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Contributed Paper

Optimizations of Hydroxyl Terminated Polymerization of Mannose Tricyclic Orthoester Toward Ready-to-use Lipomannan Backbone

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ABSTRACT

Throughout the life cycle of *mycobacterium tuberculosis (Mtb)*, its survival critically depends on its interactions with mammalian host cells. Therefore, in order to have better treatments for and preventions of tuberculosis (TB), it is important to understand the biological activity of the *Mtb* surface components. α-1,6 Polymannan, the backbone of lipomannan (LM), is present abundantly on the surface of *mycobacterium tuberculosis* and interacts with mammalian hosts. Conjugation of polymannan with thiol linker comprises an essential biochemical tool to study the *Mtb* surface interactions with mammalian hosts. A short alkyl chain with hydroxyl head group is employed to attach to the reducing end of the polysaccharide via a glycosidic bond, and with a terminal thiol group to serve as a linker. The target - conjugated polymannan with thiol linker is achieved by a rapid synthetic route, relying on a ring-opening polymerization, using a tricyclic mannosyl orthoester as monomer. The ring-opening polymerization, triggered by a Lewis acid without the presence of the linker, was previously proposed to be terminated by water at the reducing end. However, in the present study, the results suggest that the polymerization may also be initiated from the reducing end by the attack of the hydroxyl head group of a short chain alkyl thiol linker. After that, an elongation of the growing polysaccharide may be achieved via the propagation to the non-reducing end. Optimizations for the polymerization and termination in a one-pot reaction were carried out. A half gram of the synthetic target molecule is attained in a single chemical step. The target synthetic bacterial surface molecule is in a ready-to-use form to be utilized further in several applications, such as immobilization on surfaces and conjugation to carrier proteins for biological and immunological studies. This biochemical tool may lead us to a better understanding on carbohydrate-based biointerfaces between the bacteria pathogen and the human immune system.

Keywords: polymannan, carbohydrate, *Mycobacterium tuberculosis* (*Mtb*), lipomannan (LM)

1. INTRODUCTION

Annually, tuberculosis (TB) kills millions of people worldwide. This devastating disease is caused by *Mycobacterium tuberculosis (Mtb)*. After years of fighting, we have failed to eradicate TB due to the two significant causes which are the low efficacy of Bacillus Calmette-Guérin (BCG) vaccine and the spread of multidrug-resistant TB [1, 2]. TB is a very complex disease because *Mtb* can down modulate the human immune system and remains in a dormant state within the immune cells for years. Throughout the life cycle of *Mtb*, its survival critically depends on its interactions with mammalian host cells [3]. Therefore, in order to have better treatments and preventions of TB, it is indispensable for us to understand the biological activity of the *Mtb* surface components. The uniqueness of physiological and biological properties of *Mtb* surface can be characterized by several important surface molecules including mannan capped lipoarabinomannan (ManLAM), lipoarabinomannan (LAM), lipomannan (LM), and phosphatidylinositol mannosides (PIMs) [3, 4].

Lipomannan (LM) is one of the crucial macromolecules involving in infectious, virulent, and survival events of *Mtb* in mammalian host cells (Figure 1a) [5, 6]. Polymannan, the backbone of LM, is a repeating unit of mannose connected together in a linear fashion by α-1, 6 glycosidic linkages which is abundantly present on the surface of *Mtb* [3, 4]. Therefore, a thorough study on polymannan physical and chemical interactions with mammalian cells may lead to a better understanding on carbohydrate-based biointerfaces between the bacteria pathogen and the human immune system. Conjugation of polymannan with thiol linker can serve as an essential biochemical tool to study the *Mtb* surface interactions.

In the present study, we report an optimization of an efficient single-chemical-step synthesis of conjugated polymannan with thiol linker (Figure 1b). A hydroxyl head group of 6-(S-benzyl) mercapto-1-hexanol is attached to the reducing end of the newly synthesized polysaccharide via a glycosidic bond *in situ* along with the polymerization reaction (Figure 2). The linker contains a terminal thiol group to serve as an attachment point to proteins or solid supports [7-9]. The target - conjugated polymannan with thiol linker is achieved by a rapid synthetic route, relying on a ring-opening polymerization using a tricyclic mannosyl orthoester as monomer. The target synthetic bacterial surface molecule is in a ready-to-use form to be utilized further in several applications, such as immobilization on surfaces and carrier proteins for biological and immunological studies [10 -13].

