NMR (CCl_4) δ 1.24 (t, J = 7 Hz, 3 H, $CO_2CH_2CH_3$), 2.40–2.90 (m, 4 H, CH_2CH_2), 4.02 (s, 2 H, $COCH_2Cl$), 4.06 (q, J = 7 Hz, 2 H, CO_2CH_2).

Ethyl (*R*)-(+)-5-Chloro-4-hydroxypentanoate (8). To a mixture of boiled water (450 mL), glucose (30 g), $NH_4H_2PO_4$ (0.9 g), KH_2PO_4, $MgSO_4$ (0.45 g), and $CaCO_3$ (2.25 g) was added 20 g of bakers' yeast at 35 °C. After the mixture was stirred for 30 min, 2.76 g (15.5 mmol) of 7 was added and the mixture was stirred at 32 °C. Every 2 days bakers' yeast (10 g) and glucose (10 g) were added. After 13 days the mixture was extracted with ethyl acetate. The usual workup gave the crude product (1.73 g), which was chromatographed on SiO_2 [hexane/ethyl acetate (30/1)] to give 1.20 g (43%) of 8: $[\alpha]^{24}_D$ +2.93° (*c* 4.23, $CHCl_3$); 63% ee (estimated by the ^1H NMR analysis for the corresponding MTPA ester in the presence of Eu(hfc)$_3$): IR (neat) 3450, 1730, 1180, 1095 cm^{-1}; ^1H NMR (CCl_4) δ 1.25 (t, J = 7 Hz, 3 H, CH_2CH_3), 1.60–1.95 (m, 2 H, CH_2CO_2), 2.30–2.55 (m, 2 H, $CHOHCH_2$), 2.98 (br s, 1 H, OH), 3.40–4.00 (m, 3 H, $CHOHCH_2Cl$), 4.08 (q, J = 7 Hz, 2 H, CH_2CH_3). Anal. Calcd for $C_7H_{13}ClO_3$: C, 46.55; H, 7.25. Found: C, 46.34; H, 7.11.

MTPA ester of 8 was prepared by the method described in the literature.[8] A mixture of 8 (77.7 mg, 0.430 mmol), pyridine (3 mL), and (−)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetyl chloride (122 mg, 0.483 mmol) was stirred for 24 h at room temperature and then poured into ice water. The organic materials were extracted with ether, and the ethereal extracts were washed with dilute HCl, water, and dried over $MgSO_4$. Evaporation of the solvent gave the crude product, which was purified by preparative TLC [hexane/ethyl acetate (2/1)] to give 60.5 mg (35%) of the MTPA ester of 8: R_f 0.41; IR (neat) 1745, 1260, 1180, 1020 cm^{-1}; ^1H NMR (CCl_4) δ 1.23 (t, J = 7 Hz, 3 H, CH_2CH_3), 1.90–2.50 (m, 4 H, CH_2CH_2), 3.40–3.80 (m, 5 H, CH_2Cl, OCH_3), 4.07 (q, J = 7 Hz, CH_2CH_3), 5.20 (m, 1 H, CHO), 7.18–7.60 (m, 5 H, C_6H_5). Anal. Calcd for $C_{17}H_{20}O_5ClF_3$: C, 51.46; H, 5.08. Found: C, 51.49; H, 5.19.

Ethyl 3,5-Dichloro-4-oxopentanoate (9). To a solution of levulinic acid (10.7 g, 92.1 mmol) in chloroform (7 mL) was introduced chlorine gas at room temperature for 1 h. The mixture was stirred overnight, and chlorine gas was again introduced for 26 h at 35 °C. Air was bubbled into the mixture for 20 h and the precipitates were filtered off to give 4.87 g (28.6%) of 3,5-dichloro-4-oxopentanoic acid (14): IR (KBr) 3000, 1735, 1705, 1250 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.01–3.02 (m, 2 H, CH_2CO_2H), 4.41 (s, 2 H, $ClCH_2CO$), 4.80 (t, J = 6 Hz, 1 H, $CHClCH_2$), 10.2 (br s, 1 H, CO_2H). Concentration of the filtrate gave 13.7 g (81%) of 3,5-dichloro-4-oxopentanoic acid. The mixture of 14 (3.03 g, 16.4 mmol), dry ethanol (30 ml), and *p*-toluenesulfonic acid (100 mg) was heated at the reflux temperature for 13 h. After the mixture was concentrated, the organic materials were extracted with ether, washed with aqueous $NaHCO_3$ and water, and dried over $MgSO_4$. Evaporation of the solvent gave 2.95 g (85%) of 9: TLC [hexane/ethyl acetate (1/1)] R_f 0.57; IR (neat) 1730, 1205, 1020, 790 cm^{-1}; ^1H NMR (CCl_4) δ 1.24 (t, J = 7 Hz, 3 H, CH_2CH_3), 2.88–3.08 (m, 2 H, CH_2CO_2), 4.09 (q, J = 7 Hz, 2 H, CH_2CH_3), 4.38 (s, 2 H, $ClCH_2CO$), 4.77 (t, J = 7 Hz, 1 H, $CHClCH_2$). Anal. Calcd for $C_7H_{10}Cl_2O_3$: C, 39.46; H, 4.73. Found: C, 39.48; H, 4.61.

