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Insight into Binding of Calyculin A to Protein Phosphatase 1: Isolation of Hemicalyculin A and Chemical Transformation of Calyculin A

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Summary

Calyculin A isolated from the marine sponge *Discodermia calyx* is a potent inhibitor of protein phosphatases 1 and 2A. We attempted to elucidate its mode of binding to the enzymes by examining the activity of natural and chemically transformed derivatives. Ten natural derivatives including a new compound, hemicalyculin A, were provided. The structure of hemicalyculin A, which comprises the southern hemisphere of calyculin A, was firmly established by chemical methods. Six compounds were prepared by selective modifications of functional groups in calyculin A. The enzyme inhibitory activity of these compounds indicated that 17-phosphate, 13-hydroxyl, and the hydrophobic tetraene moieties were all necessary for binding to the enzymes. The derivatives lacking the peptide portion were less cytotoxic even when they possessed full enzyme inhibitory activity.

Introduction

Reversible protein serine/threonine phosphorylation regulates numerous cellular functions, including muscle contraction, neurotransmission, cell proliferation, carcinogenesis, and apoptosis [1–3]. A large number of protein kinases have been well investigated for several decades [4–6], but it has not been until recently that our understanding on protein phosphatases, namely PP1, PP2A, PP2B, and PP2C, has been considerably deepened by the discovery of natural product inhibitors [7].

PP1 and PP2A are strongly inhibited by a diverse array of natural products, e.g., polyether polyketides such as okadaic acid (**1**) and tautomycin (**2**), nonribosomal peptides represented by microcystin LR (**3**), and polyketide/nonribosomal peptides such as calyculin A (**4**) (Figure 1). Interestingly, these compounds contain acidic moieties that are in fact essential for their inhibitory activity [7]. The crystal structure of the complex of PP1c and microcystin LR clearly show the ionic, hydrophobic, and covalent interactions between the inhibitor and the enzyme, in which the Masp/Glu, Adda, and Mdha residues of microcystin LR are involved [8]. Although a number of molecular modeling studies have been done for complexes between PP1 and other inhibitors, there is no clear evidence for their modes of binding to the enzyme [9–14]. A crystal structure of the complex between okadaic acid and PP1c has recently been reported [15].

Calyculin A (**4**) was isolated from the marine sponge *Discodermia calyx* as an antitumor agent, and it was later found to inhibit PP1 and PP2A with IC_{50} values of 1.4 and 2.6 nM, respectively [16]. It is also a potent tumor promoter; this activity is ascribed to inhibition of the protein phosphatases [17]. Seventeen natural calyculin derivatives, including clavosines, 21-glycosylated derivatives, have been isolated from several marine sponges [18–24]. Initially, we reported that dephosphonocalyculin A was as potent as calyculin A in inhibition of PP1 and PP2A [23]. This is puzzling because the acidic group was considered to be essential for the inhibition of the enzymes, which was indicated by molecular modeling studies of complexes between PP1 and calyculins based on the PP1c/microcystin LR complex model [9–14]. In addition to this point, other structural requirements for the inhibition of PP1 remained unclear. To clarify these important issues, we attempted natural-products chemical approaches; isolation of new congeners, and preparation of fragments of calyculin A. We also purified natural calyculin derivatives very carefully before evaluating their biological activities.

In this paper we describe the isolation and structure of hemicalyculin A, a truncated calyculin A, from *D. calyx*, preparation of derivatives of calyculin A, and PP1 and PP2A inhibition and cytotoxicity of these derivatives and of natural calyculins previously obtained. We propose that 17-phosphate, 13-hydoxyl, and tetraene moieties are essential for inhibition of PP1 and PP2A.

Results

Isolation and Structure Elucidation of a New Compound, Hemicalyculin A (5)

In the course of bioassay-guided fractionation of the extract of *D. calyx*, we have discovered a prominent new derivative, hemicalyculin A (**5**). The organic fraction of the crude extract was chromatographed over silica gel. Fractions that inhibited PP1 were further separated by ODS flash chromatography followed by HPLC to afford hemicalyculin A (**5**, 1.2×10^{-4}% wet weight), which showed UV absorption at 340 nm but no absorption at 230 nm, indicating the lack of the oxazole unit.

Hemicalyculin A (**5**) had a molecular formula of $C_{36}H_{55}N_2O_{10}P$, as established by HRFABMS and NMR spectral data. The ^1H NMR spectrum exhibited diagnostic signals for calyculins: an *O*-methyl, five singlet *C*-methyls, and three doublet methyls, while the signals for an oxazole and *N*-methyls were missing. The ^{13}C NMR spectrum revealed the presence of a phosphate on C17 because pertinent signals (C16, C17, and H17) were doubled because of coupling to a phosphorus

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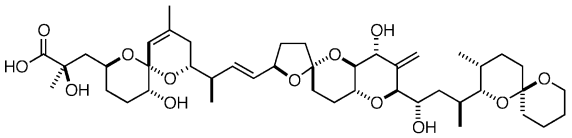
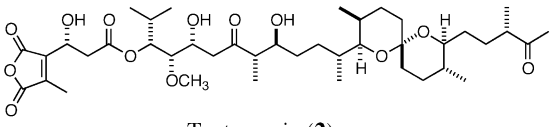
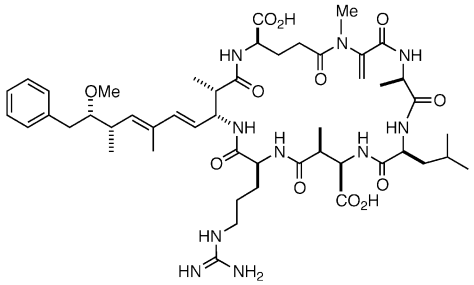


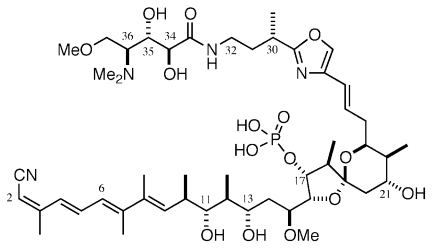
Okadaic acid (**1**)



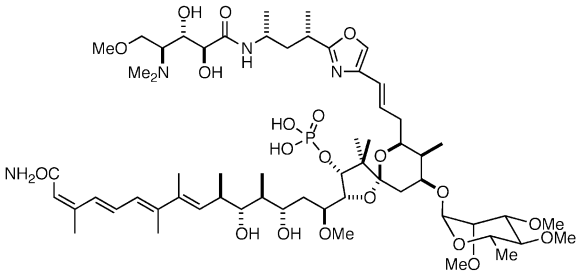
Tautomycin (**2**)



Microcystin-LR (**3**)



Calyculin A (**4**)



Clavosine A (**21**)

Figure 1. Structures of Protein Phosphatase Inhibitors

atom. Interpretation of the COSY and HMQC spectra disclosed that the structural unit C1 through C26 was identical to that of calyculin A (**4**). HMBC crosspeaks among H25, H26, and a carbon at 118.8 ppm were reminiscent of a nitrile group attached to C26. NOESY correlations implied that the geometry of the tetraene unit was identical to that in **4**.

