gradient (TEAB, 0.15–0.7 M, 1 L each) to give samples of the pure ATPγS (ca. 20–50 μmol), which were then transformed into [β-^{17}O,^{18}O]ATPβS as described below.

Enzymatic Conversion of ATPγS to ADPβS. The purified ATPγS (40–45 μmol) samples (see above) were converted individually to ADPβS samples by reaction with myokinase (750–1000 U) in solutions containing 50 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM $MgCl_2$, 1 mM DTT, and 75 mM AMP (1.5–3.0 mL, pH 8.0). The progress of each reaction was monitored by ^{31}P NMR spectroscopy. After 15 h at 25 °C, the reactions were determined to be ca. 75% complete. The product, ADPβS (15–35 μmol), was purified by DEAE-Sephadex A-25 column chromatography (0.1–0.4 M TEAB, pH 8.0, 500 mL each). The purified ADPβS samples were then converted to ATPβS as described below.

Stereoselective Conversion of [β-^{17}O,^{18}O]ADPβS to (S_P)-[β-^{17}O,^{18}O]-ATPβS. The [β-^{17}O,^{18}O]ADPβS (15–35 μmol) samples from the above reactions were converted to (S_P)-[β-^{17}O,^{18}O]ATPβS samples by reaction in solutions containing 10 mM ADPβS, 40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 380 mM KCl, 0.8 mM DTT, 15 mM PEP, and pyruvate kinase (250 U/mL). The progress of each reaction was monitored by ^{31}P NMR spectroscopy and found to be complete in 15 h. The reactions were terminated by passing the mixtures through Chelex 100 (2 mL, pH 8.0), washing with deionized water (8 mL). The solutions were concentrated in vacuo, and the residues obtained were dissolved in D_2O (0.8 mL) containing 50 mM EDTA, 10 mM DTT, and 200 mM Tris (pH 8.0). Carbon tetrachloride (0.25 mL) was added to each solution to precipitate residual enzyme. The aqueous layers were subjected to ^{31}P NMR analysis. The spectra of these substances were obtained on a Bruker AM 400 instrument at 160 MHz with a deuterium field lock; spectral width 7936 Hz, acquisition time 4.19 s, pulse width 3 μs, relaxation delay 0.5 s, number of transients, 5000–11 300 (see Figures 4 and 5 for plots of the $^{31}P_\beta$ regions of these spectra).

Acknowledgment. Financial assistance was provided by NIH Grants GM-28688 (D.D.M.) and GM-27251 (P.S.M.).

Supplementary Material Available: Tables of X-ray crystallographic data for **11B**, including fractional coordinates, bond lengths and angles, and anisotropic parameters (6 pages); table of observed and calculated structure factors (12 pages). Ordering information is given on any current masthead page.

Dimethylallyltryptophan Synthase. An Enzyme-Catalyzed Electrophilic Aromatic Substitution

John C. Gebler, Andrew B. Woodside, and C. Dale Poulter*

Contribution from the Department of Chemistry, University of Utah, Salt Lake City, Utah 84112.
Received March 19, 1992

Abstract: Dimethylallyltryptophan (DMAT) synthase catalyzes the alkylation of L-tryptophan at C(4) by dimethylallyl diphosphate (DMAPP) in the first pathway-specific step in the biosynthesis of ergot alkaloids. The mechanism of the reaction was studied with analogs of both substrates. Five 7-substituted derivatives of *N*-acetyltryptophan (**2**, Z = OCH_3, CH_3, F, CF_3, and NO_2) were synthesized. The L enantiomers of the free amino acids were obtained by selective hydrolysis of the racemate using aminoacylase from *Aspergillus*. In addition, the *E* and *Z* fluoromethyl and difluoromethyl analogs of DMAPP (**1**, Y = CH_3, CH_2F, CHF_2) were prepared. Rates of the enzyme-catalyzed reactions were measured for the dimethylallyl derivatives with L-tryptophan and for the L-tryptophan derivatives with DMAPP. In addition, the relative reactivities of the methanesulfonate derivatives of the DMAPP analogs were determined for solvolysis in aqueous acetone. A Hammett plot for the tryptophan analogs gave a good linear correlation with $\rho = -2.0$. In addition, a Hammett plot of the logarithms of the relative rates of solvolysis and enzyme-catalyzed alkylation gave a positive linear correlation. These results indicate that the prenyl-transfer reaction catalyzed by DMAT synthase is an electrophilic aromatic substitution and is mechanistically similar to the electrophilic alkylation catalyzed by farnesyl diphosphate synthase.

Prenyltransferases comprise a family of enzymes that catalyze the alkylation of electron-rich acceptors by the hydrocarbon moieties of allylic isoprene diphosphates.[1] These reactions are the major building steps in isoprene metabolism and lead to a number of essential metabolites, including sterols,[2] dolichols,[3] ubiquinones,[4] and prenylated proteins.[5] A variety of functional groups, such as carbon–carbon double bonds,[1,2] aromatic rings,[4,6,7] alcohols,[8] amines,[9] and mercaptans,[5] can serve as prenyl acceptors. Although several different mechanisms have been proposed for various prenyl-transfer reactions, all of the transformations can be explained as electrophilic alkylations of the acceptors by the allylic diphosphates.[1,10]

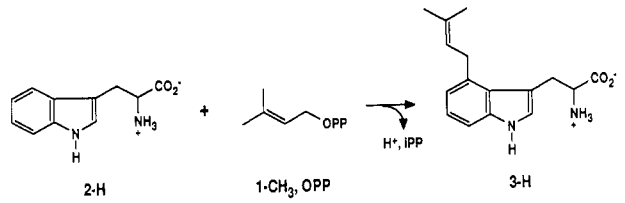
Farnesyl diphosphate synthase is the only prenyltransferase for which questions concerning the mechanism of bond formation have been extensively explored. Poulter and Rilling[11] discovered that the enzyme catalyzes hydrolysis of its allylic substrate by a direct displacement at carbon, and linear free energy studies with alternate substrates indicate that there is substantial development of positive charge in the allylic moiety during prenyl transfer.[12] Experiments with bisubstrate analogs of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) suggest that the enzyme catalyzes a stepwise reaction via carbocationic intermediates.[13]

Dimethylallyltryptophan synthase catalyzes alkylation of C(4) in L-tryptophan (**2**-H) by DMAPP (**1**-CH_3,OPP).[7,14] This is the

(1) Poulter, C. D.; Rilling, H. C. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; John Wiley & Sons: New York, 1981; Vol. 1, pp 162–224.
(2) Poulter, C. D. *Acc. Chem. Res.* **1990**, *23*, 70–77.
(3) Matsuoka, S.; Sagami, H.; Kurisaki, A.; Ogura, K. *J. Biol. Chem.* **1991**, *265*, 13157–13164.
(4) Weinstein, J. D.; Branchaud, R.; Beele, S. L.; Bement, W. J.; Sinclair, P. R. *Arch. Biochem. Biophys.* **1986**, *245*, 44–50.
(5) Seabra, M. C.; Reiss, Y.; Casey, P. J.; Brown, M. S.; Goldstein, J. L. *Cell* **1991**, *65*, 429–434.
(6) Pennock, J. F.; Threlfall, D. R. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; John Wiley & Sons: New York, 1981; Vol. 2, pp 191–304.
(7) Lee, S.-L.; Floss, H. G.; Heinstein, P. *Arch. Biochem. Biophys.* **1976**, *177*, 84–94.
(8) Zhang, D. L.; Daniels, L.; Poulter, C. D. *J. Am. Chem. Soc.* **1990**, *112*, 1264–1265.
(9) Dihanich, M. E.; Najarian, D.; Clark, R.; Gillman, E. C.; Martin, N. C.; Hopper, A. K. *Mol. Cell. Biol.* **1987**, *7*, 177–184.
(10) Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307–313.
(11) Poulter, C. D.; Rilling, H. C. *Biochemistry* **1976**, *15*, 1079–1083.
(12) Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926–3927.
(13) Davisson, V. J.; Neal, T. R.; Poulter, C. D. *J. Am. Chem. Soc.* **1985**, *107*, 5277–5279.
(14) Cress, W. A.; Chayet, L. T.; Rilling, H. C. *J. Biol. Chem.* **1981**, *256*, 10917–10923.

0002-7863/92/1514-7354$03.00/0

first pathway-specific step in the biosynthesis of ergot alkaloids from dimethylallyltryptophan (3-H, DMAT). The enzyme from



Claviceps purpurea is a 105 kDa homodimer[15] which, unlike other prenyltransferases, is active in metal-free buffers.[7,14,15] Although the structures of the substrates and products are consistent with an electrophilic aromatic substitution for the alkylation, C(4) is not the preferred site of electrophilic attack at an indole ring.[7] We now describe a series of linear free energy studies with alternate substrates for L-tryptophan and DMAPP that are consistent with an electrophilic mechanism.