Ethyl (4*S*)-(+)-3,5-Dichloro-4-hydroxypentanoate (10). To a mixture of boiled water (300 mL), glucose (6 g), $NH_4H_2PO_4$ (0.6 g), KH_2PO_4 (0.6 g), $MgSO_4$ (0.3 g), and $CaCO_3$ (1.5 g) was added bakers' yeast (10 g) at 35 °C. After 30 min, 1.78 g (8.36 mmol) of 9 was added and the mixture was stirred for 5 days at 35 °C. The organic materials were extracted with ethyl acetate. The usual workup followed by column chromatography over SiO_2 [hexane/ethyl acetate (10/1–1/1)] gave 0.862 g (48%) of 10: $[\alpha]^{25}_D$ +5.46° (*c* 5.02, $CHCl_3$); IR (neat) 3450, 1730, 1160, 1100, 950 cm^{-1}; ^1H NMR (CCl_4) δ 1.27 (t, J = 7 Hz, 3 H, CH_2CH_3), 2.70–3.25 (m, 3 H, CH_2CO_2, OH), 3.80–4.40 (m, 4 H, $ClCH_2CH(OH)CHCl$), 4.13 (q, J = 7 Hz, 2 H, CH_2CH_3). Anal. Calcd for $C_7H_{12}Cl_2O_3$: C, 39.09; H, 5.63. Found: C, 39.43; H, 5.57.

Ethyl (*R*)-(+)-(*E*)-5-Chloro-4-hydroxy-2-pentenoate (11). A mixture of 10 (862 mg, 4.01 mmol), dry ether (8 mL), and triethylamine (1.35 mL, 8.82 mmol) was stirred for 98 h at room temperature and then poured into ice water. After the mixture was acidified with dilute HCl, the organic materials were extracted with ethyl acetate. After the usual workup, the crude product (556 mg) was chromatographed on SiO_2 [hexane/ethyl acetate (10/1–1/1)] to give 306 mg (43%) of 11: $[\alpha]^{26}_D$ +6.30° (*c* 3.65, $CHCl_3$); 83% ee by the ^1H NMR analysis in the presence of Eu(hfc)$_3$; IR (neat) 3500, 1720, 1665, 1300, 1275, 1180 cm^{-1}; ^1H NMR (CCl_4) δ 1.27 (t, J = 7 Hz, 3 H, CH_2CH_3), 3.45 (br s, 1 H, OH), 3.52 (d, J = 7 Hz, 2 H, CH_2Cl), 4.11 (q, J = 7 Hz, 2 H, CH_2CH_3), 4.30 (m, 1 H, CH(OH)), 5.99 (dd, J = 1.2 and 15 Hz, 1 H, $=CHCO_2$), 6.77 (dd, J = 4.4 and 15 Hz, 1 H, $CHOHCH=$). Anal. Calcd for $C_7H_{11}ClO_3$: C, 47.07; H, 6.21. Found: C, 47.11; H, 6.35.

Hydrogenation of 11. A mixture of 11 (296 mg, 1.66 mmol), dry ethanol (3 mL), and palladium on charcoal (66 mg) was stirred for 45 h under 1 atm of hydrogen. After filtration, concentration of the solvent left 214 mg of an oil, which was chromatographed on SiO_2 [hexane/ethyl acetate (20/1–5/1)] to give 117 mg (39%) of 8: $[\alpha]^{26}_D$ +5.26° (*c* 2.93, $CHCl_3$); TLC [hexane/ethyl acetate (1/1)] R_f 0.48. The spectral data were identical with those of the sample prepared from 7.

(*R*)-5-(Chloromethyl)tetrahydro-2-furanone (12). A mixture of 8 (117 mg, 0.648 mmol), concd HCl (0.5 mL), and water (0.5 mL) was stirred for 8 h at 95 °C. The organic materials were extracted with ether and worked up as usual. Evaporation of the solvent gave 32 mg (36%) of 12: $[\alpha]^{26}_D$ −7.18° (*c* 2.20, $CHCl_3$) (lit.[9] $[\alpha]^{27}_D$ −12.9° (*c* 3.03, $CHCl_3$)); IR (neat) 1780, 1175, 1040, 920 cm^{-1}; ^1H NMR (CCl_4) δ 1.90–2.72 (m, 4 H, $(CH_2)_2$), 3.67 (d, J = 5 Hz, 2 H, CH_2Cl), 4.70 (m, 1 H, CHO). Spectral data were identical with those of the authentic sample.[9]

Ethyl (*R*)-4,5-Epoxypentanoate (13). Sodium (85 mg, 3.7 mmol) was dissolved in dry ethanol (6 mL), and 8 (552 mg, 3.06 mmol) was added at 0 °C with stirring. The mixture was stirred for 5 h, poured into ice water, and acidified with dilute HCl. The organic materials were extracted with CH_2Cl_2. The usual workup followed by column chromatography [SiO_2, hexane/ethyl acetate (20/1–1/1)] gave 92 mg (21%) of 13: $[\alpha]^{24}_D$ +4.10° (*c* 3.32, $CHCl_3$); IR (neat) 1740, 1260, 930, 880 cm^{-1}; ^1H NMR (CCl_4) δ 1.23 (t, J = 7 Hz, 3 H, CH_2CH_3), 1.78 (m, 2 H, $CH_2CH_2CO_2$), 2.20–3.00 (m, 5 H, CH_2OCH and CH_2CO_2), 4.06 (q, J = 7 Hz, 2 H, CO_2CH_2). Anal. Calcd for $C_7H_{12}O_3$: C, 58.32; H, 8.39. Found: C, 58.14; H, 8.14.

Registry No. 1, 136576-72-2; (3*R*,4*S*)-2, 136576-80-2; (3*S*,4*S*)-2, 136576-73-3; 3, 132341-85-6; 4, 112789-84-1; 5, 19041-15-7; 6, 92694-51-4; 7, 14594-24-2; 8, 136576-74-4; 8 MTPA ester, 136599-73-0; 9, 136576-75-5; (3*R*,4*S*)-10, 136576-76-6; (3*S*,4*S*)-10, 136576-82-4; 11, 136576-77-7; 12, 52813-64-6; 13, 136576-78-8; 14, 136576-79-9; 4-chloro-5-methyltetrahydro-2-furanone, 136576-81-3; 3-(ethoxycarbonyl)propanoyl chloride, 14794-31-1; levulinic acid, 123-76-2.

