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Synthesis of Fluorescently Labeled UDP-GlcNAc Analogues and Their Evaluation as Chitin Synthase Substrates

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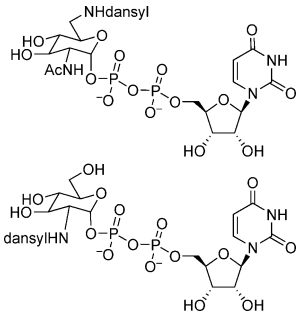

Chitin synthase (CS) polymerizes UDP-GlcNAc to form chitin (poly-β(1,4)-GlcNAc), a key component of fungal cell wall biosynthesis. Little is known about the substrate specificity of chitin synthase or the scope of substrate modification the enzyme will tolerate. Following a previous report suggesting that 6-*O*-dansyl GlcNAc is biosynthetically incorporated into chitin, we became interested in developing an assay for CS activity based on incorporation of a fluorescent substrate. We describe the synthesis of two fluorescent UDP-GlcNAc analogues and their evaluation as chitin synthase substrates.

Introduction

Chitin synthase (CS) converts cytosolic uridine diphosphoryl-*N*-acetylglucosamine (UDP-GlcNAc) to chitin, poly-β(1,4)-GlcNAc (Figure 1). CS is an attractive therapeutic target, as chitin is an integral structural component of the fungal cell wall but is not found in mammals. Current methods for CS activity determination are based on the incorporation of radiolabeled substrate into the growing chitin chains.[1,2] While these methods provide reasonably reliable and reproducible results, there is a clear need for an assay that is both faster and avoids the hazards associated with radioactivity.[3] We present here the design, synthesis, and evaluation of the first fluorescently labeled UDP-GlcNAc analogues (**3** and **4**) as part of our ongoing efforts to develop inhibitors of CS.

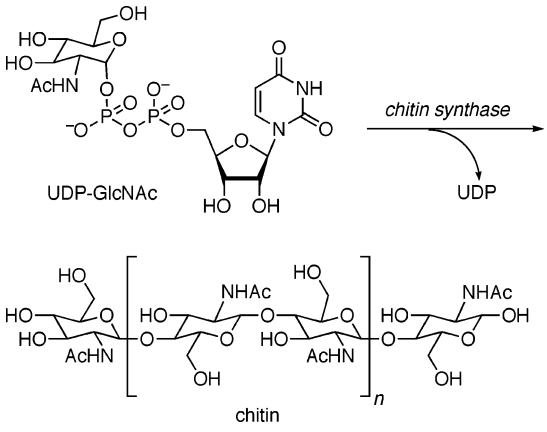

FIGURE 1. Synthesis of chitin.

Background. Most studies of chitin synthase rely on a similar assay protocol: incubation of CS (obtained as a crude membrane preparation) with a radiolabeled substrate (^{14}C-UDP-GlcNAc) results in the synthesis of radiolabeled chitin.[1,2] The chitin is isolated by precipitation and filtration of the reaction mixture, facilitated by the insolubility of chitin chains in almost all solvents.

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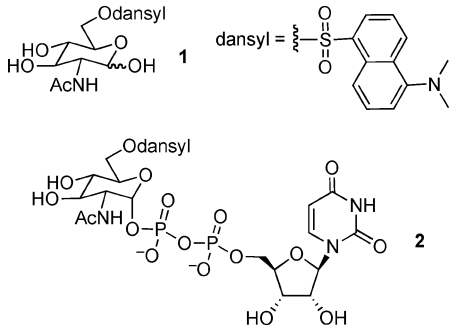
CHART 1. Fluorescently Labeled GlcNAc (1) and UDP-GlcNAc (2)



Measurement of the radioactivity content of the filter pads provides a means to quantify CS activity. Scintillation counting is the bottleneck in the assay process, limiting the total number of data points that can be collected in a day.

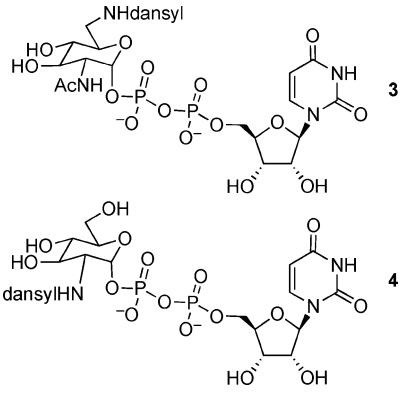
A fluorescence-based assay could dramatically increase the amount of data acquired per unit of time, in part through the use of fluorescent plate readers.[4−6] Increasing the rate of data acquisition would facilitate routine kinetic characterization of inhibitors (for which only IC_{50} values are typically reported) and would permit large libraries of compounds to be screened for activity against the enzyme, accelerating the discovery of novel inhibitors. Fluorescence-based assays have been used in this capacity for nonpolymerizing (single sugar) glycosyltransferase systems.[7]

In addition, a fluorescence-based assay would avoid the well documented hazards associated with the use of radioactive materials.[8] While it has been demonstrated that less hazardous ^{3}H-labeled UDP-GlcNAc can be used in place of ^{14}C-labeled substrate,[9,10] the use of any radioactive material introduces other obvious problems involving procurement, transportation, and secure storage.

Analogue Design. Developing a fluorescence assay for CS activity based on a fluorescent substrate analogue requires that analogues be processed by the enzyme with efficiency similar to that of UDP-GlcNAc. Carrano et al. have reported that fluorescently labeled GlcNAc analogue (**1**, Chart 1) is biosynthetically incorporated into chitin by *Candida albicans* protoplasts, with the implication that **1** is transiently converted in vivo to **2**.[10]

The indication that CS can tolerate such substitution at the 6-OH position of UDP-GlcNAc provides a basis for the design of new potential substrates. The use of analogues of UDP-GlcNAc rather than GlcNAc is appealing in that (1) it obviates the need for additional metabolic transformations prior to polymerization, providing a more direct measurement of CS activity, and (2) it provides compatibility with the most common in vitro CS assay protocol. This naturally led us to consider compounds such as **2** and **3** (Chart 2) as potential substrates. While there was no prior indication that substitution of the 2-NHAc group would be tolerated by CS,[11] **4** was prepared and evaluated on the basis of its synthetic accessibility.

CHART 2. Fluorescently Labeled UDP-GlcNAc Analogues



Synthesis of Dansyl UDP-GlcNAc Analogue 3. The susceptibility of the dansylate in **1** to nucleophilic displacement precluded the direct synthesis of the analogue **2**. As an alternative, the 6-OH group was converted to an amine and subsequently dansylated, forming a much more robust sulfonamide bond.

