J. Am. Chem. Soc. 1992, 114, 3966-3973

Structure-Activity Studies on the Retinal Rod Outer Segment Isoprenylated Protein Methyltransferase

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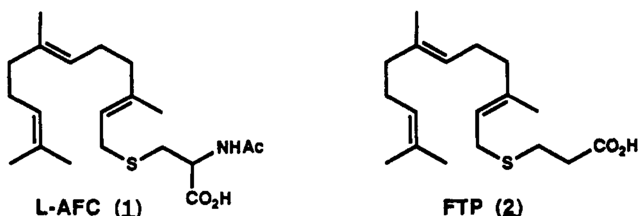
Received November 8, 1991

Abstract: Structure-activity studies were performed on the retinal rod outer segment isoprenylated protein methyltransferase that transfers a methyl group from *S*-adenosylmethionine (AdoMet) to the carboxyl group of isoprenylated (farnesylated or geranylgeranylated) cysteine residues. This methyltransferase enzyme has been shown to methylate *N*-acetyl-*S*-farnesyl-L-cysteine (L-AFC, **1**) and *S*-(farnesyl-3-thio)propionic acid (FTP, **2**). It is shown here that the enzyme does not enzymatically process D-AFC (**8**), although D-AFC (**8**) is a mixed-type inhibitor of the enzyme. Small modifications in the FTP (**2**) structural series generally lead to inactive substrates. For example, neither the *cis*- nor the *trans*-acrylate derivatives of FTP (**2**) are substrates of the enzyme, but both are inhibitors of it. Alkyl substitutions at the 3-position of FTP (**2**), moreover, lead to inhibitors of the methyltransferase. Substituents at the 2-position of FTP (**2**), as in 2-methyl-*S*-(farnesyl-3-thio)propionic acid (MFTP, **28**) or *S*-farnesyl-2-(thiomethyl)acrylic acid (FTMA, **31**), produce active substrates. Modifications at the carboxyl moiety produce neither substrates nor inhibitors of the enzyme. The conclusion from this and earlier studies is that the methyltransferase is selective for an isoprenylated thiopropionate moiety. Small deviations from this minimally essential structure lead to the abolition of substrate activity.

Introduction

Isoprenylated protein methyltransferases catalyze the transfer of methyl groups from *S*-adenosylmethionine (AdoMet) to carboxyl terminal *S*-farnesylated or *S*-geranylgeranylated cysteine residues.[1,2] The formation of the carboxyl terminal methyl ester completes the set of hydrophobic modifications which make up the isoprenylation pathway (Scheme I).[3-6] Other reactions in the pathway involve the transfer of either a farnesyl ($n = 1$) or a geranylgeranyl ($n = 2$) moiety to the cysteine thiol, followed by proteolytic cleavage of the "CAAX" box, rendering the S-prenylated cysteine carboxyl as the terminal residue.[3,4,7-18] Isoprenylation is thought to target the modified peptides to membranes where they are active.[3-5,6,19] Of the biochemical reaction shown in Scheme I, only the methylation reaction is biochemically reversible[20] and, hence, susceptible to regulation.

Isoprenyl transferases, which catalyze the first reaction in the pathway, recognize the peptide sequences in the neighborhood of the cysteine to be alkylated.[1,2] This is important because there needs to be a mechanism to distinguish between those proteins to be farnesylated and those to be geranylgeranylated. Indeed, there are distinct enzymes for farnesylation and geranylgeranylation.[21,22] On the other hand, the methyltransferase seems not to require specific peptide sequences. Simple farnesylated molecules, such as *N*-acetyl-*S*-farnesyl-L-cysteine (L-AFC, **1**) and *S*-(farnesyl-3-thio)propionic acid (FTP, **2**), are excellent substrates for the enzyme, with K_M values of 23.0 and 13.7 μM, respectively.[23] In both cases, all remnants of the protein backbone are gone, suggesting that the enzyme recognizes mainly the *S*-(farnesyl-3-thio)propionic acid moiety and not the carrier protein. Modifications of the basic FTP (**2**) moiety tend to produce molecules of lessened substrate activity. For example, deletion of a methylene carbon in FTP (**2**) generates *S*-(farnesyl-2-thio)acetic acid (FTA, **3**), a potent inhibitor of the enzyme with a measured K_I of 4.6 μm, and reduction of the farnesyl side chain generates an inactive molecule.[23] In this article, the specificity of the rod outer segment isoprenylated protein methyltransferase is further defined with respect to the nature of inhibitor and substrate structure.



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Materials and Methods

Materials. Frozen bovine retinas were obtained from W. Lawson Co. (Lincoln, NE). *S*-Adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmol and 85 Ci/mmol) and Amplify were purchased from Amersham. L-Cysteine, DL-cysteine, D-cysteine, dimethyl vinylphosphoate, *trans,trans*-farnesol, methyl 2-(bromomethyl)acrylate, (methylthio)trimethylsilane, 2-nitroethanol, D-(−)-α-phenylglycinol, potassium trimethylsilanolate, and trimethylbromosilane were acquired from Fluka Chemical. Methyl 2-nonenoate was procured from Pfaltz & Bauer. *trans,trans*-Farnesyl-L-cysteine (L-FC, **4**), *trans,trans*-farnesyl-DL-cysteine (DL-FC, **5**), and

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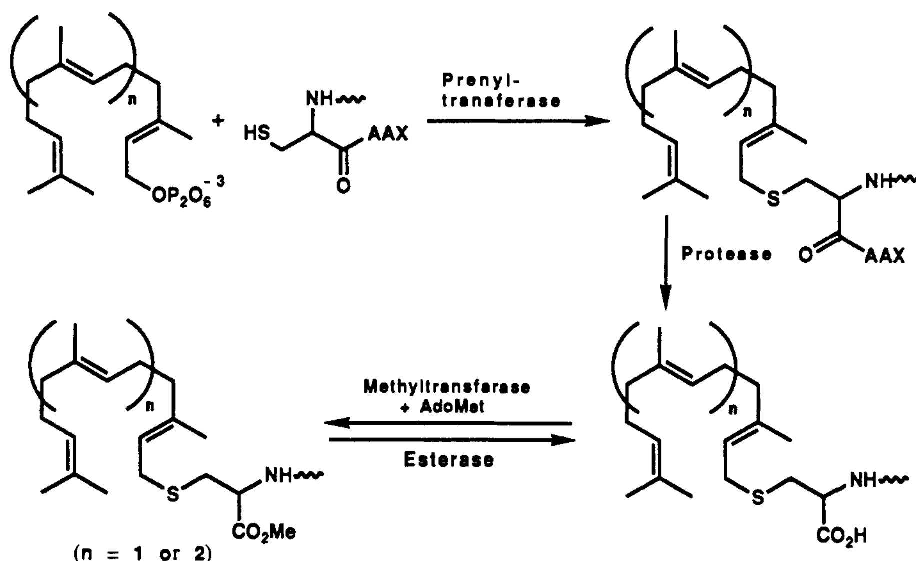
Scheme I. Biochemical Reactions in the Isoprenylation Pathway



trans,trans-farnesyl-D-cysteine (D-FC, **6**) were prepared according to the procedure of either Brown,[24] Kamiya,[25] or Yang.[26] The syntheses of *S*-(farnesyl-3-thio)propionic acid (FTP, **2**) and *S*-(farnesyl-2-thio)acetic acid (FTA, **3**) have been previously reported.[23] Other reagents, unless otherwise stated, were purchased from Aldrich Chemical. Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained either with a Varian VRX 300S or a Varian VRX 500S spectrometer operating at a proton frequency of 299.949 and 499.843 MHz, respectively. Chloroform ($CDCl_3$), dimethyl sulfoxide (DMSO-d_6), or water (D_2O) was used as the ^{1}H NMR solvent. The residual proton absorption of the deuterated solvent was used as the internal standard. All ^{1}H NMR chemical shifts are reported as δ values in parts per million (ppm), and the coupling constants (J) are given in hertz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet; dd, doublet of doublets; dt, doublet of triplets, dm, doublet of multiplets; sxt, sextet.

