Table I. Substrate Activities of Analogues[23] [a]

substrate analogue	K_M, μM	V_{max}, pmol min^{-1} mg^{-1}
1	23.1 ± 1.4	14.0 ± 0.3
2	14.0 ± 1.1	14.0 ± 0.3
4	50.6 ± 5.4	3.4 ± 0.1
5	29.0 ± 4.7	7.4 ± 0.4
7	25.8 ± 3.6	0.6 ± 0.02

[a] Methyltransferase activity was determined as described in the legend of Figure 1. Kinetic constants for **1** and **2**[19] are given here for comparison.

substrate. Intramolecular transfer of the methyl group to the free carboxyl moiety produces the neutral ester. A mechanism of this type would develop positive charge on the sulfur atom, suggesting that β-alanine derivatives like **8** be studied as possible substrates or transition-state inhibitors of the enzyme. However, **8** proved to be neither a substrate nor an inhibitor of the methyltransferase. Therefore, there is currently no evidence for a mechanism involving initial transfer of the methyl group to the sulfur atom of the substrate. Mechanisms of this type also seem unlikely since model studies suggest that an "in-line" displacement mechanism is required in methyl group transfers from sulfur.[21,22]

The studies described here demonstrate a great deal of specificity of the methyltransferase for the farnesylthiopropionic acid moiety. Here we show a clear preference for the presence of a nucleophilic sulfur atom in an active substrate. Alterations in this heteroatom progressively lead from substrate molecules to competitive inhibitors to an inert molecule in the case of **8**. Although the methyltransferase's function is to modify a large protein, in seeking the protein the enzyme looks not for the protein itself but for a small prosthetic unit and, indeed, not for the entire prosthetic unit but for a specific moiety within it.

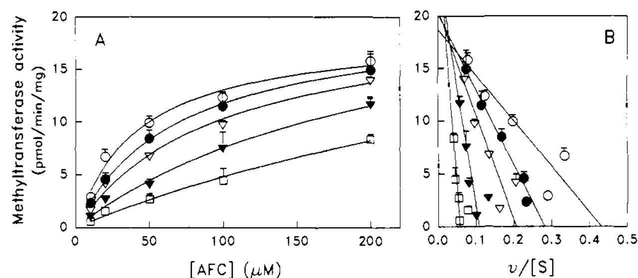

Figure 1. Inhibition of **1** methylation by **3**. Michaelis-Menten (A) and Eadie-Hofstee (B) plots of the formation of **1** [^{3}H]methyl ester as a function of **1** concentration in the presence of increasing concentrations of **3**: (○) no **3** and (●) 10, (△) 20, (▼) 50, and (□) 100 μM **3**. Assays were performed as described in ref 14. Briefly: **1** and **3**, were dissolved in Me_2SO and incubated with washed rod outer segment membranes (0.5 mg of protein/mL) and *S*-adenosyl[*methyl*-^{3}H]methionine (10 μM, 15 Ci/mmol) in 100 mM Hepes (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$ for 30 min at 37 °C. Final Me_2SO concentration in the assay was 4% (v/v). The amount of the corresponding *methyl*-^{3}H esters was determined by HPLC analysis of the chloroform extracts obtained from the incubation mixtures. Samples were injected in 15% 2-propanol/hexane on a normal-phase HPLC column (Dynamax 60) connected to an on-line Berthold radioactivity monitor and eluted with the same solvent at 1.5 mL/min. Symbols represent average values of four determinations and error bars indicate the standard deviation of mean.

Acknowledgment. This work was financially supported by U.S. Public Health Service N.I.H. Grant EY-03624. The excellent technical assistance of Ms. Mei Huei Tu is gratefully acknowledged.

