activated neutrophils and macrophages [14, 15]. It has also been reported that apidaecin 1b, a non-glycosylated proline rich AMP, differentially immunomodulates human macrophages, monocytes and dendritic cells [16]. Hence, we have analyzed the immunomodulatory effects of different glycoforms of drosocin and its non-glycosylated form on the pro-inflammatory markers, TNF-α and IL-6, in activated or un-activated murine macrophages. Comparative analysis of various properties of monosaccharide and disaccharide containing drosocins will help in understanding the effect of different sugars in glycosylated variants of the peptide.

Materials and methods

Synthesis of glycosylated threonine carrying $Ac_4Gal\beta1 \rightarrow 3Ac_2GalNAc\alpha1$ (scheme 1)

N^{α}-fluoren-9-ylmethoxycarbony-O-(2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (2)

Crude anomeric mixture of N^{α}-fluoren-9-ylmethoxycarbonyl-*O*-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester **1** (1.8 g) [3] was dissolved in dry CH_3OH (115 ml). After addition of sodium methoxide (41 ml, 100 mM solution in dry CH_3OH), the reaction mixture was stirred for 2 h at 0 °C. The progress of reaction was monitored by TLC. After neutralization with ion exchange resin (Amberlite IR50), the reaction mixture was filtered and concentrated. The crude product was purified by flash chromatography using CH_2Cl_2 as solvent A and 5% CH_3OH-CH_2Cl_2 as solvent B (gradient of 0–50% B over 40 min) to afford **2** (700 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, J = 7.5 Hz, 2H, Ar), 7.59 (dd, J = 2.1 Hz, 7.1 Hz, 2H, Ar), 7.41–7.28 (m, 9H, Ar), 5.90 (d, J = 9.4 Hz, 1H, NH), 5.29–5.13 (m, 2H, CH_2-Ph), 4.88 (d, J = 3.6 Hz, 1H, H-1), 4.47–4.29 (m, 4H, β-H, α-H, CH_2 Fmoc), 4.24–4.19 (m, 1H, CH Fmoc), 4.07 (d, J = 2.4 Hz, 1H, H-4), 3.95 (dd, J = 2.9 Hz, 10.5 Hz, 1H, H-3), 3.84–3.77 (m, 3H, H-5, H-6^{ab}), 3.47 (dd, J = 3.4 Hz, 10.1 Hz, 1H, H-2), 1.28 (d, J = 6.3 Hz, 3H, CH_3Thr); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.6, 157.0 (COFmoc), 144.1, 143.8, 141.5, 135.4, 135.1, 128.9, 128.7, 127.9, 125.4, 125.3, 124.9, 120.2, 99.4 (C-1), 76.4, 70.1, 69.9, 68.3, 68.0, 67.6, 63.1, 60.8, 59.0, 47.3, 18.8 (*C*H_3 Thr). HRMS: calculated for $C_{32}H_{34}N_4O_9Na$ $[M + Na]^+$ 641.2327; found, 641.2609. All data are in agreement with literature report [17].

N^{α}-fluoren-9-ylmethoxycarbony-O-(2-azido-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (3)

To a solution of **2** (524 mg, 0.85 mmol)) in CH_3CN (4 ml) was added α, α-dimethoxy-toluene (277 μl, 1.84 mmol), followed by *p*-toluenesulfonic acid monohydrate (40 mg, 0.21 mmol) at 0 °C. The reaction mixture was allowed to stir for 1 h at 0 °C and 3 h at room temp. The reaction mixture was neutralized using triethylamine, diluted with CH_2Cl_2 and washed thoroughly with water and dried over Na_2SO_4. The crude residue was purified using flash chromatography using a gradient of 0–60% EtOAc-Hexane over 40 min to give the titled compound **3** (395 mg, 66%) as a colourless solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (d, J = 7.4 Hz, 2H, Ar), 7.62 (d, J = 7.4 Hz, 2H, Ar), 7.49–7.46 (m, 2H, Ar), 7.43–7.27 (m, 12H, Ar), 5.77 (d, J = 9.3 Hz, 1H, NH), 5.56 (s, 1H, CHPh), 5.26 (d, J = 17.6, 2H, CH_2-Ph), 4.94 (d, J = 3.3 Hz, 1H, H-1), 4.50–4.42 (m, 3H, βHThr, CH_2Fmoc), 4.33 (dd, J = 10.3, 7.4 Hz, 1H, H-3), 4.26–

4.22 (m, 3H, H-6[a], αHThr, CHFmoc), 4.12–4.02 (m, 3H, H-6[b], H-5, H-4), 3.70 (br s, 1H, OH), 3.53 (dd, J = 10.5, 3.5 Hz, 1H, H-2), 1.29 (d, J = 6.4 Hz, 3H, CH_3Thr). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 157.0 (COFmoc), 144.1, 143.9, 141.5, 137.4, 135.2, 129.6, 128.9, 128.8, 128.6, 127.9, 127.3, 126.4, 125.4, 120.2, 101.5, 99.4 (C-1), 76.5, 75.5, 69.3, 67.9, 67.7, 67.6, 63.5, 61.4, 58.9 53.6, 47.4 (CHFmoc), 18.9 (CH_3Thr). HRMS: calculated for $C_{39}H_{39}N_4O_9$ $[M + H]^+$ 707.2718; found, 707.4371. All data are in agreement with literature report [17].

N^{α}-fluoren-9-ylmethoxycarbony-O-[2-azido-4,6-O-benzylidene-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine benzyl ester (4)

A reaction mixture of **3** (510 mg, 0.72 mmol), per-*O*-acetylated trichloroacetimidate derivative of Galactose (711 mg, 1.44 mmol) [18] and activated 4A° molecular sieves (600 mg) in dry CH_2Cl_2 (10 ml) was stirred for 30 min. at room temperature under argon. TMSOTf (26 μl, 0.14 mmol) was added to the reaction mixture at 0 °C. The reaction mixture was warmed to room temperature and stirred until completion. The reaction was quenched with a few drops of triethylamine. The reaction mixture was diluted with CH_2Cl_2 and filtered through Celite. The filtrate was successively washed with satd $NaHCO_3$, H_2O and dried over $MgSO_4$ and concentrated. Purification by flash chromatography using gradient of 0–40% EtOAc-Hexane over 40 min. Afforded **4** (500 mg, 66.8%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, J = 7.5 Hz, 2H, Ar), 7.62 (d, J = 7.4 Hz, 2H, Ar), 7.55–7.52 (m, 2H, Ar), 7.44–7.29 (m, 12H, Ar), 5.78 (d, J = 9.4 Hz, 1H, NH), 5.54 (s, 1H, $PhCH(O)_2$), 5.41 (d, J = 3.3 Hz, 1H, H-4′), 5.34–5.22 (m, 3H, H-2′, CH_2-Ph), 5.04 (dd, J = 10.4, 3.4 Hz, 1H, H-3′), 4.94 (d, J = 3.5 Hz, 1H, H-1), 4.79 (d, J = 7.9 Hz, 1H, H-1′), 4.56–4.45 (m, 2H, CH_2Fmoc), 4.36–4.32 (m, 1H, H-6[a]), 4.30–4.19 (m, 4H, βHThr, αHThr, CH Fmoc, H-6[a']), 4.16–4.08 (m, 2H, H-3, H-4), 4.04–3.91 (3H, H-6[b'], H-6[b], H-5′), 3.75 (dd, J = 10.8, 3.6 Hz, 1H, H-2), 3.65 (br s, 1H, H-5), 2.17, 2.05, 2.03, 1.99 (4 s, 3H each, 4XOAc), 1.31 (d, J = 6.3 Hz, 3H, CH_3Thr). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 170.4, 170.3, 169.6, 156.9 (CO Fmoc), 144.1, 143.8, 141.5, 137.7, 135.1, 129.2, 128.9, 128.8, 128.7128.4, 127.9, 127.3, 127.2, 126.3, 125.4, 125.3, 120.2, 102.6, 100.9 (C-1′), 99.6 (C-1), 76.3, 76.1, 75.8, 71.2, 71.1, 69.2, 68.8, 67.9, 67.5, 67.1, 63.7, 61.6, 59.3, 58.9, 47.3 (CH Fmoc), 20.9, 20.8, 19.0 (*C*H_3Thr). HRMS calculated for $C_{53}H_{56}N_4O_{18}Na$ 1059.3590; found 1059.3727.

