Automated Glycan Assembly of Oligogalactofuranosides Reveals the Influence of Protecting Groups on Oligosaccharide Stability

Narayana Murthy Sabbavarapu and Peter H. Seeberger*

Cite This: J. Org. Chem. 2021, 86, 7280−7287

ABSTRACT: Galactofurans are an important structural constituent of arabinogalactan and lipopolysaccharides (LPS) ubiquitously present on the envelopes of all Mycobacteria. Key to the automated glycan assembly (AGA) of linear galactofuranosides as long as 20-mers was the identification of thioglycoside building blocks with a fine balance of stereoelectronic and steric effects to ensure the stability of oligogalactofuranoside during the synthesis. A benzoylated galactofuranose thioglycoside building block proved most efficient for oligosaccharide construction.

Tuberculosis caused by Mycobacterium tuberculosis (M. tb.), kills more people than any other infectious disease.1 M. tb. bacteria are surrounded by an intricate network of mycolyl chains that form a dense outer hydrophobic framework that is critical for survival and pathogenicity of the organism.2 The TB cell wall consists of two major structural components, arabinogalactan (AG) and lipoarabinomannan (LAM) that are both composed of β-galactose and β-arabinose furanoses. Arabinogalactan consists of a linear galactan backbone of approximately 30 alternating β-(1 → 5)- and β-(1 → 6)-linked galactofuranose (Galf) residues.3 Furanose-containing oligosaccharides are important for microorganisms, but rarely found in humans and other primates. Therefore, the enzymes that are necessary for the construction of galactofuranosyl motifs in microorganisms are attractive targets for the development of new antituberculosis drugs.4 The low abundance of bifunctional galactofuranosyltransferase (GlfT2) and the structural heterogeneity of oligogalactofuranosides limits the access to probes for cell-wall biosynthesis and to determine substrate specificities.5

Well-defined synthetic galactofuranosides that resemble the interior portion of AG are necessary to establish structure−activity relationships for these carbohydrates.6 Solution phase syntheses of galactofuranosyl oligomers ranging from 4 to 12 Galf residues have been reported.7 A stepwise synthesis of a galactan tetramer revealed structural constraints in the trisaccharide nucleophile that resulted in drastically reduced reactivity. Therefore, a “nonreducing to reducing end” strategy relying on monosaccharide nucleophiles was employed, to prepare a tetrasaccharide galactan.7c The synthesis of longer galactofuranosyl oligomers relied on an iterative glycosylation approach.7b,8 A range of galactofuranosides were synthesized to probe substrate specificities in biological systems.9 Most oligosaccharide sequences were prepared via stepwise syntheses that require many discrete operations and multiple purifications.

Automated glycan assembly was developed to accelerate oligosaccharide synthesis.10 Over the past two decades, it has been improved to access more complex glycans.11 However, oligofuranosides were not prepared by AGA beyond short arabinofuranosides.12 To explore the utility of AGA11 to prepare oligogalactofuranosides, we wanted to test the limits of preparing linear galactans found on the surface of M. tb. Here, we disclose the automated synthesis of linear oligogalactofuranoside 20-mer 1 using building blocks with judiciously selected orthogonal protecting groups (Figure 1).

Figure 1. Retrosynthetic analysis of β-(1 → 5)- and β-(1 → 6)-linked linear galactan 20-mer 1.
The power of automated synthesis relies on the section of differentially protected monosaccharide building blocks that result in high yielding and completely selective glycosylations. Galactofuranose thioglycoside building blocks were designed to carry a temporary 9-fluorenylmethoxycarbonyl (Fmoc) protecting group at C-5 (3) or C-6 (4) respectively. A C-2 benzoate provides anchimeric assistance to ensure stereoselectivity for trans-glycosidic linkages. Regioselective benzoylation of thioglycoside 713 was followed by 3-O-benzylation and subsequent aqueous acetic acid mediated hydrolysis of acetonide protection afforded diol 8 (Scheme 1). Trans-

**Scheme 1. Synthesis of Building Blocks 3 and 4**

![Diagram](image)

