propane ring and the macrolide core and the relative configuration at the C13 stereocenter. However, the relative configuration of the sugar unit in callipeltoside A (**1**) with regard to the core has been assigned through NOE analysis.

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[25] See the Supporting Information for ^{1}H and ^{13}C NMR data of **30** and **31**. The chemical shift reported for the chlorine bearing carbon C21, within the cyclopropane ring of the callipeltosides, was $\delta = 55.4$ with the C9 methoxy group adjacent at 55.2. However, in our aglycons **30** and **31**, only one resonance is present at $\delta \approx 55$, which is assigned to the methoxy group; furthermore, HMQC (heteronuclear multiple-quantum coherence) analysis of **30** showed the C21 resonance to be at $\delta = 34.2$. In the callipeltosides a resonance at $\delta = 34.0$ was attributed to the remaining C20 methine. In actual fact, the signal of the C20 methine in the aglycons **30** and **31** lies at $\delta = 12.02$, almost coinciding with the C25 methyl signal at $\delta = 11.98$, which were resolved by a DEPT 90 NMR experiment. Thus, we conclude that the ^{13}C NMR spectra of the callipeltosides may require the reassignment of C20 ($\delta = 12.0$) and C21 ($\delta = 34.0$). For supportive ^{13}C NMR spectral data on chlorocyclopropanes, see: Y. Kusuyama, T. Kagosaku, T. Hasegawa, *Bull. Chem. Soc. Jpn.* **1990**, *63*, 2836.

Insights into the Branched-Chain Formation of Mycarose: Methylation Catalyzed by an (*S*)-Adenosylmethionine-Dependent Methyltransferase**

Huawei Chen, Zongbao Zhao, Tina M. Hallis, Zhihong Guo, and Hung-wen Liu*

Methylation is a common biotransformation that encompasses a wide variety of substrates involved in a myriad of biological processes.[1] For example, methylation of DNA has been shown to play an important role in gene regulation, and methylation of specific protein targets has been established as a general mechanism to control signal transduction or cell growth and differentiation. In addition, the biological consequences of methylation of rRNA and mRNA are also well documented.[1] The majority of biological methyl transfers are catalyzed by methyltransferases that use (*S*)-adenosylmethionine (AdoMet) as the methyl donor. It is of interest that methylation is such a prevalent process in living organisms, considering that methyl transfer from AdoMet to its acceptor is intrinsically a very slow reaction in water.[2] Although methyltransferases characteristically display low k_{cat} values, their catalysis of methyl transfer can still be considered significant compared to the uncatalyzed reaction.

While the flexibility of AdoMet as a methyl donor is apparent from its ability to use carbon, nitrogen, and oxygen as acceptors, methyltransferases that are capable of catalyzing C-methylation are much less common and therefore less well studied.[3] A specific area in which C-methyltransferases