N^{α}-fluoren-9-ylmethoxycarbony-O-[2-azido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine benzyl ester (6)

Compound **4** (402 mg, 0.39 mmol) was dissolved in 80% of acetic acid in water (12 ml) and stirred for 1.5 h at 70 °C. After completion of the reaction, the mixture was concentrated and dried to afford compound **5**. To the solution of **5** (360 mg) in pyridine (5 ml) was added acetic anhydride (2.5 ml) at 0 °C. The reaction was allowed to warm and stirred for 6 h at room temperature. The reaction mixture was concentrated and purified by flash chromatography using gradient of 0–35% EtOAc-Hexane over 30 min, to yield pure compound **6** (270 mg, 67.5%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, J = 7.5 Hz, 2H, Ar), 7.62 (d, J = 7.3 Hz, 2H, Ar), 7.44–7.29 (m, 9H, Ar), 5.67 (d, J = 9.4 Hz, 1H, NH), 5.46 (d, 1H, J = 3.1 Hz, H-4′), 5.37 (d, 1H, J = 3.3 Hz, H-4), 5.29–5.16 (m, 3H, CH_2-Ph, H-2′), 5.01 (dd, J = 10.5, 3.3 Hz, 1H, H-3′), 4.84 (d, J = 3.7 Hz, 1H, H-1), 4.70 (d, J = 7.7 Hz, 1H, H-1′), 4.54–4.40 (m, 3H, CH_2Fmoc, βH Thr), 4.37–4.31 (m, 1H, αHThr), 4.16–4.07 (m, 4H, CHFmoc, H-3, H-6[a'b']), 4.04–3.89 (m, 4H, H-5′, H-5, H-6[ab]), 3.53 (dd, J = 10.7, 3.7 Hz, 1H, H-2), 2.15, 2.13, 2.06, 2.04, 2.03, 1.99 (6 s, 3H each, 6XOAc), 1.32 (d, J = 6.4 Hz, 3H, CH_3-Thr). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.6, 170.4, 170.3, 170.2, 170.1, 169.7, 156.9 (CO Fmoc), 144.0, 143.8, 141.5, 135.1, 129.1, 128.9, 128.8, 128.7128.0, 127.3, 127.2, 125.3, 125.2, 120.2, 101.7 (*C*-1′), 99.5 (*C*-1), 75.0, 71.0 (C-3′), 69.5, 69.0 (C-4′), 68.1, 67.9, 67.6, 67.0 (C-4), 63.1, 61.2, 60.5, 59.9 (C-2), 58.9, 47.3, 21.2, 20.9, 20.8, 20.7, 18.7 (CH_3Thr). HRMS calculated for $C_{50}H_{56}N_4O_{20}Na$ 1055.3488; found 1055.327.

N^{α}-fluoren-9-ylmethoxycarbony-O-[2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine benzyl ester (7)

To a solution of compound **6** (210 mg, 0.20 mmol) in pyridine (7 ml), thiolacetic acid (7 ml) was added drop-wise at 0 °C. The reaction mixture was allowed to attain room temperature and stirred for overnight. The reaction mixture was diluted with toluene (15 ml) and the solvents were evaporated. The residue was co-concentrated thrice from toluene. Crude product was purified using flash chromatography following gradient of 0–60% EtOAc-Hexane over 40 min, which yielded **7** (170 mg, 80%) as a white amorphous solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (d, J = 7.3 Hz, 2H, Ar), 7.62 (d, J = 7.0 Hz, 2H, Ar), 7.45–7.30 (m, 9H, Ar), 5.77 (d, J = 8.8 Hz, 1H, NH), 5.53 (d, J = 9.4 Hz, 1H, NH), 5.37 (d, 2H, J = 2.4 Hz, H-4′, H-4), 5.29–5.05 (m, 3H, CH_2-Ph, H-2′), 4.96 (dd, J = 10.5, 3.0 Hz, 1H, H-3′), 4.82 (d, J = 2.4 Hz, 1H, H-1), 4.59–4.40 (m, 5H, H-1′, CH_2-Fmoc, βHThr, αHThr), 4.26–4.09 (m, 6H, H-2, H-5, H-5′, H-6[a'b'], CHFmoc), 3.99–3.87 (m, 2H, H-6[ab]), 3.81 (dd, J = 10.7, 2.2 Hz, 1H, H-3), 2.16, 2.13, 2.07, 2.04, 2.03, 1.98 (6 s, 21H, 7XOAc), 1.29–1.23 (m, 3H, CH_3Thr). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 170.6, 170.5, 170.3, 170.2, 170.1, 169.8, 156.6 (CO Fmoc), 143.9, 143.8, 141.5, 134.6, 129.2, 129.1, 128.8, 128.7, 128.6128.0, 127.3, 125.1, 120.3, 100.9 (C-1′), 100.3 (C-1),

73.3, 71.0, 70.9 (C-3′), 69.9, 69.0 (C-4′), 68.1, 68.0, 67.3, 67.0 (C-4), 63.1, 61.2, 60.6, 58.9, 48.9, 47.4 (*C*H Fmoc), 23.5, 21.2, 20.9, 20.8, 20.7, 18.5 (CH_3Thr). HRMS: calculated for $C_{52}H_{61}N_2O_{21}$ $[M + H]^+$ 1049.3767; found, 1049.5974.

N^{α}-fluoren-9-ylmethoxycarbony-O-[2-acetamido-4, 6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl] -L-threonine (I)

To a solution of **7** (660 mg, 0.63 mmol) in CH_3OH (10 ml) was added 10% Pd/C (660 mg). The mixture was stirred under one atmosphere of hydrogen gas for 2 h. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using CH_2Cl_2 as solvent A and 10% CH_3OH-CH_2Cl_2 as solvent B (gradient of 0–70% B over 50 min) to afford **I** (518 mg, 86%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 7.82 (d, J = 7.3 Hz, 2H, Ar), 7.69 (d, J = 7.3 Hz, 2H, Ar), 7.43–7.31 (m, 4H, Ar), 5.37–5.34 (m, 2H, H-4′, H-4), 4.99–4.97 (m, 2H, H-3′, H-2′), 4.63–4.49 (m, 4H, H-1, H-1′, CH_2Fmoc), 4.38–4.26 (m, 3H, βHThr, H-2, CHFmoc), 4.22–4.07 (m, 5H, αHThr, H-6^b, H-$6^{b'}$, H-5, H-5′), 3.99–3.93 (m, 2H, H-6^a, H-$6^{a'}$), 3.87 (d, J = 11.1 Hz, 3.2 Hz, 1H, H-3), 2.12, 2.09, 2.04, 2.02, 2.00, 1.96, 1.93 (7 s, 21H, 7XOAc), 1.23 (d, J = 6.6 Hz, 3H, CH_3Thr). ^{13}C NMR (75 MHz, CD_3OD): δ 172.1, 171.8, 170.9, 170.7, 170.6, 170.1, 169.6, 157.7 (CO Fmoc), 143.9, 143.8, 141.3, 127.5, 126.8, 124.7, 124.6, 119.7, 119.6 (Ar C), 101.1 (C-1′), 99.5 (C-1), 76.0, 73.6, 70.8, 70.3, 69.8, 68.7, 67.6, 67.1, 66.3, 62.9, 60.9, 58.5, 53.4, 21.8, 19.4, 19.3, 19.2, 19.1, 17.8 (CH_3Thr). HRMS: calcd for $C_{45}H_{55}N_2O_{21}$ $[M + H]^+$ 959.3297; found, 959.5693.