Preparation of Rod Outer Segment (ROS) Membranes and Methyltransferase Assay. The ROS membranes were obtained from frozen bovine retinas as previously described.[23] The membranes were used as the source of methyltransferase. Inhibitors or substrates for the methyltransferase were dissolved in DMSO and incubated in washed ROS membranes (0.5 mg of protein/mL), *S*-adenosyl-L-[methyl-^{3}H]-methionine (10 μM, 15 Ci/mmol), in 100 mM Hepes (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$ for 30 min at 37 °C. The final concentration of DMSO in the assay was 4% (vol/vol). The amount of the corresponding methyl-^{3}H ester produced was determined by HPLC analysis of the chloroform extracts obtained from the incubation mixtures as described in Perez-Sala et al. 1991.[20] The samples were injected on a normal phase HPLC column (Dynamax 60, Rainin, Woburn, MA) connected to an on-line Berthold radioactivity monitor (Berthold, Nashua, NH) and eluted at a flow rate of 1.5 mL/min, with the solvent conditions described below for each compound. In all cases, the methyl-^{3}H esters were readily separated from the corresponding parent acids.

Standard Procedure for the Schotten–Bauman[27] N-Acetylation of *trans-trans*-Farnesylcysteine.[24–26] The appropriate enantiomer of *trans,trans*-farnesylcysteine (1.0 mmol) was added in one portion to a solution of 1 N aqueous NaOH (1.0 mL) at 0 °C. After stirring for 5.0 min, 1 N aqueous NaOH (0.20 mL) and acetic anhydride (0.20 mmol) were added sequentially. The solution was stirred at 0 °C for 10 min. The addition of 1 N aqueous NaOH (0.20 mL) and acetic anhydride (0.20 mmol) was repeated five more times. The mixture was maintained alkaline (pH 9) by the periodic addition of 1 N aqueous NaOH. After the last addition, the mixture was stirred at room temperature for 30 min, then acidified (pH 3) with 3.5% aqueous HCl, and extracted with ethyl acetate (5 × 25 mL). The combined ethyl acetate layers were washed with brine (50 mL), dried ($MgSO_4$), filtered, and concentrated to give the crude product as an oil. The desired AFC was isolated by use of silica gel chromatography, eluting with 5% MeOH in EtOAc.

***N*-Acetyl-*S*-farnesyl-L-cysteine (L-AFC, 1).** L-FC (**4**) was N-acetylated by use of the standard procedure described above to afford L-AFC (**1**) as a colorless oil in 52% yield: −23.4° [*c* 0.01, MeOH]$_D$; HPLC t_R = 4.3 min (hexane/2-propanol/TFA, v/v/v, 85:15:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.54 (6 H, s), 1.61 (3 H, s), 1.62 (3 H, s), 1.84 (3 H, s), 1.83–1.92 (2 H, m), 1.96–2.05 (6 H, m), 2.60 (1 H, dd, J = 8.5, 13.5 Hz), 2.78 (1 H, J = 5.5, 13.5 Hz), 3.10 (1 H, dd, J = 7.5, 13.5 Hz), 3.18 (1 H, dd, J = 8.0, 13.5 Hz), 4.35 (1 H, dt, J = 5.0, 11.5 Hz), 5.05 (2 H, br s), 5.14 (1 H, t, J = 7.0 Hz), 8.19 (1 H, d, J = 8.0 Hz). The physical properties and spectroscopic characteristics of this compound were consistent with those reported in the literature.[23]

***N*-Acetyl-*S*-farnesyl-DL-cysteine (DL-AFC, 7).** DL-FC (**5**) was N-acetylated by use of the standard procedure described above to afford DL-AFC (**7**) as a colorless oil in 43% yield. The physical properties and spectroscopic characteristics of this compound were consistent with those reported above.

***N*-Acetyl-*S*-farnesyl-D-cysteine (D-AFC, 8).** D-FC (**6**) was N-acetylated by use of the standard procedure described above to afford D-AFC (**8**) as a colorless oil in 46% yield: +24.0° [*c* 0.01, MeOH]$_D$. The physical properties and spectroscopic characteristics of this compound were consistent with those reported above.

The methyl ester of the L-AFC (**1**) was obtained from the corresponding carboxylic acid by treatment with methanolic HCl (0.05–0.1 M).[28] The product isolated gave a ^{1}H NMR spectrum essentially identical to that of the parent compound except for a singlet resonance, equivalent to 3 protons, at δ 3.74 ppm. HPLC analysis gave a single peak with retention time, t_R = 6.8 min (hexane/2-propanol, v/v, 85:15, flow rate = 1.5 mL/min).

General Procedure for the Determination of Enantiomeric Excess of L-AFC (1) and D-AFC (8). 1-Ethyl-3-((3-dimethylamino)propyl)carbodiimide hydrochloride[29] (1.2 mmol) was added in one portion to a solution of the appropriate AFC enantiomer (1.0 mmoL), D-(−)-α-phenylglycinol[30,31] (1.5 mmol), and 1-hydroxybenzotriazole hydrate[32] (1.5 mmol) in DMF (2.5 mL) at 0 °C. The mixture was stirred at 0 °C for 2 h and then at room temperature overnight. The solution was poured into brine (20 mL) and extracted with ethyl acetate (5 × 25 mL). The combined ethyl acetate layers were washed with 3.5% aqueous HCl (3 × 25 mL), dried ($MgSO_4$), filtered, and concentrated to give the crude product as an oil. The unpurified *N*-acetyl-*S*-farnesylcysteine phenylglycinolamide (AFC–PGA) was then eluted through a short plug of silica

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gel with EtOAc to remove base line contaminants prior to ^{1}H NMR (500 MHz) analysis.

***N*-Acetyl-*S*-farnesyl-DL-cysteine-(D)-α-phenylglycinolamide (DL-AFC-PGA, 9).** DL-AFC (**7**) was derivatized as described in the procedure above to provide DL-AFC-PGA (**9**) as a waxy solid. Analysis of the diastereomeric amide by ^{1}H NMR (500 MHz, DMSO-d_6) showed two distinct peaks—a broad triplet at δ 5.16 (J = 7.0 Hz, 51%) and a broad triplet at δ 5.11 (J = 7.0 Hz, 49%) corresponding to the vinyl proton at C-2 of the farnesyl moiety. HPLC analysis gave two peaks with t_R = 7.2, and 19.8 min in a ratio of 51:49 (hexane/2-propanol, v/v, 95:5, flow rate = 1.5 mL/min) for the two diastereomers.

***N*-Acetyl-*S*-farnesyl-L-cysteine-(D)-α-phenylglycinolamide (L-AFC-PGA, 10).** L-AFC (**1**) was derivatized as described in the procedure above to provide L-AFC-PGA (**10**) as a colorless oil. Analysis of the diastereomeric amide by ^{1}H NMR (500 MHz, DMSO-d_6) showed two distinct sets of peaks—a broad triplet at δ 5.16 (J = 7.0 Hz, 96%) and a broad triplet at δ 5.12 (J = 7.0 Hz, 4%). HPLC analysis gave two peaks with t_R = 7.4 and 19.1 min in a ratio of 4:96 (hexane/2-propanol, v/v, 95:5, flow rate = 1.5 mL/min). The percentage enantiomeric excess (% ee) of L-AFC (**1**) was estimated to be 92%.

***N*-Acetyl-*S*-farnesyl-D-cysteine-(D)-α-phenylglycinolamide (D-AFC-PGA, 11).** D-AFC (**8**) was derivatized as described in the procedure above to provide D-AFC-PGA (**11**) as a colorless oil. Analysis of the diastereomeric amide by ^{1}H NMR (500 MHz, DMSO-d_6) showed two distinct sets of peaks—a broad triplet at δ 5.11 (J = 7.0 Hz, 2%) and a broad triplet at δ 5.17 (J = 7.0 Hz, 98%). HPLC analysis gave two peaks with t_R = 7.2 and 19.8 min in a ratio of 98:2 (hexane/2-propanol, v/v, 95:5, flow rate = 1.5 mL/min). The percentage enantiomeric excess (% ee) of D-AFC (**8**) was estimated to be 96%.

Synthesis of *trans*,trans-Farnesyl Mercaptan (FM, 12) from *trans*,-*trans*-Farnesyl Bromide. A solution of *trans,trans*-farnesyl bromide (5.70 g, 20.0 mmol, 1.0 equiv), thiourea[33] (1.52 mg, 20.0 mmol, 1.0 equiv) in 2-propanol (30 mL) was stirred at room temperature for 4 h. The solvent was removed under vacuum, and the residue triturated with hexane (100 mL). The resulting solid was filtered and dried under high vacuum to afford *trans,trans*-farnesyl thiouronium bromide (**13**), as a colorless solid in 89% yield (mp 100–101 °C): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.66 (3 H, s), 1.69 (3 H, s), 1.92–2.21 (8 H, m), 3.82 (2 H, d, J = 8.5 Hz), 5.07 (2 H, br t, J = 6.9 Hz), 5.22 (1 H, br t, J = 8.5 Hz), 8.71 (2 H, br s), 8.94 (2 H, br s). The product was sufficiently pure for use in the subsequent hydrolysis.

