Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 1, pp 165–177, 1997
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PII: S0968-0896(96)00199-X
0968-0896/97 $17.00 + 0.00

Combinatorial Synthesis and Biological Evaluation of Library of Small-Molecule Ser/Thr-Protein Phosphatase Inhibitors

Peter Wipf,[a,*] April Cunningham,[a] Robert L. Rice[b] and John S. Lazo[b,*]
Departments of [a]Chemistry and [b]Pharmacology, University of Pittsburgh, Pittsburgh, PA 15260, U.S.A.

Abstract—In eukaryotes, phosphorylation of serine, threonine, and tyrosine residues on proteins is a fundamental posttranslational regulatory process for such functions as signal transduction, gene transcription, RNA splicing, cellular adhesion, apoptosis, and cell cycle control. Based on functional groups present in natural product serine/threonine protein phosphatase (PSTPase) inhibitors, we have designed pharmacophore model **1** and demonstrated the feasibility of a combinatorial chemistry approach for the preparation of functional analogues of **1**. Preliminary biological testing of 18 structural variants of **1** has identified two compounds with growth inhibitory activity against cultured human breast cancer cells. In vitro inhibition of the PSTPase PP2A was demonstrated with compound **1d**. Using flow cytometry we observed that compound **1f** caused prominent inhibition in the G1 phase of the cell cycle. Thus, the combinatorial modifications of the minimal pharmacophore **1** can generate biologically interesting antiproliferative agents. Copyright © 1997 Elsevier Science Ltd

Introduction

Many eukaryotic cell functions, such as signal transduction, cell adhesion, gene transcription, RNA splicing, apoptosis, and cell proliferation, are controlled by protein phosphorylation, which is regulated by the dynamic relationship between both kinases and phosphatases.[1] Indeed, the principal role of many second messengers is to modulate kinase selectivity. In an effort to intervene early in the initiation stage of cellular events and in recognition of the tumor promoting effects of phorbol ester based protein kinase C activators, the lion's share of synthetic chemistry research in this area has focused on protein kinases.[2] However, there is substantial recent biological evidence for the multiple regulatory functions of protein phosphatases and a clear link between phosphatase inhibition and apoptosis.[3–9]

Besides some minor phosphorylation of histidine, lysine, arginine, and, in bacteria, aspartate, most eukaryotic amino acid phosphate derivatives are found on serine, threonine, and tyrosine protein residues. Generally, the primary characterization of phosphatases follows these structural guidelines: Ser/Thr protein phosphatases (PSTPases), Tyr protein phosphatases (PTPases), and dual-specificity phosphatases (DSPases).[3]

PSTPases have been classified according to their substrate specificity, metal ion dependence and sensitivity to inhibition (Table 1).[10,11] cDNA cloning has revealed at least 40 different enzymes of this type. In addition to proteins (Inhibitor-1, Inhibitor-2, DARPP-32, NIPP-1),[4] several (mostly marine) toxins have been identified as potent inhibitors (Fig. 1).[12]

Okadaic acid is produced by several species of marine dinoflagellates and reversibly inhibits the catalytic subunits of the PSTPase subtypes PP1, PP2A, and PP3.[4] SAR studies showed that the carboxyl group as well as the four hydroxyl groups were important for activity.[13,14] Calyculin A was identified as a cytotoxic component of the marine sponge *Discodermia calyx*. It has an extremely high affinity to PP1, PP2A, and PP3 with an IC_{50} in the 0.3 nM range.[4] Microcystins are potent cyclic hepta- and pentapeptide toxins of the general structure cyclo[D-Ala-X-D-*erythro*-β-methyl-*iso*-Asp-Y-Adda-D-*iso*-Glu-*N*-methyldehydro-Ala] where X and Y are variable L-amino acids.[4] They are known to promote tumors in vivo, but, with the exception of hepatocytes, are impermeable to most cells in vitro.[4]

The large number of naturally occurring microcystins makes it possible to carry out a limited SAR study.[15] Apparent IC_{50}s for microcystins range between 0.05 and 5 nM, with similar preference for PP1, PP2A, and PP3 as found for okadaic acid and calyculin A.[4] The

Table 1. Ser/Thr protein phosphatase classification[3,4]

Family	Subfamily	Characteristic
PSTPases	PP1	IC_{50} for okadaic acid 10–50 nM
	PP2A	IC_{50} for okadaic acid 0.5 nM
	PP2B (calcineurin)	Ca(II)-dependent; IC_{50} for okadaic acid >2000 nM
	PP2C	Mg(II)-dependent; not inhibited by okadaic acid
	PP3	IC_{50} for okadaic acid 4 nM

substitution of alanine for arginine has little effect on phosphatase inhibitory potency; there is, however, a difference in relative cytotoxicity.[15] The dehydroamino acid residue and the *N*-methyl substituents are also not critical. Crucial are the glutamic acid unit, since esterification leads to inactive compounds, and the overall shape of the Adda residue, since the (6*Z*)-isomer is inactive. Some variations in the Adda unit, specifically the *O*-demethyl and the *O*-demethyl-*O*-acetyl analogues, exert little effect on bioactivity, however. Considerably less information is available in the nodularin series, since fewer compounds are available;

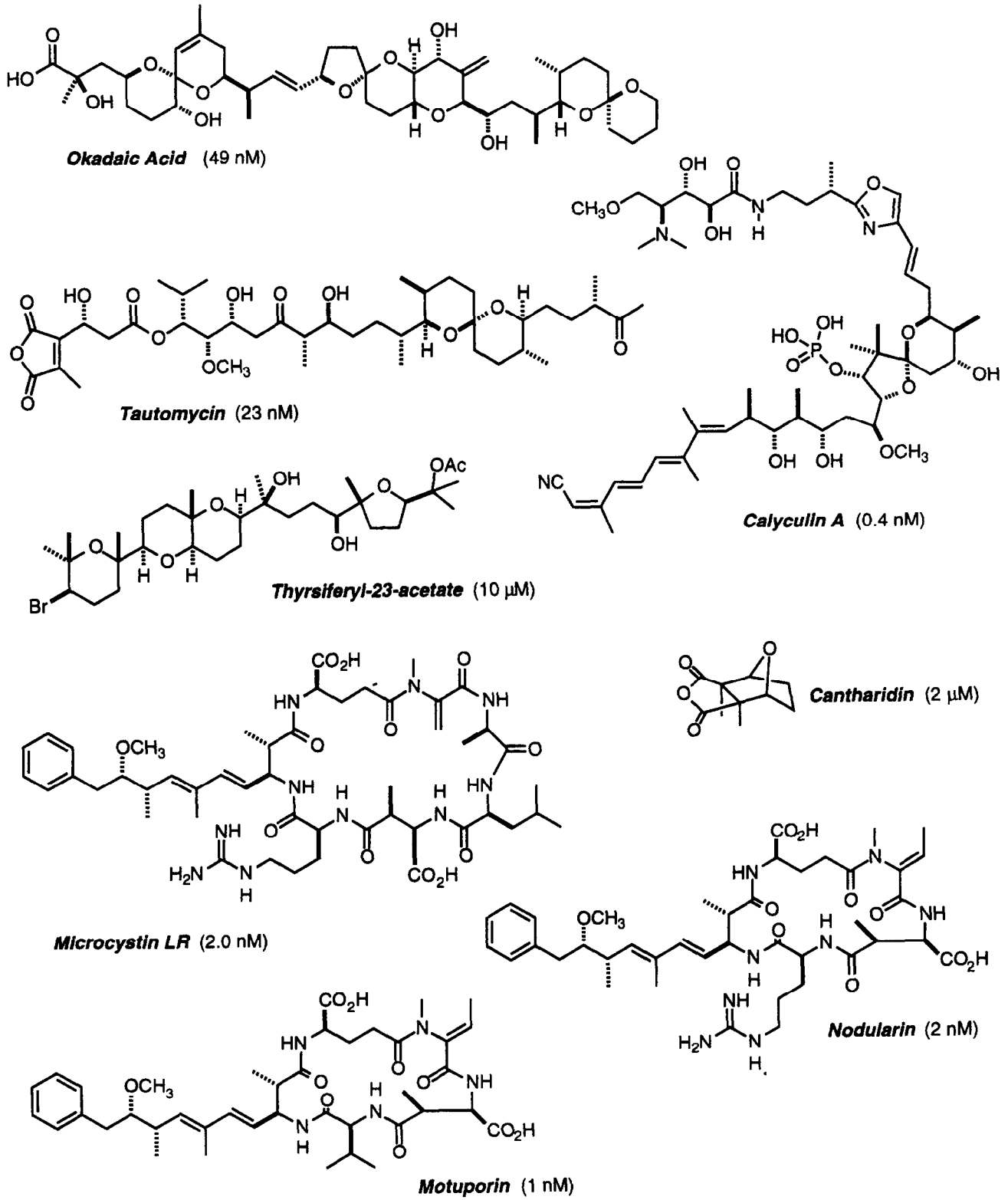


Figure 1. Natural product inhibitors of PSTPases (IC_{50} vs PP1).

