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Synthesis and Properties of Some Novel Anti-calmodulin Drugs

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Abstract—The preparation and properties of some novel inhibitors of calmodulin function are described. The compounds are cationic derivatives of phenyl-substituted thiazoles which inhibit the calmodulin stimulation of cyclic-AMP phosphodiesterase and are active against animal tumor cells in culture. These derivatives form the basis for the preparation of new, more potent inhibitors of calmodulin function which could take advantage of the reported elevated levels of calcium-bound calmodulin in tumor cells and show preferential anti-tumor activity.

Introduction

Calmodulin (CaM) is the main protein involved in calcium buffering and in the Ca^{2+}-dependent regulation of specific cell functions. It uses changes in intracellular calcium levels to modulate a variety of biological and biochemical processes, affecting no fewer than two dozen enzymes.[1–3] Higher eukaryotes have several CaM genes which are differentially regulated while encoding identical proteins. Because of the importance of Ca^{2+} in progression through the cell cycle, CaM plays a critical role in the regulation of cell proliferation.[4–6] It has been shown that disease states characterized by unregulated growth, such as cancer, are correlated with elevated levels of Ca^{2+}-bound CaM.[8–11] Thus, CaM is a promising target for the development of drugs that show preferential activity in tumors and, as a result, exhibit fewer toxic side effects in normal cells.

CaM, like other Ca^{2+}-binding proteins, contains the helix-coil-helix Ca^{2+}-binding motif, the so-called EF hand. The structure of Ca^{2+}-CaM is an elongated dumbbell in which two structurally similar domains are connected by a helical linker region,[12] with each domain containing two EF hands (i.e. 4 Ca^{2+} sites per protein molecule). LaPorte et al.[13] found that the binding of calcium to calmodulin exposes a hydrophobic surface which can bind different aromatic molecules, including the anti-psychotic drug trifluoperazine (TFP) and 8-anilino-1-naphthalenesulfonate (ANS), a fluorescence probe normally used to study protein structure. They found that cationic derivatives such as TFP, when bound to Ca^{2+}–CaM, inhibited the calmodulin activation of cyclic-AMP phosphodiesterase, whereas the anionic ANS could bind to Ca^{2+}–CaM but did not affect phosphodiesterase activity. Subsequently, various aromatic molecules have been found to act as anti-calmodulin compounds, including most of the other phenothiazine drugs,[14] the anti-estrogen agent tamoxifen,[15,16] and some synthetic naphthalenesulfonamides of α,ω-diaminoalkanes.[17]

Whereas these compounds have been shown to inhibit calmodulin function, their anti-calmodulin effects may be secondary to other properties, for example, as anti-psychotic drugs (in the case of the phenothiazines) or anti-estrogens (in the case of tamoxifen and related compounds). Little has been done to assess detailed structure–activity relationships in these anti-calmodulin drugs, with the phenothiazine drugs[14] and the relatively simple naphthalenesulfonamides[17] probably the best-characterized. Because the structures of compounds in each of these groups are similar, comparisons have been limited primarily to the role of substituent effects rather than of structural or conformational variations per se. The minimum requirements for an anti-CaM compound appear to be an aromatic group and a cationic group. The aromatic portion interacts with the hydrophobic surfaces of CaM that are exposed upon the binding of

