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Enzymatic glycosylation of the topical antibiotic mupirocin

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Abstract Mupirocin is a commercially available antibiotic that acts on bacterial isoleucyl-tRNA synthetase, thereby inhibiting protein synthesis and preventing bacterial infection. An *in vitro* glycosylation approach was applied to synthesize glycoside derivatives of mupirocin using different NDP-sugars and glycosyltransferase from *Bacillus licheniformis*. Ultra pressure liquid chromatography-photo diode array analyses of the reaction mixtures revealed the generation of product peak(s). The results were further supported by high-resolution quadruple time of flight electrospray ionization mass spectrometry analyses. The product purified from the reaction mixture with UDP-D-glucose was subjected to NMR analysis, and the structure was determined to be mupirocin 6-*O*-*β*-D-glucoside. Other glycoside analogs of mupirocin were determined based on high-resolution mass analyses. Antibacterial activity assays against *Staphylococcus aureus* demonstrated complete loss of antibacterial activity after glucosylation of mupirocin at the 6-hydroxyl position.

Keywords Mupirocin glycosides · *Bacillus* glycosyltransferase · Aminoacyl tRNA synthetases · Bacterial resistance

Introduction

Mupirocin is a broad-spectrum polyketide antibiotic, effective against primary and secondary skin infections and against methicillin-resistant *Staphylococcus aureus* ("MRSA"), resistant to mainstream antibiotics (penicillin, streptomycin, and methicillin) [14, 15, 20]. Since it was isolated as a major metabolite from *Pseudomonas fluorescens* NCIMB 10586 [34], different analogs (pseudomonic acids A, B, C, D) were discovered, with pseudomonic acid A being recognized as the major component comprising C_{17} monic acid (C_{12}+C_5 units), esterified with the C_9 saturated fatty acid, 9-hydroxynonanoic acid [10, 12] (Fig. 1). It shares no structural homology with other antibiotics currently in clinical use, and has been used primarily as an ointment with different brand names (*e.g.*, Bactoderm, Bactroban, mupirocin calcium cream) (http://www.drugbank.ca/drugs/DB00410) for specific pathogens that frequently cause secondary infections in superficial wounds [14].

MRSA strains are commonly found "superbugs," resistant to many antibiotics. *S. aureus* and *Streptococcus pyogenes* are pathogens commonly causing various skin infections [33] of vulnerable sites, such as post-operative wounds burns, and even minor cuts. However, they can ultimately lead to serious problems, such as bloodstream infections, pneumonia, osteomyelitis, sepsis, and endocarditis (http://mrsa-research-center.bsd.uchicago.edu/patients_families/faq.html). Dealing with these bacteria has become an increasing challenge due to the emergence of resistant variants [29]. Mupirocin provided a solution to this, with activity against aminoacyl tRNA-synthetase.

Mupirocin acts as an inhibitor of isoleucyl-tRNA synthetase (IleRS) and ultimately blocks the synthesis of essential bacterial proteins, reducing cell sustainability under both *in vitro* and infectious conditions [20, 29, 30]. This enzyme has established functions and serves as a global regulator of transcription, translation, and various cell signaling pathways whose inhibition is the major target in the rational design of antibacterial agents and new-generation drugs [5, 20]. Mupirocin has been in clinical use since 1985, demonstrating predominant action against Gram-positive pathogens. It is currently the world's most widely used antibiotic for control

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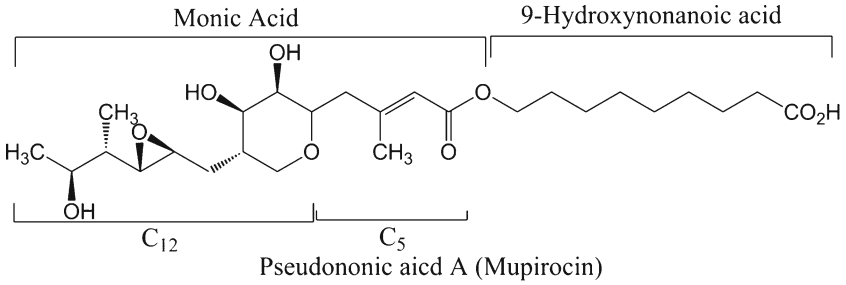


Fig. 1 The typical structure of mupirocin (Pseudomonic acid A) showing monic acid esterified with 9-hydroxynonanoic fatty acid chain

of MRSA [9, 10]. It is restricted to topical use against pathogenic infections and colonization of *S. aureus*, but the increasing rate of resistance among these species has become problematic [16]. Mupirocin is quickly hydrolyzed in body fluids to form monic acid, which is inactive against pathogens [13, 33]. In topical applications, it is used as a 2 % mupirocin ointment in a water-miscible polyethylene glycol base for skin wounds, or as a 2 % cream in a soft paraffin base for nasal application and application at surgical sites [13, 14].

Post modification of such antibiotics may provide a solution to overcome the current restrictions. Rather than discovering new drugs, relatively simple modifications of existing ones to synthesize analogs by chemical or enzymatic approaches, allowing enhancement of pharmacological potency, could bring new drugs into clinical use more quickly, bypassing some of the time-consuming and expensive steps along the way [19, 34]. Carbohydrate appendages in secondary metabolites that change their behavior in terms of cell biological recognition are well known [32]. Glycosylation is one approach used in the modification of drugs, and has been shown to broaden the biological activity of modified compounds, changing their physical and chemical properties [31]. Some natural products that are glyco-conjugates, commonly used as antimicrobial therapeutics (erythromycin A, clarithromycin, streptomycin, azithromycin) for various bacterial infections, are now being considered as anti-cancer drug candidates [30]. Vancomycin, a glycosylated natural product from *Amycolatosis orientalis*, is considered to be the 'last defense' against infections caused by methicillin-resistant Gram-positive bacteria after mupirocin. It was further glycodiversified to generate 39 additional derivatives, showing improved antibiotic activity against *S. aureus* and *Enterococcus faecium* [7, 8]. Similarly, for tylosin and erythromycin (antibiotics), altering the sugar moiety was shown to affect their molecular mechanism of action [18]. Additionally, glycoside attachment to amphotericin B (an antifungal agent) enhanced its solubility and pharmacokinetic properties [6].