however, the general SAR appears similar to the microcystins.[15] There are only slight differences in the inhibition profile; IC_{50}s for PP1 and PP3 are 2 and 1 nM, respectively, which is about 50 times higher than the IC_{50} for PP2A. The recently isolated motuporin (=[L-Val^2]nodularin) is even more potent with an IC_{50} <1 nM for PP1.[16,17] This secondary metabolite was isolated from a Papua New Guinea sponge and is the only member of the greater microcystin family that has thus far yielded to total synthesis.[18]

Tautomycin is produced by a terrestrial *Streptomyces* strain. This relatively unstable molecule inhibits PP1, PP2A and PP3 indiscriminately with an IC_{50} in the 15 nM range.[4] The remaining natural product inhibitors, thyrsiferyl-23-acetate and cantharidine, were shown to be somewhat selective, though weak (IC_{50} 0.16–10 μM) inhibitor of PP2A.[19–22]

Despite some recent total synthesis efforts,[23] no SAR for calyculin A, tautomycin[24] or thyrsiferyl acetate were reported. High toxicity, especially hepatotoxicity, is commonly found with all natural PSTPase inhibitors, often limiting the range of feasible pharmacological studies, and appears to be intrinsically associated with a non-specific phosphatase inhibition.[25] Importantly, based on kinetic and competition binding studies, okadaic acid, calyculin A, tautomycin, and the microcystins appear to bind competitively at the same site of PSTPases.[26–29] Since phosphatases are ubiquitous, precise tools in membrane and post-membrane signal transduction pathways, the development of selective inhibitors or activators of PSTPases that are cell-permeable, non-hepatotoxic, or broadly cytotoxic is of major significance for future progress in this field.

Design and Synthesis of Calyculin A Analogues

The design of our PSTPase inhibitor library was based on the SAR available for the natural product inhibitors and assumed that the presence of a carboxylate, a nonpolar aromatic function, and hydrogen-bond acceptors and donors (e.g. a peptidomimetic group) in suitable spatial arrangements are sufficient for strong and selective binding. A pharmacophore model that addresses these criteria is shown in Figure 2. Traditional computational studies by Quinn *et al.* have identified a related structural model based on molecular modeling of okadaic acid, calyculin A, and microcystin LR.[30] Whereas computational studies of the minimal structural requirements for PSTPase inhibition aim for an accurate prediction of the important conformational and electronic features of the lead structures, our combinatorial[31,32] analysis achieves this goal via a random optimization of the steric and electronic properties of the pharmacophore. Most marine natural products have evolved along an optimization of broad-range activity rather than specificity.[33] The structural variation present in a library of PSTPase inhibitors will allow the simultaneous exploration of high-affinity and high-specificity features providing selectivity beyond the natural product model.

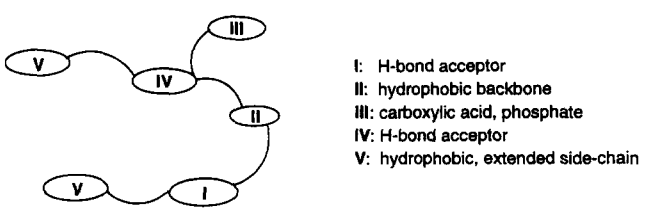


Figure 2. Pharmacophore model for PSTPase inhibitor library.

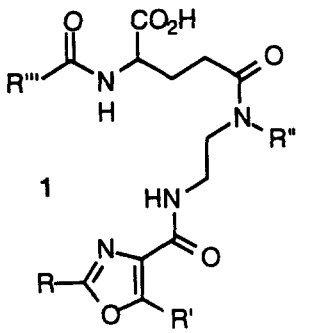


Figure 3. Parent structure for PSTPase inhibitor library synthesis.

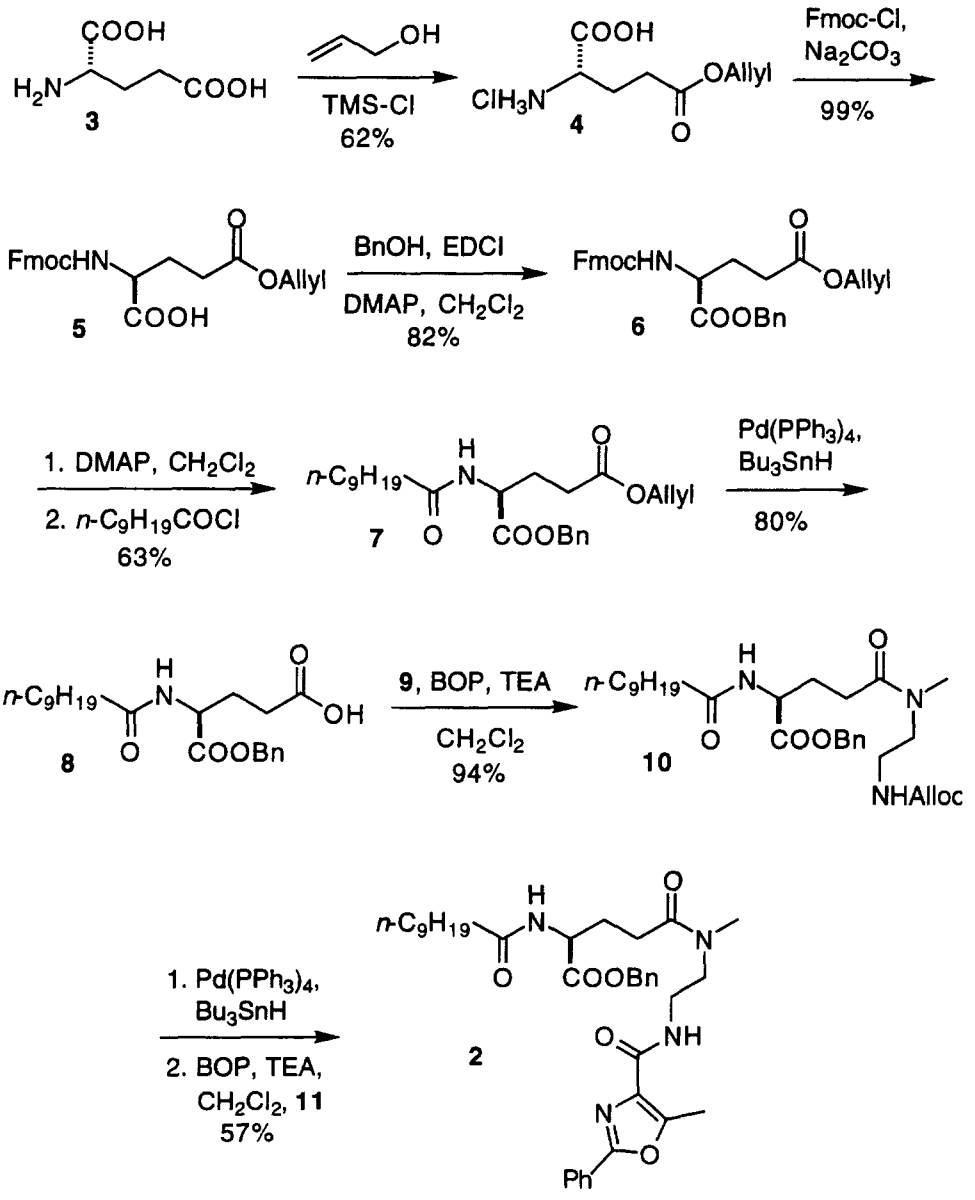
Specifically, we have designed compounds of structure **1** to provide a platform for functional group variation according to our pharmacophore model (Fig. 3). The carboxylic acid moiety, crucial for bioactivity, is derived from glutamic acid. The substituent R attached to the oxazole moiety of **1** can be varied within a broad range and should probably be mostly hydrophobic in nature. To a lesser extent, direct substitutions at the oxazole R′ are possible that will explore the tolerance for bulky residues at this site. A variable and relatively flexible diamine segment serves as the spacer between oxazole moiety and carboxylic acid side chain in place of the synthetically less readily accessible spiroketal of calyculin A. A related *N*-methyl dehydroalanine residue is found in microcystin LR. The hydrophobicity of this subunit is modulated by *N*-alkylation with residues R″. An acyl portion R‴CO is responsible for providing the molecule with a relatively rigid hydrophobic tail similar to the Adda amino acid side chain in microcystins and the tetraene cyanide in calyculin A.