Reagents and conditions: (a) (1) PhCOCl, Py., 46%; (2) Ag2O, BnBr, 78%; (3) AcOH/H2O, 80%; (b) (1) PhCH(OMe)2, CSA, 72%; (2) Et3SiH, TFA, TFAA, 82%; (3) FmocCl, Py., 87%; (c) (1) TBDPSCl, Im., 82%; (2) Ag2O, BnBr, 75%; (d) (1) TBAF, AcOH, 65%; (2) FmocCl, Py., 74%.

acetalation of 8 with benzaldehyde dimethyl acetal under acidic conditions preceded the regioselective opening of the benzylidene acetal using triethyl silane under acidic conditions before the C-5 hydroxyl was protected with Fmoc to furnish building block 3 in excellent yield. Building block 4 was prepared from 8 by selective protection as the corresponding TBDPS ether and 5-O-benzoylation to access thiofuranoside 9. Selective cleavage of the silyl ether using acetic acid buffered TBAF, followed by installation of the C-6 Fmoc provided building block 4 (Scheme 1).

With thioglycoside building blocks 3 and 4 in hand, photocleavable aminopentanol linker immobilized on poly-styrene resin 2 was placed in the reaction vessel of the automated synthesizer to prepare galactan heptamer 10 (Scheme 2). A four-step AGA process consisting of acidic wash, glycosylation, capping to mask unreacted nucleophiles, and removal of the temporary protecting group to expose the nucleophile for the next glycosylation was executed. UV irradiation using a continuous flow device released the protected oligosaccharide products from the polymer support that were analyzed using analytical HPLC and MALDI. In addition to desired galactan heptamer 10, a host of deletion sequences were obtained. A careful analysis of the deletion sequences revealed that the temporary Fmoc protecting groups remained intact even after treatment with 20% piperidine in DMF. Changing the deprotection solution on the synthesizer to triethylamine (20% in DMF), or DBU (5% in DMF) and a higher reaction temperature (60 °C) failed to cleave Fmoc. The very hydrophobic Fmoc group may interact with hydrophobic regions of the sugar scaffold during oligosaccharide assembly to result in aggregation and poor reactivity. To counteract aggregation and improve resin swelling, dichloromethane was used as solvent and the use of DBU (5% in CH2Cl2) resulted in complete Fmoc cleavage. However, AGA of galactan heptamer 10 using the improved deprotection step revealed unwanted deletion sequences with exposed hydroxyl groups. Apparently, the arming benzyl ethers at C-3, C-6 in building block 3 and C-3, C-5 positions in 4 have profound impact on the stability of the growing galactofuranoside due to intrinsic steric and stereoelectronic effects.14

On the basis of previous observations, we speculated that thiofuranosides 5 and 6 containing disarming benzoate esters may facilitate the assembly of linear oligogalactofuranosanes. Building blocks 5 and 6 were prepared from thiofuranoside 713 by benzylation and isopropylidene cleavage to afford 11. Regioselective benzoylation of 11 at low temperature and placement of Fmoc on the remaining secondary hydroxyl furnished 5. Selective silylation of the 6-hydroxyl in 11 with TBDPSCl and benzylation gave 12. Desilylation of 12 by HF/pyridine followed by Fmoc protection yielded thioglycoside 6 (Scheme 3).

**Scheme 3. Synthesis of Building Blocks 5 and 6**

![Diagram](image)

Reagents and conditions: (a) (1) PhCOCl, Py., 86%; (2) AcOH/H2O, 80%; (b) (1) PhCOCl, Py., CH2Cl2, --60 °C, 82%; (2) FmocCl, Py., 89%; (c) (1) TBDPSCl, imidazole, 85%; (2) PhCOCl, Py., 78%; (d) (1) HF/Py., 72%; (2) FmocCl, Py., 80%.

AGA of galactan heptamer 13 using thiofuranosides 5 and 6 produced a single product according to the HPLC trace of the crude product (Scheme 4 and Figures S1 and S2). This encouraging result prompted us to prepare longer galactofuranose oligomers and to evaluate the influence of the protecting groups on the building blocks (Bn vs Bz) on the stability of growing oligogalactofuranoside. Therefore, using the AGA process developed for shorter sequences, linear galactan 20-mer 1 was assembled using building blocks 5 and 6. HPLC and MALDI analysis of the crude mixture revealed that per-O-benzoylated furanoside glycosides 5 and 6 performed well. The desired product was purified by preparative HPLC and the structural integrity of protected galactofuranoside 20-mer 14 was verified.
was confirmed by $^1$H, $^{13}$C NMR, as well as MALDI mass spectrometry (Scheme 5). Fully protected galactan 14 (17 mg) was treated with sodium methoxide to cleave all benzoyl ester groups, followed by Pd(OH)$_2$/C-catalyzed hydrogenolysis in the presence of hydrogen to cleave the Cbz group furnishing linear galactan 20-mer 1 (2 mg).

