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# Synthesis of the *C1*-phosphonate analog of UDP-GlcNAc

Robert Chang, Thanh-Trang Vo and Nathaniel S. Finney*

*Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093-0358, USA*

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**Abstract**—We describe the first synthesis of the *C1*-phosphonate analog of UDP-GlcNAc, based on a new preparation of the corresponding glycosyl phosphonate. This *C*-glycosyl analog is shown to be a very weak inhibitor ($K_i > 10$ mM) of fungal chitin synthase, indicating that at least in this case the replacement of the anomeric oxygen with a methylene group is not an innocent substitution.


*Keywords:* UDP-GlcNAc; Glycosyl phosphonate; Chitin synthase; Glycosyl transferase inhibition

## 1. Introduction

Nucleotide diphosphate (NDP) sugars and glycosyl phosphates play a central role in glycobiology.[1] Accordingly, significant effort has been devoted to the development of NDP sugar mimics, such as *C*-glycosyl analogs, as inhibitors of the associated enzymes.[2] The ubiquitous NDP UDP-GlcNAc (**1**; Fig. 1) is polymerized by the fungal enzyme chitin synthase (CS) to form chitin (poly-β-(1→4)-GlcNAc), an essential component of the fungal cell wall (Fig. 2).[3] CS is absent in humans, and it is thus an attractive therapeutic target. In spite of this, relatively little progress has been made in developing CS inhibitors.[4]

In the course of our efforts to develop new CS inhibitors,[5] we became interested in obtaining **2**, the phosphonate analog of UDP-GlcNAc. *C*-Glycosyl phosphonate derivatives are well established as inert isosteres of the

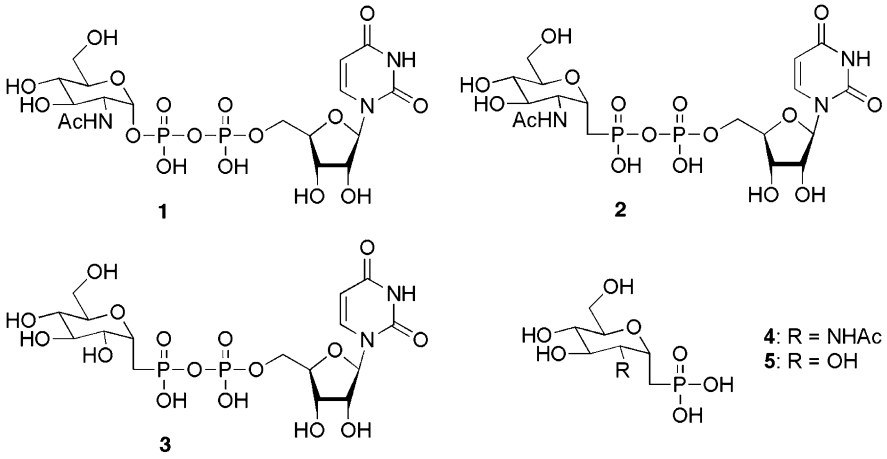


**Figure 1.** UDP-GlcNAc and related phosphonates.

* Corresponding author at present address: Organic Chemistry Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. E-mail: finney@oci.unizh.ch

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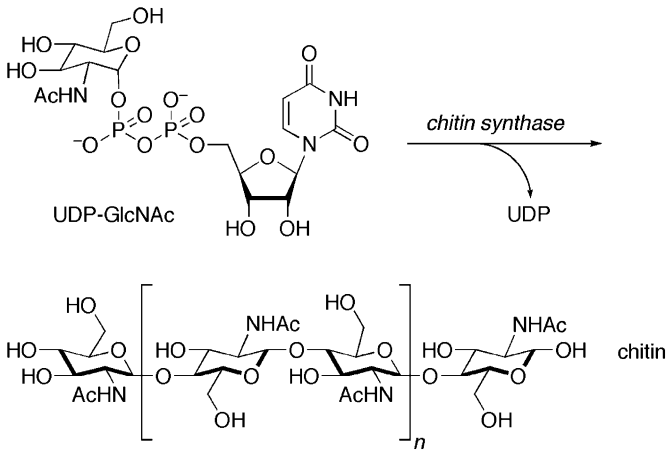