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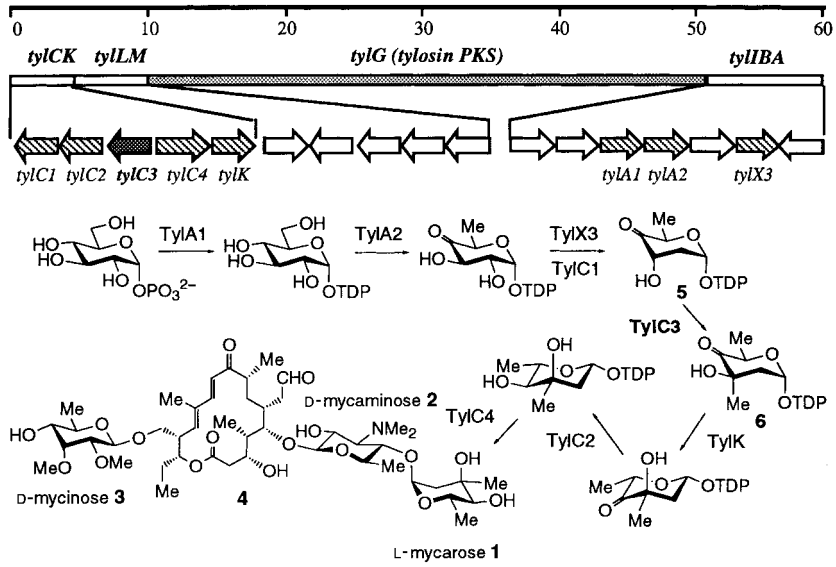
clearly play a central role is the production of methyl-branched sugars. On the basis of their biogenesis, branched-chain sugars can be divided into two groups.[4, 5] Sugars carrying a methyl or a two-carbon side chain are classified as Group I, and those bearing a hydroxymethyl or a formyl branch make up Group II. Branched chains longer than two carbon atoms are extremely rare. Formation of Group I branched-chain sugars most likely involves coupling of a one- or two-carbon unit from appropriate donors such as AdoMet or pyruvate to a diphosphonucleotidyl(NDP)-hexulose.[5] Unfortunately, information on the biosynthetic pathways of these unusual sugars, especially mechanisms of branched-chain attachment, is sparse and is mostly derived from early tracer experiments.[4, 5] This paper reports the isolation and characterization of TylC3, the first C-methyltransferase to be purified that is involved in the biosynthesis of a branched-chain sugar, namely, L-mycarose (**1**, Scheme 1). L-Mycarose, along with two other sugars, D-mycaminose (**2**) and D-mycinose (**3**), is an essential component of the macrolide antibiotic tylosin (**4**), which is produced by *Streptomyces fradiae.* Tylosin is used commercially to treat veterinary Gram-positive and mycoplasma infections, as well as to promote livestock growth.[6] L-Mycarose also forms part of a few other clinically important antibiotics including erythromycin, in which L-mycarose is methylated at O-3 (L-cladinose).



Scheme 1. Top: organization of the tylosin PKS gene cluster; bottom: biosynthesis of tylosin (**4**). **1**–**3** are shown as substituents, not as independent molecules.

Early genetic studies led to the identification of the entire gene cluster responsible for the biosynthesis of tylosin,[7] including the genes involved in the biosynthesis of L-mycarose.[8] Sequencing results and analyses identified *tylC3* as the gene likely to encode the C-methyltransferase required for the attachment of the methyl branched chain to the hexulose precursor **5** (Scheme 1). Although TylC3 did not display significant sequence homology to any characterized AdoMet-dependent methyltransferase, three localized sequences similar to the well-defined binding motifs of AdoMet-dependent enzymes were identified. One gene product that did exhibit strong homology to TylC3 was EryBIII (68% identity and 80% similarity) in the erythromycin-producing strain *Saccharopolyspora erythraea*.[9] Mutation experiments implicated *eryBIII* as the gene encoding the C-methyltransferase in the biosynthesis of L-cladinose in erythromycin.[10] However, no biochemical studies were performed on the gene product to confirm its function.

To verify the function of TylC3, the *tylC3* gene was amplified by polymerase chain reaction (PCR) from the cosmid pHJL311[11] and cloned into a pET-24b(+) vector. The resulting construct, pHC34, was used to transform *Escherichia coli* BL21(DE3), from which the expressed C-terminal His-tagged TylC3 was purified to near homogeneity by a Ni-NTA column (Qiagen). N-Terminal amino acid sequencing confirmed the identity of TylC3,[12] and the subunit molecular mass of 48 kDa, as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), correlates well with the predicted value of 46 423 Da deduced from the amino acid sequence. An M_r of 43.1 kDa, estimated by gel filtration, indicated that TylC3 exists as a monomer in solution.

Initial tests with [^3H_3C]AdoMet showed that TylC3 does bind AdoMet, even in the absence of sugar substrate. HPLC analysis[13] of an incubation of this enzyme with the expected substrate **5**[14] revealed the presence of a new compound. The NMR data identified[15] this new product as the C-3 methylated sugar **6**, and this firmly establishes the function of TylC3 as the AdoMet-dependent C-methyltransferase required for the biosynthesis of L-mycarose. A positive Nuclear Overhauser Effect (NOE) between the newly installed C-3 methyl group and the C-5 axial hydrogen atom showed the methyl group to be axial; this indicates that TylC3-catalyzed methylation proceeds with overall inversion of configuration at C-3.