^{1}H, ^{1}H-coupling constants and NOESY data showed that the relative stereochemistry of the portion from C1 to C8 and from C15 to C25 in **5** was the same as in **4**. However, the stereochemistry of the C9–C13 portion could not be firmly determined on the basis of NMR data. Accordingly, we attempted to obtain the common core portion from each compound that contained all chiral centers by oxidative cleavage of the olefinic bonds between C8 and C9 and between C25 and C26 (Figure 2). Oxidation of **4** with RuO_4, $OsO_4/NaIO_4$, or O_3 afforded inseparable mixtures. In order to circumvent the problem, we sought to fully protect calyculin A before ozonolysis. The 11,13-diol was selectively protected as an acetonide to afford **6**, whose 34,35-diol was then cleaved with $NaIO_4$, followed by reduction with $NaBH_4$ to furnish **7**. The phosphate group in **7** was protected by *p*-bromobenzylation, yielding compound **8**, ozonolysis of which proceeded smoothly to afford **9**. Hemicalyculin A (**5**) was similarly treated to give **9**, which was spectroscopically indistinguishable from its counterpart derived from **4**. Therefore, all the stereogenic centers in hemicalyculin A (**5**) were shown to have the same absolute stereochemistry as the corresponding centers in calyculin A (**4**).

After completion of the above chemical transformations, we noticed that oxidative cleavage of an oxazole affords a nitrile [25]. This reaction proceeds through an epidioxide intermediate, which then undergoes rearrangement to form a cyanide and an anhydride. In a similar fashion, calyculin A (**4**) may be converted to hemicalyculin A (**5**). In fact, calyculin A furnished **5** in 5% yield upon treatment with singlet oxygen. It should be noted that the reaction barely proceeds in the absence of methylene blue. Therefore, we believe that hemicalyculin A (**5**) is not an artifact formed from **4** during isolation work.

Chemical Transformation of Calyculin A

In order to obtain further information of the structure-activity relationships for the calyculins, we carried out several chemical transformations of calyculin A (**4**).

After several trials to protect the diol groups in caly-

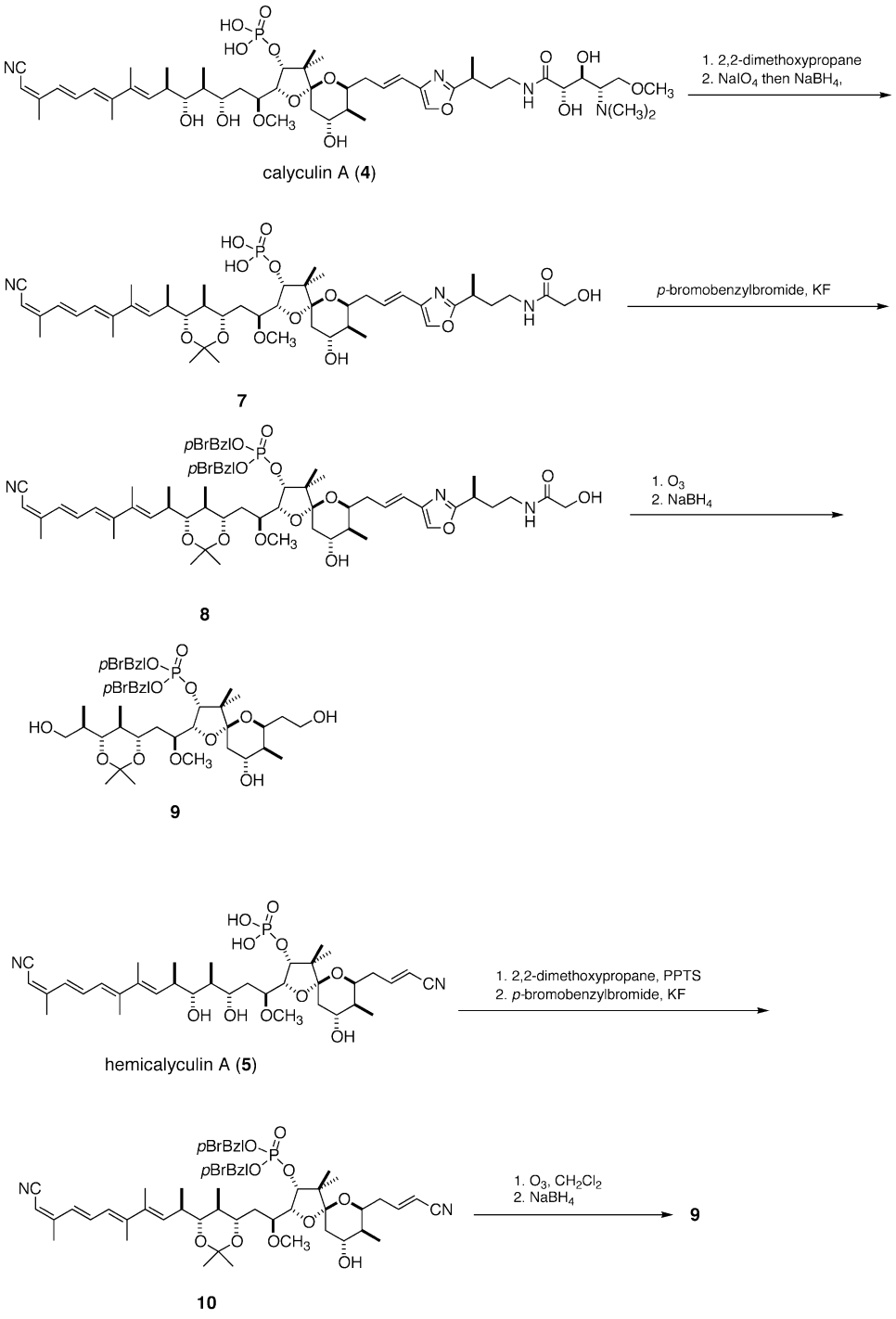
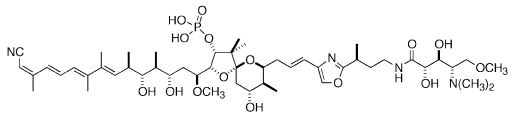
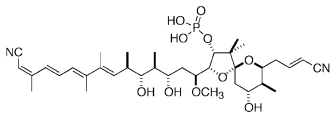
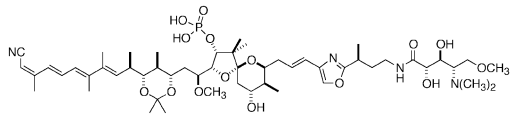
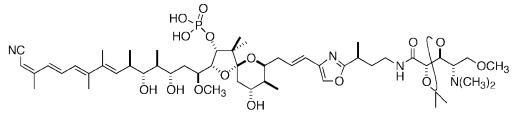
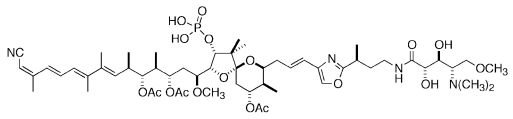
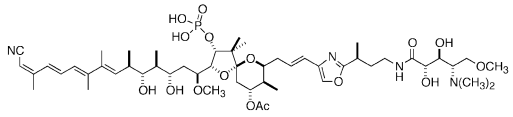
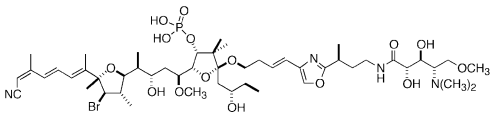
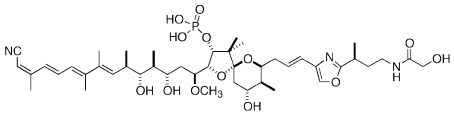


