Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 2937–2944

BIOORGANIC & MEDICINAL CHEMISTRY

2-Carboxymethylendothal Analogues as Affinity Probes for Stabilized Protein Phosphatase 2A

Charles W. Laidley,[a,†] William G. Dauben,[b,‡] Zhen R. Guo,[b,§] Joe Y. L. Lam[b,¶] and John E. Casida[a,*]

[a]*Environmental Chemistry and Toxicology Laboratory, Department of Environmental Science, Policy and Management, 115 Wellman Hall, University of California, Berkeley, CA 94720-3112, USA*

[b]*Department of Chemistry, University of California, 115 Wellman Hall, Berkeley, CA 94720-1460, USA*

Received 18 May 1999; accepted 26 July 1999

Abstract—Endothal ($\mathbf{1}_{diacid}$) and [^{3}H]cantharidic acid ([^{3}H]CA) bind with high affinity to the catalytic subunit of protein phosphatase 2A (PP2A). PP2A in liver cytosol was greatly stabilized with 30% glycerol as a preliminary step in the potential use of endothal-type derivatives for affinity chromatography. We report here the first introduction of a functionalizable group into endothal which allows retention of binding site affinity (assayed as [^{3}H]CA binding in mouse liver cytosol). 2-Carboxymethylendothal anhydride (**7**) was prepared in two steps and 97% overall yield from *cis*-aconitic anhydride and furan. The potency of **7** was retained on conversion to two 2-carboxymethyl esters but not to two 2-(*n*-alkylcarboxamidomethyl) analogues.

Introduction

The dynamic process of protein phosphorylation and dephosphorylation by protein kinases and protein phosphatases plays a major role in intracellular signal transduction.[1] Protein phosphatase 2A (PP2A) dephosphorylates serine and threonine residues in a broad range of cellular phosphoproteins and is the intracellular target for many drugs, toxins and tumor promoters.[2–4] Natural PP2A inhibitors include the exceptionally potent and structurally complex microcystin-LR[5,6] and okadaic acid[7,8] and the less active but much simpler palasonin (**2**) and cantharidin (**3**) (Fig. 1).[9-13] Microcystin[14] and a photoaffinity ligand based on okadaic acid[15] bind covalently making them valuable in characterization of the ligand binding site. Microcystin–Sepharose affinity chromatography for PP2A[16] is controversial[17] and okadaic acid-Sepharose is not useful[16] in recovering significant PP2A activity. An attractive alternative for affinity chromatography is to use compounds based on the herbicide endothal ($\mathbf{1}_{diacid}$) and its analogues **2** and **3** (Fig. 1) which display non-covalent binding characteristics.[18–25]

This study used the cantharidic acid radioligand ([^{3}H]CA) (Fig. 1) binding assay to optimize the interaction of new endothal analogues with PP2A. This procedure was previously fully validated as being directly related to its catalytic site and enzymatic activity.[11] The first goal of this study, as a prelude to affinity chromatography, was to determine the association rate and saturation conditions of [^{3}H]CA. Second, the enzyme was stabilized so that equilibration of the affinity probe could be achieved. The third goal was to introduce a functionalizable group into endothal which allowed retention of binding site affinity. This had not been achieved before since substitution at the 2- and 3-carboxyl group in the endothal series or introduction of substituents at the 1-, 4-, 5- or 6-position led to reduction in or total loss of PP2A binding activity and toxicity.[9–12,20–25] The new series examined here was the 2-substituted endothal anhydride analogues other than the 2-methyl compound (**2**). The fourth goal was to determine the effect of derivatizing the functionalizable group on

Key words: Enzymes inhibitors; herbicides; natural products; substituent effects.

* Corresponding author. Tel.: +1-510-642-5424; fax: +1-510-642-6497; e-mail: ectl@nature.berkeley.edu

† Present address: Department of Biology, Pacific University, Forest Grove, OR 97116, USA.

‡ Dedicated to the memory of Professor William G. Dauben (1919–1997) who made outstanding contributions to many areas of organic chemistry including cantharidin synthesis and action.

§ Present address: Process R&D, Bristol-Myers Squibb Pharmaceutical Research Institute, New Brunswick, NJ 08903-0191, USA.

¶ Present address: Genetic Analysis R&D, PE Applied Biosystems, Foster City, CA 94404, USA.

0968-0896/99/$ - see front matter
PII: S0968-0896(99)00239-4

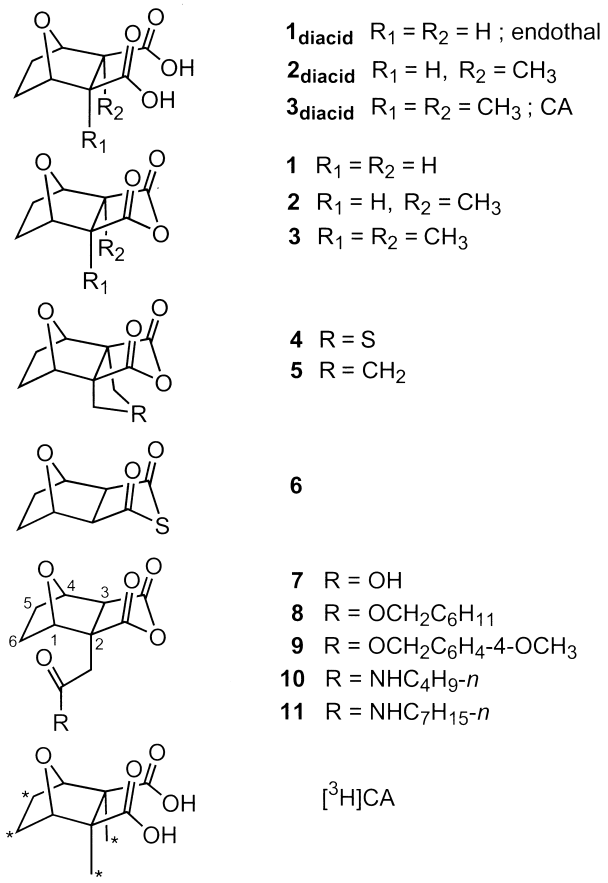


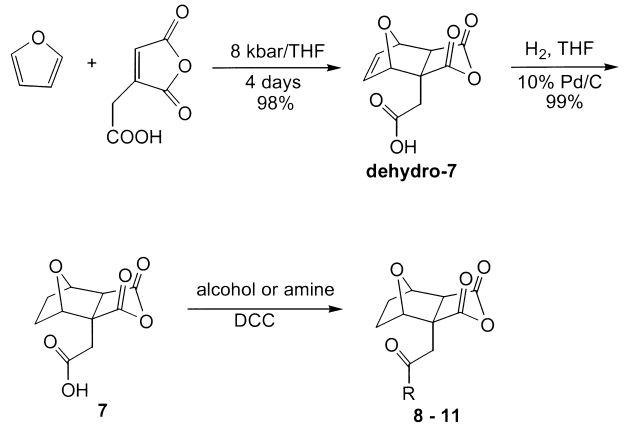
Figure 1. Structures of variously-substituted *exo,exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acids ($\mathbf{1}_{diacid}$–$\mathbf{3}_{diacid}$) and anhydrides (**1**–**11**) and of radioligand [^{3}H]CA (asterisks designate labeled positions) for the active site of protein phosphatase 2A. Palasonin is compound **2** and cantharidin is **3**. One structure gives the numbering system.

inhibitory potency. These are preliminary steps in using the endothal-type series as improved affinity probes and possibly for affinity chromatography.