An HPLC assay was developed and used to determine the kinetic parameters for the enzyme.[16] K_m for AdoMet was 1.5 ± 0.2 μM, but K_m for the sugar substrate **5** was beyond the sensitivity of the assay and could only be estimated as less than 1 μM. The small K_m values and a k_{cat} of 1.4 ± 0.1 min^{-1} are typical for methyltransferases,[2] which exhibit large catalytic accelerations as compared to the uncatalyzed reaction even though their turnover numbers are often low. Considering that purified TylC3 is UV/Vis transparent above 300 nm and its activity does not depend on the presence of metal ions,[17] the enzyme appears not to require the assistance of any cofactor to stabilize the enediolate intermediate **7**, which is likely to be the methyl acceptor in this mechanism (Scheme 2).

To gain evidence for the intermediacy of enediolate **7** in the course of TylC3-catalyzed reaction,[18] a 4,4-difluoro substrate analogue **8** was synthesized as shown in Scheme 3.[19–21] It is anticipated that upon incubation with TylC3, **8** may undergo deprotonation at C-3 followed by the elimination of a fluoride

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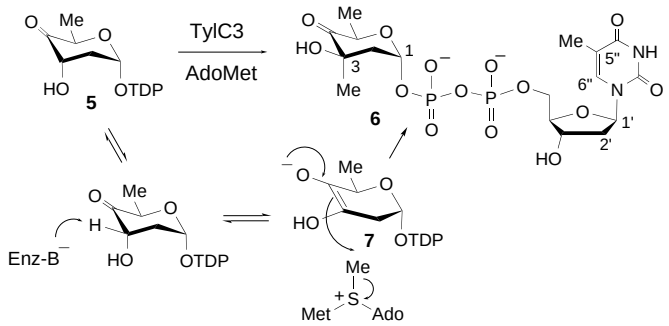
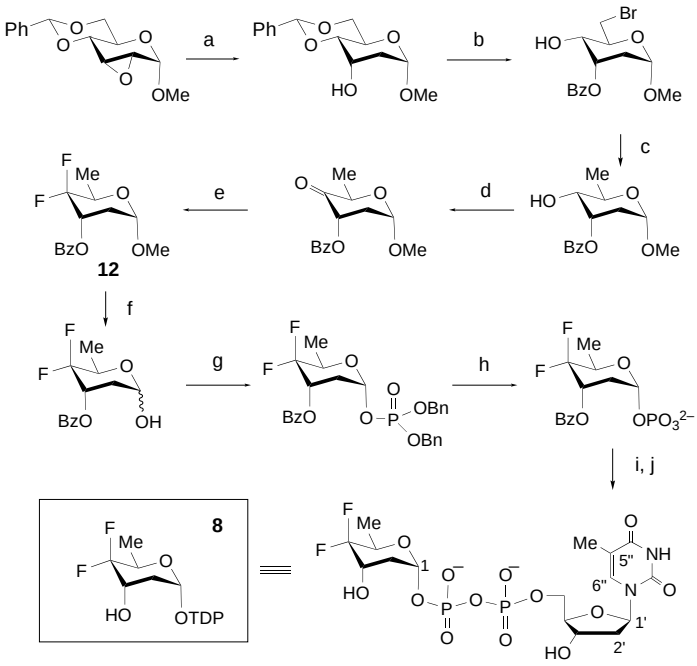


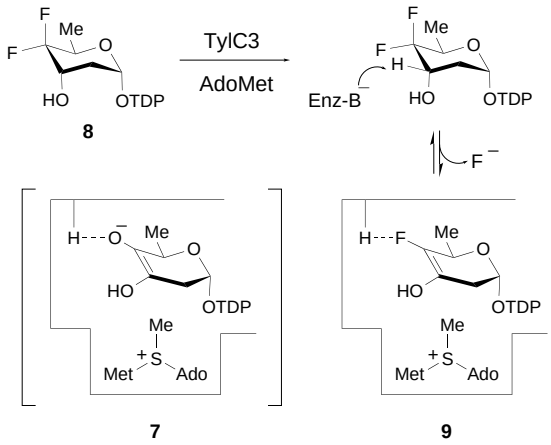
Scheme 2. Essential components of the methylation step **5** → **6**.