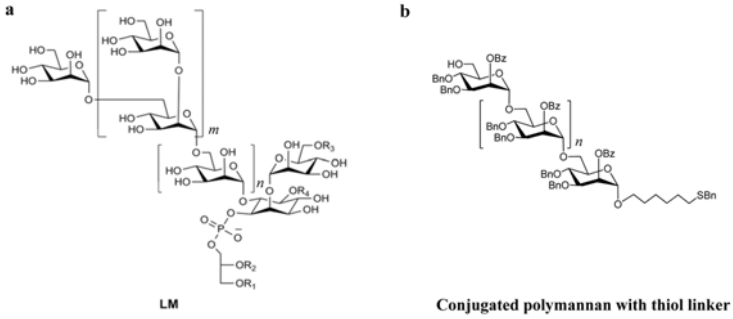


Figure 1. (a) Structure of lipomannan (LM) [10], (R_1 is tuberculostearic acid and R_2, R_3, and R_4 are various fatty acids) **(b)** Proposed structure of the conjugated polymannan with thiol linker.

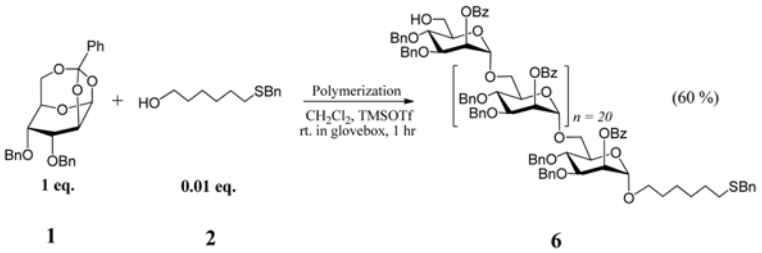


Figure 2. Optimal polymerization conditions for the conjugated polymannan with thiol linker in a single chemical step.

2. MATERIALS AND METHODS

A tricyclic mannosyl orthoester, monomer **1**, was prepared based on a previously published report [14]. 6-Mercapto-1-hexanol was purchased from Sigma-Aldrich Co. LLC. The mercapto group was protected by benzylation as done previously, to yield 6-(S-benzyl) mercapto-1-hexanol **2** [15].

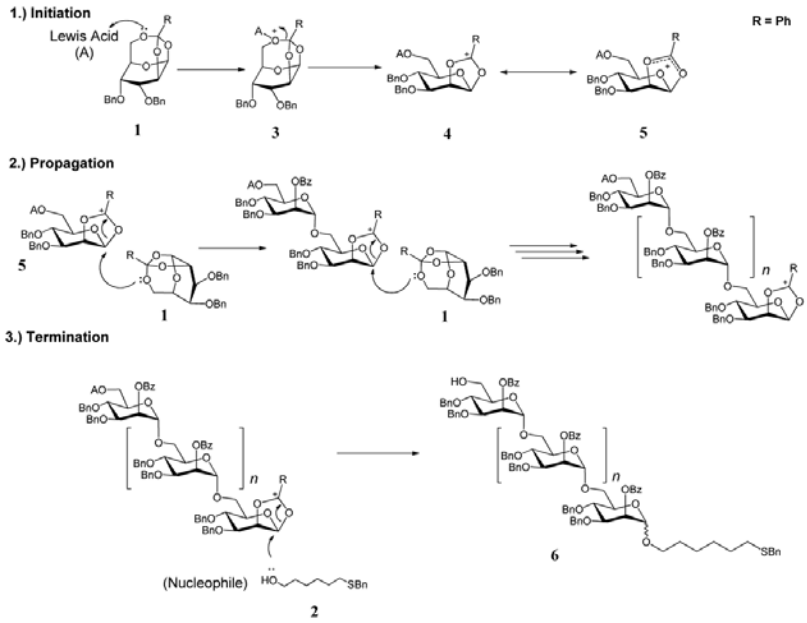
One-Pot Polymerization and Termination:

Monomer **1** and 6-(S-benzyl) mercapto-1-hexanol **2** were dried in a Kügelrohr apparatus under high vacuum at 80 °C for 4 hours prior to polymerization. Dried dichloromethane, employed as a solvent, was transferred under inert atmosphere into a glass vial capped with rubber septa. A catalytic amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) was added and the reaction temperature was kept at room temperature or -80 °C under inert atmosphere while stirring for 60 minutes. The reaction mixture was concentrated *in vacuo*. The crude product was further purified by a flash column chromatography to obtain product **6**.

