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Glycosylated analogs of formaecin I and drosocin exhibit differential pattern of antibacterial activity

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Abstract The synthetic glycopeptides are interesting model systems to study the effect of *O*-glycosylation in modulating their function and structure. A series of glycosylated analogs of two antibacterial peptides, formaecin I and drosocin, were synthesized by varying the nature of sugar and its linkage with bioactive peptides to understand the influence of structure variation of glycosylation on their antibacterial activities. Higher antibacterial activities of all glycopeptides compared to their respective non-glycosylated counterparts emphasize in part the importance of sugar moieties in functional implications of these peptides. The consequences of the unique differences among the analogs were apparent on their antibacterial activities but not evident structurally by circular dichroism studies. We have shown that differently glycosylated peptides exhibit differential effect among each other when tested against several Gram-negative bacterial strains. The change of monosaccharide moiety and/or its anomeric configuration in formaecin I and drosocin resulted into decrease in the antibacterial activity in comparison to that of the native glycopeptide, but the extent of decrease in antibacterial activity of glycosylated drosocin analogs was less. Probably, the variation in peptide conformation arising due to topological dissimilarities among different sugars in the same peptide resulting in possible modulation in binding properties appears to be responsible for differences in their antibacterial activities. Indeed, these effects of glycosylation are found to be sequence-specific and depend in the milieu of amino acid residues. Interestingly, none of the carbohydrate variants affected the basic property of these peptides, which is non-hemolytic and non-toxicity to eukaryotic cells.

Keywords Synthesis · Glycosylated peptides · Formaecin I · Drosocin · Antibacterial activity

Abbreviations

CAMPs	Cationic antimicrobial peptides
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
SDS	Sodium-dodecyl-sulfate
TFE	2,2,2-trifluoroethanol

Introduction

Carbohydrates are frequently a required component of antibiotic agents that bind to either protein or RNA target structures, or provide the requisite conformational stability to them and such glycoconjugates therefore have medicinal potential [1]. In general, protein glycosylation is a ubiquitous post-translational modification capable of transforming protein's properties in different ways. It is known that glycosylation can profoundly impact a protein's folding, stability, and intracellular trafficking [2–6]. The structures and dynamics of several glycosylated peptides have been elucidated and it has been shown that peptide conformation is stabilized and its fluctuations restricted by the glycosylation [4, 7–13]. However, a detailed understanding of the functions of glycoproteins is often hindered by the structural microheterogeneity caused by the diverse patterns of glycosylation [3].

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In insects, glycosylation is one of the important post-translational modifications of short proline rich cationic antimicrobial peptides (CAMPs) and glycosylated antimicrobial peptides are found to be *O*-glycosylated. Glycosylation seems to have important role in antimicrobial activity as removal of sugar moiety causes significant reduction in activity. Drosocin [14], formaecins [15], pyrrhocoricin [16] and lebocins [17] are examples of such proline rich short CAMPs which are glycosylated at threonine residue with an *O*-linked monosaccharide (α-GalNAc) or a disaccharide (β-galactopyranosyl-α-GalNAc) sugar moiety. Due to widespread resistance to almost all conventional antibiotics, there is an urgent need to discover or design novel antibiotics [18]. Naturally occurring antimicrobial [19] and host defense peptides are being developed as new anti-infective therapeutic agents [20]. Peptide antibiotics offer several advantages such as lower tendency to induce resistance in bacteria, specific action against bacterial membranes, and the ability to target both the surface and the intracellular milieu of bacteria [20]. However, the mode of action of the proline rich cationic antimicrobial peptides from the insects is not known completely and they are thought to kill bacteria by entering cells and binding target/targets to bring their antibacterial activity [21–23]. With enormous potential coded within in terms of configuration and nature, carbohydrate molecules are quite an interesting tool for development and designing of novel glycopeptide antibiotics. Indeed lesser understanding of sugar moiety in relation to their coupling partners is a serious limitation in their wide use as important conjugate in development of sugar linked antibiotic agents.

Earlier it has been reported that *O*-glycosylation has effect on the structure and biological activity of antibacterial peptides as compared to their non-glycosylated counterparts [15, 17, 24–27]. We have shown that conformation provided by carbohydrate can be mimicked by strategically designed amino acids substitutions in the native glycosylated antibacterial peptide and resulted into a functionally equivalent non-glycosylated analog [25]. Here we examined the effect of different *O*-linked sugars in two proline-rich glycosylated antibacterial peptides. The possible consequences of variation in sugar residue both in terms of its nature as well as configuration of linkage to amino acid residue are determined in the context of antibacterial activities. These variations are studied in two different antibacterial glycopeptides, formaecin I [GRPNPVNNKPT(αGalNAc)PHPRL] and M-drosocin [GKPRPYSPRPT (αGalNAc)SHPRPIRV], which are having differences in glycosylated threonine surrounding amino acids. Secondary structure determination using circular dichroism spectroscopy and cytotoxic activities of all the peptides were also studied.

Results

Synthesis of antibacterial glycopeptides with sugar variants

The proline rich class of antibacterial glycopeptides from insects contain conserved threonine residue, which is glycosylated by either a monosaccharide (2-acetamido-2-deoxy-D-galactopyranosyl; GalNAc) or a disaccharide (β-D-galactopyranosyl-GalNAc). These sugars are α-linked to threonine. To explore the effect of variation in the sugar moiety of antibacterial peptides, formaecin I [GRPNPVNNKPT(αGalNAc)PHPRL] and M-drosocin [GKPRPYSPRPT(αGalNAc)SHPRPIRV], on their structures and antibacterial activities, various analogs having differences in the sugar region and its linkage to peptide were synthesized. The key challenge was the synthesis of differently glycosylated threonine residues, which are the critical building blocks for synthesizing various glycosylated analogs of antibacterial peptides, formaecin I and M-drosocin. We have also synthesized non-glycosylated analogs of native formaecin I and M-drosocin to further analyze the influence of sugar moiety on their functional acivities.

Various glycosylated analogs of threonine (Fig. 1) like N^{α}-Fmoc-Thr(Ac_3-α-D-GalNAc)-OH (**Ia**), N^{α}-Fmoc-Thr(Ac_3-α-D-GlcNAc)-OH (**Ib**), N^{α}-Fmoc-Thr(Ac_3-β-D-GalNAc)-OH (**Ic**), N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNAc)-OH (**Id**), N^{α}-Fmoc-Thr(Ac_4-β-D-Gal)-OH (**Ie**), N^{α}-Fmoc-Thr(Ac_4-β-D-Glc)-OH (**If**) and N^{α}-Fmoc-Thr(Ac_4-α-D-Man)-OH (**Ig**) were synthesized. For the synthesis of **Ia** and **Ib** (Scheme 1), per-*O*-acetyl 2-azido-2-deoxy-glycopyranosyl bromides were selected as glycosyl donors containing non-participating group at C-2. The preparation of these glycosyl donors involved the azidonitration of galactal and glucal followed by their conversion to halides [28]. In case of azidonitration of glucal, the products, the mixture of 2-azido-2-deoxy-gluco and 2-azido-2-deoxy-manno derivatives [29], could not be separated by chromatography and the mixture was converted to the corresponding bromides (**1b** and **1c**). The glycosylation of N^{α}-Fmoc-threonine benzyl ester [30] with acyl-protected 2-azido glycosyl bromides (**1a**, **1b** and **1c**) afforded the glycosides **2a** [31], **2b** and **2c**. Reductive acetylation of crude **2a** by thiolacetic acid using pyridine as a solvent provided **3a** [32], whereas crude mixture of **2b** and **2c** resulted the major products **3b** and **3c,** respectively, which could be purified by flash chromatography. Deprotection of benzyl esters of **3a** and **3b**, by hydrogenation yielded the final products **Ia** [31] and **Ib**, respectively.

The synthesis of N^{α}-Fmoc-Thr(Ac_3-β-D-GalNAc)-OH (**Ic**) and N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNAc)-OH (**Id**), as shown in the Scheme 2, started from 2-amino-2- deoxy-D-glycosyl hydrochloride. The hydrochlorides (**4a** and **4b**) were converted into **5**, **6**, and **7** by well established

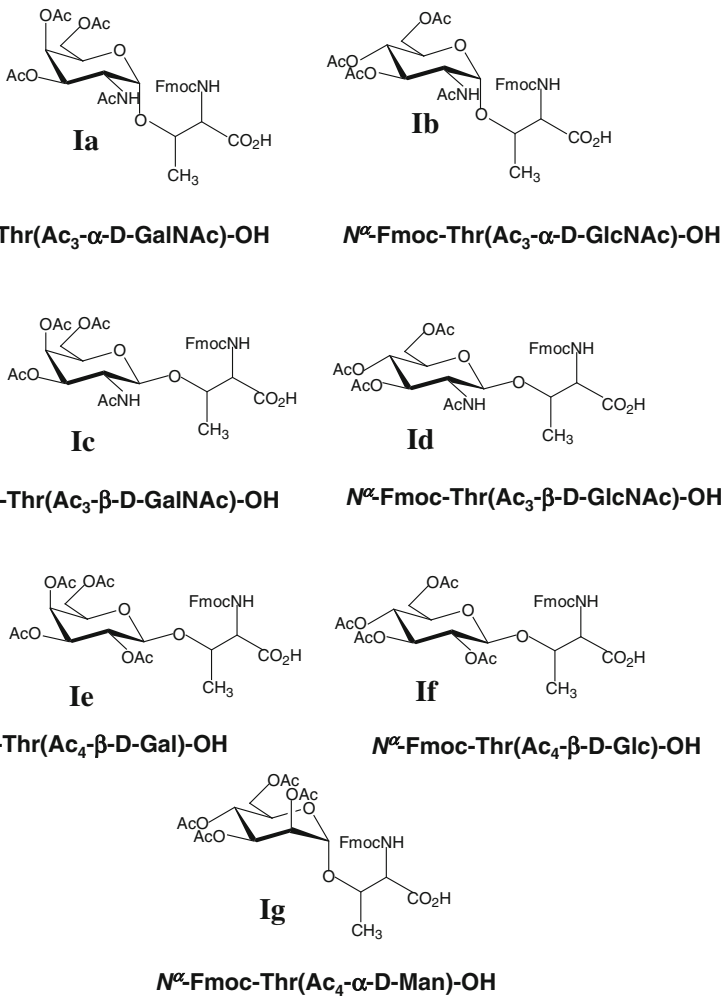


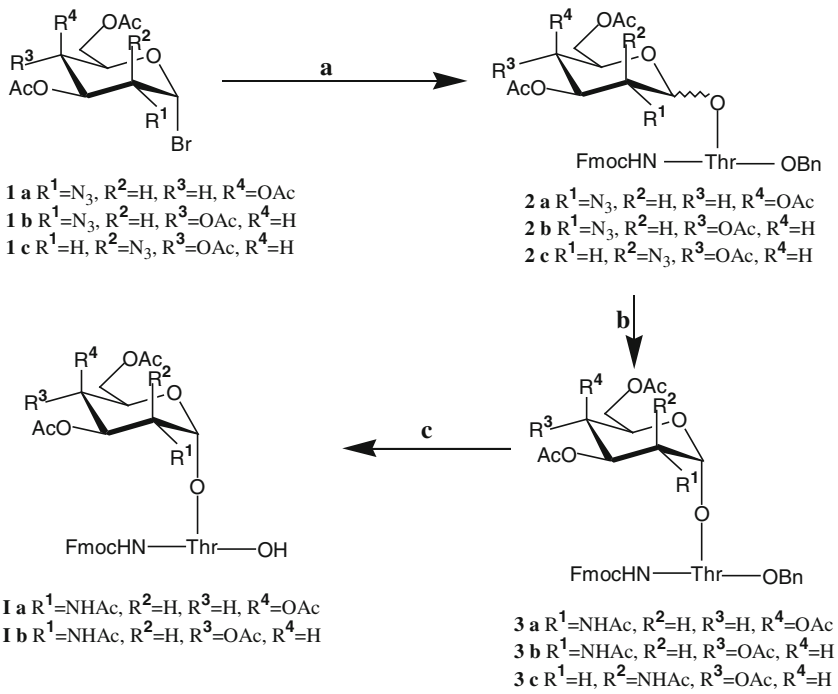
Fig. 1 Different glycosylated analogs of Threonine used for synthesizing differently glycosylated peptides

procedures [33]. Reaction of trichloroacetimidates (**7a** and **7b**) with N^{α}-Fmoc-threonine benzyl ester in the presence of catalytic amounts of trimethylsilyl trifluoromethanesulfonate (TMSOTf) provided the β-linked glycosides (**8a** and **8b**) in good yields. Subsequent replacement of the *N*-trichloroethoxycarbonyl group in **8** by the *N*-acetyl group with zinc and acetic anhydride afforded **9**. Finally, benzyl esters of **9a** and **9b** were reduced with Pd/C under H_2 atmosphere, which provided the desired products **Ic** and **Id**, respectively.