Initially, the development of an efficient approach for the combinatorial synthesis of target structures **1** focused on the optimization of the solution-phase synthesis of model compound **2** (Scheme 1). L-Glutamic acid (**3**) was protected in 62% yield as the γ-allyl ester using allyl alcohol and chlorotrimethylsilane.[34] Treatment with Fmoc-Cl followed by coupling to benzyl alcohol using 1-ethyl-3-[3-(dimethylamino-)propyl]-carbodiimide hydrochloride (EDCI) provided the tri-protected amino acid **6** in 82% yield. The Fmoc protective group was subsequently removed by exposure to DMAP and the free amine was acylated in situ with decanoyl chloride to give amide **7** in 63% yield. Pd(0)-catalyzed deprotection[35] of the allyl ester and coupling of the resulting acid **8** to ethylene diamine **9** in the presence of (1H-1,2,3-benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluoro-

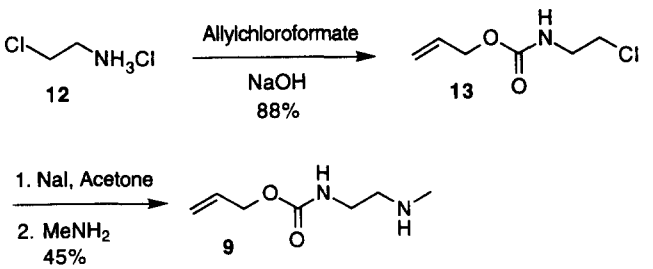
phosphate (BOP)[36] led to amide **10** in 75% yield. A versatile general route to monoprotected ethylene diamines was easily achieved by carbamoylation of 2-chloroethylamine monohydrochloride (**12**), Finkelstein reaction, and aminolysis (Scheme 2).

Deprotection of the Alloc-group gave a primary amine which was coupled, in situ, to oxazole acid (**11**) using BOP as a coupling agent. The desired amide **2** was obtained in 57% yield for the two steps. The heterocyclic moiety **11** was efficiently prepared from *N*-benzoyl threonine (**14**) by side-chain oxidation and cyclodehydration with Dess–Martin reagent and electrophilic phosphorus, respectively,[37] followed by saponification of oxazole **15** (Scheme 3).

The solution-phase preparation of calyculin analogue **2** established the necessary general protocols for the preparation of a library of structural variants of the pharmacophore model **1** on solid support. We have successfully applied this basic strategy for the parallel synthesis of 18 structural analogues (Scheme 4, Table 2). Coupling of diprotected glutamate **5** to the polystyrene-based Wang resin[38] with EDCI was performed on large scale and provided a supply of solid phase beads. The base-labile Fmoc protective group was removed by treatment with piperidine and THF, and the resin was distributed to three specially designed Schlenk filters equipped with suction adapters and inert gas inlets for maintaining steady bubbling. After the addition of solvent, hydrophobic residues R‴COCl were added to each flask, which provided three different amide derivatives **17**. After filtration and rinsing of the resin, allyl esters **17** were deprotected via Pd(0) chemistry and each batch was distributed over three modified Schlenk filters, providing nine different reaction sites for acylation. Addition of three different *N*-allyloxycarbonyl protected diamines



Scheme 1.

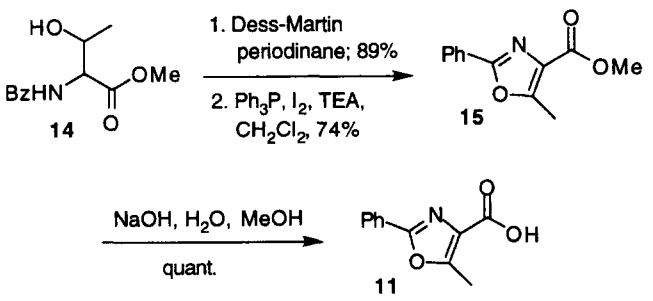


Scheme 2.

in the presence of PyBroP[39] or CloP[40] as coupling agents extended the side chain carboxyl terminus of glutamic acid toward the desired heterocyclic moiety in **1**. The resulting nine compounds (**18**) were each deprotected at the N-terminus and distributed over two additional Schlenk filters for the final segment condensation. Coupling with two different oxazole carboxylic acids in the presence of CloP and final purification by rinsing with solvent provided the phosphatase library **1** still attached to the solid support. Complete or partial cleavage with 50% trifluoroacidic acid was necessary to release the carboxylate which is required for biological activity. After filtration of the solid support and evaporation of the resulting mother liquor, the desired compounds **1** were obtained in a chemically pure and structurally well defined fashion ready for rapid throughput biological screening. In each case, the purity of the final compound was >60% according to spectroscopic analysis (^{1}H NMR, MS). The contamination was derived from incomplete couplings to the sterically hindered secondary amine moiety of Alloc-NHCH$_2$CH$_2$NH(R″). A small sample of resin had been routinely cleaved for reaction monitoring, but this coupling was difficult to drive to completion. Mass recovery was essentially quantitative. We are still in the process of further optimizing the reaction sequence and are confident that purities of >80% for the final material **1** can routinely be achieved after improvement of the coupling step.

Preliminary Biochemical and Biological Analysis of Library 1

We have begun to evaluate the ability of compounds **1a–r** to inhibit PP1 and PP2A. Initial studies were conducted in collaboration with Drs A. Boynton and D. Messner,[41] with their previously described assay.[25,29] These preliminary studies demonstrated that several members of our library inhibit protein phosphatases PP1 or PP2A by >50% at concentrations of 100 μM. We have further examined the ability of one member of the library to inhibit the catalytic activity of PP2A. As demonstrated in Figure 4, calyculin A inhibited PP2A activity at 10 nM, and compound **1d** caused 50% inhibition at 100 μM. These results document that our minimal structure retained the ability to inhibit the catalytic activity of Ser/Thr phosphatase. More comprehensive analyses are currently being conducted.



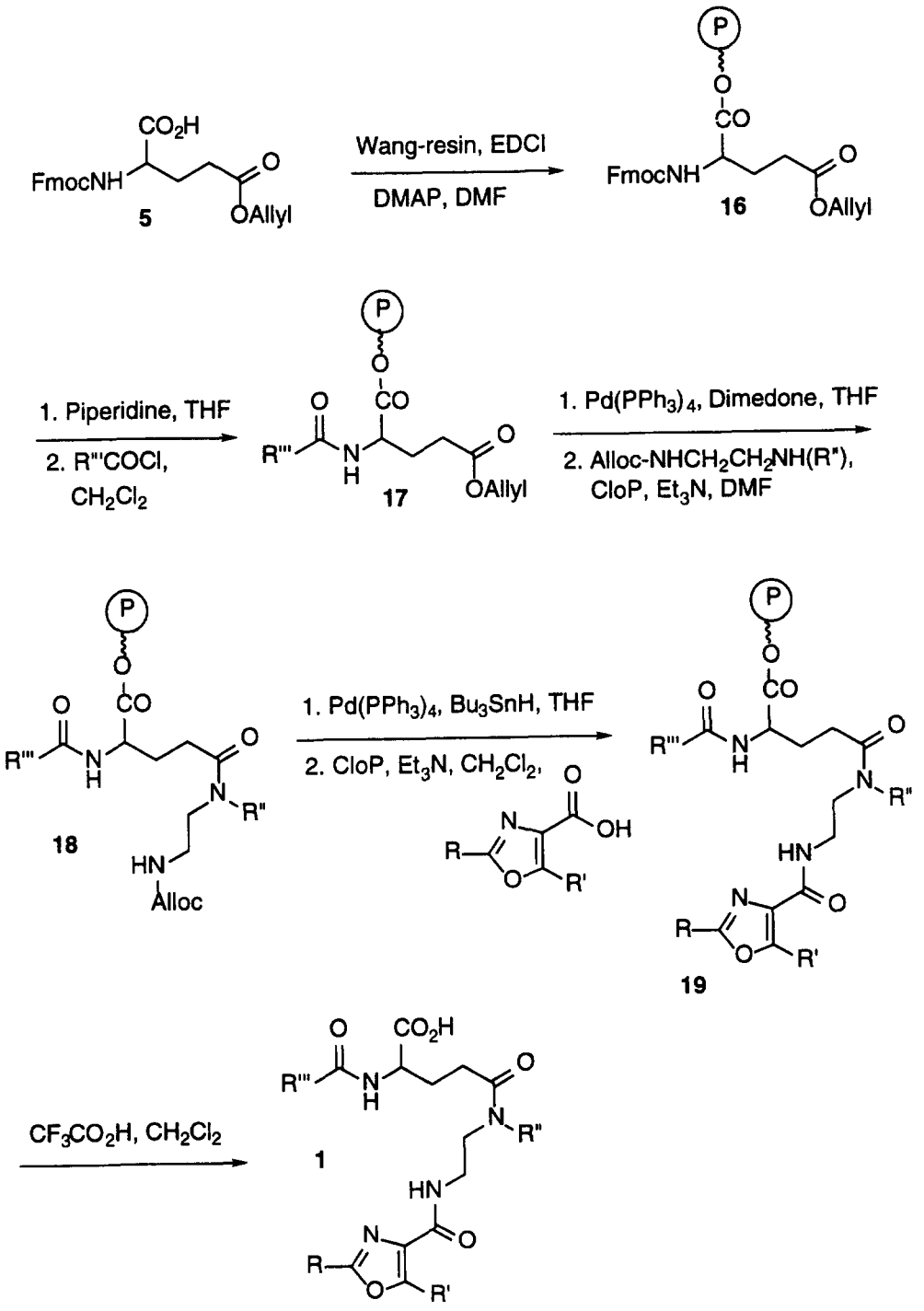
Scheme 3.