Figure 2. Preparation of 9 from Calyculin A (4) and Hemicalyculin A (5)

culin A (4), we selected the acetonide. Upon isopropylidenation, calyculin A preferentially afforded 11,13-*O*-isopropylidene-calyculin A (6); the second acetonide group at the 34,35-diol was introduced only at elevated temperatures. It should be noted that the 11,13-acetonide was removable with 80% AcOH, whereas it was not possible to deprotect the 34,35-acetonide without affecting other functionalities. A similar phenomenon was observed in the removal of the 34,35-acetonide from the fully protected calyculin A [26]. Fortunately, these different reactivities of the two acetonides allowed us to prepare 11,13-*O*-isopropylidenecalyculin A (6) and 34,35-*O*-isopropylidenecalyculin A (11).

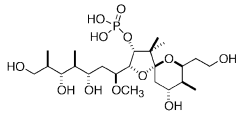
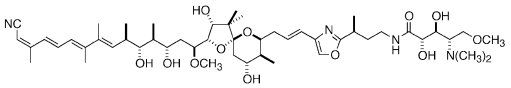
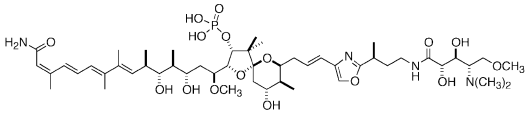
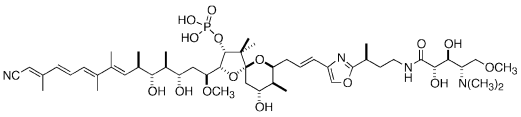
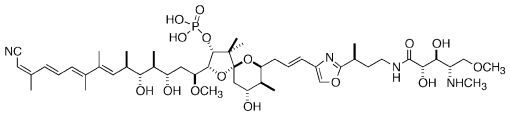
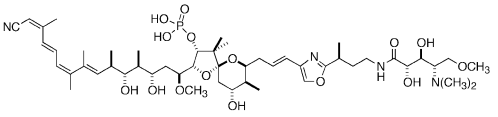
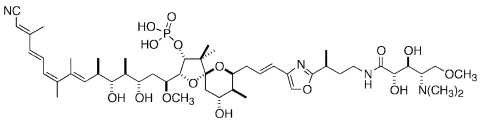
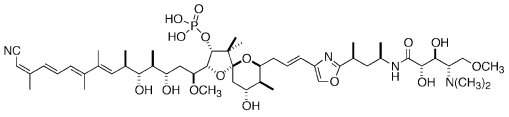
Treatment of calyculin A with Ac_2O/pyridine provided a complex mixture. FABMS and HPLC analyses of the reaction mixture indicated that calyculin A peracetate was formed. However, undesired reactions took place during work-up, as revealed by HPLC. NMR and MS analyses of the products indicated that the complexity arose from the hydrolysis of the acetates at C34 and

Table 1. Protein Phosphatase Inhibitory Activity of Calyculin Derivatives

Compound	IC_{50} (PP1γ)	IC_{50} (PP2A)
 Calyculin A (**4**)	8.2 nM	1.0 nM
 Hemicalyculin A (**5**)	14 nM	1.0 nM
 11,13-*O*-isopropylidene-calyculin A (**6**)	>10 μM	>10 μM
 34,35-*O*-isopropylidene-calyculin A (**11**)	7.8 nM	1.1 nM
 Calyculin A 11,13,21-triacetate (**12**)	>10 μM	>10 μM
 Calyculin A 21-acetate (**13**)	13 nM	2.1 nM
 Calyculin J (**14**)	105 nM	20 nM
 C1/C34-Calyculin A (**15**)	9.0 nM	1.1 nM

(*continued*)

Table 1. Continued

Compound	IC_{50} (PP1γ)	IC_{50} (PP2A)
 C9/C25-calyculin (**16**)	>10 μM	>10 μM
 Dephosphonocalyculin A (**17**)	>10 μM	>10 μM
 Calyculinamide A (**18**)	5.0 nM	1.5 nM
 Calyculin B (**19**)	112 nM	9.0 nM
 Des-*N*-methylcalyculin A (**20**)	11 nM	1.0 nM
 Calyculin E (**22**)	185 nM	14 nM
 Calyculin F (**23**)	55 nM	7.5 nM
 Calyculin C (**24**)	29 nM	2.6 nM

C35. The selectivity appeared to be due to the basicity of the neighboring dimethylamino group. When the acetylation product was kept in MeOH for 10 days, methanolysis of the 34- and 35-acetates proceeded, and the product was converged to calyculin A 11,13,21-triacetate (**12**).

To evaluate the contribution of the 21-hydroxyl group, we selectively acetylated this group. For that purpose, 11,13-*O*-isoprolylidene-calyculin A (**6**) was peracetylated, and the product was kept in MeOH for 10 days to remove the acetyl groups at C34 and C35. Removal of the 11,13-acetonide with aqueous AcOH provided calyculin A 21-acetate (**13**).

Because X-ray crystallography revealed that the 36-dimethylamino group significantly contributed to stabilization of the pseudocyclic conformation of calyculin A (**4**) by forming a salt bridge with the 17-phosphate group [18], the effect of this group on biological activities of calyculins is interesting. Although selective modification of this group was not possible, it was removed by cleavage of the bond between C34 and C35. However, upon $NaIO_4$ oxidation of the unprotected calyculin A, the cleavage of the relevant bond took place with concomitant formation of an iodinated tetrahydrofuran ring analogous to the one in calyculin J (**14**) [19]. In order to prevent this side reaction, 11,13-*O*-isopropylidenecalyculin A (**6**) was oxidized with $NaIO_4$ followed by reduction with $NaBH_4$ and deprotection of the acetonide to afford C1/C34-calyculin A (**15**).

Although proposed by modeling studies, the importance of the hydrophobic tail of calyculin A has not been demonstrated experimentally. Therefore, we prepared C9/C25-calyculin (**16**) that lacked the tetraene unit and the portion from C26 to C45. Removal of the acetonide in fragment **9** with 80% AcOH followed by hydogenolysis afforded compound **16**.

Protein Phosphatase Inhibitory Activities

The enzyme inhibitory activities of ten natural [18–20, 22, 23] and six chemically transformed derivatives are shown in Table 1. Eight compounds exhibited activity as potent as that of calyculin A; four of them were essentially inactive, and the remaining four derivatives had partly lost activity.