Potassium hydroxide (700 mg, 12.5 mmol, 2.5 equiv) in water (2.0 mL) was added in one portion to a suspension of *trans,trans*-farnesyl thiouronium bromide (**13**) (18.0 g, 4.98 mmol, 1.0 equiv), 3-*tert*-butyl-4-hydroxy-5-methylphenyl sulfide[34] (200 mg, 0.56 mmol, 0.1 equiv) in 2-propanol (30 mL). The mixture was stirred at room temperature for 4 h and then acidified (pH 5) with 3.5% aqueous HCl (10 mL). The mixture was extracted with ethyl acetate (4 × 50 mL). The combined ethyl acetate layers were washed with brine (25 mL), dried ($MgSO_4$), filtered, and concentrated to provide a yellow oil. Chromatography of the material on silica, eluting 10% ethyl acetate in hexanes, afforded FM (**12**) as a colorless oil in 73% yield: HPLC t_R = 2.7 min (hexane/2-propanol, v/v, 100:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.37 (1 H, t, J = 7.5 Hz), 1.58 (6 H, s), 1.66 (3 H, s), 1.69 (3 H, s), 1.91–2.20 (8 H, m), 3.40 (2 H, t, J = 7.5 Hz), 5.07 (2 H, br t, J = 6.9 Hz), 5.32 (1 H, br t, J = 7.5 Hz).

Synthesis of *trans,trans*-Farnesyl Mercaptan (FM, 12) from *trans*,-*trans*-Farnesol. Diisopropyl azodicarboxylate[35,36] (8.10 mL, 8.32 g, 19.8 mmol, 2.1 equiv) was added slowly to a solution of triphenylphosphine (10.8 g, 41.2 mmol, 2.1 equiv) in THF (100 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min. A mixture of *trans,trans*-farnesol (4.39 g, 19.8 mmol, 1.0 equiv) and thioacetic acid (3.09 g, 40.7 mmol, 2.1 equiv) in THF (50 mL) was then added dropwise over a 10-min period. The mixture was stirred at 0 °C for 1 h and then at room temperature for 1.5 h. The solvent was removed under vacuum and the residue triturated with hexanes (100 mL). The resulting solid was filtered and the filtrate concentrated to provide a yellow oil. Chromatography of this oil on silica, eluting with 10% ethyl acetate in hexanes, afforded *trans,trans*-farnesyl thioacetate (**14**) as a colorless oil in 92% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.66 (3 H, s), 1.69 (3 H, s), 1.94–2.10 (8 H, m), 2.33 (3 H, s), 3.53 (2 H, d, J = 7.5 Hz), 5.07 (2 H, br t, J = 6.9 Hz), 5.22 (1 H, br t, J = 7.5 Hz).

trans,trans-Farnesyl thioacetate (**14**) (1.00 g, 3.57 mmol, 1.0 equiv) in THF (2.0 mL) was added dropwise to a suspension of lithium aluminum hydride (528 mg, 14.2 mmol, 4.0 equiv) in THF (40 mL) at 0 °C. The mixture was stirred at room temperature for 1 h, and then the excess lithium aluminum hydride was destroyed by the slow addition of ethyl acetate (5.0 mL). The solution was poured into 10% aqueous HCl (50 mL) and extracted with ethyl acetate (5 × 50 mL). The combined ethyl acetate layers were washed with brine (25 mL), dried ($MgSO_4$), filtered, and concentrated to provide a yellow oil. Chromatography of this material on silica, eluting with 10% ethyl acetate in hexanes, afforded FM (**12**) as a colorless oil in 95% yield. The physical properties and spectroscopic characteristics of this compound were consistent with those listed above.

General Procedure for the Synthesis of Certain Inhibitors and Substrates by Conjugate Addition. The appropriate Michael acceptor (2.0 mmol) was added in one portion to a solution of FM (**12**) (1.0 mmol) and sodium methoxide (1.0 mmol) in methanol (5.0 mL) at 0 °C. The mixture was stirred at 0 °C for 10 min and then at room temperature for 1 h. The solvent was removed under vacuum, and the residue dissolved in ethyl acetate (150 mL). The ethyl acetate solution was washed with 3.5% aqueous HCl (25 mL) and brine (25 mL), dried (Na_2SO_4), and concentrated to an oil. Chromatography of this material on silica gave the desired product.

***S*-(Farnesyl-3-thio)propionitrile (FTPN, 15).** Acrylonitrile (Michael acceptor) was treated as described in the procedure for conjugate addition to provide FTPN (**15**) as a colorless oil in 83% yield: HPLC t_R = 3.9 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.66 (6 H, s), 1.91.2.21 (8 H, m), 2.58 (2 H, t, J = 6.3 Hz), 2.69 (2 H, t, J = 6.3 Hz), 3.21 (2 H, d, J = 8.9 Hz), 5.06 (2 H, br t, J = 6.9 Hz), 5.20 (1 H, br t, J = 8.0 Hz).

***S*-(Farnesyl-3-thio)propionamide (FTPA, 16).** Acryloamide was treated as described in the procedure for conjugate addition to provide FTPA (**16**) as a white solid in 85% yield (mp 81–83 °C): HPLC t_R = 6.5 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.55 (6 H, s), 1.61 (3 H, s), 1.63 (3 H, s), 1.91–2.20 (8 H, m), 2.45 (2 H, t, J = 7.2 Hz), 2.72 (2 H, t, J = 7.2 Hz), 3.14 (2 H, d, J = 7.6 Hz), 5.06 (2 H, br t, J = 6.0 Hz), 5.20 (1 H, br t, J = 7.6 Hz), 5.75 (1 H, br s), 6.15 (1 H, br s).

***S*-(Farnesylthio)-2-nitroethane (FTE, 17).** β-Acetylnitroethanol[37,38] was treated as described in the procedure for conjugate addition to provide FTE (**17**) as a colorless oil in 55% yield: HPLC t_R = 3.2 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.66 (6 H, s), 1.94–2.12 (8 H, m), 3.10 (2 H, t, J = 6.7 Hz), 3.18 (2 H, d, J = 7.8 Hz), 3.18 (2 H, d, J = 7.8 Hz), 4.49 (2 H, t, J = 6.7 Hz), 5.08 (2 H, m), 5.19 (1 H, br t, J = 8.0 Hz).

Methyl *S*-(Farnesyl-3-thio)butyroate (FTB-OMe, 18). Methyl crotonate was treated as described in the procedure for conjugate addition to provide FTB-OMe (**18**) as a colorless oil in 81% yield: HPLC t_R = 4.9 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (3 H, d, J = 7.9 Hz), 1.58 (6 H, s), 1.66 (6 H, s), 1.92–2.20 (8 H, m), 2.42 (1 H, dd, J = 7.4 Hz, 15.8 Hz), 2.63 (1 H, dd, J = 5.8, 15.8 Hz), 3.14 (1 H, m), 3.18 (2 H, d, J = 7.9 Hz), 3.68 (3 H, s), 5.08 (2 H, br m), 5.22 (1 H, br t, J = 7.9 Hz).

Methyl *S*-(Farnesyl-3-thio)nonanoate (FTN-OMe, 19). Methyl 2-nonenoate was treated as described in the procedure for conjugate addition to provide FTN-OMe (**19**) as a colorless oil in 63% yield: HPLC t_R = 3.1 min (hexane/2-propanol, v/v, 100:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (3 H, t, J = 8.0 Hz), 1.20–1.49 (10 H, m), 1.58 (6 H, s), 1.66 (6 H, s), 1.94–2.10 (8 H, m), 2.54 (1 H, dd, J = 8.1, 17.7 Hz), 2.89 (1 H, dd, J = 8.1, 17.7 Hz), 3.10 (1 H, m), 3.21 (2 H, d, J = 7.8 Hz), 3.69 (3 H, s), 5.08 (2 H, br m), 5.20 (1 H, br t, J = 8.1 Hz).