Experimental Section

General Procedures. ^1H and ^{13}C NMR spectra were obtained in $CDCl_3$ or D_2O (Cambridge Isotope Laboratories) and are reported in ppm downfield from either internal TMS or 3-(trimethylsilyl)-1-propanesulfonic acid (Aldrich). Silica gel flash chromatography was performed using 40 μM silica gel (Baker). Thin-layer chromatography was carried out on silica gel 60 F_{254} glass plates (Merck). Silica TLC plates were visualized under UV light or by dipping them in a 10% solution of phosphomolybdic acid (Aldrich Chemical Co.) in ethanol followed by heating. All reactions were routinely run in oven-dried glassware under a nitrogen atmosphere with magnetic stirring. Reagent grade anhydrous diethyl ether and tetrahydrofuran were dried over 3-Å molecular sieves (Baker) and distilled from Na/benzophenone ketyl. Reagent grade acetonitrile and CH_2Cl_2 were distilled from anhydrous P_2O_5. Reagent grade solvents were used without purification for flash chromatography. Distilled triethylamine and acetonitrile and deionized water were used for HPLC chromatography. Protein concentrations were determined by the Bradford procedure[16] using BSA as a standard.

Materials. The following materials were purchased from the companies indicated and were used without further purification unless otherwise stated: Dowex AG 50W-X4 cation exchange resin (100–200 mesh, Bio-Rad); sodium methylate (Fisher Scientific Co.); (2-fluorophenyl)-hydrazine hydrochloride, [2-(trifluoromethyl)phenyl]hydrazine, and diethyl acetamidomalonate (Lancaster Synthesis); 7-nitroindole and acrolein (Aldrich); (*o*-methoxyphenyl)hydrazine hydrochloride (Research Plus Inc.); acylase I (aminoacylase, *N*-acylamino acid amidohydrolase, EC 3.5.1.14) from *Aspergillus* sp., ninhydrin reagent solution, L-tryptophan, D-tryptophan, and DL-tryptophan (Sigma Chemical Company); 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce). Opti-Fluor (Packard) was used for liquid scintillation cocktail. Dimethylallyltryptophan synthase was isolated from *Claviceps purpurea*.[15]

General Procedure for Enzyme-Catalyzed Hydrolysis of *N*-Acetyltryptophans. An aqueous solution of racemic N-acetylated amino acid was titrated to pH 7.5 with LiOH and diluted to a final concentration of 50 mM. Reactions were initiated by addition of 5 mg of acylase I for each 10 mL of solution. Incubation was at 37 °C, and the progress of the reactions was monitored by assaying aliquots for free amino acid with ninhydrin. Incubations were continued until the formation of free amino acid ceased (typically 4–27 h). The pH was adjusted to 5.0 with concentrated HCl, and 500 mg of Norit was added. The suspension was heated to 60 °C for 5 min, cooled to room temperature, and filtered. The filtrate was acidified to pH 1.5 with concentrated HCl, and unreacted N-acetylated material was removed by extraction with ethyl acetate. The aqueous layer was applied to a column of Dowex 50 (H^+ form) and then rinsed with water until the eluate was neutral. The amino acid was eluted with 1 N NH_4OH. The eluate was lyophilized to yield the product.

L-**7-Methoxytryptophan (2-OCH_3)**: white solid, mp 264–267 °C dec (lit.[17] mp 266 °C); ^1H NMR (D_2O/ND_4OD) δ 7.35 (1 H, d, J = 7.9 Hz, indole), 7.20 (1 H, s, H at indole C(2)), 7.10 (1 H, t, J = 7.9 Hz, H at indole C(6)), 6.81 (1 H, d, J = 7.6 Hz, indole), 3.80 (3 H, s, OCH_3), 3.63 (1 H, m, H at C_α), 3.20 (1 H, dd, J = 14.5 Hz, J = 5.3 Hz, C_β), 3.04 (1 H, dd, J = 14.5 Hz, J = 7.3 Hz, C_β); ^{13}C NMR (D_2O/ND_4OD) δ 183.8, 148.7, 131.6, 126.9, 126.5, 122.8, 114.7, 113.4, 105.4, 59.0, 58.4, 32.3; UV (5% NH_4OH) 269 (ε 7741), 290 nm (ε 5541); MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 378 (M^+ + 2TMS, 3.2), 232 (100); HRMS calcd for $C_{18}H_{30}N_2O_3Si_2$ 378.1795, found 378.1795.

L-**7-Methyltryptophan (2-CH_3)**: off-white solid, mp 286–290 °C dec (lit.[18] mp 287 °C).

L-**7-Fluorotryptophan (2-F)**: white solid, mp 190 °C dec; ^1H NMR (D_2O/ND_4OD) δ 7.45 (1 H, d, J = 7.9 Hz, H at indole C(4)), 7.22 (1 H, s, H at indole C(2)), 7.04 (1 H, dt, J = 7.9 Hz, J_{HF} = 4.8 Hz, H at indole C(5)), 6.93 (1 H, J_{HF} = 11.5 Hz, J = 7.9 Hz, H at indole C(6)), 3.66–3.61 (1 H, m, H at C_α), 3.18 (1 H, dd, J = 14.6 Hz, J = 5.3 Hz, H at C_β), 3.04 (1 H, dd, J = 14.6 Hz, J = 7.3 Hz, H at C_β); ^{13}C NMR (D_2O/ND_4OD) δ 182.8, 152.1 (d, J_{CF} = 241.7 Hz), 133.6 (d, J_{CF} = 5.8 Hz), 127.7, 122.0 (d, J_{CF} = 6.2 Hz), 117.1 (d, J_{CF} = 3.2 Hz), 113.2, 109.2, 108.9, 58.6, 31.7; UV (5% NH_4OH) 267 (ε 6167), 287 (ε 4265) nm; MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 366 (M^+ + 2TMS, 1.3), 220 (100); HRMS calcd for $C_{14}H_{19}FN_2O_2Si$ 294.1200, found 294.1201.

L-**7-(Trifluoromethyl)tryptophan (2-CF_3)**: off-white solid, mp 201 °C dec; R_f = 0.70 (9:7:4 ethyl acetate/2-propanol/25% NH_4OH); ^1H NMR (D_2O/ND_4OD) δ 7.94 (1 H, d, J = 8.0 Hz, indole), 7.55 (1 H, d, J = 7.5 Hz, indole), 7.32 (1 H, s, H at indole C(2)), 7.24 (1 H, dd, J = 8.0 Hz, J = 7.5 Hz, H at indole C(5)), 3.63 (1 H, m, H at C_α), 3.22 (1 H, dd, J = 14.5 Hz, J = 5.3 Hz, H at C_β), 3.09 (1 H, dd, J = 14.5 Hz, J = 6.9 Hz, H at C_β); ^{13}C NMR (D_2O/ND_4OD) δ 184.1, 133.6, 131.5, 128.0, 127.3 (q, J_{CF} = 270 Hz), 125.7, 121.7 (q, J_{CF} = 4.6 Hz), 121.0, 115.5 (q, J_{CF} = 32 Hz), 113.6, 58.9, 32.2; UV (5% NH_4OH) 294 nm (ε 10 100); MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 344 (M^+ + TMS, 3.9), 198 (100); HRMS calcd for $C_{15}H_{19}F_3N_2O_2Si$ 344.1168, found 344.1126.

L-**7-Nitrotryptophan (2-NO_2)**: yellow solid, mp 238–240 °C dec (lit.[19] mp 240 °C); R_f = 0.76 (9:7:4, ethyl acetate/2-propanol/25% NH_4OH); ^1H NMR (D_2O/ND_4OD) δ 8.12 (1 H, d, J = 8.1 Hz, indole), 8.07 (1 H, d, J = 7.8 Hz, indole), 7.34 (1 H, s, H at indole C(2)), 7.21 (1 H, dd, J = 8.1 Hz, J = 7.8 Hz, H at indole C(5)), 3.57 (1 H, m, H at C_α), 3.16 (1 H, dd, J = 14.5 Hz, J = 5.6 Hz, C_β), 3.07 (1 H, dd, J = 14.5 Hz, J = 6.8 Hz, C_β); ^{13}C NMR (D_2O/ND_4OD) δ 184.5, 134.4, 133.9, 131.2, 130.2, 128.7, 121.6, 120.9, 115.0, 58.9, 32.5; UV (5% NH_4OH) 382 nm (ε 5347); MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 344 (M^+ + TMS, 3.9), 198 (100); HRMS calcd for $C_{15}H_{19}F_3N_2O_2Si$ (M^+ + TMS) 344.1168, found 344.1126.