Attempted de Novo Design, Synthesis, and Evaluation of a Ligand for the Allosteric Site of Phosphofructokinase

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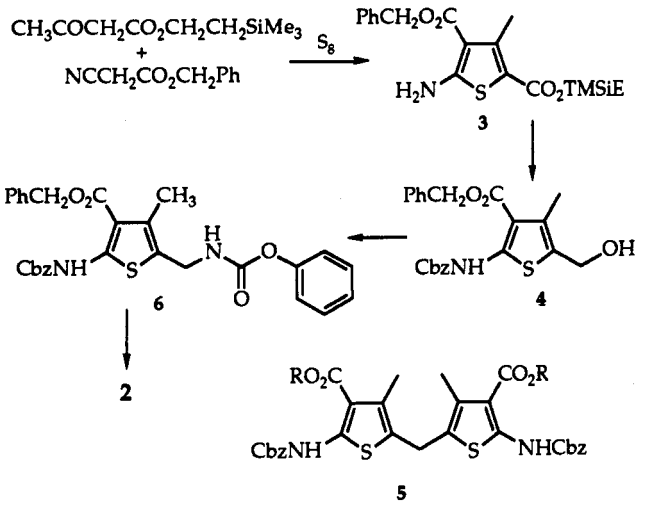
The design of small-molecule ligands for protein binding sites has traditionally focused on enzyme inhibitors, using strategies that depend on knowledge of enzymatic mechanism or modification of lead structures. With the increasing availability of structural information through X-ray crystallography and NMR methods have come attempts to design ligands directly, using a combination of intuition and automated methods to invent completely new molecules to complement the geometric and electronic characteristics of a binding site. The possibility of devising ligands for noncatalytic sites, such as receptor or allosteric sites, can now be addressed as well. This report describes our attempt to design a ligand for the allosteric effector site of phosphofructokinase (PFK).

0022-3263/91/1956-7179$02.50/0

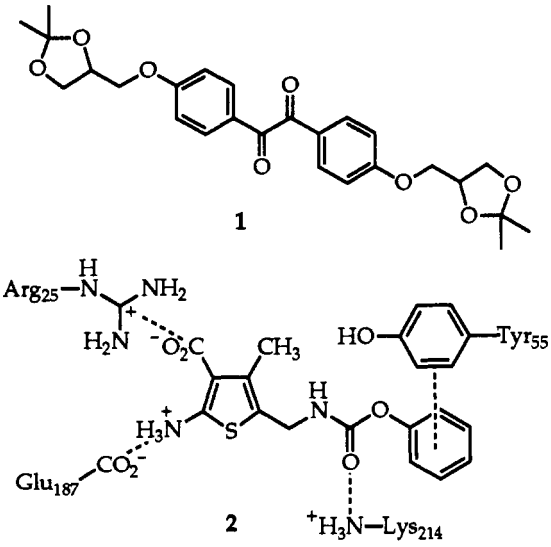
Scheme I



PFK catalyzes the phosphorylation of fructose 6-phosphate (F6P) with ATP to form fructose 1,6-diphosphate and ADP. There are two physiologically relevant allosteric effectors of the bacterial enzyme: the activator ADP and the inhibitor phosphoenolpyruvate (PEP).[1] The enzyme has been shown to conform to the classic K-type model of allostery first proposed by Monod and co-workers, in which the active and inactive forms of the enzyme differ in their conformation and the effectors simply shift the position of the equilibrium between the two forms.[2]

Crystal structures of PFK from *B. stearothermophilus* in both the active (ADP complex)[3] and inactive (2-phosphoglycolate complex)[4] forms are available, as are structures for the *E. coli* enzyme.[5] The allosteric site is surrounded by charged residues (Arg and Lys), which are responsible for interaction with the phosphate groups of the effectors. Work by Lau and Fersht has implicated a role for Glu-187 in mediating the different effects of ADP and PEP.[6] In seeking to devise a ligand for this site, we first considered shape complementarity and then attempted to introduce appropriate ionic interactions between the ligand and the above charged residues.

As the first step in the design process, the program DOCK[7] was used to search the Cambridge Structural Database (CSD)[8] to identify structures that were likely to fit within the allosteric site of the R state form of *E. coli* PFK.[9] Substructures common to the highest scoring hits included an aromatic ring that was placed in the back of the receptor cavity; the highest scoring compound from the search was the benzil derivative 1.[10] Alterations in 1 were made to remove steric interference, to trim noninteracting parts of the structure, and to incorporate functional groups to hydrogen bond with the polar amino acid side chains. At each iteration, the new design was minimized using the modified MM2 force field implemented by MACROMODEL,[11] and the molecule was fit into the receptor site manually, with appropriate single-bond rotations. DOCK was again used to determine the optimal docking orientation for the individual structures. Finally, consideration was given to reduction of stereochemical and synthetic complications and resulted in the substituted thiophene 2 as the target; the intended interactions between this structure and the allosteric site are shown.



The synthesis of thiophene 2 is outlined in Scheme I. The tetrasubstituted derivative 3 was constructed in a single step by modification of a reaction pioneered by Gewald,[12] using 2-(trimethylsilyl)ethyl acetoacetate, benzyl cyanoacetate, and elemental sulfur; transesterification was avoided by conducting this condensation in *tert*-butyl alcohol. The poorly nucleophilic amino group was protected as the carbobenzoxy (Cbz) derivative using Cbz chloride and 4-(dimethylamino)pyridine. Selective cleavage of the 2-(trimethylsilyl)ethyl ester and subsequent reduction of the carboxyl group via reaction of the acid chloride with borohydride afforded the hydroxymethyl derivative 4.