Treatment of GlcNAc with concentrated HCl in BnOH provided the anomeric benzyl compound **5** in 71% yield (Scheme 1).[12,13] Selective tosylation of the primary alcohol was effected with TsCl in cold (0 °C) pyridine, and subsequent displacement of the primary tosyl group with azide anion was achieved using NaN_3 in warm (70 °C) DMF to provide **7**. The resulting azide was reduced under Staudinger conditions (PPh_3, THF, H_2O).[14] Selective dansylation of **8** was carried out by treatment with dansyl-Cl in cold (0 °C) pyridine. Acylation of the diol (as well as the sulfonamide) was performed under standard conditions to provide **10** (95% yield).

Standard catalytic or transfer hydrogenation methods failed to remove the anomeric benzyl group.[15−22] After

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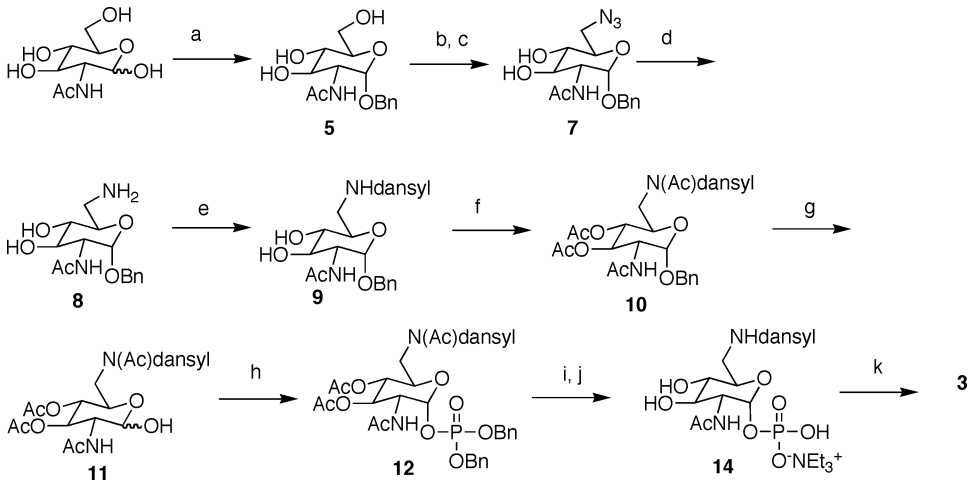
SCHEME 1. Synthesis of 3[a]



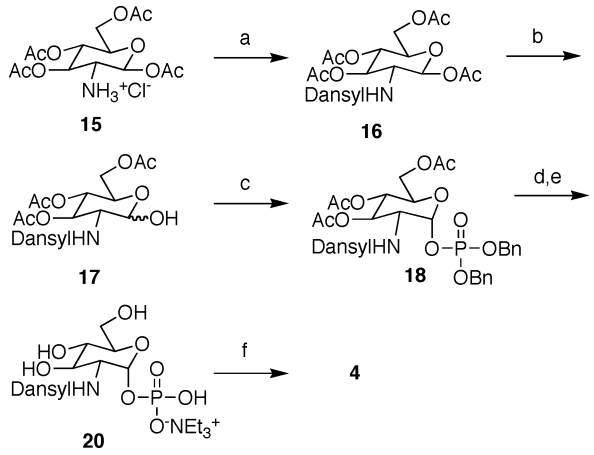
[a] Reagents and conditions: (a) BnOH, HCl (aq), 71%; (b) TsCl, Py, 0 °C, 81%; (c) NaN_3, DMF, 70 °C, 82%; (d) PPh_3, H_2O, THF, 90%; (e) dansylCl, py, 0 °C, 93%; (f) Ac_2O, NEt_3, DMAP, CH_2Cl_2, 95%; (g) $FeCl_3$, CH_2Cl_2, 45−79%; (h) $[(BnO)_2PO]_2O$, LDA, THF, 78 °C, 53%; (i) Pd/C, H_2, MeOH, 74%; (j) 7:3:1 MeOH/H_2O/NEt_3, 99%; (k) UMP-morpholidate, tetrazole, py, 3-5 days, 8%.

extensive experimentation, it was determined that only iterative treatment with excess $FeCl_3$ in CH_2Cl_2 effected cleavage to a significant extent.[22,23] Treatment of the lithium salt of **11** with tetrabenzylpyrophosphate (obtained from the DCC coupling of dibenzyl pyrophosphate)[24,25] provided the α-dibenzyl phosphate **12**. The benzyl phosphate protecting groups were easily removed by hydrogenolysis (1 atm H_2, cat. Pd/C), and global deacylation was achieved by stirring in 7:3:1 MeOH/H_2O/NEt_3.[26] The deprotected phosphate, **14**, was coupled with uridine monophosphate by stirring in minimal pyridine for several days with the morpholidate of uridine monophosphate to obtain dansylated UDP-GlcNAc (**3**).[27−29]

Synthesis of Dansyl UDP-GlcNAc Analogue 4. The synthesis of the second fluorescently labeled UDP-GlcNAc analogue, **4**, was much less complicated than that of **3** (Scheme 2). Reaction of amine **15** (obtained in three steps from glucosamine)[30,31] with dansyl-Cl provided **16** (68%). Removal of the anomeric acetate with hydrazine acetate in DMF gave **17** in near quantitative yield.[32−34] Phosphorylation, deprotection, and coupling of **17** were performed in analogy to the synthesis of **3**.

Evaluation of 3 and 4 as CS Substrates. Compounds **3** and **4** were evaluated as substrates for CS (see Experimental Section for details). To test the ability of the fluorescent analogues to replace the native substrate, assays were conducted in which a fraction of the UDP-GlcNAc (initially 1 mM) was replaced with **3** or **4**. Control experiments were performed in which the omitted UDP-GlcNAc was not replaced with **3** or **4**.

SCHEME 2. Synthesis of Compound 4[a]



[a] Reagents and conditions: (a) DansylCl, py, 68%; (b) H_2NNH_2−HOAc, DMF, 99%; (c) $[(BnO)_2PO]_2O$, LDA, THF, −78 °C, 90%; (d) H_2, Pd/C, MeOH, 99%; (e) 7:3:1 MeOH/H_2O/NEt_3, 99%; (f) UMP-morpholidate, tetrazole, py, 16%

Five different proportions (100, 90, 75, 60, and 40%) of the normal 0.66 mM per assay of UDP-GlcNAc were examined for chitin synthesis (Figure 2). Only a small decrease in activity is observed when the amount of UDP-GlcNAc is reduced (Figure 2, circles), consistent with the fact that the substrate is initially present in saturating concentrations. Experiments in which the omitted UDP-GlcNAc is replaced with fluorescently labeled substrate (Figure 2: **3**, squares; **4**, triangles) show a more pronounced decrease in CS activity. As the proportion of the fluorescent compounds is increased, the activity of the enzyme decreases: at 40% UDP-GlcNAc/60% dansyl-labeled substrate, the activity decreases 44% with **3** and 37% with **4**.

