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The First Direct Evaluation of the Two-Active Site Mechanism for Chitin Synthase

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Chitin synthase polymerizes UDP-GlcNAc to form chitin (poly-β(1,4)-GlcNAc) and is essential for fungal cell wall biosynthesis. The alternating orientation of the GlcNAc residues within the chitin chain has led to the proposal that chitin synthase possesses two active sites. We report the results of the first direct test of this possibility. Two simple uridine-derived dimeric inhibitors are shown to exhibit 10-fold greater inhibition than a monomeric control, consistent with the presence of two active sites. This observation has important implications for the development of antifungal agents, as well as the understanding of polymerizing glycosyltransferases.

Introduction

We wish to report the first direct evaluation of the two-active site mechanism for chitin synthase (CS), a glycosyltransferase essential for fungal cell wall biosynthesis.[1] Comparison of monomeric and dimeric inhibitors provides evidence of bivalent binding of a dimeric inhibitor, consistent with the presence of two active sites in close proximity. These results suggest that polymerizing transferases such as chitin synthase are mechanistically distinct from the comparatively well-understood single-sugar transferases.[2,3] In the case of chitin synthase, this mechanistic divergence has important implications for the design of new antifungal therapeutic agents.

Background. Chitin synthase converts cytosolic uridine diphosphoryl-*N*-acetylglucosamine (UDP-GlcNAc) to chitin, poly-β(1,4)-GlcNAc (Figure 1), with simultaneous extrusion of the polymer through the membrane. Chitin is an integral structural component of the fungal cell wall but is not found in mammals, and thus CS is an attractive therapeutic target. However, like other polymerizing transferases, CS is an integral membrane protein (for which no crystal structure has been determined) and exhibits relatively low affinity for its substrate ($K_M \approx 1$ mM).[1,4] These factors preclude the effective development of inhibitors by structure-based design or the synthesis of substrate analogues.[5−7]

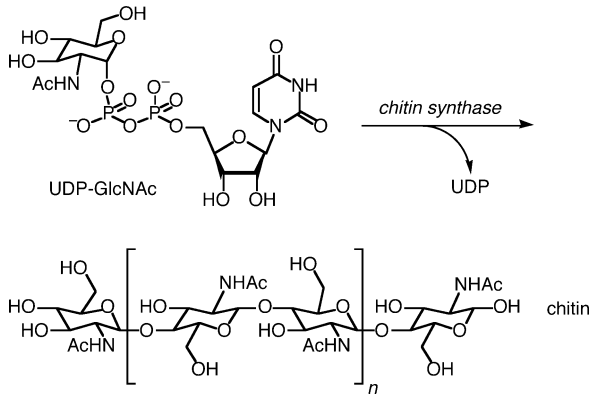

FIGURE 1. Chitin synthase polymerizes UDP-GlcNAc to form chitin.

Mechanism-Based Inhibition? In addressing this problem, we were drawn to the long-standing debate as to whether polymerizing transferases such as CS possess two active sites and are thus mechanistically distinct

(1) Representative overviews of fungal chitin synthase (EC 2.4.1.16): (a) Munro, C. A.; Gow, N. A. R. *Med. Mycol.* **2001**, *39*, 41−53. (b) Valdivieso, M. H.; Duran, A.; Roncero, C. *EXS* **1999**, *87*, 55−69. (c) Merz, R. A.; Horsch, M.; Nyhlen, L. E.; Rast, D. M. *EXS* **1999**, *87*, 9−37. (d) Bulawa, C. E. *Annu. Rev. Microbiol.* **1993**, *47*, 505−534. (e) Cabib, E. *Advances Enzymol.* **1987**, *59*, 59−101.

(2) For an overview of common glycosyltransferases, see: *Essentials of Glycobiology*; Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., Marth, J., Eds.; Cold Spring Harbor Laboratory Press: Spring Harbor, NY, 1999.

(3) The term "polymerizing glycosyltransferase" is used here to refer to processive transferases that synthesize polymeric sugars without releasing the growing chain and the term "single-sugar transferases" to refer to transferases that add a single sugar to an acceptor substrate and release the glycosylated product.

(4) We limit our discussion to fungal CS, as much less is known about CS in insects and other organisms.

(5) For previous studies on rationally designed UDP-GlcNAc analogues, see: (a) Behr, J.-B.; Gourlain, T.; Helimi, A.; Guillerm, G. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1713−1716. (b) Xie, J.; Thellend, A.; Becker, H.; Vidal-Cros, A. *Carbohydr. Res.* **2001**, *334*, 177−182. (c) Behr, J.-B.; Gautier-Lefebvre, I.; Mvondo-Evina, C.; Guillerm, G.; Ryder, N. S. *J. Enzyme Inhib.* **2001**, *16*, 107−112. (d) Grugier, J.; Xie, J.; Duarte, I.; Valery, J.-M. *J. Org. Chem.* **2000**, *65*, 979−984. (e) Schafer, A.; Thiem, J. *J. Org. Chem.* **2000**, *65*, 24−29. (f) Obi, K.; Uda, J.-I.; Iwase, K.; Sugimoto, O.; Ebisu, H.; Matsuda, A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1451−1454.

(6) Polyoxins and nikkomycins are the predominant known natural product inhibitors of chitin synthase. For a review, see: Zhang, D.; Miller, M. *Curr. Pharm. Des.* **1999**, *5*, 73.

(7) Crystal structures of several single-sugar glycosyltransferases have recently been determined. For discussion, see: (a) Davies, G. J. *Nature Struct. Biol.* **2001**, *8*, 98−100. (b) Charnok, S. J.; Henrissat, B.; Davies, G. J. *Plant Physiol.* **2001**, *125*, 527−531. (c) Thomas, S.; Yen, T.-Y.; Macher, B. A. *Glycobiology* **2002**, *12*, 4G−7G.