For synthesizing N^{α}-Fmoc-Thr(Ac_4-β-D-Gal)-OH (**Ie**) and N^{α}-Fmoc-Thr(Ac_4-β-D-Glc)-OH (**If**), first the per-*O*-acetylated glycopyranoses (**11a** and **11b**) were transformed with hydrazine acetate into their corresponding 1-*O*-unprotected pyranoses (**12a** and **12b**), which were converted to trichloroacetimidates by treatment with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as base following earlier reported protocol [34]. The glycosylation of N^{α}-Fmoc-benzyl protected threonine with the per-*O*-acetylated galactose trichloroacetimidate (**13a**) and per-*O*-acetylated glucose trichloroacetimidate (**13b**) in the presence of catalytic amounts of TMSOTf afforded high yield of β-linked glycosides **14a** and **14b**, respectively. Subsequent removal of benzyl esters by hydrogenolysis yielded the final products **Ie** and **If** (Scheme 3).

The classical Koenigs-Knorr method [35] was used for the synthesis of N^{α}-Fmoc-Thr(Ac_4-α-D-Man)-OH (**Ig**). The synthesis of **Ig** was achieved by the glycosylation of N^{α}-Fmoc-threonine benzyl ester with per-*O*-acetylated mannosyl bromide **16** [36], which afforded nearly quantitative yields of α-glycoside **17**. Careful hydrogenolysis of **17** afforded the desired product **Ig** (Scheme 4).

The resulting glycosylated amino acid building blocks were used for synthesizing antibacterial peptides, formaecin I and M-drosocin, and their various glycosylated analogs (Table 1). The glycopeptides were synthesized by the solid phase methodology. 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 was then coupled to the NH_2-peptide resin by Fmoc/HBTU protocol [37]. Further elongation of the peptide chain was carried out according to the standard Fmoc/



1 a R^1=N_3, R^2=H, R^3=H, R^4=OAc
1 b R^1=N_3, R^2=H, R^3=OAc, R^4=H
1 c R^1=H, R^2=N_3, R^3=OAc, R^4=H

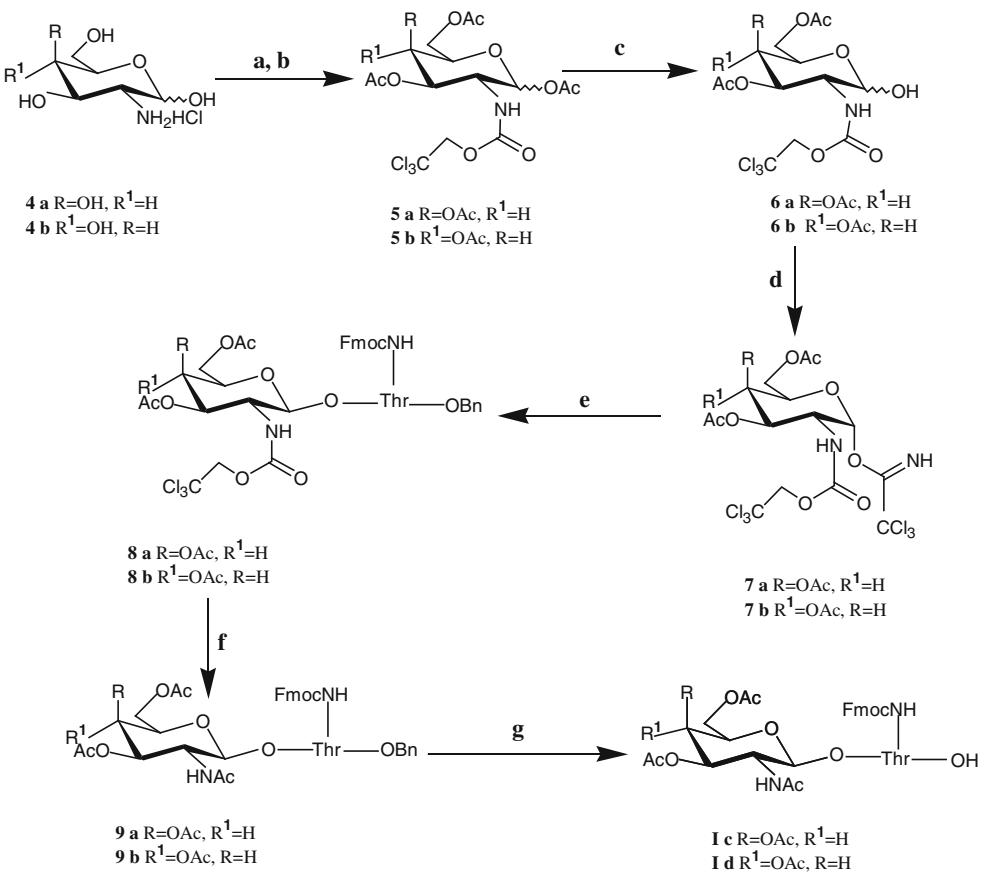
2 a R^1=N_3, R^2=H, R^3=H, R^4=OAc
2 b R^1=N_3, R^2=H, R^3=OAc, R^4=H
2 c R^1=H, R^2=N_3, R^3=OAc, R^4=H

I a R^1=NHAc, R^2=H, R^3=H, R^4=OAc
I b R^1=NHAc, R^2=H, R^3=OAc, R^4=H

3 a R^1=NHAc, R^2=H, R^3=H, R^4=OAc
3 b R^1=NHAc, R^2=H, R^3=OAc, R^4=H
3 c R^1=H, R^2=NHAc, R^3=OAc, R^4=H

Scheme 1 Reagents and conditions: (a) Ag_2CO_3, $AgClO_4$, Fmoc-Thr-OBn, CH_2Cl_2/ Toluene, 4d; (b) Pyridine, thiolacetic acid; (c) H_2, 10%Pd/C, MeOH or EtOAc

DCC-HOBt protocol. Finally, the deprotection and cleavage of peptides from resin provided the glycopeptides with acetyl groups protected sugar. The removal of acetate groups was achieved by treatment of purified peptide with 5% hydrazine hydrate. Finally, all the peptides were repurified and characterized by mass spectrometry (Table 1).



4 a R=OH, R^1=H
4 b R^1=OH, R=H

5 a R=OAc, R^1=H
5 b R^1=OAc, R=H

6 a R=OAc, R^1=H
6 b R^1=OAc, R=H

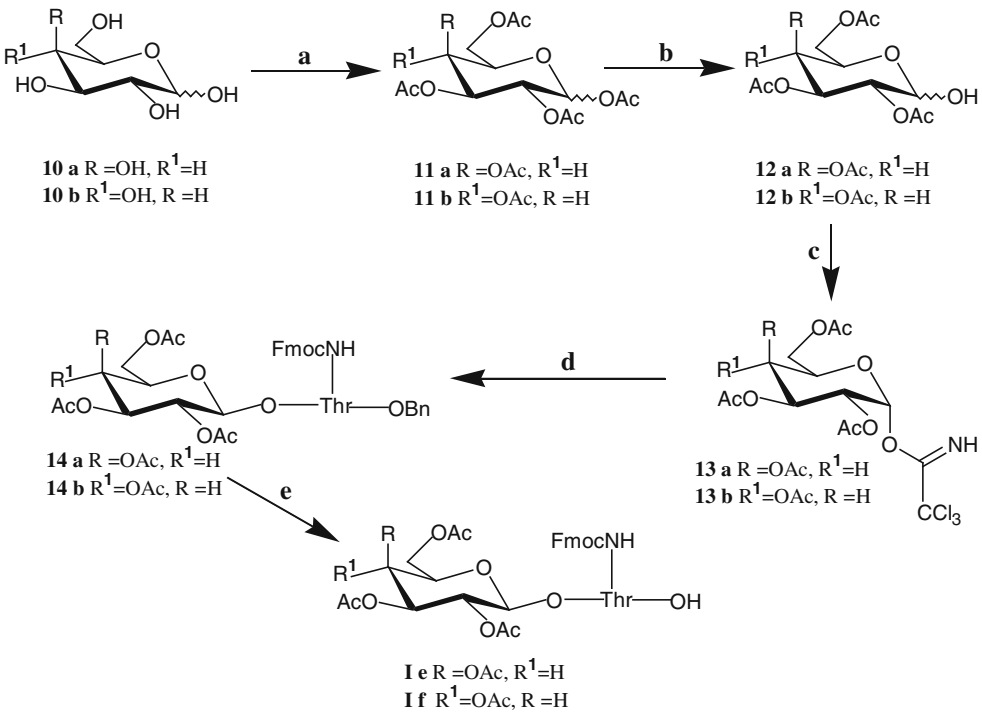
7 a R=OAc, R^1=H
7 b R^1=OAc, R=H

8 a R=OAc, R^1=H
8 b R^1=OAc, R=H

9 a R=OAc, R^1=H
9 b R^1=OAc, R=H

I c R=OAc, R^1=H
I d R^1=OAc, R=H

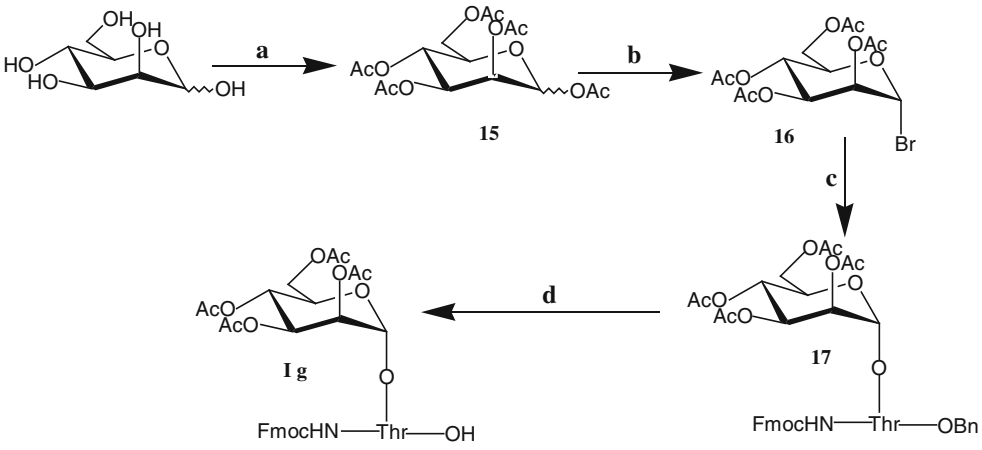
Scheme 2 Reagents and conditions: (a) Teoc-Cl, $NaHCO_3$; (b) Py, Ac_2O; (c) DMF, N_2H_4 HOAc; (d) CH_2Cl_2, CCl_3CN, DBU; (e) CH_2Cl_2, Fmoc-Thr-OBn, TMSOTf; (f) Zn, Ac_2O, rt; (g) H_2, 10%Pd/C, MeOH