The decrease in ^3H incorporation is the result expected if **3** and **4** are incorporated into the growing chitin chains. However, no fluorescence is observed upon examination

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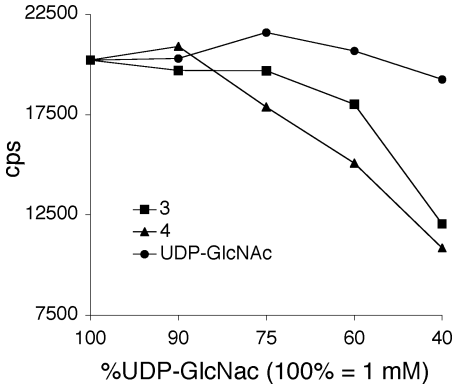


FIGURE 2. Evaluation of **3** and **4** as substrates for CS.

of the chitin isolated by filtration. We estimate that 1% incorporation of **3** or **4** should be visible,[35] and the absence of emission suggests that if **3** or **4** are incorporated, they are incorporated with <1% of the efficiency of UDP-GlcNAc. Assuming this estimate to be correct, the decrease in CS activity in the presence of **3** and **4** can have one of two explanations. The first is that **3** and **4** bind more tightly than UDP-GlcNAc but turn over only very slowly; this would account for a decrease in activity without commensurate appearance of fluorescent product. An alternative interpretation is that **3** and **4** bind to CS with moderate affinity but do not turn over at all, that is, they are inhibitors.

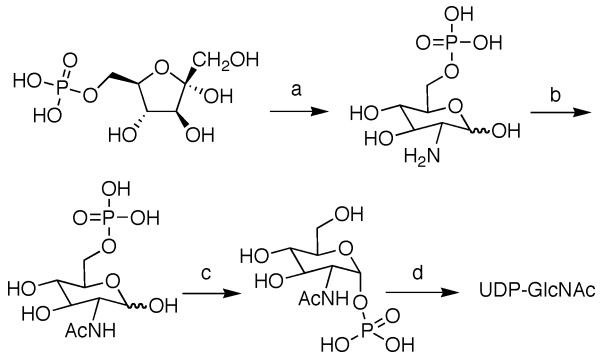
Our current data do not allow us to distinguish between these two possibilities. However, given that our objective was to develop a high-throughput assay for CS activity, the difference between an inhibitor and a substrate that binds but does not turn over within the limits of detection is essentially a semantic one.

In retrospect, the possibility that **3** and **4** are inhibitors is not entirely surprising, as the biosynthesis of UDP-GlcNAc in fungi proceeds through GlcNAc-6-phosphate rather than free GlcNAc (Scheme 3).[36] As such, if dansyl GlcNAc (**1**) really is converted in vivo to the corresponding nucleotide sugar (**2**) and then to chitin, this must proceed through adventitious exploitation of an alternative biosynthetic route. This is possible but rather improbable, and we believe it is more likely that the previous report of efficient direct fungal incorporation of **1** into chitin is in error.[10]

Conclusion

We have described the synthesis and evaluation of the first fluorescent analogues of UDP-GlcNAc. These compounds appear to act as weak inhibitors toward chitin synthase, although it is possible that they are actually substrates, albeit very inefficient ones. In either case, they are not useful for the development of a fluorescently based assay for chitin synthase activity. However, given the precedent for other glycosyltransferases accepting unnatural substrates, it remains possible that **3** and **4** will prove to be useful for developing other fluorescence-based enzyme assays.[37]

SCHEME 3. Biosynthesis of UDP-GlcNAc in Fungi[a]



[a] Enzymes and cosubstrates: (a) glutamine-fructose 6-phosphate amido transferase, glutamine; (b) glucosamine phosphate *N*-acetyltransferase; (c) 3-phospho-*N*-acetylglucosamine mutase; (d) UDP-*N*-acetylglucosamine pyrophosphorylase, uridine triphosphate.

Experimental Section

UDP-GlcNAc Analogue 3. Monophosphate **14** (0.08 g, 0.132 mmol) was azeotropically dried with THF and dissolved in dry pyridine (0.53 mL) under N_2. Tetrazole (0.03 g, 0.40 mmol) and uridine 5′-monophosphate morpholidate (4-morpholine-*N*,*N*′-dicyclohexyl-carboxamidine salt) (0.18 g, 0.26 mmol) were added, and the reaction was concentrated after 120 h. Purification by silica gel chromatography (10−15% H_2O/CH_3CN with 1% NEt_3) yielded 0.03 g, contaminated with UMP-morpholidate and starting material. This mixture was purified by size exclusion chromatography (250 mM NH_4OAc) to yield 0.02 g, still contaminated with a small amount of dansyl starting material, which was removed after purification by reverse-phase silica gel chromatography (10% CH_3CN/H_2O) to yield **3** (0.01 g, 8.2%) as a yellow film. ^{1}H NMR (400 MHz, CD_3OD), δ: 2.05 (s, 3H), 2.87 (s, 6H), 3.03 (m, 2H), 3.64 (m, 2H), 3.90 (m, 2H), 4.10 (s, 1H), 4.22 (m, 3H), 4.34 (s, 1H), 5.44 (s, 1H), 5.81 (d, 1H, J = 7.2 Hz), 5.93 (d, 1H, J = 4.4 Hz), 7.25 (d, 1H, J = 7.2 Hz), 7.55 (m, 2H), 7.99 (d, 1H, J = 8.8 Hz), 8.18 (d, 1H, J = 6.8 Hz), 8.39 (d, 1H, J = 8.4 Hz), 8.52 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 14.5, 23.1, 30.8, 45.2, 45.8, 55.3, 66.1, 71.1, 72.6, 72.7, 73.1, 75.5, 84.9, 89.7, 96.1, 103.1, 116.3, 120.6, 124.2, 128.9, 129.8, 130.8, 130.8, 131.0, 136.7, 142.4, 152.4, 152.8, 166.0, 174.1. FTIR (KBr), cm^{-1}: 3423 (br), 1684 (s), 1457 (s), 1256 (s), 1117 (s), 933 (s). HRMS(MALDI−FTMS), *m*/*z*: calcd for $C_{29}H_{38}N_5O_{18}P_2S$ (M − H)$^-$ 838.1413, found 838.1296. TLC (20% H_2O/CH_3CN), R_f: 0.55.