Activation of the hydroxyl group for eventual substitution by nitrogen nucleophiles was plagued by dimerization of the intermediates to give 5 (R = *t*Bu in initial work).[13]

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(2) Monod, J.; Wyman, J.; Changeaux, J. P. *J. Mol. Biol.* **1965**, *12*, 88–118.
(3) Evans, P. R.; Farrants, G. W.; Hudson, P. J. *Phil. Trans. R. Soc. Lond. B* **1981**, *293*, 53–62.
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(5) Rypniewski, W. R.; Evans, P. R. *J. Mol. Biol.* **1989**, *207*, 805–821.
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(9) We thank Professor P. R. Evans for providing these coordinates in advance of their release.
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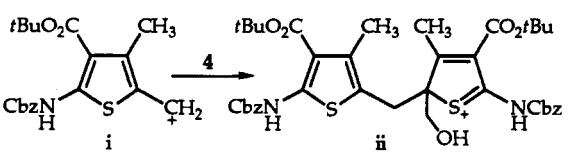
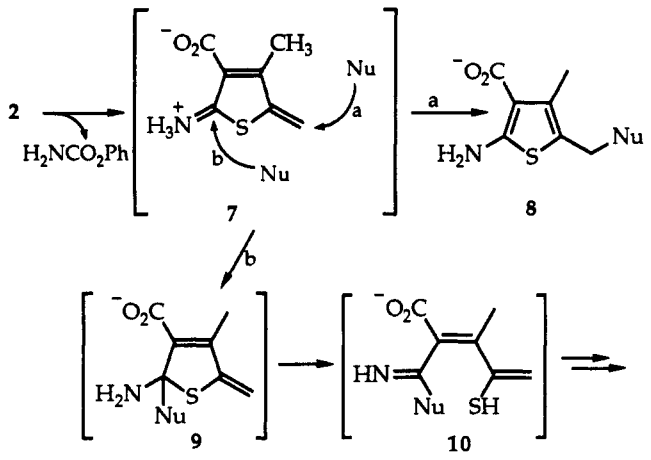
(12) Gewald, K.; Schinke, E.; Boettcher, H. *Chem. Ber.* **1966**, *99*, 94–100.
(13) Dimer 5 was characterized by ^1H and ^{13}C NMR and by mass spectrometry. It is presumably formed by ionization of the activated alcohol to the stabilized cation i, which leads to electrophilic attack at the 5-position of another molecule of 4. Loss of formaldehyde from ii would then account for the observed product. The strong propensity of thiophene derivatives to undergo substitution at the α-position is well precedented, as is the reactivity of aminothiophenes in general.[14]



(14) Scrowston, R. M. *Chem. Heterocycl. Compd.* **1986**, *44* (Part 3), 309–563. Norris, R. K. *Chem. Heterocycl. Compd.* **1986**, *44* (Part 2), 631–799.

Scheme II



However, the use of triphenylphosphine and CCl_4 in the presence of NaN_3[15] was successful, presumably because isolation of an activated intermediate was avoided. Reduction of the azide with triphenylphosphine and hydrolysis of the phosphinamide under basic conditions afforded the amine, which without purification was converted to the phenyl carbamate **6** in 50% yield from alcohol **4**. Simultaneous removal of the benzyl protecting groups was accomplished with triethylsilane and Pd(II) catalysis[16] to give the target α-aminothiophene-β-carboxylate **2** in 7% overall yield and 10 linear steps.[17]

Assays of PFK were carried out in the presence of 2.5 mM ATP (K_m = 71 μM) and 1 mM F6P (K_m = 20 μM). PFK is not inhibited by thiophene **2** at concentrations up to 2 mM, nor is there any competition with the known allosteric effector PEP. However, if **2** is incubated in buffer for as little as 5 min before the assay is initiated by addition of PFK, inactivation of the enzyme is observed, suggesting that the compound decomposes to a species that does inhibit. Furthermore, the inactivation appears to be irreversible, since dilution of the inhibited mixture 500-fold does not lead to recovery of activity. Incubation of the thiophene in buffer and addition to enzyme and substrate at various times indicated that the reactive species reaches a steady-state concentration after 10 min and is maintained for at least 7 h. It was also found that 2 mM dithiothreitol (DTT) protects the enzyme against inactivation, suggesting that the decomposition product is electrophilic.

Isolation and characterization of this species was attempted in order to confirm the above hypothesis. No new resonances appeared in the ^1H NMR spectrum during the decomposition, nor could any identifiable products, either in the presence or absence of DTT, be discerned by analytical ion exchange or reverse-phase HPLC. Mass spectrometry failed to provide evidence for a thiophene–DTT adduct. Decomposition of the thiophene was accompanied by formation of a water- and DMF-insoluble precipitate which was presumed to be polymeric in nature. A proposed mechanism for decomposition is outlined in Scheme II. Loss of the carbamate moiety affords the electrophilic quinonoid species **7**, which can undergo nucleophilic attack either at the methylene group (a) or at the α-position (b).[14] The adduct **9** formed in the latter route could lead to loss of the thiophene ring and further reaction.[18] The electrophilic species **7** may be responsible for inactivation of PFK by covalent linkage to an active site residue.

Experimental Section

Computer Modeling.[19] DOCK. A fragment of PFK was created that included all atoms with 10 Å of the allosteric site; the solvent-accessible surface of this site, including surface normals, was generated using MS.[21] This surface served as input for the subroutine SPHGEN, which created a 77-sphere cluster representing the allosteric site of PFK. This cluster was then used for a DOCK search of the separate classes from the CSD; hits from each class were reviewed on the graphics terminal for possible development into reasonable ligands, as described in the text. The best hit was CASHEJ (1) from class 17.