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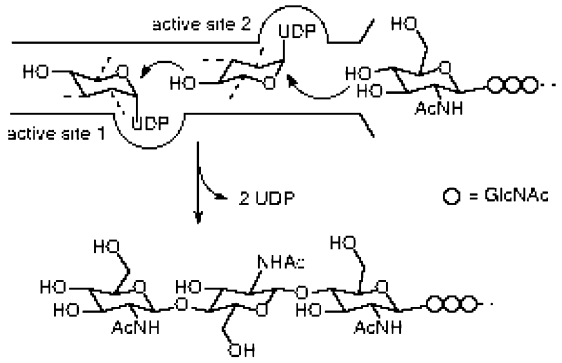


FIGURE 2. Two-active site mechanism for chitin synthase.

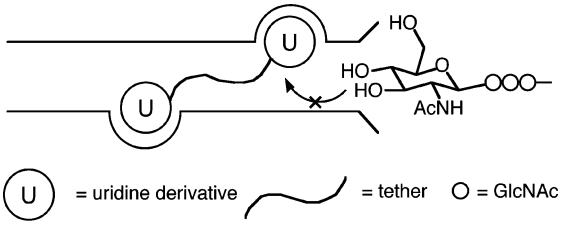


FIGURE 3. Proposed two-site mechanism can be probed with bivalent inhibitors.

from single-sugar transferases.[8] At the origin of this debate is the extended structure of polysaccharides such as chitin, in which adjacent sugar residues have opposed orientations (Figure 1). It has been proposed that CS has two active sites, one for each sugar orientation, in order to facilitate the simultaneous polymerization and extrusion of the linear polymer (Figure 2). This proposal has been pointedly debated, with arguments for and against based on the same indirect evidence: primarily protein sequence analysis but also mutagenesis and the crystal structures of several single-sugar glycosyl-transferases.[9] Significantly, no direct test of this hypothesis has been reported.

A defining characteristic of the two-active site hypothesis is that it is chemically testable: if there are two UDP-GlcNAc binding sites in close proximity, then appropriate dimeric nucleoside inhibitors should show bivalent inhibition and should be more potent than the corresponding monomers (Figure 3).[10]

Bivalent Inhibitors of Chitin Synthase. Potential bivalent inhibitors were constructed on the basis of 5′-deoxy-5′-aminouridine fragments connected by ethylene glycol-based carbamate linkers (Figure 4).[11] Compounds **1**−**4** vary in maximum uridine−uridine 5′−5′ distance

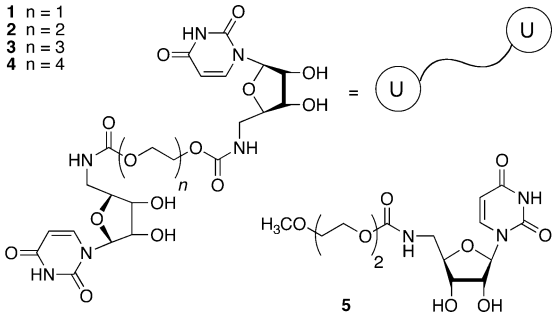


FIGURE 4. Potential bivalent inhibitors and a monomeric control.

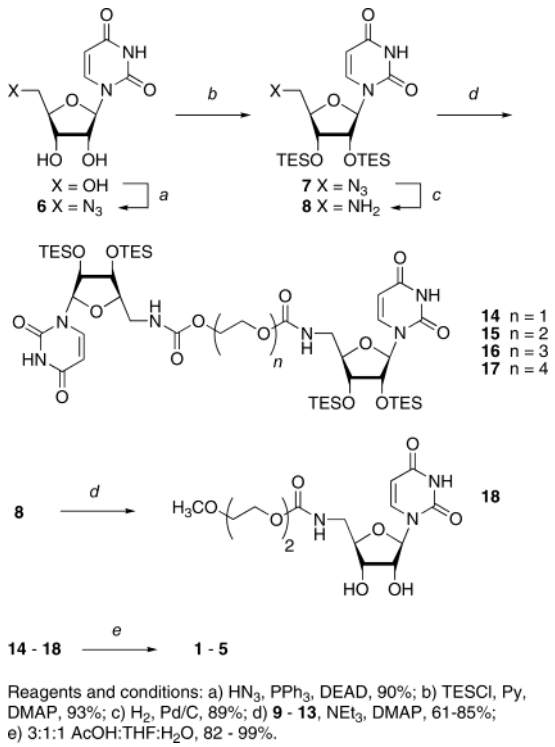


FIGURE 5. Synthesis of **1**−**5**.

from 14 to 22 Å, which spans the calculated maximum possible separation (∼18 Å) for two active sites.[12] Compound **5** is similar in structure to **1**−**4** but is a methyl-terminated monomer and serves as a control. All five compounds were prepared in five steps from uridine (Figure 5, Chart 1).

Inhibition of CS activity by **1**−**5** was determined using an established assay based on incorporation of ^{3}H-UDP-GlcNAc in precipitated chitin.[13] The shortest dimers, **1** and **2**, exhibited significant inhibition of chitin synthase at 1 mM (32 and 45%, respectively, Figure 6). The longer

(8) For one of the first formal statements of the two-active site hypothesis, see: (a) Saxena, I. M.; Brown, R. M., Jr.; Fevre, M.; Geremia, R. A.; Henrissat, B. *J. Bacteriol.* **1995**, *177*, 1419−1424. For representative further discussion, see: (b) Saxena, I. M.; Brown, R. M., Jr.; Dandekar, T. *Phytochemistry* **2001**, *57*, 1135−1148. (c) Saxena, I. M.; Brown, R. M., Jr. *Curr. Opp. Plant Biol.* **2000**, *3*, 523−531.

(9) For a summary of arguments against the two-site mechanism, see refs 7a,b and: Kamst, E.; Spaink, P. H. *Trends Glycosci. Glycotech.* **1999**, *11*, 187−199.