Discussion

The Phosphate Group on C17

All potent natural phosphatase inhibitors so far obtained contain one or two acidic groups, which are the carboxylic acid in microcystin LR (**3**), okadaic acid (**1**), and tautomycin (**2**) and the phosphate group in calyculin A (**4**) [7]. It is widely accepted that these acidic groups mimic the phosphorylated serine or threonine residues in the substrate.

In our earlier studies, dephosphonocalyculin A (**17**) inhibited both PP1 and PP2A as strongly as calyculin A, which led to the hypothesis that the structural part of calyculin A, other than the phosphate group, would participate in binding to the enzymes [23]. During purification of hemicalyculin A (**5**), we experienced that a fraction that gave one peak in ODS-HPLC with aqueous MeOH as the mobile phase was further separable by HPLC with aqueous MeCN. The dephosphonocalyculin A-rich fraction obtained during isolation of hemicalyculin A (**5**), which gave a single peak in the HPLC with aqueous MeOH and had significant enzyme-inhibitory activity, was further purified by HPLC with aqueous MeCN, which afforded a preparation of dephosphonocalyculin A (**17**) with essentially no enzyme-inhibitory activity. It is likely that the potent activity of the intermediate HPLC fraction was due to the presence of a highly active contaminant(s). However, at this moment we have no idea as to the character of the active species. We now conclude that the phosphate group in calyculin A is absolutely essential for the inhibition of PP1 and PP2A.

The 1-Cyano Group

Calyculinamide A (**18**) is as potent as calyculin A (**4**); the two compounds differ only in the terminal structure. The former has an amide terminus, whereas the latter has a cyano group. It is possible that the nitrile and amide groups in both compounds may interact with the enzyme through the formation of one or more hydrogen bonds. A significantly lower activity of calyculin B (**19**) [19] may be ascribable to either a loss of the hydrogen bonding interaction or a repulsion caused by the displaced cyano group.

The Dimethylamino Group at C36

Dimethylamino groups are found in many potent bioactive compounds and are considered to play an important role in exerting their activities [27, 28]. In our earlier study, des-*N*-methyl-calyculin A (**20**) was significantly less active than calyculin A (**4**) [22]. Reexamination with larger amounts of des-*N*-methyl-calyculin A (**20**) resulted in comparable activity to that of calyculin A, suggesting that the basicity of the dimethylamino group is less important in binding to the enzymes, which was also supported by potent activity observed in C1/C34-calyculin A (**15**).

Hydroxyl Groups

Among a total of five hydroxyl groups in calyculin A, the one on C21 is clearly not involved in binding to the enzyme, which was indicated by potent activity of clavosine A (**21**) (Figure 1), a glycosylated derivative of 21-*epi*-calyculinamide C [24]. This was supported by retained activity of calyculin A 21-acetate (**13**). 34,35-*O*-isopropylidenecalyculin A (**11**) was as active as calyculin A and thus demonstrated the irrelevance of the hydroxyl groups on C34 and C35 in enzyme inhibition. This is consistent with the potent activity of C1/C34-calyculin A (**15**), which lacks the 35-hydroxyl group.

In stark contrast to the above-mentioned derivatives, 11,13-*O*-isopropylidenecalyculin A (**6**) did not inhibit the enzyme even at 10 μg/ml. X-ray crystallography and NMR analysis of calyculin A [17] showed that the C1–C15 side chain is not in the fully extended conformation due to the repulsion of the methyl groups on C10 and C12; the chain bends at the bond between C10 and C11. It would be possible that incorporation of a rigid dioxane ring might prevent the side chain from adopting the specific conformation required for activity. In order to

Table 2. Cytotoxicity against P388

Calyculin A (**4**)	0.170
Hemicalyculin A (**5**)	450
C1/C34-calyculin A (**15**)	40
Dephosphonocalyculin A (**17**)	>5000
11,13-*O*-isopropylidenecalyculin A (**6**)	2000

IC_{50} is in ng/ml

disprove the idea, we examined calyculin A 11,13,21-triacetate (**12**). The triacetate was also inactive, substantiating the importance of the hydroxyl groups on C11 and C13. Calyculin J (**14**), which lacks the 11-hydroxyl group [22], was as active as calyculin B (**19**). If the two compounds' diminished activities compared with that of calyculin A are due to the perturbation caused by the disorientation of the hydrophobic tail with respect to the spiroketal [11], the contribution of the 11-hydroxyl group to the activity appears to be minimal.

Oxazole Ring and the C30–C32 Linker

The oxazole ring placed near the center of the molecule functions not only to force the linear molecule to bend but also to form a salt bridge with the phosphate; it thereby serves to determine the overall shape of calyculin A (**4**) in the solid [18] and liquid [29] states. In spite of its architectural importance, the oxazole ring was shown not to be required for the activity since hemicalyculin A (**5**) retained the almost identical activity as calyculin A. The potent activity of hemicalyculin A (**5**) clearly demonstrated that the densely functionalized γ-amino acid and the C30–C32 linker were also unnecessary for enzyme inhibition.

Tetraene Terminus

All potent natural inhibitors, such as okadaic acid (**1**) and microcystin LR (**3**), have a bulky hydrophobic tail, which was shown to be important for enzyme inhibition [7]. The crystal structure of a PP1c/microcystin-LR complex showed that the Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4*E*,6*E*-decadienoic acid] side chain of microcystin-LR (**3**) is accommodated in the hydrophobic groove [8]. The SAR studies of microcystins revealed that the congeners with the 6*Z*-Adda residue were less active [30], implying the importance of the shape of the hydrophobic tail for accommodating the hydrophobic groove of the enzyme [8]. In contrast with this idea, calyculins A (**4**), B (**19**), E (**22**), and F (**23**), which are geometrical isomers of olefins in the hydrophobic tail, i.e., the tetraene portion, showed similar IC_{50} values against PP1 and PP2A in our earlier report [20]. It is now conceivable that we measured the activity of a mixture of four compounds in each case because of facile photoisomerization of the double bonds [20]. We prepared calyculins by avoiding light as much as possible and determined their enzyme-inhibitory activity in the dark. Consequently, calyculins B, E, and F were shown to be significantly less active than calyculin A (Table 1). In order to examine the role of the hydrophobic tail as a whole in calyculin A (**4**), we examined the activity of the C9/C25-calyculin (**16**). It was inactive against both PP1 and PP2A, allowing us to confirm the indispensability of the hydrophobic tail in calyculins.

Structural Units Interacting with Enzymes

Five research groups have reported molecular modeling studies of the binding of calyculin A to PP1 [9–14]. Although all workers used the crystal structure of microcystin LR bound PP1c as a template, they proposed two different gross shapes for the "bound" calyculin A. A recent study on the solution structure of calyculin A has shown conformational aspects of the molecule; in $CDCl_3$ calyculin A adopts a pseudocyclic conformation, as observed in the crystal structure, whereas in CD_3OD some of polar groups are solvent exposed [29]. Therefore, it is possible that the conformation of calyculin A changes depending on the local environment of the binding site of PP1.