Peptide synthesis

Disaccharide containing drosocin [GKPRPYSPRPT(β-Gal(1 → 3)αGalNAc) SHPRPIRV; Di-drosocin] used in this study was chemically synthesized by solid-phase method on an automated peptide synthesizer (433A; Applied Biosystems, Foster City, CA, USA), employing Fmoc-methodology. For synthesizing glycosylated peptide, pre-synthesized glycosylated amino acid was added and coupled manually on the growing polypeptide chain. The peptide was cleaved from the peptide-resin by treatment with a cleavage mixture containing phenol (0.75 g), EDT (0.25 ml), TFA (10 ml), thioanisole (0.5 ml) and water (0.5 ml). The crude peptide was purified by semi-preparative HPLC (Waters, USA) using C18 column (Waters XBRIDGE™ BEH 130 reverse phase C18 column, 19 X 250 mm, 10 μm, spherical) as reported previously [8]. The purified glycosylated peptide was de-acetylated using aqueous 5% hydrazine hydrate and then purified again using HPLC. The peptide was characterized using mass spectrometry. Synthesis and characterization of monoglycosylated drosocin (M-drosocin) and non-glycosylated drosocin (n-drosocin) have been discussed previously [7].

Antibacterial activities

Minimum inhibitory concentrations (MICs) against various bacterial strains such as *E. coli* ATCC 25922, *E. coli* ATCC 11755, *E. coli* ATCC 35218, *E. coli* ATCC 8739, *E. coli* ATCC 10536, *E. coli* ATCC 9637, *E. coli* ATCC ML35p, *S. typhimurium* ATCC 14028, *S. typhi* Vi+, *S. enterica* NCTC 6017, *K. pneumoniae* ATCC 13883 and *K. pneumoniae* ATCC 700603 were determined for peptides using broth microdilution assay in Mueller Hinton broth. Bacteria growing in logarithmic growth phase were washed thrice with Phosphate Buffer (PB 10 mM, pH 7.4) and diluted in Muller Hinton broth to adjust A_{600} = 0.001 corresponding to 3-5x10^5CFU/ml. Ninety μL of this culture was aliquoted in 96 well plate and 10 μL of varying concentrations of different peptides were added. The plate was incubated at 30 °C for 22 h. The MIC is defined as lowest concentration of the peptide that inhibits measurable growth of an organism. Each test condition was set up in duplicate and the experiment was repeated at least thrice to calculate the average.

Killing kinetics

The kinetics of antibacterial activity against *E. coli* ATCC 25922 by non-glycosylated and glycosylated forms of drosocin was performed in Mueller Hinton media, 10 mM Phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) and Dulbecco's Modified Eagle's Medium (DMEM). Mid-log phase bacteria (approximately 3-4x10^5CFU/ml) were incubated at 30 °C up to 6 h or 12 h without (only cells) and with peptide. Aliquots were removed at different time intervals, diluted in PB and plated in Mueller-Hinton solid medium to allow colony counts. All assays were repeated at least once, with the difference between assays being ≤1 $\log_{10}$ CFU/ml. The results presented in Figs. 1, S1 and S2 were from a single representative experiment.

Circular-dichroism spectroscopy

The circular-dichroism (CD) spectra of all peptides (50 μM) were obtained at 25 °C in a 1 mm path-length cuvette in the range of 250–190 nm using CD spectrometer (J-815 JASCO Corporation, Japan). Five scans with a speed of 100 nm/min were averaged and analyzed using JASCO spectra analysis software. The spectra were recorded in 10 mM PB pH 7.4, 90% TFE in water and 10 mM SDS in 10 mM PB pH 7.4. Results were expressed as mean residue ellipticity in [θ] (Deg-cm^2/dmol).

Haemolytic assay

Haemolytic assay was done as described previously [7]. In brief, a 200 μl aliquot of packed rat erythrocytes volume was washed with cold PBS (10 mM PB, pH 7.4, 150 mM NaCl) at 4 °C till the supernatant was colourless, which was discarded each time. The pellet was resuspended in PBS, diluting the cells to 2% of their packed volume. The erythrocytes (90 μl) were plated in a 96-well microtiter plate (Greiner Bio-one, Wemmel, Belgium) along with 10 μl of peptide solution corresponding to final concentration of 200 μM added to the wells and the plate was incubated at 37 °C for 90 min. Any haemolytic activity would manifest as rupture of RBCs and release of haemoglobin into the solution. Subsequently, the plate was centrifuged at 4000 rpm for 10 min at 4 °C to pellet intact cells and 75 μl of supernatant was transferred to a flat bottom microtiter plate (Greiner Bio-one, Wemmel, Belgium). Its absorbance was read at 405 nm and the percentage of RBCs lysis was calculated. The haemolysis affected by only PBS and 1% TritonX-100 were considered as negative control and 100% haemolysis, respectively. Each test condition was set up in triplicate and the results have been averaged from three independent experiments.

MTT assay

Cytotoxicity of the synthetic peptides was determined by standard MTT assay following the protocol as previously described [8]. The method is based on the reduction of the salt, Methylthiazolyldiphenyl Tetrazolium Bromide (MTT) into a crystalline blue formazan product by the cellular oxidoreductases of viable cells. The synthesized peptides were incubated with murine macrophages cell lines, J774 and RAW 264.7 (2.5×10^4 cells/well) grown in DMEM. Following 24 h incubation with peptides (200 μM), cells were washed and then incubated with fresh culture media and 100 μl MTT (Sigma, 0.25 mg/ml) in 0.1 M PBS, pH 7.4 at 37 °C in a humid atmosphere with 5% CO_2 for 3 h. Media was then gently aspirated from test cultures and 100 μl of Dimethyl Sulphoxide (DMSO) was added to the wells. The plates were shaken for 2 min before taking the absorbance at 570/630 nm in a microtiter plate reader. The percentage of viability was calculated as AT/AC × 100; where AT and AC are the absorbances of treated and negative control cells, respectively. In the positive and negative controls, the peptide solution was substituted by the same volumes of ethanol and PBS, respectively. Each test condition was set up in triplicate and the results have been averaged from three independent experiments.

Effects of peptides on the secretion of TNF-α and IL-6 in murine macrophages (RAW 264.7)

The immunomodulatory effects of drosocin peptides on murine macrophages, were assessed by measuring the concentrations of pro-inflammatory cytokines TNF-α and IL-6 in the supernatants of stimulated RAW 264.7 cells by sandwich ELISA using commercially available ELISA Kits (BD Biosciences, San Diego, USA) in accordance with the manufacturer's description. Cells were seeded in 24-well tissue culture plates at a density of 5×10^5 cells/ml and incubated overnight at 37 °C in 5% CO_2. Cells were then incubated at least in triplicate for 24 h in the presence of medium alone or with *E. coli* O111:B4 LPS (20 ng/ml) (Sigma Chemical Co., St Louis, USA), different concentrations (6.25 μM, 12.5 μM, 25 μM, 50 μM and 200 μM) of synthesized peptides or a combination of LPS and peptide or polymyxin B. Polymyxin B (7.2 μM = 10 μg/ml) was used as a positive control. Supernatants were collected and stored at −20 °C until use. Each experiment was set up in triplicate and the results have been averaged from three independent experiments.

