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Triazole-Linked Glycolipids Enhance the Susceptibility of MRSA to β-Lactam Antibiotics

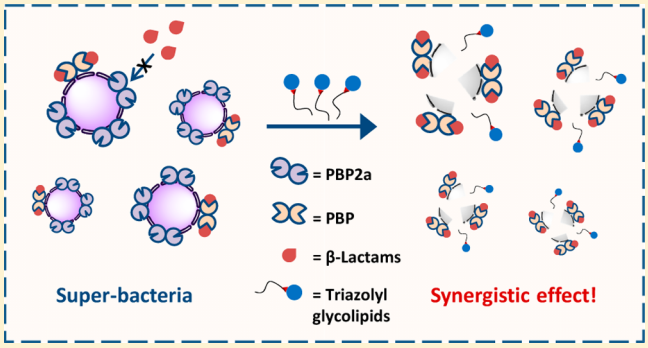
Xi-Le Hu,[†,‡] Dan Li,[†,§] Lei Shao,[§] Xiaojing Dong,[§] Xiao-Peng He,*[,‡] Guo-Rong Chen,[‡] and Daijie Chen*[,§]

[‡]Key Laboratory for Advanced Materials & Institute of Fine Chemicals, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, PR China

[§]State Key Laboratory of New Drug and Pharmaceutical Process, Shanghai Institute of Pharmaceutical Industry, China State Institute of Pharmaceutical Industry, Shanghai 200040, PR China

Supporting Information

ABSTRACT: We show here that a series of triazolyl glycolipid derivatives modularly synthesized by a "click" reaction have the ability to increase the susceptibility of a drug-resistant bacterium to β-lactam antibiotics. We determine that the glycolipids can suppress the minimal inhibitory concentration of a number of ineffective β-lactams, upward of 256-fold, for methicillin-resistant *Staphylococuss aureus* (MRSA). The mechanism of action has been preliminarily probed and discussed.



KEYWORDS: *Triazolyl glycolipid, click reaction, β-lactams, MRSA, superbacteria*

The rapid development of drug-resistant superbacteria has posed an immense threat to patients clinically. For instance, methicillin-resistant *Staphylococuss aureus* (MRSA) accounts for 60−70% of *S. aureus* infections in hospitals and causes the highest number of invasive infections among all antibiotic-resistant bacteria.[1−3] According to the report of the U.S. Centers for Disease Control and Prevention, invasive infection of MRSA had a fatality rate of 14% in 2011. In particular, the increasing prevalence of community-acquired MRSA infection has extended an even greater danger to the public.[4] Unfortunately, the development of new antibiotics has failed to keep pace with the development of drug-resistance over the past few decades.[5,6] As a consequence, use of antimicrobial drug combinations has been viewed as a critical strategy to combat multidrug resistant pathogens.[7]

To date, β-lactam antibiotics are the most commonly used drugs in the treatment of various bacterial infections, but the vast majority of them have been no longer effective in treating MRSA infection.[8] This is largely because of the specific resistance mechanisms that MRSA develops against β-lactam antibiotics.[9] For example, the main antibacterial mechanism of β-lactams is to associate with the penicillin binding proteins (PBPs) that are responsible for peptidoglycan crossing and cell wall synthesis.[10] The binding may result in blockage of the cell wall synthesis, leading to growth inhibition or cell lysis. However, to resist the drugs, penicillin binding protein 2a (PBP2a) is overexpressed by MRSA beyond the PBPs.[11] Because of the low binding affinity between PBP2a (encoded by the gene *mecA*)[12] and β-lactams, the cell wall synthesis can be renewed. This protein thereby acts as an effective equipment of the superbacterium to prevent the attack from β-lactams.