The current study focused on synthesizing diverse glycoconjugates of the topical antibiotic mupirocin [2] using *Bacillus* glycosyltransferase and different NDP-sugars as sugar donors.

Materials and methods

General procedures

UDP-D-glucose and UDP-D-galactose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). TDP-D-2-deoxyglucose was synthesized in our laboratory, and TDP-L-rhamnose, was obtained from GeneChem (Daejeon, South Korea). HPLC-grade methanol and water were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Mupirocin was purchased from Lanospharma Laboratories Co. Ltd. (Hong Kong). All other chemicals used in this study were of high-grade, and were purchased from commercial sources.

Culture conditions and heterologous protein production

A previously constructed transformant of *E. coli* BL21 (DE3) harboring pET28 (a)-YjiC (GenBank accession number AAU40842) [24] was cultured in Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin, followed by incubation in a shaking incubator at 37 °C and 150 rpm. The culture was induced with 0.8 mM (final concentration) of isopropyl-*β*-D-thiogalactopyranoside (IPTG), and incubated for 20 h at 20 °C in a shaking incubator at 150 rpm. The cell pellets were harvested *via* centrifugation at 3,000 rpm for 15 min, washed twice with buffer (50 mM Tris–HCl, 10 % glycerol, pH 7.4), and re-suspended in 1 mL of the same buffer. Sonication was performed, followed by high-speed centrifugation (12,000 rpm, 30 min, 4 °C) to obtain a clear lysate. The lysate was finally loaded on to a His-TALON metal nickel affinity resin (Takara, Japan) for 30 min, maintained at 4 °C, for purification of the recombinant protein. Using gravity flow, the resin-bound protein was eluted stepwise with increasing concentrations of imidazole (10, 100, and 200 mM in buffer containing 200 mM NaCl and 20 mM Tris–HCl, pH 7.4). The fractions were analyzed by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and further concentrated using an Amicon Ultra-15 device (Millipore, 30 K NMWL). The purified protein (~45 kDa) was stored in buffer (50 mM Tris–HCl, pH 7.4, 10 % glycerol) at -20 °C until use.

In vitro enzymatic reaction

The glycosylation reaction was carried out in the presence of the purified enzyme (YjiC) using the substrate (mupirocin) and different sugar donors (UDP-D-glucose, UDP-D-galactose, TDP-D-2-deoxyglucose and TDP-L-rhamnose). A 100 μL volume reaction was used, maintaining final concentrations of 100 mM Tris–HCl buffer at pH 8.0, 10 mM $MgCl_2.6H_2O$, 2 mM substrate (dissolved in methanol) and 4 mM of respective NDP-sugars, individually, with 30 μg/mL of appropriately diluted enzyme. The rest of the volume was made up with Milli-Q water. The mixture was incubated at 37 °C for 3 h, and then quenched by the addition of 400 μL chilled methanol (HPLC-grade). The reaction mixture without enzyme was used as a control. The quenched reactions were centrifuged (12,000 rpm, 20 min) to remove protein precipitates. Finally, the reaction mixtures were subjected to high performance liquid chromatography-photo diode array (HPLC-PDA) and ultra-pressure liquid chromatography (UPLC), followed by high- resolution liquid chromatography-electrospray ionization-quadruple-time of flight-electrospray ionization mass spectrometry (UPLC-QTOF-HR ESI/MS).

The preparative-scale reaction was carried out in a 10-mL volume with purified YjiC (30 μg/mL), 10 mM UDP-D-glucose (~56 mg), 8 mM substrate (~40 mg, dissolved in MeOH), 100 mM Tris HCl (pH 8.0) buffer, and 10 mM $MgCl_2.6H_2O$, incubated for 18 h at 37 °C. The reaction was stopped by adding a triple volume of chilled methanol. The reaction mixture was then mixed by vortexing, and was centrifuged (12,000 rpm, 30 min, 4 °C) to remove denatured proteins. Finally, the supernatant was concentrated by evaporation and lyophilized to dryness before use in experiments.