Chemistry

Compounds studied here from syntheses reported earlier are: $\mathbf{1}_{diacid}$, $\mathbf{2}_{diacid}$, $\mathbf{3}_{diacid}$ (CA), **2, 3, 4 and 6**;[18–21] the individual enantiomers of **2**, i.e. (+)-**2** and (−)-**2**.[24] Compound **5** was obtained by high pressure Diels–Alder reaction between 1-cyclopentene-1,2-dicarboxylic anhydride and furan under 8 kbar and 3 days to give a pair of *exo* and *endo* dehydro-2,3-trimethyleneendothal anhydride isomers in the ratio of 13:12 in 99% yield. The two isomers were separated by fractional recrystallization and the pure *exo* isomer was hydrogenated with 10% Pd/C in THF to give **5**.[21]

Carboxymethylendothal anhydride (**7**) was the key target compound for introducing a functionalizable group at the 2-position (Scheme 1). The [4+2] Diels–Alder reaction between furan and the commercially available *cis*-aconitic anhydride will not occur at atmospheric pressure due to the aromaticity of the furan and the sterically hindered environment in the transition state



Scheme 1. Synthesis of 2-carboxymethylendothal anhydride [**7**] and esters and amides thereof [**8**–**11**].

created by the carboxymethyl group. However, when this reaction is carried out at 25°C under 8 kbar for 4 days the conversion is 100% to give 98% yield of the Diels–Alder adduct, dehydro-2-carboxymethylendothal anhydride (dehydro-**7**), as the only product (no *endo* isomer could be detected). Hydrogenation of dehydro-**7** in THF over 10% Pd/C gave **7** in 99% yield. Compound **7** was converted to esters **8** and **9** and amides **10** and **11** using dicyclohexylcarbodiimide (DCC) for coupling the carboxylic acid and alcohol or primary amine. No attempt was made to resolve the diastereomeric acids, esters and amides **7**–**11**.

Results and Discussion

Enzyme stability and stabilization

Temperature effects on association and dissociation. The rates of [^{3}H]CA association and dissociation are highly temperature-dependent (Fig. 2). There is negligible ligand association/dissociation at 4°C and even at 21°C ligand association appears incomplete at 8 h and dissociation only reaches the $t_{1/2}$ value after 24 h. Although steady-state binding appears to have been nearly attained after 8 h at 37°C the maximal binding levels remain similar to those obtained at 21°C. Dissociation rates at 37°C are identical using $\mathbf{1}_{diacid}$, pyrophosphate or $MnCl_2$ as the competing ligand (Fig. 2) consistent with earlier observations[4] that they act competitively rather than allosterically at the [^{3}H]CA binding site. Further, the apparent rate of ligand dissociation seen at 37°C (Fig. 2) is confounded by a parallel loss of binding activity under these conditions (Fig. 3) leading to further examination of binding site stability and stabilization.

Stability of binding site. Although increasing the temperature facilitates ligand binding, prolonged exposure of cytosol to temperatures above 4°C results in loss of [^{3}H]CA binding activity. Radioligand binding in 20% cytosol is essentially stable for 24 h at 4°C but declines at 21 and 37°C with $t_{1/2}$ values of 16 and 2.6 h, respectively (Fig. 3). Similar studies using more dilute (0.5%) cytosol in imidazole buffer establish the same trends (data not shown).