UDP-GlcNAc Analogue 4. Phosphate **20** (0.04 g, 0.06 mmol) was dried overnight in vacuo and dissolved in dry pyridine (500 mL) under N_2. Uridine 5′-monophosphate mor-

(35) Under our standard assay conditions (see Experimental Section), a single assay vial produces ~5 × 10^{-8} mol of CS. 100% incorporation of **3** or **4** would thus provide ~5 × 10^{-8} mol of dansyl fluorophore distributed on a ~1 cm diameter filter pad. This in turn would be roughly equivalent to viewing 1 mL of a 10^{-5} M solution of dansyl fluorophore. Emission from such a solution, even in water, can easily be seen. Our estimate that 1% of this emission (i.e., viewing 1 mL of a 10^{-7} solution) would also be visible is based on the assumption that the final environment of the dansyl group is more like dioxane (ϕ ~ 0.66) than pure H_2O (ϕ ~ 0.07). See Supporting Information for an expanded discussion of these calculations and assumptions.

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pholidate (4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt) (0.09 g, 0.13 mmol) and tetrazole (0.01 g, 0.19 mmol) were added and allowed to stir for 153 h. The mixture was concentrated and purified by reverse-phase silica gel chromatography (2−100% CH_3CN/H_2O) to obtain 0.04 g of product, contaminated with the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt. This mixture was subjected to size exclusion chromatography (250 mM NH_4HCO_3) and again to reverse-phase silica gel chromatography to obtain **4** (0.01 g, 16%) as a pale yellow film. ^{1}H NMR (400 MHz, CD_3OD), δ: 2.86 (s, 6H), 3.20 (m, 2H), 3.60 (m, 1H), 3.65 (m, 1H), 3.81 (m, 1H), 3.92 (m, 1H), 4.12 (s, 1H), 4.25 (s, 2H), 4.35 (s, 1H), 5.60 (s, 1H), 5.80 (s, 1H), 5.94 (s, 1H), 7.23 (d, 1H, J = 7.2 Hz), 7.55 (m, 2H), 8.00 (m, 1H), 8.33 (d, 1H, J = 6.4 Hz), 8.47 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD), δ: 46.3, 60.2, 63.2, 71.6, 72.7, 73.2, 75.1, 76.1, 90.3, 103.6, 116.7, 121.7, 124.6, 129.4, 130.1, 131.1, 131.4, 131.4, 138.6, 143.0, 153.0, 166.5. FTIR (KBr), cm^{-1}: 3467 (br), 1684 (s), 1466 (s), 1309 (s), 1256 (s), 1125 (s). HRMS-(MALDI−FTMS), *m/z*: calcd for $C_{28}H_{37}N_3O_{18}P_2S$ $(M)^-$ 797.1268, found 797.1150. TLC (20% H_2O/CH_3CN), R_f: 0.52.

Benzyl-Protected GlcNAc 5. *N*-Acetyl glucosamine (6.00 g, 27.12 mmol) was dissolved in benzyl alcohol (50 mL), and concentrated HCl (2.9 mL) was added. The mixture was heated to 90 °C for 3 h, cooled to room temperature, and then poured onto 500 mL Et_2O and stored overnight at −20 °C. The resulting precipitate was recovered by filtration and rinsing with Et_2O and hexanes to yield 17.64 g of crude material, which was purified by silica gel chromatography (8−15% $MeOH/CH_2Cl_2$) to yield **5** (5.98 g, 71%) as a white foam. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.95 (s, 3H), 3.37 (t, 1H, J = 9.2 Hz), 3.68 (m, 3H), 3.82 (d, 1H, J = 10.8 Hz), 3.89 (dd, 1H, J = 3.4, 10.8 Hz), 4.61 (dd, 2H, J = 11.8, 98.4 Hz), 4.85 (s, 1H), 7.24−7.37 (m, 5H). ^{13}C NMR (100 MHz, CD_3OD), δ: 23.1, 31.4, 55.9, 63.2, 70.6, 72.9, 73.1, 74.5, 97.9, 126.4, 129.4, 129.6, 129.7, 139.3, 173.8. FTIR (film), cm^{-1}: 3301 (br), 2917 (s), 1650 (s), 1562 (s), 1387 (s), 1239 (s), 1143 (s), 1045 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{15}H_{22}N_1O_6$ $(M + H)^+$ 312.1447, found 312.1437. TLC (10% $MeOH/CH_2Cl_2$), R_f: 0.24.

Tosylate 6. Benzylated GlcNAc **5** (0.59 g, 1.90 mmol) was cooled to 0 °C in dry pyridine (6.0 mL). TsCl (0.54 g, 2.84 mmol) was dissolved in dry pyridine (5 mL) and added to the solution of **5** over 0.5 h. The mixture was allowed to stir for 14 h at 4 °C, concentrated, and purified by silica gel chromatography (10% $MeOH/CH_2Cl_2$) to obtain **6** (0.72 g, 81%) as a white foam. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.93 (s, 3H), 2.42 (s, 3H), 3.27 (t, 1H, J = 9.2 Hz), 3.62 (t, 1H, J = 8.8 Hz), 3.71−3.76 (m, 1H), 3.78−3.83 (m, 1H), 4.19 (dd, 1H, J = 5.8, 10.6 Hz), 4.29 (dd, 1H, J = 2.0, 10.4 Hz), 4.48 (dd, 2H, J = 12.4, 79.2 Hz), 4.70 (d, 1H, J = 3.2 Hz), 7.27 (m, 5H), 7.39 (d, 2H, J = 8.0 Hz), 7.78 (d, 2H, J = 8.0 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 21.8, 22.7, 55.2, 70.5, 71.0, 71.6, 72.0, 72.6, 97.5, 128.9, 129.0, 129.3, 129.4, 130.9, 134.4, 128.6, 146.4, 173.4. FTIR (film), cm^{-1}: 3388 (br), 2934 (br), 1676 (s), 1553 (s), 1370 (s), 1186 (s), 1125 (s), 1082 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{22}H_{27}N_1O_8S$ $(M)^+$ 465.1457, found 465.1458. TLC (10% $MeOH/CH_2Cl_2$), R_f: 0.3.