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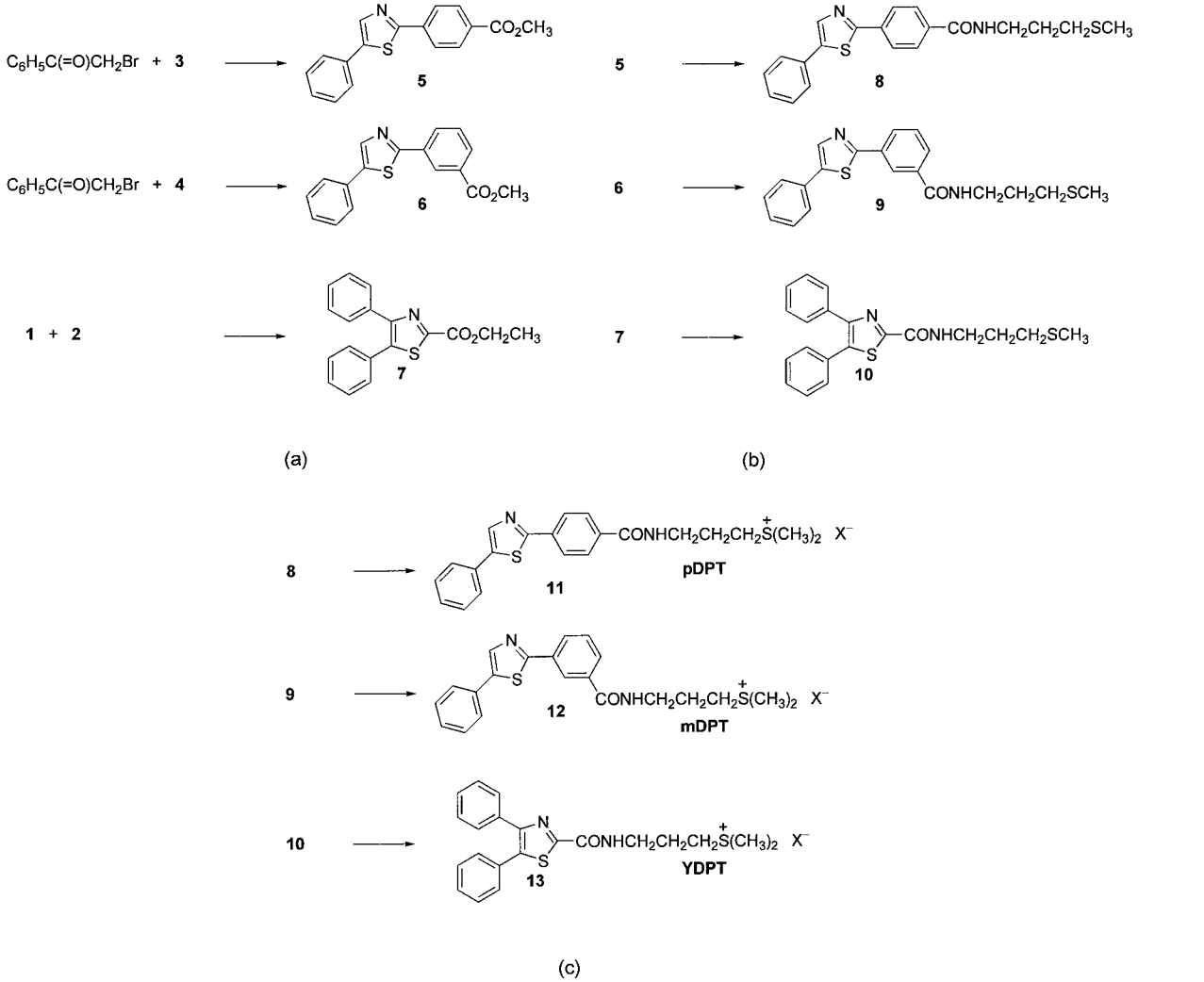
Ca^{2+} to the protein, and the cationic group presumably interacts with anionic side chains of CaM residues. It is possible that the charged group merely aids in solubilizing the compound; however, the observation that ANS, an anionic aromatic compound, binds to CaM without inhibiting the CaM stimulation of cyclic-AMP phosphodiesterase activity[13] indicates that association with CaM alone is not enough to cause an anti-CaM effect and that the nature of the ionic group is important.

As part of an on-going NMR-based drug design study, we have prepared several lead compounds as possible inhibitors of calmodulin activity and are studying the three-dimensional structures of their complexes with calmodulin. We have monitored the effects of these compounds on the stimulation by CaM of cyclic-AMP phosphodiesterase activity to measure their anti-CaM effects, as well as their effects on tumor cells in culture. This report presents an evaluation of some of the properties of these inhibitors. The detailed information obtained from these studies will aid in the design and synthesis of modified derivatives with enhanced activity against calmodulin function and tumor cell viability.

The syntheses of the candidate inhibitors is fairly straightforward and uses literature techniques[18] as well as methods developed in this laboratory.[19,20] These compounds, in fact, constitute an extension and expansion into protein inhibitors using techniques initially used in the preparation of nucleic acid-binding ligands.[19,20] The substituted thiazoles are prepared by the Hantzsch method in which the appropriate thioamide and α-haloketone are condensed[18] (Scheme 1(a)). The cationic side chain is introduced by aminolysis of the desired ester with 3-(methylthio)propylamine (Scheme 1(b), followed by methylation with iodomethane (Scheme 1(c)). For ease of identification, compound **11** is called pDPT (*para*-diphenylthiazole), compound **12** is called mDPT (*meta*-diphenylthiazole) and compound **13** is called YDPT ('Y-shaped' diphenylthiazole).