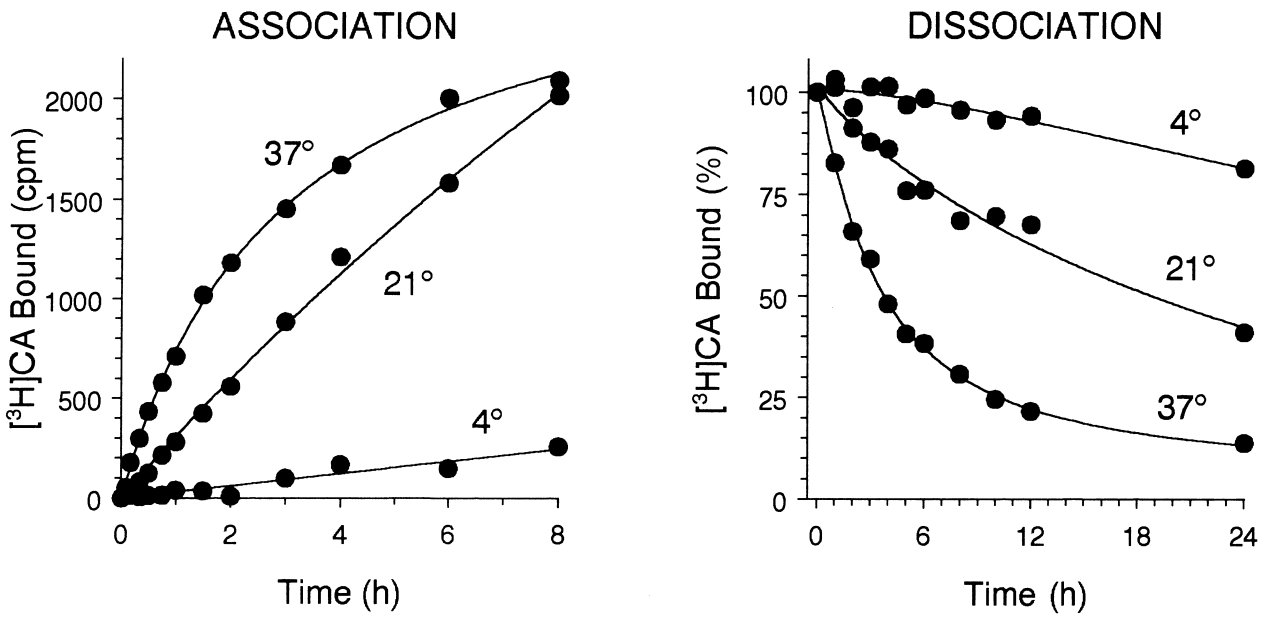


Figure 2. Temperature effects on [^{3}H]CA association and dissociation. Association kinetics determined by adding 5 nM [^{3}H]CA alone or with 10 μM unlabeled CA (for nonspecific binding) to cytosol in pH 7.0 imidazole/EDTA/EGTA buffer and incubating for up to 8 h. Dissociation kinetics determined by preincubating cytosol with 5 nM [^{3}H]CA for equilibration with dissociation initiated by $\mathbf{1}_{diacid}$ (10 μM) and monitored for 24 h. The same curve was obtained for dissociation of bound [^{3}H]CA at 37°C by $\mathbf{1}_{diacid}$ (10 μM) (illustrated) and by pyrophosphate (100 μM) or $MnCl_2$ (5 mM) (not shown).

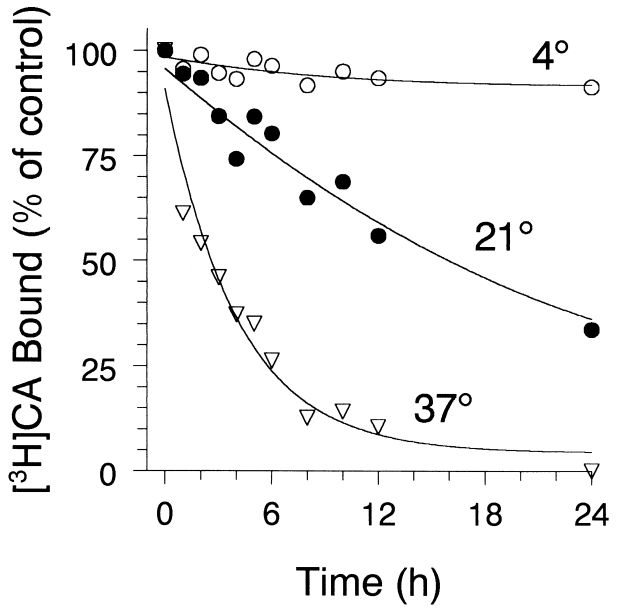


Figure 3. Temperature effects on stability of [^{3}H]CA binding site of PP2A. Cytosol (20% fresh weight equivalent in pH 7.0 imidazole/EDTA/EGTA buffer) was incubated at 4, 21 and 37°C then diluted 40-fold to 0.5% cytosol in the same buffer and assayed for [^{3}H]CA binding activity for an additional 3 h at 37°C. Cytosol maintained at 0°C was used as the control (i.e. 100% binding activity).

Stabilization of binding site. Several candidate protein stabilizing agents were tested for possibly prolonging [^{3}H]CA binding activities for cytosol in imidazole buffer with incubations for 14 h at 21 or 37°C. Little or no stabilization or enhanced binding is achieved with standard protease inhibitors (0.3 μM aprotonin, 20 μM leupeptin, and 1 μM pepstatin, tested individually), benzamidine (1 mM), EDTA (1 mM), EGTA (1 mM) and *N*-methylmaleimide (1 mM). There is some enhancement in stability with 2-mercaptoethanol (12 mM), dithiothreitol (5 mM), $MnCl_2$ (50 μM), phenylmethanesulfonyl fluoride (1 mM) and 2-phenyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide (PSCP)[26] (10 μM) (data not shown) whereas glycerol (30%) gives remarkably increased binding site stability. The imidazole/glycerol medium, designed from these observations, extends the binding activity not only at 21°C but also at 37°C where 10, 20 and 30% glycerol enhance binding by 40, 100 and 160%, respectively, relative to buffer without glycerol (Fig. 4A). Subsequent supplementation of buffer containing 30% glycerol with PSCP, 2-mercaptoethanol and Mn^{2+} results in a further increase in binding activity. The apparent rate of [^{3}H]CA association was not affected by the glycerol concentration (Fig. 4B) and accurate rates of ligand association/dissociation could now be considered for the first time. Re-examination of binding kinetics in this new 'stabilized' buffer media yielded complete stabilization after 6–8 h at 37°C (association $t_{1/2}$ of 67 min) and 90% dissociation after 33 h ($t_{1/2}$ of 114 min) (Fig. 5). Binding activity under these conditions remained stable for > 24 h (see control line) (Fig. 5). The similarity in association and dissociation rates in the presence or absence of glycerol suggests that this stabilizer acts to increase the number of binding sites from 1.1 to 2.7 pmol/mg protein without altering the binding kinetics under the standard assay conditions.

Earlier studies in the absence of stabilizers were made with incubations at 37°C without recognizing that binding site loss occurred to a major extent leading to an underestimate of maximum binding at equilibrium. These values in the imidazole/glycerol buffer are now 260% of those obtained without stabilization indicating that earlier estimates of PP2A concentration of cytosol

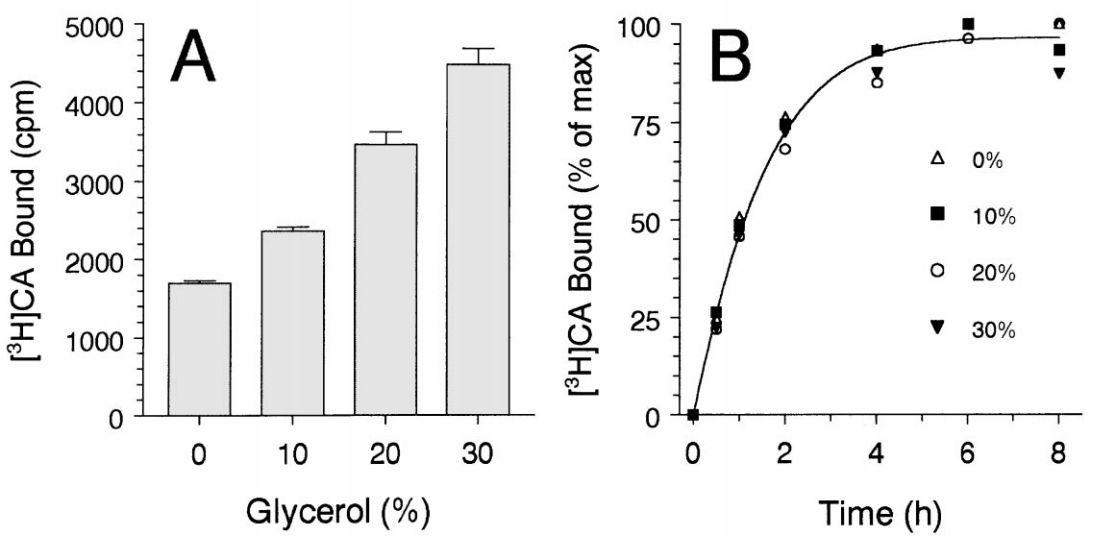


Figure 4. Glycerol enhancement of [^{3}H]CA binding at equilibrium (A) without affecting the association rate (B). Association determined as in Fig. 2 at 37°C but in pH 7.0 imidazole/glycerol buffer with 0, 10, 20 or 30% glycerol.