In conclusion, we disclose the first automated glycan assembly of oligogalactofuranosides. The identification of differentially protected benzoyl substituted galactofuranose thioglycoside building blocks was key to the successful automated synthesis of the glycans as long as 20-mers found on the cell surface of bacteria. The building blocks will be useful for the construction of many other oligofuranosides.

**EXPERIMENTAL SECTION**

**General Information.** All chemicals used were reagent grade and used as supplied unless otherwise noted. Automated syntheses were performed on a home-built synthesizer developed at the Max Planck Institute of Colloids and Interfaces.15 Merrifield resin LL (100–200 mesh, Novabiochem) was modified and used as solid support.16 Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 F$_{254}$ plates (0.25 mm). Compounds were visualized by UV irradiation or dipping the plate in a p-anisaldehyde (PAA) solution. Flash column chromatography was carried out by using forced flow of the indicated solvent on Fluka Kieselgel 60 M (0.04–0.063 mm). Analysis and purification by normal and reverse phase HPLC was performed using an Agilent 1200 series. Products were lyophilized using a Christ Alpha 2–4 LD plus freeze-dryer. $^1$H, $^{13}$C, and HSQC NMR spectra were recorded on a Varian 400-MR (400 MHz), Varian 600-MR (600 MHz), or Bruker Biospin AVANCE700 (700 MHz) spectrometer. Spectra were recorded in CDCl$_3$ by using the solvent residual peak chemical shift as the internal standard (CDCl$_3$: 7.26 ppm $^1$H, 77.16 ppm $^{13}$C) or in D$_2$O using the solvent as the internal standard in $^1$H NMR (D$_2$O: 4.79 ppm $^1$H) unless otherwise stated. High resolution mass spectra were obtained using a 6210 ESI-TOF mass spectrometer (Agilent) and a MALDI-TOF Autoflex (Bruker). MALDI and ESI mass spectra were run on IonSpec Ultima instruments.

**Automated Synthesis.** Solvents used for dissolving building blocks and preparing the activator, TMSOTf, and capping solutions were taken from an anhydrous solvent system (jcmeyer-solvent systems). Other solvents used were HPLC grade. The building blocks were coevaporated three times with toluene and dried 2 h under a high vacuum before use. Activator, deprotection, acidic wash, capping, and building block solutions were freshly prepared and kept under argon during the automation run. All yields of products obtained by AGA were calculated based on resin loading. Resin loading was determined by performing one glycosylation (Module C) with ten equivalents of building block followed by DBU promoted Fmoc-cleavage and determination of dibenzofulvene production by measuring its UV absorbance.

**Preparation of Stock Solutions.** Fully protected galactan 14 (17 mg) was treated with sodium methoxide to cleave all benzoyl ester groups, followed by Pd(OH)$_2$/C-catalyzed hydrogenolysis in the presence of hydrogen to cleave the Cbz group furnishing linear galactan 20-mer 1 (2 mg).

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was adjusted to 25 °C. Pyridine solution (2 mL, 10% in DMF) was delivered into the reaction vessel. After 1 min, the reaction solution was drained and the resin washed with CH2Cl2 (three times with 3 mL for 25 s). The capping solution (4 mL) was delivered into the reaction vessel. After 20 min, the reaction solution was drained and the resin washed with CH2Cl2 (three times with 3 mL for 25 s).