Synthesis.[22] **2-(Trimethylsilyl)ethyl Acetoacetate.**[24] The ester was prepared according to the procedure of Lawsson et al.[24] from 2-(trimethylsilyl)ethanol (5.00 mL, 35 mmol), anhydrous NaOAc (13 mg, 160 μmol), and diketene (3.3 mL, 37 mmol). The product was isolated by distillation at reduced pressure (ca. 20 mmHg); the fraction boiling from 120–124 °C was collected to give 3.63 g (95%, based on recovered starting material) of the ester: ^1H NMR δ 4.15 (d, 1, J = 8.6), 4.14 (d, 1 J = 8.6), 3.35 (s, 2), 2.11 (s, 3), 0.94 (d, 1 J = 8.6), 0.92 (d, 1, J = 8.6), -0.06 (s, 9); ^{13}C NMR δ 200.5, 167.1, 63.5, 50.1, 30.0, 17.2, -1.7; IR 1739, 1714 cm^{-1}. Anal. Calcd for $C_9H_{18}O_3Si$: C, 53.43; H, 8.97. Found: C, 53.64; H, 8.92.

2-Amino-5-[[(phenoxycarbonyl)amino]methyl]-4-methylthiophene-3-carboxylic Acid (2). To a solution of **6** (690 mg, 1.30 mmol) (see below) and palladium(II) acetate (15 mg, 65 μmol) in CH_2Cl_2 was added triethylsilane (3.1 mL, 19.5 mmol). After 1.5 h, the mixture was concentrated in vacuo, the resulting residue was dissolved in $CHCl_3$ (15 mL), and the solution was filtered (0.45-μm filter) and concentrated in vacuo. The solid obtained was triturated with CH_3CN, petroleum ether, and CH_2Cl_2 to yield 250 mg (62%) of **2**. A small amount was purified by reversed-phase C-18 HPLC (10 mm × 25 cm, 25% CH_3CN/75% CH_3CN–H_2O, 0–30 min, 10 mL/min, k' = 1.8) for analytical purposes: mp 171.0 °C dec; ^1H NMR (d_7-DMF) δ 12.18 (b, 0.5), 8.02 (s, 2.5), 7.43 (b, 1), 7.39 (dd, 2, J = 7.9, 7.9), 7.21 (dd, 1, J

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(16) Sakaitari, M.; Ohture, Y. *J. Org. Chem.* **1990**, *55*, 870–876.

(17) The initial route to this compound, via the corresponding *tert*-butyl ester, was abandoned because of the sensitivity of the benzylic position to the acidic conditions required for deprotection.

(18) Cf., inter alia: Rault, S.; Cugnon de Sevricourt, M.; Robba, M. *Recl. Trav. Chim. Pays-Bas* **1982**, *101*, 205–208. Gewald, K.; Jablokoff, H.; Hentschel, M. *J. Prakt. Chem.* **1975**, *317*, 861. Guanti, G.; Dell'Erba, C.; Leandri, G.; Thea, S. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2357–2360.

(19) **General.** All energy calculations and minimizations were performed on a μVAX II computer under μVMS v. 4.6 using MACROMODEL v. 1.2.[11] The modified version of MM2 was used for molecular mechanics minimizations of the ligand, and the modified AMBER force field[20] was used for minimizations including the protein. Database searches were performed on a VAX 11/780 running 4.3 BSD Unix using DOCK (v. 1.0)[7] and the CSD.[8] An Evans & Sutherland PS350 graphics terminal hosted by a μVAX II was used in conjunction with MOGLI (v. 2.0, Evans & Sutherland) or MACROMODEL for display and interactive modeling. The protein coordinates used were those of the R state *E. coli* PFK with products bound (2.4-Å resolution, corresponding to PDB1PFK.ENT from the Brookhaven Protein Databank).

(20) Weiner, P. K.; Kollman, P. A. *J. Comput. Chem.* **1981**, *2*, 287–303. Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagena, G.; Profeta, S., Jr.; Weiner, D. *J. Am. Chem. Soc.* **1984**, *106*, 765–784. Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. *J. Comput. Chem.* **1986**, *7*, 230–252.

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(22) **General.** Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Reactions involving reagents sensitive to moisture were conducted under an atmosphere of dry nitrogen using dry reagents and solvents. Preparative thin-layer chromatography (TLC) was performed with plates precoated with silica gel G.F. (Analtech, Inc., Newark, DE). Column chromatography was performed according to Still, Kahn, and Mitra[23] using silica gel 60 (E. Merck, Darmstadt). Melting points reported are uncorrected. ^1H NMR data are reported in the following manner: chemical shift (δ) in ppm downfield from internal tetramethylsilane (multiplicity, integrated intensity, coupling constants in hertz). ^{13}C NMR spectra were obtained using two-level ^1H decoupling or broad-band ^1H decoupling: chemical shifts (δ) are reported as ppm relative to tetramethylsilane, downfield positive. NMR spectra were recorded in $CDCl_3$, and IR spectra were recorded in $CHCl_3$, unless otherwise noted.

(23) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *42*, 2923–2925.

(24) Prepared by a procedure analogous to that described by Lawsson, S.-O.; Gronwall, S.; Sandberg, R. *Organic Syntheses*; Wiley: New York, 1973; Vol. V, pp 155–157.

= 7.4, 7.4), 7.14 (d, 2, J = 7.7), 4.31 (d, 2, J = 5.8), 2.28 (s, 3); ^{13}C NMR (d_7-DMF) δ 167.0, 163.6, 154.4, 151.1, 132.0, 129.3, 125.0, 121.8, 115.1, 104.2, 36.7, 14.7; IR 3440, 3330, 1690, 1640 cm^{-1}. Anal. Calcd for $C_{14}H_{14}N_2O_4S\cdot(H_2O)_{0.25}$: C, 54.10; H, 4.70; N, 9.01. Found: C, 54.23; H, 4.35; N, 8.52.