As a consequence, alternative strategies to develop new adjuvant agents that restore the drug-susceptibility of superbacteria are highly desirable. Instead of simply inhibiting bacteria growth, tactics that impart conventional antibiotics with a refreshed ability to kill MRSA have been believed to be more practical for treating infection clinically.[13,14]

We show here that series of modularly synthesized glycolipid derivatives can increase the susceptibility of the drug-resistant superbacterium, MRSA (both ATCC and clinical), to a panel of β-lactam antibiotics (Figure 1a). Glycolipids represent a pivotal class of cell-surface components that participate in diverse cellular events. Recent investigations suggest that both natural and synthetic glycolipids could be potential drug candidates with antiviral,[15−17] anticancer,[18−20] as well as antimicrobial activities.[21−24] However, the glycolipids identified as antimicrobial compounds have insufficient activity for killing even drug-sensitive bacteria.[21−24] Here, we have prepared three series of glycolipids by a click chemistry, and we determined that they have a strong ability to lower the minimal inhibitory concentration (MIC) of a number of conventional β-lactams, upward of 256-fold, for both ATCC and clinical MRSA strains.

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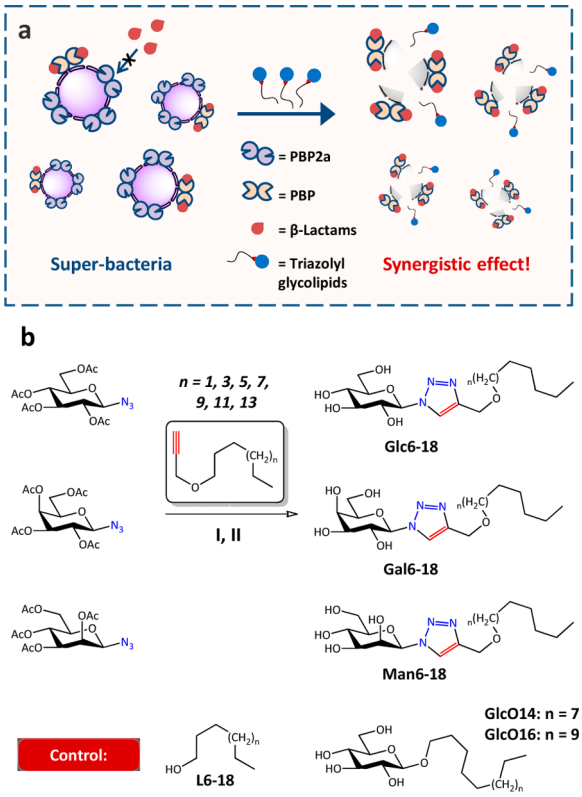

Figure 1. (a) Cartoon depicting the synergistic effect of the combined triazolyl glycolipid derivatives and β-lactam antibiotics to kill drug-resistant bacteria (PBP and PBP2a are penicillin binding proteins and penicillin binding proteins 2a, respectively). (b) Synthetic scheme of the glycolipids synthesized by a click reaction. Reagents and conditions: (I) $CuSO_4 \cdot 5H_2O$, Na ascorbate in CH_2Cl_2/MeOH; (II) $NH_3 \cdot H_2O$, MeOH.

Click chemistry has been widely used for the construction of bioactive compound libraries due to its modularity and conciseness in manipulation.[25,26] In the present study, azido β-D-glucoside,[27] β-D-galactoside,[27] and β-D-mannoside[28] were used to couple with alkynyl lipids with increasing chain length (C6, C8, C12, C14, C16, and C18) by the Cu(I)-catalyzed azide−alkyne 1,3-dipolar cycloaddition click reaction (Scheme S1).[29−31] A subsequent deacylation produced three series of "clicked" (triazole-linked) glycolipid derivatives with different glycosyl moieties in conjugation with length-varied lipid chains in high yields (Figure 1b). The raw lipid alcohols **L6−18** and glucolipids **GlcO14** and **GlcO16** without the "click" modification were used as control compounds (Figure 1b).