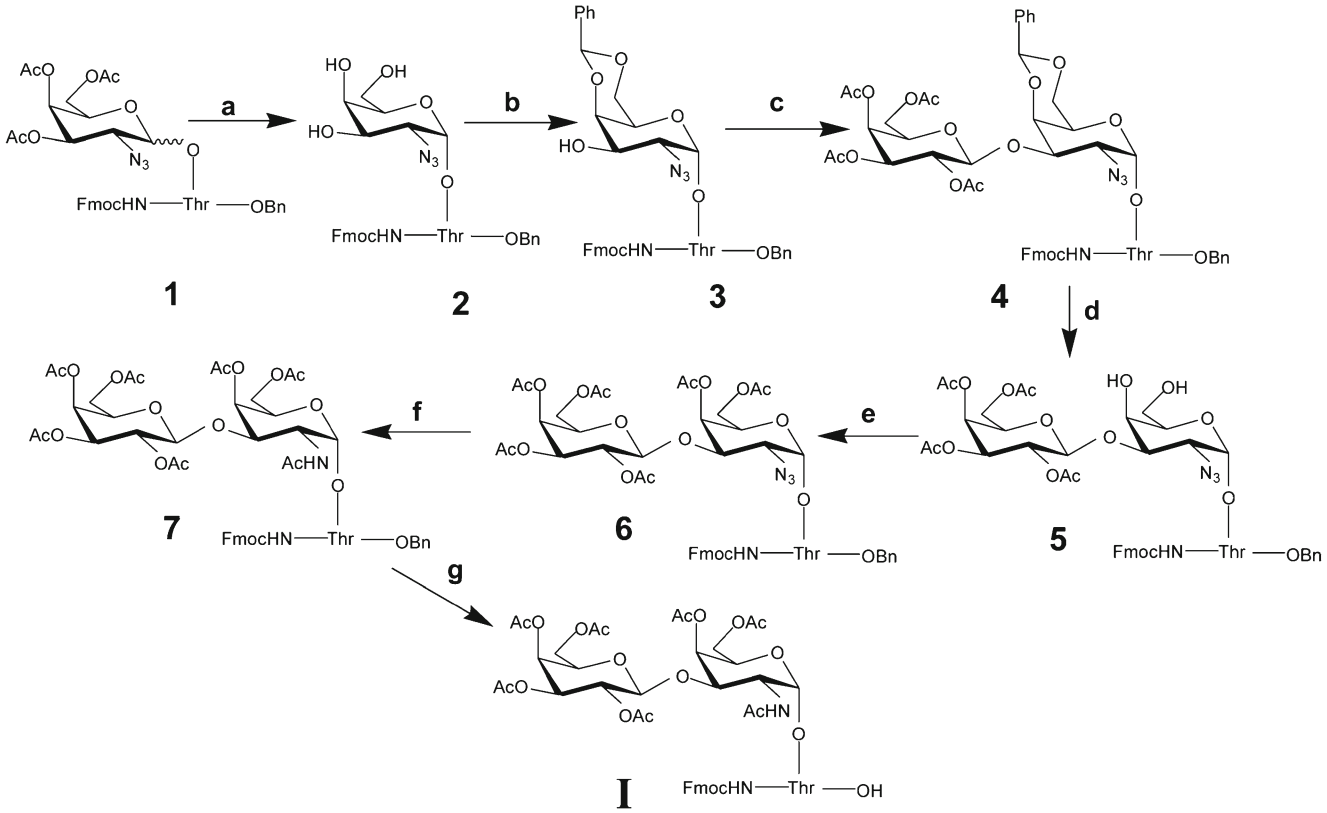
Statistical analysis

Statistical analysis of variance was performed using OriginPro 8.5 and significance of differences ($p \leq 0.01$) with respect to control values were calculated by paired t tests.

Results and discussion

Synthesis of drosocin carrying disaccharide

Two glycosylated forms of drosocin carrying monosaccharide (α-GalNAc) and disaccharide (Galβ1 → 3GalNAcα) were found to be secreted by *Drosophila* upon bacterial challenge [3]. In this report, we have compared the effect of these two sugars on the antibacterial potential of drosocin. The comparative study of other properties like haemolytic, cytotoxic and immunolmodulatory activities of these two isoforms and non-glycosylated form was also undertaken. Monosaccharide, T_n antigen containing drosocin (M-drosocin) and non-glycosylated drosocin (n-drosocin) were synthesized in the laboratory previously [7]. To synthesize drosocin carrying disaccharide, first the critical building block "N^{α}-fluoren-9-ylmethoxycarbony-*O*-[2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine" (**I**) (Scheme 1) was prepared and then incorporated onto the growing polypeptide chain as discussed earlier [7] using solid phase peptide synthesis. Briefly, the peptide chain was first assembled on the resin by standard Fmoc/DCC-HOBt protocol until the glycosylated threonine residue position. The glycosylated threonine **I** was then coupled to the NH_2-peptide resin by Fmoc/HBTU protocol [19]. Further elongation of the peptide chain was carried out according to the standard Fmoc/DCC-HOBt protocol. The deprotection and cleavage of peptide from resin provided the glycopeptide with acetyl groups protected sugar. The purified glycosylated peptide was deacetylated and



Scheme 1 Synthetic scheme for synthesis of N^{α}-*fluoren-9-ylmethoxycarbony-O-[2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine*(I) from compound 1 (N^{α}-fluoren-9-ylmethoxycarbonyl-*O*-(2-azido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester)

finally, the disaccharide containing drosocin (Di-drosocin) was repurified using HPLC and characterized using MALDI-TOF (Table 1 and see supplementary data).

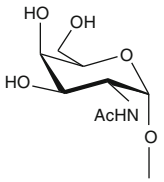
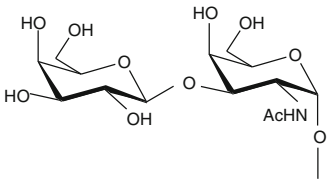
Antibacterial activity

The antibacterial activity of disaccharide containing drosocin [GKPRPYSPRPT(βGal(1 → 3)αGalNAc)SHPRPIRV; Di-drosocin] was compared with monosaccharide carrying drosocin [GKPRPYSPRPT(αGalNAc)SHPRPIRV; M-drosocin], and non-glycosylated drosocin (GKPRPYSPRPTSHPRPIRV; n-drosocin), using microbroth dilution assay (Table 2). It was observed that disaccharide containing drosocin displayed two fold higher MIC against *E. coli* ATCC 25922, *E. coli* ATCC 9637 and *S. typhimurium* ATCC14028 whereas four fold higher MIC against *E. coli* ATCC 35218, *E. coli* ATCC 10536, *S. typhi* Vi + and *S. enterica* NCTC 6017 in comparison with that of M-drosocin. Moreover, Di-drosocin exhibited eight fold higher MIC against *E. coli* ATCC 11775, *E. coli* ATCC 8739, *E. coli* ML-35p and *K. pneumoniae* ATCC 13883 and sixteen fold higher MIC against *K. pneumonia* ATCC 700603 in comparison with that of M-drosocin. However, Di-drosocin resulted around two to four times lower MIC than that of n-drosocin against *Salmonella* and some of the *E. coli* strains. Di-drosocin and n-drosocin showed the similar antibacterial activity against both of the *Klebsiella* strains and *E. coli* ATCC 11775, *E. coli* ATCC 8739 and *E. coli* ML35p. M-drosocin exhibited the lowest MIC value compared to that of Di-drosocin and n-drosocin against all the tested Gram negative bacterial strains of *E. coli*, *Salmonella* and *Klebsiella* (Table 2). Thus, aforementioned results indicated that glycosylation of the peptide as well as increase in sugar chain length affected the antibacterial activity of the peptide. In the earlier studies Bulet et al. [13] reported that monosaccharide (α-GalNAc) containing drosocin possessed lower antibacterial activity in comparison with that of drosocin bearing disaccharide against *E. coli* D22. Contrary to it, our studies showed that M-drosocin was more active than Di-drosocin against all the tested Gram negative bacterial strains. These differences in the results may arise because of the difference in the bacterial strain as it has been shown that magnitude of antibacterial activity of peptide varies in different strains.