3-Benzyl 5-[2-(Trimethylsilyl)ethyl] 2-Amino-4-methylthiophene-3,5-dicarboxylate (3). Benzyl cyanoacetate (10.6 g, 41.5 mmol), elemental sulfur (1.33 g, 41.5 mmol) and 2-(trimethylsilyl)ethyl acetoacetate (8.4 g, 41.5 mmol) were dissolved in *tert*-butyl alcohol (17 mL) and heated to 40 °C. Diethylamine (4.3 mL, 41.5 mmol) was added, and the bright red solution was heated at 70 °C for 22 h. The solution was concentrated in vacuo, filtered through 5 cm of silica gel with Et_2O, and then chromatographed (1:3 Et_2O–hexanes) to give a yellow solid. This material was recrystallized from petroleum ether and then hexanes to yield 8.5 g of **3** (52%): mp 74.5–75.0 °C; ^{1}H NMR δ 7.40–7.28 (m, 5), 6.65 (s, 2), 5.28 (s, 2), 4.29 (dd, 2, J = 8.4, 8.4), 2.70 (s, 3), 1.05 (dd, 2, J = 8.4, 8.4), 0.04 (s, 9); ^{13}C NMR δ 166.5, 165.7, 162.9, 147.8, 136.0, 128.5, 128.1, 127.9, 108.4, 107.9, 65.8, 62.6, 17.3, 16.2, −1.6; IR 1700, 1680 cm^{-1}. Anal. Calcd for $C_{19}H_{25}NO_4SSi$: C, 58.28; H, 6.44; N, 3.58; S, 8.19. Found: C, 58.30; H, 6.47; N, 3.56; S, 8.38.

3-Benzyl 5-[2-(Trimethylsilyl)ethyl] 2-[[(Phenylmethoxy)carbonyl]amino]-4-methylthiophene-3,5-dicarboxylate. A solution of **3** (7.7 g, 19.7 mmol), 4-(dimethylamino)pyridine (214 mg, 1.8 mmol), and Et_3N (4.9 mL, 35 mmol) in CH_2Cl_2 (35 mL) was cooled to 0 °C and treated with two 2.65-mL aliquots (17.5 mmol) of benzyl chloroformate at 30-min intervals. The solution was stirred for 12 h and then transferred to a separatory funnel and diluted with EtOAc (400 mL). The organic layer was washed in 1 N HCl (2 × 40 mL) and 40 mL of brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to yield a solid which was triturated with petroleum ether to give 4.5 g of the N-Cbz derivative. The remaining filtrate was chromatographed (70 mm, 1:9 Et_2O–hexanes) to give another 0.5 g of product (48% yield overall): mp 97.0–98.0 °C; ^{1}H NMR δ 10.80 (s, 1), 7.40–7.32 (m, 10), 5.32 (s, 2), 5.25 (s, 2), 4.32 (dd, 2 J = 8.5, 8.5), 2.73 (s, 3), 1.09 (dd, 2, J = 8.5, 8.5), 0.06 (s, 9); ^{13}C NMR δ 165.6, 162.8, 154.7, 152.6, 145.0, 135.2, 134.9, 128.6, 128.5; 128.4, 128.3, 128.1, 117.0, 112.9, 68.2, 66.7, 62.9, 17.4, 15.6, −1.6; IR 1730, 1700, 1675 cm^{-1}. Anal. Calcd for $C_{27}H_{31}NO_6SSi$: C, 61.69; H, 5.94; N, 2.66; S, 6.10. Found: C, 61.62; H, 5.95; N, 2.64; S, 5.91.

2-[[(Phenylmethoxy)carbonyl]amino]-4-methylthiophene-3,5-dicarboxylic Acid 3-Benzyl Ester. A solution of the above diester (4.88 g, 9.3 mmol) in THF (25 mL) was treated with 1.0 M tetrabutylammonium fluoride in THF (TBAF, 25.7 mL), stirred for 12 h, treated with another aliquot of TBAF (20.0 mL), and stirred for another 7 h. The reaction mixture was quenched with ice, and the resulting slurry was dissolved in EtOAc (600 mL). The mixture was washed with 1 N HCl (300 mL), the the EtOAc was separated and concentrated in vacuo to yield a light-green solid which was triturated with Et_2O to yield 3.56 g (98%) of the carboxylic acid as a white powder: mp 215.0–216.1 °C; ^{1}H NMR δ 10.83 (s, 1), 7.38 (m, 10), 5.33 (s, 2), 5.25 (s, 2), 2.73 (s, 3); ^{13}C NMR δ 166.4, 164.9, 155.0, 145.4, 136.9, 136.7, 129.7, 129.4, 69.1, 67.8, 15.9; IR 1730, 1700, 1645 cm^{-1}. Anal. Calcd for $C_{22}H_{19}NO_6S\cdot(H_2O)_{0.25}$: C, 61.46; H, 4.51; N, 3.26; S, 7.46. Found: C, 61.46; H, 4.46; N, 2.92; S, 7.26.

Benzyl 2-[[(Phenylmethoxy)carbonyl]amino]-5-(hydroxymethyl)-4-methylthiophene-3-carboxylate (4). A solution of the above carboxylic acid (750 mg, 1.8 mmol) in CH_2Cl_2 (20 mL) and DMF (ca. 0.5 mL) was cooled to 0 °C, and thionyl chloride (1.8 mL) was added. After 30 min, the mixture had turned clear and was concentrated in vacuo. The solid was dissolved in dioxane (20 mL), the solution was cooled to 10 °C, and sodium borohydride (1.8 g) was added as a slurry in dioxane over 5 min. The mixture was warmed to room temperature for 15 min and cooled to 10 °C, and ice (10 mL) was added slowly to the stirring mixture. After 1 h, the reaction mixture was poured onto 5 cm of silica and eluted with 1:2/EtOAc–hexanes to yield a solid which was triturated with Et_2O to yield 500 mg (69%) of **4**: mp 133.0–133.5 °C; ^{1}H NMR δ 10.57 (s, 1), 7.39–7.31 (m, 10), 5.30 (s, 2), 5.22 (s, 2), 4.66 (s, 2), 2.30 (s, 3); ^{13}C NMR δ 165.8, 152.8, 151.1, 135.6, 135.3, 132.3, 128.6, 128.5, 128.4, 128.3, 128.1, 126.4, 111.8, 68.0, 66.4, 57.3, 14.7; IR 1730, 1670 cm^{-1}. Anal. Calcd for $C_{22}H_{21}NO_5S$: C, 64.22; H, 5.14; N, 3.40; S, 7.79. Found: C, 63.87; H, 4.93; N, 3.25; S, 7.42.

