Bioconjugate Chemistry

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Synthesis of 1,2,3-Triazolo-Linked Octyl (1→6)-α-D-Oligomannosides and Their Evaluation in Mycobacterial Mannosyltransferase Assay

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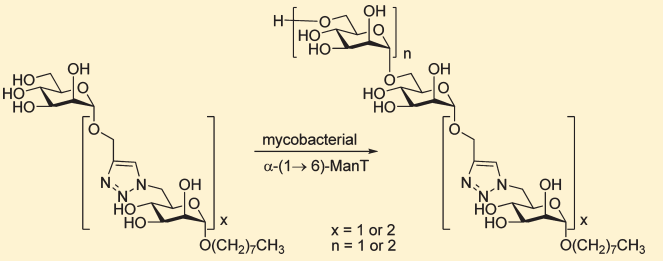
Supporting Information

ABSTRACT: The synthesis of conjugates consisting of two or three mannose units interconnected by a 1,2,3-triazole linker installed by the "click" reaction is reported. These conjugates were evaluated in mycobacterial mannosyltransferase (ManT) assay. Detailed analysis of the reaction products showed that these compounds with triazole linker between sugar moieties were tolerated by the enzyme, which elongated them by one or two sugar units with α-(1→6) linkage. The effectiveness of this transfer was reduced in comparison to that observed for the acceptor analogues containing a glycosidic linkage, but still, this is the first report on such unnatural compounds serving as substrates for mycobacterial ManT. The ability of the studied compounds to function as acceptors for the ManT suggests that the relative distance and spatial orientation of acceptor octyl hydrophobic aglycone (optimal length for the ManT) and free primary C-6 hydroxy group of the nonreducing terminal mannose unit (to which glycosyl residue is transferred by the mycobacterial ManT) are important for ManT activity, but at the same time, their variations are tolerated by the enzyme in a relatively wide range.



INTRODUCTION

The 1,3-dipolar cycloaddition reaction ("click" reaction) is a powerful means for linking two units, one with azide and the other consisting of an alkyne function. The reaction is also widely used for linking a carbohydrate with another carbohydrate or noncarbohydrate unit. Recently, Sharpless et al.[1] and Meldal et al.[2] found out that the use of Cu(I) as catalyst led to an exclusive formation of 1,4-disubstituted 1,2,3-triazole (Figure 1). This finding has raised interest in the use of this kind of chemistry, which is furthermore advantageous in terms of compatibility with various functionalities and of its broad applicability in different solvent systems.

In the field of carbohydrate chemistry, click chemistry has been used for the synthesis of a large number of compounds ranging from small molecules, such as simple glycosides, e.g., *N*-glycosyltriazoles,[3−5] through oligosaccharide mimetics[6−15] to homogeneous and heterogeneous neoglycoconjugates. The latter group of neoglycoconjugates includes glycomacrocycles,[16−18] glycoclusters,[19,20] glycodendrimers,[21−24] glycocyclodextrins,[25,26] and glycopolymers.[27]

Click chemistry has also found applications in the field of mycobacterial investigation. Carbohydrate-derived triazoles with *arabino* and *ribo*-configurations in which the triazole unit was built up at position 5 have been recently synthesized and tested as

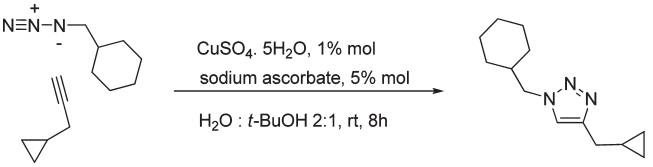


Figure 1. Example of Cu(I)-catalyzed Husigen azide−alkyne 1,3-dipolar cycloaddition reaction.

potential antitubercular agents.[28] A series of *arabino* glycosyl triazoles with varying length of the hydrophobic group has been prepared in order to evaluate their inhibitory activity against *Mycobacterium bovis*. The screening has revealed their low to moderate antimycobacterial activity, which was strongly dependent on the nature of the hydrophobic group.[29] Small molecules for screening a glycosyltransferase activity have also been prepared by the use of the "click" reaction. Thus, a diverse set of organic azides or alkynes was coupled with an appropriate mannose-based alkyne (propargyl function at C-1) or azide (at C-6) derivatives, respectively, in order to construct a library of modified compounds, which were utilized to study the substrate

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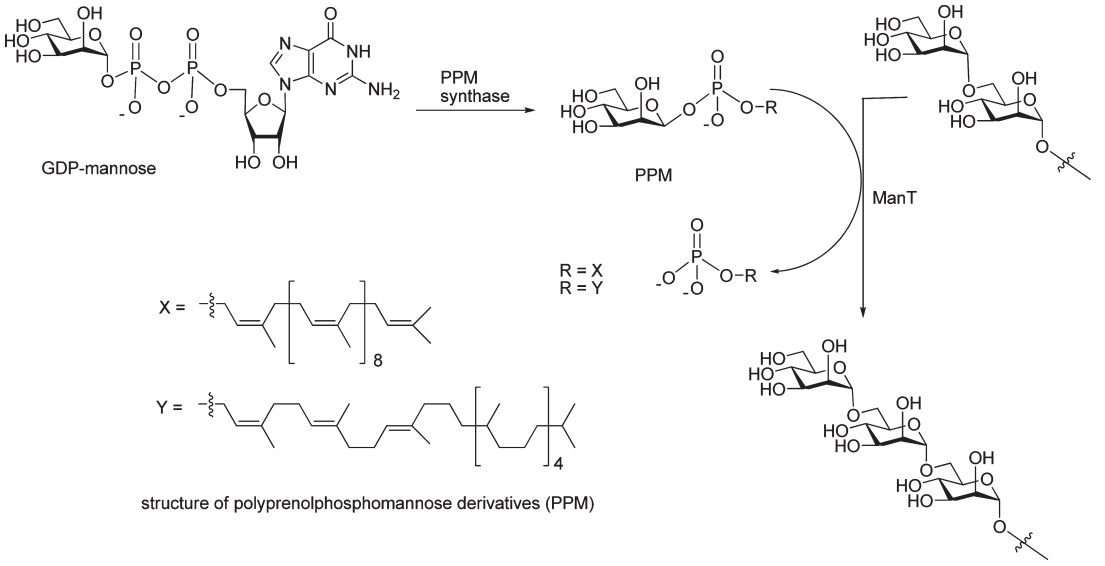


Figure 2. Reaction catalyzed by the polyprenolphosphomannose-dependent α-(1→6)-mannosyltransferase (ManT).

specificity of the leishmanian β-1,2-mannosyltransferases.[30] Very recently, Dondoni's group[31] synthesized a set of *C*-oligomannosides having the triazole ring as the interglycosidic linker and being deoxygenated at the 6-position of the nonreducing terminal mannose residue, and reported their inhibitory activity against mycobacterial ManT.

The aim of this study was to use the Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) reaction for the synthesis of 1,2,3-triazolo-linked octyl (1→6)-α-D-oligomannosides and to investigate whether they are processed by the mycobacterial mannosyltransferase (ManT), whose action is schematically shown in Figure 2. These conjugates consist of two or three mannose moieties, wherein each glycosidic linkage between mannose units is replaced by 1,2,3-triazole spacer, while a glycosidic linkage between the aglycone and the first sugar unit remained intact. Thus, the obtained novel conjugates possess structural features characteristic for the already confirmed synthetic substrates for the ManT: hydrophobic aglycone attached by a glycosidic bond and free primary hydroxyl group at C-6 atom of the nonreducing terminal mannose residue. In these terms, they are similar to octyl (1→6)-α-D-mannooligosaccharides, efficient synthetic acceptor analogues, which have been screened in ManT assay previously.[32,33]