Results and Discussion

We have initially used substituted thiazoles as the basis of our inhibitors for several reasons: (1) The 'flexible' aromatic system provided by the ability of pendant



Scheme 1. (a) Reactions to form thiazole ring systems, (b) aminolysis reactions forming methylthio ethers, and (c) methylation reactions forming sulfonium derivatives.

phenyl rings to rotate relative to the thiazole ring allows the inhibitor to more readily accommodate variations in the hydrophobic surface of CaM. This should allow greater specificity and affinity in derivatives which take advantage of this flexibility, particularly when compared to derivatives which have rigid, fused ring systems (e.g. phenothiazine, naphthalene). (2) The unambiguous positioning of substituents about the thiazole ring inherent in the chemistry of thiazole ring formation makes design of derivatives easier. (3) The synthesis of such ring systems is usually straightforward and a considerable number of possible aromatic substituents are possible. (4) In addition, we are hopeful that the natural occurrence of thiazoles in biomolecules may allow for more ready disposition of metabolic by-products of the inhibitors, hopefully minimizing toxicities which might arise from such metabolites. Similarly, the dimethylsulfonium side chain should be metabolized into non-toxic products.

The sulfonium side chain was used initially in previous work done on DNA binding ligands, and the derivatives described herein continue its use, in part for the reason indicated above. Moreover, our feeling at this point is that while a cationic group is necessary for the anti-CaM properties of a ligand, the nature of the cationic group may not be as critical as how it is disposed in the complex. However, refinements in ligand structure may lead to situations in which specific features of the charged group might be altered minutely to provide enhanced binding of the ligand to CaM.

Anti-CaM effects

The three diphenylthiazole (DPT) derivatives inhibit the calmodulin activation of cyclic-AMP phosphodiesterase (PDE) to varying degrees (Fig. 1): at 0.1 mM, pDPT and mDPT inhibit the conversion of cyclic-AMP to AMP by about 70–75%; under the same conditions, the known CaM antagonist trifluoperazine (TFP) shows about 95% inhibition. The 'Y-shaped' DPT (YDPT) is noticeably less effective than pDPT and mDPT in inhibiting the phosphodiesterase activity. Under the assay conditions no conversion of cyclic-AMP to AMP was observed for the ligands alone (no PDE or CaM), for ligand plus and CaM (no PDE), or for ligand plus PDE (no CaM), showing the inhibition is caused by the ligand–CaM combination.

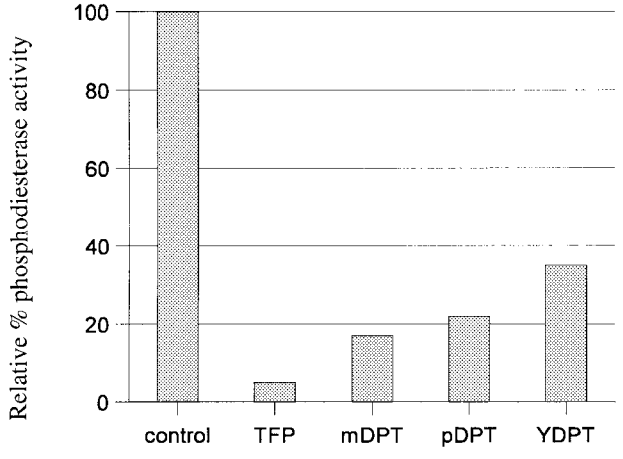


Figure 1. Effects of pDPT, mDPT and YDPT on the calmodulin-stimulation of cyclic-AMP phosphodiesterase. Control (no additions) is taken as 100%. The effect of trifluoperazine (TFP) is shown for comparison.

Stoichiometry and binding constants

The association of mDPT with CaM was monitored by the changes in the absorption spectrum of the ligand as the CaM-to-ligand ratio was changed gradually at a fixed ligand concentration (0.05 mM). The spectral intensity changes at 330 nm showed a simple saturation curve (Fig. 2), and Scatchard analysis of the data indicated a 1:1 stoichiometry with an association constant of 1.15×10^5 M (Fig. 3). The spectrophotometric behavior of pDPT is more complex: there is an initial decrease in ligand absorbance (monitored at 336 nm) (for CaM-to-ligand ratios less than about 1); subsequently, there is a gradual increase which nears saturation but has not saturated at ratios of about 4 or 5. This is suggestive of a degree of non-specific binding with CaM. Interestingly, this difference appears to be the result of a simple change in the location of the cationic side chain relative to the initial phenyl ring. The spectral changes observed upon binding of the YDPT to CaM are similar to those observed with pDPT in that there is an initial decrease in ligand absorbance (for ligand-to-CaM ratios <1), however, this is followed by a slow *linear* increase in absorbance, with no indication of saturation at a CaM-to-ligand ratio of 5 or 6. Again, this different behavior of YDPT compared to mDPT arises from a relocation of the cationic side chain relative to the orientation of the aromatic system. We have not analyzed the binding of either pDPT or YDPT any further at this time.