Analytical procedures

The reaction mixture was analyzed by reverse-phase UPLC-PDA with a C18 column (ACQUITY UPLC BEH, C18, 1.7 μm) connected to a PDA (UPLC LG 500 nm) at a UV absorbance of 222 nm. The binary mobile phases were composed of solvent A (HPLC-grade water) and solvent B (100 % acetonitrile, ACN). The total flow rate was maintained at 0.4 μL/min during the 10-min program. The flow of B (ACN) was 0 % up until 5 min, after which it increased to 100 % at 8 min, followed by constant flow of 0 % at 8-10 min. Flow was stopped at 10 min. During the pH- and time-dependent reaction, HPLC-PDA (PDA-HPLC Shimadzu, Japan; SPD-M20A Detector) with a reverse-phase C_{18} column (Mightysil RP-18 GP, 150×4.6 mm, Kanto Chemical, Japan) was used to calculate the highest yield in the binary mobile phase. The total flow rate was maintained at 1 mL/min for the 30-min program. The flow of B was maintained at 5 % until 5 min, when it increased to 50 % until 10 min, was maintained at constant flow until 15 min, and then increased to 90 % at 25 min. Finally, the flow of B was maintained at 10 % at 28 min, after which the program stopped at 30 min. The reaction mixtures were further analyzed by UPLC-QTOF-HR ESI/MS in positive ion mode on an ACQUITY (UPLC, Waters Corp., USA) coupled with a SYNAPT G2-S (Water Corp., USA). The dried reaction mixture was dissolved in 1 mL methanol and subjected to preparative HPLC (Shimadzu, Japan) with a C_{18} column (YMC-Pack ODS-AQ (150×20 mm I.D., 10 μm), connected to a UV detector for purification, and was analyzed at a UV absorbance of 222 nm. A 40-min binary program was used with a flow rate of 10 mL/min containing solvent B (100 % ACN) and A (HPLC-grade water). The flow of B was initially maintained at 0 % and increased to 5 % until 10 min, remained at 50 % from 15 to 25 min, raised to 90 % at 30 min, lowered to 50 % at 35 min, and finally stopped at 40 min.

The purified product was lyophilized, dried, and dissolved in methanol-d_4, then subjected to 900 MHz nuclear magnetic resonance (NMR) (Bruker, BioSpin) analyses, including 1-dimensional ^{1}H-NMR (proton NMR), ^{13}C-NMR (carbon NMR), and 2-dimensional NMR- correlation spectroscopy NMR (COSY), rotating-frame NOE Spectroscopy (ROESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond connectivity (HMBC) analyses, as appropriate.

Antibacterial activity test

In vitro paper disc diffusion tests were performed to assay antibacterial activity against *Staphylococcus aureus* subsp. aureus KCTC 1916, according to the previously reported guidelines [11]. Paper discs 6 mm in diameter and Muller-Hilton agar (MHA) plates were prepared aseptically. *S. aureus* subsp. *aureus* KCTC 1916 was cultured for 3 h in LB-broth medium, and then spread on the MHA plates. The discs were soaked with various concentrations (1 μg/mL, 2 μg/mL, 4 μg/mL, 16 μg/mL) of serially diluted mupirocin standard (in methanol) and placed onto the inoculated MHA agar plates at ~2 cm distances from each other. The minimum concentration of mupirocin for which a clear zone of inhibition was observed was used as a reference for comparison with mupirocin 6-*O*-*β*-D-glucoside at the same concentrations for the antibacterial assay.

Results

Production and purification of enzyme

The previously constructed pET28 (a) -YjiC was used for the production and purification of the enzyme [24]. The

recombinant *E. coli* BL21 (DE3) strain was prepared using heat shock transformation with pET28 (a)-YjiC. A 200 μL inoculum from a single transformant was then cultured in 100 mL LB medium for protein expression and purification. After incubation at 37 °C until the OD_{600} reached 0.5-0.7, the culture was induced by 0.8 mM IPTG and incubated for 20 h at 20 °C. After purification of the protein, SDS-PAGE confirmed that the purified protein was ~45 kDa in size. After concentration using an Amicon Ultra-15 (Millipore, USA; 10 K NMWL device), SDS-PAGE analysis revealed the protein to be ~90 % pure. The amount of concentrated protein was calculated using the Bradford method, and it was stored at −20 °C until use [12].

Enzyme-catalyzed glyco-conjugation

An enzymatic reaction was carried out to synthesize mupirocin glyco-conjugates in the presence of purified protein and UDP-D-glucose as a sugar donor under the conditions described above. The UPLC-PDA chromatogram analysis of the reaction mixture revealed novel peaks at retention times (t_R) 4.01 min (P_1), 3.81 min (P_2) and 3.71 min (P_3) in comparison with the standard peak at t_R=4.45 min (S) (Fig. 2). The novel peaks were further analyzed by UPLC-QTOF-HR ESI/MS in positive ion mode. The mass analysis showed peaks at $[MUP_{Glu}+H]^+$ m/z^+=663.3596, $[MUP_{Diglu}+H]^+$ m/z^+=825.4130, and $[MUP_{Diglu}+Na]^+$ m/z^+=847.3953, indicating the presence of three products in the reaction mixture: two single sugar-conjugated products and one double sugar-conjugated product (Fig. 3). Considering the high yield (~60 %), a scaled-up reaction was carried out on a preparative scale, as explained in the materials and methods. The yield of the single-sugar-conjugated mupirocin was quantified after prep-HPLC purification, lyophilization, and drying (~5.2 mM, *i.e.* ~26 mg). The glucoside was subjected to NMR analyses for structural elucidation.

Structural characterization

The ^{1}H-NMR study of the purified product showed the presence of two anomeric protons with chemical shifts of δ= 4.59 ppm (d, J=7.8 Hz, 1H, H-1″) and δ=4.68 ppm (d, J= 7.8 Hz, 1H, H-1″), representing the beta (β) configuration of the sugar moieties, while other signals of glucose moieties were observed in the sugar region, δ=3.0–4.0 ppm (Fig. S1, S2). Although the preparative HPLC showed a single purified peak, NMR analysis revealed the presence of two anomeric proton spectra, showing a mixture of two monoglucosides of mupirocin in a ratio of 2:1. Separation of the two monoglucosides was tedious, due to their identical structures and polarities. Thus, NMR analyses of the compound were performed by taking the dominant anomeric spectrum (the major product; δ=4.59 ppm), which matched the protons of the standard compound with negligible shifts (Table 1). Because of the presence of the aliphatic chain in mupirocin and the mixture of two glucosides, analysis of the minor product was difficult. Thus, the minor product was predicted based on previous studies, and the major product was identified here. The results were further supported by ^{13}C NMR analyses, where the anomeric carbon (C-1″) was present at δ=103.511 and other sugar signals were present between 60 and 80 ppm, as designated in Fig. S3. ^{1}H-^{1}H COSY showed an anomeric proton at δ=4.59 ppm in close relation with δ=3.21 ppm, which was annotated as H-6 of the aglycone (Fig. S3) from previously reported NMR data [21], while ROESY-NMR analysis showed correlation of the anomeric proton (H-1″)