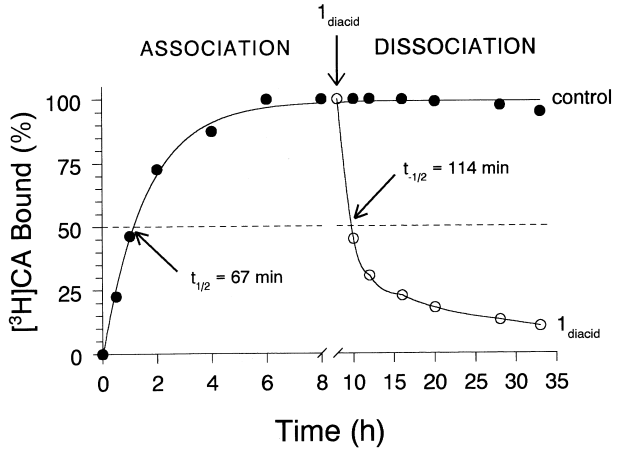


Figure 5. [^{3}H]CA association and dissociation in pH 7.0 imidazole/glycerol buffer with 30% glycerol at 37°C. Association and dissociation determined as in Fig. 2 but with dissociation initiated by $\mathbf{1}_{diacid}$ (10 μM).

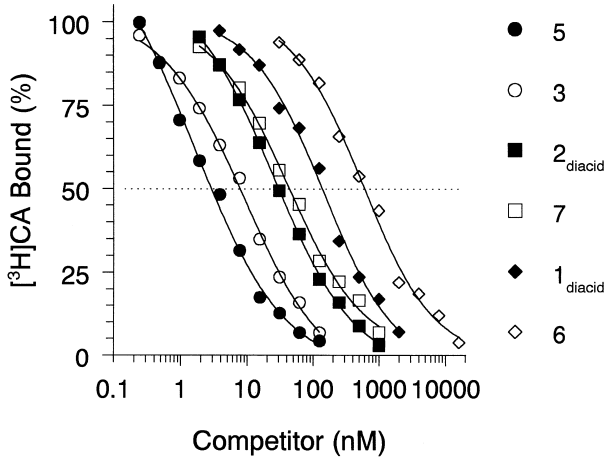


Figure 6. Competition curves showing the potency of six endothal analogues for inhibition of [^{3}H]CA binding at pH 7.25. Cytosol in pH 7.25 imidazole/glycerol buffer (see Experimental) incubated with 5 nM [^{3}H]CA alone or with competitor for 12 h at 37°C using unlabeled CA (1 μM) to correct for nonspecific binding. Binding density at 100% is 2.7 pmol/mg protein. Data can be compared with results at pH 6.0 in the same experiment (Table 1). The potency order refers to compounds designated as in Fig. 1.

by this procedure were only about 40% of the actual value. Although the mechanism by which glycerol leads to increased binding activity is not known, glycerol is thought to stabilize protein–protein interactions[27] and is used for storing commercially-purified PP2A.[28]

Inhibitor structure–activity and pH relationships

Selection of carboxymethylendothal (7). The 2- and 3-substituents greatly influence potency for inhibition of [^{3}H]CA binding in imidazole/glycerol at pH 7.25 with 12 h incubation (Fig. 6, Table 1). Relative to endothal ($\mathbf{1}_{diacid}$), a 2-methyl substituent ($\mathbf{2}_{diacid}$) increases potency by sevenfold at pH 7.25 and threefold at pH 6.0. Anhydride (±)-**2** is similar in potency to (+)-**2** and (−)-**2**, so the presence of the 2-methyl substituent is more important than its stereochemistry. A second methyl substituent in the anhydride series increases the potency a further fourfold (**3** versus **2**) and this activity level is maintained with the -CH_2SCH_2- bridge (**4**) and further increased with the -$CH_2CH_2CH_2$- linkage (**5**). The thioanhydride of **1** (compound **6**) is fourfold less potent than $\mathbf{1}_{diacid}$ itself. The potency order of $\mathbf{1}_{diacid}$ < $\mathbf{2}_{diacid}$ < **3** at pH 7.25 indicates that introduction of 2- or 2,3-substituents at the *endo* position may achieve the desired goal. The functionalizable carboxymethyl group was then introduced *endo* at C-2 of **1** to obtain **7** with an IC_{50} of 45–47 nM at pH 7.25 and 6.0 which is a potency increase of 3.1-fold at pH 7.25 and decrease of 1.7-fold at pH 6.0.

Activity of esters and amides (8–11) of carboxymethylendothal anhydride (7). The finding that **7** is of similar potency to **2** at pH 7.25, i.e. 2-carboxymethyl versus 2-methyl, led to the preparation of esters and amides as models for linking **7** to affinity matrices. Esters **8** and **9** show similar potency to the parent acid **7**, whereas

amides **10** and **11** are considerably less active. On this basis, esters **8** and **9** may hydrolyze to compound **7**, a possibility not directly examined in the present study.

Effect of pH on structure–activity relationships. The endothal derivatives differ in the effect of pH on their inhibitory activity. The pH optimum of [^{3}H]CA binding in imidazole/glycerol buffer with a 12 h incubation at 37°C is broad, with little difference in the range of 6.0 to 7.25 (Fig. 7). In contrast, [^{3}H]**6** binding is optimal at pH 6.0 with $<10\%$ of this activity at pH 7.4.[12,21] The IC_{50} values of endothal analogues were determined at two pH values (6.0 and 7.25) (Table 1) which in themselves have little effect on [^{3}H]CA binding in the absence of inhibitors (Fig. 7). This comparison reconfirms the pH effect on **6** binding noted above since we find a sixfold greater potency at pH 6.0 than at 7.25. Moreover, $\mathbf{1}_{diacid}$, $\mathbf{2}_{diacid}$, the enantiomers of **2**, and **4** follow a similar pattern with two to fivefold greater potency at pH 6.0 than at 7.25. Compounds **3**, **5** and **7** and the four esters and amides (**8**–**11**) show much less difference (0.8–1.5-fold) in potency with pH (Table 1). A more detailed examination established that the binding affinities of **3** and **7** are not greatly influenced by the pH whereas those of $\mathbf{1}_{diacid}$, **2** and **6** are highly dependent on the assay pH (Fig. 7). The mechanism by which the pH differentially affects the inhibitory potency is not known but probably involves optimal interactions of the carboxylate substituent directly ($\mathbf{1}_{diacid}$ and $\mathbf{2}_{diacid}$) or after hydrolysis[10,21,23] with the ligand binding site.