3. RESULTS AND DISCUSSION

The synthesis of polymannan with thiol linker was previously done by a ring opening polymerization terminated by a thiol linker [16]. The rapid synthesis relied on a ring-opening polymerization using a tricyclic mannosyl orthoester as a monomer [14, 16-17]. A glycosylation reaction to attach the partial protected thiol alkyl chain was proposed to be achieved in a termination step of polymerization reaction by the hydroxyl group of the partial protected thiol linker. However, this previously reported condition was only done on a small scale with 30 mg of monomer **1** [16].

In this study, we focused on optimizations of the rapid synthesis of conjugated polymannan with the linker **2** by up-scalable conditions. The polymerization was triggered by a catalytic amount of TMSOTf to provide the desired polymer products with regio- and stereoselectivity [14, 17]. The polymannan chains were allowed to grow for a period of time. Then, the reaction was terminated by the short chain alkyl linker with hydroxyl head group - 6-(S-benzyl) mercapto-1-hexanol **2**. According to the previously proposed mechanism of the cationic ring-opening polymerizations of tricyclic mannosyl orthoester with a termination by the linker **2**, the possible polymerization and termination are illustrated in Scheme 1. In order to scale up the one-pot polymerization and termination reactions, a few basic parameters including reaction temperature, reaction time before termination, and equivalent ratio of monomer **1** to linker **2** were varied to obtain the optimal reaction conditions.



Scheme 1. A possible mechanism of cationic ring-opening polymerizations of tricyclic mannosyl orthoester with a termination by 6-(S-benzyl) mercapto-1-hexanol as thiol linker **2** [16].

Optimizations of Polymerization and Terminating Reaction Conditions

For the first set of experiments (Entries 1-4, Table 1), the reactions were carried out at -80 °C. The equivalent ratio of monomer **1** to linker **2** was 1:4, to ensure that a sufficient amount of thiol linker was available to serve as a terminator of the reaction. The reaction time before the linker termination was varied to 2, 7, 30, and 60 minutes. No polymannan was obtained at the reaction times of 2 and 7 minutes. Polymannan was obtained at the reaction times of 30 and 60 minutes, however, no conjugated polymannan with thiol linker was observed. According to the results from the first set of the experiments, it can be concluded that the termination carried out at 30 and 60 minutes allowed enough time for polymerization to occur, but the terminations carried out at 2 and 7 minutes did not. And since no conjugated polymannan was observed, the termination of the polymerization was dominated by water molecules. This might happen due to the humidity, inevitably, water molecules entered the reaction system and inhibit the introduction of linker **2** into the polymer molecules.

Hence, the second set of experiments (Entries 5-6, Table 1) was conducted at room temperature in a glovebox to secure an inert atmosphere without moisture. The polymerization time was 30 and 60 minutes. The equivalent ratios of monomer **1** to linker **2** were varied to 1:4, 1:5, 1:10, and 1:20. However, no conjugated polymannan with thiol linker was observed, and only the polymannan was obtained. The chemical kinetic rate at room temperature was probably so fast that the growing polymer chain had been completed before thiol linker was introduced into the reaction. The terminating agent could be the trace amount of water molecule already present within the monomer starting material and TMSOTf catalyst.

Table 1. Overall results of polymerization reactions carried out at different experimental conditions (√ indicates observed as major product(s), - indicates unobserved product(s), N/A indicates unavailable data).