Methyl 2-Methyl-*S*-(farnesyl-3-thio)propionate (MFTP-OMe, 20). Methyl methacrylate was treated as described in the procedure for conjugate addition to provide MFTP-OMe (**20**) as a colorless oil in 83% yield: HPLC t_R = 5.0 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.18 (3 H, d, J = 6.8 Hz), 1.58 (6 H, s), 1.63 (3 H, s), 1.66 (3 H, s), 1.91–2.20 (8 H, m), 2.45 (1 H, dd, J = 6.8, 12.2 Hz), 2.57 (1 H, sxt, J = 6.8 Hz), 2.74 (1 H, dd, J = 6.8, 12.2 Hz), 3.08 (1 H, dd, J = 6.8, 13.6 Hz), 3.16 (1 H, dd, J = 6.8, 13.6 Hz), 3.68 (3 H, s), 5.06 (2 H, br m), 5.20 (1 H, br t, J = 6.8 Hz).

Dimethyl *S*-Farnesyl-2-thioethyl Phosphonate (DMFEP, 21). Dimethyl vinylphosphonate was treated as described in the procedure for

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conjugate addition to provide DMFEP (**21**) as a colorless oil in 66% yield: HPLC t_R = 6.0 min (hexane/2-propanol, v/v, 85:15, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56 (6 H, s), 1.63 (3 H, s), 1.92–2.09 (8 H, m), 2.65 (2 H, m), 3.14 (2 H, d, J = 7.8 Hz), 3.69 (3 H, s), 3.73 (3 H, s), 5.08 (2 H, br m), 5.20 (1 H, t, J = 8.0 Hz).

Methyl *S*-(Farnesyl-*cis*-3-thio)acrylate (*cis*-FTAA-OMe, 22). Methyl propiolate was treated as described in the procedure for conjugate addition to provide *cis*-FTAA-OMe (**22**) as a colorless oil in 58% yield: HPLC t_R = 4.1 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.66 (3 H, s), 1.91–2.20 (8 H, m), 3.39 (2 H, d, J = 7.3 Hz), 3.72 (3 H, s), 5.07 (2 H, br m), 5.28 (1 H, t, J = 7.3 Hz), 5.84 (1 H, d, J = 9.9 Hz), 7.06 (1 H, d, J = 9.9 Hz).

Methyl *S*-(Farnesyl-*trans*-3-thio)acrylate (*trans*-FTAA-OMe, 23). The standard procedure for conjugate addition was modified by the use of methyl propiolate and triethylamine (1.0 mmol) in THF (5.0 mL) to provide *trans*-FTAA-OMe (**23**) in 55% yield: HPLC t_R = 4.6 min (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.68 (3 H, s), 1.91–2.20 (8 H, m), 3.42 (2 H, d, J = 7.4 Hz), 3.68 (3 H, s), 5.07 (2 H, m), 5.24 (1 H, t, J = 7.4 Hz), 5.72 (1 H, d, J = 14.6 Hz), 7.63 (1 H, d, J = 14.6 Hz).

Methyl *S*-Farnesyl-2-(thiomethyl)acrylate (FTMA-OMe, 24). The standard procedure of conjugate addition was modified by the use of methyl 2-(bromomethyl)acrylate and triethylamine (1.0 mmol) in THF (5.0 mL) to provide FTMA-OMe (**24**) as a colorless oil in 81% yield: HPLC t_R = 5.2 min (hexane/2-propanol, v/v, 100:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.57 (6 H, s), 1.61 (3 H, s), 1.65 (3 H, s), 1.92–2.12 (8 H, m), 3.08 (2 H, d, J = 7.5 Hz), 3.32 (2 H, s), 3.75 (3 H, s), 5.06 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz), 5.62 (1 H, d, J = 1.2 Hz), 6.18 (1 H, d, J = 1.2 Hz).

Ethyl *S*-(Farnesyl-3-thio)pyruvate (FPA-OEt, 25). The standard procedure for conjugate addition was modified by the use of ethyl bromopyruvate and triethylamine (1.0 mmol) in THF (5.0 mL) to provide FPA-OEt (**25**) as a colorless oil in 73% yield: HPLC t_R = 5.7 min (hexane/2-propanol, v/v, 100:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.37 (3 H, t, J = 8.0 Hz), 1.59 (6 H, s), 1.67 (6 H, s), 1.92–2.12 (8 H, m), 3.09 (2 H, d, J = 8.0 Hz), 3.48 (2 H, s), 4.35 (2 H, q, J = 8.0 Hz), 5.02 (2 H, br s), 5.16 (1 H, t, J = 7.5 Hz).

Methyl Ester Hydrolysis. The carboxylic acids FTB (**26**), FTN (**27**), MFTP (**28**), *cis*-FTAA (**29**), and *trans*-FTAA (**30**) were obtained from their corresponding methyl esters (50–100 mg scale) by saponification with 10% aqueous KOH in methanol (10 mL). The reaction time for FTB-OMe (**18**), FTN-OMe (**19**), and MFTP-OMe (**20**) was 30 min at 0–5 °C, while the isomers of FTAA-OMe required 12 h at 40–50 °C. After completion of the reaction, the mixture was acidified (pH 5) with 3.5% aqueous HCl and concentrated. The residue was dissolved in ethyl acetate (150 mL), washed with 3.5% aqueous HCl (25 mL) and with brine (25 mL), dried (Na_2SO_4), filtered, and concentrated to give an oil. Chromatography of the material on silica gave the desired product. FTB (**26**), FTN (**27**), and MFTP (**28**) were obtained as colorless oils in 74, 69, and 77% yields, respectively. HPLC analyses of **26**, **27**, and **28** gave single peaks with t_R = 3.8, 2.8, and 3.9 min (hexane/2-propanol/TFA, v/v/v, 100:0.1:0.1, flow rate = 1.5 mL/min), respectively. Their NMR spectra were essentially identical to those of their methyl esters except that the singlet resonances at δ 3.68 ppm were absent.

***S*-(Farnesyl-*cis*-3-thio)acrylic Acid (*cis*-FTAA, 29).** The hydrolysis procedure described above was used to provide *cis*-FTAA (**29**) as a colorless oil in 69% yield: HPLC t_R = 3.3 min (hexane/2-propanol/TFA, v/v/v, 100:0.2:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.66 (3 H, s), 1.91–2.21 (8 H, m), 3.39 (2 H, d, J = 7.3 Hz), 5.07 (2 H, br m), 5.28 (1 H, t, J = 7.3 Hz), 5.84 (1 H, d, J = 9.9 Hz), 7.18 (1 H, d, J = 9.9 Hz).

***S*-(Farnesyl-*trans*-3-thio)acrylic Acid (*trans*-FTAA, 30).** The hydrolysis procedure described above was used to provide *trans*-FTAA (**30**) as a colorless oil in 63% yield: HPLC t_R = 3.6 min (hexane/2-propanol/TFA, v/v/v, 100:0.2:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (6 H, s), 1.65 (3 H, s), 1.68 (3 H, s), 1.90–2.20 (8 H, m), 3.42 (2 H, d, J = 7.4 Hz), 5.07 (2 H, m), 5.24 (1 H, t, J = 7.4 Hz), 5.72 (1 H, d, J = 14.6 Hz), 7.72 (1 H, d, J = 14.6 Hz).

Synthesis of *S*-Farnesyl-2-(thiomethyl)acrylic Acid (FTMA, 31). A solution of FTMA-OMe (**24**) (47 mg, 0.14 mmol, 1.0 equiv) and potassium trimethylsilanolate[39] (54 mg, 0.42 mmol, 3.0 equiv) in THF (4.0 mL) was stirred at room temperature for 16 h. The solution was diluted with ether (100 mL), washed with 3.5% aqueous HCl (2 × 25 mL) and with brine (25 mL), dried (Na_2SO_4), filtered, and concentrated to provide a light yellow oil. Chromatography of the material on silica, eluting with 40% hexanes in ethyl acetate, afforded FTMA (**31**) as a colorless oil in 88% yield: HPLC t_R = 3.5 min (hexane/2-propanol/TFA, v/v/v, 100:0.2:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.56 (6 H, s), 1.61 (3 H, s), 1.65 (3 H, s), 1.90–2.20 (8 H, m), 3.08 (2 H, d, J = 7.5 Hz), 3.32 (2 H, s), 5.06 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz), 5.75 (1 H, d, J = 1.2 Hz), 6.32 (1 H, d, J = 1.2 Hz).