Benzyl 2-[[(Phenylmethoxy)carbonyl]amino]-5-[[(phenoxycarbonyl)amino]methyl]-4-methylthiophene-3-carboxylate (6). A solution of **4** (1.17 g, 2.8 mmol), triphenylphosphine (1.49 g, 5.7 mmol), and sodium azide (555 mg, 8.5 mmol) in DMF (6 mL) was cooled to −42 °C, and CCl_4 (0.90 mL) was added over 5 min. The reaction mixture was allowed to warm to room temperature slowly and stirred for 13 h. The DMF was removed in vacuo, and the residue was dissolved in EtOAc, filtered, and concentrated in vacuo to yield the benzylic azide. The azide was immediately dissolved in pyridine (6 mL), triphenylphosphine (1.49 g, 5.7 mmoL) was added, and the mixture was stirred for 19 h. The solution was concentrated in vacuo from benzene (3 × 6 mL) and heptane (3 × 6 mL) to yield the benzylic amine as a solid. A solution of the amine in CH_2Cl_2 (6 mL) was cooled to 0 °C. Triethylamine (0.99 mL, 7.1 mmol) and phenyl chloroformate (0.74 mL, 5.7 mmol) were added, and the solution was allowed to stir for 16 h. The reaction mixture was dissolved in EtOAc (400 mL), washed with 1 N HCl (2 × 100 mL) and brine (100 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was chromatographed (1:4 EtOAc–hexanes), and the solid obtained was triturated with Et_2O to yield 766 mg of **6** (51%): mp 142.0–142.5 °C; ^{1}H NMR δ 10.59 (s, 1), 7.38–7.09 (m, 15), 5.30 (s, 2), 5.23 (s, 2), 5.17 (b, 1), 4.45 (d, 2, J = 5.5), 2.32 (s, 3); ^{13}C NMR δ 165.6, 154.1, 152.8, 150.8, 150.7, 135.5, 135.2, 132.3, 129.2, 128.6, 128.5, 128.4, 128.3, 128.1, 125.2, 123.4, 121.5, 111.6, 68.0, 66.4, 37.4, 14.7; IR 1735, 1670 cm^{-1}. Anal. Calcd for $C_{29}H_{26}N_2O_6S$: C, 65.65; H, 4.94; N, 5.28; S, 6.04. Found: C, 65.34; H, 4.78; N, 5.06; S, 5.91.

Enzymology.[25] Assays for Competitive Inhibition.[26] An assay stock solution containing all of the nonenzymic components, except the substrate to be varied, was prepared which provided the following final concentrations when added to the assay mixture: 10 mM $MgCl_2$, 10 mM NH_4Cl, 200 μM NADH, 1 mM F6P, 1 mM ATP, and 1 mM creatine phosphate. A solution of auxiliary enzymes was prepared daily which provided the following amounts in the final assay mixture: 70 μg of aldolase, 3 μg of triosephosphate isomerase, 30 μg of glyceraldehyde 3-phosphate dehydrogenase, and 10 μg of creatine kinase. Assays were conducted by charging two quartz cuvettes with the appropriate amount of substrate solution, variable substrate, inhibitor solution or DMF, and auxiliary enzyme solution. The assay was initiated by adding PFK (20 ng) to the sample cuvette and a corresponding amount of buffer to the reference cuvette.

Absorbance vs time data were collected for 10 min, and a straight line was fitted to the linear portion of the data using ENZFITTER[27] (Uvikon 860) or Uvikon software (Uvikon 930). The apparent K_m was determined for F6P and ATP in the presence of 2 mM **2** and in its absence. The effect of PEP in the presence and absence of 1.4 mM **2** was also determined using saturating concentrations of ATP and F6P (1 mM).

Assay for Irreversible Inhibition. Three incubation mixtures were prepared: (1) PFK (20 μg/mL) and **2** (2 mM) in buffer (100 mM Tris, pH 8.2); (2) PFK (20 μg/mL), **2** (2 mM), and dithiothreitol (4.4 mM) in buffer; and (3) PFK (20 μg/mL) and

(25) **General.** All enzymes and biochemicals were obtained from Boehringer Mannheim and all solutions were prepared in 0.1 M Tris, pH 8.2 buffer unless otherwise specified. A stock solution (1 mg/mL) of fructose 6-phosphate kinase (Sigma Chemical Co., lyophilized powder, type VII) was prepared in 0.5 M NaCl, 0.1 M Tris, pH 8.2; this solution was stored at −20 °C and was stable indefinitely. A dilute stock (10 μg/mL) was prepared in Tris and was stable for about 1 week. Aldolase, triosephosphate isomerase, and glyceraldehyde 3-phosphate dehydrogenase were obtained as ammonium sulfate suspensions. These suspensions were successively ultrafiltered (AMICON centricon-10 filter, M_r cutoff 10000) three times with equal volumes of Tris and diluted to final concentrations of 10, 2, and 20 mg/mL, respectively. These stock solutions were stable for about 2 weeks. A stock solution (1 mg/mL) of creatine kinase (lyophilized powder) was prepared daily in Tris. A stock solution (130 mM) of **2** was prepared in DMF, and further dilutions were also made with DMF so that addition to the assay mixture would provide a 5% DMF solution at the desired concentration of thiophene. The rate of fructose 1,6-diphosphate production was monitored spectrophotometrically with Kontron Uvikon Models 860 or 930, using a coupled assay to follow the rate of NADH oxidation at 340 nm. All incubations and assays were conducted at 31 °C.