Table 1. Structure–activity relationships for endothal analogues and carboxymethylendothal derivatives as inhibitors of [^{3}H]CA binding at pH 7.25 and pH 6.0 in imidazole/glycerol buffer with 12 h incubation at 37°C

No.[a]	IC_{50} (nM) at indicated pH[b]		IC_{50} ratio
	7.25	6.0	7.25/6.0
Endothal analogues			
$\mathbf{1}_{diacid}$	139 ± 14	28 ± 4	5.0
(±)-**2**	31 ± 5	12 ± 1	2.6
(+)-**2**	26 ± 1	9.7 ± 0.7	2.7
(−)-**2**	37 ± 3	14 ± 1	2.6
$(\pm)\text{-}\mathbf{2}_{diacid}$	19 ± 2	9.4 ± 0.8	2.0
3	8.0 ± 1.2	9.9 ± 2.2	0.8
4	10 ± 1	2.7 ± 0.0	3.7
5	3.0 ± 0.4	2.4 ± 0.5	1.3
6	608 ± 6	104 ± 3	5.9
(±)-Carboxymethylendothal derivatives			
7	45 ± 3	47 ± 5	1.0
8	42 ± 4	31 ± 6	1.4
9	48 ± 10	63 ± 12	0.8
10	251 ± 32	168 ± 18	1.5
11	206 ± 21	216 ± 15	1.0

[a] For structures see Fig. 1.
[b] Data are the means ± SE of three experiments.

Candidate affinity probes

The goal of discovering an endothal analogue combining a functionalizable substituent with reasonably high potency in the [^{3}H]CA binding assay was achieved with **7**. It was accordingly converted to two esters and two amides (Table 1). Potent esters **8** and **9** may hydrolyze during assay (despite attempted inhibition of esterases by PSCP)[26] and act as **7**. Amides **9** and **10**, with *N*-butyl and *N*-heptyl substituents, respectively, have reduced affinity or cleave more slowly. In comparison with microcystin–Sepharose affinity chromatography[16,17] the use of a carboxymethylendothal derivative potentially provides greater reversibility and ease of PP2A displacement. Affinity chromatography with an endothal analogue would require several hours equilibration time at 37°C based on the association kinetics of [^{3}H]CA in imidazole/glycerol buffer at pH 7.0 (Fig. 5). In conclusion, it is clear that the *endo* 2-position (either alone or in combination with a 3-substituent) is the site for derivatization to retain binding site activity and further structure–activity studies are required to achieve conjugates of adequate potency and stability for affinity chromatography.

Experimental

Chemicals

General. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were determined for $CDCl_3$ or THF-d_8 solutions calibrated with Me_4Si. IR spectra

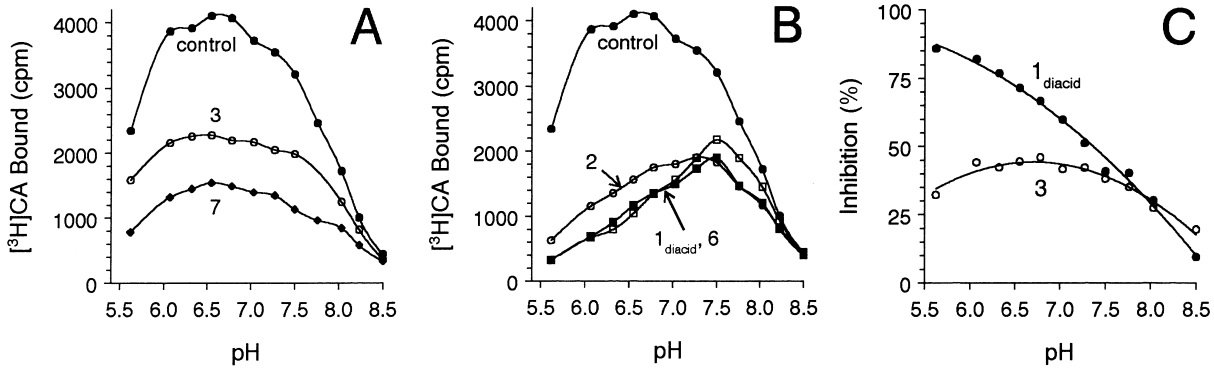


Figure 7. Effect of pH on [^{3}H]CA binding (control in A) and inhibition by endothal analogues. A and B: experimental conditions the same as Fig. 6 except pH varied from 5.5 to 8.5 and competitors included at levels that inhibited 42–64% at pH 7.0. C: data for $\mathbf{1}_{diacid}$ and **3** presented as % inhibition at each pH value.

were recorded using KBr pellets for solid samples. Melting points (mp) determined in capillary tubes are uncorrected. Et_2O and THF were distilled over CaH_2. Flash chromatography utilized 230–400 mesh silica gel at medium pressure. The high pressure apparatus and the general procedures for high pressure reactions have been described.[24]

***exo*-2,3-Trimethyleneendothal anhydride (5).** A solution of 1-cyclopentene-1,2-dicarboxylic anhydride (1.5 g, 10.9 mmol), furan (0.81 g, 12.0 mmol), and CH_2Cl_2 (1.5 mL) was pressurized to 8 kbar for 3 days at 25°C. The solvent was evaporated under reduced pressure to give a pair of *exo* and *endo* dehydro-2,3-trimethyleneendothal anhydride isomers as a white solid (2.24 g, 99% yield). ^{1}H NMR showed *exo* to *endo* in the ratio of 13:12. This mixture of the two isomers was recrystallized from hexane:EtOAc (6:4). The crystals collected were identified as *endo*-dehydro-**5**: mp 127–128°C; IR ν_{max} 2968, 2948, 1841, 1778, 1444, 1241 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.33–1.41 (m, 2H), 1.89–2.04 (m, 2H), 2.17–2.23 (m, 2H), 5.24 (t, J = 0.95 Hz, 2H), 6.66 (t, J = 1.03 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 31.2, 31.6, 69.4, 83.9, 137.0, 174.4. The mother liquor was evaporated to dryness and recrystallized five times from hexane:EtOAc (7:3) to give pure *exo*-dehydro-**5**: mp 133–134°C; IR ν_{max} 2974, 2943, 1847, 1771, 1252, 1199 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.76–1.87 (m, 3H), 2.11–2.13 (m, 1H), 2.47–2.52 (m, 2H), 4.98 (s, 2H), 6.62 (t, J = 0.82 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 30.0, 33.6, 68.1, 833.4, 136.6, 171.8.