The relative efficacies of our derivatives on CaM stimulation of phosphodiesterase activity compared to TFP presumably reflect the relative affinities for CaM ($K_a\sim1.15\times10^5\,M^{-1}$ for mDPT versus $\sim10^6\,M^{-1}$ for TFP[21]). These data indicate that relatively small changes in the structure of the ligand molecule can have relatively large effects on its properties, changing the binding properties from fairly specific (in the case of mDPT) to less specific (pDPT) to relatively nonspecific (YDPT).

Cytotoxicity of inhibitors

Preliminary measurements of the effects of these compounds on cell viability were performed on L1210 murine leukemia cells growing in spinner culture. All compounds showed modest cytotoxicity as measured by dye exclusion, with about 25% inhibition of viable cell number occurring at about 5 mM for mDPT and 5–10 mM for pDPT and YDPT. More detailed evaluations of cytotoxic effects are being conducted.

While most of the calmodulin antagonists (particularly the phenothiazines) exhibit cytotoxicity against a

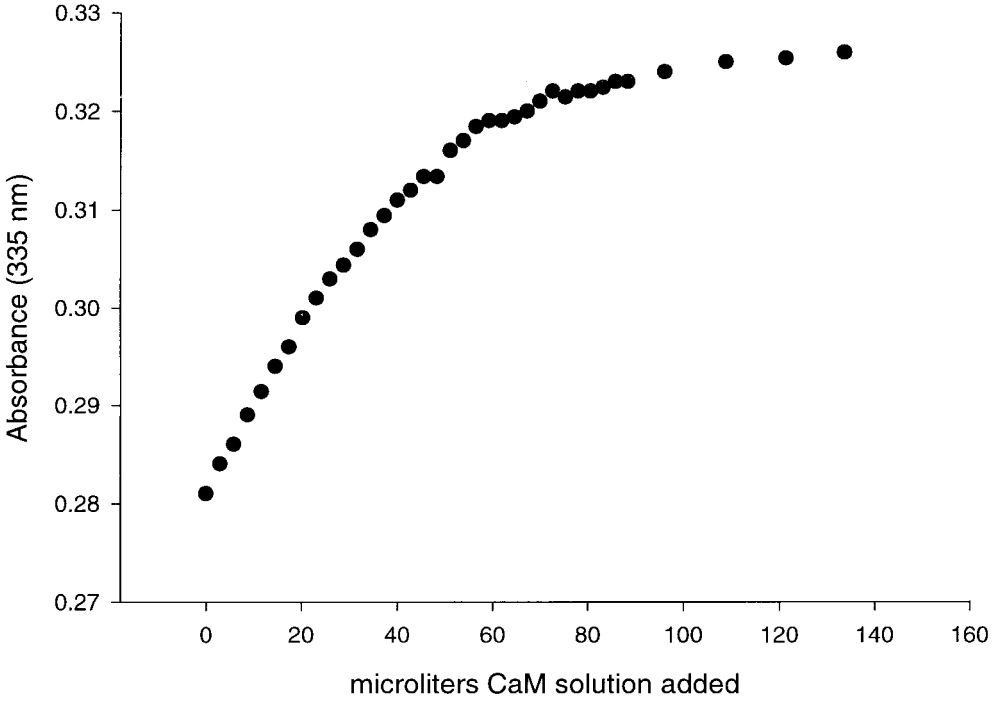


Figure 2. Changes in absorbance at 335 nm of mDPT (0.05 mM) upon addition of aliquots of a solution of calmodulin (5 mM) containing 0.05 mM mDPT.