***S*-(Farnesyl-3-thio)pyruvic Acid (FPA, 32).** The procedure described above was used to provide FPA (**32**) from FPA-OEt (**25**) as a colorless oil in 85% yield: HPLC t_R = 7.4 min (hexane/2-propanol, v/v, 92:8, flow rate = 1.5 mL/min); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (6 H, s), 1.65 (6 H, s), 1.90–2.20 (8 H, m), 3.08 (2 H, d, J = 7.5 Hz), 3.48 (2 H, s), 5.06 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz).

Synthesis of *S*-Methyl *S*-(Farnesyl-2-thio)acetothiohydroximate (FTH, 33). *n*-Butyllithium (1.60 M in hexanes, 500 μL, 0.800 mmol, 1.5 equiv) was added over a 5.0-min period to a solution of FTE (**17**) (160 mg, 0.521 mmol, 1.0 equiv) in THF (2.0 mL) at 0 °C. After stirring for 10 min, (methylthio)trimethylsilane[40] (120 μL, 0.841 mmol, 1.6 equiv) was added in portions. The solution was stirred at room temperature for 3 days. The mixture was poured into water (10 mL), then acidified (pH 6) with 3.5% aqueous HCl (10 mL), and extracted with ether (3 × 50 mL). The combined ethereal layers were washed with brine (50 mL), dried ($MgSO_4$), filtered, and concentrated to provide a yellow oil. Chromatography of the material on silica, eluting with 20% ethyl acetate in hexanes, provided FTH (**33**) as a colorless oil in 51% yield: HPLC t_R = 4.9 (hexane/2-propanol, v/v, 100:0.2, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.57 (6 H, s), 1.64 (6 H, s), 1.92–2.12 (8 H, m), 2.29 (3 H, s), 3.24 (2 H, d, J = 7.8 Hz), 3.46 (2 H, s), 5.08 (2 H, m), 5.23 (1 H, br t, J = 8.1 Hz), 7.4 (1 H, br s).

Synthesis of Sodium Methyl *S*-Farnesyl-2-thioethyl Phosphonate (SMFEP, 34). A solution of DMFEP (**21**) (41.7 mg, 0.12 mmol, 1.0 equiv) and sodium iodide[41] (16.2 mg, 0.11 mmol, 0.9 equiv) in acetone (1.0 mL) was stirred at room temperature for 18 h. The mixture was poured into water (10 mL), extracted with ether (2 × 25 mL), and the aqueous layer was concentrated to provide a white solid. Reverse phase chromatography of this material by use of a C_{18} Sep-Pak Cartridge (Millipore), eluting with water, and concentration, afforded SMFEP (**34**) as a colorless solid in 89% yield (mp > 250 °C). Reverse phase (C_{18}, Dynamax-300, Rainin) HPLC t_R = 11.1 min (H_2O/CH_3CN/TFA, v/v/v, 50:50:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, D_2O) δ 1.58 (6 H, s), 1.64 (6 H, s), 1.90–2.20 (8 H, m), 2.85 (2 H, m), 3.49 (2 H, d, J = 7.8 Hz), 3.70 (3 H, s), 5.08 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz).

Synthesis of Disodium *S*-Farnesyl-2-thioethyl Phosphonate (DSFEP, 35). Trimethylbromosilane[42] (590 μL, 4.56 mmol, 19 equiv) was added in one portion to DMFEP (**21**) (41 mg, 0.12 mmol, 1.0 equiv) in dichloromethane (1.0 mL) at room temperature. The mixture was stirred for 2 h and then concentrated. The residue was dissolved in 0.1 N aqueous Na_2CO_3 (12 mL, pH 8) extracted with ether (3 × 50 mL), and the aqueous layer was concentrated to provide a white solid. Reverse phase chromatography of this material by use of a C_{18} Sep-Pak Cartridge (Millipore), eluting with water, and concentration, afforded DSFEP (**35**) as a colorless solid in 85% yield (mp > 250 °C). Reverse phase (C_{18}, Dynamax-300, Rainin) HPLC t_R = 5.6 min (H_2O/CH_3CN/TFA, v/v/v, 50:50:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, D_2O) δ 1.60 (6 H, s), 1.69 (6 H, s), 1.90–2.20 (8 H, m), 2.89 (2 H, m), 3.44 (2 H, d, J = 7.8 Hz), 5.08 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz).

Synthesis of Sodium *S*-Farnesyl-2-thioethyl Sulfonate (SFES, 36). A solution of FM (**12**) (420 mg, 1.76 mmol, 1.0 equiv), 2-bromoethanesulfonate[43] (1.85 g, 8.76 mmol, 5.0 equiv) in water (25 mL) was heated at reflux for 2 days. The mixture was cooled and washed with ether (3 × 50 mL), and the aqueous layer was concentrated to provide a white solid. Repeated fractional recrystallization of the solid with 50% aqueous ethyl alcohol afforded SFES (**36**) as a colorless solid in 85% yield (mp > 250 °C): reverse phase (C_{18}, Dynamax-300, Rainin) HPLC t_R = 6.2 min (H_2O/CH_3CN/TFA, v/v/v, 50:50:0.1, flow rate = 1.5 mL/min); ^{1}H NMR (300 MHz, D_2O) δ 1.58 (6 H, s), 1.65 (6 H, s), 1.90–2.20 (8 H, m), 2.96 (2 H, m), 3.02 (2 H, d, J = 7.8 Hz), 3.45 (2 H, m), 5.06 (2 H, m), 5.20 (1 H, br t, J = 8.0 Hz).

Results

Stereospecificity of Substrate Processing. Initial experiments were focused on determining whether AFC is processed stereo-

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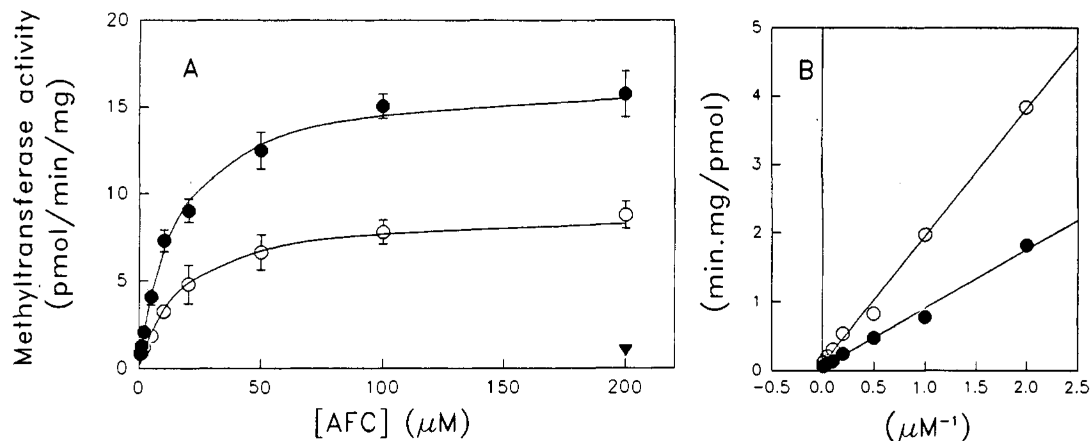


Figure 1. Methyltransferase activity in the presence of L-AFC (1) (●), DL-AFC (7) (○), and D-AFC (8) (▽). Michaelis–Menten (A) and Lineweaver (B) plots for the formation of L-AFC[³H]methyl ester as a function of increasing concentrations of L-AFC (1) (●), DL-AFC (7) (○), and D-AFC (8) (▼). Methyltransferase assays were performed as described in the Materials and Methods section. Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