Azide 7. NaN_3 (6.82 g, 104.9 mmol) was added to a solution of tosylate **6** (4.88 g, 10.49 mmol) in DMF (15 mL), and the mixture was heated to 70 °C for 20 h. Concentration and silica gel chromatography (10% $MeOH/CH_2Cl_2$) afforded **7** (2.86 g, 82%) as a white foam. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.94 (s, 3H), 3.34 (t, 1H, J = 8.8 Hz), 3.41 (dd, 1H, J = 6.2, 13.0 Hz), 3.49 (dd, 1H, J = 2.4, 13.8 Hz), 3.67 (dd, 1H, J = 8.8, 10.4 Hz), 3.79 (m, 1H), 3.91 (dd, 1H, J = 3.6, 10.8 Hz), 4.62 (dd, 2H, J = 12.4, 88.4 Hz), 7.25−7.37 (m, 5H). ^{13}C NMR (100 MHz, CD_3OD), δ: 23.1, 53.2, 55.7, 70.9, 72.9, 73.6, 97.9, 129.3, 129.6, 129.7, 139.0, 173.8. FTIR (film), cm^{-1}: 3432 (br), 3310 (s), 2943 (s), 2095 (s), 1667 (s), 1623 (s), 1562 (s), 1457 (s), 1387 (s), 1291 (s), 1047 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{15}H_{22}N_2O_5$ $(M + H)^+$ 337.1512, found 337.1508. TLC (15% MeOH /CH_2Cl_2), R_f: 0.65.

Amine 8. Triphenylphosphine (4.58 g, 17 mmol) and H_2O (5 mL) were added to a solution of azide **7** (2.86 g, 8.51 mmol) in THF (15 mL). The reaction was concentrated after 20 h. The resulting residue was dissolved in H_2O (500 mL) and washed with CH_2Cl_2 (5 × 250 mL). The aqueous layer was concentrated to yield **8** (2.37 g, 90%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.95 (s, 3H), 2.74 (dd, 1H, J = 7.4, 13.6 Hz), 2.99 (dd, 1H, J = 2.4, 13.6 Hz), 3.23 (t, 1H, J = 9.2 Hz), 3.61 (m, 1H), 3.70 (dd, 1H, J = 8.8, 10.4 Hz), 3.88 (dd, 1H, J = 3.6, 10.8 Hz), 4.61 (dd, 2H, J = 12.2, 88.4 Hz), 4.85 (d, 1H, J = 3.2 Hz), 7.24−7.38 (m, 5H). ^{13}C NMR (100 MHz, CD_3OD), δ: 23.1, 44.3, 55.9, 70.9, 72.9, 74.1, 74.5, 98.1, 129.1, 129.5, 129.7, 139.3, 173.8. FTIR (film), cm^{-1}: 3301 (br), 3091 (s), 2899 (s), 1641 (s), 1571 (s), 1457 (s), 1396 (s), 1108 (s), 1055 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{15}H_{22}N_2O_5$ $(M + H)^+$ 311.1607, found 311.1594. TLC (10% $MeOH/CH_2Cl_2$), R_f: 0.01.

Sulfonamide 9. Amine **8** (2.37 g, 7.65 mmol) was dissolved in dry pyridine (100 mL) under N_2 and cooled to 0 °C. Dansyl-Cl (3.10 g, 11.48 mmol) was dissolved in dry pyridine (100 mL) and added to the amine solution dropwise over 0.75 h. After 20 h at 4 °C, the reaction was concentrated and purified by silica gel chromatography (5−15% $MeOH/CH_2Cl_2$) to yield **9** (3.88 g, 93%) as a yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.96 (s, 3H), 2.88 (s, 6H), 3.20 (dd, 2H, J = 12.2, 98.4 Hz), 3.65 (m, 1H), 3.70 (m, 1H), 4.05 (m, 1H), 4.44 (dd, 2H, J = 11.6, 86.4 Hz), 4.77 (d, 1H, J = 3.2 Hz), 6.11 (d, 1H, J = 8.8 Hz), 7.14 (d, 1H, J = 7.2 Hz), 7.26 (m, 5H), 7.49 (m, 2H), 8.23 (d, 1H, J = 7.2 Hz), 8.35 (d, 1H, J = 8.4 Hz), 8.51 (d, 1H, J = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 23.3, 43.5, 69.6, 70.4, 71.2, 72.5, 96.9, 115.1, 118.9, 123.1, 127.8, 128.2, 128.3, 129.3, 129.5, 129.7, 130.1, 134.8, 137.0, 151.6, 171.4. FTIR (film), cm^{-1}: 3345 (br), 2951 (br), 1667 (s), 1571 (s), 1466 (s), 1326 (s), 1151 (s), 1055 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{27}H_{33}N_3O_7S$ $(M)^+$ 543.2039, found 543.2025. TLC (10% MeOH/CH_2Cl_2), R_f: 0.46.

Sulfonamide 10. DMAP (0.02 g, 0.15 mmol), NEt_3 (0.82 mL, 5.88 mmol), and acetic anhydride (0.56 mL, 5.88 mmol) were added to a solution of **9** (0.80 g, 1.47 mmol) in dry CH_2Cl_2 (2 mL), and the reaction was allowed to stir for 0.5 h. The reaction was diluted with CH_2Cl_2 (200 mL), washed with $NaHCO_3$ (1 × 200 mL) and brine (1 × 200 mL), dried (Na_2SO_4), and concentrated to yield **10** (0.93 g, 95%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.91 (s, 3H), 2.04 (s, 3H), 2.14 (s, 3H), 2.19 (s, 3H), 2.89 (s, 6H), 4.13 (dd, 1H, J = 9.6, 15.2 Hz), 4.24 (d, 1H, J = 13.2 Hz), 4.35 (m, 1H), 4.47 (d, 1H, J = 11.6 Hz), 4.90 (m, 2H), 5.02 (t, 1H, J = 9.6 Hz), 5.30 (t, 1H, J = 9.6 Hz), 5.70 (d, 1H, J = 9.6 Hz), 7.18 (d, 1H, J = 8.0 Hz), 7.37 (m, 5H), 7.56 (m, 2H), 7.82 (d, 1H, J = 8.4 Hz), 8.35 (d, 1H, J = 7.2 Hz), 8.59 (d, 1H, J = 8.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 20.8, 20.9, 23.2, 24.7, 30.4, 45.4, 47.5, 51.9, 69.1, 69.7, 70.7, 71.1, 95.5, 115.3, 117.5, 122.9, 128.1, 128.4, 128.5, 128.8, 129.4, 129.7, 131.5, 131.6, 134.1, 136.2, 152.1, 169.6, 170.1, 170.3, 170.9. FTIR (film), cm^{-1}: 3345 (br), 2951 (s), 1754 (s), 1693 (s), 1545 (s), 1370 (s), 1239 (s), 1178 (s), 1055 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{33}H_{39}N_3O_{10}NaS$ $(M + Na)^+$ 692.2248, found 692.2272. TLC (10% $MeOH/CH_2Cl_2$), R_f: 0.72.