Acid-Catalyzed Hydrolysis of N-Acetylated Tryptophans. N-Acetylated material (50 mM) was refluxed in 2 N HCl for 3 h. The solution was applied to Dowex (H^+ form), washed with water until the eluant was neutral, and eluted with 10% (v/v) NH_4OH solution. The water was removed by lyophilization. NMR and MS data were identical to those of the corresponding L enantiomers obtained by enzymatic hydrolysis.

Analysis of Tryptophan Enantiomers with Marfey's Reagent.[20] In a 1-mL microvial were placed 5 μmol of the amino acid in 100 μL of water, 200 μL of 1% (w/v) 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone, and 40 μL of 1 M $NaHCO_3$. The mixture was heated at 40 °C in a dry block heater for 1 h, treated with 20 μL of 2 M HCl, and filtered. The filtrate was applied to a C_{18} reversed-phase column and eluted with a linear gradient of 10% to 40% acetonitrile/0.05 M triethylammonium phosphate, pH 3.0 (1:10 to 2:5 v/v). The eluant was monitored at 340 nm. Each analysis was done in duplicate. Samples of D, L, and D,L amino acids were derivatized and evaluated for different D, L ratios to confirm retention times and the accuracy of the method. The sample contained derivatized tryptophans and hydrolyzed FDAA.

Ethyl (*E*)- and (*Z*)-3-(Fluoromethyl)-2-butenoate ((*E*)- and (*Z*)-4). Following a procedure similar to that reported by Machleidt et al.,[21] 16.2 g (72 mmol) of triethyl phosphonoacetate was treated with 3.47 g (72 mmol) of a 50% (w/w) dispersion of NaH, followed by addition of 5.0 g (66 mmol) of fluoroacetone. (**CAUTION**: Fluoroacetone is extremely toxic. Proper precautions should be taken when using this material.) After workup, the residue was distilled (116–119 °C, 16 mm/Hg) to give 6.48 g of a colorless oil. GLPC (12 m OV 101, 80 °C) showed two components in a ratio of 45:55 with retention times of 2.60 and 2.92 min, respectively. A 2.0-g portion of the mixture was separated by flash chromatography.

Ethyl (*Z*)-3-(fluoromethyl)-2-butenoate ((*Z*)-4): 880 mg (44%); R_f = 0.44 (ether/pentane, 1:10 (v/v)); IR (neat) 2979, 2933, 1714, 1704,

(15) Gebler, J. C.; Poulter, C. D. *Arch. Biochem. Biophys.* **1992**, *296*, 308–313.
(16) Scopes, R. *Protein Purification. Principles and Practice*; Springer Verlag: Berlin, 1982; p 266.
(17) Bergmann, E. D.; Hoffmann, E. *J. Chem. Soc.* **1962**, 2827–2829.
(18) Hoffmann, E.; Ikan, R.; Galun, A. B. *J. Heterocycl. Chem.* **1965**, *2*, 298–299.
(19) Da Settimo, A. *Ann. Chim. Rome* **1962**, *52*, 17–24.
(20) Gyula, A. S.; Mezo, G.; Hudecz, F. *J. Chromatogr.* **1988**, *144*, 115–122.
(21) Machleidt, H.; Hartmann, V.; Bunger, H. *Ann.* **1963**, *667*, 35–47.

1441, 1372, 1332, 1299, 1225, 1141, 1091, 809, 759, 723, cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.65 (1 H, m, H at C(2)), 5.38 (2 H, d, J = 48 Hz, CH_2F), 3.98 (2 H, q, J = 5.0 Hz, ethyl CH_2), 2.00 (3 H, s, H at C(4)), 1.17 (3 H, t, J = 5.0 Hz, ethyl CH_3); ^{13}C NMR 75 MHz ($CDCl_3$) δ 165.7, 151.6 (d, J_{CF} = 13.0 Hz), 114.4 (d, J_{CF} = 12.0 Hz), 84.4 (d, J_{CF} = 176.0 Hz), 59.5, 13.9 (d, J_{CF} = 9.0 Hz), 13.8; ^{19}F NMR 282 MHz ($CDCl_3$) δ −224.2 (t, J_{HF} = 49.5 Hz); MS (m/z, relative intensity) 133.1 (36.2), 118.0 (50.5), 101.0 (100.0), 98.0 (16.9), 53.1 (30.9), 32.0 (76.6), 29.0 (22.4). Anal. Calcd for $C_7H_{11}FO_2$: C, 57.52; H, 7.59. Found: C, 57.80; H, 7.61.

Ethyl (*E*)-3-(fluoromethyl)-2-butenoate ((*E*)-4): 720 mg (36%); R_f = 0.33 (ether/pentane, 1:10 (v/v)); IR (neat) 2958, 2922, 1710, 1666, 1442, 1361, 1323, 1270, 1225, 1150, 1091, 1053, 1032, 998, 955, 857 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.91 (1 H, m, CH), 4.82 (2 H, d, J_{HF} = 47.0 Hz, CH_2F), 4.17 (2 H, t, J = 7.1 Hz, ethyl CH_2), 2.09 (3 H, s, H at C(4)), 1.28 (3 H, t, J = 7.1 Hz, ethyl CH_3); ^{13}C NMR ($CDCl_3$) δ 165.6, 155.6 (d, J_{CF} = 19.8 Hz), 116.4 (d, J_{CF} = 4.0 Hz), 82.5 (d, J_{CF} = 163.0 Hz), 60.1, 20.0 (d, J_{CF} = 10.0 Hz), 14.3; ^{19}F NMR 282 MHz ($CDCl_3$) δ −218.7 (t, J_{HF} = 47.0 Hz); MS (m/z, relative intensity) 133.1 (28.6), 118.1 (11.2), 101.0 (100.0), 98.0 (38.1), 53.1 (28.1), 32.0 (57.4), 29.0 (18.0). Anal Calcd for $C_7H_{11}FO_2$: C, 57.52; H, 7.59. Found: C, 57.83; H, 7.53.

Ethyl (*E*)- and (*Z*)-3-(Difluoromethyl)-2-butenoate ((*E*)- and (*Z*)-5). Using a procedure similar to that described for (*E*)- and (*Z*)-4, 27.5 g (123 mmol) of triethyl phosphonoacetate was treated with 5.88 g (122.5 mmol) of an oil dispersion of NaH, followed by addition of 9.6 g (102.1 mmol) of 1,1-difluoroacetone. After workup, the residue was distilled (66–70 °C, 16 mmHg) to give 10.18 g of a clear colorless oil. GLPC analysis on an OV 101 column (12.5 m, 95 °C) showed two components in a ratio of 35:65 with retention times of 4.92 and 4.96 min, respectively. A 3.0-g sample of the materials was separated by flash chromatography on silica gel.

Ethyl (*Z*)-3-(difluoromethyl)-2-butenoate ((*Z*)-5): 0.78 g (26%), R_f = 0.48 (ether/pentane, 1:32 (v/v)); IR (neat) 2980, 1721, 1675, 1445, 1381, 1322, 1299, 1250, 1151, 1088, 1042, 1021, 873, 827, 810, 730, 649 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.38 (1 H, t, J_{HF} = 55.6 Hz, CHF_2), 5.96 (1 H, s, H at C(2)), 4.19 (2 H, q, J = 7.2 Hz, ethyl CH_2), 1.97 (3 H, s, H at C(4)), 1.29 (3 H, t, J = 7.2 Hz, ethyl CH_3); ^{13}C NMR ($CDCl_3$) δ 164.2, 147.5 (t, J_{CF} = 24.0 Hz), 132.0 (t, J_{CF} = 8.0 Hz), 110.0 (t, J_{CF} = 234.0 Hz), 60.7, 15.9 (t, J_{CF} = 3.4 Hz), 14.0; ^{19}F NMR ($CDCl_3$) δ −121.8 (d, J_{HF} = 55.6 Hz); MS (m/z, relative intensity) 164.1 (24.9), 136.1 (25.7), 119.0 (100.0), 71.0 (17.5), 51.0 (11.4), 39.0 (13.4), 27.2 (12.0). Anal. Calcd for $C_7H_{10}F_2O_2$: C, 51.22; H, 6.13. Found: C, 51.01; H, 6.09.