Scheme 3 Reagents and conditions: (a) Pyridine, Ac_2O; (b) DMF, N_2H_4 HOAc; (c) CH_2Cl_2, CCl_3CN, DBU; (d) CH_2Cl_2, Fmoc-Thr-OBn, TMSOTf; (e) H_2, 10%Pd/C, MeOH or EtOAc

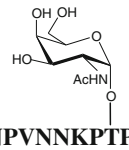
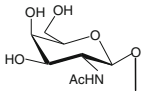
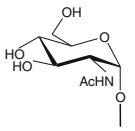
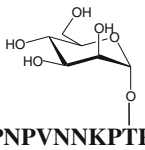
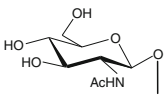
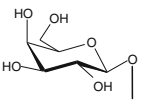
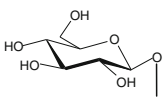
Antibacterial activity

Earlier it has been reported that formaecin I **(I)** and monoglycosylated drosocin (M-drosocin) **(IX)** have higher antibacterial activities than their non-glycosylated counterparts [14, 15, 25]. The synthetic differently glycosylated peptides of formaecin I and M-drosocin as well as their respective non-glycosylated analogs were evaluated at 25 nmoles for their antibacterial activities against Gram-negative bacterial strains of *E. coli* ATCC 25922, *E. coli* BL21λD3, *S. typhimurium*, and *S. typhi* Vi^+. It was observed that formaecin I (**I**), M-drosocin (**IX**) and their corresponding glycosylated analogs exhibited higher activities in comparison to their respective non-glycosylated peptides, **II** and **X,** against all the tested Gram-negative bacterial strains (Figs. 2 and 3). In case of formaecin I, the change of sugar linkage configuration from α- to β- resulted in a decrease of 30–40% in antibacterial activity of β-GalNAc-formaecin I (**III**) in comparison to that of α-GalNAc-formaecin I (**I**) (Fig. 2). With improved antibacterial activity of the α-GlcNAc-formaecin (**IV**) in comparison to that of **III,** the importance of α linkage is ascertained. However, **IV** did not exhibit equivalent levels of antibacterial activity to native peptide **I**, suggesting the importance of configuration of the sugar linkage to peptide as well as axial orientation of the hydroxyl group at C4. Antibacterial activity comparison between synthetic α-GlcNAc- (**IV**) and β-GlcNAc- (**VI**) containing formaecin I analogs resulted into 20–35% decrease in the activity of **VI** as compared to that of **IV,** which further emphasizes the importance of



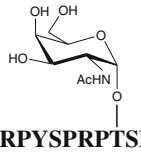
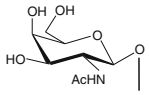
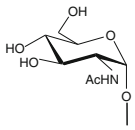
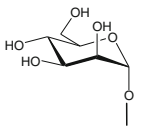
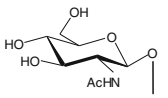
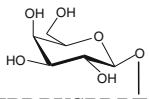
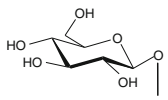
Scheme 4 Reagents and conditions: (a) Py, Ac_2O; (b) CH_2Cl_2, 33%HBr in AcOH **;** (c) CH_2Cl_2, Fmoc-Thr-OBn, Molecular sieves, Silver triflate; (d) H_2, 5%Pd/C, MeOH

Table 1 Amino acid sequences of formaecin I, M-drosocin, their non-glycosylated and different sugar containing analogs

Peptide	Sequence	MALDI-TOF MS [M+H] Calcd	MALDI-TOF MS [M+H] Exptl
Formaecin I **(I)**	 **GRPNPVNNKPTPHPRL**	1997.0691	1997.0752
Nonglycosylated formaecin I **(II)**	**GRPNPVNNKPTPHPRL**	1793.9897[a]	1793.2686
β-GalNAc-formaecin I **(III)**	 **GRPNPVNNKPTPHPRL**	1997.0691	1997.0917
α-GlcNAc-formaecin **(IV)**	 **GRPNPVNNKPTPHPRL**	1997.0691	1997.0968
α-Man-formaecin I **(V)**	 **GRPNPVNNKPTPHPRL**	1956.0425	1956.0793
β-GlcNAc-formaecin I **(VI)**	 **GRPNPVNNKPTPHPRL**	1997.0691	1997.0826
β-Gal-formaecin I **(VII)**	 **GRPNPVNNKPTPHPRL**	1956.0425	1956.0541
β-Glc-formaecin I **(VIII)**	 **GRPNPVNNKPTPHPRL**	1956.0425	1956.0729

α- configuration of the sugar linkage. The replacement of α-GalNAc- in formaecin I (**I**) with α-Man- (**V**) resulted in decrease in its antibacterial activity level up to 50%, probably because of stereochemistry at both C2 and C4 positions is different to that of α-GalNAc- present in native formaecin I. To examine the influence of equatorial

Table 1 (continued)

M-drosocin **(IX)**	 **GKPRPYSPRPTSHPRPIRV**	2401.3230	2401.3933
Nonglycosylated drosocin **(X)**	**GKPRPYSPRPTSHPRPIRV**	2198.2436[a]	2198.4484
β-GalNAc-drosocin **(XI)**	 **GKPRPYSPRPTSHPRPIRV**	2401.3230	2401.3347
α-GlcNAc-drosocin **(XII)**	 **GKPRPYSPRPTSHPRPIRV**	2401.3230	2401.3765
α-Man-drosocin **(XIII)**	 **GKPRPYSPRPTSHPRPIRV**	2360.3766	2360.3169
β-GlcNAc-drosocin **(XIV)**	 **GKPRPYSPRPTSHPRPIRV**	2401.3230	2401.3406
β-Gal-drosocin **(XV)**	 **GKPRPYSPRPTSHPRPIRV**	2360.3766	2360.3164
β-Glc-drosocin **(XVI)**	 **GKPRPYSPRPTSHPRPIRV**	2360.3766	2360.3701

calcd and exptl are abbreviations used for calculated and experimental value respectively, [a] *ESI HRMS*

orientation of hydroxyl group at C4, comparison in antibacterial activities was done between α-GalNAc- (**I**), α-GlcNAc- (**IV**) and β-GalNAc- (**III**), β-GlcNAc- (**VI**) containing formaecin analogs. The α-GlcNAc- containing formaecin analog (**IV**) showed 10–20% decrease in the antibacterial activity in comparison to that of formaecin (**I**) while β-GlcNAc- (**VI**) showed 10% lower activity than **III**, which supports the fact that axial configuration of the

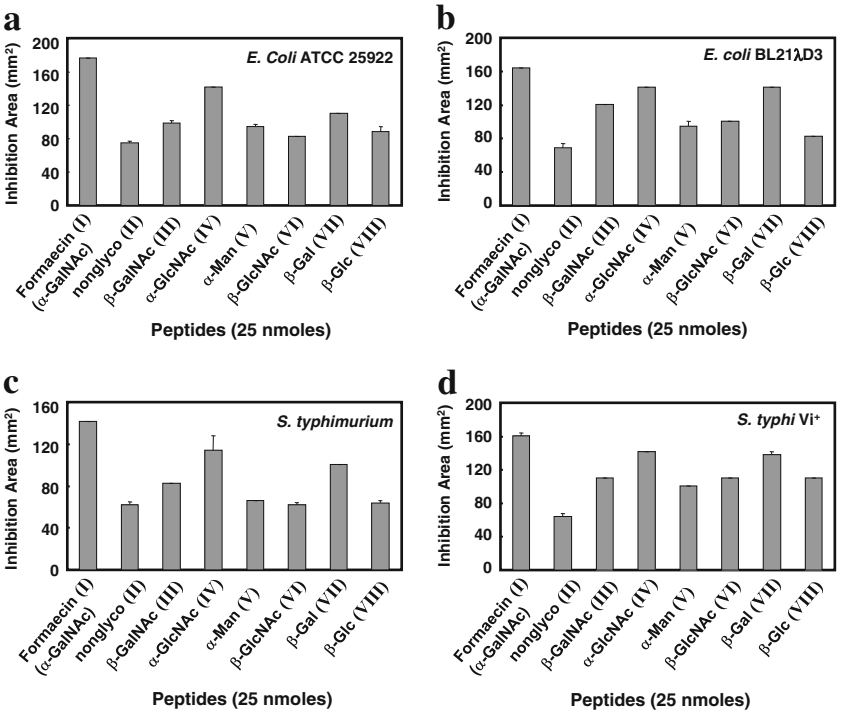


Fig. 2 Antibacterial activity data of the differently glycosylated analogs of formaecin I against *E.coli* ATCC25922 (**a**), E. coli BL21λD3 (**b**), *S. typhimurium* (**c**), and *S. typhi* Vi^+ (**d**), compared with those of the native formaecin I [GRPNPVNNKPT(α-GalNAc) PHPRL] and its non-glycosylated form (GRPNPVNNKPTPHPRL) at 25 nmoles. The results represent the mean ± S.E.M. of at least three independent experiments performed in triplicate

hydroxyl group at C4 plays an important role. Also, comparative account of antibacterial activity among β-Gal- (**VII**) and β-Glc- (**VIII**) containing formaecin analogs showed that **VII** exhibited 20–40% higher activity against all the tested strains as compared to that of **VIII** further suggesting the importance of stereochemistry of the OH group at C4 position. To probe the replacement of acetylated amide with OH group at C2, comparison between β-Gal- (**VII**) and β-GalNAc- (**III**) containing formaecin analogs was done. The β-Gal formaecin analog (**VII**) was found to have 5–15% higher antibacterial activity than that of β-GalNAc-formaecin analog

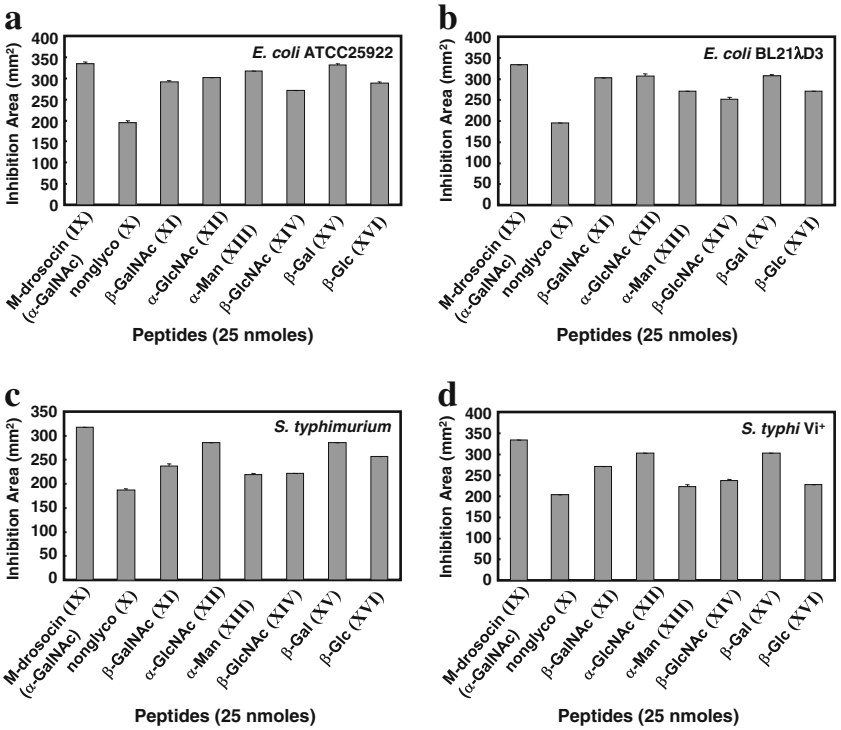


Fig. 3 Comparison of antibacterial activity of differently glycosylated analogs of M-drosocin with M-drosocin [GKPRPYSPRPT(α-GalNAc) SHPRPIRV] and its non-glycosylated form (GKPRPYSPRPTSHPRPIRV) at 25 nmoles against *E.coli* ATCC25922 (**a**), *E. coli* BL21λD3 (**b**), *S. typhimurium* (**c**), and *S. typhi* Vi^+ (**d**). The results represent the mean ± S.E.M. of at least three independent experiments performed in triplicate