Entry	Reaction Temp.	Reaction time before termination (minutes)	Equivalent ratio (monomer: thiol linker)	Product(s)			
				Conjugated monosaccharide **7**	Short chain of polymannan with thiol linker	Long chain of polymannan with thiol linker	Polymannan
1	- 80°C	2	1:4	N/A	-	-	-
2		7	1:4	N/A	-	-	-
3		30	1:4	-	-	-	√
4		60	1:4	-	-	-	√
5	Room	30	1:4, 1:20	-	-	-	√
6	temp. in	60	1:4, 1:5, 1:10	-	-	-	√
7	glovebox	1	1:4	-	-	-	√
8		3	1:4	-	-	-	√
9		5	1:4	-	-	-	√
10		10	1:4	-	-	-	√
11		Premix	1:4	√	-	-	-
12	- 80°C	2	1:4	√	-	-	-
13		6	1:4	√	-	-	-
14		10	1:4	√	-	-	√
15		16	1:4	√	-	-	√
16	Room	Premix	1:0.5	-	√	-	-
17	temp. in		1:0.33	-	-	√	-
18	glovebox		1:0.1	-	-	√	-

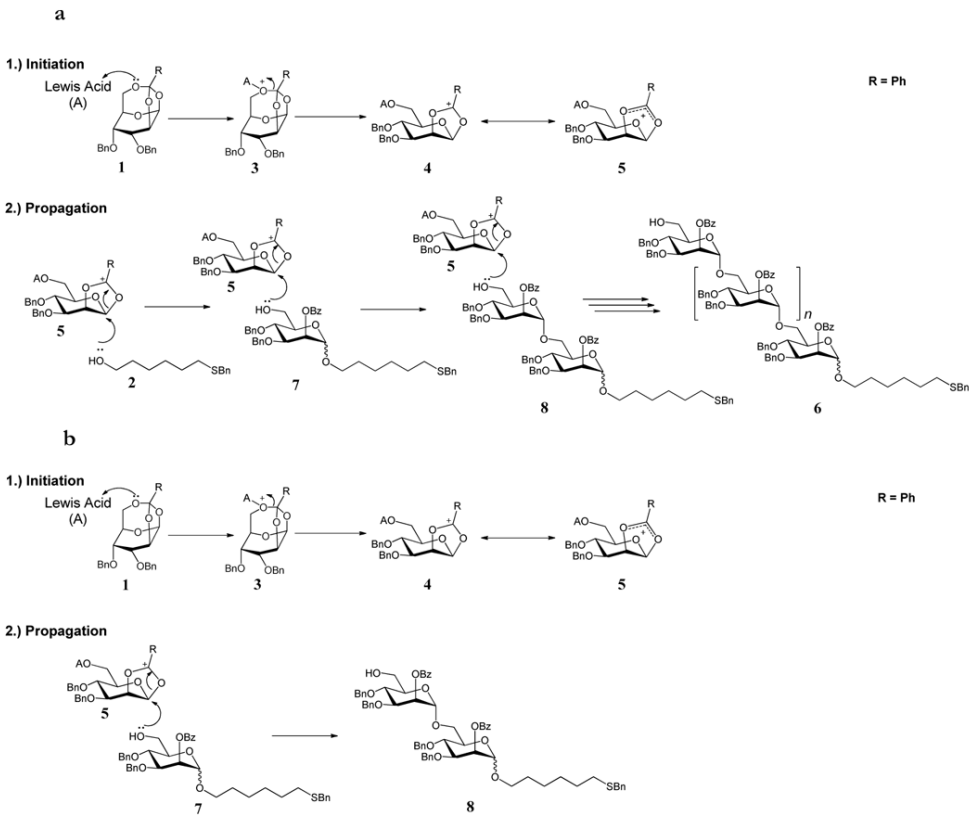
The third set of experiments (Entries 7-11, Table 1) was done at room temperature in a glovebox in a shorter reaction time. The polymerization time was set to be under 30 minutes. The polymerization time was varied to 1, 3, 5, 10, 30 minutes, and a premix condition, where the monomer **1** and linker **2** were mixed together prior to polymerization, were employed. The equivalent ratio of monomer **1** to linker **2** was kept at 1:4. From this set of experiments, only the polymannans without linker were obtained in all conditions, except in the premix conditions. In the premix conditions, only conjugated mannose monosaccharide with thiol linker (the conjugated monosaccharide **7**) was present. The result indicated that the hydroxyl group of a thiol linker was able to attack the reducing end of the monomer. Moreover, the chemical kinetic rate at room temperature may be too fast that the growing polymer chain had been completed within a minute, even before the thiol linker was introduced into the reaction.