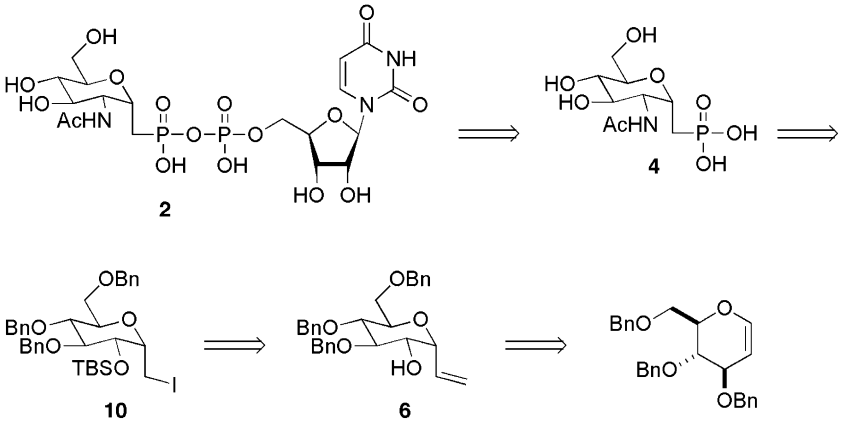

**Figure 2.** Biosynthesis of chitin from UDP-GlcNAc.

corresponding phosphates.[2] While there is only a single report of the synthesis and evaluation of the UDP-glucose analog (**3**) (arguably the simplest phosphonate isostere),[6] several syntheses of phosphonate analogs of α-D-glucose-1-phosphate have been reported, and the *C*-glycosyl analog of α-D-glucose (**5**) has been studied as a substrate-analog inhibitor of several enzymes.[7] In contrast, there are no biological studies of either **2** or the corresponding α-D-GlcNAc-1-phosphate analog **4**. This can be attributed to limited synthetic availability: to our knowledge, **2** has never been prepared, and only a single synthesis of phosphonate **4** has been achieved, although related *C*-glycosyl compounds have been synthesized.[8] The first synthesis of **2** is reported here, based on a new synthesis of a precursor to **4** and **5**.

## 2. Results and discussion

### 2.1. Overview of the synthesis of *C*-glycosyl derivatives 2 and 4

In the retrosynthetic direction, **2** was envisioned to arise from coupling of an activated uridine 5′-monophosphate (UMP) equivalent with **4** (Scheme 1). The previous reported synthesis of **4** began with 2,3,5-tri-*O*-benzyl-arabinofuranose and provided **4**, via intermediate iodide **10**, in 12 steps with a reported overall yield of 14% (Fig. 3).[8f] A report of the apparently problematic behavior of the organomercury intermediates,[7b] coupled with the emergence of direct methods for vinylation of glucal epoxides,[9] prompted us to prepare **10** from the




**Scheme 1.** Retrosynthetic overview.

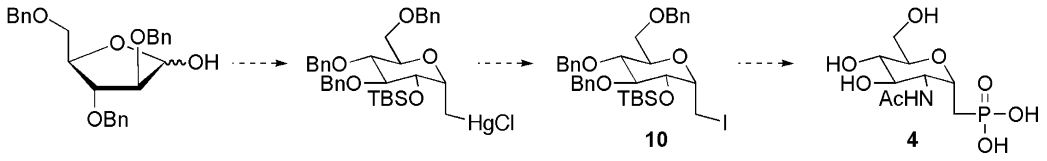



**Figure 3.** The previous synthesis of **4**.

corresponding alcohol, available in one step from the α-vinyl glycosyl compound **6**, which could in turn be prepared in two steps from the commercially available 1,5-anhydro-2-deoxy-3,4,6-tri-*O*-benzyl-D-*arabino*-hex-1-enitol (tri-*O*-benzyl-D-glucal).