PSTPases are intracellular targets and, thus, we have examined the antiproliferative effects of members of the library to indirectly assess whether our compounds might enter cells. Exponentially growing human MDA-MB-231 breast carcinoma cells were exposed to all compounds at the highest available concentrations, which ranged from 30 to 100 μM. With the exception of two compounds, all lacked significant growth inhibitory activity. Compound **1i** caused 50% growth inhibition at 20 μM but had no further cytotoxicity at higher drug concentrations. Compound **1f** caused 50% growth inhibition at 100 μM and had a clear concentration-dependency (Fig. 5). Cell proliferation is coordinated by phosphorylation of cyclin-dependent kinases and tightly regulated by both kinases and phosphatases.[42] Thus, inhibition of Ser/Thr phosphatases such as PP2A or PP1 can result in disrupted cell cycle transition with restriction at discrete points in the cell cycle. Exponentially growing human MDA-MB-231 breast cancer cell populations (population doubling time of approximately 30–35 h) typically have approximately 50% of all cells in the S or DNA synthetic phase of the cell cycle (Fig. 6A,C). In contrast, when MDA-MB-231 cells were incubated for 48 h with 88 μM compound **1f**, there was prominent accumulation in the G1 phase with a concomitant decrease in both S and G2/M phases (Fig. 6B,C). Incubation of MDA-MB-231 cells for 72 h with 88 μM **1f** also caused a prominent accumulation in the G1 phase (Fig. 6D).

Discussion

Due to the limited character of previous SAR studies of the available natural product serine/threonine phosphatase inhibitors, the design of a small-molecule pharmacophore model has to allow for considerable structural variation. The combinatorial chemistry strategy is therefore ideally suited to address this problem. Among the characteristic structural features of calyculin A and the microcystins, the presence of a carboxylate, amide, oxazole, and lipophilic moieties are important features shared with our first generation lead structure **1**. The use of traditional amide coupling protocols combined with transition metal susceptible protective groups provided the basis for the parallel synthesis of 18 analogues of **1** via a solid-phase chemistry. We have begun to test this library both for biochemical and biological activity. Figure 4 demonstrates that the basic pharmacophore that we have

identified retains the ability to inhibit Ser/Thr phosphatases. We have not yet evaluated other members of our library with this assay, but inhibition of PP2A and PP1 has been established in preliminary studies for several members of our library. Compounds **1a–r** were further subjected to an assay for cytotoxicity and apoptosis in human breast carcinoma cells, and two members (**1h** and **f**) with an IC_{50} of <100 μM were found.[43] Interestingly, **1d**, which can block PP2A activity did not appear to be cytotoxic. This lack of biological activity may be due to poor cell penetration of cellular metabolism. Compound **1h** did not suppress cell proliferation significantly more than 50% in our assay and thus was not examined further. Compound **1f**, however, exhibited a concentration-dependent inhibition in proliferation of MDA-MB-231 cells and flow cytometry data confirmed blockage in cell cycle progression at the G1 checkpoint. Although PSTPase inhibitors such as okadaic acid and calyculin A often are found to block cells in G2/M, a concentration-depended cell cycle arrest at the G1/S interface similar to that seen by us has been detected with some cells.[44] An additional attractive target



Scheme 4.

Table 2. Test library of 18 structural variants of pharmacophore model **1** prepared according to *Scheme 4*

Compound	R	R′	R″	R‴
1a	Ph	CH_3	CH_3	n-C_9H_{19}
1b	Ph	CH_3	n-C_6H_{13}	n-C_9H_{19}
1c	Ph	CH_3	Bn	n-C_9H_{19}
1d	Ph	Ph	CH_3	n-C_9H_{19}
1e	Ph	Ph	n-C_6H_{13}	n-C_9H_{19}
1f	Ph	Ph	Bn	n-C_9H_{19}
1g	Ph	CH_3	CH_3	$PhCH_2CH_2$
1h	Ph	CH_3	n-C_6H_{13}	$PhCH_2CH_2$
1i	Ph	CH_3	Bn	$PhCH_2CH_2$
1j	Ph	Ph	CH_3	$PhCH_2CH_2$
1k	Ph	Ph	n-C_6H_{13}	$PhCH_2CH_2$
1l	Ph	Ph	Bn	$PhCH_2CH_2$
1m	Ph	CH_3	CH_3	PhCH=CH
1n	Ph	CH_3	n-C_6H_{13}	PhCH=CH
1o	Ph	CH_3	Bn	PhCH=CH
1p	Ph	Ph	CH_3	PhCH=CH
1q	Ph	Ph	n-C_6H_{13}	PhCH=CH
1r	Ph	Ph	Bn	PhCH=CH

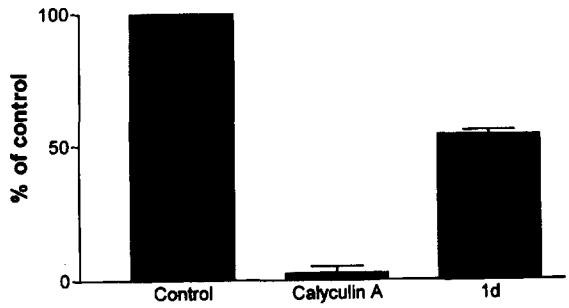


Figure 4. Inhibition of PP2A activity by compound **1d**. The catalytic subunit of PP2A was incubated with vehicle alone (control), calyculin A (10 nM), or **1d** (100 μM), and the dephosphorylation of the substrate fluorescein diphosphate determined spectrofluorometrically. Mean results to two independent experiments are shown; bars indicate the range.

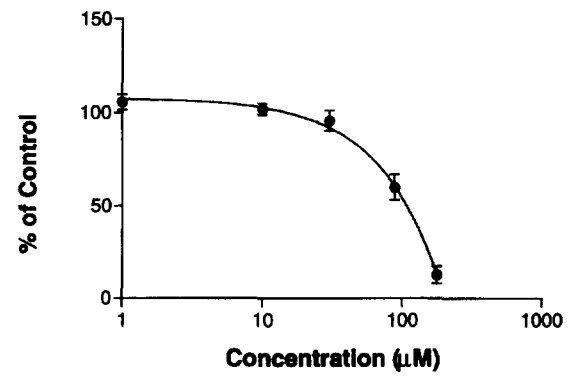


Figure 5. Antiproliferative effect of compound **1f** against human MDA-MB-231 breast cancer cells.

phosphatase that might control the G1/S transtion would be the dual specificity phophatase cdc25A.[45] Studies of the effect of **1a–r** on cdc25A and other phosphatases that may control cell cycle checkpoints are currently in progress.

These results clearly demonstrate the feasibility of using a combinatorial approach based on a natural product lead to identify novel antiproliferative and potential antineoplastic agents. Since cellular signal transduction is regulated by reversible enzymatic phosphorylation of serine, threonine and tyrosine residues on proteins, we expect that appropriately substituted, phosphatase-specific isomers of **1** will become important probes for transcription factor regulation, cell cycle control, and membrane and post-membrane signaling pathways. We are actively pursuing the synthesis and rapid-screening assays of much larger libraries based on **1** to identify more potent and more specific analogues.

Experimental Section

General methods

All glassware was dried in an oven at 150 °C prior to use. THF and dioxane were dried by distillation over Na/benzophenone under a nitrogen atmosphere. Dry CH_2Cl_2, DMF and CH_3CN were obtained by distillation from CaH_2.

2-Amino-pentanedioic acid 5-allyl ester (4). To a stirred suspension of 2.5 g (16.9 mmol) of L-glutamic acid (**3**) in 40 mL of dry allyl alcohol was added dropwise 5.4 mL (42.3 mmol) of chlorotrimethylsilane. The suspension was stirred at 22 °C for 18 h and poured into 300 mL of Et_2O. The resulting white solid was filtered off, washed with Et_2O, and dried in vacuo to provide 3.80 g (62%) of **4**: mp 133–134.5 °C (Et_2O); IR (KBr) 3152, 2972, 2557, 1738, 1607, 1489, 1450, 1289, 1366, 1264, 1223, 1177, 1146, 1121, 1084 cm^{-1}; ^1H NMR (D_2O): δ 5.8–5.7 (m, 1 H), 5.14 (dd, 1 H, J = 1.4, 17.3 Hz), 5.09 (dd, 1 H, J = 1.0, 10.4 Hz), 4.44 (d, 2 H, J = 5.6 Hz), 3.92 (t, 1 H, J = 6.8 Hz), 2.48 (t, 2 H, J = 7.0 Hz), 2.1–2.0 (m, 2 H); ^{13}C NMR (DMSO-d_6): δ 171.5, 170.6, 132.7, 117.9, 64.7, 51.2, 29.3, 25.2; MS (EI) *m/z* (relative intensity) 188 (63), 142 (72), 128 (27), 100 (21), 85 (100), 74 (32), 56 (73).