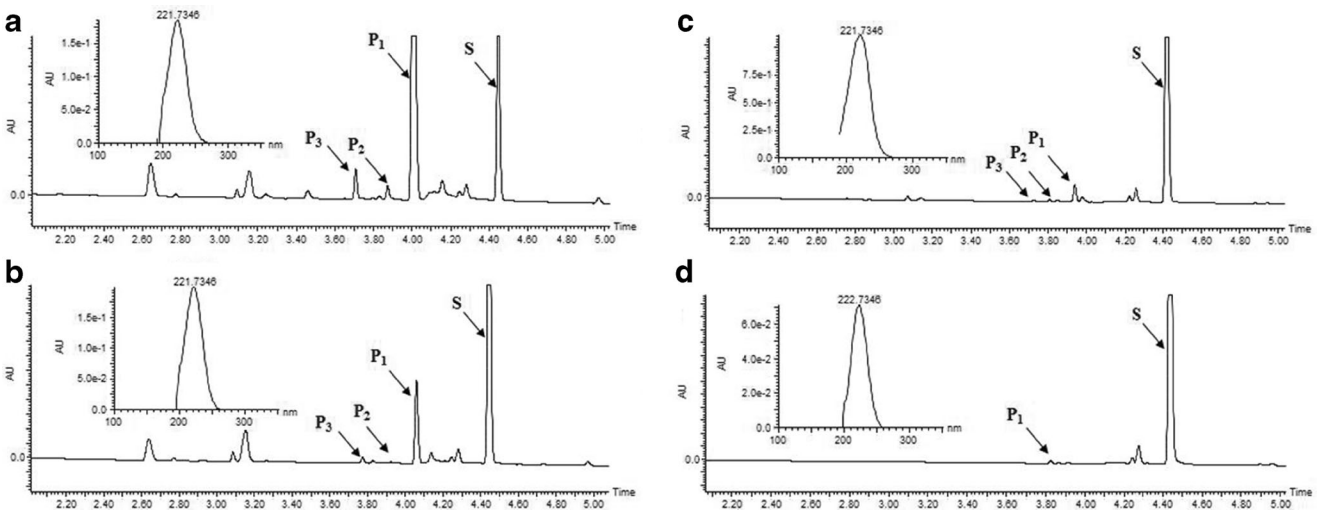


Fig. 2 The UPLC-PDA chromatogram analysis, showing product formation in the reaction mixtures of mupirocin coupled with different NDP-sugars, and their respective λ_{max}. The chromatograms represent mupirocin conjugated with: **a**. UDP-D-glucose, **b**. UDP-D-galactose, **c**. TDP-2-deoxy D-glucose and **d**. TDP-L-rhamnose. P_1 and P_2 are the mono glucosides, where P_1 is the major product, and P_3 is the di-glucoside in each case; S is the substrate peak