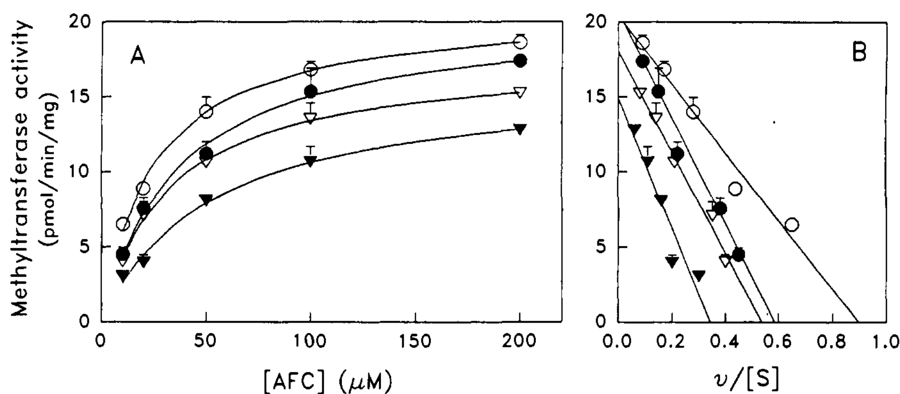


Figure 2. D-AFC (8) inhibition of L-AFC (1) methylation. Michaelis–Menten (A) and Eadie–Hofstee (B) plots for the formation of L-AFC[³H]methyl ester as a function of L-AFC (1) (20 μM) in the presence of increasing concentrations of D-AFC (8): 0 (○), 20 (●), 50 (▽), and 100 μM (▼). Methyltransferase assays were performed as described in the Materials and Methods section. Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

specifically. The importance of a chiral center in the propionate radical is not immediately obvious, since FTP (**2**) is a substrate for the enzyme. L-AFC (**1**), DL-AFC (**7**), and D-AFC (**8**) were prepared by N-acetylation of the corresponding *S*-farnesylcysteine[24-26] under Schotten–Bauman conditions.[27] The percentage enantiomeric excess (% ee) of each compound was determined by ¹H NMR spectroscopy of the chiral derivative of AFC with D-(−)-α-phenylglycinol.[30,31] By use of this method, the D-AFC (**8**) and L-AFC (**1**) obtained proved to have a % ee of 96% and 92%, respectively. D-AFC (**8**) was then assayed as a possible methyltransferase substrate and showed very slight activity (Figure 1) when compared to L-AFC (**1**) and DL-AFC (**7**). This low methyltransferase activity could be due to the 2% L-AFC (**1**) impurity present in D-AFC (**8**). Comparison of L-AFC (**1**) and DL-AFC (**7**) methyltransferase activity indicates that D-AFC (**8**) inhibits the processing of its enantiomer. When assayed as an inhibitor of L-AFC (**1**), D-AFC (**8**) inhibited the enzyme through mixed-type inhibition, with a measured K_I = 73.2 ± 4.7 μM (Figure 2 and Table I). Thus, the substrate activity of L-AFC (**1**) is stereospecific, but the D enantiomer can still bind to the enzyme.

Modifications of the Propionate Backbone. (A) Acrylate Derivatives. Initial experiments on the specificity of the retinal rod outer segment isoprenylated protein methyltransferase focused on altering the carbon backbone between the carboxyl group and the sulfur atom. Deletion of a methylene carbon from FTP (**2**) generates FTA (**3**), a competitive inhibitor.[23] The introduction of a double bond in the FTP (**2**) structure gives rise to *cis*-FTAA (**29**) and *trans*-FTAA (**30**) acrylic acid derivatives (Table II). *cis*-FTAA (**29**) and *trans*-FTAA (**30**) were readily prepared by adding *all-trans*-farnesyl mercaptan (FM, **12**) to methyl propiolate and separating the isomers by HPLC. When the Michael addition reaction was carried out in sodium methoxide (1.0 equiv) in methanol, the *cis*-FTAA-OMe (**22**) isomer predominated by a ratio of approximately 8:1. By the use of triethylamine (1.0 equiv) in THF, however, the product ratio was basically reversed to 1:7, favoring the *trans*-FTAA-OMe (**23**) isomer. Saponification of **22** and **23** with 10% aqueous KOH in methanol provided *cis*-FTAA (**29**) and *trans*-FTAA (**30**), respectively. Interestingly, neither isomer was a substrate for the methyltransferase, but they were inhibitors of the enzyme. Their apparent K_I's were virtually identical (Figures 3 and 4 and Table I), indicating a lack of enzyme binding preference for one isomer over the other.

(B) Substitutions at the 2 and 3 Positions of FTP (2). Substituted FTP derivatives were next studied for their putative substrate activities. As expected from the results with L-AFC (**1**), 2-methyl-*S*-(farnesyl-3-thio)propionic acid (MFTP, **28**) was a substrate for the enzyme (Figure 5). Correcting for the observation that the enzymatic processing is stereospecific, as found with L-AFC (**1**), the apparent K_M for MFTP (**28**) is somewhat lower than that for L-AFC (**1**) and the V_{max} of MFTP (**28**) is more than 3-fold higher (Table I). In fact, if the D enantiomer of MFTP (**28**) acts as an inhibitor of the enzyme, the V_{max} value for the L form could be even higher.

Substituents at the 3-position were also studied. Racemic *S*-(farnesyl-3-thio)butyric acid (FTB, **26**) was not a substrate for the enzyme. It proved to be a moderate mixed competitive inhibitor with a K_I of approximately 30.2 ± 3.4 μM (Figure 6 and Table I). The 3-position appeared not to be a position readily amenable to substitution, since *S*-(farnesyl-3-thio)nonanoic acid (FTN, **27**) was only a very weak inhibitor of the enzyme and, of course, not a substrate of it. It should be noted that K_I's were

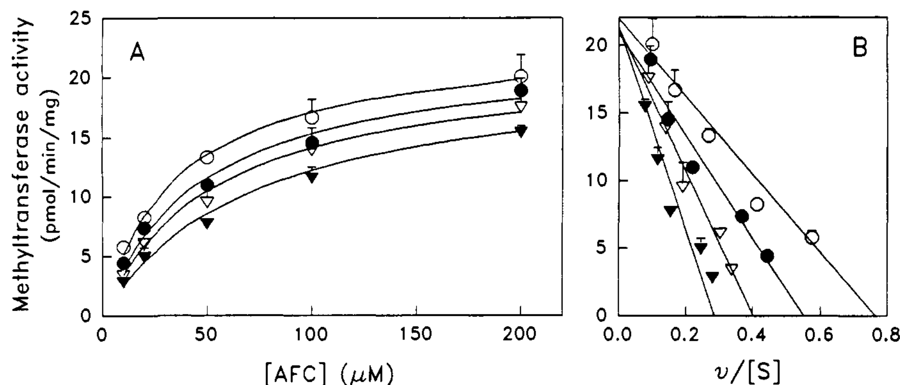


Figure 3. *cis*-FTAA (**29**) inhibition of L-AFC (**1**) methylation. Michaelis-Menten (A) and Eadie-Hofstee (B) plots for the formation of L-AFC-[^{3}H]methyl ester as a function of L-AFC (**1**) (20 μM) in the presence of increasing concentrations of *cis*-FTAA (**29**): 0 (○), 20 (●), 50 (▽), and 100 μM (▼). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

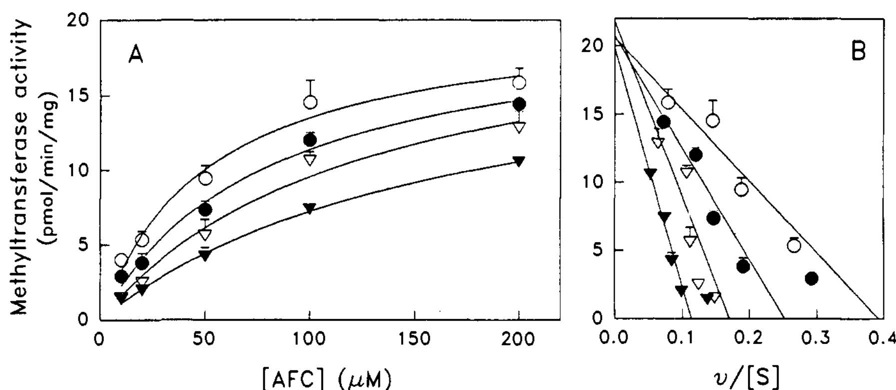


Figure 4. *trans*-FTAA (**30**) inhibition of L-AFC (**1**) methylation. Michaelis-Menten (A) and Eadie-Hofstee (B) plots for the formation of L-AFC-[^{3}H]methyl ester as a function of L-AFC (**1**) (20 μM) in the presence of increasing concentrations of *trans*-FTAA (**30**): 0 (○), 20 (●), 50 (▽), and 100 μM (▼). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