(10) For a recent review of polyvalency in protein−carbohydrate association, see: Kiessling, L. L.; Young, T.; Mortell, K. H. *Glycoscience* **2001**, *2* 1817−1861. For reviews of equally relevant bisubstrate analogue inhibition strategies, see: (a) Broom, A. D. *J. Med. Chem.* **1989**, *32*, 2−7. (b) Radzicka, A.; Wolfenden, R. In *Methods in Enzymology*; Academic Press: New York, 1995; pp 284−312.

(11) See Experimental Section. All relevant ^{1}H NMR spectra can be found in Supporting Information.

(12) Maximum 5′−5′ distances were calculated using ChemBats3D 3.0 (Cambridgesoft, Cambridge, MA). The maximum distance was determined by constructing a model of the transition state for condensation of two molecules of UDP-GlcNAc and manually adjusting dihedral angles to attain the most extended conformation possible. The ethylene glycol linkers are sufficiently flexible that they do not constrain the relative orientation of the uridine residues.

(13) Orlean, P. *J. Biol. Chem.* **1987**, *262*, 5732−5739. 100% activity/0% inhibition is typically ≈ 20 000 cps/h. A time-independent background of ≈ 500 cps/h is observed, equivalent to ∼2.5% apparent inhibition. Reported values are the average of ≥3 measurements. Experimental variation is consistently ±5%.

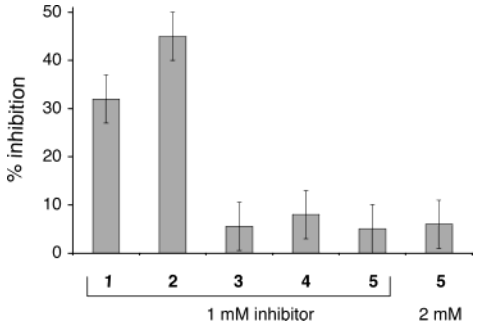
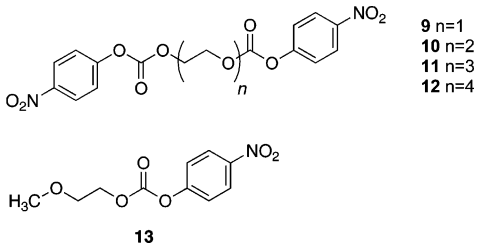


FIGURE 6. Inhibitory activity of dimers **1**−**4** and monomer **5**.

CHART 1. Linker Precursors for the Synthesis of 1−5



dimers, **3** and **4**, and the monomeric control, **5**, showed minimal activity at the same concentration. Notably, doubling the concentration of **5** ([**5**] = 2 mM) did not lead to a significant increase in inhibition, indicating that **1** and **2** are not better inhibitors simply by virtue of double the effective concentration of uridine derivative. Inhibition by dimers **1** and **2** and monomer **5** was measured over 0.05−20 mM, allowing determination of IC_{50} = 2.2 mM for **1**, IC_{50} = 1.1 mM for **2** and IC_{50} = 11.8 mM for **5**.[14] At [**2**] = 1 mM, increasing [UDP-GlcNAc] to 15 mM led to full recovery of enzymatic activity (V_{max}), indicating that inhibition by **1** is competitive.[15,16]

That dimers **1** and **2** are an order of magnitude more potent than **5** and **3** or **4** is consistent with the two-active site model for CS. Comparing **1** or **2** and **5**, the enhanced inhibition may be ascribed to simultaneous binding of the two uridine groups by two active sites. In compounds **1**−**4**, the only difference is the distance between the two covalently linked uridine groups. Thus, the inhibitory nature of **1** and **2** is consistent with the idea that these shorter linkers best match the inter-active site distance in CS while the linkers in **3** and **4** are too long. In contrast, the single-site mechanism fails to offer a rationale for either the increased efficacy of dimeric inhibitors relative to a monomer or for the tether length dependence observed in the dimeric inhibitors.[17]

(14) IC_{50} values were determined by nonlinear least-squares fitting of % inhibition vs log [inhibitor] plots using Prism3CX (Graphpad, Inc., San Diego, CA). Inhibitor concentrations were spaced evenly on the log [inhibitor] axis to minimize residual errors in fitting. Complete details are provided in Supporting Information.

(15) Segel, I. H. *Enzyme Kinetics*, John Wiley & Sons: New York, 1975. See Supporting Information for details.

(16) Intra- and interprotein variants of the two-site mechanism cannot be distinguished by the approach described here. However, the linker length dependence argues against bivalent intermolecular binding.

Conclusion

In conclusion, we have presented the first chemical evaluation of a two-active site mechanism for chitin synthase and have provided evidence that supports this mechanism. While it remains impossible to prove a mechanism, the alternative single-site mechanism provides no explanation for our observations. These observations have several important implications. First, it suggests that dimerization of more potent monomeric inhibitors should lead to high-affinity inhibitors of CS. Second, it provides for the first time a potential strategy by which CS could be selectively targeted in the presence of the numerous mammalian single-sugar GlcNac transferases. Finally, it suggests that other important polymerizing transferases such as the cellulose, heparin, and hyaluronan synthases may also function by a two-site mechanism and could be selectively inhibited by this same strategy. Efforts are underway to identify superior monomers for dimerization as well as to develop other mechanistic probes for chitin synthase.

Experimental Section.

For general experimental information, see Supporting Information.

5′-Deoxy-5′aminouridine Dimer 1. Silylated dimer **14** (0.46 g, 0.43 mmol) was dissolved in 3:1:1 AcOH/THF/H_2O (5 mL) and allowed to stir at room temperature for 9 h. The reaction was concentrated to give dimer **1** (0.25 g, 98%) as a white solid. Dimer **1** was purified by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) prior to use in chitin synthase. ^{1}H NMR (400 MHz, CD_3OD): δ 3.36 (m, 4H), 3.98 (s, 4H), 4.18 (t, 2H, J = 5.2 Hz), 4.23 (s, 4H), 5.71 (d, 2H, J = 8.0 Hz), 5.77 (d, 2H, J = 4.8 Hz), 7.64 (d, 2H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 42.2, 64.2, 70.8, 73.8, 82.8, 90.7, 102.7, 142.3, 151.8, 158.8, 166.4. IR (KBr): ν 3336 (br), 1728 (s), 1553 (s), 1475 (s), 1396 (s), 1274 (s), 1073 (s), 776 (s). HRMS calcd for $C_{22}H_{28}N_6O_{14}Na$ (MNa^+) 623.1556, found 623.1575. R_f = 0.32 (15% water/acetonitrile).

