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Focused Library with a Core Structure Extracted from Natural Products and Modified: Application to Phosphatase Inhibitors and Several Biochemical Findings

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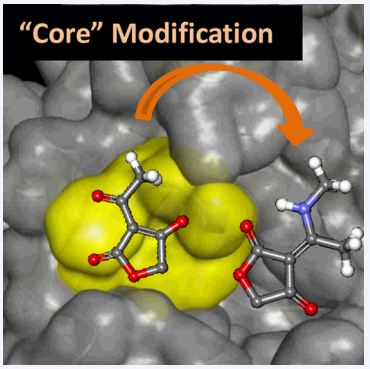
CONSPECTUS: Synthesis of a focused library is an important strategy to create novel modulators of specific classes of proteins. Compounds in a focused library are composed of a common core structure and different diversity structures. In this Account, we describe our design and synthesis of libraries focused on selective inhibitors of protein phosphatases (PPases). We considered that core structures having structural and electronic features similar to those of PPase substrates, phosphate esters, would be a reasonable choice. Therefore, we extracted core structures from natural products already identified as PPase inhibitors. Since many PPases share similar active-site structures, such phosphate-mimicking core structures should interact with many enzymes in the same family, and therefore the choice of diversity structures is pivotal both to increase the binding affinity and to achieve specificity for individual enzymes.



Here we present case studies of application of focused libraries to obtain PPase inhibitors, covering the overall process from selection of core structures to identification and evaluation of candidates in the focused libraries. To synthesize a library focused on protein serine-threonine phosphatases (PPs), we chose norcantharidin as a core structure, because norcantharidin dicarboxylate shows a broad inhibition profile toward several PPs. From the resulting focused library, we identified a highly selective PP2B inhibitor, NCA-01. On the other hand, to find inhibitors of dual-specificity protein phosphatases (DSPs), we chose 3-acyltetronic acid extracted from natural product RK-682 as a core structure, because its structure resembles the transition state in the dephosphorylation reaction of DSPs. However, a highly selective inhibitor was not found in the resulting focused library. Furthermore, an inherent drawback of compounds having the highly acidic 3-acyltetronic acid as a core structure is very weak potency *in cellulo*, probably due to poor cell membrane permeability. Therefore, we next modified the core structure from acidic to neutral by transformation to the enamine derivative and constructed a second-generation focused library (RE derivatives). The resulting compounds showed dramatically improved cell membrane permeability and inhibitory selectivity and included VHR (*vaccinia* VH1-related)-selective RE12 and CDC25A/B (cell division cycle 25A/B)-selective RE44. These inhibitors act on target enzymes *in cellulo* and do not generate reactive oxygen species, which is a potential problem with quinoid-type inhibitors of CDC25s. The cellular activity of RE12 was further improved by replacement of the side chain to afford RE176, which showed more potent antiproliferative activity than RE12 against HeLa cells.

The dramatic change of inhibitory selectivity obtained by core structure modification from 3-acyltetronic acid to its enamine derivative was associated with a change in the mode of action. Namely, RE derivatives were found to be noncompetitive inhibitors with respect to a small-molecular substrate of CDC25A/B, whereas RK-682 was a competitive inhibitor of VHR. We identified the binding site of RE derivatives on the CDC25A as a pocket adjacent to the active site; this appears to be a promising target site for development of further novel inhibitors of CDC25s.

1. INTRODUCTION

Screening of chemical libraries is an important approach for discovery of new biologically active compounds, and construction of various libraries is currently one of the most important tasks of synthetic chemists in the fields of chemical biology and medicinal chemistry. In the 1990s, combinatorial chemistry was introduced as a tool to synthesize large numbers of compounds by combining various building blocks. To avoid the need for multiple separation/purification processes, solid-phase synthesis and simple, high-yield reactions have often been used (Figure 1A).[1] Consequently, the chemical space[2] occupied by such libraries was rather limited. Since then, organic chemists have developed efficient synthetic strategies for libraries of more complex, natural product-like molecules, in which compounds with desired biological effects are expected