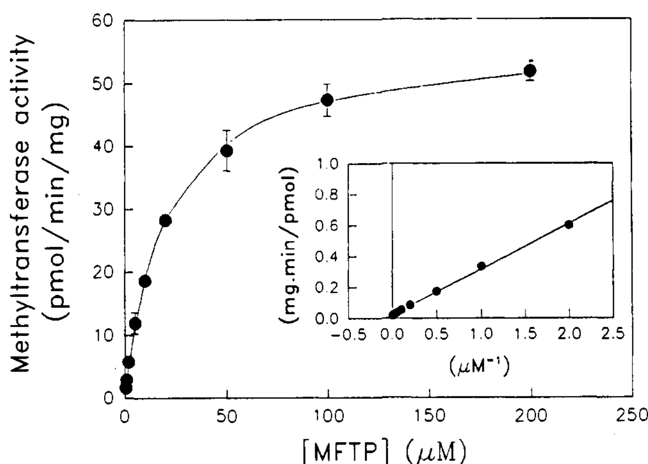
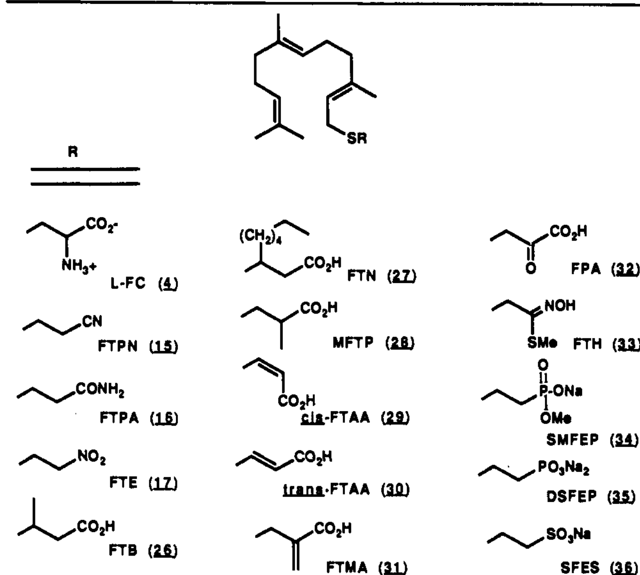


Figure 5. Methyltransferase activity in the presence of MFTP (**28**). Michaelis-Menten and Lineweaver (inset) plots for the formation of MFTP[^{3}H]methyl ester as a function of increasing concentrations of MFTP (**28**) (●). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

Table I. Kinetic Constants of Substrates and Inhibitors for Retinal Rod Outer Segment Isoprenylated Protein Methyltransferase

	K_M (μM)	V_{max} (pmol/min/mg)	K_I (μM)
L-AFC (**1**)	22.3 ± 2.9	19.4 ± 0.6	
D-AFC (**8**)			73.2 ± 4.7
FTB (**26**)			30.2 ± 3.4
MFTP (**28**)	21.6 ± 1.4	49.0 ± 2.3	
cis-FTAA (**29**)			40.4 ± 1.9
trans-FTAA (**30**)			38.3 ± 2.6
FTMA (**31**)	18.2 ± 2.4	44.0 ± 3.5	
FPA (**32**)			28.4 ± 1.5

Table II. Synthetic Substrates and Inhibitors of Retinal Rod Outer Segment Isoprenylated Protein Methyltransferase



not measured on relatively weak inhibitors because we were concerned with nonspecific inhibition effects of the molecules when assayed at concentrations above 200 μM. Therefore, we simply report K_I's of compounds which inhibited greater than 50% methyltransferase activity of L-AFC (**1**) methylation (20 μM) at 200 μM concentration of inhibitor.

Electronic effects were also studied with the α-methylene derivative, *S*-farnesyl-2-(thiomethyl)acrylic acid (FTMA, **31**). FTMA (**31**) was an excellent substrate, with a K_M = 18.2 ± 2.4 μM and a V_{max} = 44.0 ± 3.5 pmol/min/mg (Figure 7 and Table

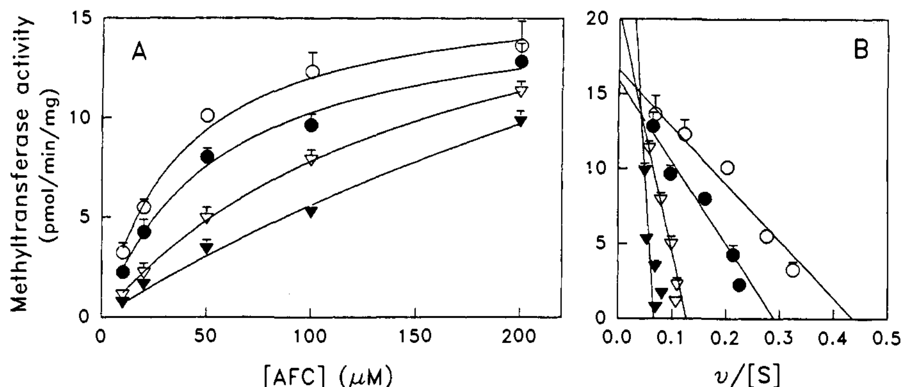


Figure 6. FTB (**26**) inhibition of L-AFC (**1**) methylation. Michaelis–Menten (A) and Eadie–Hofstee (B) plots for the formation of L-AFC[^{3}H]methyl ester as a function of L-AFC (**1**) (20 μM) in the presence of increasing concentrations of FTB (**26**): 0 (○), 20 (●), 50 (▽), and 100 μM (▼). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

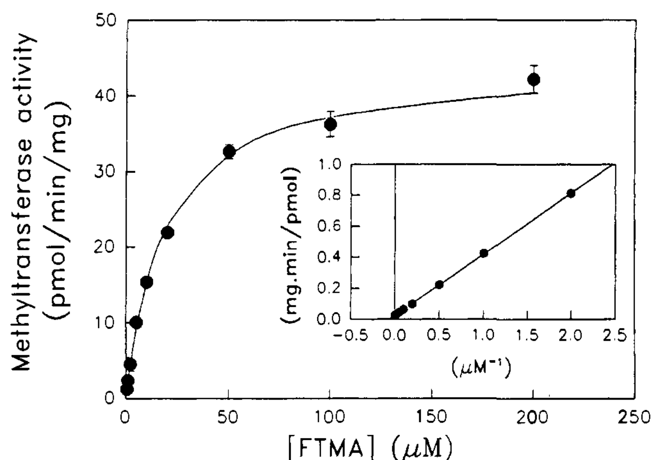


Figure 7. Methyltransferase activity in the presence of FTMA (**31**). Michaelis–Menten and Lineweaver (inset) plots for the formation of FTMA[^{3}H]methyl ester as a function of increasing concentrations of FTMA (**31**) (●). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

I). On the other hand, the α-keto derivative *S*-(farnesyl-3-thio)pyruvic acid (FPA, **32**) proved to be not a substrate but rather a potent inhibitor of the enzyme, with a K_I of 28.4 ± 1.5 μM (Figure 8 and Table I). *S*-Farnesyl-L-cysteine (L-FC, **4**), which contains a positively charged nitrogen at the 2 position, was not a substrate for the enzyme and only a weak inhibitor of it.

Modifications at the Carboxyl Group. Structure–activity studies were performed to determine the role of the carboxyl group in whether a molecule is a substrate or an inhibitor of this enzyme. Substitution of the carboxyl group for either a phosphonate—disodium *S*-farnesyl-2-thioethyl phosphonate (DSFEP, **35**)—or a sulfonate moiety—sodium *S*-farnesyl-2-thioethyl sulfonate (SFES, **36**)—led to compounds which were not substrates but only weak inhibitors of the enzyme. The monomethyl phosphonate (sodium methyl *S*-farnesyl-2-thioethyl phosphonate (SMFEP, **34**)) of DSFEP (**35**) also proved to be a weak inhibitor of the enzyme. The thiohydroximate (*S*-methyl *S*-(farnesyl-2-thio)acetothiohydroximate (FTH, **33**)) was also a weak inhibitor. Thus the enzyme discriminates at the carboxyl moiety with respect to both substrates and inhibitors.

Substitution of a neutral functional group for the ionized carboxyl moiety would not be expected to generate substrates but could produce potent methyltransferase inhibitors. Neither the carboxamide FTP (**2**) derivative (*S*-(farnesyl-3-thio)propionamide (FTPA, **16**)), the corresponding nitrile (*S*-(farnesyl-3-thio)-propionitrile (FTPN, **15**)), nor the nitro compound (*S*-(farnesylthio)-2-nitroethane (FTE, **17**)) proved to be potent methyltransferase inhibitors. Thus, a negative charge at the carboxyl terminal appears to be quite important for the binding of analogues to the enzyme active-site.