To a solution of *exo*-dehydro-**5** (0.51 g, 2.5 mmol) in THF (20 mL) was added 10% Pd/C (0.15 g). The resulting mixture was stirred under hydrogen (3 L inflated balloon) for 20 h and the reaction mixture was filtered. The filtrate was evaporated to give **5** as a white solid (0.50 g, 98%). A sample was recrystallized from hexane:EtOAc (6:4) to give **5** as white crystals; mp 171–172°C; IR ν_{max} 2983, 2900, 1859, 1779 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.40 (m, 1H), 1.66–1.82 (m, 4H), 1.86–1.95 (m, 3H), 2.49–2.54 (m, 2H), 4.59 (dd, J = 2.31, J = 2.28 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 25.9, 26.4, 35.8, 70.3, 81.9, 172.6. HRMS calcd for $C_{11}H_{12}O_4$: 208.0736. Found: 208.0736.

Dehydro-2-carboxymethylendothal anhydride (dehydro-7). A solution of *cis*-aconitic anhydride (1.0 g, 6.4 mmol), furan (0.48 g, 7.1 mmol), and dry THF (2 mL) was pressurized to 8 kbar for 4 days at 25°C. The solvent was evaporated under reduced pressure to give dehydro-**7** as an oil (1.41 g, 98%) and ^{1}H NMR showed 100% conversion and >95% purity. The oily product was stirred with anhydrous CH_2Cl_2, filtered and then evaporated to give white crystals of dehydro-**7** (1.22 g, 5.4 mmol) in 85% yield: mp 122–123°C; ^{1}H NMR (THF-d_8) δ 2.21, 3.21 (AB quartet, J = 18 Hz, 2H), 2.94 (s, 3H), 5.09 (m, 1H), 5.26 (m, 1H), 6.59 (dd, J = 1.73, 5.75 Hz, 1H), 6.65 (dd, J = 1.77, 5.76 Hz, 1H); ^{13}C NMR (THF-d_8) δ 37.8, 52.8, 58.4, 84.1, 85.1, 136.5, 138.7, 171.2, 172.9, 175.0. Anal. calcd for $C_{10}H_8O_6$: C, 53.58; H, 3.60. Found: C, 53.24; H, 3.74.

2-Carboxymethylendothal anhydride (7). To a solution of dehydro-**7** (1.22 g, 5.4 mmol) in dry THF (30 mL) was added 10% Pd/C (0.8 g), and the mixture was stirred under hydrogen (3 L inflated balloon) for 20 h and filtered. The filtrate was evaporated to give a colorless oil which crystallized out upon addition of CH_2Cl_2 to give **7** (1.22 g, 5.4 mmol) in 99% yield. A sample was recrystallized from hexane–EtOAc: mp 224–225°C; IR ν_{max} 3067 (broad), 3003, 1860, 1777, 1714 cm^{-1}; ^{1}H NMR (THF-d_8) δ 1.59–1.92 (m, 4H), 2.74, 3.05 (AB quartet, J = 18 Hz, 2H), 2.99 (s, 1H), 4.66 (d, J = 4.7 Hz, 1H), 4.80 (d, J = 5.4 Hz, 1H); ^{13}C NMR (THF-d_8) δ 25.5, 28.2, 35.8, 55.6, 57.4, 82.2, 83.8, 172.2, 173.1, 176.3. Anal. calcd for $C_{10}H_{10}O_6$: C, 53.10; H, 4.46. Found: C, 52.72; H, 4.47.

2-(Cyclohexylmethoxycarboxymethyl)endothal anhydride (8). To a solution of **7** (1.0 g, 4.4 mmol) in THF (30 mL) was added DCC (0.91 g, 4.4 mmol) in THF (5 mL) dropwise at room temperature, and the mixture was stirred for 5 min and cooled in an ice-bath. Cyclohexylmethanol (0.51 g, 4.5 mmol) in THF (5 mL) was added dropwise, and the mixture was stirred for 3 h at 0°C. The white precipitate was filtered, and the filtrate was evaporated to give a white solid which was recrystallized from 40% EtOAc in hexanes to give **8** (1.28 g, 4.0 mmol) in 90% yield; mp 125–127°C; IR ν_{max} 2924, 2842, 1846, 1778, 1721, 1445, 1408, 1357, 1268, 1206, 1144, 998, 958 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.90–0.96 (m, 2H), 1.15–1.25 (m, 2H), 1.56–1.61 (m, 2H), 1.64–1.73 (m, 6H), 1.79–1.83 (m, 2H), 1.90–1.94 (m, 1H), 2.60, 3.12 (AB quartet, J = 18 Hz, 2H), 2.84 (s, 1H), 3.12 (d, J = 17.77 Hz, 1H), 3.90 (m, 2H), 4.78 (s, 1H), 4.95 (d, J = 5.4 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 24.7, 25.5, 26.2, 27.7, 29.5, 35.7, 36.8, 55.0, 55.3, 71.2, 81.0, 82.6, 170.3, 170.8, 175.0. HRMS calcd for $C_{17}H_{22}O_6$: 322.1416. Found: 322.1419.

2-(4-Methoxybenzyloxycarboxymethyl)endothal anhydride (9). To a solution of **7** (0.80 g, 3.5 mmol) in THF (35 mL) was added DCC (0.72 g, 3.5 mmol) in THF (5 mL) dropwise at room temperature, and the mixture was stirred for 5 min and cooled in an ice-bath. 4-Methoxybenzyl alcohol (0.49 g, 3.5 mmol) in THF (10 mL) was added dropwise, and the mixture was stirred for 1 h at 0°C, then overnight at room temperature. The white precipitate was filtered, and the filtrate was evaporated to give a white solid which was recrystallized from 40% EtOAc in hexanes to give **9** (1.10 g, 3.2 mmol) in 91% yield; mp 135–136°C; IR ν_{max} 2926, 2849, 1850, 1779, 1724, 1518, 1406, 1357, 1253, 1139, 998, 909, 821 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.53–1.57 (m, 1H), 1.76–1.82 (m, 2H), 1.90–1.95 (m, 1H), 3.13, 2.60 (AB quartet, 2H, J = 17.81 Hz), 2.82 (s, 1H), 3.81 (s, 3H), 4.78 (d, 1H, J = 4.17 Hz), 4.93 (d, 1H, J = 5.39 Hz), 5.06, 5.05 (AB quartet, 2H, J = 11.77 Hz), 6.90 (m, 2H), 7.24–7.31 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 24.7, 27.7, 35.8, 55.0, 55.3, 56.3, 67.7, 81.0, 82.6, 114.1, 126.7, 130.6, 160.0, 170.1, 170.8, 175.0. HRMS calcd for $C_{18}H_{18}O_7$: 346.1053. Found: 346.1059.