### 2.2. Synthesis of iodide 10

Our synthesis of **10** began with tri-*O*-benzyl-D-glucal (Scheme 2). Epoxidation with 3,3-dimethyldioxirane (DMDO),[10] followed by aluminum-mediated vinylation provided **6** in 75% yield over two steps.[9] TBS protection of the C-2 hydroxyl group was successfully achieved with TBSOTf (TBSCl proving ineffective), affording silyl ether **7** in high yield. Subsequent ozonolysis and in situ reduction with $NaBH_4$ furnished the alcohol **8**. While direct iodination of **9** with iodine and triphenylphosphine was unsuccessful, **8** could be quantitatively converted to the corresponding mesylate **9** and treated with tetrabutylammonium iodide (TBAI) in DMF at 120 °C, providing the desired iodide **10** in six operations and 28% overall yield from tri-*O*-benzyl-D-glucal.[11] In comparison, the previous report of **10** describes its preparation in three steps and 80% yield from 2,3,5-tri-*O*-benzyl-β-D-arabinofuranose. However, the new synthesis described here is operationally simple and avoids toxic and potentially problematic organomercury intermediates.

### 2.3. Synthesis of *C*-glycosyl derivative 4

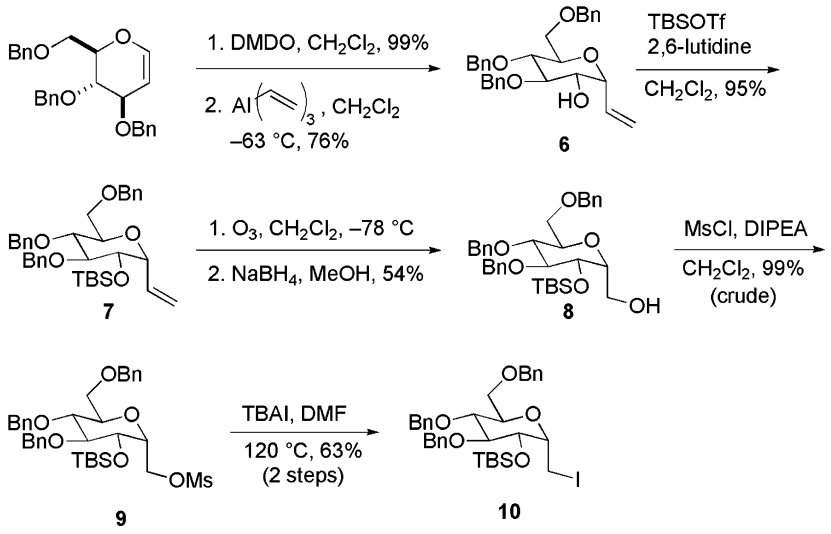
*C*-Glycosyl compound **4** was prepared from **10** by the known route (Scheme 3).[8i] Iodide **10** is also an established precursor to **5**.

### 2.4. Synthesis of the nucleoside phosphonate 2

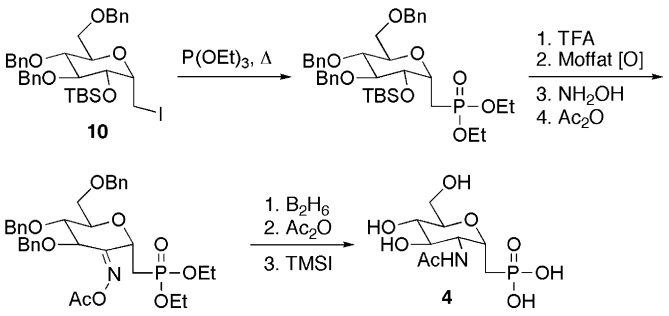
The synthesis of **2** was effected via the Moffatt–Wong protocol (Scheme 4),[11] by which treatment of **4** with uridine 5′-monophosphomorpholidate in the presence of 1-*H*-tetrazole provided **2** in 37% yield after purification.