2-(9-H-Fluoren-9-ylmethoxycarbonlyamino)-pentanedioic acid 5-allyl ester (5). To 20 mL of dioxane was added 1.5 g (6.7 mmol) of ester **4**. The resulting suspension was treated with 16.8 mmol (17.7 mL of a 10% soln) of Na_2CO_3 at 0 °C, stirred for 5 min and treated with 1.74 g (6.7 mmol) of Fmoc-Cl dissolved in 10 mL of dioxane. The reaction mixture was warmed to 22 °C, stirred for 3 h, poured into 50 mL of H_2O and extracted with Et_2O (2 × 25 mL). The aq layer was cooled to 0 °C, acidified to pH 1 with conc HCl, and extracted with EtOAc (3 × 25 mL). The resulting organic layer was dried (Na_2SO_4) and concd in vacuo to give 2.72 g (99%) of **5** as a viscous oil: $[\alpha]_D$ +8.5° (*c*

2.8, $CHCl_3$, 21 °C); IR (neat) 3312, 3061, 2951, 2361, 2349, 2332, 1725, 1528,1447, 1414, 1325, 1254, 1117, 1078, 1049 cm^{-1}; ^1H NMR: δ 11.09 (br s, 1 H), 7.73 (d, 2H, J= 7.5 Hz), 7.57 (d, 2H, J=5.1 Hz), 7.4–7.25 (m, 4H), 6.0–5.85 (m, 1 H), 5.76 (d, 1H, J=8.1 Hz), 5.30 (d, 1H, J=19.5 Hz), 5.21 (d, 1H, J=10.5 Hz), 4.6–4.35 (m, 5H), 4.19 (t, 1H, J=6.6 Hz), 2.5–2.2 (m, 4H); ^{13}C NMR: δ175.6, 172.6, 156.2, 143.7, 143.5, 141.2, 131.7, 127.6, 127.0, 125.0, 119.9, 118.4, 67.1, 65.4, 53.1, 46.9, 30.2, 27.1; MS (EI) *m/e* (rel. int.) 409 (7), 351 (19), 338 (12), 280 (11), 239 (11), 196 (12), 178 (100), 165 (40); HRMS (EI) calcd for $C_{23}H_{23}NO_6$: 409.1525, found: 409.1501.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester 1-benzyl ester (6). To a soln of 1.5 g (36.6 mmol) of **5** in 5 mL of CH_2Cl_2 was added 0.42 mL (40.3 mmol) of benzyl alcohol, 0.912 g (47.6 mmol) of EDCI, and 45 mg (3.66 mmol) of dimethylaminopyridine (DMAP). The reaction mixture was stirred at 22 °C for 6 h, diluted with 20 mL of CH_2Cl_2, and extracted with H_2O (1 × 15 mL), 0.1 M HCl (2 × 15 mL), and brine (2 × 10 mL). The organic layer was dried (Na_2SO_4), concd in vacuo, and chromatographed on SiO_2 (hexanes:EtOAc, 5:1) to give 1.83 g (82%) of **6** as a white solid: mp 66.2–67.1 °C (EtOAc:hexanes); $[\alpha]_D$ +1.4° (*c* 1.64, $CHCl_3$, 21 °C); IR (neat) 3314, 1726, 1682, 1527, 1443, 1414, 1383, 1254, 1173, 1099, 1082, 980, 754, 735 cm^{-1}; ^1H NMR: δ 7.75 (d, 2 H, J=7.4 Hz), 7.59 (d, 2H, J=7.1 Hz), 7.41–7.27 (m, 9H), 5.95–5.85 (m, 1H), 5.44 (d, 1H, J=8.2 Hz), 5.34–5.19 (m, 4H), 4.56 (d, 2H, J=5.6 Hz), 4.5–4.4 (m, 3H), 4.21 (t, 1H, J=7.0 Hz), 2.5–2.0 (m, 4H); ^{13}C NMR: δ 172.2, 171.6, 155.8, 143.7, 143.5, 141.1, 135.0, 131.8, 128.5, 128.3, 128.1, 127.6, 126.9, 124.9, 119.8, 118.3, 67.2, 66.9, 66.2, 53.3, 47.0, 28.0, 27.3; MS (FAB, MNBA/MeOH) *m/z* (rel. int.) 500 ($[M+H]^+$, 40), 465 (8), 448 (14), 433 (12), 413 (8), 386 (38), 371 (24), 349 (9), 324 (16), 309 (26), 293 (11), 265 (10), 247 (24), 231 (56), 215 (39), 202 (26), 191 (24), 179 (67), 165 (48), 154 (67), 143 (31), 133 (71), 117 (100).

2-Decanoylamino-pentanedioic acid 5-allyl ester 1-benzyl ester (7). To a suspension of 1 g (2.0 mmol) of **6** in 10 mL of CH_2Cl_2 was added 1 g (8.2 mmol) of DMAP. The reaction mixture was stirred at 22 °C for

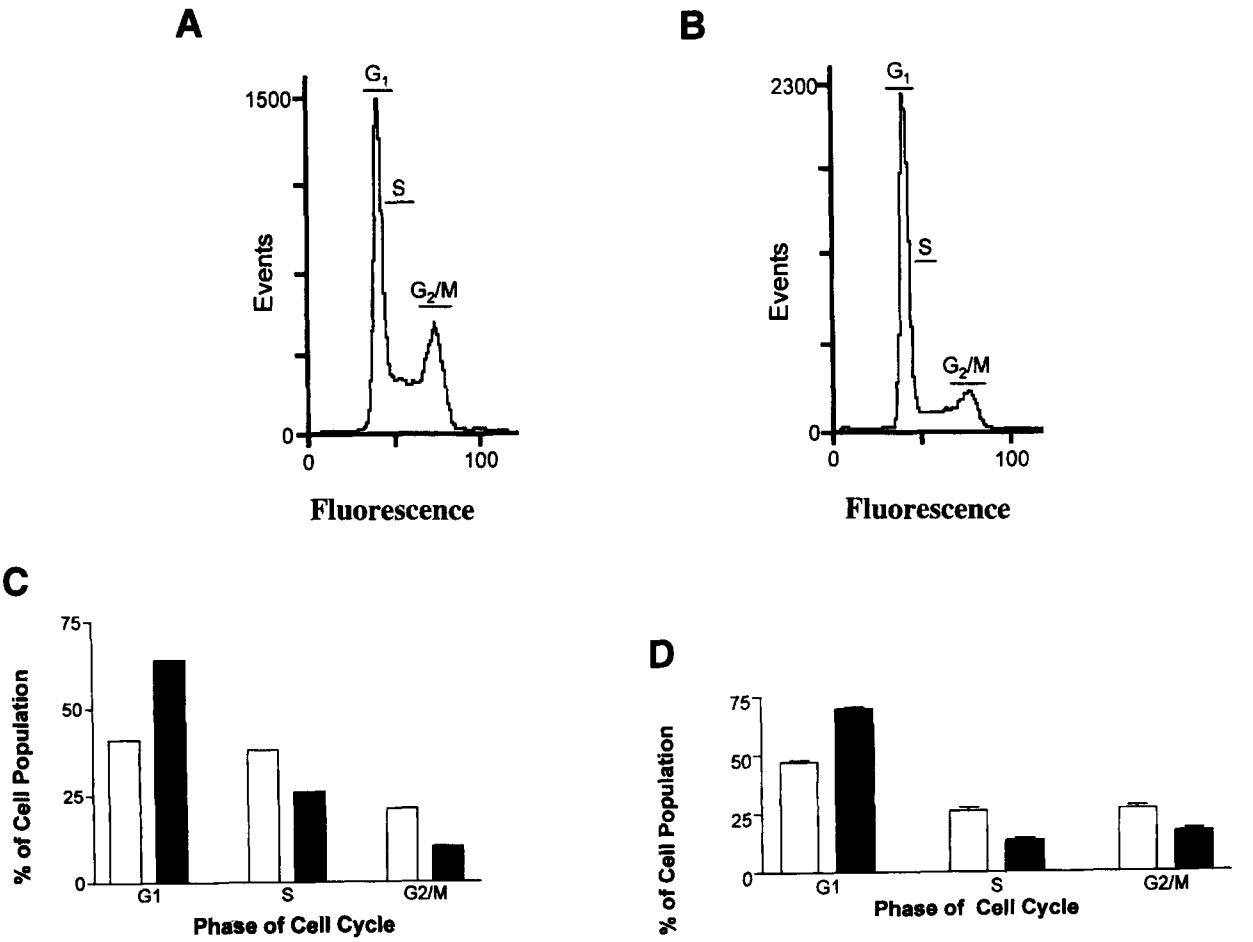


Figure 6. Cell cycle distribution of human breast cancer cells after treatment with compound **1f** determined by flow cytometry. Panel A. Flow cytometry analysis of MDA-MB-231 cells treated with vehicle alone. Panel B. Flow cytometry analysis 48 h after treatment with 88 μM compound **1f**. Fluorescence channel measures intracellular propidium iodide concentration, an index of DNA content. Horizontal bars are the gating positions that allow for cell cycle analysis. Panel C. MDA-MB-231 cell cycle distribution 48 h after continuous treatment with 88 μM compound **1f**. This is the result of one experiment. Open bars are control cells and black bars are cells treated with **1f**. Panel D. Cell cycle distribution 72 h after continuous treatment with 88 μM **1f**. The mean values were obtained from three independent determinations. Open bars are control cells and black bars are cells treated with 88 μM **1f**. The SE of the mean are displayed.