Ethyl (*E*)-3-(difluoromethyl)-2-butenoate ((*E*-5): 1.19 g (40%), R_f = 0.37 (ether/pentane, 1:32 (v/v)); IR (neat) 2979, 1715, 1667, 1442, 1401, 1366, 1350, 1330, 1270, 1226, 1170, 1095, 1033, 957, 882, 832, 667, 635 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.06 (1 H, s, H at C(2)), 5.99 (1 H, t, J_{HF} = 55.6 Hz, CHF_2), 4.22 (2 H, q, J = 7.2 Hz, ethyl CH_2), 2.19 (3 H, s, H at C(4)), 1.30 (3 H, t, J = 7.2 Hz, ethyl CH_3); ^{13}C NMR ($CDCl_3$) δ 165.0, 146.7 (t, J_{CF} = 22.3 Hz), 121.0 (t, J_{CF} = 9.9 Hz), 115.3 (t, J_{CF} = 239.5 Hz), 60.4, 14.0, 11.3 (t, J_{CF} = 2.6 Hz); ^{19}F NMR ($CDCl_3$) δ −119.2 (d, J_{HF} = 55.6 Hz); MS (m/z, relative intensity) 164.1 (20.6), 136.0 (17.0), 119.0 (100.0), 116.0 (42.7), 71.0 (21.6), 68.0 (32.7), 58.1 (20.5), 50.9 (15.6), 43.1 (60.2), 39.0 (19.2), 32.0 (66.5), 29.0 (25.6), 27.2 (19.8). Anal. Calcd for $C_7H_{10}F_2O_2$: C, 51.22; H, 6.13. Found: C, 51.12; H, 6.06.

General Procedure for Synthesis of Fluorinated Allylic Alcohols. The butenoate ester (10 mmol) was dissolved in CH_2Cl_2 and cooled to −78 °C, followed by addition of diisobutylaluminum hydride (21 mmol, 1.0 M solution in cyclohexane), while the temperature of the reaction was maintained below −60 °C. After the addition was complete, the resulting pale yellow solution was allowed to warm to −40 °C for 1 h. The reaction mixture was cooled to −78 °C and quenched by the addition of methanol. The mixture was then allowed to warm to room temperature for 2 h before the addition of saturated NH_4Cl solution. The resulting suspension was filtered through a Soxhlet extraction filter that contained 5.0 g of 1:1 anhydrous $MgSO_4$ and Celite. The filtrate and solids were transferred to a Soxhlet extractor and continuously extracted with diethyl ether for 4 h. Ether was removed by rotary evaporation to afford a clear, pale yellow oil. Yields were greater than 90%.

(*Z*)-3-(Fluoromethyl)-2-butenol ((*Z*)-1-CH_2F,OH): IR (neat) 3345, 2967, 2940, 2883, 1676, 1641, 1449, 1378, 1250, 1192, 1097, 980, 840, 786 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.53 (1 H, m, H at C(2)), 4.82 (2 H, d, J_{HF} = 47.4 Hz, CH_2F), 4.12 (3 H, m, OH and H at C(1)), 1.77 (3 H, s, H at C(4)); ^{13}C NMR ($CDCl_3$) δ 133.0 (d, J_{CF} = 14.8 Hz), 128.6 (d, J_{CF} = 7.9 Hz), 80.5 (d, J_{CF} = 160.9 Hz), 57.3, 19.8 (d, J_{CF} = 5.2 Hz); ^{19}F NMR ($CDCl_3$) δ −216.2 (t, J_{HF} = 47.4) Hz.

(*E*)-3-(Fluoromethyl)-2-butenol ((*E*)-1-CH_2F,OH): IR (neat) 3325, 2922, 2880, 1678, 1651, 1452, 1367, 1231, 1184, 1100, 1037, 972, 850, 782 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.67 (1 H, m, H at C(2)), 4.72 (2 H, d, J_{HF} = 47.4 Hz, CH_2F), 4.18 (2 H, t, J = 5.2 Hz, H at C(1)), 3.63 (1 H, s, OH), 1.70 (3 H, s, H at C(4)); ^{13}C NMR ($CDCl_3$) δ 133.0 (d, J_{CF} = 14.2 Hz), 127.3 (d, J_{CF} = 11.4 Hz), 87.1 (d, J_{CF} = 165.9 Hz, CH_2F), 58.2, 12.8 (d, J_{CF} = 3.3 Hz); ^{19}F NMR ($CDCl_3$) δ −214.0 (t, J_{HF} = 47.4 Hz).

(*Z*)-3-(Difluoromethyl)-2-butenol ((*Z*)-1-CHF_2,OH): IR (neat) 3350, 2979, 2930, 2880, 1681, 1450, 1392, 1368, 1198, 1112, 1008, 815 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.49 (1 H, t, J_{HF} = 56.2, CHF_2), 5.77 (1 H, m, H at C(2)), 4.23 (2 H, m, H at C(1)), 3.18 (1 H, s, OH), 1.82 (3 H, s, H at C(4)); ^{13}C NMR ($CDCl_3$) δ 132.5 (t, J_{CF} = 9.0 Hz), 131.1 (t, J_{CF} = 21.7 Hz), 112.0 (t, J_{CF} = 234.2 Hz, CHF_2), 57.5, 15.6 (t, J_{CF} = 3.0 Hz); ^{19}F NMR ($CDCl_3$) δ −117.3 (d, J_{HF} = 56.2 Hz).

(*E*)-3-(Difluoromethyl)-2-butenol ((*E*)-1-CHF_2,OH): IR (neat) 3328. 2970, 2930, 2872, 1685, 1442, 1362, 1200, 1111, 1010, 868, 812, 660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.94 (1 H, t, J_{HF} = 56.2 Hz, CHF_2), 5.87 (1 H, m, H at C(2)), 4.23 (2 H, m, H at C(1)), 3.39 (1 H, s, OH), 1.73 (3 H, s, H at C(4)); ^{13}C NMR ($CDCl_3$) δ 131.7 (t, J_{CF} = 9.4 Hz), 131.1 (t, J_{CF} = 21.5 Hz), 117.0 (t, J_{CF} = 236.0 Hz, CHF_2), 58.1, 9.2 (t, J_{CF} = 2.3 Hz); ^{19}F NMR ($CDCl_3$) δ −116.1 (d, J_{HF} = 56.2 Hz).

3-Methyl-2-butenyl Methanesulfonate (1-CH_3,OMs). 3-Methyl-2-butenol (5.8 mmol), triethylamine (8.7 mmol), and 10 mL of diethyl ether were cooled to −78 °C. Methanesulfonyl chloride (5.5 mmol) was added dropwise, and the reaction mixture was allowed to warm until triethylamine hydrochloride began to precipitate. The suspension was maintained at 4 °C for 15 min, cooled to −78 °C, and rapidly filtered through a cold (−78 °C) fine-fritted glass funnel under N_2. The filtrate was rapidly concentrated under vacuum at 4 °C, and the resulting clear colorless oil was stored at −70 °C: ^1H NMR ($CDCl_3$, −10 °C) δ 5.50 (1 H, t, J = 6.6 Hz, H at C(2)), 4.81 (2 H, d, J = 6.6 Hz, H at C(1)), 3.05 (3 H, s, sulfonyl CH_3), 1.83 (3 H, s, CH_3), 1.80 (3 H, s, CH_3).

(Mono- and Difluoromethyl)dimethylallyl Methanesulfonates. The reaction and workup were performed as described for 1-CH_3,OMs, except the solvent was removed under vacuum at room temperature.

(*Z*)-3-(Fluoromethyl)-2-butenyl methanesulfonate ((*Z*)-1-CH_2F,-OMs): IR (neat) 3018, 2979, 2938, 2695, 2500, 1656, 1471, 1450, 1349, 1245, 1205, 1188, 1170, 1056, 1037, 971, 920, 809, 787, 776, 748 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.62 (1 H, t, J = 6.6 Hz, H at C(2)), 4.94 (2 H, J_{HF} = 47.4 Hz, CH_2F), 4.79 (2 H, d, J = 6.6 Hz, H at C(1)), 3.00 (3 H, s, sulfonyl CH_3), 1.86 (3 H, s, CH_3); ^{19}F NMR ($CDCl_3$) δ −217.8 (t, J_{HF} = 47.4 Hz).

(*E*)-3-(Fluoromethyl)-2-butenyl methanesulfonate ((*E*)-1-CH_2F, OMs): IR (neat) 3010, 2990, 2939, 2660, 2500, 1735, 1686, 1626, 1448, 1350, 1250, 1190, 1173, 1120, 1058, 1010, 980, 937, 878, 812, 745, 720, 659 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.92 (1 H, t, J_{HF} = 55.0 Hz, CH_2F), 5.92 (1 H, m, H at C(2)), 4.82 (2 H, m, H at C(1)), 3.00 (3 H, s, sulfonyl CH_3), 1.81 (3 H, s, CH_3); ^{19}F NMR ($CDCl_3$) δ −116.9 (t, J_{HF} = 55.0 Hz).