5′-Deoxy-5′-aminouridine Dimer 2. Silylated dimer **15** (0.24 g, 0.22 mmol) was dissolved in 3:1:1 AcOH/THF/H_2O (25 mL) and allowed to stir at room temperature for 9 h. The reaction was concentrated to give dimer **2** (0.14 g, 99%) as a white solid. Dimer **2** was purified by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) prior to use in chitin synthase. ^{1}H NMR (400 MHz, D_2O): δ 3.34 (m, 4H), 3.61 (s, 4H), 3.93 (m, 2H), 3.97 (m, 2H), 4.05 (m, 4H), 4.18 (m, 2H), 5.65 (m, 2H), 5.72 (d, 2H, J = 8.0 Hz), 7.50 (d, 2H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 43.5, 65.2, 70.5, 72.1, 74.8, 84.2, 91.4, 102.8, 142.7, 152.1, 158.9, 165.9. IR (KBr): ν 3388, 1702, 1545, 1457, 1265 cm^{-1}. HRMS: calcd for $C_{24}H_{32}N_6O_{15}Na$ ($M - Na^+$) 667.1823, found 667.1805. R_f = 0.50 (20% H_2O/CH_3CN).

5′-Deoxy-5′-aminouridine Dimer 3. Silylated dimer **16** (1.73 g, 1.51 mmol) was dissolved in 3:1:1 AcOH/THF/H_2O (50 mL) and allowed to stir at room temperature for 5 h. The mixture was concentrated to give dimer **3** (1.01 g, 98%) as a white solid. Dimer **3** was purified by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) prior to use in chitin synthase assays. ^{1}H NMR (400 MHz, D_2O): δ 3.30 (dd, 2H, J = 6.0, 14.8 Hz), 3.40 (dd, 2H, J = 3.2 Hz), 3.55 (s, 4H), 3.61 (m, 4H), 3.94 (m, 2H), 3.99 (t, 2H, J = 5.4 Hz), 4.06 (m, 4H), 4.2 (t, 2H, J = 4.6 Hz), 5.67 (d, 2H, J = 4.0 Hz), 5.74 (d, 2H,

(17) It is possible that the enhanced binding could reflect adventitious association of the second uridine with a portion of the enzyme that normally binds GlcNAc. While this possibility cannot be excluded, three factors weigh against it: (1) GlcNAc itself exhibits no inhibitory activity; (2) uridine and GlcNAc are not particularly similar in structure; and (3) GlcNAc is present at 40 mM in the assay.

8.0 Hz), 7.53 (d, 2H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 43.6, 65.3, 70.5, 71.5, 71.5, 72.2, 74.7, 84.2, 91.5, 102.8, 142.7, 152.1, 158.9, 165.8. IR (KBr): ν 3380, 3091, 2951, 1693, 1562, 1466, 1274 cm^{-1}. HRMS calcd for $C_{26}H_{36}N_6O_{15}Na$ (M − Na$^+$) 711.2085, found 711.2087. R_f = 0.65 (20% H_2O/CH_3CN).

5′-Deoxy-5′-aminouridine Dimer 4. Silylated dimer **17** (1.26 g, 1.06 mmol) was dissolved in 3:1:1 AcOH/THF/H_2O (50 mL) and allowed to stir at room temperature for 5 h. The mixture was concentrated to give dimer **4** (0.78 g, 99%) as a white solid. Dimer **4** was purified by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) prior to use in chitin synthase. ^{1}H NMR (400 MHz, D_2O): δ 3.30 (m, 2H), 3.40 (m, 2H), 3.54 (m, 12H), 3.60 (t, 4H, J = 3.8 Hz), 3.96 (m, 4H), 4.07 (m, 4H), 4.20 (t, 2H, J = 5.0 Hz), 5.67 (d, 2H, J = 4.0 Hz), 5.74 (d, 2H, J = 8.0 Hz), 7.553 (d, 2H, J = 8.4 Hz). ^{13}C NMR (100.594 MHz, CD_3OD): δ 43.6, 65.3, 70.5, 71.5, 71.5, 72.2, 74.7, 84.2, 91.4, 102.8, 142.7, 152.1, 158.9, 165.8. IR (KBr): ν 3401, 3107, 2943, 1739, 1543, 1469, 1395, 1264 cm^{-1}. HRMS calcd for $C_{28}H_{40}N_6O_{17}Na$ (M − Na$^+$) 755.2347, found 755.2334. R_f = 0.50 (20% H_2O/CH_3CN).

5′-Deoxy-5′-aminouridine Monomer 5. Silylated monomer **18** (0.91 g, 1.47 mmol) was dissolved in 3:1:1 AcOH/THF/H_2O (5 mL) and allowed to stir at room temperature for 20 h. The mixture was concentrated and purified by silica gel, chromatography (15% CH_3OH/CH_2Cl_2) to give monomer **5** (0.47 g, 82%) as a white solid. Monomer **5** was purified by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) prior to use in chitin synthase. ^{1}H NMR (400 MHz, D_2O): δ 3.22 (s, 3H), 3.32 (dd, 1H, J = 5.8, 15.2 Hz), 3.41 (dd, 1H, J = 4.0, 21.6 Hz), 3.46 (m, 3H), 3.55 (m, 2H), 3.60 (t, 2H, J = 4.0 Hz), 3.97 (m, 2H), 4.09 (m, 2H), 4.21 (t, 1H, J = 4.4 Hz), 5.69 (d, 1H, J = 4.4 Hz), 5.75 (s, 1H, 8.0 Hz), 7.54 (s, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 43.5, 65.2, 70.5, 71.3, 72.1, 74.7, 84.2, 91.4, 102.8, 142.7, 152.1, 158.9, 165.8. IR (KBr): ν 3397, 3318, 3205, 3065, 2978, 2917, 1684, 1553, 1483, 1335, 1256 cm^{-1}. HRMS calcd for $C_{15}H_{23}N_3O_9Na$ (M − Na$^+$) 412.1332, found 412.1331. R_f = 0.58 (20% H_2O/CH_3CN).