### 2.5. Biological evaluation of 2

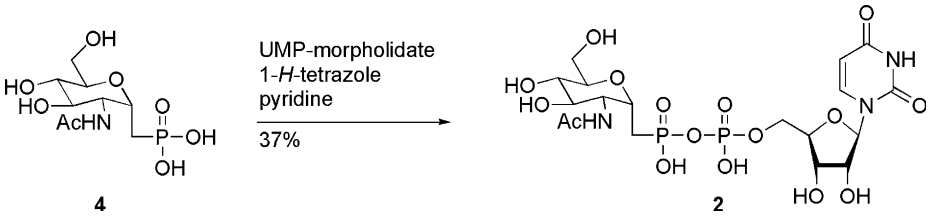
We have conducted chitin synthase inhibition assays with **2** (see Section 4 for details). Our standard chitin synthase assay is conducted using freshly isolated




**Scheme 2.** Synthesis of intermediate iodide **10**.




**Scheme 3.** Preparation of *C*-glycosyl derivative **4**.




**Scheme 4.** Conversion of **4** to **2**.

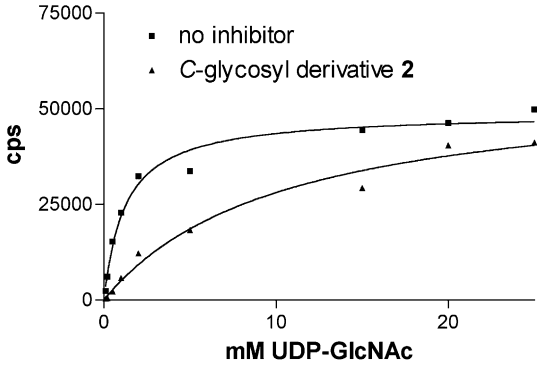



**Figure 4.** Inhibition of CS by **2**.

chitin synthase from *Saccharomyces cerevisiae*, in pH 7.6 buffer, using a combination of UDP-GlcNAc and $^{3}$H-UDP-GlcNAc (ca. $5 \times 10^{-8}$ mol/assay vial, combined).[5,12] At a fixed time (typically 1 h), the reaction is terminated and the chitin is precipitated by addition of trichloroacetic acid and ethanol. The precipitated chitin is isolated by filtration, and chitin formation is quantified by scintillation counting of the filter pad. Carrying out the standard radioactivity-based assay for CS activity in the absence and presence of **2** (4 mM) and several concentrations (1–25 mM)[†] of UDP-GlcNAc revealed that **2** is best a weak inhibitor of CS ($K_i > 10$ mM; Fig. 4). While the inhibition curve is qualitatively consistent with competitive inhibition, this could not be definitively demonstrated—full activity was not recovered at 25 mM UDP-GlcNAc, and at higher substrate concentrations the assay becomes unreliable.

It is instructive to compare the observed $K_i$ (>10 mM) with the $K_M$ for UDP-GlcNAc (~1 mM) and the $K_i$ for UDP itself (~1 mM).[3] Treating $K_M$ as a qualitative measure of substrate binding, this comparison indicates that the majority of binding affinity derives from the nucleotide portion of the substrate. This is not in itself surprising, given that GlcNAc has no inhibitory effect on chitin synthase activity. However, it also indicates that binding affinity is not simply a matter of having a negatively charged nucleoside diphosphate present, since replacement of the anomeric oxygen does not alter the formal charge of the *C*-glycosyl compound **2**.

[†] In order to conserve material (i.e., **2**), we opted to carry out inhibition studies at fixed inhibitor concentration and variable substrate concentration. While this is not the conventional method, we have previously shown it to be adequate for $K_i$ determination (Ref. 5). In principle, this approach also allows demonstration of competitive inhibition—even at fixed inhibitor concentration, addition of excess substrate should eventually lead to recovery of full activity if the inhibitor is competitive.