With the compounds in hand, their antimicrobial activity was tested using the CLSI (clinical and laboratory standards institute) broth protocol (a representative image is shown in Figure S1). We first determined that the glyclipids alone showed weak antimicrobial effect with MIC of around 256 μg mL^{-1} against a MRSA ATCC43300. In contrast, while used in combination with oxacillin that is resistant by MRSA (MIC = 64−128 μg mL^{-1}, Table 1), five glycolipid derivatives (25% MIC) showed an evident synergistic effect, lowering the oxacillin MIC by 16- to 256-fold (Table 1). We note that the glycolipids with relatively longer lipid chain lengths (C12−16) showed better synergistic effect than those with shorter chains (C6−10). The glycosyl type of the compounds also impacted the synergy. For example, while **Glc12**/**Man12** and **Glc14**/**Gal14** were active, the corresponding isomers **Gal12** and **Man14** did not show a comparable activity.

Table 1. MICs of Oxacillin Alone and Used in Combination with Triazolyl Glycolipids against MRSA ATCC43300

compd	MIC (μg mL^{-1}, alone)	MIC (μg mL^{-1}, combined)[a]	fold of reduction
Oxacillin	64−128		
Glc12	256	1−2	128−256
Glc14	256	4	64
Gal14	256	8	32
Gal16	256	16	16
Man12	256	4	64

[a]Oxacillin combined with 32 μg mL^{-1} glycolipid.

Glc12 with the best synergistic effect was selected for additional experiments. Notably, the unmodified lipid alcohols **L6−18** and control glucolipids **GlcO14** and **GlcO16** without the triazole ring (Figure 1b) did not show an evident synergistic effect when used in combination with oxacillin for MRSA (Table 2). We also determined that the critical micelle concentrations (CMCs) of **Glc12** and **GlcO14** were 43 and 60 μM, respectively (Figure S2). These values are smaller than their concentrations used for killing MRSA (85 and 74 μM for **Glc12** and **GlcO14**, respectively). This probably suggests the formation of micelles of both glycolipids, which might facilitate the encapsulation of the antibiotic, enhancing the internalization of the drug.[32] Moreover, with a concentration above its MIC (141 μM), a non-carbohydrate amphiphile **L12** (the lipid starting material of **Glc12**) showed much weaker synergistic effect than **Glc12**. These results suggest that the CuAAC modification forming the triazolyl glycolipids is essential for the activity. The suppression growth of **Glc12** for two mammalian cell lines (human kidney cell line HEK293 and mouse embryo fibroblast MEF) was tested using an MTS cell-counting assay. The results showed that the glycolipid was not toxic to both cell lines (Figure S3).

Table 2. MICs of Oxacillin Alone and Used in Combination with Triazolyl Glycolipids against MRSA ATCC43300

compd	MIC (μg mL^{-1}, alone)	MIC (μg mL^{-1}, combined)[a]
Oxacillin	64−128	
L6	>256	>32
L8	>256	>32
L10	>256	>32
L12	>256	32
L14	>256	>32
L16	>256	32
L18	>256	>32
GlcO14	>256	>32
GlcO16	>256	>32
Glc12	256	1−2

[a]Oxacillin combined with 32 μg mL^{-1} glycolipid.

The synergy of **Glc12** with oxacillin against MRSA ATCC43300 was evaluated by an Epsilometer test. While oxacillin alone did not produce an evident inhibitive effect against the bacterial strain, the combination of the glycolipid enlarged remarkably the inhibition zone (Figure 2a). In the meanwhile, a time-kill assay was carried out. The presence of 2 mg mL^{-1} oxacillin or 32 mg mL^{-1} glycolipid alone caused slight growth suppression of MRSA, which is similar to that of control

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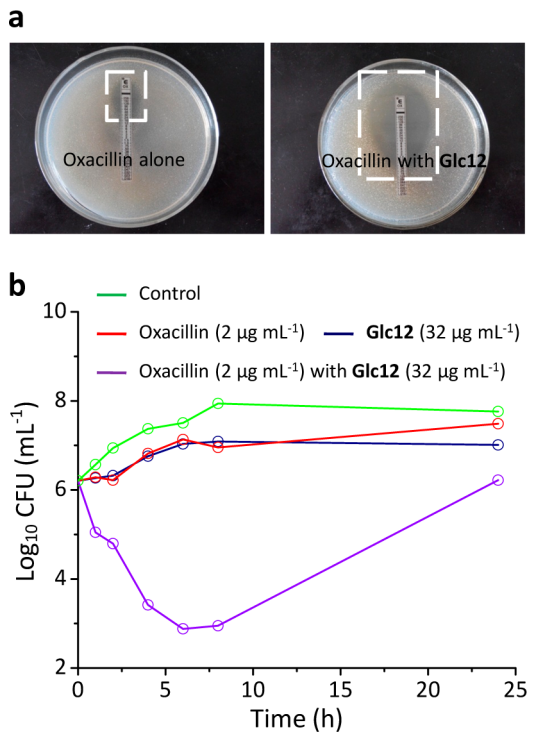