(*Z*)-3-(Difluoromethyl)-2-butenyl methanesulfonate ((*Z*)-1-CHF_2,OMs): IR (neat) 3010, 2980, 2930, 2650, 2495, 1714, 1450, 1397, 1350, 1247, 1188, 1172, 1095, 1057, 1015, 971, 933, 875, 804, 740, 717 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.40 (1 H, t, J_{HF} = 54.0 Hz, CHF_2), 5.79 (1 H, t, J = 6.6 Hz, H at C(2)), 4.81 (2 H, d, J = 6.6 Hz, H at C(1)), 3.00 (3 H, s, sulfonyl CH_3), 1.88 (3 H, s, CH_3); ^{19}F NMR ($CDCl_3$) δ −118.0 (d, J_{HF} = 54.0 Hz).

(*E*)-3-(Difluoromethyl)-2-butenyl methanesulfonate ((*E*)-1-CHF_2,OMs): IR (neat) 3010, 2990, 2939, 2660, 2500, 1735, 1686, 1626, 1448, 1350, 1250, 1190, 1173, 1120, 1058, 1010, 980, 937, 878, 812, 745, 720, 659 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.95 (1 H, t, J_{HF} = 55.0 Hz, CHF_2), 5.92 (1 H, m, H at C(2)), 4.82 (2 H, m, H at C(1)), 3.00 (3 H, s, sulfonyl CH_3), 1.81 (3 H, s, CH_3); ^{19}F NMR ($CDCl_3$) δ −116.9 (d, J_{HF} = 55.0 Hz).

Kinetic Measurements for 3-Methyl-2-butenyl Methanesulfonates. A 35-mL conductivity cell, equipped with a magnetic stirrer and a rubber septum cap, was filled with a solution of acetone/water, and 9.2 mg (85 μmol) of 2,6-lutidine was added. The cell was placed in a constant-temperature bath and allowed to equilibrate before the addition of 20 μmol of methanesulfonate. Conductance was recorded as a function of time. All determinations were in triplicate.

Assay for Dimethylallyltryptophan Synthase. The final composition of the assay buffer was 500 μM amino acid (except for 2-OCH_3, which was 3 mM), 500 μM [^{3}H]DMAPP (specific activity 2 μCi/μmol), 4 mM $CaCl_2$, 50 mM Tris-HCl, 20 mM 2-mercaptoethanol, 10% glycerol (v/v) pH 7.8, and enzyme in a total volume of 200 μL. Incubations were carried out for 10 min at 30 °C. After incubation, the reaction was stopped by the addition of distilled water (3 mL, 4 °C). The mixture was transferred to a 10-mL syringe barrel containing 3 mL of Dowex 50W-X4 cation exchange resin (H^+ form) and washed three times with 10 mL of water. The product was eluted into a glass scintillation vial with $NH_4OH/H_2O/CH_3OH$ (2:3:5). The vials were placed in a dry

block heater (90 °C), and the solvent was removed under a stream of N_2. To the resulting residue were added 1 mL of water and 10 mL of scintillation cocktail. The contents of the vials were thoroughly mixed, and radioactivity was determined by liquid scintillation spectrometry.

Kinetic Constants. Michaelis–Menten constants were determined under steady-state conditions. Rates of reactions were measured within the linear range (product formation below 10%) using the standard assay for DMAT synthase. Incubation times were 15 min at 30 °C except for **2**-CF_3 and **2**-NO_2, which required 30 min.

Stock solutions consisting of 10 mM amino acid in assay buffer (50 mM Tris-HCl, 20 mM 2-mercaptoethanol, 10% glycerol (v/v) pH 7.8) and 10 mM [1-^{3}H]-**1**-CH_3,OPP (specific activity = 30 μCi/μmol) in 25 mM NH_4HCO_3 were prepared. These were combined and diluted to the necessary concentrations with assay buffer. All measurements were made in the presence of 4 mM $CaCl_2$. Data points were the mean of four determinations.

Product Studies. General. In a plastic screw cap vial were placed **1**-CH_3,OPP (1 mM), [1-^{3}H]-**1**-CH_3,OPP (70 μM, specific activity = 31 μCi/μmol), amino acid (1 mM), $CaCl_2$ (4 mM) DMAT synthase (30–75 μg, specific activity ~500 nmol min^{-1} mg^{-1}), and assay buffer to a total volume of 1.4 mL. The mixture was incubated at 30 °C. After 24 h, the resulting suspension was filtered, and the filtrate was lyophilized. The residue was dissolved in 0.1% trifluoroacetic acid and 10% acetonitrile and applied to a C_{18} reversed-phase HPLC column. The column was eluted with a 1:10 to 4:5 (v/v) linear gradient of acetonitrile/0.1% aqueous TFA in 48 mL. Fractions were collected, radioactivity was determined, and those fractions containing radioactivity were lyophilized. Samples used for NMR analysis were exchanged in D_2O three times.

7-Methyl-4-(dimethylallyl)tryptophan (3-CH_3). DMAPP and **2**-CH_3 were incubated with 75 μg of DMAT synthase to give a single radioactive product: t_R 34.5 min; MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 430 (M^+ + 2TMS, 2.8) 73 (100); HRMS calcd for $C_{23}H_{38}N_2O_2Si_2$ 430.2467, found 430.2460; ^{1}H NMR (D_2O/ND_4OD) δ 7.28 (1 H, s, H at indole C(2)), 6.99 (1 H, d, J = 7.2 Hz, indole H), 6.89 (1 H, d, J = 7.2 Hz, indole H), 5.31 (1 H, m, H at C(2″)), 3.83 (1 H, dd, J = 9.4 Hz, J = 4.7 Hz, H at C_α), 3.74 (2 H, m, H at C(1″)), 3.57 (1 H, dd, J = 15.5 Hz, J = 4.6 Hz, H at C_β), 3.15 (1 H, dd, J = 15.5 Hz, J = 9.4 Hz, H at C_β), 2.45 (3 H, s, CH_3 at indole C(4)).

7-Methoxy-4-(dimethylallyl)tryptophan (3-OCH_3). DMAPP and **2**-OCH_3 were incubated with 60 μg of DMAT synthase to give a single radioactive product: t_R 33.9 min; MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 446 (M^+ + 2TMS, 1.8), 73 (100).

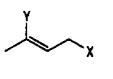
7-Fluoro-4-(dimethylallyl)tryptophan (3-F). DMAPP and **2**-F were incubated with 75 μg of DMAT synthase to give a single product: t_R 33.4 min; MS(EI) (*m/z*, bis-TMS derivative, relative intensity) 434 (M^+ + 2TMS, 1.7) 288 (100); HRMS calcd for $C_{22}H_{35}FN_2O_2Si_2$ 434.2221, found 434.2213.

7-Nitro-4-(dimethylallyl)tryptophan (3-NO_2). DMAPP and **2**-NO_2 were incubated with 30 μg of DMAT synthase to give a single radioactive product: t_R 35.0 min.

7-(Trifluoromethyl)-4-(dimethylallyl)tryptophan (3-CF_3). DMAPP and **2**-CF_3 were incubated with 30 μg of DMAT synthase to give a single radioactive product: t_R 37.5 min.

Results

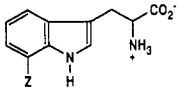
Synthesis of Substrates. Tryptophan Analogs. A series of racemic 7-substituted *N*-acetyltryptophans were prepared by acetylation of commercially available material (7-methyltryptophan (**2**–CH_3)), of compounds prepared by the Fisher indole synthesis[22,23] (7-methoxytryptophan[17] (**2**–OCH_3), 7-fluorotryptophan (**2**–F), and 7-(trifluoromethyl)tryptophan (**2**–CF_3)), or of material synthesized by attachment of an amino acid side chain to 7-substituted indoles[24] (7-nitrotryptophan[19] (**2**-NO_2)).



1

X = OH, OMS, OPP

Y = CH_3, CH_2F, CHF_2



2

Z = H, OCH_3, CH_3, F, CF_3, NO_2

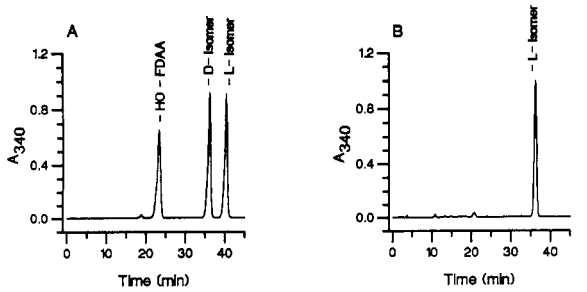


Figure 1. Chromatographic traces of FDAA-derivatized samples of 7-methyltryptophan (**2**-CH_3): (A) D,L mixture; (B) L-**2**-CH_3 isolated from acylase I hydrolysate of racemic **2**-CH_3.