All modeling studies suggested that the tetraene portion of calyculin A plays the same role as the Adda side chain in microcystin LR. However, the importance of the phosphate group was not emphasized by some groups [10, 14] because of a previous report on the potent activity of dephosphonocalyculin A. Although not involved in binding to microcystin LR, the acidic groove of PP1 was suggested to play an important part in binding to calyculin A through interactions concerning the 36-dimethylamino group [7, 11]. When the phosphate function in calyculin A binds to the catalytic site of the enzyme and if calyculin A adopts an extended conformation, the 36-dimethylamino group is suitably placed in the vicinity of the acidic groove of PP1 to effect ionic interaction. However, this proposal is inconsistent with the potent activity of calyculin A toward the PP1 mutants whose acidic groove residues are replaced by basic or neutral residues [31]. In any case, the molecular modeling studies of calyculin A did not converge either in the bound conformation of calyculin A or in important functional groups. This is partly because of the lack of precise SAR data for the calyculins.

If we presume that a structural unit whose modification abolishes the inhibitory activity is involved in binding to the enzyme, three such units could be pointed out for calyculin A: 17-phosphate, 13-hydroxyl, and the tetraene moieties. These functional groups are all located in the polyketide portion. When the three groups interact with the enzyme at the same time, the central spiroketal portion must be placed apart from the peptide portion by the breakdown of the intramolecular salt bridges. Additionally, the interaction of calyculin A (**4**) with the acidic groove is shown not to be important because hemicalyculin A (**5**) and C1/C34-calyclulin A (**15**), both of which lack the dimethylamino group, are as potent inhibitors as calyculin A (**4**).

Cytotoxicity

Finally, we compared the enzyme inhibitory and cytotoxic activities of selected calyculin derivatives (Table 2). Three compounds with comparable enzyme inhibitory activity (calyculin A [**4**], hemicalyculin A [**5**], and C1/C34-calyculin A [**15**]) and two inactive derivatives (dephosphonocalyculin A [**17**] and 11,13-*O*-isopropylidenecalyculin A [**6**]) were chosen. The cytotoxicity of the potent

Table 3. ^{1}H and ^{13}C NMR data for **5** (in CD_3OD, 300 K)

Carbon Number	^{13}C, ppm	^{1}H, ppm (mult,*J*(Hz))	Carbon Number	^{13}C, ppm	^{1}H, ppm (mult,*J*(Hz))
1	118.1	---	20a	30.6	1.60 (dd,14.4,2.7)
2	96.2	5.26 (s)	20b	---	1.86 (dd,14.4,3.9)
3	158.8	---	21	71.9	3.97 (q,3.0)
4	129.1	6.77 (d,15)	22	38.9	1.61 (m)
5	135.5	7.15 (dd,15,11)	23	67.4	4.42 (dt,9.3,2.8)
6	125.3	6.38 (d,11)	24a	37.9	2.20 (ddd,3.5,9.2,13.7)
7	145.7	---	24b	---	2.46 (dddd,1.8,5.3,10.0,15.0)
8	136.9	---	25	156.0	7.06 (ddd,16.0,9.2,5.4)
9	132.6	6.02 (d,10)	26	101.9	5.66 (d,16.0)
10	37.4	2.80 (ddq,6.9,2.3)	27	118.8	---
11	79.0	3.44 (dd,9.6,2.7)	42	10.5	0.88 (d,7.3)
12	44.1	1.67 (ddq,5.3,7.0,9.0)	43	23.0	0.98 (s)
13	71.8	4.12 (dd,5.7,10.4)	44	18.1	1.20 (s)
14a	35.2	1.48 (dd,9.8,13.6)	45	60.8	3.63 (s)
14b	---	1.80 (dd,11.2,14.6)	46	11.9	0.79 (d,6.0)
15	80.3	3.81 (dt,9.6,0.7)	47	18.5	1.06 (d,6.9)
16	85.9	4.14 (dd,9.6,5.7)	48	14.2	1.89 (s)
17	85.2	4.16 (dd,10.0,4.6)	49	14.9	2.05 (s)
18	51.7	---	50	19.2	2.10 (s)
19	109.8	---			

The NMR data were recorded at 600 MHz for ^{1}H NMR and at 150 MHz for ^{13}C NMR. The chemical shifts are recorded as δ ppm.

enzyme inhibitors were in the order of calyculin A (**4**) >> C1/C34-calyculin A (**15**) > hemicalyculin A (**5**). Hemicalyculin A (**5**) lacked two of the basic groups, the dimethylamino and oxazole groups, whereas C1/C34-calyculin A lacked only the dimethylamino group. Therefore, the cytotoxicity of the potent enzyme inhibitory compounds was in the reverse order of their acidity, demonstrating that both dimethylamino and oxazole groups served to cancel the negative charges of the phosphate group by forming salt bridges as revealed by the crystal structure and that they facilitated membrane permeation. The morphological change of 3Y1 fibroblast cells occurred in 7 min when the cells were exposed to 100 nM of calyculin A (**4**), whereas no change was observed for 3 hr in those treated with 1 μM of hemicalyculin A (**5**). **6** and **17**, two apparently membrane-permeable compounds with no enzyme-inhibitory activities, exhibited essentially no cytotoxic activity.

Significance

Reversible serine/threonine phosphorylation regulates countless cellular processes. The recent discovery of natural product inhibitors, including okadaic acid, calyculin A, and microcystin LR, has shed light on the functions of protein phosphatases, e.g., PP1 and PP2A. Inhibitors of these enzymes are believed to be potential therapeutic agents, particularly antitumor agents [32]. To obtain insight into the mode of interaction between calyculin A and the enzymes, we have examined the PP1 and PP2A inhibitory activity of a new calyculin derivative, hemicalyculin A (5), and chemically transformed calyculin derivatives as well as 10 natural calyculins, which provided significant structure-activity relationships for the calyculins and thereby suggested that the 17-phosphate, 13-hydroxyl, and tetraene moieties are involved in binding to PP1 and PP2A. This new finding is consistent with the binding model of microcystin LR to PP1c that was obtained by X-ray crystallography of the microcystin LR/PP1c complex. It is also demonstrated that the cytotoxic activity of the calyculins is ascribed to their inhibition of PP1 and PP2A. Our new finding will contribute not only to the design of inhibitors of enzymes but also to the development of anticancer agents.

Experimental Procedures

Ultraviolet spectra were measured on a Hitachi 330 spectrophotometer. Optical rotations were determined with a JASCO DIP-1000 polarimeter. FAB mass spectra were measured with a JEOL SX-102 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-A600 NMR spectrometer. The sponge *D. calyx* was collected by hand with SCUBA at depths of 15–20 min off the Izu Peninsula and Sikine-jima island. Specimens were immediately frozen and kept frozen at $-20^{\circ}C$ until processed. A protein phosphatase inhibitory assay was carried out as described in the literature [33]. The activity was expressed by the IC_{50} value, which is affected by the enzyme concentration in the assay solution. Therefore, the difference of the activities of compounds with IC_{50} values around 1 nM was not evaluated precisely.