Figure 2. (a) Epsilometer test of the antimicrobial activity of oxacillin alone (2 μg mL^{-1}) and used in combination with 32 μg mL^{-1} **Glc12** against MRSA ATCC43300. (b) Time-kill curve established for oxacilline, **Glc12**, and the combination against MRSA ATCC43300.

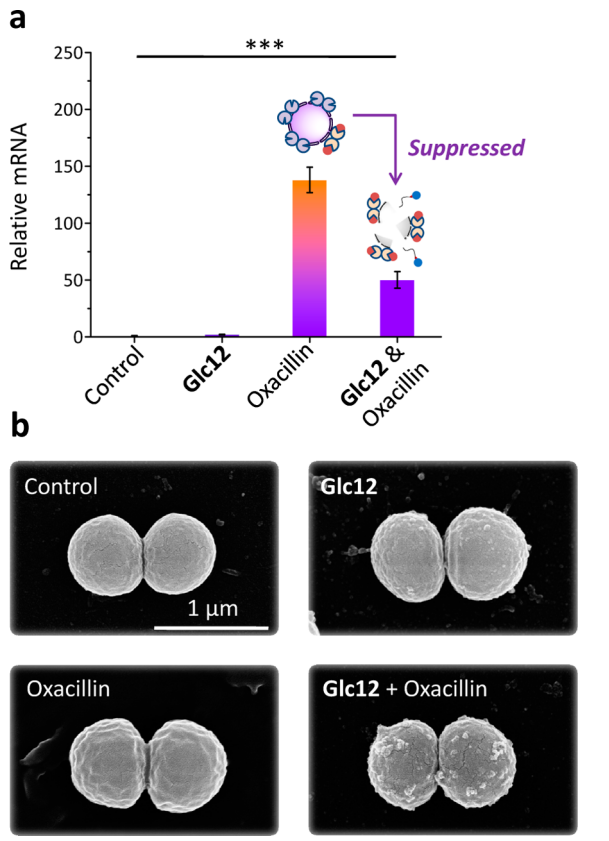

Figure 3. (a) RT PCR analysis and (b) scanning electronic microscope imaging of MRSA ATCC43300 in the absence and presence of oxacillin (2 μg mL^{-1}) or **Glc12** (32 μg mL^{-1}) alone and the combination (***$P < 0.05$). All experiments were performed at least three times. Statistical analysis was performed with *t* tests. Error bars show the standard error of the mean.

(Figure 2b). In contrast, the combination of both inhibited significantly the bacteria growth, and the maximum killing rate was reached within 6 h of cultivation.

Next, the synergy of **Glc12** in combination with a range of β-lactam antibiotics used clinically was tested against MRSA ATCC43300 using the checkerboard method. To our delight, the presence of **Glc12** lowered the MIC of all the antibiotics used by up to 256-fold (Table S1). This suggests that the triazolyl glycolipid has the ability to restore the susceptibility of MRSA over a wide spectrum of β-lactams.

With the above promising observations, we sought to probe preliminarily the mechanism of action underlying the synergy. Since the expression of PBP2a represents the major mechanism by which MRSA resists β-lactam antibiotics, we analyzed the expression level of the PBP2a-coding gene, *mecA*, by MRSA ATCC43300 in the absence and presence of oxacillin and/or **Glc12** using quantitative real-time PCR. The result suggested that *mecA* expression can be sharply enhanced by oxacillin, but not **Glc12** alone (Figure 3a). Interestingly, the combination of both evidently lowered the gene expression, suggesting that the synergistic mechanism is likely related to the suppression of PBP2a.