L enantiomers were obtained by enantioselective hydrolysis of the *N*-acetyl groups with acylase I from *Aspergillus* according to the procedure of Chenault et al.[25] The reactions were followed by detection of free amino acid with ninhydrin reagent,[26] and incubation was discontinued when progress curves indicated hydrolysis had ceased. The enantiomeric purity of the products was determined by HPLC. The amino acids were derivatized with Marfey's reagent[20] (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), and the resulting disastereomers were separated on a C_{18} reversed-phase column. A typical profile is shown in Figure 1 for 7-methyltryptophan. The first component to elute was hydrolyzed Marfey's reagent, followed in turn by the L- and D-tryptophan derivatives. This pattern has been observed for a variety of other amino acids and was rationalized by formation of stronger intramolecular hydrogen bonds for the later eluting diastereomer.[27] Integration of peak areas for the diastereomeric tryptophan derivatives indicated that the L-tryptophan analogs obtained from the acylase-catalyzed hydrolysis were greater than 99% enantiomerically pure.

Dimethylallyl Analogs. Fluorinated alcohols (*E*)- and (*Z*)-3-(fluoromethyl)-2-butenol (**1**-CH_2F,OH) and (*E*)- and (*Z*)-3-(difluoromethyl)-2-butenol (**1**-CHF_2,OH) were prepared from the corresponding ketones by a Horner–Emmons Wittig condensation with ethyl phosphonoacetate followed by reduction of the α,β-unsaturated esters with diisobutylaluminum hydride (DIBAL). The isomeric esters were separated by flash chromatography before treatment with DIBAL, and the stereochemistry of the double bonds was established by ^{1}H NMR spectroscopy. As is common for isomeric α,β-unsaturated esters, the group cis to the carbonyl unit was deshielded relative to the trans isomer.[28] Thus, the hydrogens in the monofluoromethyl and difluoromethyl moieties of (*Z*)-**4** and (*Z*)-**5** resonated downfield (5.38 and 7.38 ppm, respectively) from similar hydrogens in (*E*)-**4** and (*E*)-**5** (4.82 and 5.99 ppm, respectively). Correspondingly, the methyl groups in the *Z* monofluoromethyl and difluoromethyl derivatives resonated upfield (2.00 and 1.97 ppm) from the methyls in the *E* isomer (2.09 and 2.19 ppm).

Dimethylallyl methanesulfonate (**1**-CH_3,OMs) was unstable when concentrated at room temperature, and we were unable to purify the material prior to solvolysis experiments. We typically used a slight excess of alcohol and triethylamine to prepare the methanesulfonate in order to consume all of the methanesulfonyl chloride, which could interfere with kinetic measurements. All of the methanesulfonates used in this study were prepared in a similar manner. The samples were analyzed by ^{1}H NMR to insure that methanesulfonyl chloride had been consumed, and the materials were used immediately for kinetic runs.

The synthesis of the allylic diphosphates from the corresponding alcohols was described previously.[29] Radioactive material was

(22) Warner, D. T.; Moe, O. A. *J. Am. Chem. Soc.* **1948**, *70*, 2765–2767.
(23) van Pee, K.-H.; Salcher, O.; Lingens, F. *Liebigs Ann. Chem.* **1981**, *2*, 233–239.
(24) Snyder, H. R.; Smith, C. W. *J. Am. Chem. Soc.* **1949**, *66*, 350–351.
(25) Chenault, H. K.; Dahmer, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 6354–6364.
(26) Moore, S. *J. Biol. Chem.* **1968**, *243*, 6281–6283.
(27) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
(28) Poulter, C. D.; Satterwhite, D. M. *Biochemistry* **1977**, *16*, 5470–5478.
(29) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768–4779.

Table I. First-Order Rate Constants for Solvolysis of Allylic Methanesulfonates

reactant	T, °C	solvent (acetone/water)	k_s, s^{-1}	k_s^{rel}
1-CH_3,OMS	20	23:2	$(1.5 \pm 0.2) \times 10^{-2}$	1
(Z)-1-CH_2F,OMs	20	23:2	$(1.9 \pm 0.2) \times 10^{-5}$	1.3×10^{-3}
(E)-1-CH_2F,OMs	20	23:2	$(2.2 \pm 0.3) \times 10^{-5}$	1.5×10^{-3}
(Z)-1-CHF_2,OMs	30	1:1	$(3.5 \pm 0.4) \times 10^{-5}$	
	50	1:1	$(2.5 \pm 0.2) \times 10^{-4}$	
	60	1:1	$(5.8 \pm 0.5) \times 10^{-4}$	
	20	23:2	9.0×10^{-7} [a]	6.0×10^{-5} [a]
(E)-1-CHF_2,OMs	30	1:1	$(3.3 \pm 0.1) \times 10^{-5}$	
	50	1:1	$(2.3 \pm 0.2) \times 10^{-4}$	
	60	1:1	$(5.3 \pm 0.2) \times 10^{-4}$	
	20	23:2	9.2×10^{-7} [a]	6.1×10^{-5} [a]

[a] Estimated from rate constants determined in 1:1 acetone/water at higher temperatures.

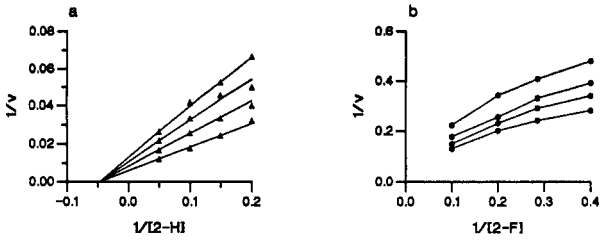


Figure 2. Double reciprocal kinetic plots for DMAT synthase: (a) varied 2-H at fixed concentrations of 1-CH_3,OPP; (b) varied 2-F at fixed concentrations of 1-CH_3,OPP.

prepared by oxidation of the alcohols to α,β-unsaturated aldehydes with MnO_2, followed by reduction with $NaBT_4$. Specific activities were determined for the naphthoate esters according to the protocol of Davisson et al.,[30] and the alcohols were then phosphorylated.

Mechanistic Studies. First-order rate constants for solvolysis of methanesulfonate esters were calcualted from conductance versus time measurements, and the data are summarized in Table I. In order to compare relative reactivities for the parent and fluorinated systems, the rate constants for (E)- and (Z)-1-CHF_2,OMs were estimated at 20 °C in 23:2 (v/v) acetone/water, using the Eyring and Winstein–Grunwald equations as previously described.[12,31] The effect of replacing hydrogens in the methyl group at C(3) by fluorine was similar to that reported for geranyl derivatives.[12,31] A single fluorine decreased the rate 770-fold, and a second fluorine gave an additional 22-fold depression. Thus, fluorine exerts a powerful electron-withdrawing effect that destabilizes the developing allylic cation with a concomitant decrease in the rate of solvolysis.

DMAT synthase was obtained from *Claviceps purpurea*.[15] The freshly purified enzyme had a specific activity of 0.5 μmol min^{-1} mg^{-1} and was stable at −20 °C in glycerol-containing buffer for several months. The assay for enzyme activity measured incorporation of radioactivity from [^{3}H]dimethylallyl diphosphate into DMAT. A similar procedure was used to determine kinetic constants for DMAT synthase when derivatives of DMAPP and tryptophan were used as alternate substrates. The normal substrates showed good Michaelis–Menten behavior in the presence of Mg^{2+}, and kinetic constants were obtained by a Lineweaver–Burke analysis (see Figure 2a). However, the 7-substituted analogs gave curved double reciprocal plots reminiscent of those obtained for tryptophan in the absence of metal ion, where the enzyme shows negative allosteric regulation.[14,15] (A typical profile is shown for 2-F in Figure 2b.) As a result, kinetic constants for each substrate were evaluated from double reciprocal plots in the presence of saturating concentrations of the nonvaried substrate where the curvature was minimal. A similar procedure was used to determine V_{max} for the analogs of DMAPP.

(30) Davisson, V. J.; Zabriskie, T. M.; Poulter, C. D. *Bioorg. Chem.* **1986**, *14*, 46–54.
(31) Poulter, C. D.; King, C.-H. R. *J. Am. Chem. Soc.* **1982**, *104*, 1422–1424.