**Module E: Fmoc Deprotection (14 min)**. The resin was washed with DMF (three times with 2 mL for 25 s) and the temperature of the reaction vessel was adjusted to 25 °C. Fmoc deprotection solution (2 mL) was delivered into the reaction vessel. After 5 min, the reaction solution was drained and the resin washed with DMF (three times with 3 mL for 25 s) and CH2Cl2 (five times each with 2 mL for 25 s). The temperature of the reaction vessel is decreased to −20 °C.
(2S,3R,4R,5R)-4-(Benzyloxy)-2-((5-(tert-Butyl)-2-methylphenyl)thio)-5-((R)-1,2-dihydroxyethyl)tetrahydrofuran-3,4-diyl dibenzoate (9a). To a stirred solution of silyl ether 8a (7.81 g, 10.08 mmol) in anhydrous CH2Cl2 containing 4 Å molecular sieves, was added silver oxide (3.0 g, 4.78 mmol) and BnBr (3.59 mL, 30.22 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was diluted with CH2Cl2 and washed with 1 M HCl, aq. NaHCO3, brine. The combined organic layers were dried over MgSO4, concentrated and purified by column chromatography using silica gel (ethyl acetate/n-hexanes: 20/80) to give 4 in 74% yield (3.0 g, 4.78 mmol) as a white foam. H NMR (400 MHz, CDCl3) δ 7.97–7.90 (m, 2H), 7.73–7.68 (m, 2H), 7.57–7.47 (m, 4H), 7.34 (d, J = 7.7, 3.2 Hz, 4H), 7.31–7.20 (m, 7H), 7.14–7.08 (m, 6H), 7.04 (d, J = 8.0 Hz, 1H), 5.58 (s, 1H), 5.49 (s, J = 1.6 Hz, 1H), 4.72 (d, J = 11.9 Hz, 1H), 4.59 (s, J = 11.4 Hz, 1H), 4.51 (d, J = 6.1, 3.2 Hz, 1H), 4.44–4.23 (m, 6H), 4.15 (s, J = 7.4 Hz, 1H), 4.07 (d, J = 6.1, 1.3 Hz, 1H), 3.80 (dod, J = 6.9, 5.1, 3.2 Hz, 1H), 2.33 (s, 3H), 1.21 (s, 9H), 1.13 (s, 9H). 13C{1H} NMR (100 MHz, CDCl3) δ 165.5, 154.0, 149.7, 137.0, 137.6, 137.5, 137.2, 137.1, 136.5, 135.6, 135.6, 133.6, 133.2, 132.6, 130.2, 130.1, 129.9, 129.3, 128.6, 128.5, 128.4, 128.2, 128.1, 127.9, 125.2, 91.1, 83.1, 83.1, 82.5, 73.1, 72.5, 62.5, 34.5, 31.4, 20.5; ESI HR-MS m/z [M + Na]+ calcd. for C47H54NaO6SSi: 781.3281, found 781.3293.

(2R,3R,4R,5R)-5-((R)-(2H-Furoyl-9-yl)ethoxy)carbonyl)-1-(benzyloxy)ethyl-4-((benzyloxy)-2-((5-(tert-butyl)-2-methylphenyl)thio)tetraketahydrofuran-3-yl benzoate (11a). To a solution of 7a (5.0 g, 13.07 mmol) in pyridine was added PhCOCl (3.34 mL, 28.75 mmol) dropwise at 0 °C, and the resulting mixture was gradually warmed to room temperature. The reaction mixture was stirred for 4 h at the same temperature, at the end of which time TLC indicated it was finished. The reaction was quenched with MeOH, diluted with CH2Cl2, and the mixture was washed with 1 M HCl, aq. NaHCO3, brine and dried over MgSO4. The combined organic layers were filtered, and concentrated. The residue was purified by silica gel column chromatography (ethyl acetate/n-hexanes: 20/80) to afford corresponding 2,3-O-benzoylated derivative in 86% yield (6.64 g) as a glasy liquid.