## 3. Conclusion

We have reported the first synthesis of **2**, as well as a mercury-free synthesis of **4**. This is significant in its own right, as GlcNAc transferases are ubiquitous and both **2** and **4** should prove to be valuable tools for studying these enzymes. However, as a cautionary note, we have shown that **2** is, at best, a very weak inhibitor ($K_i > 10$ mM) of chitin synthase. This indicates that, at least for this enzyme, the replacement of the anomeric phosphonate oxygen with a methylene group—a widespread strategy in the synthesis of sugar phosphate and NDP sugar analogs—is not an innocent substitution.

## 4. Experimental

### 4.1. General

All reactions were carried out in oven or flame dried glassware, under an atmosphere of $N_2$, unless otherwise noted. THF and $CH_2Cl_2$ were dried by passage through an activated column of alumina, and pyridine was distilled from $CaH_2$. All other reagents were used as received unless otherwise stated. Thin layer chromatography was performed on Silica Gel 60 plates ($F_{254}$, EM Science) and visualized with UV light or stained with $KMnO_4$, ninhydrin, or phosphomolybdic acid. Flash column chromatography was performed using silica gel (Selecto Scientific, 32–63 μm) or reverse phase silica gel (EM Science, Silica Gel 60, RP-18) as indicated. IR spectra were recorded using a Nicolet 550 spectrometer. $^{1}$H NMR data were acquired on a Varian HG-400 (400 MHz) spectrometer and are reported in ppm relative to residual monoprotium solvent ($CHCl_3$ at 7.26 ppm, $CHD_2OD$ at 3.30 ppm, HOD at

4.67 ppm). Proton decoupled $^{13}C$ NMR spectra were obtained on a Varian HG-400 (100 MHz) spectrometer and are reported in ppm relative to solvent as internal standard ($CDCl_3$ at 77.0 ppm, $CD_3OD$ at 49.0 ppm, added $CH_3OH$ at 49.5 ppm for $D_2O$). High resolution mass spectra were obtained on an Ionspec Ultima FTMS (MALDI-FTMS) at the Scripps Research Institute, La Jolla, CA. 1-Deoxy-1-α-ethenyl-3,4,6-tri-*O*-benzyl-D-glucose (**6**) was prepared by the method of Rainier et al.[9] Iodide **10** was converted to phosphonate **4** by the method of Casero et al.[8i] The identity of known intermediates was confirmed by comparison to reported analytical data. The chitin synthase assay protocol used is based on the procedure of Orlean,[12] modified after helpful discussions with the author.[5] *S. cerevisiae* strains (PP-1D, wild type) were kindly provided by Professor Orlean and were stored at −70 °C on freezer stabs.

### 4.2. *C*-Glycosyl analog of UDP-GlcNAc (2)

A soln of **4** (0.034 g, 0.11 mmol, 1 equiv) in dry pyridine (0.5 mL) was added to a soln of 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (0.023 g, 0.33 mmol, 3.0 equiv), and 1-*H*-tetrazole (0.031 g, 0.44 mmol, 4.0 equiv) in dry pyridine (0.5 mL) and stirred for 5 d at room temperature. The soln was then concentrated under diminished pressure and applied to size-exclusion gel (Bio-Rad, Bio-Gel, P-2 Fine, 2.5 × 100 cm), eluted with 0.25 M $NH_4HCO_3$ and lyophilized to obtain **2** (0.026 g, 0.04 mmol, 37%) as a fluffy white solid; $^1H$ NMR (400 MHz, $D_2O$): δ 7.78 (d, *J* 8.0 Hz, 1H), 5.79 (m, 2H), 4.26–4.00 (m, 6H), 3.82 (m, 1H), 3.67 (m, 1H), 3.54 (m, 3H), 3.29 (t, *J* 9.0 Hz, 1H), 2.16 (m, 2H), 1.86 (s, 3H); $^{13}C$ NMR (100 MHz, $D_2O$): δ 175.1, 166.9, 152.4, 142.3, 103.2, 89.1, 83.8, 74.4, 74.2, 73.4, 71.2, 70.2, 65.3, 61.3, 54.2, 49.5, 25.8 ($J_{C-P}$ 144.9 Hz), 22.6; HR-MALDI-FTMS: calcd for $C_{18}H_{28}N_3O_{16}P_2$ [M−H$^-$]: 604.0950. Found: *m*/*z* 604.0949. TLC (33% 1 M aq $NH_4OAc$–*i*-PrOH), $R_f$: 0.55.