Isolation of Hemicalyculin A (5)

The sponge *D. calyx* was collected at a depth of 5 m off Sikine-jima island, 100 km south of Tokyo. Specimens were immediately frozen and kept frozen at $-20^{\circ}C$ until processed. After epibionts were removed, the frozen sponge (10 kg) was homogenized and extracted with ethanol (2 $\times$ 6 liters) and methanol (2 $\times$ 6 liters). The combined extracts were concentrated and partitioned between CH_2Cl_2 and H_2O. The organic phase (29 g) was subjected to silica gel flash chromatography with $CHCl_3$/MeOH solvent pairs. Enzyme-inhibitory fractions containing the $CHCl_3$/MeOH (98:2), $CHCl_3$/MeOH (95:5), and $CHCl_3$/MeOH (9:1) fractions were subjected to ODS flash chromatography with aqueous methanol. All 30 fractions were monitored by the diode-array-detected HPLC with ODS column. The fractions containing hemicalyculin A were further purified by ODS HPLC with 68% MeOH containing 50 mM $AcONH_4$ and then with 45% MeCN containing 50 mM $AcONH_4$ to yield **5** (12 mg).

Hemicalyculin A (**5**) colorless solid: $[\alpha]^{20}_{D}$ -107° (*c* 0.5, MeOH); UV λ_{max} (MeOH), 237 nm (ϵ 3700), 340 nm (ϵ 19000); HRFABMS [*m/z*

705.3513 (M-H)$^-$, $C_{36}H_{55}N_2O_{10}P$ (Δ −0.3 mmu)]; for ^1H and ^{13}C NMR data, see Table 3.

Preparation of 9 from Calyculin A (4)

To a solution of calyculin A (**4**, 10 mg) in CH_2Cl_2 (500 μl) was added 2,2-dimethoxypropane (300 μl) and pyridinium *p*-toluenesulfonate (PPTS, 12 mg), and the mixture was stirred at 40°C for 20 hr, followed by the addition of three drops of triethylamine and evaporation. The residue was purified by ODS HPLC with 75% MeOH containing 50 mM $AcONH_4$ to furnish 11,13-*O*-isopropylidene-calyculin A (**6**). To a solution of **6** in 80% MeOH (1 ml) was added $NaIO_4$ (5 mg), and the mixture was stirred at room temperature for 2 hr. Then the reaction mixture was treated with $NaBH_4$ (5 mg) and stirred at room temperature for 2 hr. The reaction was quenched by addition of AcOH (50 μl), and the reaction mixture was diluted with H_2O followed by extraction with EtOAc to afford **7**. The material was dissolved in DMF (500 μl) and treated with KF (5 mg) and *p*-bromobenzylbromide (30 mg) at 50°C overnight. The reaction mixture was diluted with H_2O and desalted through an ODS short column. The MeOH eluate from the column was purified by ODS HPLC with 88% MeCN containing 50 mM $AcONH_4$ to afford **8**. Compound **8** was ozonized for 5 min at −78°C in CH_2Cl_2, and the reaction mixture was reduced with $NaBH_4$ (8 mg) and MeOH (800 μl) and stirred at room temperature for 5 hr. The reaction was stopped by the addition of AcOH, and the mixture was evaporated, then purified by ODS HPLC with 90% MeOH containing 50 mM $AcONH_4$ to furnish **9** (2.8 mg; $[\alpha]^{20}_D$ −33° [*c* 0.1, MeOH]).

Preparation of 9 from Hemicalyculin A (5)

To a solution of hemicalyculin A (**5**, 2 mg) in CH_2Cl_2 (300 μl) were added 2,2-dimethoxypropane (300 μl) and pyridinium *p*-toluenesulfonate (5 mg), and the mixture was stirred at 40°C for 20 hr. The reaction solution was basified by the addition of three drops of triethylamine and evaporated. The residue was purified by ODS HPLC with 82% MeOH containing 50 mM $AcONH_4$ to furnish 11,13-*O*-isopropylidene-hemicalyculin A. The product was dissolved in DMF (500 μl) and treated with KF (3 mg) and *p*-bromobenzylbromide (30 mg) at 50°C overnight. The reaction mixture was diluted with H_2O and desalted on an ODS short column. The MeOH eluate from the column was purified by ODS HPLC with 95% MeOH containing 50 mM $AcONH_4$ to afford **10**. Compound **10** was ozonized for 5 min at −78°C in CH_2Cl_2. The product was treated with $NaBH_4$ (6 mg) and MeOH (600 μl) at room temperature for 5 hr. The reaction was stopped by the addition of AcOH, and the mixture was evaporated, then purified by ODS HPLC with 90% MeOH containing 50 mM $AcONH_4$ to furnish **9** (1.4 mg; $[\alpha]^{20}_D$ −31° [*c* 0.1, MeOH]).

Photo-Oxidation of Calyculin A

Calyculin A (**4**, 10 mg) was irradiated in CH_2Cl_2 (500 μl) under two 500 W flood lamps at −78°C for 1 hr with bubbling O_2 gas in the presence of methylene blue as a sensitizer. The reaction mixture was evaporated. The residue was dissolved in MeOH and passed through a short column packed with 2 ml of Dowex 50 W (Na^+ form). The MeOH eluent was further purified by ODS HPLC with 68% MeOH containing 50 mM $AcONH_4$ and then with 45% MeCN containing 50 mM $AcONH_4$ to yield hemicalyculin A (**5**, 0.5 mg).

Preparation of 34,35-*O*-Isopropylidene-Calyculin A (11)

To a solution of calyculin A (**4**, 2 mg) in CH_2Cl_2 (300 μl) were added 2,2-dimethoxypropane (300 μl) and pyridinium *p*-toluenesulfonate (5 mg), and the mixture was stirred at 60°C for 24 hr. The reaction solution was basified by the addition of three drops of triethylamine and evaporated. The product was purified by ODS HPLC with 55% MeCN containing 50 mM $AcONH_4$ to furnish calyculin A bisacetonide. The bisacetonide was dissolved in 80% AcOH (500 μl) and stirred at 50°C overnight. The reaction mixture was purified by ODS HPLC with 48% MeCN containing 50 mM $AcONH_4$ to afford 34,35-*O*-isopropylidene-calyculin A (**11**, 1.2 mg).