Discussion

Previously we had demonstrated that simple molecules such as L-AFC (**1**) and FTP (**2**) are excellent substrates for the retinal rod outer segment isoprenylated protein methyltransferase.[23] These results suggest that the enzyme does not require recognition of the peptide portion of the natural substrates. Substantial specificity, however, is directed at the L-AFC (**1**) moiety, because the cysteine analogue, *N*-acetyl-*S*-farnesyl-L-homocysteine (AFHC, **37**), is not a substrate for the enzyme.[23] However, removal of the *N*-acetyl group in L-AFC (**1**) produces FTP (**2**), which is an excellent substrate for the enzyme. In FTP (**2**), all vestiges of the natural peptide backbone have been removed. Modifications of the farnesyl moiety generally produce analogues with substantially reduced substrate or inhibitory activities.[23]

The oxidation state of the sulfur atom is an important factor for substrate activity, because conversion to the sulfoxide generates

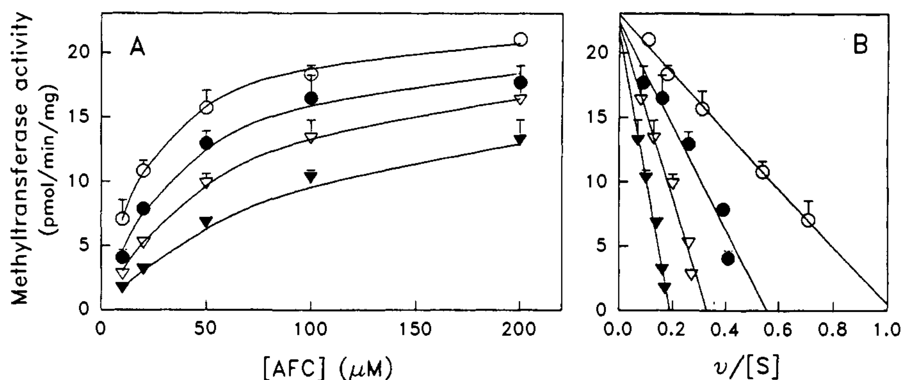


Figure 8. FPA (**32**) inhibition of L-AFC (**1**) methylation. Michaelis–Menten (A) and Eadie–Hofstee (B) plots for the formation of L-AFC[^{3}H]methyl ester as a function of L-AFC (**1**) (20 μM) in the presence of increasing concentrations of FPA (**32**): 0 (○), 20 (●), 50 (▽), and 100 μM (▼). Symbols represent average values of triplicate experiments, and error bars represent the standard deviation of mean.

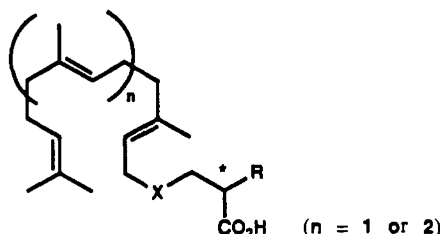


Figure 9. Active methyltransferase substrates are obtained only when X = S or Se, when R is an H, an alkyl, or an *N*-acyl group, and when the absolute stereochemistry at * is L.

a competitive inhibitor.[44] Moreover, the substitution of the sulfur for a selenium or an oxygen atom progressively decreases substrate activity, while having little effect on the binding constant.[44] The substitution of nitrogen for sulfur generates a zwitterionic species; the positive charge on nitrogen abolishes both substrate and inhibitor binding to the enzyme.[44] Therefore, the sulfur heteroatom is an important element in the interactions of the substrate with the methyltransferase.

In this article, the structure–activity studies were extended to further map the methyltransferase substrate binding site. These experiments demonstrated that only the L enantiomer of AFC was an active substrate, although D-AFC (**8**) was a mixed-type inhibitor of the enzyme. Further small structural changes in the substrate led to inhibitory molecules. The introduction of a double bond into FTP (**2**) led to the *cis*- and *trans*-acrylate derivatives, which were inert as substrates but were both competitive inhibitors with similar K_I's. The fact that the acrylates are not substrates could mean that neither the cis nor trans conformation approximates the substrate conformation at the active-site of the enzyme. Alternatively, it is possible that transmethylation from AdoMet initially occurs at the substrate's sulfur, with the molecule bound in a transoid conformation which subsequently becomes cisoid in order for the intramolecular transfer of the methyl group to occur. The possibility of an initial transmethylation to the substrate sulfur from AdoMet would also be consistent with the observed heteroatom requirement described above.[23] The possibility of a mechanism of this type could be investigated by following the stereochemical fate of the methyl group of chiral methyl labeled AdoMet during the reaction.[45,46] It should also be noted, however, that the acrylate derivatives are significantly different electronically from their saturated counterpart FTP (**2**).

The introduction of a methyl group at the 2-position of FTP (**2**) led to an active substrate, MFTP (**28**), an expected result given that L-AFC (**1**) is an excellent substrate for the enzyme. Along these lines, the 2-methylene derivative, FTMA (**31**), also proved to be an excellent substrate for the enzyme. Electronic effects may be operative as well in this series, since the 2-keto analogue, FPA (**32**), proved to be an inhibitor but not a substrate for the methyltransferase. Generally, it is expected that the 2-position of FTP (**2**) will be open for modification allowing for the preparation of potent inhibitors rather than substrates of the methyltransferase. Interestingly, even the introduction of a single methyl group, as in 3-methyl FTP, produced an inhibitor.

It is noteworthy that the small changes in structure described above led to substantial differences in substrate activities, although the molecules were still bound to the enzyme active-site. Similar observations were made on analogues where the sulfur atom was substituted for sulfoxide, oxygen, and finally selenium; the K_M's (K_I's) remained relatively constant, whereas the V_{max}'s plummeted.[44]

The results of the propionate backbone modifications described above can be contrasted with those modifications made at the carboxyl group. Neutralization of the presumptive negative charge, as in the nitrile, FTPN (**15**), the amide, FTPA (**16**), and the nitro compound, FTE (**17**), led to molecules that were only weakly bound to the enzyme and, of course, were not substrates of it. This supports the argument that the enzyme binds the charged state of the substrate. The negatively charged carboxylate group is then involved in a displacement reaction during methylation.[47,48] Phosphonates and sulfonates were not substrates for the enzyme but were relatively weak inhibitors. Therefore, a great deal of specificity appears to be directed at the carboxyl moiety, as might be expected.

The structure–activity studies described here and elsewhere show a substantial amount of specificity directed at the *S*-(farnesyl-3-thio)propionic moiety. It is likely that little binding affinity is directed at the peptide backbone, although we have not directly compared farnesylated cysteine derivatives with their peptide analogues. However, there is a published study on the methyltransferase processing of farnesylated peptides based on the ras sequence.[49] It has been reported that hexapeptide analogues terminating with a farnesylated cysteine residue are substrates for the liver methyltransferase.[49] In this case, the apparent K_M was measured to be 2 μM, which is approximately 10-fold lower than that observed with FTP (**2**), namely 13.7 μM.[23] This would suggest that some binding to the peptide may also be involved. However, it should be noted that both the assay conditions used and the source of enzyme were different from that reported here.

The limited interaction of the methyltransferase with the peptide backbone must be contrasted to what is found with the isoprenyl transferases.[15,21] Here, peptide sequence discrimination is important, as would be expected since most cysteine residues are not targeted for isoprenylation. Furthermore, farnesyl and geranylgeranyl transferases appear to recognize different peptide sequences.[50] Peptide sequence information becomes unimportant in the case of the methyltransferases, because all of the information necessary for directing the methylation reaction is found in the isoprenylated cysteine residue. The specificity of the methyltransferase with respect to the thiopropionate moiety is substantial.

In summary, the studies reported in this paper map the substrate binding-site of the retinal rod outer segment isoprenylated protein methyltransferase. The composite minimal structure shown in Figure 9 shows where permissible structural modifications can be made in the basic FTP (**2**) structure so that substrate activity is retained. As shown in Figure 9, methyltransferase substrates are only obtained under a limited set of circumstances. As implied in Figure 9, an *all-trans*-geranylgeranyl or *all-trans*-farnesyl substitution is also permitted (unpublished experiments). As indicated here and elsewhere,[23] competitive inhibitors are readily obtained by diverse modifications.

Acknowledgment. This work was financially supported by the U.S. Public Health Service N.I.H. Grant EY-03624. B.A.G. acknowledges the support of U.S. Public Health Service N.I.H. Grant EY-06345. The excellent technical assistance of Ms. Mei Tu is also gratefully acknowledged.

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