5′-Deoxy-5′-azidouridine (6). A solution of hydrazoic acid (HN_3) was prepared by the slow addition of 14.4 mL of sulfuric acid to a suspension of NaN_3 (33.8 g, 520 mmol) in 16.9 mL H_2O and 206 mL of toluene at 0 °C. (Caution: HN_3 and NaN_3 are toxic and potentially explosive. The reaction temperature must be maintained at or below 0 °C during addition.) The mixture was stirred for 30 min, at which time Na_2SO_4 precipitated. (Note: the precipitate may hinder stirring.) The toluene layer was decanted, dried over Na_2SO_4, and stored over Na_2SO_4 until used. Uridine (5.00 g, 20.48 mmol) was suspended in 350 mL of dry THF under an atmosphere of nitrogen. Triphenylphosphine (16.53 g, 61.40 mmol) and 125 mL of fresh HN_3 (~2.5 M in toluene) were added, and the mixture was cooled to 0 °C. Diethylazodicarboxylate (DEAD, 10.92 mL, 61.40 mmol) was slowly added dropwise, and the reaction was allowed to warm to room temperature. After 15 h, the reaction was concentrated under reduced pressure. (Note: the temperature was maintained at or below 45 °C during concentration.) The resulting viscous solid was dissolved in minimal CH_2Cl_2 (~500 mL) and extracted into water (2 × 200 mL). After washing with with CH_2Cl_2 (6 × 300 mL), the product was obtained by concentrating the aqueous phase. (Note: the temperature was maintained at or below 45 °C during evaporation.) Column chromatography (10−15% CH_3OH/CH_2Cl_2) afforded **6** (4.95 g, 90% yield) as a white foam. ^{1}H NMR (400 MHz, CD_3OD): δ 3.59 (dd, 1H, J = 4.6, 13.4 Hz), 3.68 (dd, 1H, J = 3.0, 13.4 Hz), 4.04 (m, 1H), 4.09 (t, 1H, J = 5.6 Hz), 4.21 (t, 1H, J = 5.0 Hz), 5.73 (d, 1H, J = 8.0 Hz), 5.83 (d, 1H, J = 4.4 Hz), 7.72 (d, 1H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 53.5, 72.1, 75.2, 84.1, 92.1, 103.4, 142.9, 152.5, 166.3. IR (KBr): ν 3231, 2104, 1728,1667, 1483 cm^{-1}. HRMS calcd for $C_9H_{12}N_5O_5$ (M + H$^+$) 270.0833, found 270.0833. R_f = 0.51 (15% CH_3OH/CH_2Cl_2).

Bis(silyl ether) 7. Azide **6** (2.04 g, 7.58 mmol) was azeotropically dried with THF and dissolved in dry pyridine (12 mL) under N_2. DMAP (0.01 g) was added; the solution was cooled to 0 °C, and TES-Cl (7.30 mL, 45.48 mmol) was added. After stirring for 6 h, the reaction mixture was concentrated and purified by silica gel chromatography (40% EtOAc/hexanes) to afford bis(silyl ether) **7** (3.50 g, 93%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.61−0.65 (m, 12H), 0.94 (m, 18H), 3.60 (dd, 1H, J = 3.4, 13.4 Hz), 3.80 (dd, 1H, J = 3.4, 13.4 Hz), 3.96 (t, 1H, J = 5.2 Hz), 4.12 (m, 1H,), 4.20 (t, 1H, J = 3.8 Hz), 5.64 (d, 1H, J = 3.2 Hz), 5.77 (d, 1H, J = 8.0 Hz), 7.66 (d, 1H, J = 8.4 Hz), 9.51 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.9, 4.9, 6.8, 6.9, 51.2, 71.2, 75.1, 81.3, 90.8, 102.2, 140.1, 149.9, 163.2. IR (Film): ν 2960, 2890, 2113, 1457, 1387, 1169 cm^{-1}. HRMS calcd for $C_{21}H_{39}N_5O_5Si_2Na$ (M − Na$^+$) 520.2382, found 520.2372. R_f = 0.76 (40% EtOAc/hexanes).

5′-Deoxy-5′-aminouridine (8). To a solution of bis(silyl ether) **7** (4.84 g, 9.72 mmol) in CH_3OH (20 mL) was added 10% w/w Pd/C (0.09 g). The flask was evacuated and backfilled with H_2 three times and allowed to stir under H_2 (1 atm) for 7 h. The reaction was filtered through Celite (rinsing with CH_3OH) and concentrated to provide 5′-deoxy-5′-aminouridine (**8**) (4.09 g, 89%) as a white foam. When necessary, further purification was effected by silica gel chromatography (8−15% CH_3OH/CH_2Cl_2). ^{1}H NMR (400 MHz, CD_3OD): δ 0.59−0.71 (m, 12H), 0.95 (t, 9H, J = 7.8 Hz), 1.00 (t, 9H, J = 7.8 Hz), 2.87 (m, 2H), 3.98 (m, 1H), 4.03 (t, 1H, J = 3.8 Hz), 4.41 (t, 1H, J = 5.4 Hz), 5.74 (d, 1H, J = 8.0 Hz), 5.81 (d, 1H, J = 6.0 Hz), 7.74 (d, 1H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 5.8, 6.0, 7.2, 7.3, 44.7, 74.6, 75.4, 87.2, 91.2, 103.0, 143.3, 152.2, 165.9. IR (KBr): ν 3388, 2969, 2873, 1693, 1475, 1379, 1265, 1151 cm^{-1}. HRMS calcd for $C_{21}H_{41}N_3O_5Si_2Na$ (M − Na$^+$) 494.2477, found 494.2484. R_f = 0.37 (15% CH_3OH/CH_2Cl_2).