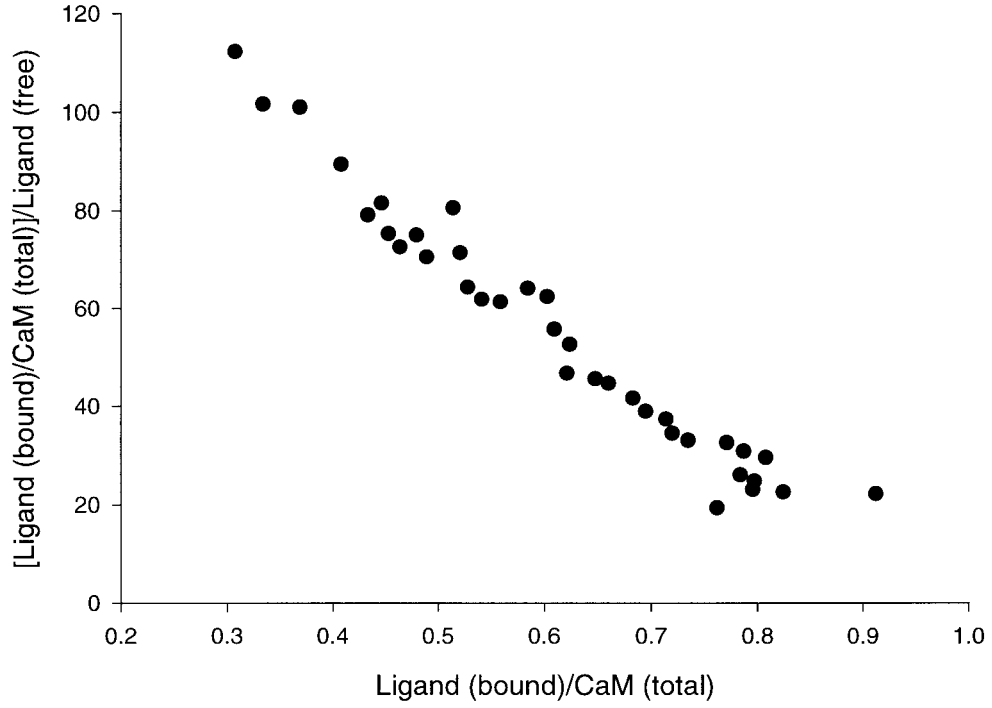


Figure 3. Scatchard analysis of data in Figure 2.

variety of cells, the overall similarity of structures and properties makes meaningful evaluation of structure–activity relationships very difficult. We have begun to develop some new inhibitors of calmodulin function which will provide the basis for a more detailed study of structure–activity relationships in calmodulin antagonists. The data obtained in this study indicate that relatively small changes in the placement of substitutents in this group of compounds have noticeable effects on their binding to CaM as well as on their anti-CaM effects. Lesser perturbations are noticeable on the toxicities. We are hopeful that these differences will be

evident in the three-dimensional structures of complexes with Ca^{2+}–CaM and, thus, provide a starting point for subsequent alterations in the structure of the inhibitor.

Experimental

All materials were obtained from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO) or Fisher Scientific (Norcross, GA) unless otherwise noted. Melting points were determined on a Laboratory Devices Mel-Temp apparatus. ^{1}H NMR measurements were made on a Bruker WH-400 spectrometer with chemical shifts referenced to internal tetramethylsilane (organic solvents) or internal sodium 4, 4-dimethyl-2,2,3,3-tetradeuterio-4-silapentanoate (aqueous solvents). Thin-layer chromatography (TLC) was run on silica gel GF_{254} plates. All analytical samples were homogenous upon TLC with ethyl acetate, ethyl acetate:petroleum ether (bp 35–60°C) (4:1, v/v) or chloroform:methanol (3:1, v/v) as solvent systems. Combustion analyses were performed by Galbraith Laboratories (Knoxville, TN). All compounds gave combustion analyses and/or NMR spectra consistent with the indicated structures. Recombinant *Drosophila melanogaster* calmodulin was expressed in *Escherchia coli* cells received from Dr. Kate Beckingham (Rice University) and purified using procedures obtained from her.

Ethyl thiocarbamoylmethanoate (1). A solution of ethyl cyanomethanoate (31.4 g, 0.32 mol) (Aldrich) in 50 mL of benzene was cooled to −10°C in a stainless steel reaction vessel and saturated with H_2S. Diethylamine (0.5 mL) was added and the addition of H_2S continued at a rate to maintain the temperature of the solution between 25 and 30°C. After approximately 4 h, the solution was cooled to 5°C and again saturated with H_2S. The vessel was sealed and allowed to stand for 18 h at room temperature. The crystals which formed were collected and washed with carbon tetrachloride; yield 24.5 g (58%), mp 60–63.5°C.

α-Bromodeoxybenzoin (α-Bromobenzylphenyl ketone) (2). Deoxybenzoin (19.6 g, 0.1 mol) (Aldrich) was brominated with bromine in acetic acid as described for 4-bromoacetophenone[22] in 95% yield, mp 55–57°C. This material was used without further purification.