24 h, treated with 0.62 mL (3.0 mmol) of decanoyl chloride, stirred for 2 h at 22 °C, and extracted with satd Na_2CO_3 (2 × 10 mL). The organic layer was dried (Na_2SO_4), evapd to dryness, and the residue was chromatographed on SiO_2 (hexanes:EtOAc, 5:1) to give 548 mg (63%) of **7** as a viscous oil: IR (neat) 3293, 3063, 2924, 2855, 1740, 1649, 1534, 1453, 1379, 1175, 986, 930 cm^{-1}; ^1H NMR: δ 7.26 (s, 5H), 6.68 (d, 1H, J = 7.8 Hz), 5.85–5.75 (m, 1H), 5.22 (d, 1H, J = 17.3 Hz), 5.14 (d, 1H, J = 10.4 Hz), 5.08 (s, 2H), 4.63–4.57 (m, 1H), 4.48 (d, 2H, J = 5.6 Hz), 2.38–2.28 (m, 2H), 2.2–2.1 (m, 3H), 2.0–1.9 (m, 1H), 1.55 (t, 2H, J = 6.9 Hz), 1.20 (bs, 12H), 0.82 (t, 3 H, J = 5.9 Hz); ^{13}C NMR δ 173.0, 172.1, 171.6, 135.0, 131.7, 128.2, 128.1, 127.8, 117.9, 66.8, 64.9, 51.3, 36.0, 31.6, 29.9, 29.1, 29.0, 26.8, 25.3, 22.3, 13.8; MS (EI) *m/z* (rel. int.) 431 (12), 319 (21), 296 (51), 142 (100), 124 (31), 91 (91); HRMS (EI) *m/z* calcd for $C_{25}H_{37}NO_5$: 431.2672, found: 431.2673.

2-Decanoylamino-pentanedioic acid 1-benzyl ester (8). To a soln of 752 mg (1.74 mmol) of 2-decanoylamino-pentanedioic acid **7** in 10 mL of CH_2Cl_2 was added 100 mg (0.087 mmol) of tetrakistriphenylphosphine Pd(0) followed by 0.52 mL (1.9 mmol) of tributyltin hydride. After 15 min, the reaction mixture was quenched with 10 mL of a 10% HCl soln. The aq layer was reextracted with 15 mL of CH_2Cl_2 and the organic layer dried (Na_2SO_4), concd in vacuo, and chromatographed on SiO_2 (hexanes:EtOAc, 9:1) to provide 545 mg (79.9%) of **8** as a thick oil: $[\alpha]_D$ +2.8° (*c* 1.2, $CHCl_3$, 21 °C); IR (neat) 3351, 3064, 2995, 2852, 1738, 1712, 1657, 1536, 1454, 1380, 1364, 1265, 1209, 1183, 1121, 739 cm^{-1}; ^1H NMR: δ 10.9–10.7 (br s, 1 H), 7.22 (s, 5 H), 6.58 (d, 1H, J = 7.8 Hz), 5.09 (s, 2H), 4.63 (dd, 1H, J = 8.1, 12.9 Hz), 2.4–2.25 (m, 2H), 2.2–2.1 (m, 3H), 2.0–1.9 (m, 1H), (m, 6H), 1.53 (t, 2H, J = 6.6 Hz), 1.19 (br s, 12H), 0.81 (t, 3H, J = 6.0 Hz); ^{13}C NMR: δ 176.9, 174.0, 171.8, 134.9, 128.5, 128.4, 128.1, 67.3, 51.4, 36.2, 31.7, 29.9, 29.3, 29.2, 29.1, 27.0, 25.5, 22.5, 14.0; MS (EI) *m/z* (rel. int.) 391 (54), 373 (62), 279 (13), 256 (19), 178 (27), 178 (23), 155 (13), 146 (6), 130 (7), 102 (100); HRMS (EI) *m/z* calcd for $C_{22}H_{33}NO_5$: 391.2358, found: 391.2350.

4-[(2-Allyloxycarbonylamino-ethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid benzyl ester (10). To a soln of 526 mg (1.3 mmol) of **8** in 10 mL of CH_2Cl_2 was added 225 μL (1.61 mmol) of triethylamine and 320 mg (2.0 mmol) of secondary amine **9**. The solution was stirred at 22 °C for 5 min, treated with 710 mg (1.61 mmol) of benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent), stirred at 22 °C for 10 min, concd in vacuo, dissolved in 15 mL of EtOAc, and extracted with 2 M HCl soln. The organic layer was chromatographed on SiO_2 (hexanes:EtOAC, 1:3) to give 715 mg (94%) of **10** as a clear oil: $[\alpha]_D$ +5.3 (*c* 0.58, $CHCl_3$, 21 °C); IR (neat) 3420, 3250, 2924, 1713, 1680, 1657, 1642, 1632, 1537, 1495, 1470, 1455, 1252, 845 cm^{-1}; ^1H NMR: δ 7.35–7.2 (br s, 5 H), 6.97 (d, 0.3H, J = 7.5 Hz), 6.82 (d, 0.7 H, J = 7.3 Hz), 5.9–5.6 (m, 2H), 5.3–5.1 (m, 4H), 4.65–4.5 (m, 1H), 4.50 (d, 2H, J = 4.9 Hz); 3.55 (t, 1H, J = 7.0 Hz), 3.35–3.1 (m, 3H), 2.85 (s, 3H), 2.4–1.8 (m, 6H), 1.65–1.5 (m, 2H), 1.22 (bs, 12H), 0.84 (t, 3H, J = 6.1 Hz); ^{13}C NMR (MeOD): δ 176.4, 176.3, 174.4, 174.2, 173.2, 158.6, 137.1, 134.3, 134.2, 132.9, 129.5, 129.2, 129.1, 117.6, 117.4, 67.8, 66.3, 66.2, 53.5, 53.3, 39.6, 39.3, 36.7, 36.6, 34.2, 32.9, 30.5, 30.4, 30.3, 30.2, 29.7, 27.6, 26.8, 23.6, 14.5; MS (EI) *m/z* (rel. int.) 531 (16), 473 (37), 418 (16), 396 (26), 374 (38), 361 (17), 338 (87), 220 (54), 184 (52), 155 (36), 130 (29), 101 (37), 91 (100); HRMS (EI) *m/z* calcd for $C_{29}H_{45}N_3O_6$: 531.3308, found: 531.3316.

2-Decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyl)-butyric acid benzyl ester (2). To a soln of 193 mg (0.363 mmol) of **10** in 15 mL of CH_2Cl_2 was added 20 mg (0.018 mmol) of tetrakistriphenylphosphine Pd(0), 127 μL (0.472 mmol) of tributyltin hydride, and 20 μL of H_2O. The reaction mixture was stirred at 22 °C for 5 min, filtered through a plug of basic Al_2O_3 and treated with 150 mg (0.726 mmol) of oxazole **11**, 60 mL (0.436 mmol) of triethylamine, and 192 mg (0.436 mmol) of BOP reagent. The reaction mixture was stirred for 30 min at 22 °C, diluted with 10 mL of CH_2Cl_2, and extracted with satd $NaHCO_3$ soln, 1 M HCl, and brine. The organic layer was concd in vacuo and chromatographed on SiO_2 (hexanes:EtOAc, 1:1) to give 131 mg (57%) of **2** as a viscous oil: $[\alpha]_D$ −0.8° (*c* 1.32, $CHCl_3$, 21 °C); IR (neat) 3476, 3415, 3311, 3065, 2925, 2854, 1741, 1649, 1526, 1491, 1379, 1338, 1264, 1240, 1200, 1174, 1070, 711 cm^{-1}; ^1H NMR: δ 8.0–7.95 (m, 2H), 7.5–7.4 (m, 2H), 7.33 (br s, 6 H), 6.93 (d, 0.3H, J = 7.0 Hz), 6.85 (d, 0.7H, J = 7.2 Hz), 5.18–5.07 (m, 2H), 4.65–4.55 (m, 1H), 3.7–3.3 (m, 4H), 2.98 (s, 1H), 2.96 (s, 2H), 2.71 (d, 3H, J = 2.6 Hz), 2.6–2.0 (m, 6H), 1.58 (t, 2H, J = 6.8 Hz), 1.3–1.1 (br s, 12H), 0.86 (t, 3H, J = 6.9 Hz); ^{13}C NMR: δ 173.3, 172.8, 172.0, 171.9, 182.5, 158.6, 153.2, 152.8, 135.9, 130.7, 130.6, 129.7, 128.8, 128.5, 128.3, 128.2, 126.7, 126.5, 126.2, 66.9, 52.2, 52.1, 48.9, 47.6, 37.2, 37.1, 36.4, 36.3, 36.2, 34.1, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 26.8, 26.6, 25.5, 22.8, 14.1, 11.8; MS (EI) *m/z* (rel. int.) 632 (38), 497 (9), 405 (18), 374 (22), 260(21), 220 (42), 186 (56), 105 (18), 91 (100); HRMS calcd for $C_{36}H_{48}N_4O_6$: 632.3574, found: 632.3572.