34,35-*O*-isopropylidene-calyculin A (11) : HRFABMS (positive; NBA) *m/z* 1049.5842 ($C_{53}H_{86}O_{15}N_4P$, Δ +1.5 mmu); ^1H NMR (MeOH-d_4 at 300 K) δ 0.76 (3H, d, H46), 0.90 (3H, s, H42), 0.98 (3H, s, H43), 1.06 (3H, d, H47), 1.24 (3H, s, H44), 1.44 (3H, d, H41), 1.36 (3H, s, acetonide-Me), 1.53 (3H, s, acetonide-Me), 1.53 (1H, m H14a), 1.60 (1H, m, H20a), 1.61 (1H, m, H12), 1.65 (1H, m, H22), 1.85 (1H, m, H31a), 1.86 (1H, m, H20b), 1.89 (1H, m, 14b), 1.89 (3H, s, H48), 2.04 (3H, s, H49), 2.07 (1H, m, H31b), 2.09 (1H, m, H24a), 2.10 (3H, s, H50), 2.12 (1H, m, H24a), 2.47 (1H, m, H24b), 2.55 (6H, s, H39,40), 2.79 (1H, m, H10), 3.09 (1H, m, H30), 3.28 (3H, s, H38), 3.36 (1H, m, H36), 3.40 (1H, m, H37a), 3.47 (1H, m, H11), 3.58 (3H, s, H45), 3.63 (1H, m, H37b), 3.85 (1H, m, H21), 3.90 (1H, m, H13), 3.91 (1H, m, H15), 4.12 (1H, dd, H16), 4.40 (1H, m, H17), 4.41 (1H, m, H23), 4.66 (1H, m, H35), 5.27 (1H, s, H2), 6.02 (1H, d, H9), 6.25 (1H, d, H26), 6.38 (1H, d, H4), 6.61 (1H, m, H25), 6.68 (1H, d, H6), 7.15 (1H, dd, H5), 7.90 (1H, s, H28).; ^{13}C NMR (MeOHd_4 at 300 K) δ 10.8 (C42), 12.2 (C46), 12.5 (C48), 14.3 (C48), 15.0 (C49), 18.1 (C44), 18.5 (C47), 18.5 (C50), 23.1 (C43), 24.5 (acetonide-Me), 27.0 (acetonide-Me), 31.1 (C20), 32.9 (C30), 35.2 (C14), 37.2 (C10), 37.2 (C24), 38.0 (C32), 38.8 (C22), 41.9 (C39,40), 44.3 (C12), 59.7 (C38), 60.3 (C45), 64.0 (C36), 68.2 (C23), 72.4 (C21), 75.8 (C34), 76.9 (C35), 79.4 (C11), 80.8 (C15), 84.0 (C17), 85.9 (C16), 95.2 (C2), 132.9 (C9), 120.4 (C26), 125.5 (C4), 129.3 (C6), 135.4 (C5), 136.3 (C28).

Preparation of Calyculin A 11,13,21-Triacetate (12)

Calyculin A (**4**, 10 mg) was dissolved in acetic anhydride/pyridine (1:1), and the mixture was stirred at room temperature overnight. The reaction mixture was evaporated and then dissolved in MeOH. After standing for a week, the MeOH solution was purified by ODS HPLC with 62% MeCN containing 50 mM $AcONH_4$ to afford calyculin A triacetate (**12**, 5.2 mg).

Calyculin A 11,13,21-triacetate (12): HRFABMS (positive; NBA) *m/z* 1135.5826 ($C_{56}H_{88}O_{18}N_4P$, Δ −0.5 mmu); ^1H NMR (MeOH-d_4 at 300 K) δ 0.94 (3H, d, H46), 0.96 (3H, d, H47), 0.97 (3H, d, H42), 0.97 (3H, s, H43), 1.29 (3H, s, H44), 1.35 (3H, d, H41), 1.40 (1H, m, H14a), 1.69 (1H, m, H22), 1.83 (1H, m, H31a), 1.84 (1H, m, H20b), 1.88 (3H, s, H48), 1.96 (3H, s, Ac), 1.98 (3H, s, H49), 2.04 (3H, s, Ac), 2.06 (1H, m, H24a), 2.06 (3H, s, Ac), 2.08 (1H, m, H12), 2.08 (1H, m, H31b), 2.09 (3H, s, H50), 2.45 (1H, m, H14b), 2.45 (1H, m, H24b), 2.92 (6H, s, H39, 40), 2.98 (1H, m, H10), 3.08 (1H, m, H32a), 3.23 (1H, m, H30), 3.38 (1H, m, H15), 3.42 (3H, s, H38), 3.47 (3H, s, H45), 3.68 (1H, m, H32b), 3.84 (1H, m, H37a), 3.85 (1H, m, H36), 3.87 (1H, m, H35), 3.95 (1H, m, H37b), 3.95 (1H, m, H16), 4.38 (1H, m, H23), 4.38 (1H, m, H17), 4.44 (1H, d, H34), 4.83 (1H, m, H11), 4.88 (1H, m, H21), 5.26 (1H, m, H13), 5.30 (1H, s, H2), 5.78 (1H, d, H9), 6.23 (1H, d, H26), 6.39 (1H, d, H6), 6.78 (1H, d, H4), 6.88 (1H, ddd, H25), 7.13 (1H, dd, H5), 7.70 (1H, s, H28).; ^{13}C NMR (MeOH-d_4 at 300 K) δ 10.8 (C42), 11.8 (C46), 13.8 (C48), 14.2 (C49), 16.2 (C41), 17.3 (C47), 18.2 (C44), 18.7 (C50), 19.0 (Ac), 21.7 (Ac), 22.0 (Ac), 22.1 (Ac), 22.4 (C43), 27.8 (C20), 29.9 (C14), 30.3 (C30), 35.1 (C10), 35.3 (C22), 35.3 (C31), 35.3 (C24), 35.3 (C32), 42.2 (C39,40), 59.0 (C38), 61.0 (C45), 66.4 (C36), 66.7 (C37), 68.1 (C23), 72.5 (C34), 72.5 (C13), 74.2 (C35), 75.0 (C21), 79.8 (C11), 79.8 (C15), 84.1 (C17), 87.5 (C16), 95.7 (C2), 119.2 (C26), 126.3 (C6), 130.0 (C4), 132.1 (C9), 134.0 (C5), 135.2 (C25), 136.2 (C28).

Preparation of Calyculin A 21-Acetate (13)

11,13-*O*-isopropylidene-calyculin A (**6**, 10 mg) was dissolved in acetic anhydride/pyridine (1:1) and stirred at room temperature overnight. The reaction mixture was evaporated and then dissolved in MeOH. After standing for 10 days, the MeOH solution was evaporated and dissolved in 80% AcOH (500 μl), and this solution was stirred at 50°C overnight. The product was purified by ODS HPLC with 90% MeOH containing 50 mM $AcONH_4$ to afford calyculin A C21-acetate (**13**, 4.4 mg).

Calyculin A 21-acetate (13): HRFABMS (positive; NBA) *m/z* 1051.5673 ($C_{52}H_{84}O_{16}N_4P$, Δ +5.3 mmu); ^1H NMR (MeOH-d_4 at 300 K) δ 0.83 (3H, d, H46), 0.93 (3H, s, H43), 0.98 (3H, d, H42), 1.04 (3H, d, H47), 1.23 (3H, s, H44), 1.30 (3H, d, H41), 1.50 (1H, m, H14a), 1.57 (1H, m, H12), 1.69 (1H, m, H22), 1.72 (1H, m, H20a), 1.80 (1H, m, H31a), 1.82 (1H, m, H20b), 1.86 (1H, m, H14b), 1.86 (3H, s, H48), 2.01 (1H, m, H31b), 2.03 (3H, s, H49), 2.03 (3H, s, Ac), 2.08 (1H, m, H24a), 2.09 (3H, s, H50), 2.43 (1H, m, H24b), 2.81 (1H, m, H10), 2.86 (6H, s, H39,40) 3.06 (1H, m, H32a), 3.20 (1H, m, H32b), 3.42 (3H, s, H38), 3.51 (1H, dd, H11), 3.55 (1H, m, H13), 3.60 (3H, s, H45), 3.79 (1H, m, H15), 3.80 (1H, m, H35), 3.83 (1H, m, H32), 3.94 (1H, m, H36), 4.00 (1H, dd, H16), 4.24 (1H, dd, H17), 4.39 (1H, m, H23), 4.39 (1H, m, H34), 4.84 (1H, m, H21), 5.27 (1H, s, H2), 6.13 (1H, d, H9), 6.22 (1H,