### 4.3. 1-Deoxy-1-α-ethenyl-2-*O*-(*tert*-butyldimethylsilyl)-3,4,6-tri-*O*-benzyl-D-glucose (7)

TBSOTf (0.10 mL, 0.48 mmol, 2.0 equiv) was added to a soln of 1-deoxy-1-α-ethenyl-3,4,6-tri-*O*-benzyl-D-glucose (**6**, 0.11 g, 0.24 mmol, 1 equiv),[9] 2,6-lutidine (0.08 mL, 0.69 mmol, 2.9 equiv) and $CH_2Cl_2$ (2 mL) at 0 °C under argon. The reaction was allowed to warm to room temperature and was stirred for 2 h. The soln was then diluted with $CH_2Cl_2$ (20 mL), washed with 1 × 20 mL satd aq $NaHCO_3$, and back-extracted with $CH_2Cl_2$ (1 × 20 mL). The extracts were combined and washed with brine (1 × 40 mL), dried over $Na_2SO_4$, and concentrated under diminished pressure. Purification by silica gel chromatography (1:20 EtOAc–hexane) afforded **7** (0.13 g, 0.22 mmol, 95%) as a pale yellow oil; $^1H$ NMR (400 MHz, $CDCl_3$): δ 7.38–7.24 (m, 13H), 7.10 (m, 2H), 6.16 (ddd, *J* 18.0, 11.2, 4.4 Hz, 1H), 5.44 (m, 2H), 4.93 (d, *J* 11.2 Hz, 1H), 4.79 (m, 2H), 4.70 (d, *J* 12.8 Hz, 1H), 4.50 (m, 4H), 3.96 (m, 1H), 3.83 (m, 1H), 3.66 (m, 3H), 0.98 (s, 9H), 0.06 (s, 6H); $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 138.9, 138.2, 138.0, 131.9, 128.4, 128.3, 128.2, 128.0, 127.9, 127.6, 127.5, 127.4, 127.3, 119.2, 83.6, 78.4, 76.3, 75.4, 75.0, 73.5, 73.2, 71.9, 68.9, 25.8, 17.9, −4.6, −4.7; FTIR (thin film, $cm^{-1}$): 2954, 2928, 2896, 2857, 1154, 1360, 1252, 1156, 1116, 1088, 1073, 1028, 1005, 865, 838; HR-MALDI-FTMS: calcd for $C_{35}H_{46}O_5SiNa$ [M−Na$^+$] 597.3007. Found: *m*/*z* 597.3006. TLC (1:5 EtOAc–hexane), $R_f$: 0.54.