EXPERIMENTAL PROCEDURES

General Methods. TLC was performed on aluminum sheets precoated with silica gel 60 F_{254} (Merck). Flash column chromatography was carried out on silica gel 60 (0.040−0.060 mm, Merck) with distilled solvents (hexanes, ethyl acetate dichloromethane, chloroform, methanol). Reaction solvents were dried and distilled before use. All reactions containing sensitive reagents were carried out under argon atmosphere. ^{1}H NMR and ^{13}C NMR spectra were recorded at 25 °C with a VNMRS 400 MHz Varian spectrometer; chemical shifts are referenced to either TMS (δ 0.00, $CDCl_3$ for ^{1}H) or HOD (δ 4.87, CD_3OD for ^{1}H) and to internal $CDCl_3$ (δ 77.23) or CD_3OD (δ 49.15) for ^{13}C. The assignment of resonances in the ^{1}H and ^{13}C NMR spectra was made by two-dimensional homonuclear and heteronuclear shift correlation experiments. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 20 °C. High-resolution mass spectra (HRMS) and CID spectra were recorded on a MALDI-QqTOF mass spectrometer operating in positive ion mode. Typically, the samples were spotted onto 2,5-dihydroxybenzoic acid matrix predeposited on the surface of a MALDI target. MS/MS spectra were interpreted manually. For the assignment of fragment ions, the general nomenclature established by Domon and Costello was followed.[34] ESI-MS experiments were carried out on a Varian 500 LC-MS Ion Trap equipped with an ESI source. The samples were introduced with carrying solvent acetonitrile−water (1:1). The nebulizing gas pressure was 50 psi and drying gas pressure was 39 psi at 350 °C. Mass spectra were recorded in the positive mode with a scan rate of 3 s/scan. Online LC-MS analysis was performed using an HPLC system (Varian, ProStar). Eluent A was 5% acetonitrile in water and eluent B was 0.01 M formic acid in 90% acetonitrile in water. The proportion of 5% B was maintained for 5 min and then linearly increased to 40% over 55 min. The sample was separated at a flow rate of 0.4 mL/min on Vydac 218-TP54 C18 analytical column (Grace, Hesperia, USA). The column effluent was introduced directly into the mass spectrometer equipped with an electrospray ionization source operated in positive ion mode.

*Octyl 2,3,4-tri-O-acetyl-6-O-tosyl-α-D-mannopyranoside (**2**).* To a stirred solution containing (**1**)[35,36] (0.52 g, 1.78 mmol) in pyridine (7 mL) cooled down in an ice bath, tosyl chloride (0.44 g, 2.31 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The resulting mixture was brought to rt and the stirring was continued for 16 h. The reaction mixture was diluted with CH_2Cl_2 (30 mL); washed with 1 M HCl (5 × 10 mL), satd $NaHCO_3$ (3 × 10 mL), and water (20 mL); dried with anhydrous Na_2SO_4; filtered; and concentrated. The crude product was dissolved in pyridine (4 mL) and cooled down in an ice bath. Acetic anhydride (7 mL) was added dropwise. The resulting mixture was brought to rt and the stirring was continued for 16 h. The reaction mixture was diluted with water (10 mL) and CH_2Cl_2 (30 mL). The organic phase was separated, washed with 1 M HCl (3 × 15 mL) and satd $NaHCO_3$ (3 × 10 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (hexanes/EtOAc 5:1→2:1) to give (**2**) (0.52 g, 51% over 2 steps) as an oil. $[\alpha]_D$ +42 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (d, 2H, *J* 8.2 Hz, Ts), 7.35 (d,

2H, *J* 8.1 Hz, Ts), 5.32 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3), 5.19 (dd, 1H, $J_{2,3}$ 3.4 Hz, H-2), 5.14 (t, 1H, $J_{4,5}$ 10.0 Hz, H-4), 4.72 (d, 1H, $J_{1,2}$ 1.4 Hz, H-1), 4.13−4.11 (m, 2H, H-6a, H-6b), 4.02 (m, 1H, H-5), 3.62 (dt, 1H, *J* 6.7, 9.6 Hz, $OCH_2C_7H_{15}$), 3.37 (dt, 1H, *J* 6.6, 9.6 Hz, $OCH_2C_7H_{15}$), 2.45 (s, 3H, CH_3 (Ts)), 2.12, 1.99, 1.98 (each s, each 3H, 3 × CH_3CO), 1.58−1.53 (m, 2H, $OCH_2CH_2C_6H_{13}$), 1.33−1.27 (m, 10H, $OCH_2CH_2(CH_2)_5CH_3$), 0.89 (t, 3H, *J* 6.8 Hz, $O(CH_2)_7CH_3$). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.3, 170.1, 170.0 (3 × CH_3CO), 145.1, 133.0, 130.0, 128.3 (Ar), 97.6 (C-1), 69.8 (C-2), 69.2 (C-3), 68.8 ($OCH_2C_7H_{15}$), 68.5 (2×) (C-5, C-6), 66.7 (C-4), 32.0, 29.5, 29.4 (2×), 26.2, 22.9 ($OCH_2(CH_2)_6CH_3$), 21.9 (CH_3(Ts)), 21.1, 20.8 (2×) (3 × CH_3CO), 14.3 ($O(CH_2)_7CH_3$). HRMS (MALDI): *m*/*z* calcd for $[C_{27}H_{40}O_{11}S]Na^+$: 595.2189. Found: 595.2199.

*Octyl 2,3,4-tri-O-acetyl-6-azido-6-deoxy-α-D-mannopyranoside (**3**).* Compound (**2**) (0.50 g, 0.87 mmol) and NaN_3 (0.28 g, 4.36 mmol) in DMF (5 mL) were stirred at 80 °C for 40 h. The reaction mixture was cooled to rt, diluted with CH_2Cl_2 (40 mL), and extracted with water (3 × 10 mL) and brine (3 × 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated. Purification by column chromatography (hexanes/EtOAc 5:1→3:1) afforded (**3**) (0.31 g, 80%) as an oil. $[\alpha]_D$ +56 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3), 5.23 (dd, 1H, $J_{2,3}$ 3.5 Hz, H-2), 5.21 (t, 1H, $J_{4,5}$ 10.1 Hz, H-4), 4.80 (d, 1H, $J_{1,2}$ 1.2 Hz, H-1), 3.95 (ddd, 1H, $J_{5,6a}$ 2.4 Hz, H-5), 3.72 (dt, 1H, *J* 6.8, 9.5 Hz, $OCH_2C_7H_{15}$), 3.47 (dt, 1H, *J* 6.6, 9.6 Hz, $OCH_2C_7H_{15}$), 3.37 (dd, 1H, $J_{5,6b}$ 6.9 Hz, $J_{6a,6b}$ −13.3 Hz, H-6b), 3.27 (dd, 1H, H-6a), 2.16, 2.05, 2.00 (each s, each 3H, 3 × CH_3CO), 1.63−1.60 (m, 2H, $OCH_2CH_2C_6H_{13}$), 1.37−1.25 (m, 10H, $OCH_2CH_2(CH_2)_5CH_3$), 0.89 (t, 3H, *J* 6.9 Hz, $O(CH_2)_7CH_3$). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.3, 170.1 (2 ×) (3× CH_3CO), 97.6 (C-1), 70.1 (C-5), 69.9 (C-2), 69.2 (C-3), 68.9 ($OCH_2C_7H_{15}$), 67.6 (C-4), 51.4 (C-6), 32.0, 29.5 (2×), 29.4, 26.3, 22.9 ($OCH_2(CH_2)_6CH_3$), 21.1, 21.0, 20.9 (3 × CH_3CO), 14.3 ($O(CH_2)_7CH_3$). HRMS (MALDI): *m*/*z* calcd for $[C_{20}H_{33}N_3O_8]Na^+$: 466.2166. Found: 466.2183.

*2-Propynyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (**5**).* To a stirred solution containing 1,2,3,4,6-penta-*O*-acetyl-D-mannopyranose (**4**) (1.50 g, 3.84 mmol) in CH_2Cl_2 (20 mL) propargyl alcohol (1.08 g, 1.12 mL, 19.21 mmol) was added. The reaction mixture was stirred for 20 min, cooled down on an ice bath, and $BF_3 \cdot OEt_2$ (5.46 g, 4.86 mL, 38.43 mmol) was added dropwise. The resulting mixture was stirred for 15 min, brought to rt, and the stirring was continued for 24 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and poured into ice-cold water (150 mL) under stirring. The organic phase was separated, washed with satd $NaHCO_3$ (3 × 100 mL) and water (100 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (hexanes/EtOAc 4:1→2.5:1) to give (**5**) as white crystals (1.34 g, 90%). mp = 103−104 °C; lit.[30] 104−106 °C; $[\alpha]_D$ +68 (*c* 1, chloroform); lit.[37] +66.5 (c 0.77, chloroform). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3), 5.26 (t, 1H, $J_{3,4}$ 10.0 Hz, H-4), 5.23 (dd, 1H, $J_{2,3}$ 3.3 Hz, H-2), 5.01 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.32−4.26 (m, 3H, H-6b, $CH_2C{\equiv}CH$), 4.11 (dd, 1H, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ −12.1 Hz, H-6a), 4.00 (ddd, 1H, $J_{5,6b}$ 5.2 Hz, H-5), 2.48 (t, 1H, *J* 2.4 Hz, $CH_2C{\equiv}CH$), 2.16, 2.11, 2.05, 2.00 (each s, each 3H, 4 × CH_3CO). ^{13}C NMR (100 Mz, CD_3OD): δ 170.6, 169.9, 169.8, 169.7 (4 × CH_3CO), 96.2 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ 175.0 Hz), 77.9 ($OCH_2C{\equiv}CH$), 75.6 ($OCH_2C{\equiv}CH$), 69.3 (C-2), 69.0 (C-3), 68.9 (C-5), 66.0 (C-4), 62.3 (C-6), 54.9 ($OCH_2C{\equiv}CH$), 20.8, 20.7, 20.6 (2×) (4 × CH_3CO). HRMS (MALDI): *m*/*z* calcd for $[C_{17}H_{22}O_{10}]Na^+$ 409.1110. Found 409.12.