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(23) **4** was prepared from DL-selenocystine by esterification followed by N-acetylation, reduction with zinc, S-alkylation with *trans,trans*-farnesyl bromide, and saponification. Chromatography of the ester on a flash silica column, eluting with hexane/ethyl acetate (4:1-1:2), gave the methyl ester of **4**, as a light yellow oil. [NMR (300 MHz, $CDCl_3$) δ 1.57 (6 H, s), 1.63 (3 H, s), 1.65 (3 H, s), 1.8-2.1 (8 H, m), 2.01 (3 H, s), 2.79 (1 H, dd, J = 5.6, 12.9 Hz), 2.89 (1 H, dd, J = 5.6, 12.9 Hz), 3.20 (2 H, m), 3.73 (3 H, s), 4.85 (1 H, dt, J_d = 7.5 Hz, J_t = 5.1 Hz), 5.05 (2 H, br t, J = 6.5 Hz), 5.26 (1 H, t, J = 8.1 Hz), 6.28 (1 H, br d, J = 7.5 Hz)]. The methyl ester of **4** (0.10 g, 0.2 mmol) was treated with 5% potassium hydroxide/methanol (10 mL) for 30 min, and the mixture was acidified to approximately pH 5 with 2 M HCl, diluted with 30 mL of water, and extracted with chloroform. The organic extract was dried, concentrated to a small volume, and chromatographed on a silica preparative layer, eluting with ethyl acetate/methanol (4:1) to give **4** as a waxy solid. The NMR spectrum of **4** was essentially identical with that of the methyl ester except that the singlet resonance at δ 3.73 ppm was absent. **5** was prepared by treating *trans,trans*-farnesyl bromide with selenourea, hydrolysis of the selenoronium salt, and conjugate addition of the selenol to methyl acrylate. Chromatography of the ester on silica eluting with ethyl acetate/hexane (1:5) gave the methyl ester of **5** as a colorless oil [NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.66 (3 H, s), 1.9-2.2 (8 H, m), 2.6-2.8 (4 H, m), 3.24 (2 H, d, J = 8.2 Hz), 3.67 (3 H, s), 5.07 (2 H, br t, J = 6.2 Hz), 5.31 (1 H, t, J = 8.2 Hz)]. Saponification of the methyl ester as described above and chromatography on silica, eluting with ethyl acetate/hexane (1:4-1:1), gave the product as a thick colorless oil. The NMR spectrum of **5**, was identical with that of the methyl ester except that the singlet resonance at δ 3.67 ppm was absent. **6** was prepared by a conjugate addition of *trans,trans*-farnesol to methyl acrylate followed by saponification. This ester was chromatographed on flash silica eluting with ethyl acetate/hexane (1:9-1:3). The methyl ester was obtained as a colorless oil [NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.66 (3 H, s), 1.9-2.2 (8 H, m), 2.57 (2 H, t, J = 6.3 Hz), 3.68 (3 H, s), 3.68 (2 H, t, J = 6.3 Hz), 3.98 (2 H, d, J = 6.6 Hz), 5.07 (2 H, m), 5.31 (1 H, t, J = 6.6 Hz)]. Saponification of the methyl ester (50 mg) was carried out as described above. Chromatography on silica eluting with ethyl acetate/hexane (1:4-1:1) gave the product as a thick colorless oil. The NMR spectrum of **6** was identical with that of the methyl ester except that the singlet resonance at δ 3.68 ppm was absent. **7** was prepared by formation of the Schiff base between β-alanine methyl ester and *trans,trans*-farnesal followed by reduction with sodium borohydride and saponification. This ester was chromatographed on a preparative silica layer, eluting with methanol/ethyl acetate (1:2). The methyl ester of **7** had an R_f of 0.5 and was isolated as a colorless oil in 19% yield [NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.75 (6 H, s), 1.9-2.1 (8 H, m), 2.47 (2 H, t, J = 7.5 Hz), 2.83 (2 H, br t, J = 6.5 Hz), 3.18 (2 H, br d, J = 6.0 Hz), 3.64 (3 H, s), 5.05 (2 H, br m), 5.22 (1 H, br t, J = 6.0 Hz)]. Saponification of the methyl ester using the procedure described above and chromatography on a preparative silica layer, eluting with methanol/ethyl acetate (1:1), gave the product (R_f = 0.3) as a waxy solid [NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.75 (6 H, s), 1.9-2.1 (8 H, m), 2.88 (2 H, br t, J = 7.5 Hz), 3.18 (2 H, br m), 3.65 (2 H, br d, J = 5.9 Hz), 5.05 (2 H, br m), 5.39 (1 H, br t, J = 5.9 Hz)].