Scheme 3. a) Super-Hydride, THF, 97 %;[19] b) NBS, $BaCO_3$, CCl_4;[20] c) Bu_3SnH, AIBN, benzene, two steps, 30 %; d) PCC, CH_2Cl_2, 69 %; e) DAST, CH_2Cl_2, 82 %; f) Dowex-50W(H^+), MeCN, H_2O, 85 %; g) BuLi, THF, then dibenzyl phosphorochloridate, 66 %; h) 5 % Pd/C, H_2, MeOH, 93 %; i) 1*H*-tetrazole, pyridine, TMP-morpholidate; j) 2 M LiOH, THF, two steps, 47 %. AIBN = azobisisobutyronitrile, Bn = benzyl, Bz = benzoyl, DAST = (diethylamino)sulfur trifluoride, NBS = *N*-bromosuccinimide, PCC = pyridinium chlorochromate, TMP = thymidine 5′-trihydrogenphosphate, TDP = thymidine 5′-dihydrogenphosphate.

ion from C-4 to yield a new intermediate **9** (Scheme 4).[22] Since **9** closely resembles the putative enediolate intermediate **7**, binding of **9** to the active site of TylC3 may lead to enzyme inhibition. However, to our disappointment, when compound **8** was exposed to TylC3, no release of fluoride ion could be detected by ^{19}F NMR, and no inhibition of TylC3 was observed. Considering that **8** is not even a competitive inhibitor for TylC3 at a concentration 25 times higher than that of the substrate in a competition experiment, **8** must either have little affinity toward TylC3 or have difficulty fitting into the active site. Clearly, a full elucidation of the catalytic mechanism of TylC3 must await further experiments.

Nevertheless, since TylC3 is a prototypical C-methyltransferase involved in methyl-branched sugar formation, insights gleaned from this study should be applicable to the biosynthesis of other methyl-branched sugars such as L-vinelose, D- and L-virenose, D-evalose, L-nogalose, L-chromose B, D-evermicose, and L-axenose.[4, 5, 23] The present studies on TylC3 not only expand our knowledge of AdoMet-dependent enzymes, but also add to the tools available for the genetic manipulation of biosynthetic pathways of deoxy sugars. Since the sugar components of macrolide antibiotics are known to be essential for specificity and activity of the parent drug, the ability to genetically engineer microorganisms to produce sugars that contain various structural alterations, such as branched chains, provides an innovative approach to the discovery of clinically useful compounds.



Scheme 4. Expected effect of TylC3 on the 4,4-difluoro substrate analogue **8**.

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[11] Cosmid pHJL311 was a generous gift from Lilly Research Laboratories.

[12] N-Terminal amino acid sequencing confirmed that the first 20 residues (MIISACRVCGNRELLPVLDL) of this protein are identical to the translated TylC3 sequence.

[13] Analysis by HPLC was performed with an Adsorbosphere SAX column (5 μ, 4.6 × 250 mm) with a gradient from 140 mM to 320 mM potassium phosphate buffer, pH 3.5, over 20 min, followed by a 5-min wash with 500 mM potassium phosphate buffer, pH 3.5.

[14] Substrate **5** was prepared as described previously: H. Chen, G. Agnihotri, Z. Guo, N. L. S. Que, X. H. Chen, H.-w. Liu, *J. Am. Chem. Soc.* **1999**, *121*, 8124 – 8125.