Consequently, the next set of experiments (Entries 12-15, Table 1) was done at -80 °C to slow the chemical kinetic rate down, with the reaction times of 2, 6, 10, and 16 minutes. No conjugated polymannan with thiol linker was observed at all. For the shorter reaction times at 2 and 6 minutes, the reaction conditions were similar to entries 1 and 2 but the conjugated monosaccharide **7** was also monitored as one of the main possible products for these entries (12-15). Certainly, these results indicated that hydroxyl group of a thiol linker was able to attack the reducing end of the remaining available monomer (the monomers that had not been incorporated into the growing chain of polymannan), and that 6 minutes of reaction time was not sufficient for the polymannan to occur. On the other hand, for the reaction times of

10 and 16 minutes, the conjugated monosaccharide **7** was present along with the polymannans. Interestingly, these results indicated that the hydroxyl group of the thiol linker was able to attack the reducing end of the remaining available monomer, but not the growing polymer chain.

After attempts in various polymerization conditions to scale up the polymerization and termination reactions, it turned out to be difficult to try to terminate the growing polymer chain with the linker. Nevertheless, an interesting outcome was noticeable. It was the event that the hydroxyl group of a thiol linker was able to attack the reducing end of the available monomer, but not the growing polymer chain.

If we look closely at the proposed mechanism of polymerization of mannose with a termination of thiol linker (Scheme 1), as long as the growing polymer chains were still present in the reaction, the linker should be able to compete with a trace amount of water molecules and terminate the growing polymer chains. Although four equivalents of the linker were available to compete with trace amount of water molecules, none of the linker molecule was able to terminate the reducing end of the growing polymer chain. This leads us to propose an alternative mechanism, where the growing polymer chain was initiated from the reducing end and propagate toward non-reducing end, as shown in Scheme 2a.



Scheme 2. (a) A possible mechanism of cationic ring-opening polymerizations of tricyclic mannosyl orthoester with 6-(S-benzyl)mercapto-1-hexanol as thiol linker. **(b)** A possible mechanism of cationic ring-opening glycosylation of tricyclic mannosylorthoester with the conjugated monosaccharide **7**.

Therefore, to support the alternative mechanism proposed in Scheme 2a, another set of experiments was conducted. In this experiment, monomer **1** and the conjugated monosaccharide **7** were premixed together prior to the polymerization. The equivalent ratio of monomer to the conjugated monosaccharide **7** was 1:2. The reaction was done at room temperature in a glovebox. As anticipated, the conjugated disaccharide with thiol linker **8** was obtained as a product. This result showed that the C6-hydroxy group of the non-reducing end of the monosaccharide could attack the monomer to form disaccharide **8** (Scheme 2b).

Further experiments (Entries 16-18, Table 1) were done with the premix conditions using monomer **1** and linker **2** with the equivalent ratios of 1:0.5, 1:0.33, and 1:0.1. A short chain of conjugated polymannans with thiol linker were obtained. In addition, by varying the equivalent ratios of monomer to linker, it was found that as the ratio of monomer to thiol linker increased, a longer chain of conjugated polymannan with thiol linker was obtained. These results suggest that it is also possible for the polymer chain to propagate from the reducing end to the non-reducing end of the polysaccharides (Scheme 2a). The ring-opening polymerization is initiated by a Lewis acid. The hydroxyl head group of the linker attacks the reducing end of the positively charged bicyclic orthoester intermediate **5** to initiate the polymerization. After that, an elongation of the polysaccharide may be achieved via the propagation toward the non-reducing end.

The premix conditions were done by the addition of the thiol linker to the same pot as monomer prior to triggering of polymerization. The conditions were employed in this current work and found to be more reliable, whereas the termination strategy first developed in our previous work [16] was carried out by the addition of the thiol linker to the growing chain at 60 minutes after the polymerization was triggered. The major drawback of the termination strategy was that the reaction time for polymerization to take place before terminating with the linker varied according to the scales of the reaction and the monomer concentrations. Consequently, when the reaction conditions were altered for the purpose of scale up, it was rather difficult to terminate the polymerization with the linker at the most suitable reaction time, resulting in unsuccessful incorporation of the linker into the polysaccharide. Often times, only the polysaccharides without the linker were obtained as products.