Methyl 4-thiocarbamoylbenzoate (3). Methyl 4-cyanobenzoate (5.0 g, 31.0 mmol) (Aldrich) was dissolved in 25 mL of anhydrous *N,N*-dimethylformamide and the resulting solution was saturated with H_2S. The solution was brought to 60–65°C, 0.1 mL of diethylamine was added and heating was continued for 1 h. The solution was poured into a mixture of 100 g ice and 100 mL water with vigorous stirring. The resulting solid was collected and recrystallized from ethanol, total yield 5.6 g (93%), mp 189–191°C. NMR ($CDCl_3$) δ 8.06 (d, 2, phenyl $C_{2,6}$ H′s), 7.90 (d, 2, phenyl $C_{3,5}$H′s), 7.69 (broad s, 1, NH?), 7.21 (broad s, 1, NH?), 3.95 (s, 3, OCH_3). Anal. calcd for $C_9H_9NO_2S$ (195.24): C, 55.37; H, 4.65; N, 7.17. Found: C, 55.43; H, 4.69; N, 7.14.

Methyl 3-thiocarbamoylbenzoate (4). Dimethyl isophthalate (Aldrich) was converted to methyl 3-cyanobenzoate in 4 steps: (i) hydrolysis to methyl hydrogen isophthalate with aqueous hydrochloric acid; (ii) conversion to the acid chloride with thionyl chloride; (iii) amidation to methyl isophthalamate with concentrated ammonium hydroxide; and (iv) dehydration of the amide with phosphorus pentoxide. The nitrile (9.21 g, 57.1 mmol) was dissolved in 35 mL of anhydrous *N,N*-dimethylformamide and the solution was heated to 65–70°C; H_2S was slowly bubbled through the solution and 1.0 mL of diethylamine was added. After 1 h, the reaction mixture was poured into 250 mL of water and the precipitate which formed was collected and dried in vacuo, yield 9.8 g (88%), mp 133–135°C. A small portion was recrystallized from ethanol to give an analytical sample, mp 137–138.5°C. NMR ($CDCl_3$) δ 8.44 (s, 1, phenyl C_2H), 8.18 (d, 2, phenyl C_4H, C_6H), 7.52 (t, 1, phenyl C_5H), 6.46 (s, 2, NH_2), 3.95 (s, 3, OCH_3) ppm. Anal. calcd for $C_9H_9NO_2S$ (195.24): C, 55.37; H, 4.65; N, 7.17. Found: C, 55.44; H, 4.67; N, 7.15.

Methyl 4-[(4-phenyl)thiazol-2-yl]benzoate (5). A solution of **3** (0.83 g, 4.2 mmol) and 2-bromoacetophenone (0.83 g, 4.1 mmol) in 2 mL of anhydrous *N,N*-dimethylformamide was heated at 60–65°C for 2 h. The crystals that formed upon cooling the reaction solution were collected by filtration, washed with water and dried in vacuo; yield 1.04 g, mp 156–158°C. Concentration of the filtrate provided an additional 0.20 g, mp 154–156°C; total yield 97%. A portion was crystallized from ethanol to give an analytical sample, mp 156.5–157°C. NMR ($CDCl_3$) δ 8.13 (s, 4, phenyl C_2H, C_3H, C_5H, C_6H), 8.00 (d, 2, phenyl $C_{2',6'}$H′′s), 7.56 (s, 1, thiazole C_5H), 7.46 (t, 2, phenyl $C_{3',5'}$H′s), 7.37 (d, 1, phenyl $C_{4'}H$) ppm. Anal. calcd for $C_{17}H_{13}NO_2S$ (295.36): C, 69.13; H, 4.44; N, 4.74. Found: C, 69.06; H, 4.46; N, 4.73.

Methyl 3-[(4-phenyl)thiazol-2-yl]benzoate (6). A solution of **4** (0.95 g, 4.9 mmol) in 1 mL of anhydrous *N,N*-dimethylformamide was added to a solution of 2-bromoacetophenone (0.95 g, 4.8 mmol) in 1 mL of the same solvent. The solution was heated at 60–65°C for 2 h and then cooled to room temperature. The solution was poured into 25 mL of water and the resulting solid was filtered, washed with water and dried in vacuo, yield 1.17 g (82%), mp 80–81°C. NMR ($CDCl_3$) δ 8.66 (s, 1, phenyl C_2H), 8.27 (d, 1, phenyl C_6H), 8.11 (d, 1, phenyl C_4H), 8.01 (d, 2, phenyl $C_{2',6'}$H′s), 7.56 (t, 1, phenyl C_5H), 7.52 (s, 1, thiazole C_5H), 7.46 (t, 2, phenyl $C_{3',5'}$ H′s), 7.37 (d, 2, phenyl $C_{4'}H$), 3.98 (s, 3, OCH_3) ppm. Anal. calcd for $C_{17}H_{13}NO_2S$ (295.36): C, 69.13; H, 4.44, N, 4.74. Found: C, 69.05; H, 4.45; N, 4.75.