2-(*n*-Butylcarboxamidomethyl)endothal anhydride (10). To a solution of **7** (1.0 g, 4.4 mmol) in THF (30 mL) was added DCC (0.908 g, 4.4 mmol) in THF (5 mL) dropwise at room temperature. The mixture was stirred for 5 min and then cooled in an ice-bath. *n*-Butylamine (0.323 g, 4.4 mmol) in THF (5 mL) was added dropwise, and the mixture was stirred for 5 h at 0°C. The white precipitate was filtered, and the filtrate was evaporated

to give a white solid **10** (1.15 g, 4.1 mmol) in 93% yield. IR ν_{max} 3175 (broad), 2967, 1775, 1702, 1407, 1210, 1138, 1051, 990; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 7.23 Hz), 1.49 (m, 2H), 1.57 (m, 2H), 1.64 (m, 2H), 1.80 (m, 2H), 2.60 (s, 1H), 2.81 (d, 1H, J = 18.26 Hz), 2.94 (d, 1H, J = 18.41 Hz) 3.42 (m, 2H), 4.43 (s, 1H), 5.05 (d, 1H, J = 4.47 Hz); ^{13}C NMR ($CDCl_3$) δ 13.6, 19.9, 24.9, 27.8, 31.4, 37.0, 39.6, 55.3, 56.8, 80.9, 82.7, 168.8, 171.5, 176.0. HRMS calcd for $C_{14}H_{19}O_5N$: 281.1263. Found: 281.1268.

2-(*n*-Heptylcarboxamidomethyl)endothal anhydride (11). To a solution of **7** (0.86 g, 3.8 mmol) in THF (30 mL) was added DCC (0.78 g, 3.8 mmol) in THF (5 mL) dropwise at room temperature. The mixture was stirred for 5 min and then cooled in an ice-bath. *n*-Heptylamine (0.461 g, 4.0 mmol) in THF (5 mL) was added dropwise, and the mixture was stirred for 5 h at 0°C, then stirred at room temperature overnight. The white precipitate was filtered, and the filtrate was evaporated to give a white solid **11** (1.17 g, 3.6 mmol) in 95% yield. IR ν_{max} 3187 (broad), 2931, 1774, 1702, 1407, 1181; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3H, J = 7.05 Hz), 1.24 (m, 8H), 1.53 (m, 4H), 1.81 (m, 2H), 2.61 (s, 1H), 2.82 (d, 1H, J = 18.31 Hz), 2.94 (d, 1H, J = 18.02 Hz), 3.41 (m, 2H), 4.44 (s, 1H), 5.06 (d, 1H, J = 4.44 Hz); ^{13}C NMR ($CDCl_3$) δ 14.0, 22.6, 26.4, 26.8, 27.4, 28.8, 31.7, 38.2, 39.1, 40.1, 55.9, 60.7, 78.3, 81.8, 174.0, 174.8, 179.0. HRMS calcd for $C_{17}H_{25}O_5N$: 323.1733. Found: 323.1733.

Protein phosphatase 2A

Assay of [^{3}H]CA binding site of PP2A. The procedure requires the radioligand, a PP2A source, and an appropriate buffer. [^{3}H]**3** (34 Ci/mmol) was repurified by silica gel chromatography with CH_2Cl_2 as required to maintain a radiochemical purity of > 95%.[4] It was diluted to 25 nM in 50 mM imidazole buffer at pH 7.4 and held for at least 1 h at 25°C to allow hydrolysis to [^{3}H]CA for use in binding assays.[4] To prepare the PP2A source, mouse liver was homogenized, using polytron and Potter–Elvehjem procedures, in 4 volumes of ice-cold 50 mM imidazole buffer at pH 7.0 (20% fresh weight equivalent) and the homogenate was centrifuged at 15,000×g for 15 min, and the resulting supernatant at 105,000×g for 60 min to obtain the cytosol which was stored at −70°C. Two other supplemented buffers were also used for comparison. The imidazole/EDTA/EGTA buffer at pH 7.0 consisted of 50 mM imidazole, 1 mM each of EDTA and EGTA, 10 μM *N*-methylmaleimide and 10 mM PSCP. The imidazole/glycerol buffer at pH 6.0, 7.0 or 7.25 (or as specified) contained 50 mM imidazole, 50 mM NaCl, 12 mM 2-mercaptoethanol, 10 μM PSCP, 10 mM $MnCl_2$ and 30% (v/v) glycerol. Ligand binding assays were conducted in 96-microwell plates as previously described.[4] Cytosol was diluted 20-fold in imidazole buffer (or imidazole/EDTA/EGTA or imidazole/glycerol buffer) and incubated with 5 nM [^{3}H]CA in the absence (total binding tubes) and presence of 10 μM unlabeled CA (non-specific binding tubes). Specific binding was calculated as the difference between total and nonspecific binding. Assays were terminated by filtration through glass-fiber filters presoaked in 0.3% polyethylenimine, and rapidly rinsed (3×1 mL) with imidazole buffer at 5°C, using a Wallac TomTec cell harvester (Harvester 96 Mach II, Gaithersburg, MD). Filtermats were dried overnight and impregnated with MeltiLex melt-on scintillator sheets (Wallac) and the bound radioactivity was quantified using a Wallac Betaplate (model 1205) flatbed counter.[4]

Association and dissociation kinetics for [^{3}H]CA binding site. Association rates were determined at 4, 21 and 37°C by mixing cytosol with 5 nM [^{3}H]CA (total binding) and 10 μM unlabeled CA (non-specific binding) and incubating for periods of 5 min to 8 h at each temperature. Ligand dissociation rates were determined by preincubating cytosol in imidazole/EDTA/EGTA buffer with 5 nM [^{3}H]CA for 6 h at 37°C followed by the addition of either $\mathbf{1}_{diacid}$ (10 μM), pyrophosphate (100 μM) or $MnCl_2$ (5 mM) for periods ranging from 1 to 24 h at temperatures of 4, 21 and 37°C. The kinetics of [^{3}H]CA binding were re-examined in imidazole/glycerol buffer to determine if stabilization was associated with reduced rates of ligand association and dissociation.