Time killing assay

The time-killing experiments were carried out in Muller Hinton (MH) media to follow kinetics of inactivation of

Table 1 Amino acid sequences and mass characterization of different forms of drosocin

Peptide Name	Sequence	MALDI-TOF MS [M+H]Calcd*	MALDI-TOF MS [M+H]Exptl*
n-drosocin	**GKPRPYSPRPTSHPRPIRV**	2198.2436	2198.4417
M-drosocin	 **GKPRPYSPRPTSHPRPIRV**	2401.3230	2401.3713
Di-drosocin	 **GKPRPYSPRPTSHPRPIRV**	2563.3757	2563.3860

*Calcd and Exptl are abbreviations used for calculated and experimental values, respectively

E.coli ATCC 25922 by different glycosylated forms of drosocin and non-glycosylated drosocin and to establish whether these peptides are bacteriostatic or bactericidal. A time course analysis of the inhibitory activity showed that M-drosocin was the most active peptide, eliminating *E. coli* at MIC over a period of 120 min. At MIC value, the number of viable bacteria decreased tenfold within 10 min by M-drosocin and within 80 min by Di-drosocin. At 2 fold MIC, M-drosocin and Di-drosocin required 40 and 120 min, respectively, to completely kill the bacteria. The non-glycosylated drosocin acted more slowly. It took 120 min to reduce the viable cells counts to 50% at its MIC value and even at 2 fold MIC, non-glycosylated drosocin failed to totally eliminate *E. coli* bacteria up to 360 min of treatment. The results showed

Table 2 Minimum inhibitory concentrations (in μM) of different glycosylated forms of drosocin and non-glycosylated drosocin against Gram negative bacteria

Bacterial strain	n-drosocin	M-drosocin	Di-drosocin
E. coli ATCC 25922	4	1	2
E. coli ATCC 11775	2	0.25	2
E. coli ATCC 35218	2	0.25	1
E. coli ATCC 8739	2	0.25	2
E. coli ATCC 10536	4	0.5	2
E. coli ATCC 9637	6	2	4
E. coli ML35p	2	0.25	2
S. typhimurium ATCC 14028	4	0.5	1
S. typhi Vi+	4	0.25	1
S. enterica NCTC 6017	4	0.5	2
K. pneumoniae ATCC 13883	4	0.5	4
K. pneumoniae ATCC 700603	16	1	16

that all the three peptides were bactericidal and M-drosocin had lower MIC and faster bactericidal activity in comparison to that of Di-drosocin and n-drosocin (Fig. 1).

In our earlier studies [8] we have shown the correlation between the antibacterial activity of the glycopeptides and their internalization into the bacterial cells. Antibacterial peptide possessing lower MIC value was internalized faster in comparison to the antibacterial peptide having higher MIC value. The differences observed in the antibacterial activity of different forms of drosocin may be due to differences in their rate of internalization in the bacterial cells. It has been reported that small changes in carbohydrate composition are sufficient to induce conformational changes in the peptide [20]; hence, the variation in the antibacterial activity of M-drosocin and Di-drosocin can also be attributed to the dissimilarities in their conformation and resulting differences in their binding affinity for target molecules [21, 22]. Thus, size of the sugar may play a key role in modulating the biological activity of glycopeptide.

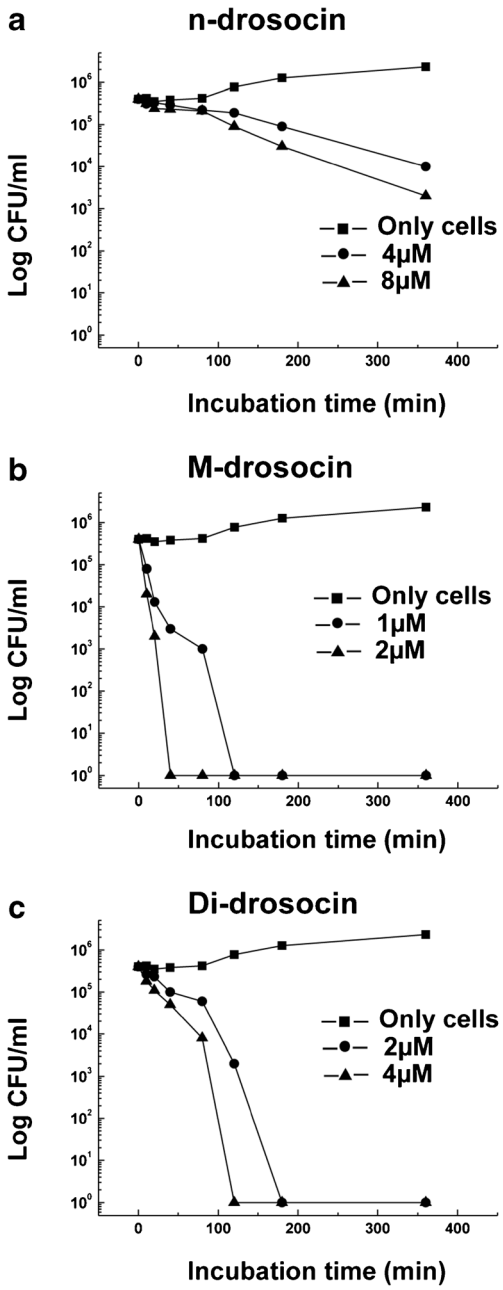


Fig. 1 Killing kinetics of different forms of drosocin against *E. coli* ATCC 25922 in MHB. Kinetics and dose response effect of the bactericidal activity of (**a**) n-drosocin, (**b**) M-drosocin and (**c**) Di-drosocin on *E. coli* at their 1 X MIC and 2 X MIC values. M-drosocin exhibits faster bactericidal activity in comparison with that of Di-drosocin and n-drosocin

Effect of sugar chain length on antimicrobial activity of drosocin corroborates the earlier observations where stimulatory ability of T-helper cell epitope was found to be hampered with extended sugar chain [23]. On the contrary, for CLV3 glycopeptide, its biological activity increased progressively as the sugar chain length increased [24]. Previous studies on glycosylated enkephalin based opioid analgesics peptides, proved that peptides with disaccharide exhibited better permeability across the blood brain barrier and showed increased potency when administered intravenously [25]. Whereas studies on modified IgG molecules showed that even a monosaccharide residue at the glycosylation site was sufficient to exhibit IgG subclass activity in the absence of further extended glycosylation and branching [26].

Circular dichroism spectroscopy

The comparative circular dichroism (CD) spectral analysis was carried out to measure the conformational effects of monosaccharide and disaccharide on the drosocin backbone in different solvents like PB (10 mM, pH 7.4), 90% TFE/water, and 10 mM SDS (Fig. 2). The CD spectra of glycosylated and non-glycosylated forms of drosocin in PB are shown in Fig. 2a. The overall spectral profiles for all the peptides were similar whereas the absolute values of the mean residue ellipticities varied among the peptides. The CD spectra of all the different forms of drosocin displayed random coil secondary structure in PB, with a strong negative band at around 200 nm. In 90% TFE/water and 10 mM SDS, both the glycosylated forms and non-glycosylated peptide exhibited spectral alterations as shown by the deviation in the curves to a more positive ellipticity (Fig. 2b and c). All the peptides were unable to acquire the properly ordered secondary structure even in 90% TFE/water as well as 10 mM SDS. The CD spectra of both glycopeptides indicated absence of any regular secondary structure and displayed similar spectral characteristics and were found to be similar to CD spectra of non-glycosylated drosocin.