*2-Propynyl α-D-mannopyranoside (**6**).* Compound **5** was dissolved in MeOH/CH_2Cl_2 (7.5 mL/2 mL) and 1 M MeONa (0.5 mL) was added. The reaction mixture was stirred for 24 h at rt, neutralized with DOWEX 50 H^+-form, filtered, and concentrated. The crude product was purified by column chromatography (CH_2Cl_2/MeOH 10:1→5:1) to give (**6**) as white crystals (0.75 g, 89%). mp = 117−118 °C; lit. 122−123 °C. $[\alpha]_D$ +123 (*c* 1, methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 5.00 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1), 4.31 (d, 2H, *J* 2.4 Hz, $OCH_2C{\equiv}CH$), 3.86 (dd, 1H, $J_{5,6a}$ 2.2 Hz, $J_{6a,6b}$ −11.8 Hz, H-6a), 3.82 (dd, 1H, $J_{2,3}$ 3.0 Hz, H-2), 3.75 (dd, 1H, $J_{5,6b}$ 5.9 Hz, H-6b), 3.72 (dd, 1H, $J_{3,4}$ 9.3 Hz, H-3), 3.65 (t, 1H, $J_{4,5}$ 9.3 Hz, H-4), 3.54 (m, 1H, H-5), 2.88 (t, 1H, *J* 2.4 Hz, $OCH_2C{\equiv}CH$). ^{13}C NMR (100 Mz, CD_3OD): δ 99.9 (C-1), 80.2 ($OCH_2C{\equiv}CH$), 76.1 ($OCH_2C{\equiv}CH$), 75.2 (C-5), 72.6 (C-3), 72.2 (C-2), 68.6 (C-4), 63.0 (C-6), 54.9 ($OCH_2C{\equiv}CH$). HRMS (MALDI): *m*/*z* calcd for $[C_9H_{14}O_6]Na^+$ 241.0688. Found 241.0675.

*2-Propynyl 2,3,4-tri-O-acetyl-6-O-tosyl-α-D-mannopyranoside (**7**).* To a stirred solution containing (**6**) (0.12 g, 0.55 mmol) in pyridine (4 mL) cooled down on an ice bath, tosyl chloride (0.31 g, 1.65 mmol) in CH_2Cl_2 (2 mL) was added dropwise. The resulting mixture was brought to rt and the stirring was continued for 16 h. The reaction mixture was diluted with CH_2Cl_2 (20 mL); washed with 1 M HCl (5 × 10 mL), satd $NaHCO_3$ (3 × 15 mL), and water (20 mL); dried with anhydrous Na_2SO_4; filtered; and concentrated. The crude product was dissolved in pyridine (3 mL) and cooled down on an ice bath. Acetic anhydride (4 mL) was added dropwise. The resulting mixture was brought to rt and stirring was continued for 16 h. The reaction mixture was diluted with water (10 mL) and CH_2Cl_2 (30 mL). The organic phase was separated, washed with 1 M HCl (3 × 15 mL) and satd $NaHCO_3$ (3 × 10 mL), dried with anhydrous Na_2SO_4, filtered, and concentrated. The crude product was purified by column chromatography (hexanes/EtOAc 2:1→1:1) to give (**7**) (0.13 g, 47% over two steps) as an oil. $[\alpha]_D$ +54 (*c* 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, 2H, *J* 8.2 Hz, Ts), 7.35 (d, 2H, *J* 8.1 Hz, Ts), 5.30 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3), 5.23 (dd, 1H, $J_{2,3}$ 3.4 Hz, H-2), 5.16 (t, 1H, $J_{4,5}$ 10.0 Hz, H-4), 4.94 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.21 (d, 2H, *J* 2.1 Hz, $OCH_2C{\equiv}CH$), 4.13−4.11 (m, 2H, H-6a, H-6b), 4.03 (m, 1H, H-5), 2.47−2.44 (m, 4H, CH_3(Ts), $OCH_2C{\equiv}CH$), 2.13, 1.99, 1.98 (each s, each 3H, 3 × CH_3CO). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.1, 170.0, 169.9 (3 × CH_3CO), 145.2, 132.9, 130.1, 128.3 (Ar), 96.2 (C-1), 78.1 ($OCH_2C{\equiv}CH$), 75.9 ($OCH_2C{\equiv}CH$), 69.4 (C-2), 69.1 (C-5), 68.9 (C-3), 68.4 (C-6), 66.4 (C-4), 55.2 ($OCH_2C{\equiv}CH$), 21.9 (CH_3(Ts)), 21.0, 20.8 (2×) (3 × CH_3CO). HRMS (MALDI): *m*/*z* calcd for $[C_{22}H_{26}O_{11}S]Na^+$ 521.1093. Found 521.1096.

*4-(2,3,4,6-Tetra-O-acetyl-α-D-mannopyranosyloxymethyl)-1-(octyl-2,3,4-tri-O-acetyl-6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (**8**).* Compound (**3**) (0.12 g, 0.26 mmol) and compound (**5**) (0.10 g, 0.26 mmol) were dissolved in DMF/H_2O (3 mL/1 mL). Copper sulfate (17.2 mg, 0.11 mmol) and sodium ascorbate (42.8 mg, 0.22 mmol) were added, and the resulting mixture was stirred at rt for 4 h. The mixture was poured into satd. NH_4Cl/H_2O solution (10 mL, 1:1) and extracted with EtOAc (4 × 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated. Purification by column chromatography (hexanes/EtOAc 5:1→1:5) afforded (**8**) as a colorless oil (0.19 g, 89%). $[\alpha]_D$ +28 (*c* 0.5, $CHCl_3$).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (s, 1H, C*H*N), 5.36 (dd, 1H, $J_{2,3}$ 3.4 Hz, $J_{3,4}$ 10.0 Hz, H-3), 5.32−5.29 (m, 2H, H-3′, H-4′), 5.24−5.22 (m, 2H, H-2, H-2′), 5.14 (t, $J_{4,5}$ 10.0 Hz, H-4), 4.95 (d, 1H, $J_{1',2'}$ 1.5 Hz, H-1′), 4.87 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.75 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.67 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.62 (dd, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ −14.3 Hz, H-6a), 4.40 (dd, $J_{5,6b}$ 8.9 Hz, H-6b), 4.30 (dd, $J_{5',6'b}$ 5.1 Hz, H-6′b), 4.18 (dd, H-5), 4.13 (dd, $J_{5',6'a}$ 2.4 Hz, $J_{6'a,6'b}$ −11.9 Hz, H-6′a), 4.05 (m, 1H, H-5′), 3.22 (m, 2H, OCH_2C$_7$H$_{15}$), 2.16, 2.15, 2.12 (2×), 2.04, 2.00, 1.98 (each s, each 3H, 7 × CH_3CO), 1.46−1.40 (m, 2H, OCH$_2$CH_2C$_6$H$_{13}$), 1.31−1.17 (m, 10H, OCH$_2$CH$_2$(CH_2)$_5$CH$_3$), 0.88 (t, 3H, J 6.9 Hz, O(CH$_2$)$_7$CH_3). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.9, 170.4, 170.2, 170.1, 170.0 (2×), 169.9 (7 × CH_3CO), 143.7 (NC=CH), 124.7 (NC=*C*H), 97.6 (C-1), 97.2 (C-1′), 69.8, 69.6, 69.3, 69.2, 69.0 (2×), 67.9, 66.2 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′), 68.7 (OCH_2C$_7$H$_{15}$), 62.6 (C-6′), 61.4 (OCH_2C=CH), 51.3 (C-6), 32.0, 29.5, 29.4, 29.3, 26.2, 22.8 (OCH$_2$(CH_2)$_6$CH$_3$), 21.1, 21.0, 20.9 (2×), 20.8, 20.7 (2×) (7 × CH_3CO), 14.3 (O(CH$_2$)$_7$-CH_3). HRMS (MALDI): m/z calcd for [$C_{37}H_{55}N_3O_{18}$]H$^+$ 830.3558. Found 830.3550.