Bis(*p*-nitrophenyl carbonate) 9. Ethylene glycol (0.13 mL, 2.07 mmol) was azeotropically dried with THF and dissolved in dry pyridine (2 mL) under N_2. The reaction was cooled to 0 °C and *p*-nitrophenylchloroformate (1.30 g, 6.21 mmol) was added. After 40 h, the reaction was concentrated and purified by silica gel chromatograpy (2% EtOAc/hexanes) to yield **9** (0.38 g, 47%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.60 (s, 4H), 7.37 (d, 4H, J = 5.2 Hz), 8.26 (d, 4H, J = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 66.1, 121.6, 125.2, 145.3, 152.1, 155.0. IR (Film): ν 3677 (br), 3135 (s), 3109 (s), 1781 (s), 1623 (s), 1588 (s), 1527 (s), 1483 (s), 1352 (s), 1213 (s), 863 (s) cm^{-1}. HRMS calcd for $C_{16}H_{12}N_2O_{10}$ (M$^+$) 392.0492, found 392.0509. R_f = 0.64 (5% EtOAc/CH_2Cl_2).

Bis(*p*-nitrophenyl carbonate) 10. Diethylene glycol (0.40 mL, 4.21 mmol) was azeotropically dried with THF and dissolved in dry pyridine (3 mL) under N_2. The reaction was cooled to 0 °C and *p*-nitrophenyl chloroformate (2.04 g, 10.10 mmol) was added. After 40 h, the reaction was concentrated and purified by silica gel chromatography silica (2% EtOAc/hexanes) to yield **10** (0.84 g, 46%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.85 (m, 4H), 4.47 (m, 4H), 7.38 (d, 4H, J = 8.0 Hz), 8.25 (d, 4H, J = 8.0 Hz). ^{13}C NMR (100.594 MHz, $CDCl_3$): δ 67.9, 68.6, 121.6, 125.1, 145.2, 152.2, 155.1. IR (Film): ν 3677, 3135, 3083, 2969, 1781, 1623, 1588, 1527, 1483, 1352, 1221 cm^{-1}. HRMS calcd for $C_{18}H_{16}N_2O_{11}Na$ (M − Na$^+$) 459.0646, found 459.0642. R_f = 0.52 (5% EtOAc/CH_2Cl_2).

Bis(*p*-nitrophenyl carbonate) 11. Triethylene glycol (1.00 mL, 7.49 mmol) was azeotropically dried with THF, dissolved in dry CH_2Cl_2 (5 mL), and cooled to 0 °C under N_2. Pyridine (2.40 mL, 30.00 mmol) and *p*-nitrophenyl chloroformate (3.80 g, 18.70 mmol) were added. After stirring for 24 h, the reaction mixture was concentrated and purified by silica gel chromatography (3−7% EtOAc/hexanes) to provide **11** (2.93 g, 81%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.73 (s, 4H), 3.83 (m, 4H), 4.45 (m, 4H), 7.37 (d, 4H, J = 8.4 Hz), 8.26 (d, 4H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 68.2, 68.6, 68.7, 70.7, 121.6, 125.1, 145.2, 152.2, 155.2. IR (Film): ν 3677, 3117, 3083, 2873, 1772, 1623, 1588, 1536, 1492, 1352, 1231

cm^{-1}. HRMS calcd for $C_{20}H_{20}N2O_{12}Na$ (M − Na^+) 503.0908, found 503.0914. R_f = 0.44 (5% EtOAc/CH_2Cl_2).

Bis(*p*-nitrophenyl carbonate) 12. Tetraethylene glycol (0.51 mL, 2.63 mmol) was azeotropically dried with THF, dissolved in dry CH_2Cl_2 (8 mL), and cooled to 0 °C under N_2. Pyridine (0.85 mL, 10.50 mmol) and *p*-nitrophenyl chloroformate (0.99 g, 6.58 mmol) were added. After stirring at room temperature for 21 h the reaction was concentrated and purified by silica gel chromatography (7−10% EtOAc/hexanes) to afford **12** (0.85 g, 61%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.67 (s, 8H), 3.79 (m, 4H), 4.41 (m, 4H), 7.38 (d, 4H, J = 9.2 Hz), 8.28 (d, 4H, J = 9.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 68.2, 68.5, 70.5, 70.6, 121.6, 125.0, 145.0, 152.1, 155.2. IR (Film): ν 3135, 3083, 2917, 1772, 1615, 1588, 1527, 1492, 1352, 1265, 1221 cm^{-1}. HRMS calcd for $C_{22}H_{24}N_2O_{13}Na$ (M − Na^+) 547.1171, found 547.1158. R_f = 0.32 (10% EtOAc/CH_2Cl_2).

***p*-Nitrophenyl Carbonate 13.** 2-(2-Methoxyethoxy)ethanol (0.77 mL, 6.42 mmol) was azeotropically dried with THF and dissolved in dry CH_2Cl_2 (6 mL) under N_2. Triethylamine (1.30 mL, 9.63 mmol) and *p*-nitrophenyl chloroformate (1.45 g, 9.63 mmol) were added. After stirring at room temperature for 3 h, the reaction was concentrated and purified by silica gel chromatography (4−5% EtOAc/hexanes) to afford **13** (1.62 g, 88%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.37 (s, 3H), 3.56 (t, 2H, J = 4.8 Hz), 3.66 (t, 2H, J = 4.8 Hz), 3.79 (t, 2H, J = 4.8 Hz), 4.43 (t, 2H, J = 4.4 Hz), 7.36 (d, 2H, J = 8.8 Hz), 8.25 (d, 2H, J = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 29.7, 59.0, 68.2, 68.6, 70.5, 71.8, 121.6, 125.1, 145.1, 152.2, 155.2. IR (Film): ν 3659, 3528, 3135, 3083, 2899, 1772, 1623, 1588, 1536, 1457, 1352, 1221 cm^{-1}. HRMS calcd for $C_{12}H_{15}NO_7Na$ (M − Na^+) 308.0741, found 308.0745. R_f = 0.35 (5% EtOAc/CH_2Cl_2).