A Highly Efficient Multienzyme System for the One-Step Synthesis of a Sialyl Trisaccharide: In Situ Generation of Sialic Acid and *N*-Acetyllactosamine Coupled with Regeneration of UDP-Glucose, UDP-Galactose, and CMP-Sialic Acid[1]

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Increasing interests in sialyl oligosaccharides[2] necessitate the development of practical methodology for the synthesis of this class of molecules. Although chemical methods for the synthesis of sialosides have been highly developed,[3] the necessary multistep protection/deprotection procedure makes large-scale process

(1) Supported by NIH GM44154. All enzymes used in this study are commercially available except CMP-NeuAc synthetase.[6]
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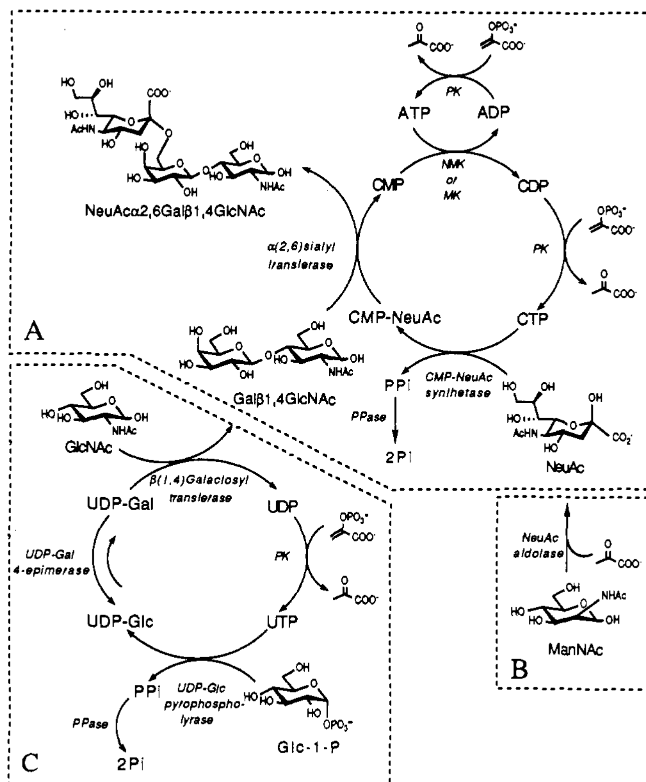

Figure 1. Abbreviations: Glc-1-P, glucose 1-phosphate; GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannosamine; NeuAc, N-acetylneuraminic acid; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; CMP, cytidine 5'-monophosphate; CDP, cytidine 5'-diphosphate; CTP, cytidine 5'-triphosphate; CMP-NeuAc, cytidine 5'-monophospho-N-acetylneuraminic acid; UDP, uridine 5'-diphosphate; UTP, uridine 5'-triphosphate; UDP-Glc, uridine 5'-diphosphoglucose, UDP-Gal, uridine 5'-diphosphogalactose; PPi, inorganic pyrophosphate; Pi, inorganic phosphate; PK, pyruvate kinase; NMK, nucleoside monophosphate kinase; MK, myokinase; PPase, inorganic pyrophosphorylase.