(2-Chloro-ethyl)-carbamic acid allyl ester (13). A soln of 2.5 g (22 mmol) of chloroethylamine hydrochloride in 10 mL of 6 M NaOH was cooled to 0 °C and treated dropwise with 2.7 mL (25.9 mmol) of allyl chloroformate while keeping the pH at 9 by addition of 6 M NaOH soln. The reaction was then warmed to 22 °C, stirred for 2 h, and extracted with THF. The organic layer was dried (Na_2SO_4), concd in vacuo, and chromatographed on SiO_2 (hexanes:EtOAc, 9:1) to give 3.1 g (88%) of **13** as a yellow oil: IR (neat) 3333, 2949, 2348, 1705, 1647, 1529, 1433, 1368, 1248, 1190, 1144, 1061, 991, 929, 776 cm^{-1}; ^1H NMR: δ 6.05–5.85 (m, 1H), 5.55–5.35 (br s, 1H), 5.26 (dd, 1H, J = 1.5, 17.1 Hz), 5.18 (dd, 1H, J = 1.0, 10.4), 4.54 (d, 2H, J = 5.5 Hz), 3.57 (t, 2H, J = 5.5 Hz), 3.5–3.35 (m, 2H); ^{13}C NMR: δ 156.0, 132.5, 117.7, 65.6, 43.8, 42.7.

(2-Methylamino-ethyl)-carbamic acid allyl ester (9). A soln of 14 g (86 mmol) of **13** and 25 g (172 mmol) of NaI in 40 mL of acetone was refluxed for 18 h, concd in vacuo, dissolved in H_2O, and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and cooled to 0 °C. Methyl amine was bubbled through the reaction mixture until the solution was satd. The reaction mixture was warmed to 22 °C, stirred for 36 h, concd in vacuo and chromatographed on SiO_2 (EtOAc) to produce 6.14 g (45%) of **9** as a yellow oil: IR (neat) 3306, 2938, 2313, 1844, 1703, 1651, 1525, 1460, 1383, 1256, 1144, 995, 927, 775 cm^{-1}; ^1H NMR: δ 5.95–5.8 (m, 1 H), 5.28 (dd, 1H, $J=1.4$, 17.3 Hz), 5.18 (d, 1H, $J=10.4$ Hz), 4.54 (d, 2H, $J=5.3$ Hz), 4.9–4.6 (br s, 1 H), 3.34 (q, 2H, $J=5.6$ Hz), 2.79 (t, 2H, $J=5.6$ Hz), 2.47 (s, 3H); ^{13}C NMR: δ 157.2, 132.8, 117.6, 65.5, 50.7, 39.7, 35.4; MS (EI) *m/e* (rel. int.) 158 (32), 138 (17), 129 (25), 101 (13), 84 (12), 73 (13), 57 (100).

5-Methyl-2-phenyl-oxazole-4-carboxylic acid methyl ester (15). A soln of 750 mg (3.2 mmol) of **14** in 10 mL of CH_2Cl_2 was treated with 1.61g (3.8 mmol) of Dess–Martin reagent. The reaction was stirred at 22 °C for 10 min, concd in vacuo, and chromatographed on SiO_2 (hexanes:EtOAc, 3:2) to give 658 mg (89%) of 2-benzoylamino-3-oxo-butyric acid methyl ester. Alternatively, a soln of 9.12 g (38 mmol) of **14** in 80 mL of CH_2Cl_2 was cooled to −23 °C and treated with 16.1 mL (115 mmol) of triethylamine and a soln of 18.3 g (115 mmol) of SO_3–pyridine complex in 60 mL of dry DMSO. The reaction mixture was warmed to 22 °C, stirred for 30 min, then cooled to −48 °C and quenched with 20 mL of satd $NaHCO_3$. The soln was extracted with 50 mL of hexanes:EtOAc (2:1). The aq layer was reextracted with hexanes:Et_2O (2:1) and the combined organic layers were washed with brine, dried (Na_2SO_4), and chromatographed (hexanes:EtOAc, 3:2) to give 7.1 g (79%) of 2-benzoylamino-3-oxo-butyric acid methyl ester as a white solid: mp 112.7–113.3 °C (hexanes:EtOAc); IR (neat) 3402, 1734, 1662, 1599, 1578, 1510, 1478, 1435, 1354, 1269, 1156, 1121, 912, 804, 714 cm^{-1}; ^1H NMR: δ 8.2–8.1 (br s, 1H), 8.0–7.4 (m, 5H), 5.49 (s, 1H), 3.86 (s, 3H), 2.33 (s, 3H); ^{13}C NMR: δ 168.2, 167.2, 132.6, 132.5, 132.1, 128.7, 127.3, 83.9, 54.2, 23.2; MS (EI) *m/e* (rel. int.) 235 (13), 208 (18), 192 (8), 121 (7), 105 (100), 77 (58).

A soln of 277 mg (1.06 mmol) of triphenylphosphine, 268 mg (1.06 mmol) of iodine, and 0.29 mL (2.11 mmol) of triethylamine in 5 mL of CH_2Cl_2 was cooled to −48 °C and treated with a soln of 124 mg (0.528 mmol) of 2-benzoylamino-3-oxo-butyric acid methyl ester in 5 mL of CH_2Cl_2. The reaction mixture was warmed to 22 °C, stirred for 20 min, transferred to a separatory funnel and extracted with aq $Na_2S_2O_7$ followed by satd Na_2CO_3. The organic layer was concd in vacuo and chromatographed on SiO_2 (hexanes: EtOAc, 9:1) to give 84.4 mg (74%) of **15** as a white solid: mp 89.3–89.9 °C (hexanes:EtOAc); IR (neat) 3025, 1717, 1610, 1561, 1485, 1436, 1348, 1323, 1302, 1285, 1235, 1188, 1103, 1072, 1057, 1022 cm^{-1}; ^1H NMR 8.1–7.95 (m, 2H), 7.5–7.3 (m, 3H), 3.92 (s, 3H), 2.68 (s, 3H); ^{13}C NMR: δ 162.7, 159.5, 156.3, 130.8, 128.8, 128.6, 128.3, 126.4, 51.9, 11.98; MS (EI) *m/z* (relative intensity) 231 (6), 217 (51), 185 (55), 105 (100), 77 (41), 44 (64); HRMS (EI) *m/z* calcd for $C_{12}H_{11}NO_3$: 217.0739, found: 217.0729.

5-Methyl-2-phenyl-oxazole-4-carboxylic acid (11). A solution of 2.07 g (9.5 mmol) of **15** in 20 mL of 3 M NaOH and 12 mL of MeOH was stirred at 22 °C for 2 h and extracted with Et_2O. The aq layer was acidified to pH 1 with concd HCl and extracted with EtOAc. The organic layer was dried (Na_2SO_4), and concd in vacuo to give 1.84 g (95%) of **11** as an off-white solid: mp 182.3–182.6 °C (EtOAc:hexanes); IR (neat) 3200, 2950, 2932, 2890, 2363, 2336, 1694, 1682, 1611, 1563, 1483, 1450, 1337, 1255, 1192, 1117, 1053, 1020 cm^{-1}; ^1H NMR: δ 10.2–9.9 (br s, 1H), 8.2–7.9 (m, 2H), 7.6–7.4 (m, 3H), 2.75 (s, 3H); ^{13}C NMR: (CD_3OD) δ 164.6, 160.7, 157.4, 131.9, 129.8, 129.6, 127.3, 127.2, 12.1; MS (EI) *m/z* (rel. int.) 203 (53), 185 (24), 157 (13), 116 (17), 105 (100), 89 (21), 77 (33), 63 (16); HRMS calcd for $C_{11}H_9NO_3$: 203.0582, found: 203.0583.

Solid-phase chemistry

Step 1, 5→16. In a medium porosity Schlenk filter aparatus was placed 750 mg Wang resin (0.96 mmol/g, 0.72 mmol of active sites). The resin was suspended in 12 mL of dry DMF and a stream of nitrogen was forced up through the filter at a rate which allowed the solvent to gently bubble. To this reaction mixture was added 1.47 g (3.6 mmol) of **5**. The suspension was agitated for 5 min and treated with 26 mg (0.216 mmol) of DMAP and 550 mg (2.88 mmol) of EDCI, agitated at 22 °C for 18 h and filtered, and the resin was washed with DMF (2 × 10 mL), H_2O (3 × 10 mL), THF (3 × 10 mL), and CH_2Cl_2 (3 × 10 mL). The resin was dried under vacuum and the remaining active sites were capped by addition of 10 mL of CH_2Cl_2 and 10 mL of acetic anhydride along with 26 mg (2.88 mmol) of DMAP to the resin. Bubbling was continued at 22 °C for 3 h and the resin was then washed with CH_2Cl_2 (6 × 15 mL) and dried in vacuo. To test the loading on the resin, 30 mg of resin was removed and suspended in 2 mL of trifluoroacetic acid for 5 min at 22 °C, filtered and washed (3 × 3 mL) with CH_2Cl_2. The filtrate was concentrated in vacuo to give 7.3 mg (85%) of **5**.