Ethyl 4,5-diphenylthiazole-2-carboxylate (7) was prepared from **1** and **2** as described for **6** except that the product was extracted into dichloromethane. Removal of the solvent provided the product as an oil, yield 40%; recrystallization from ethanol gives a low melting solid (mp <10°C) which was used without further purification. NMR ($CDCl_3$) δ 7.51–7.53 (m, 4, 4- and 5-phenyl $C_{2,6}$H′s), 7.33–7.37 (m, 2, 4- and 5-phenyl

C_4H's), 7.27–7.30 (m, 4, 4- and 5-phenyl $C_{3,5}H'$s), 4.51 (q, 2, OCH_2), 1.45 (t, 3, CH_3) ppm.

General procedure for the preparation of 3-(methylthio)-propylamides. The appropriate ester (usually 2–5 mmol) is dissolved in 2-5 mL of 3-(methylthio)propylamine (Eastman) and the solution heated at 70–75°C for 2–3 h. The solution is allowed to cool to room temperature and then diluted to 50 mL with dichloromethane. The organic solution is extracted with N HCl (3×50 mL) and water (3×50 mL) and dried with anhydrous magnesium sulfate. Evaporation of the solvent in vacuo provides the crude product.

Methyl 3-{[4-(4-phenyl)thiazol-2-yl]benzamido}propyl sulfide (8). Yield 98%; crystallized from ethanol, mp 143–144°C. NMR ($CDCl_3$) 8.12 (d, 2, phenyl $C_{2,6}H'$s), 8.00 (d, 2, phenyl $C_{2',6'}H'$s), 7.87 (d, 2, phenyl $C_{3,5}H'$s), 7.54 (s, 1, thiazole C_5H), 7.46 (t, 2, phenyl $C_{3',5'}H'$s), 7.37 (d, 1, phenyl $C_{4'}H$), 6.56 (broad t, 1, NH), 3.62 (q, 2, NCH_2), 2.65 (t, 2, SCH_2), 2.14 (s, 3, SCH_3), 1.99 (p, 2, internal CH_2) ppm. Anal. calcd for $C_{20}H_{20}N_2OS_2$ (368.52): C, 65.18; H, 5.47; N, 7.60. Found: C, 65.00; H, 5.49; N, 7.52.

Methyl 3-{[3-(4-phenyl)thiazol-2-yl]benzamido}propyl sulfide (9). Yield 78%; recrystallized from ethanol, mp 125–126°C. NMR ($CDCl_3$) 8.50 (s, 1, phenyl C_2H), 8.16 (d, 1, phenyl C_6H), 8.00 (d, 2, phenyl $C_{2',6'}H'$s), 7.87 (d, 1, phenyl C_4H), 7.55 (t, 1, phenyl C_5H), 7.52 (s, 1, thiazole C_5H), 7.46 (t, 2, phenyl $C_{3',5'}H'$s), 7.37 (d, 1, phenyl $C_{4'}H$), 6.67 (broad t, 1, NH), 3.70 (q, 2, NCH_2), 2.65 (t, 2, SCH_2), 2.15 (s, 3, SCH_3), 1.99 (p, 2, internal CH_2) ppm. Anal. calcd for $C_{20}H_{20}N_2OS_2$ (368.52): C, 65.18; H, 5.47; N, 7.60. Found: C, 65.23; H, 5.48; N, 7.56.

Methyl 3-(4,5-diphenylthiazole-2-carboxamido)propyl sulfide (10). Yield 82%, mp 118–119°C after recrystallization from ethanol. NMR ($CDCl_3$) 7.48–7.51 (m, 4, 4- and 5-phenyl $C_{2,6}H'$s), 7.33–7.36 (m, 2, 4-and 5-phenyl C_4H's), 7.30–7.32 (m, 4, 4- and 5-phenyl $C_{3,5}H'$s), 3.60 (q, 2, NCH_2), 2.61 (t, 2, SCH_2), 2.13 (s, 3, SCH_3), 1.96 (p, internal CH_2) ppm.

General procedure for the preparation of dimethylsulfonium derivatives. The appropriate methyl sulfide (1–2 mmol) is dissolved in 5 mL of methanol:iodomethane (1:1 by volume). The solution is allowed to stand in the dark for 24 h at room temperature. Removal of the solvent in a stream of dry nitrogen affords the crude product as the iodide salt. Generally, the iodide derivatives proved intractable due to discoloration or decomposition and they were converted to the chloride derivatives by passage of an aqueous solution through a column (1×8 cm) of Dowex 1×8 (chloride form). Lyophilization of the eluate provides the chloride derivative.