difficult. Enzymatic synthesis of sialosides has been also extensively studied[4] but usually requires a separate preparation of expensive and unstable sugar nucleotides, and the process often suffers from product inhibition by the released nucleoside di- or monophosphates. A practical solution to these problems was illustrated in a large-scale synthesis of N-acetyllactosamine (LacNAc) with in situ regeneration of nucleoside diphosphate sugars.[5] Recently, we reported the enzyme-catalyzed synthesis of sialyl-LacNAc, NeuAcα(2,6)Galβ(1,4)GlcNAc, starting from sialic acid (NeuAc), LacNAc, phospho(enol)pyruvate (PEP), and catalytic amounts of ATP and CMP, with in situ regeneration of CMP-NeuAc, CTP, and ATP (A in Figure 1).[6] Now, we report herein two efficient enzymatic procedures for the synthesis of sialyl-LacNAc: one starting from N-acetylmannosamine (ManNAc) and N-acetyllactosamine (LacNAc) and another from three monosaccharides, N-acetylglucosamine (GlcNAc), N-acetylmannosamine (ManNAc), and glucose 1-phosphate (Glc-1-P).[7] The first procedure involves the in situ generation of NeuAc catalyzed by NeuAc aldolase coupled with regeneration of CMP-NeuAc (A + B in Figure 1). The second procedure involves the in situ generation of NeuAc and LacNAc coupled with regeneration of UDP-Glc, UDP-Gal, and CMP-NeuAc (A + B + C in Figure 1). The two multienzyme systems operate very efficiently without problems of product inhibition.

The enzymatic aldol reaction (B in Figure 1) was first introduced to the previously investigated regeneration system (A in Figure 1): ManNAc was converted to NeuAc catalyzed by NeuAc aldolase[8] (EC 4.1.3.3) in the presence of pyruvic acid. Although NeuAc aldolase also catalyzes the reverse reaction (NeuAc to ManNAc and pyruvate), the produced NeuAc is irreversibly incorporated into cycle A via CMP-NeuAc catalyzed by CMP-sialic acid synthetase[6] coupled with inorganic pyrophosphatase (PPase) catalyzed decomposition of the released inorganic pyrophosphate. The sialyl-LacNAc was obtained in 89% yield after Bio-Gel P-2 column chromatography. The experimental procedure is as follows:

To 1.65 mL of HEPES buffer (200 mM, pH 7.5) were added ManNAc (43 mg, 180 μmol), LacNAc (22 mg, 60 μmol), CMP (2.0 mg, 6 μmol), ATP (0.32 mg, 0.6 μmol), PEP sodium salt (56 mg, 240 μmol), $MgCl_2\cdot6H_2O$ (12.2 mg, 60 μmol), $MnCl_2\cdot4H_2O$ (3.0 mg, 16 μmol), KCl (4.4 mg, 60 μmol), pyruvic acid sodium salt (33 mg, 300 μmol), NeuAc aldolase (EC 4.1.3.3; 45 U), MK (EC 2.7.4.3; 100 U), PK (EC 2.7.1.40; 120 U), PPase (EC 3.6.1.1; 6 U), mercaptoethanol (0.22 μL), CMP-NeuAc synthetase (EC 2.7.7.43; 0.3 U in 1 mL of 0.1 M Tris buffer, pH 9), and α-(2,6)sialyltransferase (EC 2.4.99.1; 0.08 U). The final volume of the reaction mixture was 3 mL. The reaction was conducted at room temperature for 2 days under argon. After disappearance of the starting material as judged by TLC on silica (R_f: LacNAc, 0.63; NeuAc, 0.31; sialyl-LacNAc, 0.30; CMP-NeuAc, 0.19 in 1:2.4 (v/v) 1 M NH_4OAc/iPrOH), the reaction mixture was directly applied on a Bio-Gel P-2 (200–400 mesh) column (2 × 36 cm) and eluted with water. The trisaccharide-containing fractions were pooled and lyophilized to give sialyl-LacNAc (37 mg, 89%) with ^1H NMR data identical with those reported.[6]

We then combined the LacNAc synthesizing cycle[5] (C in Figure 1) and the above-mentioned cycle (A + B in Figure 1). This system requires only three monosaccharide components, GlcNAc, ManNAc, and Glc-1-P, for the sialyl-LacNAc synthesis. UDP-Glucose pyrophosphorylase converted Glc-1-P to UDP-Glc, which was epimerized to UDP-Gal by UDP-Gal 4-epimerase.[5] The galactose moiety of UDP-Gal was coupled with GlcNAc to generate LacNAc, which was incorporated into cycle A in Figure 1 to produce sialyl-LacNAc. The experimental procedure is as follows:

To 2.6 mL of HEPES buffer (200 mM, pH 7.5) were added ManNAc (43 mg, 180 μmol), GlcNAc (13.3 mg, 60 μmol), Glc-1-P (21.5 mg, 60 μmol), CMP (2.0 mg, 6 μmol), UDP (2.8 mg, 6 μmol), ATP (0.32 mg, 0.6 μmol), PEP sodium salt (75 mg, 320 μmol), $MgCl_2\cdot6H_2O$ (16.3 mg, 80 μmol), $MnCl_2\cdot4H_2O$ (4.0 mg, 20 μmol), KCl (6.0 mg, 80 μmol), pyruvic acid sodium salt (33 mg, 300 μmol), NeuAc aldolase (45 U), MK (100 U), PK (120 U), PPase (12 U), mercaptoethanol (0.33 μL), galactosyltransferase (EC 2.4.1.22; 1 U), UDP-Glc pyrophosphorylase (EC 2.7.7.9; 1 U), UDP-Gal 4-epimerase (EC 5.1.3.2; 1 U), CMP-NeuAc synthetase (0.3 U in 1 mL of 0.1 M Tris buffer, pH 9), and α(2,6)sialyltransferase (0.08 U). The final volume of the reaction mixture was 4 mL. The reaction was stopped in 2 days, and pure sialyl-LacNAc (9 mg; 22%) was isolated based on the above-mentioned procedure.[9]

In summary, we have demonstrated two new procedures for the efficient synthesis of a sialyl trisaccharide: one starting from ManNAc and LacNAc and another from GlcNAc, ManNAc, Glc-1-P, and catalytic amounts of CMP, UDP (0.1 equiv each), and ATP (0.01 equiv) with no tedious separate preparations of sugar nucleotides, which are regenerated in situ. The pyruvate generated from PEP is used as a substrate in the NeuAc aldolase reaction. These procedures illustrate the advantages of multi-

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enzyme-catalyzed organic synthesis with in situ cofactor regeneration[10] and should be applicable to the synthesis of many novel biologically important sialosides and oligosaccharides starting from unactivated monosaccharides.

Acknowledgment. We thank Dr. J. C. Paulson (Cytel Corp., La Jolla, CA) for providing us with α(2,6)sialyltransferase.

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Surface-Enhanced Raman Spectroscopy of C_{60} on Gold: Evidence for Symmetry Reduction and Perturbation of Electronic Structure in the Adsorbed Molecule

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The recent discovery of methods for preparing and purifying C_{60} in significant yield[1-3] has facilitated new studies of its structure and photophysical properties. Recent electrochemical studies reveal that C_{60} and C_{70} have very similar pyracylene-type electronic character,[4] in apparent contradiction to the theoretical prediction of quite different highly delocalized excited-state orbitals in the two molecules.[5] This behavior, along with the recently reported observation that salts of the C_{60} radical anion behave as semiconductors,[6] prompts questions of whether the C_{60} structure and electronic properties will be perturbed at interfaces. Low-resolution STM images of C_{60} reveal spheroidal molecules that are mobile on gold surfaces, indicating that they are not strongly bound.[7,8] The detailed interpretation of the STM results remains specious, however, as it is not clear to what extent the STM probe tip perturbs the electronic structure of the adsorbed molecule.[7] We have therefore used surface-enhanced Raman (SER) spectroscopy to obtain a clearer picture of the surface interactions and structure of C_{60} on gold. Differences between the SER and Raman spectra provide evidence for both a loss of symmetry and a change in the electronic structure of C_{60} upon adsorption.