### 4.4. 1-Deoxy-1-α-hydroxymethyl-2-*O*-(*tert*-butyldimethylsilyl)-3,4,6-tri-*O*-benzyl-D-glucose (8)

Ozone was bubbled through a soln of **7** (0.13 g, 0.23 mmol, 1 equiv) in $CH_2Cl_2$ (2 mL) at −78 °C until it appeared blue (15 min). $N_2$ was subsequently bubbled through the reaction mixture to remove excess ozone, and the soln was warmed to 0 °C. MeOH (2 mL) and $NaBH_4$ (0.02 g, 0.46 mmol, 2.0 equiv) were subsequently added, and the reaction mixture was allowed to warm slowly to rt. After 4 h, the reaction was concentrated under diminished pressure, dissolved in $CH_2Cl_2$ (20 mL), washed with satd $NH_4Cl$ (1 × 20 mL), and back-extracted with $CH_2Cl_2$ (1 × 20 mL). The extracts were combined, dried over $Na_2SO_4$, and concentrated under diminished pressure. Purification using silica gel chromatography yielded **8** (0.07 g, 0.12 mmol, 54%) as a colorless oil; $^1H$ NMR (400 MHz, $CDCl_3$): δ 7.35–7.24 (m, 13H), 7.06 (m, 2H), 4.87 (d, *J* 11.2 Hz, 1H), 4.81 (d, *J* 11.2 Hz, 1H), 4.76 (d, *J* 10.8 Hz, 1H), 4.62 (d, *J* 12.0 Hz, 1H), 4.52 (d, *J* 12.0 Hz, 1H), 4.45 (d, *J* 10.8 Hz, 1H), 4.09 (m, 1H), 3.98 (m, 2H), 3.88 (dd, *J* 12.4, 4.0 Hz, 1H), 3.72–3.57 (m, 5H), 0.82 (s, 9H), 0.12 (s, 3H), 0.05 (s, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 138.6, 137.9, 137.8, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.4, 127.3, 83.4, 78.0, 76.6, 75.4, 75.0, 73.6, 72.5, 72.0, 68.9, 58.1, 25.8, 17.9, −4.6, −4.8; FTIR (thin film, $cm^{-1}$): 2952, 2928, 2885, 2857, 1454, 1360, 1254, 1124, 1094, 1071, 1028, 1005, 860, 837; HR-MALDI-FTMS: calcd for $C_{34}H_{47}O_6Si$ [M−H$^+$] 579.3136. Found: 579.3137; TLC (4:6 EtOAc–hexane), $R_f$: 0.47.

### 4.5. 1-Deoxy-1-α-iodomethyl-2-*O*-(*tert*-butyldimethylsilyl)-3,4,6-tri-*O*-benzyl-D-glucose (10)

Methanesulfonyl chloride (0.004 μL, 0.052 mmol, 1.2 equiv) was added to a soln of **8** (0.026 g, 0.045 mmol, 1 equiv) and diisopropylethyl amine (0.016 μL, 0.090 mmol, 2.0 equiv), in $CH_2Cl_2$ (0.5 mL) at 0 °C under

argon. The reaction was stirred for 5 min, then diluted with $CH_2Cl_2$ (20 mL), washed with satd $NH_4Cl$ (1 × 5 mL), and back-extracted with $CH_2Cl_2$ (1 × 5 mL). The extracts were combined, dried over $Na_2SO_4$, and concentrated to dryness under diminished pressure to afford the mesylate **10** (0.029 g crude weight, 0.044 mmol, 99%). Crude **10** was dissolved in DMF (0.5 mL); TBAI (0.170 g, 0.460 mmol, 10.2 equiv) was subsequently added, and the reaction mixture was heated to reflux (120 °C). After 12 h, the reaction was diluted with EtOAc (5 mL) and washed with brine (3 × 5 mL), and back-extracted with EtOAc (1 × 15 mL). The extracts were combined, dried over $Na_2SO_4$, and concentrated under diminished pressure. Purification by silica gel chromatography afforded **11** (0.020 g, 0.028 mmol, 63% over two steps) as a pale yellow oil. Spectral data were identical to those reported by Nicotra and coworkers.[8i]

### 4.6. Chitin synthase assays

Active yeast cultures were temporarily maintained on agar plates, stored at 4 °C. Cells were cultured in 200 mL YEPG (1% yeast extract, 2% bactopeptone, 2% glucose) medium at 30 °C and allowed to grow to saturation. An aliquot (10–12 mL) of the saturated medium was transferred to 400 mL of YEPG medium to give an optical density of 0.15–0.20 and allowed to grow to an optical density of 0.65–0.70. The cells were washed with cold water and TM buffer (50 mM tris·HCl, 2.5 mM $MgCl_2$, pH 7.5) by suspension and centrifugation (15 min, 2000*g*). The wet weight of the cells at this point was typically around 1 g; this weight was used to determine the vol of buffer in which the final pellet was suspended (vide infra).