Step 2, 16→17. A suspension of 690 mg (0.576 mmol) of 2-(9H-fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester linked to Wang resin (**16**) in 15 mL of THF was treated with 6 mL (57.6 mmol) of piperidine, agitated by bubbling for 30 min, filtered and washed with CH_2Cl_2 (6 × 10 mL). The resin was dried in vacuo. A suspension of this resin in 10 mL of CH_2Cl_2 was treated with 0.48 mL (2.31 mmol) of decanoyl chloride and 14 mg (0.115 mmol) of DMAP. The reaction mixture was agitated at 22 °C for 6 h, filtered and the resin was washed with CH_2Cl_2 (6 × 10 mL) and dried in vacuo.

Step 3, 17→18. A suspension of 690 mg (0.576 mmol) of 2-decanoylamino-pentanedioic acid 5-allyl ester linked to Wang resin (**17**) in 10 mL of THF was treated with 67 mg (0.0576 mmol) of tetrakis(triphenylphosphine)palladium(0) and 806 mg (5.75 mmol) of dimedone, and agitated by bubbling at 22 °C for 18 h. The resin was then filtered, washed with THF (2 × 10 mL), CH_2Cl_2 (2 × 10 mL), MeOH (2 × 10 mL), H_2O (2 × 10 mL), 1% HOAc soln (2 × x10 mL), H_2O (2 × 10 mL), MeOH (2 × 10 mL), CH_2Cl_2 (2 × 10 mL), and dried in vacuo. Cleavage and examination of 40 mg of resin by ^{1}H NMR showed full deprotection of the allyl ester.

A suspension of this resin in 12 mL of DMF was treated with 0.22 mL (1.572 mmol) of triethylamine and 414.1 mg (2.62 mmol) of Alloc-$NHCH_2CH_2NHMe$. After agitating the reaction mixture for 5 min to ensure proper mixing, 540 mg (1.572 mmol) of CloP was added. The reaction mixture was agitated with bubbling for 18 h at 30 °C, cooled to 22 °C, and the resin was filtered and washed with DMF (2 × 10 mL), CH_2Cl_2 (2 × 10 mL), MeOH (2 × 10 mL), H_2O (2 × 10 mL), THF (2 × 10 mL), and CH_2Cl_2 (2 × 10 mL). The resin was dried in vacuo and 40 mg of resin was cleaved with CF_3CO_2H. The ^{1}H NMR of the residue showed that coupling had occurred to nearly 100%.

Step 5, 18→19. A suspension of 200 mg (0.192 mmol) of 4-[(2-allyloxycarbonylamino-ethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid linked to Wang resin (**18**) in 6 mL of CH_2Cl_2 was treated with 12 mg (0.0096 mmol) of tetrakistriphenylphosphine Pd(0), 62 ml (0.230 mmol) of tributyltin hydride, and 10 µl of H_2O. The reaction mixture was agitated with bubbling N_2 for 15 min, filtered, and the resin was washed with 10 mL portions of CH_2Cl_2, THF, acetone, MeOH, H_2O, acetone, EtOAc, hexanes, THF, and CH_2Cl_2. The resin was then dried in vacuo and 15 mg was removed for testing. The ^{1}H NMR of the TFA-cleaved residue showed full deprotection as well as full removal of all tin side products.

A suspension of 185 mg (0.190 mmol) of this resin in 8 mL of CH_2Cl_2 was treated with 117 mg (0.576 mmol) of oxazole carboxylic acid, 198 mg (0.576 mg) of CloP, and 80 µl (0.576 mmol) of triethylamine. The reaction mixture was agitated by bubbling with N_2 for 3 h, filtered, and washed with 20 mL of CH_2Cl_2, acetone, water, acetone, and CH_2Cl_2. The resin was dried in vacuo and 15 mg was removed for testing. The ^{1}H NMR of the residue showed that the reaction had gone to 60% completion. The resin was subsequently submitted to a second coupling cycle.

Step 6, 19→1. A suspension of 115 mg (0.12 mmol) of 2-decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyl)-butyric acid linked to Wang resin (**19**) in 3 mL of TFA was stirred for 5 min, filtered, and washed with 5 mL of CH_2Cl_2. The extract was concd in vacuo to provide 33.1 mg (100% for step 2 to step 6) of **1**. A ^{1}H NMR showed the product to be 66% pure with 2-acylamino-pentanedioic acid as the major impurity. Acid **1a** was dissolved in 3 mL of CH_2Cl_2 and treated with 0.016 mL (0.138 mmol) of benzyl bromide and 0.02 mL (0.138 mmol) of DBU to provide material identical with the benzyl ester **2** prepared by solution phase chemistry.

Cell culture

Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection at passage 28 and were maintained for no longer than 20 passages. The cells were grown in RPMI-1640 supplemented with 1% penicillin (100 µg/mL) and streptomycin (100 µg/mL), 1% L-glutamate, and 10% fetal bovine serum in a humidified incubator at 37 °C under 5% CO_2 in air. Cells were routinely found free of mycoplasma. To remove cells from the monolayer for passage or flow cytometry, we washed them two times with phosphate buffer and briefly (< 3 min) treated the cells with 0.05% trypsin/2 mM EDTA at room temperature. After the addition of at least two volumes of growth medium containing 10% fetal bovine serum, the cells were centrifuged at 1000 ***g*** for 5 min. Compounds were made into stock solns using DMSO, and stored at −20 °C. All compounds and controls were added to obtain a final concn of 0.1–0.2% (v/v) of the final soln for experiments.

PP2A assay

The activity of the catalytic subunit of bovine cardiac muscle PP2A (Gibco-BRL, Gaithersburg, MD) was measured with fluorescein diphosphate (Molecular Probes, Inc., Eugene, OR) as a substrate in 96-well microtiter plates. The final incubation mixture (150 µL) comprised 25 mM Tris (pH 7.5), 5 mM EDTA, 33 µg/mL BSA, and 20 µM fluorescein diphosphate. Inhibitors were resuspended in DMSO, which was also used as the vehicle control. Reactions were initiated by adding 0.2 units of PP2A and incubated at room temperature overnight. Fluorescence emission from the product was measure with Perseptive Biosystems Cytoflour II (exciton filter, 485 nm; emission filter, 530 nm) (Framingham, MA).

Cell proliferation assay

The antiproliferative activity of newly synthesized compounds was determined by our previously described method.[46] Briefly, cells (6.5×10^3 cells/cm^2) were plated in 96 well flat bottom plates for the cytotoxicity studies and incubated at 37 °C for 48 h. The plating medium was aspirated off 96 well plates and 200 µL of growth medium containing drug was added per well. Plates were incubated for 72 h, and then washed 4× with serum free medium. After washing, 50 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide soln (2 mg/mL) was added to each well, followed by 150 µL of complete growth medium. Plates were then incubated an additional 4 h at 37 °C. The soln was aspirated off, 200 µL of DMSO added, and the plates were shaken for 30 min at room

temperature. Absorbance at 540 nm was determined with a Titertek Multiskan Plus plate reader. Biologically active compounds were tested at least three independent times.

Measurement of cell cycle kinetics

Cells (6.5×10^5/cm^2) were plated and incubated at 37 °C for 48 h. The plating medium was then aspirated off, and medium containing a concentration of compound **1f** that caused approximately 50% growth inhibition (88–100 μM) was added for 48–72 h. Untreated cells at a similar cell density were used as control populations. Single cell preparations were fixed in ice-cold 1% paraformaldehyde, centrifugation at 1000 ***g*** for 5 min, resuspended in Puck's saline, centrifuged, and resuspended in ice-cold 70% ethanol overnight. The cells were removed from fixatives by centrifugation (1000 ***g*** for 5 min) and stained with a 5 μg/mL propidium iodide and 50 μg/mL RNase A solution. Flow cytometry analyses were conducted with a Becton Dickinson FACS Star. Single parameter DNA histograms were collected for 10,000 cells, and cell cycle kinetic parameters calculated using DNA cell cycle analysis software version C (Becton Dickinson). Experiments at 72 h were performed at least three independent times.

Acknowledgment

Funding was provided by the American Cancer Society (Junior Faculty Research Award to P.W.), Upjohn Co., the Alfred P. Sloan Foundation. and a United States Army Breast Cancer Predoctoral Fellowship. We thank Drs Boynton and Messner for their preliminary biochemical evaluation of **1a-r**.

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(*Received in U.S.A.* 2 *February* 1996; *accepted* 31 *May* 1996)