Silylated Dimer 14. 5′-Deoxy-5′-aminouridine (**8**) (0.88 g, 1.60 mmol) and bis(*p*-nitrophenyl carbonate) **9** (0.29 g, 0.73 mmol) compounds were azeotropically dried with THF and dissolved in dry CH_2Cl_2 (3 mL) under N_2. Triethylamine (0.22 mL, 1.60 mmol), and (dimethylamino)pyridine (0.01 g, 0.07 mmol) were added, and the reaction mixture was allowed to stir for 20 h, after which it was concentrated and dissolved in CH_2Cl_2 (500 mL). After washing with aqueous $NaHCO_3$ (saturated, 2 × 300 mL) and H_2O (1 × 300 mL), the organic phase was dried over Na_2SO_4 and concentrated and purified by silica gel chromatography (2−4% CH_3OH/CH_2Cl_2) to yield silylated dimer **14** (0.52 g, 31%) as a white solid. H^1 NMR (400 MHz, $CDCl_3$): δ 0.54−0.68 (m, 24H), 0.93 (t, 18H, J = 7.6 Hz), 0.98 (t, 18H, J = 7.6 Hz), 1.28 (t, 4H, J = 7.2 Hz), 3.49 (s, 2H), 4.04 (s, 2H), 4.22 (m, 4H), 4.71 (s, 2H), 5.25 (d, 2H, J = 5.3 Hz), 5.78 (d, 2H, J = 8.0 Hz), 7.24 (d, 2H, J = 7.6 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.8, 5.0, 6.8, 7.0, 42.0, 62.3, 63.5, 72.5, 72.6, 84.4, 96.2, 102.6, 144.1, 150.5, 156.5, 163.4. IR (Film): ν 3345 (br), 3196 (br), 2969 (s), 2873 (s), 1719 (s), 1597 (s), 1518 (s), 1466 (s), 1335 (s), 1248(s), 1169 (s), 1082(s). HRMS calcd for $C_{46}H_{84}N_6O_{14}Si_4Na$ $(MNa)^+$, 1079.5020, found 1079.5000. R_f = 0.35 (8% MeOH/CH_2Cl_2).

Silylated Dimer 15. 5′-Deoxy-5′-aminouridine (**8**) (0.49 g, 1.05 mmol) and bis(*p*-nitrophenyl carbonate) **10** (0.22 g, 0.50 mmol) compounds were azeotropically dried with THF and dissolved in dry CH_2Cl_2 (3 mL) under N_2. Triethylamine (0.31 mL, 1.10 mmol) and DMAP (0.01 g, 0.03 mmol) were added, and the reaction mixture was allowed to stir for 20 h, after which it was concentrated and purified by silica gel chromatography (1−8% CH_3OH/CH_2Cl_2) to provide silylated dimer **15** (0.47 g, 85%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.52−0.65 (m, 24H), 0.90 (t, 18H, J = 8.0 Hz), 0.96 (t, 18H, J = 7.8 Hz), 3.45 (t, 4H, J = 5.4 Hz), 3.69 (s, 4H), 3.99 (t, 4H, J = 3.6 Hz), 4.06 (t, 4H, J = 4.4 Hz), 4.21 (d, 4H, J = 4.4 Hz), 4.60 (t, 4H, J = 5.4 Hz), 5.41 (d, 2H, J = 5.6 Hz), 5.76 (d, 2H, J = 8.0 Hz), 5.88 (s, 2H), 7.51 (d, 2H, J = 8.0 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.6, 4.6, 4.8, 4.9, 6.7, 6.8, 6.9, 42.7, 64.2, 69.4, 72.9, 84.1, 93.2, 102.3, 142.5, 150.1, 156.5, 163.4. IR (Film): ν 3318, 3196, 2951, 2873, 1702, 1536, 1457, 1414, 1379, 1265, 1169, 1073 cm^{-1}. HRMS calcd for $C_{48}H_{88}N_6O_{15}Si_4Na$ (M − Na^+) 1123.5277, found 1123.5277. R_f = 0.4 (10% CH_3OH/CH_2Cl_2).

Silylated Dimer 16. 5′-Deoxy-5′-aminouridine (**8**) (0.94 g, 1.95 mmol) and bis(*p*-nitrophenyl carbonate) **11** (2.20 g, 4.70 mmol) were azeotropically dried with THF and dissolved in dry CH_2Cl_2 (8 mL) under N_2. Triethylamine (1.10 mL, 4.10 mmol) and DMAP (0.01 g, 0.10 mmol) were added, and the reaction mixture was allowed to stir for 20 h. It was then diluted with CH_2Cl_2 (200 mL), washed with water (6 × 100 mL), dried over Na_2SO_4, and concentrated, and the resulting solid was purified by silica gel chromatography (3% CH_3OH/CH_2Cl_2) to provide silylated dimer **16** (1.73 g, 78%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.48−0.63 (m, 24H), 0.86−0.96 (m, 36H), 3.41 (s, 4H), 3.63 (m, 8H), 3.95 (s, 2H), 4.03 (s, 2H), 4.20 (m,4H), 4.54 (s, 2H), 5.43 (d, 2H, J = 5.6 Hz), 5.73 (d, 2H, J = 8.0 Hz), 5.84 (s, 2H), 7.31 (d, 2H, J = 8.0 Hz), 9.79 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.7, 4.8, 4.9, 5.0, 6.7, 6.8, 6.9, 6.9, 42.8, 64.3, 69.4, 70.4, 72.9, 73.0, 84.2, 93.5, 102.3, 142.8, 150.0, 156.5, 163.3. IR (Film): ν 3371, 3109, 2934, 2873, 1693, 1553, 1466, 1387, 1256 cm^{-1}. HRMS calcd for $C_{50}H_{92}N_6O_{16}Si_4Na$ (M − Na^+) 1167.5539, found 1167.5520. R_f = 0.65 (15% CH_3OH/CH_2Cl_2).