Thus, CD studies of disaccharide containing drosocin showed that its secondary structure was not affected by increase in sugar chain length as CD spectra of Di-drosocin were similar to that of M-drosocin in different environments. The

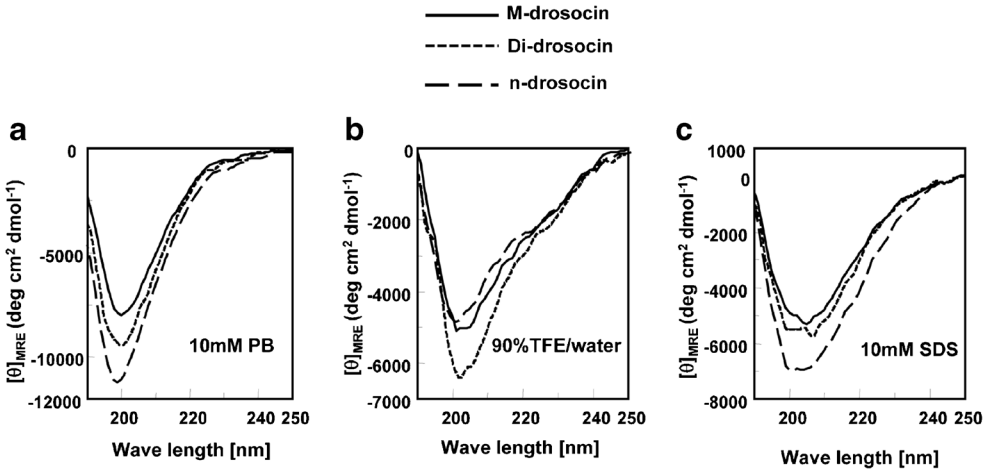


Fig. 2 Circular Dichroism spectra of drosocin analogs showed characteristic random coil secondary structure in different environments and are unaltered with change in the chain length of sugar. Circular Dichroism spectra of M-drosocin, Di-drosocin, n-drosocin in (**a**) 10 mM PB pH 7.4 (**b**) 90% TFE/water (**c**) 10 mM SDS. CD spectra of all three peptides show random coiled conformation, which was observed to be unaltered in presence of different environments. Increase in sugar length did not show any conformational variation as observed by CD

structural studies of a mucin fragment from CD43 by NMR [27] also suggested that the distal sugar residue did not influence the overall peptide conformation. Our results indicated that increase in carbohydrate chain length in drosocin can modulate the antibacterial activity but its effect on conformation of peptide could not be resolved using CD.

Haemolytic activity

Haemolytic activity of drosocin carrying disaccharide was examined on murine erythrocytes and was compared with that of M-drosocin and n-drosocin. Di-drosocin displayed less than 10% haemolytic activity at the concentration of 200 μM when tested on 2% (vol/vol) suspensions of rat blood cells, similar to that of M-drosocin and n-drosocin (Fig. 3). All the different forms of drosocin were found to be non-toxic to rat blood cells even at the concentration of 200 μM which is quite higher than the concentrations at which drosocin forms displayed bactericidal activity against *E. coli* ATCC 25922 in PBS (Fig. S1). Moreover, Di-drosocin behaved similar to that of the monoglycosylated drosocin and thus, increasing the length of sugar chain did not affect the haemolytic activity of drosocin.

Cytotoxic activity

The cytotoxic potential of the disaccharide containing drosocin was also compared with that of monoglycosylated and non-glycosylated forms of drosocin by following incubation of murine macrophages cell lines, J774 and RAW 264.7 in DMEM for 24 h with these peptides using MTT assay. All the different forms of drosocin caused no decrease in cell viability of J774 and RAW 264.7 in comparison with that of control group (no peptide) (Fig. 4) even at the concentration of 200 μM at which both the glycosylated and non-glycosylated forms of drosocin displayed the bactericidal activity against *E. coli* ATCC 25922 in DMEM (Fig. S2). Thus, increasing sugar chain length in Di-drosocin was observed to be non-toxic up to a concentration of 200 μM to mammalian erythrocytes tested as well as to macrophages tested similar to that of M-drosocin and n-drosocin.

Thus, we observed that M-drosocin exhibited higher antibacterial activity compared to Di-drosocin and non-

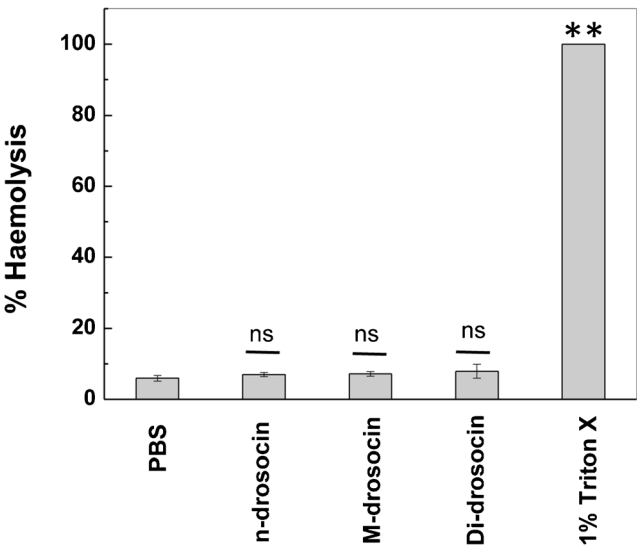


Fig. 3 Haemolytic activity of glycosylated as well as non-glycosylated forms of drosocin on murine erythrocytes. Percentage haemolysis was calculated after exposure of murine erythrocytes to 200 μM of n-drosocin, M-drosocin and Di-drosocin. Erythrocytes incubated with 10 mM PBS alone served as the negative control and cells lysed using 1% Triton X-100 were used to measure 100% lysis, which served as a positive control. All the peptides studied do not show haemolytic activity. The results represent the mean ± S.E.M. of at least three independent experiments performed in triplicate. **: $p < 0.01$ compared to the PBS control; 'ns' means there was no statistical significance from the PBS control

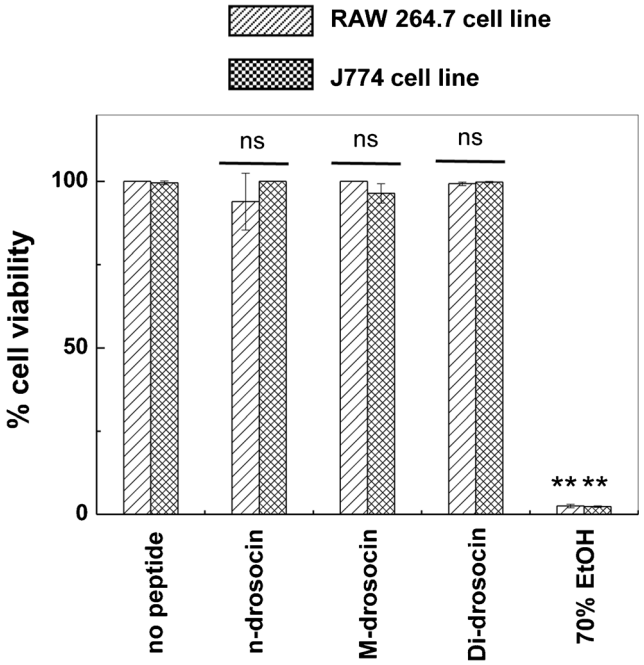


Fig. 4 Cytotoxic activity of drosocins on macrophage cell lines. Percentage cell viability was calculated for n-drosocin, M-drosocin and Di-drosocin against murine macrophage cell lines, J774 and RAW 264.7 at concentrations of 200 μM following standard MTT assay considering cell viability in presence of only PBS as 100% as depicted by no peptide. Macrophages showed less than 5% cell viability in presence of 70% ethanol which served as a positive control. The results represented in the bar graph depict the mean ± S.E.M. of at least three independent experiments performed in triplicate. Two asterisks (**) means statistically different ($p < 0.01$) from the no-peptide control while 'ns' means there was no statistical significance from the no-peptide control

glycosylated drosocin. Increased sugar chain length affected the antibacterial activity of drosocin but neither altered the random coil conformation of drosocin according to CD spectra nor changed the toxicity against mammalian cells tested.