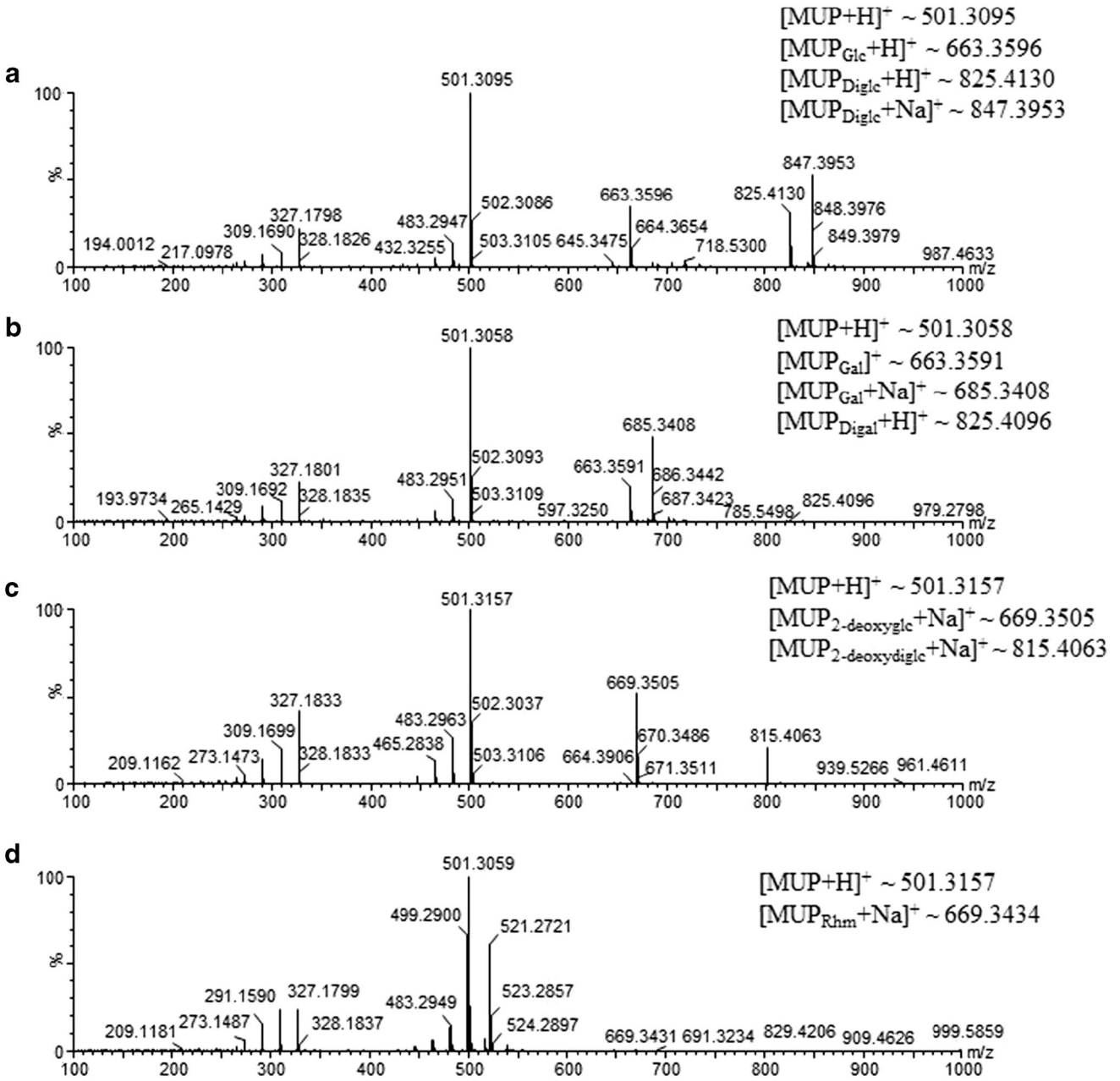


Fig. 3 UPLC-QTOF-HR ESI/MS analysis of different reaction mixtures. Suspected glycoside peaks detected in UPLC-PDA were confirmed with their respective mass

with H-6 (δ=3.24 ppm (*i.e.* δ=3.22 and δ=3.29 ppm in ^{1}H-NMR) as well as H-6 with H-7 (δ=3.25 ppm and δ=3.28 ppm), H-16 ax (δ=3.85 ppm), H-5″, and H-3″ (δ=3.67 ppm).

These data predicted that the sugar should be attached at the C-6 hydroxyl position of mupirocin. Further evidence was collected from HSQC (Fig. S3) analysis, revealing a direct connection between the anomeric proton H-1″ (δ=4.59 ppm) and the anomeric carbon C-1″ (δ =103.49 ppm), as well as correlation of H-6 with the anomeric carbon C-1″ (δ=3.21 ppm), C-5 (δ=74.07) and C-2″ (δ=76.58), whereas HMBC showed relationships between the anomeric proton H-1″ (δ=4.59 ppm), C-5 (δ=75.43 ppm), and C-6 (δ=68.11 ppm); and similarly, H-6 with C-6″ (δ=61.27 ppm). These analytical results confirmed the product to be mupirocin 6-*O*-β-D-glucoside, a novel glucoside derivative of mupirocin that has not yet been reported. The other glucoside is likely mupirocin 7-*O*-β-D-glucoside, as the aglycone contained two

Table 1 Comparison of ^{1}H-NMR of mupirocin with mupirocin 6-*O*-*β*-D-glucoside