(**III**) giving the assessment of the effect of acetamido group in **III.**

The antibacterial activity levels of synthetic M-drosocin (**IX**) and its analogs (**X-XVI**) were compared using above Gram-negative bacterial strains (Fig. 3). It was observed that the change of configuration of sugar linkage from α-GalNAc- (**IX**) to β-GalNAc- (**XI**) in M-drosocin resulted in 10–25% decrease in its antibacterial activity. Also, comparison between α-GlcNAc-drosocin (**XII**) and β-GlcNAc-drosocin (**XIV**) exhibited 10–20% decrease in the antibacterial activity of **XIV** to that of **XII**. The 5–30% lower levels of activity of α-Man-drosocin **(XV)** as compared to **IX,** when tested against various Gram-negative strains**,** showed the influence of stereochemistry at C2 and C4 hydroxyl groups of sugar on its antibacterial activity. To assess the importance of axial orientation of hydroxyl group at C4, the antibacterial activities of α-GalNAc-drosocin (**IX**) and β-GalNAc-drosocin (**XI**) were compared with α-GlcNAc-drosocin (**XII**) and β-GlcNAc-drosocin (**XIV**), respectively. The activity levels of **XII** were found to be 10% lower than that of α-GalNAc-drosocin **(IX)** whereas 10–15% decrease in antibacterial activity was observed for drosocin analog containing β-GlcNAc- (**XIV**) as compared to that of β-GalNAc-drosocin (**XI**). Also, the equatorial orientation of the OH- group at C4 in β-Glc-drosocin (**XVI**) resulted into 10–20% decrease in its antibacterial activity in comparison to that of β-Gal-drosocin **(XV).** To assess the effect of the acetamido group, the comparison of antibacterial activity of β-Gal-drosocin **(XV)** and β-GalNAc-drosocin **(XI)** exhibited 15% more activity in β-Gal-drosocin than β-GalNAc-drosocin (**XI**).

Though native formaecin I, M-drosocin and their all glycosylated analogs showed higher antibacterial activities as compared to their respective non-glycosylated counterparts, they showed significant differences among each other. The pattern of antibacterial activities of different glycosylated analogs of drosocin (**XI–XVI**) was comparable to that of formaecin analogs (**III–VIII**) but the extent of decrease in the antibacterial activity of glycosylated drosocin analogs as compare to that of M-drosocin was less.

Thus, overall comparison of the antibacterial activities of formaecin I, M-drosocin and their analogs against Gram-negative bacterial strains showed the following order **:** α-GalNAc- > α-GlcNAc- > β-Gal- > β-GalNAc- > β-GlcNAc- ≈ β-Glc- ≈ α-Man- > non-glycosylated- with the difference much more pronounced in different glycosylated analogs of formaecin I than of M-drosocin.

Conformational studies

The comparative conformational properties of formaecin I, M-drosocin and their glycosylated analogs were analyzed by CD spectroscopy in water, sodium-dodecyl-sulfate (10 mM SDS), and 2,2,2- trifluoroethanol (90% TFE).

The CD spectra of formaecin I, M-drosocin and their glycosylated analogs in water are shown in Figs. 4a and 5a. The overall spectral profiles for all the glycosylated analogs

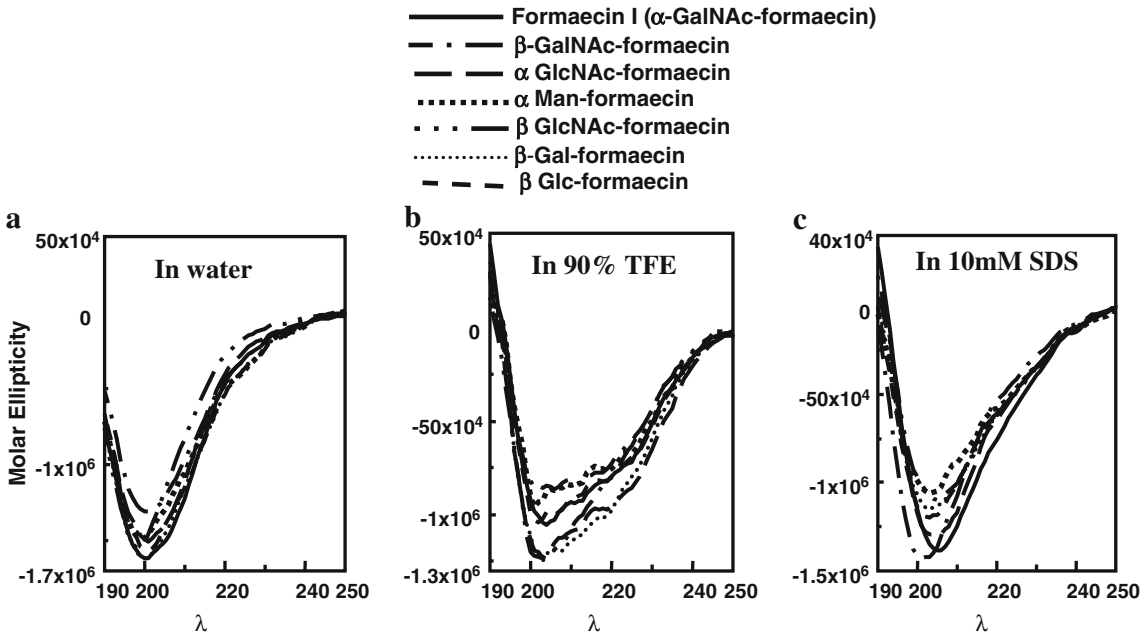


Fig. 4 CD spectra of the differently glycosylated analogs of formaecin I in water (**a**), in 90% TFE (**b**), and in 10 mM SDS (**c**) compared with native formaecin I [GRPNPVNNKPT(α-GalNAc) PHPRL], suggesting that the consequences of the sugar variation are not distinguishable by circular dichroism studies

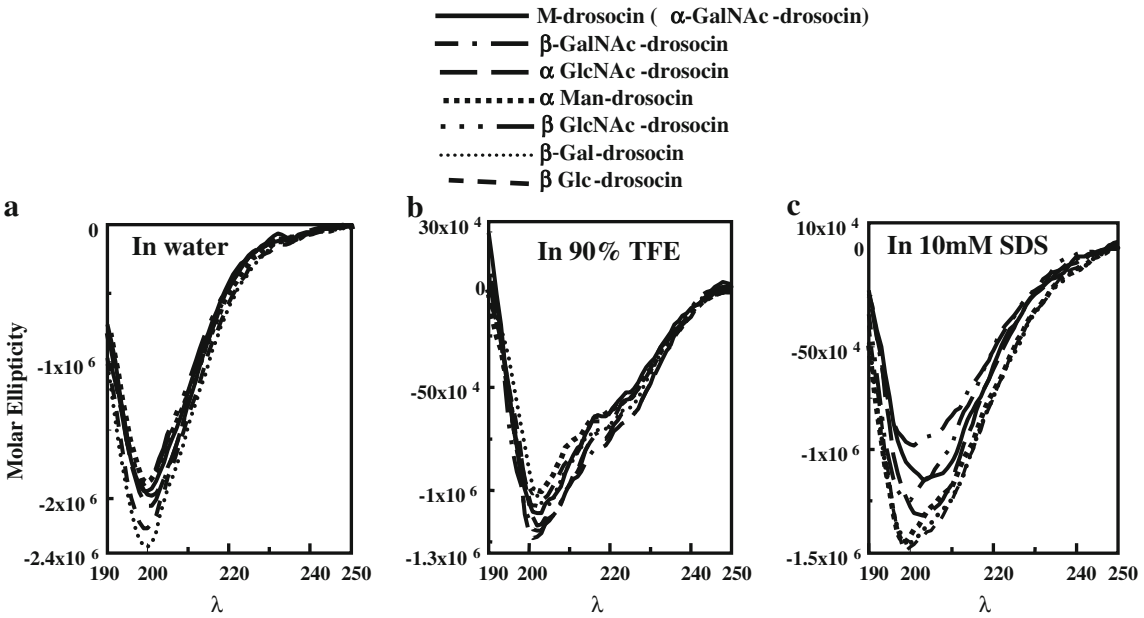


Fig. 5 Circular dichroism profiles of M-drosocin [GKPRPYSPRPT(α-GalNAc)SHPRPIRV] and its differently glycosylated analogs in water (**a**), in 90% TFE (**b**), and in 10 mM SDS (**c**). All the glycopeptides show similar pattern in different environments

of formaecin and M-drosocin were generally similar whereas the absolute values of the molar ellipticities varied slightly among the analogs. The CD spectra of all the glycopeptides showed a minima around 200 nm and a negative shoulder at around 235 nm, which are the characteristics of a disordered structure. All the glycosylated peptides displayed basically random conformations in water. In lipophilic solvent (90% TFE), all the peptides exhibited spectral alterations (Figs. 4b and 5b). Formaecin I, M-drosocin and their glycosylated analogs showed characteristic two negative bands at about 203 nm and 225 nm and a positive band around 190 nm, similar to the pattern, which has been assigned to the presence of type-I (III) β-turns or β-turn mixtures [38, 39] with significant type-I character also reported for drosocin and its deglycosylated form [26]. In micellar SDS environment (Figs. 4c and 5c), all the glycopeptides exhibited similar CD spectra which were characterized by a broad negative band at around 202 nm somewhat red shifted and less intense than that observed in water, suggesting a minor stabilization of the turn structure without clear effect on the peptide conformation.

Thus, conformational studies by CD of formaecin I, M-drosocin and their glycosylated analogs did not show any peculiar difference among each other. In general they displayed random conformations in water. In the membrane mimicking environments like TFE and SDS, all these peptides could not acquire any appreciable ordered structure except for the presence of β-turns as exhibited by their CD spectra.

Cytotoxicity of glycosylated peptides

In vitro cytotoxic assays are useful to evaluate the intrinsic ability of the peptide to cause cell death as a consequence of interference in several cellular functions. To investigate the *in vitro* cytotoxicity of these antimicrobial peptides on eukaryotic cells, the hemolytic activity and MTT assays were done. The effects of formaecin I, M-drosocin, their differently glycosylated analogs and respective non-glycosylated analogs on murine erythrocytes and SP2/O cell lines were evaluated.

The hemolytic activities of formaecin I, M-drosocin and their respective analogs **II–VIII** and **X–XVI** were determined as the percentage of lysis of rat erythrocytes. It was observed that formaecin I, its non-glycosylated form (**II**) and glycosylated analogs (**III–VIII**) showed less than 5% hemolytic activity at the concentration of 100 μM, when tested on 2% (vol/vol) suspensions of rat erythrocytes. All these peptides were non-toxic to rat blood cells. Similar results were obtained with M-drosocin and its analogs **X–XVI**, when tested for their hemolytic activities on rat erythrocytes at a concentration of 100 μM used. Thus, hemolytic activity was absent in formaecin I, M-drosocin and in their respective non-glycosylated peptides as well as their differently glycosylated analogs (Fig. 6a and b).