Silylated Dimer 17. 5′-Deoxy-5′-aminouridine (**8**) (1.59 g, 3.36 mmol) and bis(*p*-nitrophenyl carbonate) **12** (0. 85 g, 1.60 mmol) were azeotropically dried with THF and dissolved in dry CH_2Cl_2 (11 mL) under N_2. Triethylamine (0.98 mL, 3.52 mmol) and DMAP (0.01 g, 0.10 mmol) were added, and the reaction was allowed to stir for 20 h. It was then diluted with CH_2Cl_2 (400 mL), washed with water (6 × 300 mL), dried over Na_2SO_4, and concentrated, and the resulting solid was purified by silica gel chromatography (3% CH_3OH/CH_2Cl_2) to afford silylated dimer **17** (2.21 g, 61%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.49−0.67 (m, 24H), 0.87−0.99 (m, 36H), 3.42 (s, 4H), 3.65 (m, 12H), 3.96 (s, 2H), 4.05 (s, 2H), 4.21 (m,4H), 4.54 (s, 2H), 5.42 (d, 2H, J = 5.6 Hz), 5.75 (d, 2H, J = 8.0 Hz), 5.90 (s, 2H), 7.31 (d, 2H, J = 8.0 Hz), 9.83 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.7, 4.9, 4.9, 6.7, 6.9, 6.9, 42.7, 64.3, 69.4, 70.5, 70.6, 72.9, 73.0, 84.3, 93.6, 102.3, 142.8, 150.1, 156.6, 163.3. IR (Film): ν 3321, 3214, 3065, 2951, 2890, 1719, 1553, 1457, 1379, 1256, 1169 cm^{-1}. MS calcd for $C_{52}H_{96}N_6O_{17}Si_4Na$ (M − Na^+) 1211.5801, found 1211.5816. R_f = 0.42 (10% CH_3OH/CH_2Cl_2).

Silylated Monomer 18. 5′-Deoxy-5′-aminouridine (**8**) (0.22 g, 0.46 mmol) was dissolved in CH_2Cl_2 (3 mL) under N_2, and triethylamine (0.14 mL, 0.50 mmol) and DMAP (0.01 g, 0.03 mmol) were added. *p*-Nitrophenyl carbonate **13** (0.14 g, 0.5 mmol) in dry pyridine (5 mL) was added, and the reaction mixture was allowed to stir for 18 h, after which it was concentrated and purified by silica gel chromatography (5% CH_3OH/CH_2Cl_2) to yield silylated monomer **18** (0.24 g, 85%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.51−0.65 (m, 12H), 0.63−0.96 (m, 18H), 3.36 (s, 3H), 3.42 (m, 2H), 3.53 (s, 2H), 3.62 (m, 4H), 3.93 (t, 1H, J = 4.0 Hz), 4.03 (s, 1H), 4.23 (m, 2H), 4.49 (t, 1H, J = 5.2 Hz), 5.44 (d, 2H, J = 5.6 Hz), 5.60 (s, 1H), 5.74 (d, 2H, J = 8.0 Hz), 7.30 (d, 2H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 4.7, 4.9, 6.7, 6.9, 42.9, 59.0, 64.2, 69.4, 70.3, 71.8, 73.0, 73.1, 84.0, 93.3, 102.3, 142.3, 149.9, 156.5, 163.2. IR (Film): ν 3318, 1763, 1615, 1536, 1352, 1256, 1213 cm^{-1}. HRMS calcd for $C_{27}H_{51}N_3O_9Si_2Na$ (M − Na^+) 640.3056, found 640.3085. R_f = 0.4 (10% CH_3OH/CH_2Cl_2).

Chitin Synthase Assay. The assay protocol used is based on the procedure of Orlean,[13] modified after helpful discussions with Prof. Peter Orlean (University of Illinois) and Dr. Enrico Cabib (NIH).[18] *Saccharomyces cerevisiae* strains (PP-1D, wild type) were kindly provided by Professor Orlean and stored at −70 °C on freezer stabs. 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.

(18) Orlean, P.; Cabib, E. Personal correspondence.

An aliquot (10−12 mL) of the saturated medium was transferred to 400 mL of YEPG medium to give an optical density at 600 nm 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 volume of buffer in which the final pellet was suspended (vida 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 volume 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} of TM buffer and homogenized throughly with a glass Dounce homogenizer. The membranes were pretreated 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 solution for every 5 μL of membrane preparation. Individual assays were performed in 1.5 mL eppendorf centrifuge tubes.

The assay solution used contained UDP-GlcNAc (1.0 mM), GlcNAc (40mM), and digitonin (0.2% w/v) dissolved in pH 7.5 tris buffer (50 mM) containing $MgCl_2$ (5.0 mM). Radioactive substrate (typically 0.125 μCi, transferred to the eppendorf as a solution that was then evaporated to dryness under vacuum) in 40 μL of assay solution (containing the 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 of cold (0 °C) aqueous trichloroacetic acid (10% v/v), filtered onto glass fiber filter disks (Whatman GF/C, 25 mm), and rinsed with 7:3 EtOH/1 M acetic acid (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 25000−30000 cpm/h, with a time-independent background of 500−600 cpm. Under these conditions, enzyme activity was linear (on the basis of plots of incorporated radioactivity vs time) to at least 3 h. Control reactions run in 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.

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Supporting Information Available: Copies of ^1H and ^{13}C NMR spectra for all compounds; IC_{50} plots for **1**, **2**, and **5**; and a V_{max} recovery plot for **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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