Alcohol 11. $FeCl_3$ (0.94 g, 5.74 mmol) was added to a solution of **10** (0.39 g, 0.57 mmol) in dry CH_2Cl_2 (2 mL) and allowed to stir for 18.5 h, at which time an additional $FeCl_3$ (0.50 g, 3.03 mmol) was added. After 24 h, the mixture was diluted with EtOAc (600 mL) and washed with brine (2 × 400 mL). The brine layers were combined and back-extracted with EtOAc (2 × 300 mL). The organic layers were combined and washed with saturated EDTA (3 × 400 mL) and brine (2 × 200 mL), dried (Na_2SO_4), and concentrated. Purification by silica gel chromatography (4% $MeOH/CH_2Cl_2$) provided **11** (0.26 g, 45−79%) as a yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.98 (s, 3H), 2.05 (s, 3H), 2.14 (s, 3H), 2.20 (s, 3H), 2.88 (s, 6H), 4.01 (dd, 1H, J = 9.4, 15.2 Hz), 4.21 (d, 1H, J = 15 Hz), 4.31 (m, 1H), 4.46 (t, 1H, J = 9.8 Hz), 4.98 (t, 1H, J = 9.8 Hz), 5.20 (d, 1H, J = 3.2 Hz), 5.35 (t, 1H, J = 10.2 Hz), 5.91 (d, 1H,

J = 9.2 Hz), 7.17 (d, 1H, J = 7.2 Hz), 7.56 (m, 2H), 7.81 (d, 1H, J = 8.4 Hz), 8.32 (d, 1H, J = 7.2 Hz), 8.57 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 20.9, 21.0, 23.3, 24.8, 45.4, 47.7, 52.3, 68.8, 70.7, 70.8, 91.3, 115.2, 117.5, 123.0, 128.7, 129.5, 129.7, 131.5, 131.6, 132.2, 134.0, 152.1, 169.9, 170.2, 170.7, 171.0. FTIR (film), cm^{-1}: 3388 (br), 2951 (s), 2812 (s), 1737 (s), 1676 (s), 1545 (s), 1370 (s), 1239 (s), 1186 (s), 1047 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{26}H_{33}N_3O_{10}S$ $(M)^+$ 579.1887, found 579.1871. TLC (10% MeOH/CH_2Cl_2), R_f: 0.73.

Dibenzyl Phosphate 12. Lithium diispropylamide (0.72, 2.00 M in THF, 1.44 mmol) was added to a solution of **11** (0.42 g, 0.72 mmol) in THF (4 mL) at −78 °C, and the reaction mixture was stirred for 0.5 h. Tetrabenzyl pyrophosphate (0.70 g, 1.29 mmol) was added and stirring continued at −78 °C for 1 h. The reaction was allowed to stir at 4 °C for 10 h and then was diluted with EtOAc (300 mL) and washed with $NaHCO_3$ (2 × 200 mL) and brine (1 × 200 mL). The organic fraction was dried (Na_2SO_4) and concentrated to 0.81 g, which was purified by silica gel chromatography (0−1% MeOH/$CHCl_3$) to yield **12** (0.31 g, 53%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.72 (s, 3H), 2.05 (s, 3H), 2.14 (s, 6H), 2.88 (s, 6H), 4.16 (m, 2H), 4.39 (m, 1H), 4.47 (m, 1H), 5.16 (m, 5H), 5.71 (m, 2H), 7.18 (d, 1H, J = 7.6 Hz), 7.35 (m, 5H), 7.55 (m, 2H), 7.86 (d, 1H, J = 8.4 Hz), 8.20 (d, 1H, J = 7.2 Hz), 8.57 (d, 1H, J = 9.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 20.8, 20.9, 22.8, 24.8, 45.4, 47.3, 51.9, 52.0, 69.9, 70.0, 70.1, 70.2, 71.0, 95.5, 95.5, 115.3, 117.6, 122.9, 128.0, 128.1, 128.5, 128.5, 128.6, 128.8, 129.3, 129.8, 130.5, 131.5, 134.3, 135.2, 135.3, 135.4, 135.4, 152.0, 169.8, 170.0, 170.2, 170.7. FTIR (film), cm^{-1}: 3301 (br), 2943 (s), 1763 (s), 1684 (s), 1562 (s), 1457 (s), 1361 (s), 1239 (s), 1151 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{40}H_{46}N_3O_{13}PSNa$ (MNa^+) 862.2381, found 862.2372. TLC (10% MeOH /CH_2Cl_2), R_f: 0.77.

Phosphate 13. To a solution of phosphate **12** (0.12 g, 0.14 mmol) in MeOH (1 mL) was added Pd/C (0.02 g, 10% w/w Pd/C). The reaction was flushed with H_2 (three times) and stirred under H_2 (1 atm) for 4 h. NEt_3 (0.02 mL, 0.14 mmol) was added, and the mixture was filtered through Celite, rinsing with MeOH. Concentration of the filtrate yielded **13** (0.08 g, 74%) as a pale yellow foam. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.30 (t, 9H, J = 7.2 Hz), 1.95 (s, 3H), 1.98 (s, 3H), 2.06 (s, 3H), 2.19 (t, 3H), 2.87 (s, 6H), 4.28 (m, 3H), 4.84 (m, 1H), 5.04 (t, 1H, J = 10.0 Hz), 5.33 (t, 1H, J = 10.0 Hz), 5.52 (dd, 1H, J = 3.4, 7 Hz), 7.25 (d, 1H, J = 7.2 Hz), 7.58 (t, 1H, J = 8.2 Hz), 7.65 (t, 1H, J = 8.0 Hz), 7.86 (d, 1H, J = 8.4 Hz), 8.31 (d, 1H, J = 7.2 Hz), 8.59 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 9.3, 20.8, 21.0, 22.7, 24.9, 45.7, 47.5, 53.2, 70.9, 72.2, 72.6, 94.7, 116.4, 118.7, 124.3, 129.7, 130.3, 130.8, 132.2, 135.7, 153.3, 171.4, 171.7, 172.8, 173.3. FTIR (KBr), cm^{-1}: 3398 (br), 2985 (s), 2944 (s), 2803 (s), 1747 (s), 1665 (s), 1557 (s), 1458 (s), 1343 (s), 1235 (s), 1169 (s), 1046 (s). HRMS-(MALDI−FTMS), *m/z*: calcd for $C_{26}H_{34}N_3O_{13}PSNa$ (MNa^+) 682.1442, found 682.1447. TLC (15% H_2O/CH_3CN), R_f: 0.24.