To a stirred solution of 2,3-O-benzoylated derivative (6.64 g, 11.24 mmol) in 80% aqueous acetic acid was stirred at 80 °C for 5 h. After completion of the reaction, the reaction mixture was concentrated and the residue was purified by column chromatography on silica gel (ethyl acetate/n-hexanes: 60/40) to give 11 in 80% yield (4.95 g) as a colorless syrup. H NMR (400 MHz, CDCl3) δ 8.17–8.11 (m, 2H), 8.09–8.03 (m, 2H), 7.69–7.55 (m, 3H), 7.53–7.42 (m, 4H), 7.30–7.23 (m, 1H), 7.18 (d, J = 8.0 Hz, 1H), 5.74 (s, J = 1.6 Hz, 1H), 5.70 (d, J = 4.3, 1.5 Hz, 2H), 4.59 (dd, J = 5.2, 3.0, 0.9 Hz, 1H), 4.18 (q, J = 4.2 Hz, 1H), 3.96–3.70 (m, 2H), 2.73 (s, J = 7.9 Hz, 1H), 2.46 (s, 3H), 1.30 (s, 9H). 13C{1H} NMR (100 MHz, CDCl3) δ 166.1, 165.4, 149.9, 137.6, 133.9, 133.8, 131.9, 131.0, 130.3, 130.2, 129.0, 128.7, 128.7, 125.7, 91.4, 84.3, 82.1, 78.2, 77.3, 70.5, 64.4, 34.5, 31.4, 20.6; ESI HR-MS m/z [M + Na]+ calcd. for C63H52NaO7S: 1107.3923, found 1107.3923.
pyridine (2.02 mL, 25.15 mmol) was added and stirred for 5 min. Then, PhCOCI (0.65 mL, 5.63 mmol) was added dropwise and stirred for 30 min at 60 °C. The reaction progress was monitored by TLC. After 0.5 h, the reaction was completed and MeOH was added to quench the reaction. The reaction mixture was diluted with CHCl₃ and washed with 1 M HCl,aq. NaHCO₃, brine and dried over MgSO₄. The combined organic layers were filtered, and concentrated. The residue was purified by silica gel column chromatography (ethyl acetate/THF: 20/80) to afford 11a in 82% yield (2.70 g) as a light-brown sticky liquid.

1H NMR (400 MHz, CDCl₃) δ 8.17–8.11 (m, 2H), 7.98–7.92 (m, 2H), 7.75 (d, J = 7.6 Hz, 2H), 7.68 (d, J = 2.1 Hz, 1H), 7.65–7.58 (m, 2H), 7.54–7.43 (m, 3H), 7.42–7.32 (m, 4H), 7.31–7.19 (m, 5H), 7.16 (d, J = 8.0 Hz, 1H), 5.80 (d, J = 1.4 Hz, 2H), 5.77 (dd, J = 7.8, 3.9 Hz, 1H), 5.67 (dt, J = 4.8, 1.3 Hz, 1H), 4.87 (t, J = 4.3 Hz, 1H), 4.75 (d, J = 11.9, 4.0 Hz, 1H), 4.65 (dd, J = 12.0, 7.7 Hz, 1H), 4.38 (dd, J = 10.4, 8.1 Hz, 1H), 4.25 (d, J = 10.4, 7.3 Hz, 1H), 4.18–4.09 (m, 1H), 2.46 (s, 3H), 1.30 (s, 9H); 13C{1H} NMR (100 MHz, CDCl₃) δ 166.0, 165.7, 165.4, 155.0, 149.9, 143.5, 143.1, 141.3, 141.2, 137.4, 133.8, 133.7, 133.2, 130.2, 130.0, 129.1, 129.5, 129.0, 128.9, 128.7, 128.6, 128.4, 127.9, 127.3, 127.3, 125.3, 125.4, 125.3, 120.1, 121.5, 121.5, 121.4, 115.6, 77.6, 74.6, 75.4, 5.4, 6.4, 20.6; ESI HR-MS m/z [M + Na]+ calcld. for C₃₂H₃₄NaO₈S: 899.2866, found 899.2877.

To a stirred solution of 2,3,5-oligosaccharide (17 mg) in methanol:CH₂Cl₂ (1:1), and stirred at 4 °C for 3 h. After completion of the reaction as indicated by TLC, the reaction mixture was diluted with ethyl acetate, aq. NaHCO₃ was added to quench the excess of acid and the colorless liquid was obtained in 82% yield (2.70 g) as a white foam: 1H NMR (400 MHz, CDCl₃) δ 7.89–7.83 (m, 2H), 7.77–7.69 (m, 2H), 4.87 (t, J = 4.3 Hz, 1H), 4.75 (d, J = 11.9, 4.0 Hz, 1H), 4.65 (dd, J = 12.0, 7.7 Hz, 1H), 4.38 (dd, J = 10.4, 8.1 Hz, 1H), 4.25 (d, J = 10.4, 7.3 Hz, 1H), 4.18–4.09 (m, 1H), 2.46 (s, 3H), 1.30 (s, 9H); 13C{1H} NMR (100 MHz, CDCl₃) δ 166.0, 165.7, 165.4, 155.0, 149.9, 143.5, 143.1, 141.3, 141.2, 137.4, 133.8, 133.7, 133.2, 130.2, 130.0, 129.1, 129.5, 129.0, 128.9, 128.7, 128.6, 128.4, 127.9, 127.3, 127.3, 125.3, 125.4, 125.3, 120.1, 121.5, 121.5, 121.4, 115.6, 77.6, 74.6, 75.4, 5.4, 6.4, 20.6; ESI HR-MS m/z [M + Na]+ calcld. for C₃₂H₃₄NaO₈S: 899.2866, found 899.2877.