No	^{1}H NMR of mupirocin aglycone	^{1}H NMR of mupirocin glucoside
2	5.73 (d, 1.7 Hz, 1H)	5.80 (dd, 2.3, 1.2 Hz, 1H), 5.77 (dd, 9.0, 1.2 Hz, 1H)
2′	2.26 (d, 7.3 Hz, 2H)	Not available
3′	1.58 (d, 6.9 Hz, 2H)	1.60 (t, 7.3 Hz, 9H)
4 eq.	2.61 (d, 2.6 Hz, 1H)	2.53 (tt, 11.7, 6.1 Hz, 1H)
4ax	2.21 (d, 4.8Hz, 1H)	2.34 (d, 4.6 Hz, 1H), 2.33 (m, 1H)
4′	1.35 (m, 2H)	1.40 – 1.35 (m, 2H), 1.36 – 1.33 (m, 2H)
5	3.72 (m, 1H)	3.76 (d, 3.1 Hz, 1H)
5′	1.37 – 1.32 (m, 2H)	1.40 – 1.35 (m, 2H), 1.36 – 1.33 (m, 2H)
6	3.35 (dd, 8.9, 3.1 Hz, 1H)	3.22 (d, 9.1 Hz, 1H), 3.29 (d, 7.8 Hz, 1H)
6′	1.37 – 1.32 (m, 2H)	1.40 – 1.35 (m, 2H), 1.36 – 1.33 (m, 2H)
7	3.86 (t, 3.7 Hz, 1H)	3.86 (dd, 4.2, 2.2 Hz, 1H), 3.85 (dd, 4.4, 2.4 Hz, 1H)
7′	1.37 – 1.32 (m, 2H)	1.40 – 1.35 (m, 2H), 1.36 – 1.33 (m, 2H)
8	1.99 – 1.89 (m, 1H)	1.97 – 1.93 (m, 1H), 1.91 (dd, 11.2, 5.7 Hz, 1H)
8′	1.63 (d, 6.2 Hz, 2H)	1.76 – 1.65 (m, 2H)
9	Not available	4.08 (t, 6.8 Hz, 2H), 1.71 (m, 2H)
9′	4.06 (t, 6.6 Hz, 2H)	Not available
9a	1.74 – 1.66 (m, 2H)	Not available
9b	1.74 – 1.66 (m, 2H)	Not available
10	2.81 (td, 5.7, 2.1 Hz, 1H)	2.77 (dd, 14.2, 10.4 Hz, 0H), 2.73 (dd, 14.5, 10.6 Hz, 1H)
11	2.71 (dd, 7.6, 2.3 Hz, 1H)	2.60 (dd, 1H)
12	1.46 – 1.38 (m, 1H)	1.40 – 1.35 (m, 1 H), 1.36 – 1.33 (m, 1H)
13	3.78 (dd, 6.7, 5.0 Hz, 1H)	3.77 (d, 3.6 Hz, 1H), 3.75 (d, 2.6 Hz, 1H)
14	1.20 (d, 6.4 Hz, 3H)	1.22 (d, 6.4 Hz, 3H), 1.20 (d, 6.4 Hz, 3H)
15	2.17 (d, 1.3 Hz, 3H)	2.20 – 2.19 (m, 3H)
16 eq.	3.53 (d, 2.0 Hz, 1-H)	3.47 (d, 2.7 Hz, 1H), 3.46 (d, 2.7 Hz, 1H)
16ax	3.83 (d, 2.9 Hz, 1H)	3.86 (dd, 4.2, 2.2 Hz, 1H), 3.85 (dd, 4.4, 2.4 Hz, 1H
17	0.94 (d, 7.1 Hz, 3H)	0.93 (d, 7.0 Hz, 2H), 0.90 (d, 7.0 Hz, 3H), 0.85 (d, 6.9 Hz, 4H
1″	Sugar region	4.68 (d, 7.8 Hz, 1H), 4.59 (d, 7.8 Hz, 1H)
2″	Sugar region	3.71(m, 2H)
3″	Sugar region	3.67 (ddd, 12.0, 5.7, 3.3 Hz, 4H)
4″	Sugar region	3.64 – 3.59 (m, 2H)
5″	Sugar region	3.67 (ddd, 12.0, 5.7, 3.3 Hz, 4H)
6a″	Sugar region	4.04 – 4.00 (m, 1H)
6b″	Sugar region	3.99 – 3.95 (m, 1H)

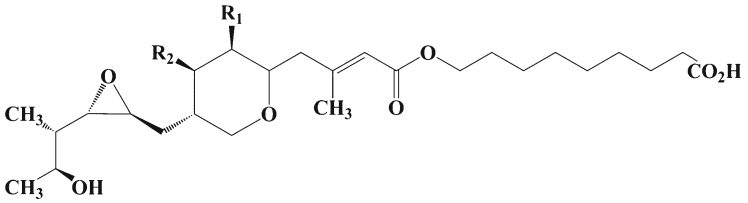
[1] H-NMR of the mupirocin standard was performed at 300 MHz in CD_3OD, whereas the [1] H-NMR of mupirocin 6-*O*-*β*-D- glucoside was determined at 900 MHz in CD_3OD. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), qn (quintet), m (multiplet) and br (broad) where necessary. Chemical shifts are reported in parts per million (ppm), and coupling constants *J* are given in Hz when appropriate

reactive hydroxyl positions, while the diglucoside observed in mass spectrometry could be mupirocin 6,7-*O*-*β*-D-glucoside.

Effects of reaction time and pH on glucoside conversion

Because mupirocin stability is affected by pH and temperature [2], we attempted to ascertain the maximum conversion of glucoside at various temperature and pH ranges. Identical reactions were carried out as explained in the materials and methods. Reaction samples were taken out at different time intervals (0, 1, 2, 3. 4, 5, and 7 h) and the absorbance at 222 nm was analyzed by HPLC-PDA in the time-dependent study. Mupirocin with UDP-D-glucose as a sugar donor was chosen to study these parameters, where the major product was considered to be the glucoside product. 100 mM Tris–HCl buffers at different pHs (pH 3.0, 4.5, 5.5, 6.5, 7, 7.5, 8.8, and 10) were prepared and used in the reaction mixture to determine the maximum conversion of the product. The reaction mixtures were analyzed by HPLC-PDA. Over the study time interval, maximum conversion was achieved between 3

Fig. 4 Glyco-conjugates generated during *in vitro* reactions



1. R_1=Glucose, R_2=OH — Mupirocin 6-*O*-*β*-D-glucoside
2. R_1=OH, R_2=Glucose — Mupirocin 7-*O*-*β*-D-glucoside
3. R_1=Glucose, R_2=Glucose — Mupirocin 6, 7-*O*-*β*-D-di-glucoside
4. R_1=Galactose, R_2=OH — Mupirocin 6-*O*-*β*-D-galactoside
5. R_1=OH, R_2=Galactose — Mupirocin 7-*O*-*β*-D-galactoside
6. R_1=Galactose, R_2=Galactose — Mupirocin 6, 7-*O*-*β*-D-di-galactoside
7. R_1=2-deoxy-glucose, R_2=OH — Mupirocin 6-*O*-*β*-2-deoxy-D-glucoside
8. R_1=OH, R_2=2-deoxy-glucose — Mupirocin 7-*O*-*β*-2-deoxy-D-glucoside
9. R_1=2-deoxy-glucose, R_2=2-deoxy-glucose — Mupirocin 6, 7-*O*-*β*-2-deoxy-D-di-glucoside
10. R_1=Rhamnose, R_2=OH — Mupirocin 6-*O*-*β*-L-rhamnoside

and 7 h of incubation (~50-65 % at 37 °C; Fig. 5a), while pHs of 7.5 to 8.8 (~58.4 % and ~58.9 %) were favorable for maximum conversion (Fig. 5b). Product formation declined after 5 h and above pH 8.8.