Effect of peptides on secretion of TNF-α and IL-6 in macrophages

We have observed that all the drosocin forms are non-toxic to murine macrophages. So, the effect of different concentrations of these peptides on secretion of pro-inflammatory markers, TNF-α and IL-6, in murine macrophage cells was investigated in presence and absence of LPS. None of the drosocin forms could stimulate macrophages to secrete TNF-α and IL-6 up to the concentration of 200 μM (Figs S3 and S4). LPS is known to activate the macrophages to secrete the pro-inflammatory markers. Polymyxin B was used as a positive control which inhibited LPS-induced activation of macrophages. The different drosocin forms did not modulate the LPS induced secretion of TNF-α and IL-6 in murine macrophages (Fig. 5). Even their 200 μM concentration, at which all the drosocin forms displayed the bactericidal activity against *E. coli* ATCC

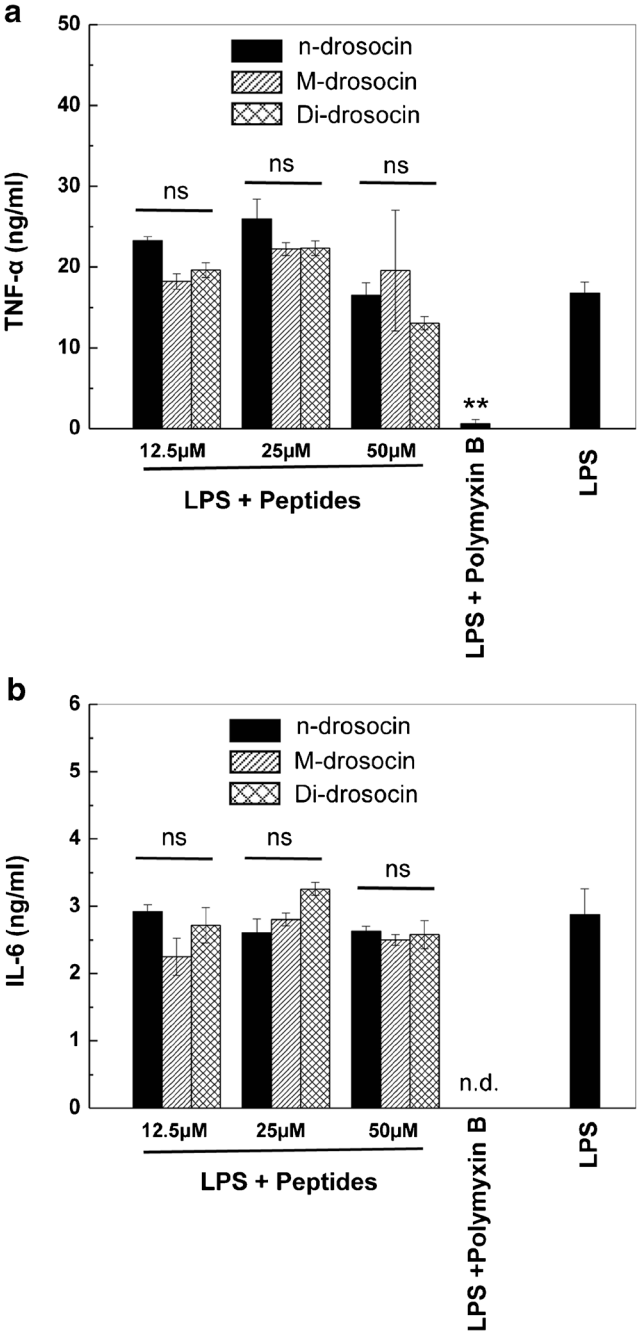


Fig. 5 Effect of drosocins on LPS stimulated secretion of TNF-α and IL-6 in macrophages. RAW 264.7 cells were treated with LPS (20 ng/ml) in the absence or presence of different concentrations of peptides (12.5 μM, 25 μM and 50 μM) and polymyxin B (7.2 μM) for 24 h. Concentrations of (**a**) TNF-α and (**b**) IL-6 in the cell supernatant were measured by ELISA. Levels of cytokines present in the cell supernatant of macrophages with medium alone were subtracted from treatments as background cytokine levels. Polymyxin B served as a positive control which inhibited the LPS induced secretion of cytokines. Data shown are representative values of three independent experiments performed in triplicate. n.d. (not detectable) indicates that cytokine levels were below detection level. **: $p < 0.01$ compared to the LPS; 'ns' means there was no statistical significance from the LPS

25922 in DMEM, could not influence LPS-induced secretion of TNF-α and IL-6 in murine macrophages (Fig. S4). Thus, increasing the sugar chain length in drosocin doesn't affect secretion of TNF-α and IL-6 in LPS activated murine macrophages. Apidaecin 1b, a proline rich antimicrobial peptide from the same class, has been earlier observed to stimulate the human macrophages [16]. This peptide was also found to partially inhibit TNF-α/IL-6 from LPS activated macrophages at suboptimal concentration [16]. On the contrary, recent report from Fritsche et al. [28] could not observe any such immunomodulatory effects of apidaecin 1b and its derivatives on any pro-inflammatory markers produced by murine macrophages stimulated with or without LPS. Similarly, our results exhibit the absence of induction of cytokines, TNF-α and IL-6, and inhibition of LPS-dependent effects for non-glycosylated and glycosylated forms of drosocin when studied in murine macrophages. It has been reported in the literature [29] that cell surface pattern recognition receptors (PRRs) vary in immune cells of different species and these PRRs are responsible for the induction of a pro-inflammatory immune response. Therefore, this might be one of the reasons for the differences in the results arising due to use of immune cells from different sources. Absence of immunomodulation or specifically pro-inflammatory responses has also been observed in other AMPs [30].