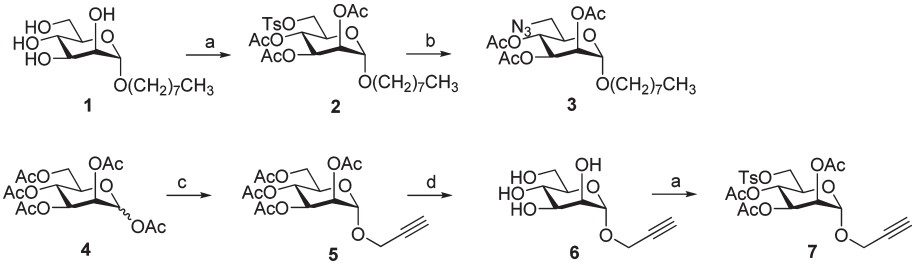
*4-(α-D-Mannopyranosyloxymethyl)-1-(octyl 6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (**9**).* Compound (**8**) (67 mg, 0.08 mmol) was dissolved in MeOH (5 mL), and 1 M MeONa (0.2 mL) was added. The reaction mixture was stirred at rt for 16 h, neutralized with DOWEX 50 H$^+$-form, filtered, and concentrated. The crude product was purified by column chromatography ($CHCl_3$/MeOH 20:1→3:1). Evaporation and lyophilization gave the title compound (**9**) (40 mg, 93%). [α]$_D$ +55 (*c* 0.2, methanol). ^{1}H NMR (400 MHz, CD_3OD): δ 8.05 (s, 1H, C*H*N), 4.89−4.85 (m, 2H, H-1′, H-6a), 4.80 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.68 (d, 1H, $J_{1,2}$ 1.4 Hz, H-1), 4.64 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.50 (dd, 1H, $J_{5,6b}$ 8.7 Hz, $J_{6a,6b}$ −14.1 Hz, H-6b), 3.87 (dd, $J_{5',6'a}$ 1.9 Hz, $J_{6'a,6'b}$ −11.8 Hz, H-6′a), 3.82 (dd, $J_{5,6a}$ 2.2 Hz, H-5), 3.79−3.76 (m, 2H, H-2, H-2′), 3.72 (dd, 1H, $J_{5',6'b}$ 6.0 Hz, H-6′b), 3.69−3.56 (m, 4H, H-3, H-3′, H-4′, H-5′), 3.51 (t, 1H, $J_{3,4}$ 9.4 Hz, $J_{4,5}$ 9.8 Hz, H-4), 3.22 (m, 2H, OCH_2C$_7$H$_{15}$), 1.45−1.20 (m, 12H, OCH$_2$-(CH_2)$_6$CH$_3$), 0.90 (t, 3H, J 6.9 Hz, O(CH$_2$)$_7$CH_3). ^{13}C NMR (100 MHz, CD_3OD): δ 145.1 (NC=CH), 127.1 (NC=CH), 101.6 (C-1), 100.6 (C-1′), 75.1, 73.1, 72.6, 72.5, 72.2, 72.1, 69.8, 68.8 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′), 68.7 (OCH_2C$_7$H$_{15}$), 63.1 (C-6′), 60.6 (OCH_2C=CH), 52.6 (C-6), 33.1, 30.6 (2×), 30.5, 27.5, 23.9 (OCH$_2$(CH_2)$_6$CH$_3$), 14.6 (O(CH$_2$)$_7$CH_3). HRMS (MALDI): m/z calcd for [$C_{23}H_{41}$-N_3O_{11}]H$^+$ 536.2814. Found 536.2811.

*4-(2,3,4-Tri-O-acetyl-6-O-tosyl-α-D-mannopyranosyloxymethyl)-1-(octyl 2,3,4-tri-O-acetyl-6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (**10**).* Compound (**3**) (0.09 g, 0.20 mmol) and compound (**7**) (0.1 g, 0.20 mmol) were dissolved in DMF/H_2O (3 mL/1 mL). Copper sulfate (12.8 mg, 0.08 mmol) and sodium ascorbate (31.8 mg, 0.16 mmol) were added, and the resulting mixture was stirred at rt for 4 h. The mixture was poured into satd. NH_4Cl/H_2O solution (10 mL, 1:1) and extracted with EtOAc (5 × 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (hexanes/EtOAc 6:1→1:5) to give (**10**) as a colorless oil (0.16 g, 85%). [α]$_D$ +38 (*c* 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (s, 1H, C*H*N), 7.80 (d, 2H, J 8.2 Hz, Ts), 7.37 (d, 2H, J 8.1 Hz, Ts), 5.36 (dd, 1H, $J_{3,4}$ 9.9 Hz, H-3), 5.27 (dd, 1H, $J_{2',3'}$ 3.4 Hz, $J_{3',4'}$ 10.0 Hz, H-3′), 5.22 (dd, 1H, $J_{2,3}$ 3.4 Hz, H-2), 5.19−5.11 (m, 3H, H-2′, H-4, H-4′), 4.88 (d, 1H, $J_{1',2'}$ 1.3 Hz, H-1′), 4.80 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.76 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.67 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.62 (dd, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ −14.2 Hz, H-6a), 4.43 (dd, $J_{5,6b}$ 8.9 Hz, H-6b), 4.17 (m, 1H, H-5), 4.11−4.04 (m, 3H, H-5′, H-6′a, H-6′b), 3.22 (m, 2H, OCH_2C$_7$H$_{15}$), 2.46 (s, 3H, CH_3 (Ts)), 2.15, 2.11 (2×), 2.00, 1.98, 1.96 (each s, each 3H, 6 × CH_3CO), 1.45−1.39 (m, 2H, OCH$_2$CH_2C$_6$H$_{13}$), 1.30−1.16 (m, 10H, OCH$_2$CH$_2$(CH_2)$_5$CH$_3$), 0.88 (t, 3H, J 6.9 Hz, O-(CH$_2$)$_7$CH_3). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.4, 170.2, 170.0, 169.9 (2×), 169.8 (6 × CH_3CO), 162.8 (NC=CH), 145.3, 132.8, 130.1, 128.3 (Ar), 125.0 (NC=CH), 97.5 (C-1), 97.1 (C-1′), 69.8, 69.5, 69.2, 69.1, 69.0 (2×), 68.7, 66.5 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′), 68.5 (OCH_2C$_7$H$_{15}$), 67.8 (C-6′), 61.3 (OCH_2C=CH), 51.3 (C-6), 32.0, 29.5, 29.4, 29.3, 26.2, 22.8 (OCH$_2$(CH_2)$_6$CH$_3$), 21.9 (CH_3(Ts)), 21.1, 21.0 (2×), 20.9, 20.8 (2×) (6 × CH_3CO), 14.3 (O(CH$_2$)$_7$CH_3). HRMS (MALDI): m/z calcd for [$C_{42}H_{59}N_3O_{19}S$]H$^+$ 942.3536. Found 942.3526.

*4-(2,3,4-Tri-O-acetyl-6-azido-6-deoxy-α-D-mannopyranosyloxymethyl)-1-(octyl 2,3,4-tri-O-acetyl-6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (**11**).* To a solution of compound (**10**) (0.14 g, 0.15 mmol) in DMF (4 mL), NaN_3 (52 mg, 0.80 mmol) and 15-crown-5 (0.18 g, 0.16 mL, 0.8 mmol) were added. The reaction mixture was stirred at 60 °C for 16 h. The reaction mixture was cooled to rt, diluted with CH_2Cl_2 (30 mL), and extracted with water (3 × 10 mL) and brine (1 × 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated. Purification by column chromatography (hexanes/EtOAc 5:1→1:1) gave (**11**) (0.11 g, 92%) as an oil. [α]$_D$ +36 (*c* 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (s, 1H, C*H*N), 5.36 (dd, 1H, $J_{2,3}$ 3.4 Hz, $J_{3,4}$ 9.9 Hz, H-3), 5.32−5.26 (m, 2H, H-3′, H-4′), 5.25−5.21 (m, H-2, H-2′), 5.14 (t, 1H, $J_{4,5}$ 10.0 Hz, H-4), 4.95 (d, 1H, $J_{1',2'}$ 1.3 Hz, H-1′), 4.90 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.74 (d, 1H, $J_{1,2}$ 1.1 Hz, H-1), 4.69 (d, 1H, J −12.3 Hz, OCH_2C=CH), 4.62 (dd, $J_{5,6a}$ 2.4 Hz, $J_{6a,6b}$ −14.2 Hz, H-6a), 4.40 (dd, $J_{5,6b}$ 8.9 Hz, H-6b), 4.17 (m, 1H, H-5), 4.02 (m, 1H, H-5′), 3.38−3.34 (m, 2H, H-6′a, H-6′b), 3.22 (m, 2H, OCH_2C$_7$H$_{15}$), 2.15 (2×), 2.12, 2.04, 2.00, 1.98 (each s, each 3H, 6 × CH_3CO), 1.46−1.39 (m, 2H, OCH$_2$CH_2C$_6$H$_{13}$), 1.32−1.17 (m, 10H, OCH$_2$CH$_2$(CH_2)$_5$CH$_3$), 0.88 (t, 3H, J 6.9 Hz, O(CH$_2$)$_7$CH_3). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.4, 170.2, 170.1, 170.0, 169.9 (2×) (6 × CH_3CO), 143.5 (NC=CH), 124.8 (NC=CH), 97.6 (C-1), 96.9 (C-1′), 70.5, 69.8, 69.6, 69.3, 69.0, 68.9, 67.8, 67.2 (C-2, C-2′, C-3, C-3′, C-4, C-4′, C-5, C-5′), 68.7 (OCH_2C$_7$H$_{15}$), 61.3 (OCH_2C=CH), 51.3, 51.2 (C-6, C-6′), 32.0, 29.5, 29.4, 29.3, 26.2, 22.8 (OCH$_2$(CH_2)$_6$CH$_3$), 21.1−20.9 (6 × CH_3CO), 14.3 (O(CH$_2$)$_7$CH_3). HRMS (MALDI): m/z calcd for [$C_{35}H_{52}N_6O_{16}$]H$^+$ 813.3512. Found 813.3538.