The cytotoxic potential of all the synthesized peptides was also determined by following incubation of exponentially growing SP2/O cells with these peptides using MTT assay. No difference was found between the cells in the control group and the cells incubated with formaecin I, M-drosocin and their

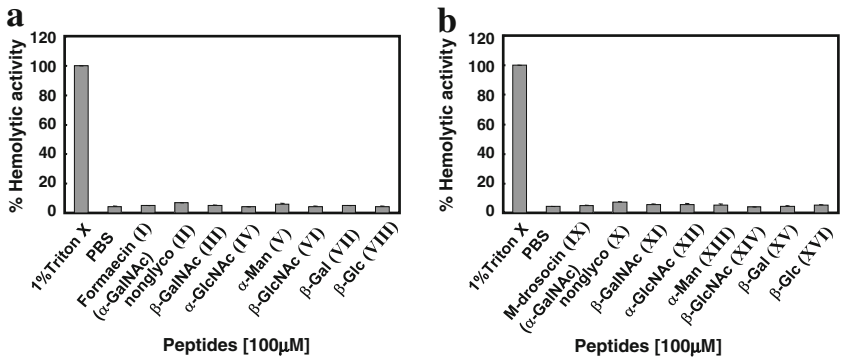


Fig. 6 Hemolytic potential of formaecin I [GRPNPVNNKPT(α-GalNAc)PHPRL], its non-glycosylated (GRPNPVNNKPTPHPRL) and differently glycosylated analogs (**a**), and M-drosocin [GKPRPYSPRPT(α-GalNAc)SHPRPIRV], its non-glycosylated (GKPRPYSPRPTSHPRPIRV) and various glycosylated analogs (**b**) at concentration of 100 μM, on rat erythrocytes following 1 h exposure. The results represent the mean ± S.E.M. of at least three independent experiments performed in duplicate

non-glycosylated and glycosylated analogs for 24 h (Fig. 7a and b). All the tested peptides caused no decrease in cell viability even with the concentration of 50 mg/ml. Thus, all the tested glycosylated peptides did not show cytotoxicity on SP2/O cells in the present study.

Discussion

The importance of protein glycosylation in a variety of biological processes is known. However, the specific contribution of carbohydrate moieties often remains elusive. The issues related to biological function of glycoconjugates can be evaluated, provided suitable quantities of rational model systems can be made available. The chemical syntheses of homogenous glycopeptides have allowed generating a diverse array of carbohydrate structures for studying their functions and local perturbations in a systematic fashion. The synthetic antibacterial glycopeptides are the useful model system for studying structural and physiological consequences of protein glycosylation.

The proline-rich class of antibacterial glycopeptides such as formaecins, drosocins, pyrrhocoricin, diptericin and lebocins from insects contain conserved glycosylated threonine residue [14, 15]. The integrity of sugar moieties is necessary for maximum activity of most of these antimicrobial peptides. We have synthesized a variety of *O*-linked glycosylated threonine residues carrying common sugar moieties present in eukaryotic proteoglycans with variable anomeric configuration and incorporated into two naturally occurring glycosylated antimicrobial peptides, formaecin I and M-Drosocin, and studied their functional implications.

A variety of glycopeptides have been studied to elucidate the impact of *N*- linked or *O*-linked sugar chains on their function as well as on the conformation and flexibility of peptides by NMR [8, 10, 13, 40, 41]. It has been shown that *O*-linked carbohydrates alter the peptide-core conformation

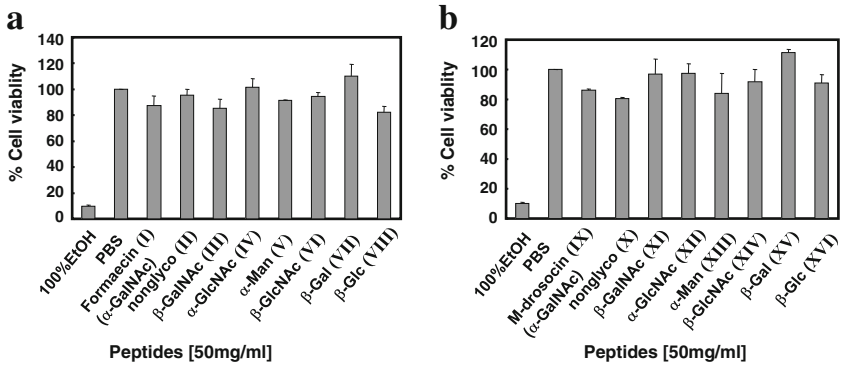


Fig. 7 Effect of formaecin I [GRPNPVNNKPT(α-GalNAc)PHPRL], its non-glycosylated (GRPNPVNNKPTPHPRL) and differently glycosylated analogs (**a**), and M-drosocin [GKPRPYSPRPT(α-GalNAc) SHPRPIRV], its non-glycosylated (GKPRPYSPRPTSHPRPIRV) and various glycosylated analogs (**b**) at concentration of 50 mg/ml on the viability of SP2/O cells following 24 h incubation at 37°C using the MTT assay. The results represent the mean ± S.E.M. of at least three independent experiments performed in duplicate

and reduce the local mobility around the glycosylation site [10, 41–43]. In case of drosocin, the comparative NMR studies [24] of glycosylated and non-glycosylated forms suggest that glycosylation provides a more extended conformation around the glycosylation site and modifies turns at other parts of the molecule. However, these structural differences were not evident with CD analysis as circular dichroism spectra gave information of overall structure, not of local elements, which were random in both cases. But the differences in antibacterial activities between glycosylated and non-glycosylated drosocin were significant. In the present analysis, CD spectra of differently glycosylated peptides reveal no evidence of regular secondary structures in different solvents and are therefore not distinguishable. However, all the glycosylated analogs of formaecin I and drosocin exhibit noticeable differences in antibacterial activities among each other despite having the same peptide sequence. This variation may be due to dissimilarities in the conformations induced by different sugar moieties among the individual glycopeptide and/or topological differences among sugars influencing binding to yet unknown receptor/receptors. We also observed that magnitude of variation in antibacterial activity was much more pronounced in formaecin analogs than in glycosylated analogs of M-drosocin. Previous findings indicate that conformational change of glycosylation also depends on the context of peptide sequence [44]. So, the difference in the variation of antibacterial activities between formaecin I glycosylated analogs and M-drosocin glycosylated analogs, can be best explained by sequence-specific glycosylation induced conformational change. All the antibacterial glycopeptides studied here show higher antibacterial activities as compared to their non-glycosylated counterparts. It appears that sugar conjugation provides higher conformational stability as compared to non-glycosylated analogs [8, 10, 13, 40, 41, 45].

In our study we found the attribute of carbohydrate attachment to peptide (via α- or β-glycosidic bond) within a locus has a contemplative effect on the antibacterial activity against Gram-negative bacteria. β-linked derivatives in both formaecin I and M-drosocin show significant differences in their antibacterial activities as compared to α-linked glycopeptides of formaecin I and M-drosocin, respectively. In general, the pattern of β-linked sugar in the simplified cases [44, 46, 47] exhibits considerable flexibility in the peptide backbone with dynamic and relatively unstrained conformations unlike their α-linked analogs, which are confined to limited conformations with remarkable stability and extended structures. The observed reduction in activity by changing the anomeric configuration of sugar from α- to β- in case of formaecin I and M-drosocin glycosylated analogs is in accordance with the work of Coltart *et al.* [46] and Wu *et al.* [44]. All β-derivatives studied here show activities lower than those of the corresponding α-derivatives.

The antibacterial activity comparison among the glycopeptides with α-GalNAc, α-GlcNAc and β-GalNAc, β-GlcNAc and β-Gal, β-Glc exhibited slightly higher activity of peptide derivatives linked with GalNAc/Gal moiety than GlcNAc/Glc moiety. This variation in activity can be attributed to the difference in the stereochemistry at C-4 with axial and equatorial OH-group in GalNAc/Gal and GlcNAc/Glc, respectively. The comparative antibacterial activity profile of β-Gal and β-GalNAc containing analogs reflected the slightly higher activity of β-Gal derivative than that of β-GalNAc derivative. The approach and participation of acetamide protons of β-GalNAc causing hindrance in binding to the target molecule might be an explanation of comparatively higher activity of β-Gal-derivative.

Effect of glycosylation seems to be dependent on configuration of the sugar linkage as well as nature and stereochemistry of substituents at the C-2 and C-4 position and in the milieu of amino acid residues of peptides as well. Change in conformational stability and/or modulation in binding properties due to stereochemical differences at C-2 and C-4 can be best possibly assigned for the minimum activity of a mannose containing analog. Thus, our analysis indicates that antibacterial activity of formaecin I and M-drosocin against Gram-negative strains studied here can be achieved maximally by glycosylation with a GalNAc sugar moiety appended in α-glycosidic linkage to threonine residue. The antibacterial activity determination of various glycosylated analogs in relation to two different peptide sequences provides a good model system to study functional consequences of sugar variants.

The comparative analyses of cell cytotoxicity examined by hemolytic activity and MTT assay for formaecin I, M-drosocin and their glycosylated analogs showed no surprising results by change of sugar as both native and their differently glycosylated derivatives were found to be devoid of any kind of toxicity.

Conclusions

The synthesis of *O*-linked antibacterial glycopeptides, formaecin I and M-drosocin, with varied carbohydrate moieties permitted access to structurally diverse molecules, which are explored for the resulting impact on their antibacterial activity. All the differently glycosylated analogs were found to be more potent than their non-glycosylated counterpart. We have shown that the glycosylated peptides differing in nature of sugar and its linkage to the peptide of same amino acid sequences exhibited different antibacterial activities most likely due to differential topological effect of glycosylation in particular and

peptide conformation in general. Indeed, these effects are found to be sequence-specific and therefore, also depend on the context of peptide sequence. The high-resolution conformational analyses of these glycopeptides are required to address the precise effect of different *O*-linked sugars. The cytotoxicity experiments showed that native antibacterial peptides or their designed analogs are non-hemolytic and non-toxic to eukaryotic cells. Our findings will be useful for understanding the effect of sugar variants in glycopeptide antibiotics and designing of glycosylated antibacterial agents.

Experimental section

Material and methods HMP (4-hydroxymethyl phenoxymethyl polystyrene) resin, solvents and reagents used for synthesis were supplied by Applied Biosystems Inc. Fmoc amino acid derivatives were procured from Bachem Feinchemikalein AG. Agrose I (Biotechnology grade) was obtained from Amresco, and tryptic soy broth (TSB) was from Himedia Laboratories Pvt. Ltd. All reactions sensitive to air and/or moisture were carried out under argon atmosphere with anhydrous solvents. NMR spectra were recorded on a BRUKER 300 Ultrashield™ NMR spectrometer. Molecular mass determinations were made by using ESI-MS system (Applied Biosystems Mariner System 5220) operating in positive mode. Chromatographic separations were performed on a silica gel column by flash chromatography (Combi Flash Companion, Teledyne ISCO). Yields are given after purification, unless differently stated.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-galactopyranosyl)-L-threonine benzyl ester **(3a)** To a solution of *N*-Fmoc-L-threonine benzyl ester (2 g, 4.63 mmol) in dry CH_2Cl_2-Toluene(1:1, 20 ml) was added 4A° molecular sieves (3 g) and Ag_2CO_3 (3.83 g, 13.9 mmol) and stirred at room temperature for 45 min. $AgClO_4$ (1.2 g, 5.78 mmol) was added and stirred for another 20 min. A solution of azidobromide **1a** (3.28 g, 8.33 mmol) in dry CH_2Cl_2-Toluene(1:1) was slowly added and the reaction mixture was stirred at room temperature for 4 days. The reaction mixture was diluted with CH_2Cl_2 and filtered over celite. The celite was rinsed with CH_2Cl_2 thoroughly until no product was retained. The filtrate was washed with saturated $NaHCO_3$ and H_2O. All the aqueous washes were back extracted with CH_2Cl_2. The pooled organic layers were dried over Na_2SO_4 and concentrated to get the product **2a** (crude 3.38 g). To a solution of **2a** (3.38 g) in pyridine (35 ml), thiolacetic acid (35 ml) was added. The reaction mixture was allowed to stir for 4 h at room temperature. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The crude product was purified by flash chromatography using (gradient of 0–60% EtOAc-Hexane over 40 min). The appropriate fractions were combined and concentrated to give desired product **3a** (2.28 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (d, *J*=7.2 Hz, 2H, Ar), 7.63 (d, *J*=7.2 Hz, 2H, Ar), 7.43–7.30 (m, 9H, Ar), 5.81 (d, *J*=9.9 Hz, 1H, NH), 5.67 (d, *J*=9.3 Hz, 1H, NH), 5.37 (br s, 1H, H-4), 5.21–5.05 (m, 3H, H-3, OCH_2Ph), 4.79 (d, *J*=3.3 Hz, 1H, H-1), 4.56–4.43 (m, 4H, β-H, $FmocCH_2$, α-H), 4.28–4.05 (m, 5H, FmocCH, H-6^{a,b}, H-2, H-5), 2.16, 2.02, 2.00, 1.96 (4 s, each 3H, 4Ac), 1.29 (d, *J*=6.0 Hz, 3H, Thr-CH_3). All data are in agreement with literature reports [32]. ESI HRMS: calcd for $C_{40}H_{44}N_2O_{13}Na[M+Na]^+$ m/z, 783.2741; found, 783.5074.

