Supporting Information
**Materials and Methods**

**Protein Purification.** TylM1 was cloned, over-expressed, and purified as described. The purified protein was dialyzed against 10 mM Tris (pH 8) and 200 mM NaCl and concentrated to 20 mg/mL.

**Preparation of dTDP-sugars.** dTDP-3-amino-3,6-dideoxyglucose and dTDP-3-amino-3,6-dideoxygalactose were enzymatically synthesized as previously reported. For the preparation of dTDP-o-mycaminose (dTDP-3-N,N-dimethylamino-3,6-dideoxyglucose), a typical 50 mL reaction was set up containing 50 mM HEPPS, 1.5 mM dTDP-3-amino-3,6-dideoxyglucose, and 4.5 mM S-adenosylmethionine. The pH was adjusted to 8.5, and 30 mgs of TylM1 were added. The reaction was incubated for 18 hours at 37°C. Subsequently, TylM1 was removed via filtration through an Amicon 10kD ultrafiltration membrane. The filtrate was diluted to 500 mL and loaded onto a 50 mL 26/10 Resource-Q column (GE LifeSciences). The products were separated with a 20-column volume gradient from 0 to 250 mM ammonium bicarbonate at pH 8.5. dTDP-o-mycaminose eluted at an ammonium bicarbonate concentration of approximately 100 mM. Mass spectroscopic analysis of the product (negative ion mode) gave the expected mass of 574 for an N,N-dimethylated sugar product. dTDP-3-N-methylamino-3,6-dideoxygalactose was prepared and purified in an analogous manner, using dTDP-3-amino-3,6-dideoxygalactose as the starting substrate. Mass spectrophotometric analysis of the product (negative ion mode) gave the expected mass of 560 for an N-monomethylated sugar product. No evidence of a dimethylated sugar was observed in the spectra obtained from this reaction.
**Crystallization and X-ray Data Collection.** Prior to crystallization trials, the protein was incubated with 5 mM S-adenosylhomocysteine and 10 mM dTDP-d-mycaminose. Crystallization conditions were then surveyed by the hanging drop method of vapor diffusion using a sparse matrix screen developed in the laboratory.

Single crystals of TylM1 were subsequently grown at room temperature via batch experiments by mixing in a 1:1 ratio the protein/S-adenosylhomocysteine/dTDP-d-mycaminose solution with a precipitant solution composed of 30-40% poly(ethylene glycol) 3400, 400 mM NaCl, and 100 mM CHES (pH 9). Crystallization was initiated by macro-seeding. The crystals were frozen for X-ray data collection by transferring them to a cryo-protectant solution composed of 25% poly(ethylene glycol) 3400, 350 mM NaCl, 5 mM S-adenosylhomocysteine, 10 mM dTDP-d-mycaminose, 100 mM CHES (pH 9), and 15% ethylene glycol. The crystals belonged to the monoclinic space group \(P2_1\) with unit cell dimensions of \(a = 73.5\ \text{Å}, \ b = 92.1\ \text{Å},\) and \(c = 80.2\ \text{Å}\) and \(\beta = 106.1^\circ\). The asymmetric unit contained two dimers.

Crystallization in the presence of dTDP-3-N-methylamino-3,6-dideoxygalactose was accomplished in an analogous manner to that described above. The crystallization process was initiated with seed crystals of the TylM1/S-adenosylhomocysteine/dTDP-d-mycaminose complex. The crystals also belonged to the monoclinic space group \(P2_1\), but with unit cell dimensions of \(a = 39.9\ \text{Å}, \ b = 92.9\ \text{Å},\) and \(c = 77.9\ \text{Å}\) and \(\beta = 97.3^\circ\). The asymmetric unit contained one dimer. These crystals showed significant anisotropic diffraction properties.

X-ray data from the TylM1/S-adenosylhomocysteine/dTDP-d-mycaminose complex crystals were collected at 100K at the Structural Biology Center Beamline 19-BM
Relevant X-ray data collection statistics are listed in Table S1. The structure was solved with PHASER using the coordinates for TylM1 (PDB code 3PFH) as a search model. Iterative rounds of model-building with COOT and refinement with REFMAC reduced the $R_{\text{work}}$ and $R_{\text{free}}$ to 18.1% and 21.6%, respectively, from 30 – 1.6 Å resolution. Relevant refinement statistics are listed in Table S2.

X-ray data from the TylM1/S-adenosylhomocysteine/dTDP-3-N-methylamino-3,6-dideoxygalactose complex crystals were collected at 100K with a Bruker AXS Platinum 135 CCD detector controlled by the Proteum software suite (Bruker AXS Inc.). The X-ray source was Cu K$\alpha$ radiation from a Rigaku RU200 X-ray generator equipped with Montel optics and operated at 50 kV and 90 mA. These X-ray data were processed with SAINT version 7.06A (Bruker AXS Inc.) and internally scaled with SADABS version 2005/1 (Bruker AXS Inc.). Relevant X-ray data collection statistics are listed in Table S1. The structure was solved with PHASER using the coordinates of the refined structure in complex with S-adenosylmethionine and dTDP-\(d\)-mymcaminose as a search probe. Iterative rounds of model building with COOT and refinement with REFMAC reduced the $R_{\text{work}}$ and $R_{\text{free}}$ to 21.9% and 27.7%, respectively, from 30 – 2.2 Å resolution. Relevant refinement statistics are listed in Table S2.