Considering that oxacillin can interfere with the cell wall synthesis, scanning electron microscopy (SEM) was used to visualize the outer surface change of MRSA in the presence of the compounds. While the cell surface in the presence of oxacillin or **Glc12** alone did not change obviously compared with that of control, the combined use of the two compounds produced a much rougher bacterial surface on which evident secretions were observed (Figure 3b). By a HPLC analysis we further observed that intracellular **Glc12** increased while oxacillin was present (Figure S4). This implies that the presence of oxacillin may facilitate the cell permeability (likely by binding to the PBPs, loosening the cell wall) of triazolyl glycolilpids that might thereby hamper the expression of PBP2a and/or activate additional signaling pathways within the cell, restoring the activity of the drug.

Eventually, we tested the synergistic effect of **Glc12** in combination with oxacillin against a panel of clinical isolates of MRSA. We observed that the MIC of the antibiotic could be lowered for 10 out of 12 clinical isolates by up to 64-fold (Table 3), which suggests the practicality of the clicked glycolipid for clinical samples.

In summary, we have unraveled that series of "clicked" triazolyl glycolipid derivatives have the ability to disarm a superbacterium, MRSA, in combination with a wide spectrum of β-lactam antibiotics used clinically. Importantly, the synergistic effect was observed over a panel of clinical isolates of MRSA. The triazole ring was determined to play a crucial role to increase the susceptibility of MRSA for antibiotic drugs since other control amphiphiles without the triazole did not show an evident synergistic effect. While triazoles have long been used as antifungal agents,[33] we have disclosed here that triazolyl glycolipids may also be potential adjuvants for restoring the antibiotic susceptibility of superbacteria. We note that similar observations have been reported by Melander and colleagues, but with different 1,4-disubstituted triazole structures.[13,14,34] The synergistic mechanism was preliminarily probed and suggested to be correlated with, but probably not limited to, a typical PBP2a suppression pathway. The

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Table 3. MICs of Oxacillin Alone and Used in Combination with Triazolyl Glycolipids against Clinically Isolated MRSA

strain code	MIC (μg mL^{-1}, alone)	MIC (μg mL^{-1}, combined)	fold of reduction
R15[a]	256−512	64−128	2−4
R16[a]	≥256	64	4
R36[a]	128	8−16	8−16
R49[b]	8−16	<1	8−16
R50[b]	8−16	<1	8−16
R53[b]	8−16	<1	8−16
R302[a]	512	128	4
R304[a]	512	256	2
R306[a]	512	32	16
R308[a]	1024	256	4
R313[a]	16	8−16	1−2
R314[a]	512−1024	16	32−64

[a]Oxacillin combined with 32 μg mL^{-1} glycolipid. [b]Oxacillin combined with 16 μg mL^{-1} glycolipid.

elaboration of the exact mechanism of action and structural optimization is currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures and table, experimental section, and original spectral copies of new compounds. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00142.

■ AUTHOR INFORMATION

Corresponding Authors

*Xiao-Peng He (xphe@ecust.edu.cn).
*Daijie Chen (hccb001@163.com).