N^{α}-fluoren-9-ylmethoxycarbony-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-galactopyranosyl)-L-threonine **(Ia)** To a solution of **3a** (982 mg, 1.29 mmol) in methanol (30 ml) was added 10% Pd/C (390 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 $CHCl_3$ as solvent A and 50% MeOH-$CHCl_3$ as solvent B (gradient of 0–30% B over 35 min) to give the titled compound **Ia** (600 mg, 69%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.82 (d, *J*=7.2 Hz, 2H, Ar), 7.71–7.67 (m, 2H, Ar), 7.42–7.29 (m, 4H, Ar), 5.39 (app d, *J*=3.0 Hz, 1H, H-4), 5.07 (dd, *J*=3.2 Hz, *J*=11.5 Hz, 1H, H-3), 4.94 (d, *J*=3.8 Hz, 1H, H-1), 4.62–4.35 (m, 4H, $FmocCH_2$, β-H, H-6^a), 4.31–4.23 (m, 3H, FmocCH, α-H, H-6^b), 4.14–4.05 (m, 2H, H-5, H-2), 2.14, 2.03, 1.94, 1.93 (4 s, each 3H, 4Ac), 1.24 (d, *J*=6.4 Hz, 3H, Thr-CH_3); The ^{1}H NMR data is in accord with the literature [31]. ^{13}C NMR (75 MHz, CD_3OD): δ 172.3, 170.8, 170.7, 170.6 (*C*OCH_3), 157.7 (*C*O Fmoc), 144.0, 143.7, 141.3, 127.4, 126.8, 124.8, 124.6, 119.6, 119.5 (Ar *C*), 99.3 (C-1), 76.3 (β-C), 68.3 (C-3), 67.4 (C-4), 66.8, 66.3, 61.9, 58.6 (*C*H_2 Fmoc, C-6, α-C, C-5, C-2), 47.1 (*C*H Fmoc), 21.5, 19.3, 19.2, 19.1 (CO*C*H_3), 17.8 (*C*H_3 Thr). ESI HRMS: calcd for $C_{33}H_{38}N_2O_{13}Na[M+Na]^+$ m/z, 693.2271, found, 693.3944.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl)-L-threonine benzyl ester **(3b)** A mixture of **1b** and **1c** (2.16 g, 5.48 mmol) and Fmoc-Thr-OBn (1.3 g, 3.01 mmol), was treated as described for the synthesis of **2a**. The crude mixture of azido-glycosides (**2b** and **2c)** was taken directly for the next reaction. The mixture (2.1 g) was dissolved in pyridine (30 ml) and thiolacetic acid (30 ml) was added to it. The mixture was stirred at room temperature for overnight. The reaction mixture was diluted with toluene and concentrated under reduced pressure. The residue was purified by flash chromatography using (gradient of 0–60% EtOAc-Hexane

over 40 min) to give two major compounds, **3b** (880 mg, 38%) and **3c** (800 mg, 35%). The ^{1}H NMR confirmed **3b** to be the title compound and **3c** was characterized as N^{α}-fluoren-9-ylmethoxycarbonyl-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl)-L-threonine benzyl ester. **3b** ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (d, *J*= 7.4 Hz, 2H, Ar), 7.66 (d, *J*=7.2 Hz, 2H, Ar), 7.44–7.30 (m, 9H, Ar), 5.94 (d, *J*=9.5 Hz, 1H, NH), 5.72 (d, *J*= 9.4 Hz 1H, NH), 5.21–5.05 (m, 4H, H-3, H-4, OCH_2Ph), 4.71 (d, *J*=3.6 Hz, 1H, H-1), 4.50–4.42 (m, 3H, α-H, $FmocCH_2$), 4.33–4.06 (m, 5H, H-6^{a,b}, β-H, FmocCH, H-2), 4.02–3.97 (m, 1H, H-5), 2.08, 2.05, 2.04, 1.97 (4 s, each 3H, 4Ac), 1.30 (d, *J*=6.3 Hz, 3H, Thr-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.3, 170.7, 170.6, 170.3 (*C*OCH3), 169.2, 156.5 (*C*O Fmoc), 143.8, 143.7, 141.3, 134.4, 129.0, 128.9, 128.6, 127.8, 127.1, 125.1, 120.0 (Ar *C*), 99.3 (C-1), 77.3 (β-C), 71.2 (C-3), 68.3, 67.8, 67.4 (C-4, C-5, O*C*H_2Ph, *C*H_2 Fmoc), 62.1, 58.6, 51.6 (C-6, α-C, C-2), 47.2 (*C*H Fmoc), 23.0, 20.7, 20.6 (CO*C*H3), 18.3 (*C*H_3 Thr). ESI HRMS: calcd for $C_{40}H_{45}N_2O_{13}$ $[M+H]^+$ m/z: 761.2933; found, 761.4794. **3c** ^{1}H NMR (300 MHz, $CDCl_3$): δ7.70 (d, *J*=7.4 Hz, 2H, Ar), 7.57 (*d*=7.3 Hz, 2H, Ar), 7.36–7.23 (m, 9H, Ar), 5.67 (d, *J*=9.4 Hz, 1H, NH), 5.65 (d, *J*=8.4 Hz, 1H, NH), 5.24–5.15 (m, 3H, H-3, OCH_2Ph), 4.98 (t, *J*=9.9 Hz, 1H, H-4), 4.73 (br s, 1H, H-1), 4.46–4.16 (m, 6H, β-H, $FmocCH_2$, α-H, FmocCH, H-6^{a}), 4.08–3.97 (m, 3H, H-6^{b}, H-5, H-2), 2.01, 1.99, 1.97, 1.94 (4 s, each 3H, 4Ac), 1.24 (d, *J*=6.3 Hz, 3H, Thr-CH_3). ^{13}C NMR ($CDCl_3$): δ 170.4, 169.9, 169.8 (*C*OCH_3), 156.6 (*C*O Fmoc), 143.8, 143.7, 141.2, 135.1, 128.6, 128.5, 128.4, 127.6, 127.1, 127.0, 125.2, 119.9 (Ar *C*), 100.1 (C-1), 77.2 (β-C), 68.8 (C-3), 68.6, 67.8, 67.5, 66.2, 62.5, 58.6, 50.6 (O*C*H_2Ph, C-4, C-5, α-C, *C*H_2 Fmoc, C-6, C-2), 47.1 (*C*H Fmoc), 23.3, 20.7, 20.6 (CO*C*H_3), 17.8 (*C*H_3 Thr). ESI HRMS: calcd for $C_{40}H_{44}N_2O_{13}Na$ $[M+Na]^+$ m/z, 783.2741, found, 783.4616.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl)-L-threonine **(Ib)** To a solution of **3b** (680 mg, 0.89 mmol) in ethyl acetate (20 ml) was added 10% Pd/C (340 mg). The mixture was stirred under one atmosphere of hydrogen gas until the reaction was complete. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using DCM as solvent A and 10% MeOH-DCM as solvent B (gradient of 0–50% B over 40 min) to afford pure **Ib** (396 mg, 66%). The ^{1}H-NMR and ^{13}C-NMR of **Ib** showed the presence of two rotamers in $CDCl_3$ and one in CD_3OD. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, *J*=7.4 Hz, Ar), 7.60 (m, Ar), 7.50 (app t, *J*=7.8 Hz, Ar), 7.41–7.28 (m, Ar), 7.17 (d, *J*=9.0 Hz, NH), 7.03 (d, *J*=9.9 Hz, NH), 6.29 (d, *J*= 9.6 Hz, NH), 6.00 (d, *J*=9.6 Hz, NH), 5.30–5.03 (m), 5.01 (d, *J*=3.8 Hz, H-1), 4.92 (d, *J*=3.3 Hz, H-1), 4.74–4.61 (m), 4.55–4.44 (m), 4.34–4.18 (m), 4.13–3.98 (m), 3.92–3.81 (m), 2.09 (s, Ac), 2.08 (s, Ac), 2.06 (s, Ac), 2.01(s, Ac), 2.00 (s, Ac), 1.96 (s, Ac), 1.90 (s, Ac), 1.26 (d, *J*= 5.9 Hz, Thr-CH_3), 0.91 (d, *J*=6.2 Hz, Thr-CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 99.3 (C-1), 98.3 (C-1). ^{1}H NMR (75 MHz, CD_3OD) δ7.82 (d, *J*=7.4 Hz, 2H, Ar), 7.70 (app t, *J*=7.0 Hz, 2H, Ar), 7.43–7.29 (m, 4H, Ar), 5.20 (dd, *J*= 9.5 Hz, *J*=10.7 Hz, 1H, H-3), 4.97 (t, *J*=9.7 Hz, 1H, H-4), 4.89 (H-1 overlap with HOD), 4.63–4.39 (m, 3H, Fmoc-CH_2, β-H), 4.29–4.05 (m, 6H, α-H, FmocCH, H-6^{a,b}, H-2, H-5), 2.05, 2.04, 1.98, 1.92 (4 s, each 3H, 4Ac), 1.22 (d, *J*= 6.4 Hz, 3H, Thr-CH_3). ^{13}C NMR (75 MHz, CD_3OD) δ 172.0, 171.0, 170.8, 169.9 (*C*OCH_3), 157.8 (*C*O Fmoc), 144.0, 143.8, 141.3, 141.3, 127.4, 126.8, 124.8, 124.7, 119.6, 119.5 (Ar *C*), 98.7 (C-1), 76.4 (β-C), 71.0, 68.9, 67.9 (C-3, C-4, C-5), 66.3 (*C*H2 Fmoc), 62.1 (C-6), 58.3 (α-C), 51.3 (C-2), 46.8 (*C*H Fmoc), 21.4, 19.2 (CO*C*H_3), 17.8 (*C*H_3 Thr). ESI HRMS: calcd for $C_{33}H_{39}N_2O_{13}$ $[M+H]^+$ m/z, 671.2452; found, 671.4193.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-[3,4,6-Tri-O-acetyl-2-deoxy-2-(2,2,2-trichloro- ethoxy-carbonylamino)-β-D-galactopyranosyl]-L-threonine benzyl ester **(8a)** A reaction mixture of Fmoc-Thr-OBn (1.7 g, 3.93 mmol), trichloroacetimidate **7a** (3.68 g, 5.895 mmol) and activated 4A° molecular sieves in dry CH_2Cl_2 was stirred at room temperature for 30 min. under argon. TMSOTf (11 μl, 0.06 mmol) was added at 0°C. The reaction mixture was warmed to room temperature and stirred until completion indicated by TLC. The mixture was diluted with CH_2Cl_2 and quenched with a few drops of triethylamine. The solution was filtered, washed with saturated $NaHCO_3$, H_2O and dried over MgSO4. Purification by flash chromatography (gradient of 0–40% EtOAc-Hexane over 35 min) afforded **8a** (2.62 g, 75%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (d, *J*=7.5 Hz, 2H, Ar), 7.65 (m, 2H, Ar), 7.42–7.29 (m, 9H, Ar), 5.83 (d, *J*=9.0 Hz, 1H, NH), 5.32 (d, *J*=2.8 Hz, 1H, H-4), 5.23–5.09 (m, 4H, H-3, NH, OCH_2Ph), 4.78–4.65 (m, 2H, $TrocCH_2$), 4.56–4.42 (m, 4H, $FmocCH_2$, H-1, β-H), 4.33–4.23 (m, 2H, α-H, FmocCH), 4.14–3.99 (m, 2H, H-6^{a,b}), 3.81–3.69 (m, 2H, H-5, H-2), 2.14, 2.04, 2.02 (3 s, each 3H, 3Ac), 1.24 (d, *J*=6.3 Hz, 3H, Thr-CH_3). ESI HRMS: calcd for $C_{41}H_{44}N_2O_{14}Cl_3$ $[M+H]^+$ m/z, 893.1860; found, 893.3827.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-galactopyranosyl)-L-threonine benzyl ester **(9a)** To the solution of **8a** (2 g, 2.24 mmol) in Ac_2O (5 ml) was added Zn powder (1.25 g). The reaction mixture was stirred at room temperature and monitored by TLC until complete. It was then filtered and concentrated *in vacuo*. The crude residue was purified by flash chromatog-