Phosphate 14. Tetraacetate **13** (0.02 g, 0.02 mmol) was dissolved in 7:3:1 MeOH/H_2O/NEt_3 and stirred for 36 h. The mixture was concentrated, redissolved in MeOH, and filtered through Celite. Concentration of the filtrate yielded **14** (0.01 g, 99%) of the diol as a pale yellow foam. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.98 (s, 3H), 2.85 (s, 6H), 2.96 (dd, 1H, J = 6.8, 12.6 Hz), 3.13 (d, 1H, J = 7.2 Hz), 3.23 (t, 1H, J = 9.2 Hz), 3.65 (t, 1H, J = 9.2 Hz), 3.89 (m, 2H), 5.37 (s, 1H), 7.23 (d, 1H, J = 7.6 Hz), 7.55 (m, 2H), 8.18 (d, 1H, J = 7.2 Hz), 8.37 (d, 1H, J = 8.4 Hz), 8.52 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 9.7, 23.4, 45.7, 46.2, 46.3, 48.0, 55.8, 55.9, 73.1, 73.2, 73.5, 95.4, 116.8, 121.0, 124.6, 129.4, 130.3, 131.3, 131.3, 131.5, 137.2, 153.3, 174.1. FTIR (KBr), cm^{-1}: 3432 (br), 1658 (s), 1562 (s), 1475 (s), 1326 (s), 1151 (s), 942 (s), 793 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{20}H_{28}N_3O_{10}PSNa$ (MNa^+) 556.1125, found 556.1129. TLC (20% H_2O/CH_3CN), R_f: 0.22.

Sulfonamide 16. Amine **14** (0.38 g, 0.99 mmol)[30,31] was added to a solution of dansyl-Cl (0.40 g, 1.48 mmol) in dry pyridine (1 mL) and allowed to stir for 11 h. Concentration followed by silica gel chromatography (1−5% MeOH/CH_2Cl_2) provided **16** (0.39 g, 68%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.31 (s, 3H), 1.74 (s, 3H), 2.01 (s, 3H), 2.05 (s, 3H), 2.86 (s, 6H), 3.69 (t, 1H, J = 9.2 Hz), 3.80 (m, 1H), 4.03 (dd, 1H, J = 2.0, 12.6 Hz), 4.23 (dd, 1H, J = 2.0, 12.6 Hz), 5.07 (t, 1H, 9.4 Hz), 5.17 (t, 1H, J = 10.0 Hz), 5.59 (d, 1H, J = 8.8 Hz), 7.16 (d, 1H, J = 7.2 Hz), 7.50 (m, 2H), 8.08 (d, 1H, J = 8.8 Hz), 8.27 (d, 1H, J = 7.2 Hz), 8.52 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 19.9, 20.4, 20.7, 20.8, 45.4, 57.0, 61.5, 67.7, 72.5, 72.7, 92.1, 115.2, 119.0, 123.3, 128.3, 129.2, 129.3, 129.8, 130.3, 135.9, 151.7, 169.0, 170.3, 171.3. FTIR (film), cm^{-1}: 3315 (br), 2952 (s), 2853 (s), 2787 (s), 1755(s), 1590 (s), 1450 (s), 1384 (s), 1235 (s), 1079 (s), 1054 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{26}H_{32}N_2O_{11}S$ (M^+) 580.1721, found 580.1727. TLC (10% MeOH/CH_2Cl_2), R_f: 0.60.

Alcohol 17. Tetraacetate **16** (0.39 g, 0.63 mmol) was azeotropically dried with THF and dissolved in dry DMF (1 mL). Hydrazine acetate (0.07 g, 0.75 mmol, azeotropically dried with THF) was added and the reaction allowed to stir for 16 h. The mixture was diluted with EtOAc (300 mL) and washed with brine (2 × 300 mL). The aqueous fractions were pooled and back-extracted with EtOAc (1 × 300 mL). Organic fractions were combined, dried (Na_2SO_4), and concentrated to yield **17** (0.36 g, 99%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 1.09 (s, 3H), 1.93 (s, 3H), 2.06 (s, 3H), 2.86 (s, 6H), 3.42 (m, 1H), 4.02 (d, 1H, J = 10.4 Hz), 4.18 (m, 2H), 4.88, (t, 1H, J = 9.6 Hz), 5.19 (t, 1H, J = 9.8 Hz), 5.28 (m, 1H), 5.56 (d, 1H, J = 10.4 Hz), 7.17 (d, 1H, J = 7.6 Hz), 7.54 (m, 2H), 8.18 (d, 1H, J = 8.4 Hz), 8,27 (d, 1H, J = 7.4 Hz), 8.54 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 19.7, 20.6, 20.8, 45.4, 56.2, 61.9, 67.1, 68.4, 69.7, 92.2, 115.1, 119.1, 123.2, 128.3, 129.2, 129.7, 129.8, 130.7, 134.5, 151.7, 169.3, 170.3, 170.6. FTIR (film), cm^{-1}: 3439 (br), 3266 (br), 2952 (s), 1747 (s), 1665(s), 1582 (s), 1458 (s), 1376 (s), 1334 (s), 1244 (s), 1153 (s), 1046 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{24}H_{30}$-$N_2O_{10}S$ (MNa^+) 561.1513, found 561.1522. TLC (10% MeOH/CH_2Cl_2), R_f: 0.51.