*4-{2,3,4-Tri-O-acetyl-6-deoxy-6-[4-(2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyloxymethyl)-1H-1,2,3-triazol-1-yl]-α-D-mannopyranosyloxymethyl}-1-(octyl 2,3,4-tri-O-acetyl-6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (**12**).* Compound (**5**) (44 mg, 0.11 mmol) and compound (**11**) (93 mg, 0.11 mmol) were dissolved in DMF/H_2O (2.1 mL/0.7 mL). Copper sulfate (3.6 mg, 0.023 mmol) and sodium ascorbate (9.0 mg, 0.046 mmol) were added, and the resulting mixture was stirred at rt for 4 h. The mixture was poured into satd. NH_4Cl/H_2O solution (10 mL, 1:1) and extracted with EtOAc (5 × 10 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (hexanes/EtOAc 4:1→1:9) to give (**12**) as a colorless oil (0.11 g, 80%).

Scheme 1[a]



[a] Reagents and conditions: (a) 1. TsCl, CH_2Cl_2, py, 16 h, rt; 2. Ac_2O, py, 16 h, rt, 51% over 2 steps for **2**; 47% for **7**; (b) NaN_3, DMF, 80 °C; 40 h, 80%; (c) $BF_3 \cdot OEt_2$, propargyl alcohol, CH_2Cl_2, 24 h, rt, 90%; (d) MeONa, $MeOH/CH_2Cl_2$, 24 h, rt, 89%.

$[\alpha]_D$ +40 (*c* 0.5, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 7.86, 7.67 (each s, each 1H, 2 × C*H*N), 5.35 (dd, 1H, $J_{3,4}$ 10.0 Hz, $J_{2,3}$ 3.6 Hz, H-3), 5.31−5.27 (m, 3H, H-3′, H-3″, H-4″), 5.22 (dd, 1H, H-2), 5.20−5.18 (m, H-2′, H-2″), 5.16 (t, 1H, $J_{3',4'}$ 9.2 Hz, $J_{4',5'}$ 9.6 Hz, H-4′), 5.12 (t, 1H, $J_{4,5}$ 9.8 Hz, H-4), 4.95 (d, 1H, $J_{1'',2''}$ 1.6 Hz, H-1″), 4.91 (d, 1H, $J_{1',2'}$ 1.2 Hz, H-1′), 4.87 (d, 1H, *J* −12.2 Hz, OCH_2C═CH), 4.75 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.68 (d, 1H, *J* −12.3 Hz, OCH_2C═CH), 4.62 (dd, $J_{5,6a}$ 2.6 Hz, $J_{6a,6b}$ −14.1 Hz, H-6a), 4.58 (dd, 1H, $J_{5',6'a}$ 2.7 Hz, $J_{6'a,6'b}$ −13.9 Hz, H-6′a), 4.47 (dd, 1H, $J_{5',6'b}$ 8.6 Hz, H-6′b), 4.43 (d, 2H, *J* −11.8 Hz, OCH_2C═CH), 4.40 (dd, 1H, $J_{5,6b}$ 8.6 Hz, H-6b), 4.32−4.27 (m, 2H, H-5′, H-6″b), 4.16 (m, 1H, H-5), 4.12 (dd, 1H, $J_{5'',6''a}$ 2.2 Hz, $J_{6''a,6''b}$ −12.3 Hz, H-6″a), 4.06 (m, 1H, H-5″), 3.22 (m, 2H, OC$H_2C_7H_{15}$), 2.15, 2.14, 2.13, 2.12, 2.11 (2×), 2.03, 2.00, 1.98, 1.97 (each s, each 3H, 10 × CH_3CO), 1.47−1.40 (m, 2H, OC$H_2CH_2C_6H_{13}$), 1.32−1.19 (m, 10H, $OCH_2CH_2(CH_2)_5CH_3$), 0.88 (t, 3H, *J* 6.9 Hz, $O(CH_2)_7CH_3$). ^{13}C NMR (100 Mz, $CDCl_3$): δ 170.9, 170.3−169.9 (9×) (10 × CH_3CO), 143.8, 143.2 (2 × NC═CH), 125.0, 124.9 (2 × NC═*C*H), 97.5 (C-1), 97.1 (C-1″), 96.8 (C-1′), 69.8, 69.6, 69.4 (2×), 69.2, 69.1, 69.0, 68.9, 68.8, 67.8, 67.6, 66.2 (C-2, C-2′, C-2″, C-3, C-3′, C-3″, C-4, C-4′, C-4″, C-5, C-5′, C-5″), 68.7 ($OCH_2C_7H_{15}$), 62.6 (C-6″), 61.2, 60.9 (2 × OCH_2C═CH), 51.2 (2×) (C-6, C-6′), 32.0, 29.5, 29.3 (2×), 26.2, 22.8 (6 × $OCH_2(CH_2)_6CH_3$), 21.1−20.8 (10 × CH_3CO), 14.3 ($O(CH_2)_7CH_3$). HRMS (MALDI): *m*/*z* calcd for $[C_{52}H_{74}N_6O_{26}]H^+$ 1199.4725. Found 1199.4728.

4-{6-Deoxy-6-[4-(α-D-mannopyranosyloxymethyl)-1H-1,2,3-triazol-1-yl]-α-D-mannopyranosyloxymethyl}-1-(octyl 6-deoxy-α-D-mannopyranoside-6-yl)-1H-1,2,3-triazole (***13***). Compound (**12**) (80 mg, 0.067 mmol) was dissolved in $MeOH/CH_2Cl_2$ (2.2 mL: 0.25 mL) and 1 M MeONa (0.12 mL) was added. The reaction mixture was stirred for 16 h, then neutralized with DOWEX 50 H^+-form, filtered, concentrated, and lyophilized to give the title compound (**13**) (42 mg, 81%). $[\alpha]_D$ +50 (*c* 0.2, methanol). ^1H NMR (400 MHz, CD_3OD): δ 8.10, 7.83 (each s, each 1H, 2 × CHN), 4.85−4.73 (m, 3H), 4.72 (d, 1H, *J* −12.3 Hz, OCH_2C═CH), 4.71 (d, 1H, *J* 1.0 Hz), 4.61 (d, 1H, *J* 0.9 Hz), 4.57 (d, 1H, *J* −12.3 Hz, OCH_2C═CH), 4.48−4.40 (m, 2H), 4.28 (q, 2H, *J* −12.3 Hz, OCH_2C═CH), 3.81−3.37 (m, 14H), 3.14 (m, 2H, OC$H_2C_7H_{15}$), 1.35−1.11 (m, 12H, $OCH_2(CH_2)_6CH_3$), 0.80 (t, 3H, *J* 6.9 Hz, $O(CH_2)_7CH_3$). ^{13}C NMR (100 MHz, CD_3OD): δ 145.3, 144.7 (2 × NC═CH), 127.1, 126.9 (2 × NC═CH), 101.8, 100.9, 100.8 (C-1, C-1′, C-1″), 75.2, 73.3, 73.0, 72.6 (2×), 72.5, 72.3, 72.1, 72.0, 69.8 (2×), 68.8 (C-2, C-2′, C-2″, C-3, C-3′, C-3″, C-4, C-4′, C-4″, C-5, C-5′, C-5″), 68.8 ($OCH_2C_7H_{15}$), 63.2 (C-6″), 60.7 (2×) (2 × OCH_2C═CH), 52.7 (2×) (C-6, C-6′), 33.1, 30.6 (2×), 30.5, 27.5, 23.9 ($OCH_2(CH_2)_6CH_3$), 14.6 ($O(CH_2)_7CH_3$). HRMS (MALDI): *m*/*z* calcd for $[C_{32}H_{54}N_6O_{16}]H^+$ 779.3669; found 779.3673.