Dimethyl 3-{4-[(4-phenyl)thiazol-2-yl]benzamido}propylsulfonium chloride (11). Yield 86%, amorphous. NMR (D_2O) 8.00 (d, 2, phenyl $C_{2,6}H'$s), 7.90 (d, 2, phenyl $C_{2',6'}H'$s), 7.84 (s, 1, thiazole C_5H), 7.80 (d, 2, phenyl $C_{3,5}H'$s), 7.53 (t, 2, phenyl $C_{3',5'}H'$s), 7.46 (d, 1, phenyl $C_{4'}H$), 3.54 (t, 2, NCH_2), 3.38 (t, 2, SCH_2), 2.94 (s, 6, $S(CH_3)_2$), 2.14 (p, 2, internal CH_2) ppm. Anal. calcd for $C_{21}H_{23}ClN_2OS_2 \cdot 0.75\ H_2O$ (432.52): C, 58.32; H, 5.71; N, 6.48. Found: C, 58.28; H, 5.72; N, 6.42.

Dimethyl 3-{3-[(4-phenyl)thiazol-2-yl]benzamido}propylsulfonium chloride (12). Yield 72%, amorphous. NMR (D_2O) 8.29 (s, 1, phenyl C_2H), 8.16 (d, 1, phenyl C_6H), 7.95 (d, 2, phenyl $C_{2',6'}H'$s), 7.88 (d, 1, phenyl C_4H), 7.85 (s, 1, thiazole C_5H), 7.66 (t, 1, phenyl C_5H), 7.56 (t, 2, phenyl $C_{3',5'}H'$s), 7.49 (d, 1, phenyl $C_{4'}H$), 3.60 (t, 2, NCH_2), 3.41 (t, 2, SCH_2), 2.95 (s, 6, $S(CH_3)_2$), 2.19 (p, 2, internal CH_2) ppm. Anal. calcd for $C_{21}H_{23}ClN_2OS_2 \cdot H_2O$ (437.02): C, 57.72; H, 5.77; N, 6.41. Found: C, 57.44, H, 5.85; N, 6.37.

Dimethyl 3-(4,5-diphenylthiazole-2-carboxamido)propylsulfonium chloride (13). Yield 72%, amorphous hydrate. NMR (D_2O) 7.49–7.52 (m, 4, 4- and 5-phenyl $C_{2,6}H'$s), 7.39–7.43 (m, 6, 4- and 5-phenyl$C_{3,4,5}H'$s), 3.63 (t, 2, NCH_2), 3.41 (t, 2, SCH_2), 2.92 (s, 6, $S(CH_3)_2$), 2.19 (p, 2, internal CH_2) ppm.

Inhibition of CaM-dependent cyclic-AMP phosphodiesterase activity. The conversion of cyclic-AMP to AMP by calmodulin-activated bovine brain cyclic-AMP phosphodiesterase was monitored by TLC of reaction aliquots on silica gel GF_{254} and visualizing the chromatogram under low wavelength UV light. Typically, reaction mixtures of 100 μL contained 0.002–0.005 unit of phosphodiesterase, 0.3 nmol Ca^{2+}–CaM, 5 nmol $CaCl_2$, 10 nmol of cyclic-AMP, and 100 nmol of ligand (where used), in a buffer of 0.01 M HEPES (pH 7.5). After incubation for various times (0.5 to 1.0 h), the reaction mixtures were treated with an equal volume of ice-cold *iso*propanol and centrifuged. The supernatant is taken to dryness on a Speed-Vac and the residue was dissolved in 10 μL of water followed by 10 μL ethanol. Aliquots of this mixture were applied to silica gel GF_{254} plates and the applied material completely dried under a stream of nitrogen gas. Using the solvent system acetonitrile:*iso*propanol:0.1 M ammonium acetate (pH 7.4): concentrated ammonia: 6:1:2:1, there is a clear separation of cyclic-AMP ($R_f = 0.55$) and AMP ($R_f = 0.05$–0.10).[23] Quantitation is carried out by scraping the spots, eluting with 50% aqueous ethanol and measuring the absorbancies using a molar extinction coefficient of $12\times10^3\ M^{-1}\ cm^{-1}$ [24] at 262 nm.

Cell culture. Murine leukemia cells (L1210) were maintained at 37°C in spinner flasks in Fischer's medium containing 10% newborn bovine serum and equilibrated with a hydrated atmosphere containing 5% CO_2. Cultures were exposed to various concentrations of the compounds being tested and cell viability was monitored by the ability of cells to exclude Trypan Blue. Cells were counted using a hemacytometer.

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