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DMF (5%) in buffer. Each mixture was combined in a polyethylene tube and incubated at 25 °C. The rates of inactivation were followed by removing 20-μL aliquots from the incubation mixtures at various times, diluting into excess substrate and auxiliary enzymes (final volume 1 mL), and determining the remaining enzyme activity. In addition, incubation mixture 1 was diluted 10-fold and assayed for remaining activity in an identical manner.

Assay for Inhibition by Decomposition Product of 2. The nonenzymic solution and **2** were mixed as described above for the competitive inhibition experiments. At various times, two 800-μL aliquots were removed, auxiliary enzymes (as for the competitive inhibition experiments) were added to each, and the assay was initiated by addition of PFK (20 ng) to the sample cuvette and buffer to the reference cuvette. In a separate experiment, auxiliary enzyme and dithiothreitol (final concentration 2 mM) were added to both the sample and the reference cuvette before initiation of the assay.

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Registry No. **2**, 136667-88-4; **3**, 136667-89-5; **4**, 136667-90-8; **6**, 136667-91-9; phosphofructokinase, 9001-80-3; 2-(trimethylsilyl)ethyl acetoacetate, 17165-45-6; 3-benzyl 5-[2-(trimethylsilyl)ethyl] 2-[[(phenylmethoxy)carbonyl]amino]-4-methylthiophene-3,5-dicarboxylate, 136667-92-0; 3-benzyl 2-[[(phenylmethoxy)carbonyl]amino]-4-methylthiophene-3,5-dicarboxylate, 136667-93-1; benzyl cyanoacetate, 14447-18-8.

Synthesis of Photopolymerizable Long-Chain Conjugated Diacetylenic Acids and Alcohols from Butadiyne Synthons

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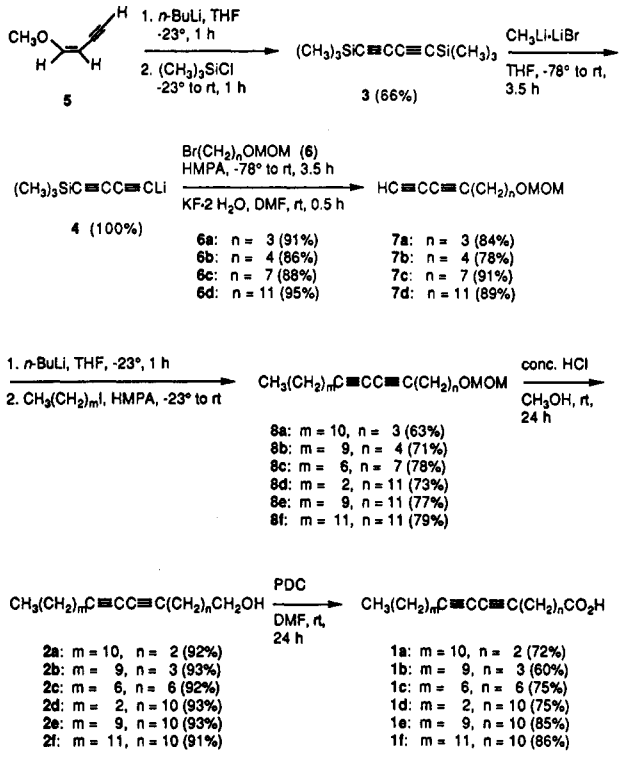
Conjugated diacetylenic chains have been incorporated into various polymerizable phospholipids for use in model membranes; ultraviolet irradiation induces cross-linking, resulting in a phospholipid polymer in the membrane bilayer.[1] In general, unsymmetrical diynoic acids **1** have been prepared by coupling of a 1-haloalkyne (usually the iodoalkyne) with a metal alkynoic acid (the Cadiot–Chodkiewicz reaction, eq 1).[2] Diynoic acids have also been

$$CH_3(CH_2)_mC{\equiv}CI + HC{\equiv}C(CH_2)_nCO_2H \xrightarrow[EtNH_2]{HONH_3^+Cl^-,\ Cu_2Cl_2} \underset{\mathbf{1}}{CH_3(CH_2)_mC{\equiv}CC{\equiv}C(CH_2)_nCO_2H} \quad (1)$$

prepared by the Cu_2Cl_2-catalyzed oxidative coupling of

Scheme I. Syntheses of Diacetylenic Alcohols 2a–f and Diacetylenic Acids 1a–f



ω-alkynoic acids with terminal alkynes.[3]

This acetylenic-coupling reaction has also been used to prepare long-chain conjugated (enyne[4] and diyne[5]) alcohols **2**. Disadvantages of this method of preparation of **1** and **2** are as follows: (a) higher ω-alkynoic acids are obtained in low yields (generally <50%),[2,3] (b) preparation of 1-haloalkynes is required in the Cadiot–Chodkiewicz reaction, and (c) unsymmetrical ω-diynoic acids are frequently contaminated by symmetrical diynoic acids.[3] The report by Zweifel and Rajagopalan[6] that the nucleophilic butadiyne synthons 1,4-bis(trimethylsilyl)-1,3-butadiyne (**3**) and 4-lithio-1-(trimethylsilyl)butadiyne (**4**) can be prepared from (*Z*)-1-methoxybut-1-en-3-yne[7] (**5**) via a series of metalation–elimination–metalation reactions suggested to us that **5** would be useful for the preparation of diacetylenic acids and alcohols **1** and **2**.

Membranes stabilized by photopolymerization have a wide range of potential applications.[8] Because of the current interest in the behavior of poly(diacetylene) chains in long-chain phospholipids as stabilizing units in membranes,[1] we have focused our attention on the efficient preparation of long-chain conjugated diacetylenic alcohols and acids. The position of the diacetylene along the hydrocarbon chain has been varied, making possible a future study of the structural requirements for efficient polymerization of lipid diacetylenes in multilayer films.

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