Conclusion

We have synthesized drosocin with β-Gal(1 → 3)α-GalNAc attached to threonine, which is one of the native glycosylated forms of drosocin and compared its antibacterial potential with monoglycosylated drosocin having αGalNAc attached to threonine as well as with non-glycosylated drosocin. We observed that there was two or more than two fold decrease in the antibacterial activity of drosocin after increasing the sugar chain length confirming the effect of sugar chain length on the function of this antibacterial peptide probably due to differential local conformation effects of the mono- and di-saccharide on drosocin peptide backbone. M-drosocin exhibited higher antibacterial activity than that of Di-drosocin and non-glycosylated drosocin against the tested strains of *E. coli*, *Salmonella* and *Klebsiella* confirming the role of monosaccharide in the antibacterial activity of drosocin. Increase in sugar chain length did not change secondary structure of peptide analog as characterized by circular dichroism studies. The disaccharide containing drosocin was found to be non-haemolytic and non-cytotoxic up to the concentration of 200 μM similar to M-drosocin and n-drosocin. Although all the three forms of drosocin were observed to have reduced antibacterial activity in DMEM probably due to the neutralisation of peptides in this cell cultue medium, M-drosocin and Di-drosocin or n-drosocin could exhibit the bactericidal effect at 50 μM and 200 μM, respectively. Di-drosocin did not modulate the expression of pro-inflammatory markers, TNF-α and IL-6 in the tested murine macrophages in presence and absence of LPS *in vitro* similar to that of M-drosocin and n-drosocin. This study provides a systematic comparison of differently glycosylated forms of drosocin and insights for understanding the effect of sugar variants in drosocin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Ganz, T.: Role of antimicrobial peptides in innate immunity. Integr Comp Biol. **43**(2), 300–304 (2003)
2. Casteels, P., Tempst, P.: Apidaecin - type peptide antibiotics function through a non - poreforming mechanism involving stereospecificity. Biochem Biophys Res Commun. **199**, 339–345 (1994)
3. Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., Hoffmann, J.A.: A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. J Biol Chem. **268**(20), 14893–14897 (1993)
4. Cociancich, S., Dupont, A., Hegy, G., Lanot, R., Holder, F., Hetru, C., Hoffmann, J.A., Bulet, P.: Novel inducible antibacterial peptides from a hemipteran insect, the sap-sucking bug Pyrrhocoris apterus. Biochem J. **300**, 567–575 (1994)
5. Mackintosh, J.A., Veal, D.A., Beattie, A.J., Gooley, A.A.: Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. J Biol Chem. **273**(11), 6139–6143 (1998)
6. Bulet, P., Hetru, C., Dimarcq, J.L., Hoffmann, D.: Antimicrobial peptides in insects; structure and function. Dev Comp Immunol. **23**(4–5), 329–344 (1999)
7. Talat, S., Thiruvikraman, M., Kumari, S., Kaur, K.J.: Glycosylated analogueues of formaecin I and drosocin exhibit differential pattern of antibacterial activity. Glycoconj J. **28**, 537–555 (2011)
8. Lele, D.S., Dwivedi, R., Kumari, S., Kaur, K.J.: Effect of distal sugar and interglycosidic linkage of disaccharides on the activity of proline rich antimicrobial glycopeptides. J Pept Sci. **21**(11), 833–844 (2015)
9. Pratt, M.R., Bertozzi, C.R.: Synthetic glycopeptides and glycoproteins as tools for biology. Chem Soc Rev. **34**, 58–68 (2005)
10. Chen, L., Drake, M.R., Resch, M.G., Greene, E.R., Himmel, M.E., Chaffey, P.K., Beckham, G.T., Tan, Z.: Specificity of O-glycosylation in enhancing the stability and cellulose binding affinity of family-1 carbohydrate binding modules. Proc Natl Acad Sci. **111**(21), 7612–7617 (2014)
11. Tachibana, Y., Fletcher, G.L., Fujitani, N., Tsuda, S., Monde, K., Nishimura, S.-I.: Antifreeze glycoproteins: elucidation of structural

motifs that are essential for antifreeze activity. Angew Chem. **116**(7), 874–880 (2004)
12. Sola, R.J., Griebenow, K.: Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci. **98**(4), 1223–1245 (2009)
13. Bulet, P., Urge, L., Ohresser, S., Hetru, C., Otvos Jr., L.: Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of Drosophila. Eur J Biochem. **238**, 64–69 (1996)
14. Wan, M., van der Does, A.M., Tang, X., Lindbom, L., Agerberth, B., Haeggström, J.Z.: Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J Leukoc Biol. **95**(6), 971–981 (2014)
15. Alalwani, S.M., Sierigk, J., Herr, C., Pinkenburg, O., Gallo, R., Vogelmeier, C., Bals, R.: The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. Eur J Immunol. **40**(4), 1118–1126 (2010)
16. Tavano, R., Segat, D., Gobbo, M., Papini, E.: The honeybee antimicrobial peptide apidaecin differentially immunomodulates human macrophages, monocytes and dendritic cells. J Innate Immun. **3**(6), 614–622 (2011)
17. Kuduk, S.D., Schwarz, J.B., Chen, X.T., Glunz, P.W., Raghupathi, G., Livingston, P.O., Danishefsky, S.J.: Synthetic and immunological studies on clustered modes of mucin-related Tn and TF O-linked antigens: the preparation of a glycopeptide-based vaccine for clinical trials against prostate cancer. J Am Chem Soc. **120**(48), 12474–12485 (1998)
18. Ren, T., Liu, D.: Synthesis of targetable cationic amphiphiles. Tetrahedron Lett. **40**, 7621–7625 (1999)
19. Knorr, R., Trzeciak, A., Bannwarth, W., Gillessen, D.: New coupling reagents in peptide chemistry. Tetrahedron Lett. **30**(15), 1927–1930 (1989)
20. O'Conner, S.E., Imperiali, B.: A molecular basis for glycosylation induced conformational switching. Chem Biol. **6**(8), 427–437 (1998)
21. Otvos Jr., L., Rogers, O.I., Consolvo, M.E., Condie, P.J., Lovas, B.A., Bulet, S., Blaszczyk-Thurin, P.M.: Interaction between heat shock proteins and antimicrobial peptides. Biochemistry. **39**(46), 4150–4159 (2000)
22. Mardirossian, M., Grzela, R., Giglione, C., Meinnel, T., Gennaro, R., Mergaert, P., Scocchi, M.: The host antimicrobial peptide Bac71-35 binds to bacterial ribosomal proteins and inhibits protein synthesis. Chem Biol. **21**(12), 1639–1647 (2014)
23. Cudic, M., Ertl, H.C.J., Otvos Jr., L.: Synthesis, conformation and T-helper cell stimulation of an O-linked glycopeptides epitope containing extended carbohydrate side-chains. Bioorg Med Chem. **10**(12), 3859–3870 (2002)
24. Shinohara, H., Matsubayashi, Y.: Chemical synthesis of Arabidopsis CLV3 glycopeptide reveals the impact of Hydroxyproline arabinosylation on peptide conformation and activity. Plant Cell Physiol. **54**(3), 369–374 (2013)
25. Li, Y., Lefever, M., Muthu, D., Bidlack, J., Bilsky, E., Polt, R.: Opioid glycopeptides analgesics derived from endogenous enkephalins and endorphins. Future Med Chem. **4**(2), 205–226 (2012)
26. Kao, D., Danzer, H., Collin, M., Andrea, G., Stambuk, J., Lauc, G., Lux, A., Nimmerjahn, F.: A monosaccharide residue is sufficient to maintain mouse and human IgG subclass activity and directs IgG effector functions to cellular fc receptors. Cell Rep. **13**, 2736–2385 (2015)
27. Hoffmann, D., Florke, H.: A structural role for glycosylation: lessons from the hp model. Fold Des. **3**, 337–343 (1998)
28. Fritsche, S., Knappe, D., Berthold, N., von Buttlar, H., Hoffmann, R., Alber, G.: Absence of *in vitro* innate immunomodulation by insect-derived short proline-rich antimicrobial peptides points to direct antibacterial action *in vivo*. J Pept Sci. **18**(10), 599–608 (2012)
29. Bryant, C.E., Monie, T.P.: Mice, men and the relatives: cross-species studies underpin innate immunity. Open Biol. **2**(4), 120015 (2012)
30. Teixeira, L.D., Silva, O.N., Migliolo, L., Fensterseifer, I.C., Franco, O.L.: *In vivo* antimicrobial evaluation of an alanine-rich peptide derived from *Pleuronectes americanus*. Peptides. **42**, 144–148 (2013)