The cells were suspended in 2 mL of TM buffer in a 50 mL plastic centrifuge tube, and glass beads (0.45 mm) were added until the vol of beads reached about 3 mm below the liquid's surface. The tube was then vortexed 20 × 30 s, with 30 s of cooling on ice between each vortex; vortexing was performed in a 4 °C cold room. The broken cells were removed from the bottom of the tube with a glass Pasteur pipet and the glass beads were rinsed 5–7 × 1.5 mL with TM buffer. The pooled rinsings were centrifuged at 2000*g* for 4 min, the supernatant was removed, and the remaining cell wall precipitate was washed once more with TM buffer. The cell-wall free supernatants were combined and centrifuged at 60,000*g* for 1 h.

The enzyme pellet was suspended in 1.6 mL·{gram wet weight of cells} TM buffer and homogenized thoroughly with a glass Dounce homogenizer. The membranes were treated with trypsin (quantified by weight, 10 min, 30 °C) and then treated with 3.0 × {mass of trypsin} trypsin inhibitor. Typically, four different concentrations of trypsin/trypsin inhibitor were tested for each new membrane preparation, and the combination with the highest activity at 30 min was used in the assay. Concentrations of trypsin typically tested were 0.5, 1.0, 2.0, and 4.0 mg/mL, and trypsin was added to the membrane preparation at a concentration of 1 μL of trypsin soln for every 5 μL of membrane preparation. Individual assays were performed in 1.5 mL Eppendorf centrifuge tubes.

The standard assay soln used contained UDP-GlcNAc (1.0 mM), GlcNAc (40 mM), and digitonin (0.2% w/v) dissolved in pH 7.5 tris buffer (50 mM) containing $MgCl_2$ (5.0 mM). For radioactivity based assays, radioactive substrate (typically 0.125 μCi, transferred to the Eppendorf as a soln which was then evaporated to dryness under diminished pressure) in 40 μL assay soln (containing necessary inhibitor) was transferred to each tube. Trypsin-treated membrane (20 μL) was then added and the mixture was incubated for 1 h at 30 °C. The reaction was stopped by the addition of 1 mL cold (0 °C) aq trichloroacetic acid (10% v/v) and filtered onto glass fiber filter disks (Whatman GF/C, 25 mm), rinsed with 7:3 EtOH–1 M aq AcOH (4 × 1 mL) and the remaining radioactivity on the filter paper was measured by scintillation counting.

Enzyme activity (in the absence of inhibitor) was typically 25–30,000 cpm/h, with a time-independent background of 500–600 cpm. Under these conditions, the enzyme activity was linear (based on plots of incorporated radioactivity vs. time) to at least 3 h. Control reactions run in the presence of 0.1 mM polyoxin D or nikkomycin Z, both of which were known competitive inhibitors ($K_i \approx 10$ μM) of chitin synthase, always showed ⩾99% inhibition.

Radioactivity based assays evaluating **2** as an inhibitor of CS were carried out using 4 mM **2** in pH 7.5 tris buffer, with $^3$H-UDP-GlcNAc added as described above. Inhibition assays were carried out at [UDP-GlcNAc] = 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 15, 20, and 25 mM. Competition curves were subjected to nonlinear least squares fitting using the Prism3 software package (GraphPad, Inc., San Diego, CA; see Table 1).

**Table 1.** Fitting parameters for CS inhibition by **2**

| Best-fit values | Activity in the absence of **2** | Activity in the presence of **2** |
|---|---|---|
| $V_{max}$ | 48,884 | 57,177 |
| $K_M$ (app) | 1.23 | 10.35 |
| $R^2$ | 0.98 | 0.98 |

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