(2S,3R,4R,5R)-2-((5-(tert-Butyl)-2-methylphenyl)thio)-5-((R)-2-methyl-5,8-diethyltetrahydrofuran-3,4-diyl dibenzoate (11b). Compound 11 (2.7 g, 4.54 mmol) was dissolved in anhydrous CHCl₃ and cooled to 0 °C. tert-Butylidenephosphinyl chloride (1.29 mL, 4.99 mmol) was added dropwise, followed by the addition of imidazole (0.77 g, 11.35 mmol). The reaction mixture was allowed to attain the room temperature under stirring, and the reaction was monitored by TLC, which indicated the completion after 3.5 h. The reaction was diluted with CHCl₃ and water, and the two layers were separated. The aqueous layer was thoroughly washed with CHCl₃ and the combined organic layers were washed with brine solution and dried over anhydrous MgSO₄. The solvent was evaporated to dryness and the residue was subjected to column chromatography (ethyl acetate/THF: 20/80) to afford 11b in 88% yield (3.21 g) as a light yellow liquid.
room temperature for 16 h, neutralized with Amberlite ion exchange (H⁺) resin, filtered and concentrated in vacuo and carried forward directly into hydrolysis without purification. The Zemplén methanolysis product was dissolved in EtOAc−BuOH:H₂O (2:1:1) and transferred to cylindrical vials. Pd(OH)₂/C (10%), (100 wt %) was added and the reaction mixture was stirred in hydrogen reactor with 5 bar pressure for 4 h. The reaction mixture was filtered through a pad of Celite and washed with methanol and water. The filtrates were concentrated in vacuo and purified on size exclusion chromatography (Method B) Synergi Hydro RP18 column and lyophilized to give a pure compound 1 in 40% yield over two steps (2 mg) as a white fluffy solid. Analytical data for 1: ¹H NMR (700 MHz, D₂O) δ 5.38–5.13 (m, 17H), 5.08–4.97 (m, 3H), 4.34 (d, J = 3.4 Hz, 2H), 4.21–4.12 (m, 48H), 4.11–4.06 (m, 8H), 4.03–3.96 (m, 17H), 3.92 (dd, J = 14.3, 8.5, 3.2 Hz, 4H), 3.83 (t, J = 4.8 Hz, 33H), 3.79–3.59 (m, 10H), 3.09–3.00 (m, 2H), 1.80–1.63 (m, 4H), 1.53–1.43 (m, 2H); ¹³C (¹H) NMR (175 MHz, D₂O) δ 76.4, 76.0, 75.5, 70.6, 70.5, 68.1, 62.8, 62.8, 61.1, 60.9, 39.4, 28.1, 26.4, 22.2; [MALDI-TOF m/z [M + Na]+ calcd. for C₁₂₅H₂₁₁N₃NaO₁₀₁: 3367.1460, found 3367.1450.

ASSOCIATED CONTENT

- Supporting Information
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.1c00505.

  Copies of ¹H NMR, ¹³C NMR, HPLC chromatograms, and MALDI (PDF)

AUTHOR INFORMATION

Corresponding Author
Peter H. Seeberger – Department of Biomolecular Systems, Max-Planck-Institute of Colloids and Interfaces, 14476 Potsdam, Germany; Freie Universität Berlin, Institute of Chemistry and Biochemistry, 14195 Berlin, Germany;

orcid.org/0000-0003-3394-8466;

Email: peter.seeberger@mpikg.mpg.de

Author
Narayana Murthy Sabbavarapu – Department of Biomolecular Systems, Max-Planck-Institute of Colloids and Interfaces, 14476 Potsdam, Germany

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.joc.1c00505

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous financial support of the Max-Planck Society. All of the acknowledged work was performed at the Max Planck Institute of Colloids and Interfaces.

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