Table II. Initial Velocities for Dimethylallyl Diphosphate and Fluorinated Analogs

substrate[a]	initial velocity, μmol mg^{-1} min^{-1}	relative velocity
1-CH_3,OPP	0.32	1.0
(Z)-1-CH_2F,OPP	1.4×10^{-2}	4.4×10^{-2}
(E)-1-CH_2F,OPP	3.6×10^{-3}	1.1×10^{-2}
(Z)-1-CHF_2,OPP	1.2×10^{-3}	3.8×10^{-3}
(E)-1-CHF_2,OPP	2.2×10^{-3}	6.9×10^{-3}

[a] [tryptophan] = [allylic substrate] = 100 μM, [Ca^{2+}] = 20 mM.

Table III. Kinetic Constants for DMAT Synthase with Tryptophan and Related Analogs

	K_M^{DMAPP}, μM	$K_M^{Tryptophan}$, μM	V_{max}, μmol min^{-1} mg^{-1}
2-H	8 ± 3	17 ± 2	0.5 ± 0.03
2-OCH_3	9 ± 1	368 ± 147	0.35 ± 0.09
2-CH_3	4 ± 0.5	14 ± 2	0.23 ± 0.05
2-F	4 ± 1	3 ± 0.5	$(4.7 \pm 0.3) \times 10^{-2}$
2-CF_3	1 ± 0.2	0.6 ± 0.2	$(9 \pm 0.7) \times 10^{-3}$
2-NO_2	3 ± 0.3	1.6 ± 0.2	$(5 \pm 1) \times 10^{-3}$

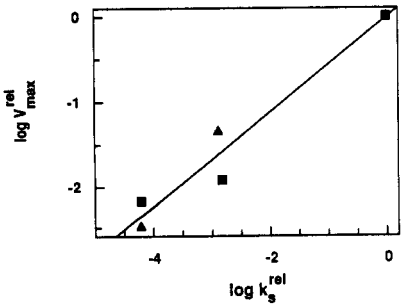


Figure 3. Hammett plot of log V_{max}^{rel} versus log k_s^{rel} for derivatives for 1 where Y = CH_3, CH_2F, and CHF_2.

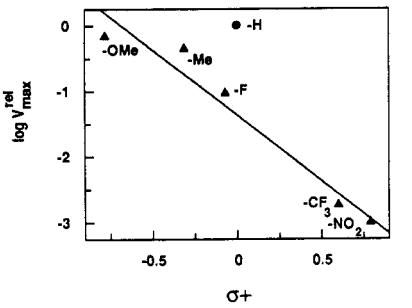


Figure 4. Hammett plot of log V_{max}^{rel} versus σ^+ for derivatives of 2 where Z = H, OCH_3, CH_3, F, CF_3, and NO_2.

Maximal velocities for the alkylation of tryptophan by DMAPP and the fluorinated analogs are given in Table II. The concentration of both substrates was 120 μM. These values were sufficient to saturate the enzyme for the normal substrates. At concentrations of allylic substrate above 120 μM, the incubation mixture became turbid, presumably because of precipitation of calcium pyrophosphate as the reaction proceeded. The mono- and difluoro analogs were substantially less reactive than DMAPP in the prenyl-transfer reaction. A Hammett plot of the logarithms of the relative rates for solvolysis and initial velocities for alkylation of tryptophan by the analogs is shown in Figure 3. The linear correlation indicates that the enzymatic reactions proceed through highly electrophilic transition states, although the DMAT synthase catalyzed reaction appears to be considerably less sensitive to substitution of hydrogen by fluorine than the solvolysis reaction.

Kinetic parameters for the tryptophan analogs are given in Table III. While the Michaelis constants for DMAPP are similar for each analog, K_M's for the analogs themselves vary considerably. K_M for the normal substrate lies approximately equidistant between the 600-fold variation for 2-OCH_3 and 2-CF_3. Maximal velocities generally decreased as the substituent at C(7) became increasingly

Table IV. Retention Ratios[a] for Substituted Tryptophans and Dimethyallyltryptophans[b]

tryptophan	retention ratio	dimethylallyltryptophan	retention ratio
2-OCH_3	6.8	3-OCH_3	13.8
2-CH_3	6.8	3-CH_3	13.6
2-F	6.3	3-F	14.0
2-CF_3	8.8	3-CF_3	14.0
2-NO_2	6.9	3-NO_2	15.6

[a] Retention ratio = volume of elution/void volume. [b] HPLC on a C_{18} reversed-phase column.

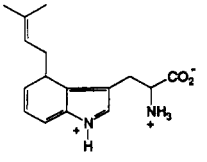
electron-withdrawing. The exception was tryptophan itself, which was slightly more reactive than the methoxy and methyl derivatives. A Hammett plot of V_{max} for the tryptophans versus σ^+ is shown in Figure 4. Except for L-tryptophan, the aromatic substrates gave a good linear correlation with a negative slope, consistent with an electrophilic substitution.

In a separate set of experiments, DMAT synthase was incubated with [^{3}H]DMAPP and tryptophans **2**-H, **2**-OCH_3, **2**-CH_3, **2**-F, **2**-CF_3, and **2**-NO_2. The radioactive products were separated from starting materials by reversed-phase HPLC. Dimethylallyltryptophan[15] (**3**-H) and **3**-CH_3 were identified from their ^{1}H NMR and mass spectra. Samples of **3**-OCH_3 and **3**-F were converted to bis-TMS derivatives and analyzed by high-resolution mass spectrometry. Both samples gave ions with the expected molecular masses for the TMS adducts. Although the enzymatic reactions with **2**-CF_3 and **2**-NO_2 gave small amounts of products, the presence of **3**-CF_3 and **3**-NO_2 could be inferred from radio-HPLC traces with characteristic retention times for the dimethylallyl derivatives on a C_{18} column (see Table IV).

Discussion

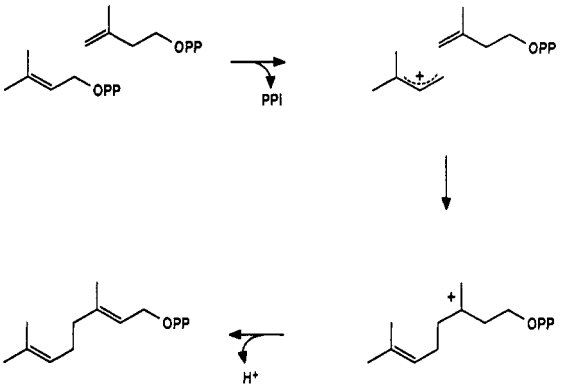
Prenyltransferases are ubiquitous enzymes that catalyze the alkylation of prenyl acceptors by the alkyl moieties of allylic isoprene diphosphates. The prenyl acceptor can be any one of a variety of nucleophilic residues, including alkenes (farnesyl diphosphate synthase,[10] squalene synthase[2]), aromatic rings (dimethylallyltryptophan synthase,[7] *p*-hydroxybenzoic acid: hexaprenyl diphosphate transferase[4]), amino groups (tRNA:dimethylallyl diphosphate transferase[9]), and sulfhydryl groups (protein:farnesyl diphosphate transferase[5]). Although different mechanisms have been proposed for various prenyl-transfer reactions, a minimal mechanism that suffices for all prenyltransferases is an electrophilic alkylation.

Farnesyl diphosphate synthase is the only prenyltransferase whose mechanism has been extensively studied. Several lines of evidence point to an electrophilic alkylation (Scheme IA). These include enzyme-catalyzed solvolysis of its allylic substrates,[11] linear free energy correlations that establish a direct relationship between the reactivity of allylic analogs during solvolysis and prenyl transfer,[12,28,32] and observation of products of carbocationic rearrangements for bisubstrate analogs during catalysis.[13] A similar mechanism for DMAT synthase would formally constitute an electrophilic aromatic substitution, as shown in Scheme IB. Although C(4) in tryptophan is meta to the indole nitrogen, it is nonetheless an activated position,[33] as illustrated by the resonance structure shown below for the σ complex formed upon alkylation at C(4). Thus, as pointed out by Lee et al.,[7] electrophilic aromatic substitution is a plausible mechanism for

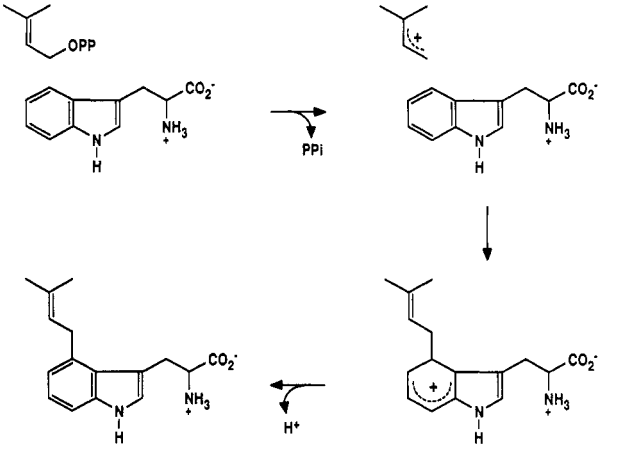


Scheme I. Electrophilic Mechanism for Prenyl-Transfer Reactions

A. Electrophilic Alkylation Catalyzed by FPP Synthase



B. Electrophilic Aromatic Substitution Catalyzed by DMAT Synthase



DMAT synthase if one assumes that the regiochemistry of the reaction is dictated by binding interactions which place C(1) of DMAPP adjacent to C(4) of tryptophan. In addition, Shibuya et al.,[34] recently reported that the configuration at C(1) of DMAPP is inverted upon alkylation of tryptophan, whereas the stereochemistry of the allylic double bond is preserved. Their results are consistent with an electrophilic aromatic substitution, which they conclude is the most plausible mechanism.