In vitro synthesis of diverse mupirocin glycosides

After the structural characterization of mupirocin 6-*O*-*β*-D-glucoside, we attempted to use other rare NDP sugars to synthesize further glyco-conjugates. Identical reactions were carried out and analyzed by UPLC-PDA coupled with QTOF HR-ESI/MS (Fig. 2). Analyses revealed the presence of glycosylated products in all of the independent reaction mixtures, including for UDP-D-galactose, TDP-D-2-deoxyglucose, and TDP-L-rhamnose (Figs. 2 and 4). Other products analyzed and confirmed by high-resolution mass spectra (Figs. 2 and 3) were mupirocin 7-*O*-*β*-D-glucoside (P_2: t_R: 3.81 min, $[MUP_{Diglc}+H]^+$ m/z^+=663.3596 and t_R: 3.71 min), mupirocin 6,7-*O*-*β*-D-diglucoside (P_3: t_R: 3.91 min, $[MUP_{Diglc}+H]^+$ m/z^+=825.4130 and $[MUP_{Diglc}+Na]^+$ m/z^+=847.3953), mupirocin 6-*O*-*β*-D-galactoside (P_1: t_R: 4.31 min, $[MUP_{Gal}+H]^+$ m/z^+=663.3591), mupirocin 7-*O*-*β*-D-galactoside (P_2: t_R: 3.92 min, $[MUP_{Gal}+Na]^+$ m/z^+=685.3408), mupirocin 6,7-*O*-*β*-D-digalactoside (P_3: t_R: 3.79 min, $[MUP_{Digal}+H]^+$ m/z^+=825.4096), mupirocin 6-*O*-*β*-D-2-deoxyglucoside (P_1: t_R: 3.93 min, $[MUP_{2\text{-deoxyglc}}+H]^+$ m/z^+=669.3505), mupirocin 7-*O*-*β*-D-2-deoxyglucoside (P_2: t_R: 3.82 min, $[MUP_{2\text{-deoxyglc}}+H]^+$ m/z^+=669.3505), mupirocin 6,7-*O*-*β*-D-2-deoxydiglucoside (P_3: t_R: 3.72 min, $[MUP_{2\text{-deoxydiglc}}+H]^+$ m/z^+=815.4063) and mupirocin 6-*O*-*β*-L-rhamnoside (P_1: t_R: 3.83 min, $[MUP_{Rhm}+H]^+$ m/z^+=669.3431). These glycoside derivatives of mupirocin are also novel compounds.

Antibacterial activity assay of mupirocin glucoside

Antibacterial activity of standard mupirocin and its newly synthesized glucoside analogue, mupirocin 6-*O*-*β*-D-glucoside, was tested against *S. aureus* subsp. *aureus* KCTC 1916, according to the previously reported method [11]. The minimum inhibitory concentration of mupirocin was first calculated using different concentrations through the paper disc diffusion method. The resulting minimum concentration generating an inhibition zone was found to be ~2 μg/mL. For subsequent experimental comparison, 2 μg/mL and 4 μg/mL concentrations of mupirocin and mupirocin 6-*O*-*β*-D-glucoside were prepared, and the diameter of the inhibition zone generated at identical incubation time intervals were compared. The standard mupirocin showed clear inhibition zone at 2 μg/mL and 4 μg/mL concentrations, however mupirocin 6-*O*-*β*-D-glucoside did not exhibit inhibitory activity confirming the complete loss of antibacterial property of mupirocin after glucosylation (Fig. S4). The increased concentrations of mupirocin 6-*O*-*β*-D-glucoside (50 to 750 μg/mL) also did not show antibacterial activity against the same strain (Fig. S4). The diverse sugar-conjugated mupirocin analogues could also exhibit altered biological activity to that of mupirocin standard which is yet to be determined.

Discussion

Since the discovery of penicillin by Alexander Fleming in 1928, the development of antibiotics has revolutionized the treatment of infectious diseases. Many clinically important antibiotics were discovered from the 1940s to 1970s. However, the rapid use of these antibiotics resulted in significant increases in resistance, which has become a major challenge to current scientists for drug discovery [10]. Mupirocin is an antibiotic that has different levels of resistance in Gram-positive pathogens (*S. aureus*, *S. epidermidis*) and other *Streptococcus* species, including methicillin-resistant ones [1, 33]. Widespread use of mupirocin for the treatment of such infections has also led to the development of high resistance