Stability and stabilization of [^{3}H]CA binding site. [^{3}H]CA association and equilibrium binding is best achieved at 37°C under conditions where stability must be considered. For stability studies, 20% cytosol was incubated at 4, 21 or 37°C for periods ranging from 1 to 24 h then diluted 40-fold and assayed for binding activity. Various potential stabilizing factors were examined including specific protease inhibitors (detailed earlier), thiol protective and derivatizing agents, PSCP, divalent ion chelators, and Mn^{2+}, a potential cofactor.[4] Compounds were initially tested for stabilizing effects on [^{3}H]CA binding activity by overnight (14 h) incubations at 21 and 37°C, followed by dilution in imidazole/glycerol buffer and assay of [^{3}H]CA binding activity.

Structure–activity and pH relationships for inhibitors of [^{3}H]CA binding. Incubations consisted of [^{3}H]CA (5 nM final concentration) and unlabeled competitor each added in dimethyl sulfoxide (5 μL) to 0.5% cytosol (20 μg protein, Bradford method)[29] in imidazole/glycerol buffer or other buffer as specified (200 μL). IC_{50} values were determined by four parameter logistic curve fitting. Data presented in figures represent typical single experiments. The effect of pH on [^{3}H]CA binding and inhibitor potency was examined in imidazole/glycerol buffer adjusted to pHs ranging from 5.5 to 8.5 in 0.5 pH increments, with the pH confirmed by direct measurement at the end of the incubation period.

Acknowledgements

The project described was supported by grant P01 ES00049 to J.E.C. from the National Institute of Environmental Health Sciences, National Institutes of Health, and by Elf-Atochem North America, Inc. (Philadelphia, PA). Research in the Department of Chemistry was supported by National Science Foundation Grant No. 8618303 to W.G.D. Advice and assistance were provided by Gary Quistad, Phillip Jefferies and

Mahmoud Mahajna of the Environmental Chemistry and Toxicology Laboratory.

References

1. Cohen, P. *Annu. Rev. Biochem* **1989**, *58*, 453.
2. MacKintosh, C.; MacKintosh, R. W. *Adv. Protein Phosphatases* **1994**, *8*, 343.
3. MacKintosh, C.; MacKintosh, R. W. *Trends Biochem. Sci.* **1994**, *19*, 444.
4. Laidley, C. W.; Cohen, E.; Casida, J. E. *J. Pharmacol. Exp. Ther*. **1997**, *280*, 1152.
5. Carmichael, W. W. In *Handbook of Natural Toxins, Vol. 3, Marine Toxins and Venoms*; Tu, A., Ed.; Marcel Dekker: New York, 1998; pp 121–147.
6. MacKintosh, C.; Beattie, K. A.; Klumpp, S.; Cohen, P.; Codd, G. A. *FEBS Lett*. **1990**, *264*, 187.
7. Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuchi, H.; Van Engen, D.; Clardy, J.; Gopichand, Y.; Schmitz, F. J. *J. Am. Chem. Soc*. **1981**, *103*, 2469.
8. Bialojan, C.; Takai, A. *Biochem. J*. **1988**, *256*, 283.
9. Graziano, M. J.; Waterhouse, A. L.; Casida, J. E. *Biochem. Biophys. Res. Commun*. **1987**, *149*, 79.
10. Graziano, M. J.; Pessah, I. N.; Matsuzawa, M.; Casida, J. E. *Mol. Pharmacol*. **1988**, *33*, 706.
11. Li, Y.-M.; Casida, J. E. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 11867.
12. Li, Y.-M.; MacKintosh, C.; Casida, J. E. *Biochem. Pharmacol* **1993**, *46*, 1435.
13. Honkanen, R. E. *FEBS Lett*. **1993**, *330*, 283.
14. MacKintosh, R. W.; Dalby, K. N.; Campbell, D. G.; Cohen, P. T. W.; Cohen, P.; MacKintosh, C. *FEBS Lett*. **1995**, *371*, 236.
15. Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Ojika, M.; Yamada, K.; Sugimura, T. *Biochem. Biophys. Res. Commun*. **1990**, *170*, 1359.
16. Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Nishiwaki-Matsushima, R.; Sugimura, T. *FEBS Lett*. **1991**, *279*, 115.
17. Moorhead, G.; MacKintosh, R. W.; Morrice, N.; Gallagher, T.; MacKintosh, C. *FEBS Lett*. **1994**, *356*, 46.
18. Dauben, W. G.; Kessel, C. R.; Takemura, K. H. *J. Am. Chem. Soc*. **1980**, *102*, 6893.
19. Dauben, W. G.; Gerdes, J. M.; Smith, D. B. *J. Org. Chem*. **1985**, *50*, 2576.
20. Matsuzawa, M.; Graziano, M. J.; Casida, J. E. *J. Agric. Food Chem*. **1987**, *35*, 823.
21. Kawamura, N.; Li, Y.-M.; Engel, J. L.; Dauben, W. G.; Casida, J. E. *Chem. Res. Toxicol*. **1990**, *3*, 318.
22. Erdödi, F.; Tóth, B.; Hirano, K.; Hirano, M.; Hartshorne, D. J.; Gergely, P. *Am. J. Physiol*. **1995**, *269*, C1176.
23. McCluskey, A.; Taylor, C.; Quinn, R. J.; Suganuma, M.; Fujiki, H. *Bioorg. Med. Chem. Lett*. **1996**, *6*, 1025.
24. Dauben, W. G.; Lam, J. Y. L.; Guo, Z. R. *J. Org. Chem*. **1996**, *61*, 4816.
25. Sodeoka, M.; Baba, Y.; Kobayashi, S.; Hirukawa, N. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1833.
26. Casida, J. E.; Eto, M.; Baron, R. L. *Nature* **1961**, *191*, 1396.
27. Dignam, J. D. In *Methods in Enzymology, Vol. 182, Preparation of Extracts from Higher Eukaryotes*; Deutscher, M. P., Ed.; Academic Press: San Diego, 1990; pp 194–203.
28. Calbiochem/Novabiochem. Technical Data Sheet for Protein Phosphatase 2A, Bovine Kidney, 1996.
29. Bradford, M. M. *Anal. Biochem*. **1976**, *72*, 248.