Author Contributions

[†]X.-L.H. and D.L. contributed equally.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Taubes, G. The bacteria fight back. *Science* **2008**, *321*, 356−361.
(2) Addicks, J. P.; Uibel, S.; Jensen, A.-M.; Bundschuh, M.; Klingelhoefer, D.; Groneberg, D. A. MRSA: a density-equalizing mapping analysis of the global research architecture. *Int. J. Environ. Res. Public Health* **2014**, *11*, 10215−10225.
(3) Chambers, H. F.; DeLeo, F. R. Waves of resistance: *staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* **2009**, *7*, 629−641.
(4) Morens, D. M.; Fauci, A. S. Emerging infectious diseases: threats to human health and global stability. *PLoS Pathog.* **2013**, *9*, e1003467.
(5) Williams, A. J.; Harland, L.; Groth, P.; Pettifer, S.; Chichester, C.; Willighagen, E. L.; Evelo, C. T.; Blomberg, N.; Ecker, G.; Goble, C.; Mons, B. Open PHACTS: semantic interoperability for drug discovery. *Drug Discovery Today* **2012**, *17*, 1188−1198.
(6) Laudano, J. B. Ceftaroline fosamil: a new broad-spectrum cephalosporin. *J. Antimicrob. Chemother.* **2011**, *66* (Suppl. 3), iii11−iii18.
(7) Chait, R.; Craney, A.; Kishony, R. Antibiotic interactions that select against resistance. *Nature* **2007**, *446*, 668−671.
(8) Łęski, T. A.; Tomasz, A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J. Bacteriol.* **2005**, *187*, 1815−1824.
(9) Jia, J.; Zhu, F.; Ma, X.; Gao, Z. W.; Li, Y. X.; Chen, Y. Z. Mechanisms of drug combinations: interaction and network perspectives. *Nat. Rev. Drug Discovery* **2009**, *8*, 111−128.
(10) Yocum, R. R.; Rasmussen, J. R.; Strominger, J. L. The mechanism of action of penicillin. Penicillin acylates the active site of *bacillus stearothermophilus* D-alanine carboxypeptidase. *J. Biol. Chem.* **1980**, *255*, 3977−3986.
(11) Yocum, R. R.; Waxman, D. J.; Rasmussen, J. R.; Strominger, J. L. Mechanism of penicillin action: penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2730−2734.
(12) Zapun, A.; Contreras-Martel, C.; Vernet, T. Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol. Rev.* **2008**, *32*, 361−385.
(13) Harris, T. L.; Worthington, R. J.; Melander, C. Potent small-molecule suppression of oxacillin resistance in methicillin-resistant *staphylococcus aureus*. *Angew. Chem., Int. Ed.* **2012**, *51*, 11254−11257.
(14) Su, Z.; Yeagley, A. A.; Su, R.; Peng, L.; Melander, C. Structural studies on 4,5-disubstituted 2-aminoimidazole-based biofilm modulators that suppress bacterial resistance to β-lactams. *ChemMedChem* **2012**, *7*, 2030−2039.
(15) Stanley, M.; Cattle, N.; McCauley, J.; Martin, S. R.; Rashid, A.; Field, R. A.; Carbain, B.; Streicher, H. 'TamiGold': phospha-oseltamivir-stabilised gold nanoparticles as the basis for influenza therapeutics and diagnostics targeting the neuraminidase (instead of the hemagglutinin). *Med. Chem. Commun.* **2012**, *3*, 1373−1376.
(16) Papp, I.; Sieben, C.; Sisson, A. L.; Kostka, J.; Böttcher, C.; Ludwig, K.; Herrmann, A.; Haag, R. Inhibition of influenza virus activity by multivalent glycoarchitectures with matched sizes. *ChemBioChem* **2011**, *12*, 887−895.
(17) Morales-Serna, J. A.; Boutureira, O.; Serra, A.; Matheu, M. I.; Díaz, Y.; Castillón, S. Synthesis of hyperbranched β-galceramide-containing dendritic polymers that bind HIV-1 rgp120. *Eur. J. Org. Chem.* **2010**, 2657−2660.
(18) Li, C.; He, X.-P.; Zhang, Y.-J.; Li, Z.; Gao, L.-X.; Shi, X.-X.; Xie, J.; Li, J.; Chen, G.-R.; Tang, Y. Click to a focused library of benzyl 6-triazolo(hydroxy)benzoic glucosides: novel construction of PTP1B inhibitors on a sugar scaffold. *Eur. J. Med. Chem.* **2011**, *46*, 4212−4218.
(19) Zhang, H.-L.; He, X.-P.; Sheng, L.; Yao, Y.; Zhang, W.; Shi, X.-X.; Li, J.; Chen, G.-R. *Mol. Divers.* **2011**, *15*, 889−900.
(20) Song, S.-X.; Zhang, H.-L.; Kim, C.-G.; Sheng, L.; He, X.-P.; Long, Y.-T.; Li, J.; Chen, G.-R. Synthesis of novel 6-triazologlycolipids via click chemistry and their preliminary cytotoxicity assessments. *Tetrahedron* **2010**, *66*, 9974−9980.
(21) Poláková, M.; Beláňová, M.; Mikušová, K.; Lattová, E.; Perreault, H. Synthesis of 1,2,3-triazolo-linked octyl (1→6)-α-D-oligomannosides and their evaluation in mycobacterial mannosyltransferase assay. *Bioconjugate Chem.* **2011**, *22*, 289−298.
(22) Azim, A.; Shah, V.; Doncel, G. F.; Peterson, N.; Gao, W.; Gross, R. Amino acid conjugated sophorolipids: a new family of biologically active functionalized glycolipids. *Bioconjugate Chem.* **2006**, *17*, 1523−1529.
(23) Ding, N.; Zhang, Z.; Zhang, W.; Chun, Y.; Wang, P.; Qi, H.; Wang, S.; Li, Y. Synthesis and antibacterial evaluation of a series of oligorhamnoside derivatives. *Carbohydr. Res.* **2011**, *346*, 2126−2135.
(24) Abalos, A.; Pinazo, A.; Infante, M. R.; Casals, M.; García, F.; Manresa, A. Physicochemical and antimicrobial properties of new rhamnolipids produced by *pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir* **2001**, *17*, 1367−1371.