Eventually, the optimum reaction conditions were obtained by premixing monomer **1** with linker **2** at the ratio of 1:0.1 (Entry 18, Table 1). The reaction was performed at room temperature in glovebox at a 500-mg scale. The conjugated polymannan products consisted of approximately 20 units of mannose linked via α-1,6 glycosidic bonds. The yield of the desired conjugated polymannan with the linker was 60% (Figure 2).

The structure of the target molecule is confirmed by ^{1}H NMR and ^{13}C NMR. The glycan part of the molecules shows NMR signals that are essentially identical to ^{1}H and ^{13}C NMR signals of the published results [14, 17]. In addition, the ^{1}H and ^{13}C NMR signals of the hydrophobic thiol linker were evidently present.

The characterization of the conjugated polymannan with thiol linker are as follows (Figure 3) :^{1}H NMR (300 MHz, $CDCl_3$) δ 1.25-1.37 (m, 4H), δ 1.47-1.55 (m, 4H), δ 2.36-2.41 (t, 2H), δ 3.30-4.15 (m, 100H), δ 3.64-3.68 (m, 2H), δ 3.66-3.70 (s, 2H), δ 4.25-4.50 (m, 40H), δ 4.72-4.95 (m, 40H), 5.03 (bs, 20H), δ 5.83 (bs, 20H), δ 7.00-7.37 (m, 205H), δ

7.40-7.60 (m, 60H), δ 8.00-8.27 (m, 40H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.90, 28.70, 29.13, 29.15, 30.33, 36.10, 61.83, 65.74, 68.38, 70.91, 71.31, 73.72, 75.02, 78.21, 98.56, 127.02, 127.32, 127.38, 127.71, 128.07, 128.16, 128.36, 128.52, 128.68, 128.97, 129.89, 130.01, 133.35, 137.51, 138.50, 165.55

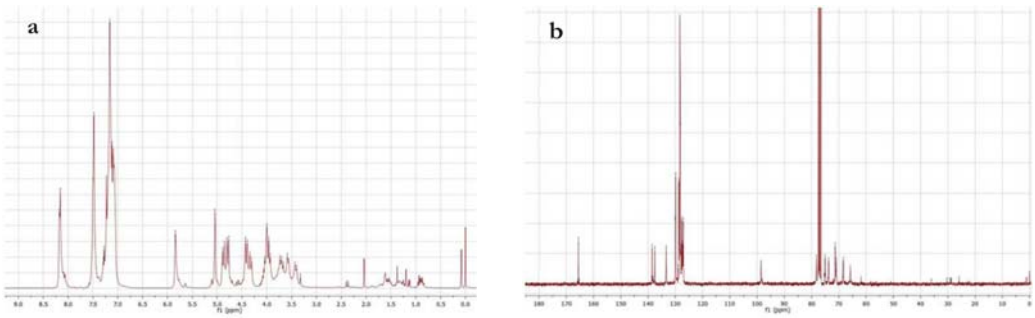


Figure 3. NMR spectra of the conjugated polymannan with thiol linker: **(a)** ^{1}H NMR and **(b)** ^{13}C NMR. (Signals of the hydrophobic thiol linker ^{1}H NMR δ 1.25-1.37 (m, 4H), δ 1.47-1.55 (m, 4H), δ 2.36-2.41 (t, 2H), δ 3.64-3.68 (m, 2H), δ 3.66-3.70 (s, 2H); ^{13}C NMR δ 25.90, 28.70, 29.13, 29.15, 30.33, 36.10, 61.83).