Dibenzyl Phosphate 18. Compound **17** (0.30 g, 0.55 mmol) was azeotropically dried with THF and cooled to −78 °C in dry THF (4 mL), and LDA (0.41 mL of 2 M THF solution) was added. After 10 min, tetrabenzyl pyrophosphate (0.42 g, 0.77 mmol) was added. The reaction was allowed to stir for 40 min and then warmed to 4 °C and allowed to stir 12 h. The mixture was then concentrated, diluted with EtOAc (500 mL), washed with $NaHCO_3$ (1 × 300 mL) and brine (1 × 300 mL), and then dried (Na_2SO_3) and concentrated to 0.55 g of crude material. Purification by silica gel chromatography (1−2% MeOH/CH_2-Cl_2) provided **18** (0.40 g, 90%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 0.97 (s, 3H), 1.92 (s, 3H), 1.99 (s, 3H), 2.84 (s, 6H), 3.5 (s, 1H), 3.85 (d, 1H, J = 11.8 Hz), 4.07 (d, J = 10.4 Hz), 4.14 (dd, 1H, J = 3.6, 12.4 Hz), 4.90 (t, 1H, J = 9.8 Hz), 5.11 (m, 5H), 5.87 (dd, 1 H, J = 3.2, 6.0 Hz), 6.05 (s, 1H), 7.12 (d, 1H, J = 7.6 Hz), 7.35 (m, 10H), 7.47 (t, 1H, J = 7.2 Hz), 7.53 (t, 1H, J = 8.0 Hz), 8.16 (d, 1H, J = 8.8 Hz), 8.26 (d, 1H, J = 7.2 Hz), 8.53 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$), δ: 19.4, 20.5, 20.7, 45.4, 55.8, 61.0, 67.7, 69.0, 69.1, 69.8, 70.1, 97.1, 115.1, 119.1, 123.2, 129.7, 128.1, 128.2, 128.3, 128.4, 128.5, 128.5, 129.2, 129.6, 129.7, 130.5, 134.7, 135.2, 135.2, 135.3, 135.3, 151.7, 169.0, 170.0, 170.2. FTIR (film), cm^{-1}: 3135 (br), 2947 (s), 2834 (s), 2781 (s), 1750 (s), 1592 (s), 1457 (s), 1374 (s), 1336 (s), 1246 (s). HRMS(MALDI−FTMS), *m/z*: calcd for $C_{38}H_{43}N_2O_{13}PSNa$ (MNa^+) 821.2116, found 821.2119. TLC (10% MeOH/CH_2Cl_2), R_f: 0.83.

Phosphate 19. Dibenzyl phosphate **18** (0.39 g, 0.49 mmol) was dissolved in MeOH (2 mL); Pd/C (0.10 g, 10% w/w Pd/C) was added, and the mixture was flushed with H_2 (three times) and allowed to stir under H_2 (1 atm) for 8 h. NEt_3 (0.0 mL, 0.49 mmol) was added, and the mixture was filtered through a pad of Celite and concentrated to yield **19** (0.29 g, 99%) as a pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD), δ: 0.69 (s, 3H), 1.31 (t, 9H, J = 7.2 Hz), 1.85 (s, 3H), 2.00 (s, 3H), 3.17 (q, 6H,

J = 7.6, 14.2 Hz), 3.45 (m, 1H), 3.57 (d, 1H, J = 10.8 Hz), 4.05 (m, 1H), 4.22 (m, 1H), 4.8 (t, 1H, J = 10.0 Hz), 5.16 (t, 1H, J = 10.0 Hz), 5.68 (m, 1H), 7.26 (d, 1H, J = 7.2 Hz), 7.57 (m, 2H), 8.24 (m, 2H), 8.21 (d, 1H, J = 8.8 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 9.7, 20.0, 21.1, 21.2, 46.3, 47.8, 57.8, 57.9, 63.2, 69.7, 70.6, 71.7, 96.9, 97.1, 116.8, 121.2, 124.8, 129.5, 130.4, 131.1, 131.4, 137.9, 153.3, 171.3, 171.3, 172.4. FTIR (film), cm^{-1}: 3441 (br), 2995 (s), 2943 (s), 1754 (s), 1580 (s), 1457 (s), 1379 (s), 1335 (s), 1248 (s) cm^{-1}. HRMS(MALDI−FTMS), *m/z*: calcd for $C_{24}H_{31}N_2O_{13}PSNa$ (MNa^+) 641.1177, found 641.1173. TLC (10% MeOH/CH_2Cl_2), R_f: 0.11.

Phosphate 20. Phosphate **19** (0.05 g, 0.06 mmol) was dissolved in 7:3:1 MeOH/H_2O/NEt_3 and allowed to stir for 72 h. After concentration, compound **20** (0.04 g, 99%) was obtained as a pale yellow solid. ^{1}H NMR (400 MHz, CD_3OD), δ: 1.27 (t, 9H, J = 7.2 Hz), 1.90 (s, 3H), 2.86 (s, 6H), 3.11 (q, 6H, J = 7.2, 14.4 Hz), 3.60 (m, 3H), 3.75 (d, 1H, J = 11.6 Hz), 3.85 (m, 1H), 5.50 (dd, 1H, J = 3.6, 6.6 Hz), 7.24 (d, 1H, J = 7.6 Hz), 7.57 (m, 2H), 8.32 (d, 1H, J = 7.2 Hz), 8.42 (d, 1H, J = 8.4 Hz), 8.51 (d, 1H, J = 8.4 Hz). ^{13}C NMR (100 MHz, CD_3OD), δ: 9.2, 24.2, 45.8, 46.9, 60.0, 60.1, 62.6, 72.2, 73.3, 74.0, 95.7, 95.8, 116.0, 121.0, 124.2, 128.7, 129.5, 130.5, 130.9, 130.9, 138.2, 152.7, 179.7. FTIR (KBr), cm^{-1}: 3458 (br), 2943 (s), 2750 (s), 2681 (s), 2497 (s), 1580 (s), 1483 (s), 1317 (s), 1151 (s) cm^{-1}. HRMS(MALDI−FTMS), *m/z*: calcd for $C_{18}H_{25}N_2O_{10}PSNa$ (MNa^+): 515.0860, found 515.0882. TLC (20% H_2O/CH_3CN), R_f: 0.40.

Chitin Synthase Assay. The assay protocol used is based on the procedure of Orlean,[1,9] modified after helpful discussions with Prof. Peter Orlean (University of Illinois) and Dr. Enrico Cabib (NIH).[1] *Saccharomyces cerevisiae* strains (PP-1D, wild type) were kindly provided by Prof. Orlean and were 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 of 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 volume 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 volume of beads reached about 3 mm below the liquid's surface. The tube was then vortexed 20 times for 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 times with 1.5 mL of 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} of 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 in 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 mL of trypsin solution for every 5 mL 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 mL of assay solution (containing necessary inhibitor) was transferred to each tube. Trypsin-treated membrane (20 mL) 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 25 000−30 000 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 ($K_i \approx 10\ \mu$M) of chitin synthase, always showed ≥99% inhibition.

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Supporting Information Available: General experimental details and copies of ^{1}H NMR spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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