[15] **6**: ^{1}H NMR (500 MHz, ^2H_2O): $\delta = 1.11$ (d, $^3J(H,H) = 6.0$ Hz, 3H; 5-Me hydrated form), 1.15 (d, $^3J(H,H) = 6.0$ Hz, 3H; 5-Me keto form), 1.37 (s, 3H; 3-Me hydrated form), 1.45 (s, 3H; 3-Me keto form), 1.82 (s, 3H; 5″-Me), 1.92 (m, 2H; 2-H hydrated form), 2.23 – 2.32 (m, 2H; 2′-H), 2.40 (m, 2H; 2-H keto form), 4.01 – 4.10 (m, 3H; 5-H hydrated form, 4′-H, 5′-H), 4.47 – 4.53 (m, 1H; 3′-H), 4.72 (q, $^3J(H,H) = 6.0$ Hz, 1H; 5-H keto form), 5.49 (m, 1H; 1-H hydrated form), 5.64 (m, 1H; 1-H keto form), 6.24 (t, $^3J(H,H) = 4.2$ Hz, 1H; 1′-H), 7.61 (s, 1H; 6″-H); ^{13}C NMR (75 MHz, ^2H_2O, hydrated form): $\delta = 11.6$, 12.0, 23.5, 38.3, 40.9 (d, $^3J(C,P) = 7.5$ Hz; C-2), 65.3 (d, $^2J(C,P) = 5.6$ Hz; C-5′), 68.1, 70.7, 71.8, 84.7, 85.0 (d, $^3J(C,P) = 7.5$ Hz; C-4′), 94.0 (d, $^2J(C,P) = 5.3$ Hz; C-1), 94.3, 111.6, 137.2, 151.6, 166.5. The ratio of the hydrated form to the keto form is approximately 3:1.

[16] The HPLC assay used for determining the kinetic parameters was performed on an Adsorbosphere SAX column (5 μ, 4.6 × 250 mm), which was eluted isocratically with 50 mM potassium phosphate buffer, pH 3.5. The peak integrations of (*S*)-adenosylmethionine and (*S*)-adenosylhomocysteine were used to determine the product conversion.

[17] Inductively coupled plasma (ICP) analysis for metal ions indicated the presence of approximately 0.4 mol of Zn^{II} per mole of TylC3. However, the zinc does not appear to be important for activity, since dialysis of the enzyme against 5 mM 1,10-phenanthroline for 4 d did not reduce the activity, although ICP analysis indicated that approximately half of the zinc was removed. Attempts to reconstitute the enzyme with zinc also failed to increase the activity. Likewise, the addition of Mg^{II} did not increase the activity of TylC3.

[18] Enediolates are common intermediates in many biotransformations. For a few examples, see: a) R. V. J. Chari, J. W. Kozarich, *J. Am. Chem. Soc.* **1983**, *105*, 7169 – 7171; b) A. E. Johnson, M. E. Tanner, *Biochemistry* **1998**, *37*, 5746 – 5754; c) C. J. Jeffery, B. J. Bohnson, W. Chien, D. Ringe, G. A. Petsko, *Biochemistry* **2000**, *39*, 955 – 964.

[19] H. H. Bear, H. R. Hanna, *Carbohydr. Res.* **1982**, *110*, 19 – 41.

[20] S. Hanessian, N. R. Plessas, *J. Org. Chem*. **1969**, *34*, 1035 – 1044.

[21] **8**: ^{1}H NMR (300 MHz, ^2H_2O): $\delta = 1.10$ (d, $^3J(H,H) = 6.6$ Hz, 3H; 5-Me), 1.73 (s, 3H; 5″-Me), 1.86 (m, 1H; 2-H_{ax}), 2.08 (m, 1H; 2-H_{eq}), 2.18 (m, 2H; 2′-H), 3.98 (m, 4H; 3-, 3′- and 5′-H), 4.06 (dq, $^3J(H,F) = 21.6$, $^3J(H,H) = 6.6$ Hz, 1H; 5-H), 4.44 (m, 1H; 4′-H), 5.40 (m, 1H; 1-H), 6.16 (m, 1H; 1′-H), 7.57 (s, 1H; 6″-H); ^{13}C NMR (75 MHz, ^2H_2O): $\delta = 11.6$, 12.0, 23.5, 38.3, 40.9 (d, $^3J(C,P) = 7.5$ Hz; C-2), 65.3 (d, $^2J(C,P) = 5.6$ Hz; C-5′), 68.6 (dd, $^1J(C,F) = 31.7$, $^1J(C,F) = 24.1$ Hz; C-4), 70.9, 84.8, 85.2 (d, $^3J(C,P) = 9.0$ Hz; C-4′), 93.6 (d, $^2J(C,P) = 4.6$ Hz; C-1), 111.6, 137.3, 151.6, 166.5; ^{19}F NMR (282 MHz, ^2H_2O): $\delta = -125.9$ (d, $^2J(F,F) = 253$ Hz), −128.3 (dd, $^2J(F,F) = 253$, $^3J(H,F) = 21.2$ Hz); ^{31}P NMR (121 MHz, ^2H_2O): $\delta = -11.2$ (d, $^2J(P,P) = 20.7$ Hz), −12.1 (d, $^2J(P,P) = 20.7$ Hz); HRMS (ESI) calcd for $C_{16}H_{23}F_2N_2O_{13}P_2$ $[M-H]^-$: 551.0649; found: 551.0669.