In Vitro Mannosyltransferase Assay and Preparation of the Enzyme Reaction Products for Structural Characterization. ManT reactions were performed with mycobacterial membranes and cell wall fractions as the source of the enzyme, as described.[36] The reaction mixtures contained 1.5 mg membrane protein, 1 mg cell wall protein, 0.05 μCi GDP-[^{14}C]mannose (Amersham, 275 mCi/mmol), 60 μM ATP, DMSO in final concn of 0.8% (v/v), 4 mM synthetic acceptors, and buffer A (50 mM MOPS pH 7.9, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) in the final volume of 160 μL. The reactions were incubated for 1 h at 37 °C and then stopped by an addition of 1 mL of 96% ethanol. The reaction products were obtained by *n*-butanol/water partitioning, as described before.[36] The butanol extracts were dried under N_2 and resuspended in *n*-butanol. 10% of these extracts were analyzed by TLC as described below for evaluation of possible inhibitory effect of the tested compounds on the production of the natural mannolipids: PIMs and polyprenylphosphoryl mannoses in the *in vitro* assay. The rest of the sample was subjected to mild acid and mild alkali hydrolyses[36] to reveal the products formed from the synthetic substrates. TLC analysis of the reaction products was performed on aluminum-coated silica 60 F_{254} plate (Merck) developed in $CHCl_3/MeOH/conc\ NH_4OH/H_2O$ (65:25:0.5:4; by vol.). The TLC plates were exposed to X-ray film (Kodak Bio-Max MR) and incorporation of radioactive label to the individual products was quantified by scraping the silica from the corresponding bands to scintillation vials and measuring the radioactivity, as described.[36]

Milligram-scale reactions were carried out with 4 mM compounds (**9**) and (**13**) using the same conditions as described above in the volume of 320 μL, using 1.5 mg membrane protein, 1 mg cell wall protein, and 200 μM cold GDP-Man instead of the radioactive substrate. Dried ethanol extracts collected from 3 reactions, in the case of **13**, and 5 reactions, in the case of **9**, were redissolved in 2 mL of water and loaded on a C18 Sep-Pak cartridge (Waters) preequilibrated with methanol and water. Then, the cartridge was first thoroughly washed with water and finally eluted with methanol. Methanol fractions containing carbohydrate compounds were pooled, dried under nitrogen, and analyzed by mass spectrometry.

RESULTS AND DISCUSSION

Synthesis of the Conjugates. The synthetic route to azido and alkyne building blocks used in the CuAAC reaction is depicted in Scheme 1.

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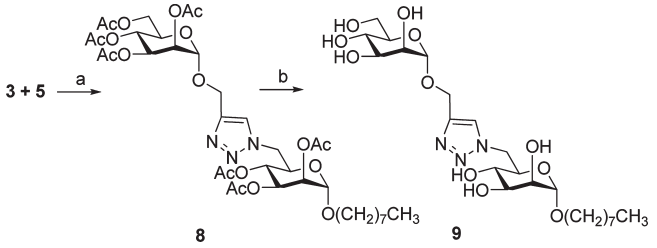
Scheme 2[a]



[a] Reagents and conditions. (a) $CuSO_4$, sodium ascorbate, DMF/H_2O 3:1, 4 h, rt, 89%; (b) MeONa, MeOH, 16 h, rt, 93%.

The alkyne building block, 2-propynyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**5**)[30,37] was accessible with a yield of ~90% in one step by glycosylation of propargyl alcohol with mannose pentaacetate (**4**) in the presence of $BF_3 \cdot OEt_2$ at rt. The synthesis of azide (**3**) bearing hydrophobic octyl aglycone was accomplished by a three-step protocol. Octyl α-D-mannopyranoside (**1**) was converted to acetylated 6-*O*-tosyl derivative (**2**) by a similar procedure as we described recently[36] followed by a treatment of the latter with an excess of NaN_3 in DMF at 80 °C providing the azido building block (**3**). On the basis of previous reports describing a successful improvement of the CuAAC reaction protocol, the coupling of **3** and **5** was carried out at rt for 4 h in DMF/H_2O (3:1) solvent mixture using copper(II) sulfate and sodium ascorbate.[38] Under these conditions, the conjugate (**8**) was obtained in good yield (89%) as a single product (Scheme 2). Its structure was identified based on the characteristic signals in the ^{1}H NMR spectrum: olefinic proton associated with the 1,2,3-triazole moiety at δ = 7.74 as a singlet, and a pair of proton doublets at δ = 4.87 and δ = 4.67 assigned to methylene group linking *O*-1′ atom to the triazole moiety. In the ^{13}C NMR spectrum, the following signals were observed: at δ = 97.6 and δ = 97.2 for two anomeric carbons, and at δ = 124.7 and δ = 143.7 assigned to olefinic carbons. Characteristic signals for two hydrogens ($OCH_2C_7H_{15}$) of octyl chain were slightly shifted and detected as a multiplet at δ = 3.22, while in the starting azide (**3**), they gave a characteristic pattern of dt at δ = 3.72 and δ = 3.47 (each 1H). Removal of acetyl groups from **8** with MeONa in MeOH yielded the unprotected conjugate (**9**), wherein the olefinic proton showed a slight downfield shift (singlet at δ = 8.05).

The construction of conjugate (**12**) consisting of three mannose units interconnected through the 1,2,3-triazole linkers was achieved by a strategy outlined in Scheme 3, starting from a tosylated intermediate conjugate (**10**), since tosyl function remains intact in the CuAAC reaction and can be subsequently subjected to a nucleophilic substitution. The tosylated alkynyl building block (**7**) was prepared by a protocol used for the synthesis of **2**, i.e., conversion of the primary hydroxyl group at C-6 of **6** to the tosylate followed by conventional acetylation of secondary hydroxyl groups (Ac_2O/py). The CuAAC reaction between azide (**3**) and alkyne (**7**) was accomplished under the same experimental conditions as mentioned above giving conjugate (**10**) in high yield (85%). Its structure was clearly established as follows: In the ^{1}H NMR spectrum, the olefinic proton was identified as a singlet at δ = 8.02. The signals of tosyl function were seen as two doublets (2H each) at δ = 7.80 and δ = 7.37 and as a singlet at δ = 2.46 corresponding to a methyl group. In the ^{13}C NMR spectrum, signals for aromatic carbons at δ = 128−145 and for methyl group of the tosyl function at δ = 21.9 were present. In the next step, the tosyl group of **10** was smoothly replaced by reaction with sodium azide under a promotion of 15-crown-5 in DMF at 60 °C providing conjugate (**11**) in almost quantitative yield (92%). In this compound, the chemical shift of C-6′ ($\delta \approx 51$) was markedly upfielded which clearly indicated the introduction of azide. Repetition of the CuAAC reaction utilizing azido (**11**) and alkyne (**5**) building blocks led to a formation of conjugate (**12**) consisting of three mannose units interconnected with 1,2,3-triazole linkers. The presence of two 1,2,3-triazole linkers was assigned from two singlets (δ = 7.86 and δ = 7.67) of the olefinic protons. Final Zemplen deprotection of **12** proceeded smoothly giving the target conjugate (**13**).