**Kinetic Analysis.** The kinetic constants for the methylation reactions were determined using the SAM510:SAM Methyltransferase Assay Kit (G Biosciences). The assay was continuously monitored with a Beckman DU 640B spectrophotometer. The SAM510 assay protocol was followed with minor alterations. Specifically, the kit components (assay buffer, enzyme mix, colorimetric mix, and S-adenosylmethionine)
totaling 100 µl were incubated at 37°C for 15 min and the solution then cooled to ambient temperature (~23°C). The dTDP-sugar substrates and water were added for a total volume of 10 µl, and the reaction initiated by the addition of 5 µl of TylM1. This yielded a total final reaction volume of 115 µl and a concentration of TylM1 of 9 µg/mL. The production of S-adenosylhomocysteine was monitored spectrophotometrically at 510 nm every 2 seconds over 20 min. dTDP-3-amino-3,6-dideoxyglucose was used in ten different concentrations ranging from 0.025 to 0.7 mM, whereas dTDP-3-amino-3,6-dideoxygalactose was used in 14 different concentrations ranging from 0.1 to 10.0 mM. Note that tests were performed to verify that the concentration of SAM was saturating.

Originally a discontinuous HPLC assay that monitored the consumption of the dTDP-sugar substrates was employed. Unfortunately, this approach was not sensitive enough for the low concentrations of dTDP-3-amino-3,6-dideoxyglucose required. As such, we shifted to the colorimetric assay described above. At the higher substrate concentrations tested in the HPLC assay, the rate of consumption of the dTDP-3-amino-3,6-dideoxyglucose was comparable to the rate of S-adenosylhomocysteine production observed using the SAM510 kit. The kinetic parameters are listed in Table S3.
Table S1: X-ray Data Collection Statistics.

|                           | TylM1/S-adenosylhomocysteine/dTDP-D-mycaminose complex | TylM1/S-adenosylhomocysteine/dTDP-3-N-methylamino-3,6-dideoxygalactose |
|---------------------------|-------------------------------------------------------|------------------------------------------------------------------------|
| resolution limits (Å)     | 50-1.6 (1.63-1.60)^b                                    | 50-2.20 (2.30-2.20)                                                   |
| number of independent     | 123048 (5330)                                          | 24812 (2554)                                                          |
| reflections               |                                                        |                                                                        |
| completeness (%)          | 91.6 (79.6)                                            | 86.5 (71.8)                                                           |
| redundancy                | 4.3 (2.4)                                             | 3.2 (1.2)                                                             |
| avg I/avg σ(I)            | 43.2 (4.3)                                            | 14.0 (3.7)                                                            |
| R_{sym} (%)^a             | 6.9 (17.6)                                            | 5.7 (14.0)                                                            |

^aR_{sym} = (\Sigma |I| - \bar{I}) / \Sigma |I| x 100.

^bStatistics for the highest resolution bin.
Table S2: Refinement Statistics.

|                        | TylM1/S-adenosylhomocysteine/dTDP-D-mycaminose complex | TylM1/S-adenosylhomocysteine/dTDP-3-N-methylamino-3,6-dideoxygalactose |
|------------------------|--------------------------------------------------------|-------------------------------------------------------------------------|
| resolution limits (Å)  | 50.0 - 1.6                                             | 30.0 – 2.2                                                              |
| \( R\)-factor (overall)%/no. reflections | 18.2/123048                                           | 21.9/24794                                                             |
| \( R\)-factor (working)%/no. reflections | 18.1/116898                                           | 21.5/23543                                                             |
| \( R\)-factor (free)%/no. reflections | 21.6/6150                                              | 27.7/1251                                                              |
| number of protein atoms | 7345                                                   | 3659                                                                   |
| number of heteroatoms  | 899                                                    | 336                                                                    |
| **average B values**   |                                                        |                                                                         |
| protein atoms (Å\(^2\)) | 24.1                                                   | 38.6                                                                   |
| ligand (Å\(^2\))      | 17.8                                                   | 30.5                                                                   |
| solvent (Å\(^2\))      | 30.0                                                   | 36.0                                                                   |
| **weighted RMS deviations from ideality** |                                                          |                                                                         |
| bond lengths (Å)       | 0.010                                                  | 0.013                                                                  |
| bond angles (º)        | 2.03                                                   | 1.70                                                                   |
| planar groups (Å)      | 0.010                                                  | 0.006                                                                  |
| **Ramachandran regions (%)** |                                                          |                                                                         |
| most favored           | 92.0                                                   | 90.3                                                                   |
| additionally allowed   | 8.0                                                    | 9.4                                                                    |
| generously allowed     | 0                                                       | 0.2                                                                    |
| disallowed             | 0                                                       | 0                                                                       |

\(^{a}\) \( R\)-factor = (\( \sum |F_O - F_C| \) / \( \sum |F_O| \)) x 100 where \( F_O \) is the observed structure-factor amplitude and \( F_C \) is the calculated structure-factor amplitude.

\(^{b}\) Distribution of Ramachandran angles according to PROCHECK.\(^7\)
### Table S3. Kinetic Parameters.

| Substrate                          | $K_m$ (mM)   | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|------------------------------------|--------------|----------------------|----------------------------------|
| dTDP-3-amino-3,6-dideoxyglucose    | 0.079 ± 0.015| 0.75 ± 0.09          | 9.5 x 10$^{-4}$                 |
| dTDP-3-amino-3,6-dideoxygalactose | 1.54 ± 0.08  | 0.61 ± 0.07          | 4.0 x 10$^{-2}$                 |
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