Our results with analogs of both DMAPP and tryptophan support an electrophilic mechanism for DMAT synthase. The Hammett plot in Figure 3 for the allylic substrates shows a positive correlation between the rates of the enzymatic alkylation and a model solvolysis reaction. The Hammett plot shown in Figure 4 also supports alkylation by an electrophilic aromatic substitution. In this case, the 7-substituted analogs gave a good linear correlation with $\rho = -2.0$. This value is, however, at the low end of the range normally seen for benzene derivatives.[35,36] Likewise, the slope of the plot in Figure 3 indicates that the enzymatic alkylation is less sensitive to substituent effects than solvolysis. These results suggest that there is less development of positive charge at the transition state for the enzymatic reaction than the reactions used for comparisons in the linear free energy correlations. The lower sensitivity toward electron-withdrawing substituents in the indole moiety may be explained by an enhanced ability of the indole nitrogen to stabilize developing positive charge in the ring. This could be accomplished by a specific hydrogen bond between the indole NH and a basic acceptor in DMAT synthase.

As shown in Figure 4, tryptophan is substantially more reactive than predicted from the behavior of the 7-substituted tryptophans.

(32) Poulter, C. D.; Argyle, J. C.; Mash, E. A. *J. Biol. Chem.* **1978**, *253*, 7227–7233.
(33) Floss, H. G.; Mothes, U.; Onderka, D.; Hornemann, U. *Naturforsch. Ser. B* **1965**, *20*, 133–136.
(34) Shibuya, M.; Chou, H.-M.; Fountoulakis, M.; Hassam, S.; Kim, S.-U.; Kobayashi, K.; Otsuka, H.; Togalska, E.; Cassady, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1990**, *112*, 297–304.
(35) Stock, L. M.; Brown, H. C. *Adv. Phys. Org. Chem.* **1963**, *1*, 36–154.
(36) Stock, L. M. *Prog. Phys. Org. Chem.* **1976**, *12*, 21–47.

This behavior cannot be directly attributed to a K_M effect since the Michaelis constant for **2**-H is in the middle of the range of K_M's for the analogs, and rates were determined at sufficiently high concentrations of substrate to reflect maximal values. However, a steric impediment in the vicinity of C(7) could alter the orientation of the analogs in the E·S complex relative to that of tryptophan in a manner to produce an impact on k_{cat}.

Currently, little is known about the structures of prenyl transferases beyond the primary sequences of several enzymes deduced from their genes. Sequence comparisons for several different prenyl transferases reveal conserved asparate-rich regions. Ashby and Edwards[37] proposed that one of the asparate-rich motifs, which they designate domain 2, binds the allylic substrate and catalyzes rupture of the carbon–oxygen linkage to the diphosphate. This domain is conserved for a wide variety of prenyl transferases, including enzymes whose normal substrates encompass a wide variety of prenyl acceptors, and its occurrence is consistent with a common mechanism for the various prenyltransfer reactions.

Linear free energy correlations show that both FPP synthase[12] and DMAT synthase catalyze electrophilic alkylations of their respective acceptors. From the viewpoint of mechanistic organic chemistry, electrophilic alkylations are highly attractive for those prenyltransferases that alkylate weakly nucleophilic acceptors such as carbon–carbon double bonds and aromatic rings. It is, however, less clear that enzymes which alkylate the more potent hydroxyl, amino, and sulfhydryl acceptors also catalyze electrophilic additions via carbocationic species or that the mechanisms shift to a nucleophilic displacement at C(1) in the allylic substrates.

Acknowledgment. This investigation was supported by Grant GM 21328 from the National Institutes of Health.

(37) Ashby, M. N.; Spear, D. H.; Edwards, P. A. *Molecular Biology of Atherosclerosis*; Attie, A. D. Ed.; Elsevier: Amsterdam, 1990; pp 27–34.

Design, Synthesis, and Study of Simple Monocyclic Conjugated Enediynes. The 10-Membered Ring Enediyne Moiety of the Enediyne Anticancer Antibiotics†

K. C. Nicolaou,*,‡ G. Zuccarello, C. Riemer, V. A. Estevez, and W.-M. Dai

Contribution of the Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Chemistry, University of California, San Diego, La Jolla, California 92093. *Received March 4, 1992*

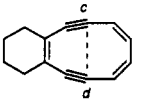
Abstract: Following the discovery of the enediyne anticancer antibiotics, investigations were initiated directed toward the design, synthesis, and study of simple monocyclic conjugated enediynes. In this article the synthesis of the parent 10-membered ring enediyne found in the enediyne natural products and its properties are described. In addition to the parent hydrocarbon **27**, the synthetic methodology developed based on the Ramberg–Bäcklund reaction delivers a series of higher ring homologs (**17–22**) and the water soluble version of the 10-membered ring compound **47**. Molecular mechanics calculations on these systems led to a number of geometrical parameters which correlated well with their tendencies to undergo the Bergman cycloaromatization reaction. Kinetic studies on the Bergman cycloaromatization of the 10-membered ring enediynes **27** and **47** led to the following thermodynamic values: **27**, energy of activation (E_a) = 23.8 kcal/mol, $\Delta G^{\ddagger}$ (37 °C) = 24.6 kcal/mol; **47**, energy of activation (E_a) = 31.5 kcal/mol, $\Delta G^{\ddagger}$ (37 °C) = 24.8 kcal/mol. The designed enediyne **47** showed potent DNA-cleaving properties becoming the first synthetic molecule to mimic the action of the naturally occurring enediynes in this regard.

Introduction

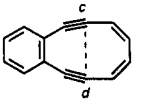
In 1987, the structures of two novel families of natural products, the calicheamicins [e.g., calicheamicin γ_1^I (**1**), Scheme I] and the esperamicins [e.g., esperamicin A_1 (**2**), Scheme I] were reported by investigators from Lederle Laboratories[1] and Bristol Myers,[2] respectively. A common and most unusual structural feature of these molecules was the 10-membered ring containing a conjugated enediyne system embedded in their skeletons. The phenomenal biological activity of these substances against bacteria and tumor cells was attributed to their ability to cause DNA cleavage. The enediyne moiety was considered crucial to their mode of action which was postulated to involve a Bergman cyclization reaction[3] leading to damaging benzenoid diradicals (Scheme II). Today, the enediyne class[4] includes, in addition to the calicheamicins and esperamicins, neocarzinostatin chromophore[5] and the dynemicins.[6]

Soon after these disclosures, and in order to test the proposed hypothesis for the mechanism of action of the naturally occurring calicheamicins and esperamicins and to probe the idea of mimicking their chemistry and biological action with simple synthetic

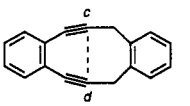
Chart I. Cyclic Conjugated Enediynes Spontaneously Undergoing Bergman Cycloaromatization at Ambient Temperature[7,8]



4a: *cd* 2.99 Å [MM2]



4b: *cd* 3.01 Å [MM2]



5: *cd* 3.03 Å [MM2]

† This work was initiated and partially carried out in the Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104. Partially taken from the Ph.D. Thesis of G. Zuccarello, University of Pennsylvania, 1989.

‡ The Scripps Research Institute.

(1) Lee, M. D.; Dunne, T. S.; Siegel, M. M.; Chang, C. C.; Morton, G. O.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3464. Lee, M. D.; Dunne, T. S.; Chang, C. C.; Ellestad, G. A.; Siegel, M. M.; Morton, G. O.; McGahren, W. J.; Borders, D. B. *J. Am. Chem. Soc.* **1987**, *109*, 3466.

0002-7863/92/1514-7360$03.00/0