raphy (gradient of 0–80% EtOAc-Hexane over 60 min) to afford **9a** (1.14 g, 67%). ^{1}H NMR (300 MHz, $CDCl_3$) δ7.77 (d, *J*=7.5 Hz, 2H, Ar), 7.66 (*d*=7.3 Hz, 2H, Ar), 7.43–7.29 (m, 9H, Ar), 5.82 (d, *J*=9.1 Hz, 1H, NH), 5.36 (d, *J*=8.4 Hz, 1H, NH), 5.24–5.18 (m, 4H, H-4, H-3, OCH_2Ph), 4.64 (d, *J*=8.3 Hz, 1H, H-1), 4.48–4.23 (m, 5H, α-H, β-H, $FmocCH_2$, FmocCH), 4.13–3.99 (m, 2H, H-6^{a,b}), 3.89–3.79 (m, 1H, H-2), 3.70 (t, *J*=6.7 Hz, 1H, H-5), 2.13, 2.03, 2.02, 1.95 (4 s, each 3H, 4Ac), 1.22 (d, *J*=6.3 Hz, 3H, Thr-CH_3), ESI HRMS: calcd for $C_{40}H_{44}N_2O_{13}Na$ $[M+Na]^+$ m/z, 783.2741; found, 783.5063.

N$^{\alpha}$-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-galactopyranosyl)-L-threonine **(Ic)** To a solution of **9a** (1 g, 1.32 mmol) in methanol (60 ml) was added 10% Pd/C (250 mg). The mixture was stirred under one atmosphere of hydrogen gas for 1 h. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using DCM as solvent A and 20% MeOH-DCM as solvent B (gradient of 0–80% B over 35 min) to afford **Ic** (680 mg, 77%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.79 (d, *J*=7.3 Hz, 2H, Ar), 7.70 (m, 2H, Ar), 7.41–7.30 (m, 4H, Ar), 5.33 (d, *J*= 3.0 Hz, 1H, H-4), 5.08 (dd, *J*=3.4 Hz, *J*=11.3 Hz, 1H, H-3), 4.60 (d, *J*=8.4 Hz, 1H, H-1), 4.44–4.34 (m, 3H, β-H, $FmocCH_2$), 4.32–3.96 (m, 6H, FmocCH, α-H, H-6^{a,b}, H-2, H-5), 2.10, 2.01, 1.96, 1.95 (4 s, each 3H, 4Ac), 1.20 (d, *J*= 6.3 Hz, 3H, Thr-CH_3).^{13}C NMR (75 MHz, CD_3OD): δ 172.5, 170.8, 170.7, 170.3 (*C*OCH_3), 157.6 (*C*O Fmoc), 143.9, 143.7, 141.2, 127.4, 126.8, 124.9, 119.5 (Ar *C*), 100.2 (C-1), 75.4 (β-C), 70.5, 70.2 (C-3, C-5), 66.8 (C-4), 66.5, 60.9, 58.6 (*C*H_2 Fmoc, C-6, α-C), 50.3 (C-2), 47.0 (*C*H Fmoc), 21.6, 19.2 (CO*C*H_3), 16.6 (*C*H_3 Thr). ESI HRMS: calcd for $C_{33}H_{38}N_2O_{13}Na$ $[M+Na]^+$ m/z, 693.2271; found, 693.4538.

N$^{\alpha}$-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-L-threonine benzyl ester **(9b)** A mixture of Fmoc-Thr-OBn (1.82 g, 4.22 mmol) and **7b** (3.95 g, mmol) was treated as described for the preparation of **8a**. The crude product **8b** (3.5 g) was taken for the next reaction. Compound **9b** was prepared from **8b** as described for preparation of **9a**. Purification of crude residue by flash chromatography (gradient of 0–80% EtOAc-Hexane over 40 min) afforded **9b** (2.25 g, 70%) as an amorphous white solid. ^{1}H NMR (300 MHz, CD_3OD): δ 7.79 (d, *J*=7.5 Hz, 2H, Ar), 7.66 (d, *J*= 7.2 Hz, 2H, Ar), 7.40–7.25(m, 9H, Ar), 5.28–4.93 (m, 4H, H-3, H-4, OCH_2Ph), 4.62 (d, *J*=8.4 Hz, 1H, H-1), 4.45–4.19 (m, 6H, β-H, $FmocCH_2$, H-6^a, α-H, FmocCH), 3.96 (dd, *J*=2.0 Hz, *J*=12.2 Hz, H-6^b), 3.80 (dd, *J*=8.5 Hz, *J*= 10.4 Hz, 1H, H-2), 3.58 (m, 1H, H-5), 1.99, 1.93 (2 s, each 6H, 4Ac), 1.18 (d, *J*=6.3 Hz, 3H, Thr-CH_3), ESI HRMS: calcd for $C_{40}H_{44}N_2O_{13}Na$ $[M+Na]^+$ m/z, 783.2741; found, 783.5068.

N$^{\alpha}$-fluoren-9-ylmethoxycarbonyl-O-(2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-L-threonine **(Id)** To a solution of **9b** (804 mg, 1.06 mmol) in methanol (50 ml) was added 10% Pd/C (200 mg). The mixture was stirred under one atmosphere of hydrogen gas for 1 h. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using DCM as solvent A and 20% MeOH-DCM as solvent B (gradient of 0–35% B over 25 min) to afford the titled compound **Id** (480 mg, 71%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (d, *J*=7.4 Hz, 2H, Ar), 7.64 (m, 2H, Ar), 7.42–7.28 (m, 4H, Ar), 6.06 (d, *J*=8.5 Hz, 1H, NH), 5.90 (d, *J*=9.0 Hz, 1H, NH), 5.29 (app t, *J*=8.0 Hz, 1H, H-3), 5.05 (t, *J*=9.6 Hz, 1H, H-4), 4.78 (d, *J*=8.3 Hz, 1H, H-1), 4.47–4.09 (m, 7H, β-H, $FmocCH_2$, H-6^a, FmocCH, α-H, H-6^b), 3.84–3.75 (m, 1H, H-2), 3.72–3.64 (m, 1H, H-5), 2.07 (s, 3H, Ac), 2.01(s, 6H, 2Ac), 1.93 (s, 3H, Ac), 1.23 (d, *J*=7.0 Hz, 3H, Thr-CH_3).^{13}C NMR (75 MHz, $CDCl_3$): δ 172.6, 171.7, 170.9, 169.5 (*C*OCH_3), 156.9 (*C*O Fmoc), 143.9, 143.7, 141.3, 127.7, 127.1, 125.2, 120.0 (Ar *C*), 99.4 (C-1), 75.6 (β-C), 72.1, 71.6 (C-3, C-5), 68.7 (C-4), 67.3, 61.9, 58.2, 54.8 (*C*H_2 Fmoc, C-6, α-C, C-2), 47.1 (*C*H Fmoc), 23.2, 20.8, 20.7, 20.6 (CO*C*H_3), 17.6 (*C*H_3 Thr). The NMR data is in accord with the literature [48]. ESI HRMS: calcd for $C_{33}H_{39}N_2O_{13}$ $[M+H]^+$ m/z, 671.2452; found, 671.4766.