DOI: 10.1021/acsmedchemlett.5b00142
ACS Med. Chem. Lett. 2015, 6, 793−797

(25) Thirumurugan, P.; Matosiuk, D.; Jozwiak, K. *Chem. Rev.* **2013**, *113*, 4905−4979.
(26) He, X.-P.; Xie, J.; Tang, Y.; Li, J.; Chen, G.-R. CuAAC click chemistry accelerates the discovery of novel chemical scaffolds as promising protein tyrosine phosphatases inhibitors. *Curr. Med. Chem.* **2012**, *19*, 2399−2405.
(27) Salunke, S. B.; Babu, N. S.; Chen, C.-T. Iron(III) chloride as an efficient catalyst for stereoselective synthesis of glycosyl azides and a cocatalyst with Cu(0) for the subsequent click chemistry. *Chem. Commun.* **2011**, *47*, 10440−10442.
(28) Cosgrove, K. L.; Bernhardt, P. V.; Ross, B. P.; McGeary, R. P. Determination of the anomeric configurations of 2,3,4,6-Tetra-*O*-Acetyl-D-mannopyranosyl azide. *Aust. J. Chem.* **2006**, *59*, 473−476.
(29) Rostovstsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599.
(30) Tornøe, C. W.; Christensen, C.; Meldal, M. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.* **2002**, *67*, 3057−3064.
(31) Meldal, M.; Tornøe, C. W. Cu-catalyzed azide-alkyne cycloaddition. *Chem. Rev.* **2008**, *108*, 2952−3015.
(32) Hu, F.-Q.; Zhang, Y.-Y.; You, J.; Yuan, H.; Du, Y.-Z. pH triggered doxorubicin delivery of PEGylated glycolipid conjugate micelles for tumor targeting therapy. *Mol. Pharmaceutics* **2012**, *9*, 2469−2479.
(33) Hitchcock, C. A.; Barrett-Bee, K. J.; Russell, N. J. The lipid composition and permeability to the triazole antifungal antibiotic ICI 153066 of serum-grown mycelial cultures of *Candida albicans*. *J. Gen. Microbiol.* **1989**, *135*, 1949−1955.
(34) Rogers, S. A.; Huigens, R. W., III; Cavanagh, J.; Melander, C. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. *Antimicrob. Agents Chemother.* **2010**, *54*, 2112−2118.

DOI: 10.1021/acsmedchemlett.5b00142
ACS Med. Chem. Lett. 2015, 6, 793−797