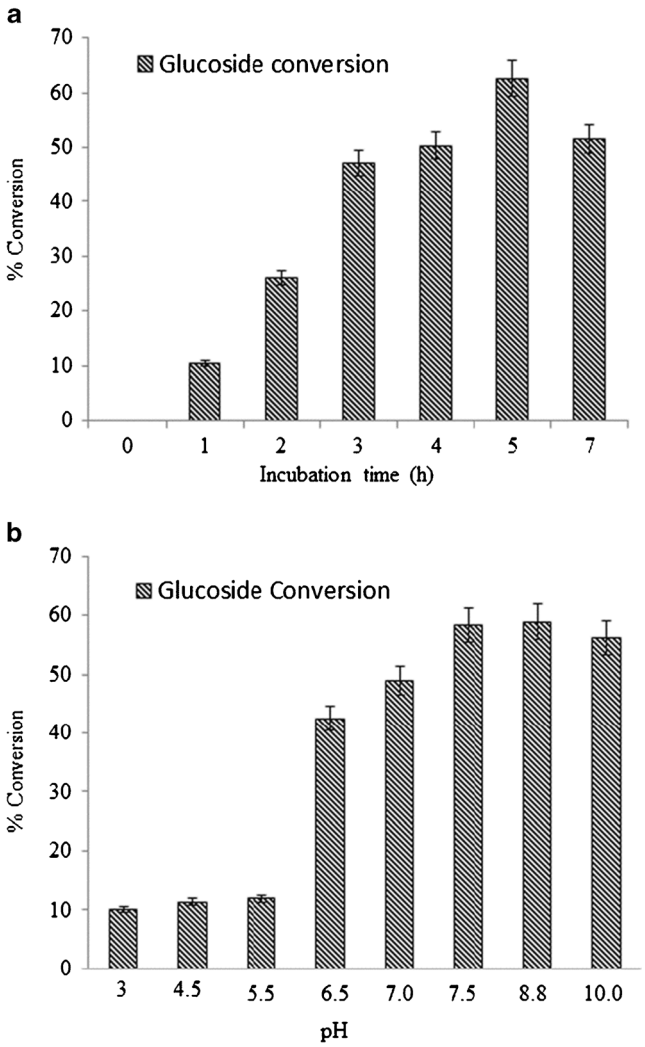


Fig. 5 Time and pH dependent *in vitro* enzymatic assay to evaluate favorable incubation time and pH range for maximum glucoside conversion. The study was based on the glucoside formation at **a** different time intervals and **b** different pH ranges in buffer conditions

(the record level reported in 1987 [3] was>500 μg/mL, and, more commonly, lower levels of 8–256 μg/mL are observed). The emergence of high-level resistance to mupirocin in both outbreak and non-outbreak settings among diverse groups is a cause of concern, showing a lack of efficacy [2].

Attention towards the aminoacyl-tRNA synthetases has led to a research focus on the synthesis of bacterial inhibitors by modifying existing ones, through modifications such as glycosylation, methylation, prenylation, and hydroxylation, or by synthesizing and screening potential new inhibitors from chemical libraries. Glycosylation is considered to be an important synthetic methodology for the generation of glycoside antibiotics [35]. Indeed, large numbers of antibiotics naturally possess sugar residues naturally, which have been experimentally proven to be crucial to their pharmacokinetic parameters [4, 34]. This synthetic approach has also been applied to modify natural products from different sources (to add or remove sugar moieties) which may or may not contain sugar moieties parentally, the products of which have been reported to have enhanced solubility, stability, and lower toxicity and cell recognition capacity, based on synthetic exercises [17]. However, we cannot ignore the experimental evidence that have shown positive results through such modification process as explained in the introduction of vancomycin derivatives [7, 8]. Some synthetic compounds may yield negative results experimentally. For example, sialic acid and galactose-conjugated vancomycin glycoside derivatives have shown reduced effectiveness against methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), vancomycin intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *Enterococcus faecalis* (VSEF) compared to the parent compounds in minimum inhibitory concentration assays [22]. Although it is worth the synthesis of analogues of potential antibiotics, the result cannot be known unless it is tested experimentally.

Thus, we generated 10 new analogs of mupirocin in this study using an *in vitro* glycosylation approach with *Bacillus* glycosyltransferase, which has been shown to accept a diverse class of natural products (*e.g.*, antibiotics, flavonoids, macrolides) as the aglycone, and NDP-D/L-sugars as the sugar donor [23–27] (Fig. 4). Engineering of the sugar moiety in such antibiotics has added novel therapeutic value by enhancing the properties of the parent molecule, accelerating functional and biological recognition [4]. Although mupirocin is widely used, focusing on the treatment of primary and secondary skin infections, it has limitations due to its low penetration into the skin, hydrolysis in the blood, and high levels of microbial resistance, which have restricted its use. The approach of conjugating sugar appendages may affect its potency. To check further potency of the generated analogues, a bio-assay was performed against *S. aureus*. With reference to previous reports, the minimum inhibitory concentration of mupirocin against *S. aureus* (reported≤4 μg/mL, ~8 μg/mL) [28] was first examined, but the minimum inhibitory concentration was found to be 2 μg/mL. To obtain a clear zone of inhibition in the paper diffusion method during bioassay, we considered both concentrations of 2 μg/mL and 4 μg/mL of mupirocin and mupirocin 6-*O*-*β*-D-glucoside. The concentrations were made through serial dilution based on the standard curve of mupirocin. The zone of inhibition was measured to be 12 mm in diameter at 2 μg/mL concentrations of mupirocin, including the paper disc diameter (6 mm), while a 14 mm zone was observed at concentrations of 4 μg/mL. Unfortunately, no inhibition zone was observed for mupirocin glucoside, even at the higher concentration of 750 μg/mL (Fig. S4). This could be due to knock down of the antibiotic activity of mupirocin glucoside. Meanwhile, while purifying the mupirocin glucoside, several organic dissolutions of the reaction mixture were performed with fluctuation in

temperature during rotary evaporator drying. Therefore, the reason could also be breakdown of the mupirocin structure and loss its antibiotic properties. Only the antibiotic activity of mupirocin 6-*O*-*β*-D-glucoside was examined. Although no activity against *S. aureus* was found even at 750 μg/mL, it may be possible to obtain positive results from the other generated glycosides with the same strains, including other Gram-positive pathogens. Therefore, at this point, we cannot be absolutely certain about the complete knock down of antibiotic properties when potential antibiotics are conjugated with sugar moieties.

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