N$^{\alpha}$-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-L-threonine benzyl ester **(14a)** A mixture of Fmoc-Thr-OBn (1.82 g, 4.22 mmol), **13a** (3.25 g, 6.6 mmol) and activated 4A° molecular sieves in dry CH_2Cl_2 was stirred at room temperature for 30 min under argon. After the addition of TMSOTf (15.38 μl, 0.0844 mmol) at 0°C, the reaction mixture was stirred at room temperature until completion indicated by TLC. The mixture was diluted with CH_2Cl_2 and quenched with triethylamine. The reaction mixture was filtered, washed with saturated $NaHCO_3$, H_2O and dried over $MgSO_4$. Purification by flash chromatography (gradient of 0–50% EtOAc-Hexane over 40 min) afforded **14a** (2.3 g, 72%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ7.76 (d, *J*= 7.4 Hz, 2H, Ar), 7.63 (m, 2H, Ar), 7.41–7.28 (m, 9H, Ar), 5.68 (d, *J*=9.1 Hz, 1H, NH), 5.33 (d, *J*=2.9 Hz, 1H, H-4), 5.27–5.11 (m, 3H, OCH_2Ph, H-2), 4.95 (dd, *J*=3.4 Hz, *J*= 10.5 Hz, 1H, H-3), 4.48–4.33 (m, 5H, α-H, β-H, H-1, $FmocCH_2$), 4.27–4.22 (m, 1H, FmocCH), 4.14–4.01 (m, 2H, H-6^{a,b}), 3.72–3.67 (m, 1H, H-5), 2.13, 2.04, 2.03, 1.99 (4 s, each 3H, 4Ac), 1.22 (d, *J*=6.1 Hz, 3H, Thr-CH_3). ESI HRMS: calcd for $C_{40}H_{43}NO_{14}Na$ $[M+Na]^+$ m/z, 784.2580; found, 784.5383.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-L-threonine **(Ie)** To a solution of **14a** (1 g, 1.31 mmol) in methanol (50 ml) was added 10% Pd/C (200 mg). The mixture was stirred under one atmosphere of hydrogen gas for 1 h. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using DCM as solvent A and 10% MeOH-DCM as solvent B (gradient of 0–40% B over 35 min) afforded **Ie** (670 mg, 76%) as a white crystalline solid. ^{1}H NMR (300 MHz, CD_3OD) δ7.78 (d, *J*=7.3 Hz, 2H, Ar), 7.68 (m, 2H, Ar), 7.41–7.29 (m, 4H, Ar), 5.38 (d, *J*=2.4 Hz, 1H, H-4), 5.16–5.04 (m, 2H, H-2, H-3), 4.64 (d, *J*=7.3 Hz, 1H, H-1), 4.42–3.99 (m, 8H, α-H, β-H, $FmocCH_2$, FmocCH, H-6^{a,b}, H-5), 2.09, 2.03, 2.00, 1.96 (4 s, each 3H, 4Ac), 1.20 (d, *J*=6.2 Hz, 3H, Thr-CH_3). ^{13}C NMR (75 MHz, CD_3OD) δ 171.9, 170.7, 170.6, 170.2 (*C*OCH_3), 170.1, 157.4 (*C*O Fmoc), 143.9, 143.7, 141.2, 127.4, 126.8, 125.0, 119.6 (Ar *C*), 99.4 (C-1), 75.43 (β-C), 70.83, 70.3, 69.1, 67.2 (C-3, C-5, C-2, C-4), 66.8, 60.7, 58.4 (*C*H_2 Fmoc, C-6, α-C), 47.0 (*C*H Fmoc) 19.5, 19.2, 19.1 (*C*OCH_3), 16.6 (*C*H_3 Thr). ESI HRMS: calcd for $C_{33}H_{38}NO_{14}$ $[M+H]^+$ m/z, 672.2292; found, 672.4792.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-L-threonine benzyl ester **(14b)** A mixture of Fmoc-Thr-OBn (3.64 g, 8.44 mmol) and **13b** (5.41 g, 10.97 mmol) was treated as described for synthesizing **14a**. Purification of the crude mixture by flash chromatography (gradient of 0–40% EtOAc-Hexane over 30 min) afforded **14b** (5 g, 79%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ7.76 (d, *J*=7.4 Hz, 2H, Ar), 7.57 (d, *J*=7.1 Hz, 2H, Ar), 7.42–7.28 (m, 9H, Ar), 5.63 (d, *J*=9.2 Hz, 1H, NH), 5.29–5.11 (m, 3H, OCH_2Ph, H-2), 5.06–4.99 (t, *J*=9.6 Hz, 1H, H-3), 4.94–4.88 (m, 1H, H-4), 4.46–4.33 (m, 4H, α-H, β-H, $FmocCH_2$), 4.44 (d, *J*=8.2 Hz, 1H, H-1), 4.26–4.01 (m, 3H, FmocCH, H-6^{a,b}) 3.49–3.43 (m, 1H, H-5), 2.04, 2.02, 2.01, 2.00 (4 s, each 3H, 4Ac), 1.22 (d, *J*=6.3 Hz, 3H, Thr-CH_3). ESI HRMS: calcd for $C_{40}H_{43}NO_{14}Na$ $[M+Na]^+$ m/z, 784.2580; found, 784.4357.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-L-threonine **(If)** To a solution of **14b** (2.18 g, 2.86 mmol) in EtOAc (70 ml) was added 10% Pd/C (1 g). The mixture was stirred under one atmosphere of hydrogen gas until the reaction was complete. The catalyst was removed by filtration and after solvent evaporation, the residue was purified by flash chromatography using DCM as solvent A and 20% MeOH-DCM as solvent B (gradient of 0–60% B over 30 min) to afford compound **If** (1.7 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.77 (d, *J*=7.4 Hz, 2H, Ar), 7.65 (m, 2H, Ar), 7.43–7.30 (m, 4H, Ar), 5.74 (d, *J*=9.4 Hz, 1H, NH), 5.24–4.94 (m, 3H, H-2, H-3, H-4), 4.55 (d, *J*=7.9 Hz, 1H, H-1), 4.45–4.24 (m, 6H, α-H, β-H, $FmocCH_2$, FmocCH, H-6^{a}), 4.15 (dd, *J*=4.1 Hz, *J*=12.2 Hz, 1H, H-6^{b}), 3.69–3.63 (m, 1H, H-5), 2.10, 2.05, 2.03, 2.02 (4 s, each 3H, 4Ac), 1.24 (d, *J*=6.3 Hz, 3H, Thr-CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.2, 171.8, 170.4, 169.5 (*C*OCH_3), 169.3, 156.9 (*C*O Fmoc), 143.9, 143.7, 141.3, 127.7, 127.1, 125.2, 119.9 (Ar *C*), 99.6 (C-1), 76.0 (β-C), 72.6, 71.7, 71.2, 68.4 (C-3, C-5, C-2, C-4), 67.4, 61.7, 58.1 (*C*H_2 Fmoc, C-6, α-C), 47.1 (*C*H Fmoc), 20.8, 20.6 (*C*OCH_3), 17.6 (*C*H_3 Thr). ESI HRMS: calcd for $C_{33}H_{37}NO_{14}$ $[M+H]^+$ m/z, 672.2292; found, 672.4753.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl)-L-threonine benzyl ester **(17)** To a solution of completely dried Fmoc-Thr-OBn (2 g, 4.64 mmol) in dry CH_2Cl_2 (20 mL) were added AgOTf (1.67 g, 6.489 mmol) and flame dried 4 A° molecular sieves. A solution of bromide **16** (3.247 g, 7.90 mmol) in CH_2Cl_2 (5 mL) was slowly added at 0°C under the constant supply of argon gas. The reaction was stirred overnight at room temp. and quenched with DIPEA (807 μL, 6.952 mmol). The reaction mixture was filtered through celite. The filtrate was washed with conc. $Na_2S_2O_3$, conc. Na_2CO_3 and brine. All the aqueous washes were back extracted with CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated. The purification of the residue by flash chromatography (gradient of 0–60% EtOAc-Hexane over 40 min) afforded **17** (2.82 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (d, *J*=7.4 Hz, 2H, Ar), 7.65 (d, *J*=7.3 Hz, 2H, Ar), 7.44–7.31 (m, 9H, Ar), 5.58 (d, *J*=9.6 Hz, 1H, NH), 5.37–5.19 (m, 4H, H-3, H-4, OCH_2Ph), 5.07 (dd, *J*=1.7 Hz, *J*=3.8 Hz, 1H, H-2), 4.86 (br s, 1H, H-1), 4.54–4.34 (m, 4H, α-H, β-H, $FmocCH_2$), 4.31–4.02 (m, 4H, FmocCH, H-6^{a,b}, H-5), 2.16, 2.08, 2.07, 2.02 (4 s, each 3H, 4Ac), 1.32 (d, *J*=6.4 Hz, 3H, Thr-CH_3). ESI HRMS calculated for $C_{40}H_{43}NO_{14}Na$ $[M+Na]^+$ m/z, 784.258; found, 784.442.

N^{α}-fluoren-9-ylmethoxycarbonyl-O-(2,3,4,6-tetra-O-α-D-mannopyranosyl)-L-threonine **(Ig)** Compound **17** (1 g, 1.31 mmol) and 5%Pd/C (200 mg) were stirred in methanol (50 mL) under H_2 (1 atm) for 45 min. The catalyst was filtered through celite and the filtrate was concentrated. The residue was purified by flash chromatography using DCM as solvent A and 10% MeOH-DCM as solvent B (gradient of 0–60% B over 40 min) to yield pure **Ig** (625 mg, 71%). ^{1}H NMR (300 MHz, MeOD) δ7.80 (d, *J*=7.4 Hz, 2H, Ar), 7.73–7.70 (m, 2H, Ar), 7.42–7.29 (m, 4H, Ar), 5.30 (dd, *J*=3.3 Hz, *J*=10.0 Hz, 1H, H-3), 5.24–5.17 (m, 2H, H-2, H-4), 4.97 (br s, 1H, H-1), 4.48–4.34 (m, 2H, α-H, β-H), 4.32–4.08 (m, 6H, H-6^{a}, H-6^{b}, $FmocCH_2$, FmocCH, H-5), 2.11, 2.05, 2.04, 1.96 (4 s, each 3H, 4Ac), 1.30 (d, *J*=6.4, 3H, Thr-CH_3). ^{13}C NMR (75 MHz, CD_3OD) δ 170.9, 170.3, 170.2, 170.1 (*C*OCH_3), 143.9, 143.9, 141.2, 127.4, 126.8,

124.9, 124.9, 119.5 (Ar *C*), 98.9 (C-1), 77.1 (β-C), 69.4, 69.3, 68.9 (C-3, C-4, C-2), 66.8, 66.1, 62.4, 59.1 (C-5, α-C, CH_2 Fmoc, C-6), 47.0 (*C*H Fmoc), 19.2 ($CO\mathit{C}H_3$), 17.5 ($\mathit{C}H_3$ Thr). ESI HRMS: calcd for $C_{33}H_{37}NO_{14}Na$ $[M+Na]^+$ m/z, 694.2111; found, 694.4171.

Peptide synthesis All peptides used were synthesized by the solid-phase method using an automated peptide synthesizer (433A; Applied Biosystems Inc.), employing Fmoc-methodology. The peptides were cleaved from the resin by treatment with TFA/thioanisole/phenol/water/EDT in ratio as recommended by Applied Biosystems Inc. The crude peptides were purified using C-18 column (Deltapak -100 Å, 15μ, spherical, 19x300mm, Waters). The sugar residue of purified glycosylated peptide was deacetylated by treatment with 5% hydrazine-hydrate and finally again purified by reverse phase column chromatography using C-18 column. The synthesized peptides were determined to be >95% pure by HPLC. Molecular mass determinations were made by using MALDI TOF/TOF 4800 (Applied Biosystems MDS SCIEX) with α- cyano-4-hydroxycinnamic acid as the matrix and ESI-MS system (Applied Biosystems Mariner System 5220) both operating in positive mode.

Antibacterial activity assay by radial diffusion The antibacterial activity of all the peptides was ascertained by a radial diffusion assay using double-layered agarose as described previously [49]. The bacterial strains used for this experiment were: *Escherichia coli* ATCC25922, *Escherichia coli* BL21λD3, *Salmonella typhi* Vi^+, and *Salmonella typhimurium*. The 5 mM peptide solution corresponding to amount of 25 nmoles of each peptide was used to determine the comparative antibacterial activity. The assay was done in triplicate and repeated thrice to calculate the average and the standard error.

Circular-Dichroism spectroscopy The circular-dichroism (CD) spectra of all peptides were obtained at 25°C in a 1 mm pathlength cuvette in the range of 250–190 nm using CD spectrometer (J-815 JASCO Corporation, Japan) with a 1.0 nm bandwith at 0.1 nm resolution and 1.0 s of response time. 5 scans with a speed of 200 nm/min were accumulated and averaged. The spectra were recorded in distilled water, 10 mM SDS in 10 mM PB, pH 7.2, 90% TFE in water. Results were expressed as mean residue ellipticity in [θ] (Deg-cm^2/dmol).

Hemolytic activity assay The hemolytic response was measured spectrophotometrically by analyzing % lysis of freshly isolated murine red blood cells [50]. A 200 μl aliquot of packed erythrocyte volume was washed thrice with cold phosphate-saline buffer (10 mM PB, pH 7.4, 150 mM NaCl) by centrifugation for 5 min at 1,150 g, 4°C. The pellet was resuspended in 10 ml PBS, diluting the cells to 2% of their packed volume. For each test condition, 90 μl of the erythrocytes were plated in a 96-well microtiter plate (Greiner Bio-one, Wemmel, Belgium) along with 10 μl of peptide solution serially diluted in the same buffer with a starting concentration of 100 μM added to the wells and the plate was incubated at 37°C for 90 min. Any hemolytic activity would manifest as rupture of RBCs and release of hemoglobin into the solution. The resuspension buffer, PBS, was used as negative control while RBCs resuspended in 1% TritonX-100, which results in their complete lysis, was used as positive control. Subsequently, the plate was centrifuged at 4,000 rpm for 10 min to pellet intact cells and 75 μl of supernatant was transferred to a flat bottom polystyrene microtiter plate (Greiner Bio-one, Wemmel, Belgium). Its absorbance was read at 405 nm and the percentage of RBC lysis was calculated. The hemolysis affected by only PBS and 1% TritonX-100 was considered as negative control and 100% hemolysis, respectively. Each test condition was set up in duplicate and the results have been averaged from three independent experiments.

MTT assay The cytotoxic potential of synthesized peptides was determined following incubation of rat myeloma SP2/O (1×10^5 cell/ml) cell line using the MTT assay. This 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. Following 24 h incubation, cells were washed and then incubated with fresh culture media and 50 μl MTT (5 mg/ml) in 0.1 M PBS, pH 7.4 at 37°C in a humid atmosphere with 5% CO_2 for 4 h. Media were then gently aspirated from test cultures and 50 μl of dimethyl sulfoxide (DMSO) was added to all wells. The plates were then shaken for 2 min and the absorbance was read at 540 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 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.

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