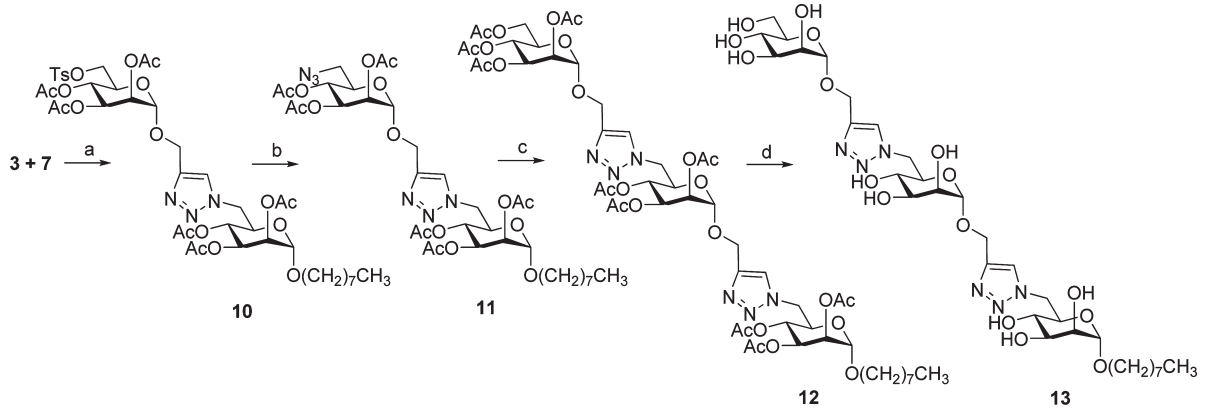
The CuAAC reaction provided simple and regioselective access to the conjugates in which the 1,2,3-triazole ring acts as a bridge between positions 1 and 6 of neighboring mannose units. Keeping structural similarity with natural acceptors, these conjugates are promising compounds for evaluation in ManT assay. In our recent study,[36] octyl α-D-mannopyranoside (**14**) along with octyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (**15**) were recognized as the acceptor substrates in ManT assay, and both compounds (Figure 3) were chosen as the reference synthetic substrates.

Biological Evaluation of the Synthesized Compounds. The synthetic conjugates (**9**) and (**13**) and the reference synthetic substrates (**14**) and (**15**) were tested in the *in vitro* assay monitoring ManT activity of the mycobacterial membranes. First, we evaluated the possible inhibitory effect of these compounds on ManT activity by TLC analysis of the full range of radioactive lipids having incorporated [^{14}C]-Man from GDP-[^{14}C]-Man. Similarly to our previous observation for **14** and **15**,[36] the presence of **9** or **13** in the reaction mixtures did not affect production of labeled phosphatidylinositol mannosides or polyprenylphosphoryl mannoses (data not shown). In a recent study of Lo Conte et al.,[31] the inhibitory effect of triazolo-linked *C*-oligomannosides (dimer to hexadecamer) were, on the contrary, examined in the ManT assay using synthetic octyl (1→6)-α-D-mannodisaccharide as the acceptor substrate and *M. smegmatis* membranes as the enzyme source. Hexamer and octamer were the most potent conjugates showing about 95% inhibition at 1 mM concentration. While elongation of these compounds could not be expected due to their deoxygenation at position 6 of the nonreducing terminal mannose residue, the novel triazolo-linked oligomannosides (**9**) and (**13**) presented in our study possess free hydroxyl group at position 6, which is necessary for their elongation by the mycobacterial α-(1→6)-ManT. Therefore, we aimed to investigate this possibility.

Interestingly, both conjugates (**9**) and (**13**) served as acceptor substrates for the ManT, although their acceptor ability was significantly different. In fact, incorporation of the radioactive label into compound (**13**) was hardly detectable by TLC (Figure 4). In two separate experiments, the conjugate (**9**) having two mannose units was about a 20× less effective acceptor than the disaccharide analogue (**15**), but about a 2× better acceptor than the monosaccharide (**14**). In addition, TLC analysis of the reaction mixture containing conjugate (**9**) showed a ladder-like profile suggesting stepwise mannose addition (Figure 4).

Since TLC analysis indicated that both conjugates (**9**) and (**13**) were transformed by the ManT, the enzymatic reactions of **9** and **13** with unlabeled GDP-Man were conducted on a larger scale to obtain the reaction products in an amount sufficient for detailed

Scheme 3[a]



[a] Reagents and conditions. (a) $CuSO_4$, sodium ascorbate, DMF/H_2O 3:1, 4 h, rt, 85%; (b) NaN_3, 15-crown-5, DMF, 60 °C; 40 h, 92%; (c) (**3**), $CuSO_4$, sodium ascorbate, DMF/H_2O 3:1, 4 h, rt, 80%; (d) MeONa, MeOH/CH_2Cl_2, 16 h, rt, 81%.

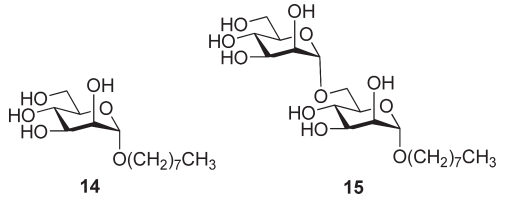


Figure 3. Structures of the reference synthetic substrates **14** and **15**.

structural characterization. HPLC analysis of the products isolated from the reaction mixture containing **9** showed three peaks (Figure 5, a). The mass spectrum obtained from a dominant chromatographic peak (marked as peak 3) with retention time of 24.5 min gave molecular $[M+H]^+$ ion for conjugate (**9**) at m/z 536.5 (Figure 5, a,b3). The chromatographic peak with elution time of 23.5 min (marked as peak 2) produced a mass spectrum with molecular $[M+H]^+$ ion at m/z 698.5, consistent with an expected monomannosylated product (Figure 5, a,b2). Moreover, the chromatographic peak eluting prior to the peaks mentioned above (marked as peak 1, retention time of 22 min) produced an $[M+H]^+$ ion at m/z 860.5 corresponding to a transfer of two mannosyl residues to the acceptor (**9**) (Figure 5, a,b1). The detection of the latter doubly mannosylated product identified by LC/MS is in agreement with the ladder-like TLC profile of the reaction products obtained with this acceptor. Similarly, LC-MS analysis carried out for the reaction mixture of **13** gave three chromatographic peaks with retention times of 23.5, 22.6, and 22.1 min. The molecular $[M+H]^+$ ions obtained from these peaks at m/z values 779.6, 941.8, and 1103.8 (Figure S1, see Supporting Information) clearly indicated the presence of **13** and its monomannosylated and dimannosylated products. The doubly mannosylated **13** detected by LC-MS but not apparent from TLC thus gives additional information about the process activity of the mycobacterial ManT.

In our recent study,[36] in which the same enzyme preparations exhibiting ManT activity were used, we observed that 6-deoxygenated derivatives were not elongated. Therefore, the ManT reaction can be hypothesized to occur at position 6 of the nonreducing terminal mannose unit of conjugates (**9**) and (**13**). This assumption is also supported by previously published reports,[32,33] wherein α-(1→6)-ManT activity of the enzyme was demonstrated.

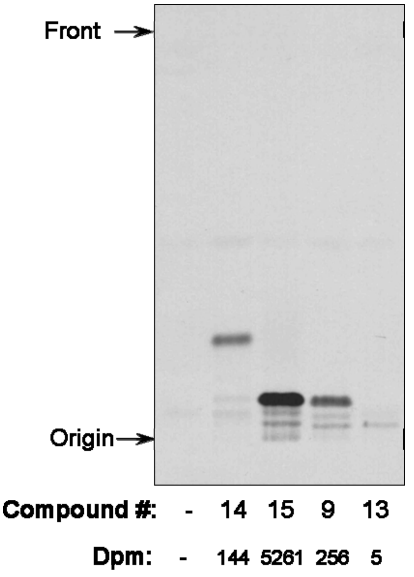


Figure 4. TLC analysis of the reaction products formed from synthetic acceptors **9**, **13**, **14**, and **15** by mycobacterial ManT. The profile is a representation of two replicates of the experiment carried out at 4 mM concentration of the tested compounds. "-" stands for control experiment without any acceptor.