[22] The difluoromethylene moiety is a strongly electron-withdrawing group which can stabilize the corresponding β-anion both by induction and by negative hyperconjugation: B. E. Smart in *Chemistry of Organic Fluorine Compounds II* (Eds.: M. Hudlicky, A. E. Pavlath), American Chemical Society, Washington, **1995**, pp. 979 – 1010. Although the pK_a data of protons adjacent to the difluoromethylene group are not available, examples of α-anion-induced β-fluoride elimination in similar structures are well known: A. M. Kornilov, I. B. Kulik, A. E. Sorochinsky, V. P. Kukhar, *Tetrahedron Asymmetry* **1995**, *6*, 199 – 206; D. Schirlin, S. Baltzer, J. M. Altenburger, C. Tarnus, J. M. Remy, *Tetrahedron* **1996**, *52*, 305 – 318.

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Oxathiaphospholane Approach to the Synthesis of P-Chiral, Isotopomeric Deoxy(ribonucleoside phosphorothioate)s and Phosphates Labeled with an Oxygen Isotope**

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Introduced by Eckstein, phosphorothioate analogues of nucleotides have become an indispensable tool for studying the metabolism of nucleic acids.[1] Standard chemical methods for the synthesis of oligo(deoxyribonucleoside phosphorothioate)s (PS-Oligos) provide a mixture of 2^n diastereoisomers, where n is the number of phosphorothioate linkages.[2] The enzymatic synthesis of stereodefined PS-Oligos is limited to the preparation of (all-R_P)-oligomers because of the stereoselectivity of available DNA and RNA polymerases. The first method for stereocontrolled chemical synthesis of PS-Oligos was elaborated in our group,[3] and several alternative methods were recently reported.[4, 5] Stereodefined PS-Oligos were used for studying the mode of action of several bacterial and human enzymes[6–8] and the stereodependent avidity of PS-Oligos toward complementary DNA or RNA.[9] However, the presence of a sulfur atom affects the properties of internucleotide bonds, mostly due to the different steric requirements of sulfur atoms (P–S vs P–O bond length), different affinity towards metal ions, and changes in the distribution of the negative charge in the phosphorothioate anion.[10] Therefore, the hydration pattern of PS-Oligos is different from that of natural oligonucleotides,[11] and this obstructs the evaluation of kinetic data of "rescue effects" of thiophilic metal ions, and makes analysis of direct or water-mediated contacts between metal ions and phosphate groups much more difficult. These inconveniences could be avoided by using P-chiral isotopomeric phosphates.[12] Here we describe the synthesis of stereodefined oligo(deoxyribonucleoside [^{18}O]phosphorothioate)s (PS^{18}O-Oligos) and oligo(deoxyribonucleoside [^{18}O]phosphate)s (P^{18}O-Oligos), in which both of the nonbridging oxygen atoms of the internucleotide bond were replaced by S and ^{18}O, or one of them was replaced by ^{18}O, respectively. Oligonucleotides containing a single P-chiral [^{16}O,^{18}O] internucleotide bond were first used by Eckstein[13] in studies on Eco RI endonuclease. Stereodefined P^{18}O-Oligos can be used to investigate the interaction of particular oxygen atoms with other molecules or metal ions, given analytical methods that allow the isotopic effect to be measured with satisfactory accuracy.[14]

To obtain stereodefined PS^{18}O-Oligos, we synthesized 5′-*O*-DMT-nucleoside-3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-

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