C_{60} was prepared as described previously.[2] The IR[1] and Raman[9,10] spectra of bulk C_{60} confirm that it has an icosahedral structure. Only four of 174 normal modes are IR active, and 10 or 11 bands are observed in the Raman spectrum (see footnote *d*, Table I).[9-11] In the SER spectrum of pure C_{60} on gold with no applied potential (Figure 1a), we can clearly identify 22 bands,[12] which are compared with bands in the Raman spectra of bulk C_{60} and C_{70} in Table I. In addition to bands similar to those in the bulk C_{60} Raman spectrum, we observe a new band at 340 cm^{-1} that may correspond to a metal–molecule vibration. The other new bands in the C_{60} SER spectrum agree closely with bands in the C_{70} Raman spectrum.[10,11,13] [C_{70} is predicted to have 53 Raman-active modes, 21 of which have been observed (Table I).]

Table I. SER and Raman Bands (cm^{-1}) of C_{60}

SER	Raman[b]	
C_{60}[a]	C_{60}	C_{70}
175 w		
256 sh		261 (28, 4)
270 s	273 (20, 10)	
340 m		
398 w		400 (sh, 0)
419 m		411 (7, 0)
	437 (9, 3)	
		459 (7, 0)
488 s	496 (43, 3)	501 (6, 0)
510 w–m		
551 w		
575 w		573 (6, 0)
700 m, br	710 (6, 2)	704 (11, 2)
730 m		739 (12, 4)
766 m	774 (10, 3)	770 (5, 0)
962[c] w, br	~970[d] (~2)	
		1062 (19, 5)
1088 w		
1186 w	1099 (2, 0)	1186 (46, 8)
1235 w		1231 (51, 8)
	1250 (2, 0)	1260 (5, 0)
1307 vw		1298 (sh, 0)
		1317 (sh, 0)
		1336 (13, 0)
		1370 (13, 0)
1393 w		
1422 w–m	1428 (14, 3)	
1452 vs		1448 (52, 9)
	1470 (100, 6)	1471 (25, 0)
		1517 (18, 8)
1559[c] s	1575 (9, 3)	1569 (100, 19)

[a] Abbreviations: w = weak, m = medium, s = strong, v = very, br = broad, sh = shoulder. [b] Raman data for C_{60} and C_{70} from ref 10. Intensities for (parallel, perpendicular) polarizations are relative to the 1470-cm^{-1} band for C_{60} and to the 1569-cm^{-1} band for C_{70}. [c] Reassigned in this work. [d] Observed in the reported spectrum[10] but not tabulated or included in the analysis.

[†] University of Pittsburgh. Present address: University of California at Los Angeles.
[‡] University of California at Los Angeles.

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(12) The surface cleaning procedure, spectrochemical cell, and Raman spectrometer are described in the following: Garrell, R. L.; Beer, K. D. *Langmuir* **1989**, *5*, 452–458. The electrode was roughened by 20 oxidation/reduction cycles between −0.600 V and +1.200 V at a scan rate of 0.500 V/s, with pauses of 8 s at −0.600 V and 1.2 s at +1.200 V. The electrode was removed from the roughening cell and rinsed with doubly distilled water to ensure the removal of residual chloride ion. To obtain spectrum 1a, 1 μL of 1.1 × 10^{-4} M C_{60} in CCl_4 was deposited on the electrode surface. This corresponds to a surface coverage of 1–2 monolayers on a 4-mm-diameter smooth surface. For spectra 1b and 1c, ~0.3 monolayer was deposited. The C_{60}-coated electrode was immersed in N_2-purged water (which served as a heat sink to minimize laser heating of the adlayer) or in N_2-purged aqueous 0.1 M KCl supporting electrolyte, and the solution was blanketed with N_2 throughout the experiment. Single-scan spectra (scan rate 1 cm^{-1}/s, 2-cm^{-1} band-pass) were obtained with 20 mW of 676.4-nm radiation (Lexel Ramanlon krypton ion laser). Repeat scans were identical, indicating that no laser-induced degradation was occurring. Spectra 1b and 1c have been smoothed with a five-point polynomial smoothing function.

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