4. CONCLUSIONS

Half a gram of the synthetic target molecule, the conjugated polymannan with thiol linker **6**, was attained in a single chemical transformation. The reaction conditions were optimized to obtain the desired product in a one-pot reaction, which allows for a much shorter cycle time and less amount of reagents used when compared with a series of step-wise reactions. The rapid synthetic protocol relied on the ring opening polymerization of a tricyclic orthoester. The results suggest that the polymerization may also be initiated from the reducing end by the attack of the hydroxyl head group of a short chain alkyl thiol linker. After that, an elongation of the polysaccharide may be achieved via propagation toward the non-reducing end. The developed methodology could provide a half gram of the polymannan, the backbone of lipomannan found on the surface of *Mycobacterium Tuberculosis*, already equipped with a thiol linker. The synthetic molecule can be directly utilized further in several applications, such as immobilization on surfaces and carrier proteins for biological and immunological studies. This biochemical tool may lead us to a better understanding of carbohydrate-based biointeractions between the bacteria pathogen and the human immune system.

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REFERENCES

[1] Russell D.G., *Nat. Rev. Mol. Cell. Biol.*, 2001; **2**: 569-577. DOI 10.1038/35085034.

[2] Sharma S.K. and Mohan A., *Chest*, 2006; **130**: 261-272. DOI 10.1378/chest.130.1.261.

[3] Neyrolles O. and Guilhot C., *Tuberculosis*, 2011; **91(3)**: 187-195. DOI 10.1016/j.tube.2011.01.002.

[4] Hutacharoen P., Ruchirawat S. and Boonyarattanakalin S., *J. Carbohyd. Chem.*, 2011; **30(7-9)**: 415-437. DOI 10.1080/07328303.2011.621041.

[5] Briken V., Porcelli S.A., Besra G.S. and Kremer L., *Mol. Microbiol.*, 2004; **53**: 391. DOI 10.1111/j.1365-2958.2004.04183.x.

[6] Nigou J., Gilleron M., Rojas M., Garcia L.F., Thurnher M. and Puzo G., *Microbes Infect.*, 2002; **4**: 945. DOI 10.1016/S1286-4579(02)01621-0.

[7] Pimpang P., Sutham W., Mangkorntong N., Mangkorntong P. and Choopun S., *Chiang Mai J. Sci.*, 2008; **35(2)**: 250-257.

[8] Pimpang P. and Choopun S., *Chiang Mai J. Sci.*, 2011; **38(1)**: 31-38.

[9] Saipanya S. and Sarakonsri T., *Chiang Mai J. Sci.*, 2010; **37(1)**: 99-105.

[10] Boonyarattanakalin S., Liu X., Michieletti M., Lepenies B. and Seeberger P.H., *J. Am. Chem. Soc.*, 2008; **130(49)**: 16791-16799. DOI 10.1021/ja806283e.

[11] Dietrich P.M., Horlacher T., Girard-Lauriault P.L., Gross T., Lippitz A., Min H., Wirth T., Castelli R., Seeberger, P.H. and Unger W.E.S., *Langmuir*, 2011; **27(8)**: 4808-4815. DOI 10.1021/la104038q.

[12] Vericat C., Vela M.E., Benitez G., Carro P. and Salvarezza R.C., *Chem. Soc. Rev.*, 2010; **39(5)**: 1805-1834. DOI 10.1039/B907301A.

[13] Naka K., Itoh H. and Chujo Y., *Langmuir*, 2003; **19(13)**: 5496-5501. DOI 10.1021/la026898i.

[14] Yongyat C., Ruchirawat S. and Boonyarattanakalin S., *Bioorg. Med. Chem.*, 2010; **18(11)**: 3726-3734. DOI 10.1016/j.bmc.2010.04.014.

[15] Meester W.J.N., van Maarseveen J.H.; Kirchsteiger K., Hermkens P.H.H., Schoemaker H.E., Hiemstra H. and Rutjes F.P.J.T., *Arkivoc*, 2004; 122-151. DOI 10.3998/ark.5550190.0005.209.

[16] Leelayuwapan H., Siwanartnusorn K., Wattanasiri C., Ruchirawat S. and Boonyarattanakalin S., *Proceedings of the 6th Pure and Applied Chemistry International Conference (PACCON 2012)*, Chiang Mai, Thailand, 11-13 January 2012; 1745-1748.

[17] Yongyat C., Ruchirawat S. and Boonyarattanakalin S., *e-Polymers*, 2014; **14(6)**: 383-390. DOI 10.1515/epoly-2014-0129.