To determine the structure of the monomannosylated product detected in the reaction mixture of **9**, its molecular ion was subjected to fragmentation. The product under MALDI-MS conditions gave $[M+NH_4-H_2O]^+$ molecular ion at m/z 697.33 (calculated monoisotopic mass is 697.32), which yielded the MS/MS fragmentation pattern shown in Figure 6. Y- and Z-fragment ions at m/z 535.23 (Y_4), 373.14 (Y_3), and m/z 357.15 (Z_3) resulted from a characteristic loss of monosaccharide residue(s). The most abundant fragments originated from the mannose cross-ring cleavages (X and A-type fragment ions) and provided additional information on the structure. The majority of the X_4 cross ring ions is isobaric and cannot be used to easily distinguish the linkage. In this regard, the most important ion is that observed at m/z 505.25 ($^{5,6}X_4$), which in association with the Z_4 fragment ion observed at m/z 519.24 could be unambiguously assigned to a

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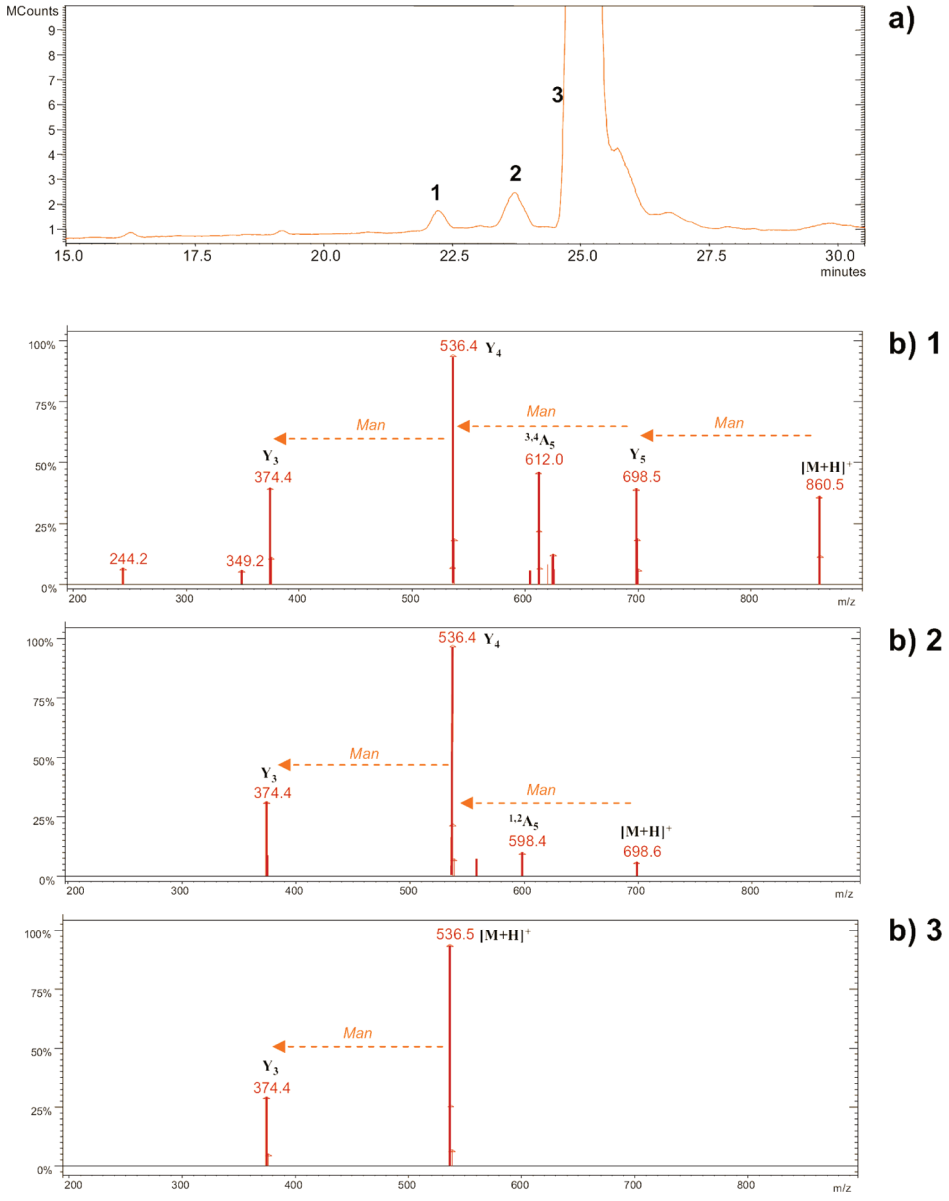

Figure 5. LC-MS analysis of the enzymatic reaction mixture of **9**. Panel a: HPLC profile. Panel b: ESI mass spectra obtained from peaks **1**, **2**, and **3**. For assignment of the fragment ions, see upper part of Figure 6.

new (1→6) linkage in the monomannosylated product of **9** formed by the ManT reaction.

The size of the carbohydrate unit of the acceptor undoubtedly plays an important role in ManT catalysis. As has been previously shown by Lowary et al.,[32] a disaccharide appears to be preferred by the ManT, and octyl (1→6)-α-D-mannodisaccharide (**15**) has been found to serve as the best synthetic acceptor. The enlargement of the acceptor to trisaccharide did not lead to a significant increase in activity, since it was recognized as the acceptor with similar efficiency as the disaccharide (**15**).[32] In the case of triazolo-linked oligomannosides, **13** bearing three mannose units is a much poorer acceptor than **9**, which comprises two mannoses. The latter is elongated by the ManT slightly better than monosaccharide (**14**) but markedly worse than disaccharide (**15**). Hence, the acceptor ability increases in the order (**13**) ≪ (**14**) < (**9**) < (**15**) ≅ octyl (1→6)-α-D-mannotrisaccharide. All these data can be explained by the size of the acceptors, since the triazole linker is much bulkier than a single glycosidic oxygen atom. In addition, the difference in the number of atoms connecting the sugar residues may also result in an alteration of their spatial orientation, which might affect interaction with the enzyme.

It seems that the natural glycosidic linkage is important for keeping the ability of the compound to function as an efficient mannosyl acceptor for the ManT. On the other hand, the transformation of both conjugates performed by the ManT demonstrates that the enzyme has a certain freedom to catalyze the transfer of glycosyl residue(s). The enzyme has at least two structural requirements for the acceptors: hydrophobic aglycone and free primary hydroxyl group at the nonreducing terminal mannose residue. Both these requirements are met not only with the best synthetic acceptors, octyl (1→6)-α-D-Man_2 (**15**) and octyl (1→6)-α-D-Man_3, but also with conjugates (**9**) and (**13**). The differences in the ability of the four evaluated compounds to function as acceptors for the ManT suggest that the relative

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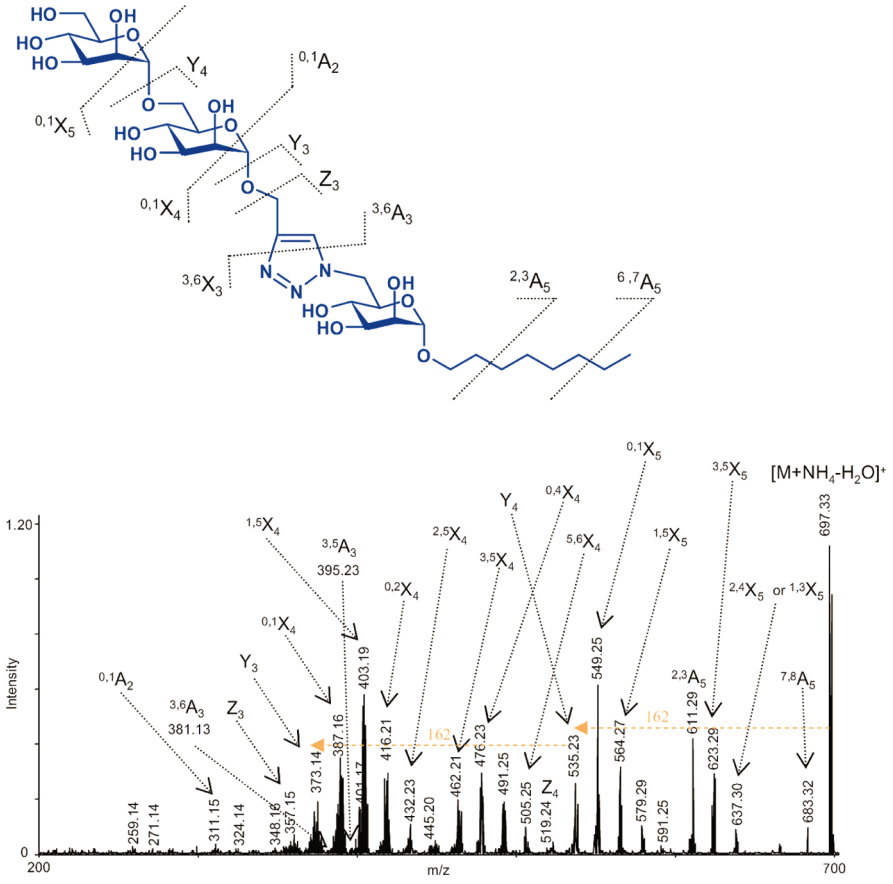

Figure 6. MALDI-MS/MS spectrum and the proposed fragmentation scheme for the monomannosylated product (MW 697.33) generated from **9** by the ManT.

distance and spatial orientation of these structural requirements are important for ManT activity, but at the same time, their variations are tolerated by the enzyme in a relatively wide range.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR and ^{13}C NMR spectra of compounds **2**, **3**, **7**, **8**, **9**, **10**, **11**, **12**, and **13**. Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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