d, H26), 6.18 (1H, d, H4), 6.77 (1H, d, H6), 6.88 (1H, ddt, H25), 7.15 (1H, dd, H5), 7.67 (1H, s, H25).; ^{13}C NMR (MeOH-d_4 at 300 K) δ 10.8 (C42), 12.2 (C46), 14.5 (C48), 14.8 (C49), 18.0 (C41), 18.1 (C44), 18.5 (C47), 18.7 (C50), 23.5 (C43), 38.5 (C20), 30.5 (C30), 35.0 (C31), 36.0 (C22), 36.0 (C22), 37.0 (C10), 37.9 (C24), 38.2 (C14), 44.0 (C12), 59.5 (C38), 61.8 (C45), 66.0 (C36), 66.3 (C37), 68.7 (C23), 72.2 (C35), 73.4 (C23), 75.6 (C21), 75.8 (C13), 79.2 (C15), 81.5 (C11), 84.0 (C17), 86.1 (C16), 95.2 (C2), 118.2 (C26), 125.0 (C4), 128.8 (C6), 132.4 (C9), 134.7 (C5), 135.8 (C28).

Preparation of C1/C34-Calyculin A (15)

To a solution of **6** in 80% MeOH (1 ml) was added $NaIO_4$ (4 mg), and the mixture was stirred at room temperature for 3 hr. Subsequently, the reaction mixture was stirred with $NaBH_4$ (5 mg) at room temperature for 2 hr. The reaction was quenched by the addition of AcOH (50 μl), and the reaction mixture was diluted with H_2O followed by extraction with EtOAc. The organic extract was dissolved in 80% AcOH (500 μl) and stirred at 50°C overnight. The reaction mixture was separated by ODS HPLC with 45% MeCN containing 50 mM $AcONH_4$ to afford C1/C34-calyculin A (**15**, 1.8 mg).

C1/C34-calyculin A (15): HRFABMS (positive; NBA) *m/z* 878.4537 ($C_{44}H_{69}O_{13}N_3P$, Δ −3.1 mmu); ^{1}H NMR (MeOH-d_4 at 300 K) δ 0.74 (3H, d, H46), 0.90 (3H, d, H42), 0.99 (3H, s, H43), 1.06 (3H, d, H47), 1.24 (3H, s, H44), 1.33 (3H, d, H41), 1.50 (1H, m, H14a), 1.60 (1H, m, H12), 1.61 (1H, m, H20), 1.65 (1H, m, H22), 1.80 (1H, m, H31a), 1.85 (1H, m, H20b), 1.87 (1H, m, H14b), 2.08 (1H, m, H24a), 2.09 (1H, m, H31b), 2.44 (1H, m, H24b), 2.78 (1H, m, H10), 3.13 (1H, m, H30), 3.22 (1H, m, H32a), 3.41 (1H, m, H32b), 3.47 (1H, m, H11), 3.84 (1H, m, H15), 3.86 (1H, m, H13), 3.86 (1H, m, H21), 3.89 (1H, m, H14b), 3.94 (1H, d, H34a), 4.02 (1H, d, H34b), 4.10 (1H, dd, H16), 4.37 (1H, dd, H17), 4.40 (1H, dt, H23), 5.27 (1H, s, H2), 6.02 (1H, d, H9), 6.27 (1H, d, H26), 6.38 (1H, d, H6), 6.71 (1H, m, H25), 6.77 (1H, d, H4), 7.15 (1H, dd, H5), 7.79 (1H, s, H28); ^{13}C NMR (MeOH-d_4 at 300 K) δ 10.9 (C42), 12.2 (C46), 14.2 (C48), 15.1 (C49), 18.2 (C44), 18.6 (C41), 18.6 (C47), 19.4 (C50), 23.0 (C43), 30.8 (C20), 32.2 (C30), 35.1 (C31), 36.0 (C14), 37.3 (C10), 37.3 (C24), 37.6 (C32), 38.7 (C22), 44.2 (C12), 51.5 (C18), 60.9 (C45), 62.5 (C34), 68.2 (C23), 72.5 (C13), 72.5 (C21), 79.2 (C11), 80.1 (C15), 83.9 (C17), 85.8 (C16), 95.2 (C2), 109.8 (C19), 119.6 (C26), 125.4 (C6), 129.4 (C4), 132.3 (C25), 132.7 (C9), 135.3 (C5), 136.0 (C28). 136.7 (C8), 139.7 (C27), 145.9 (C7), 158.9 (C3), 170.0 (C29), 175.3 (C33).

Preparation of C9/C25-Calyculin (16)

Compound **9** was dissolved in 80% AcOH (500 μl) and stirred at 50°C overnight. The reaction mixture was purified by ODS HPLC and subjected to hydogenolysis (Pd/C-H_2) to afford C9/C25-calyculin (**16**, 1.6 mg).

C9/C25-calyculin (16): HRFABMS (positive; NBA+NaCl) *m/z* 567.2532 ($C_{23}H_{45}O_{12}PNa$, Δ −1.4 mmu); ^{1}H NMR (MeOH-d_4 at 300 K) δ 0.88 (3H, d, H46), 0.89 (3H, d, H42), 0.98 (3H, s, H43), 1.40 (3H, d, H47), 1.19 (3H, s, H44), 1.52 (1H, m, 14Ha), 1.58 (1H, m, H22), 1.60 (1H, m, H20a), 1.86 (1H, m, H20b), 1.88 (1H, m, H10), 1.89 (1H, m, H12), 1.93 (1H, m, H14b), 3.41 (1H, br, H11), 3.53 (1H, dd, H9a), 3.70 (1H, m, H9b), 3.70 (3H, s, H45), 3.85, (1H, m, H21), 3.87 (1H, m, H15), 3.88 (1H, m, H16), 4.00 (1H, br, H13), 4.15 (1H, br, H17).; ^{13}C NMR (MeOH-d_4 at 300 K) δ 10.8 (C42), 12.0 (C46), 15.6 (C47), 18.1 (C44), 23.2 (C43), 30.4 (C20), 36.2 (C14), 38.7 (C10), 39.0 (C22), 43.2 (C12), 52.3 (C18), 60.9 (C45), 64.7 (C9), 72.0 (C15), 72.1 (C13), 78.9 (C11), 80.1 (C16), 86.0 (C17), 109.3 (C19).

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Note Added in Proof

A crystal structure of a complex between calyculin A and PP1γ was recently determined (Kita, A., Matsunaga, S., Takai, A., Kataiwa, H., Wakimoto, T., Fusetani, N., Isobe, M., and Miki, K. (2002). Crystal structure of the complex between calyculin A and the catalytic subunit of protein phosphatase 1. Structure, in press).