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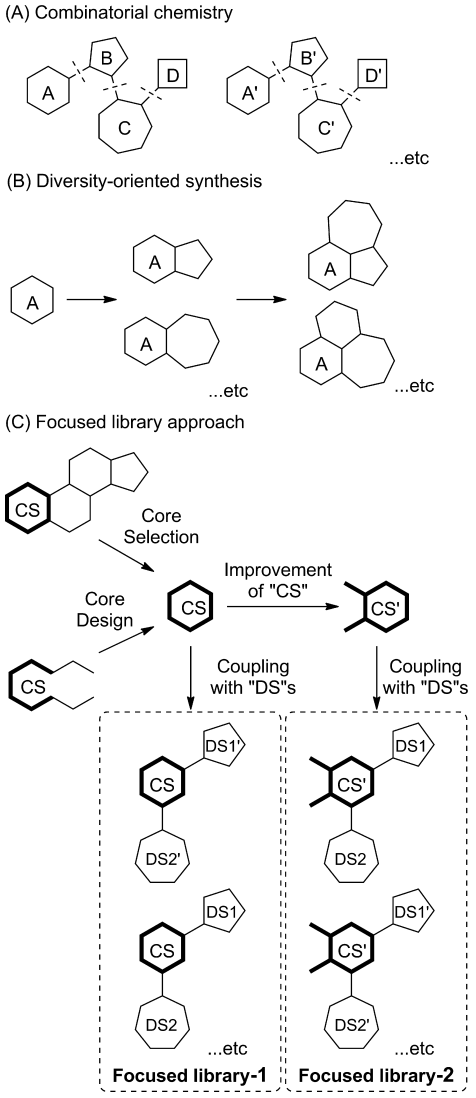

Figure 1. Schematic representation of various approaches to synthesizing chemical libraries: (A) combinatorial chemistry; (B) diversity-oriented synthesis; (C) focused library approach starting from natural products. CS, core structure; DS, diversity structure.

to be present with high probability. These approaches are embraced by the concept of diversity-oriented synthesis (DOS), in which a set of compounds with diverse and complex structures is obtained from simple starting materials without focusing on any specific class of target protein (Figure 1B).[2,3] This concept was later extended to DOS starting from natural products.[4] The aim of DOS is generally to achieve a high degree of structural variation and complexity, and sometimes to construct architectures that resemble biologically active natural products. Recently a more function-oriented approach, that is, biology-oriented synthesis based on bio- and chemoinformatic data, has been proposed.[5]

In contrast to such diversity-oriented approaches, a "focused library" is designed to target a specific class of proteins and usually consists of a relatively small number of compounds.[6−8] Compounds in a focused library normally have a common "core structure" (Core), which is a key structure for interaction with a specific class of proteins, together with a range of "diversity structures" (Figure 1C). It is unlikely that natural products or synthetic molecules identified by screening would have sufficient potency, selectivity, solubility, and cell permeability for direct application in biological research, and thus the focused library approach is important for creating second-generation molecules having superior properties. The most important issue in library construction is the selection of a Core. The Core could be simply extracted from a lead compound based on the structure of a cocrystal with the target protein, if available, or could be based on preliminary structure−activity relationship (SAR) data on a limited number of derivatives. Alternatively, a Core might be designed based on physiological ligands or substrates.[9] Such a Core could be further optimized, if necessary (Figure 1C). Diversity structures could be selected randomly or could include structures expected to modulate the target activity and selectivity. In general, convergent synthesis of target libraries by coupling reactions of the Core with diversity structures, avoiding late-stage construction of the Core, would be a reasonable strategy.

Focused libraries have often been utilized in the development of enzyme inhibitors.[10] Enzyme inhibitors with high specificity for a target enzyme are useful for investigating the biological role of the enzyme and may also be useful as lead compounds for therapeutic agents. For example, enzymes involved in post-translational modifications of proteins and in signal transduction, such as proteases, protein kinases, and phosphatases (PPases), are promising therapeutic targets. However, these classes of enzymes contain large families that consist of many enzymes (or subtypes) that catalyze the same reaction of different substrates; enzymes in such a family have highly homologous active sites in terms of amino acid sequence and three-dimensional shape but have distinct biological functions. The focused library approach is particularly suitable for finding selective inhibitors of individual enzymes in such families. For example, if an enzyme inhibitor identified by screening binds to the active-site pocket of the enzyme competitively with respect to the substrate, a partial structure of the parent inhibitor may mimic a structural feature of the substrate or the transition state of the enzymatic reaction, and would be suitable as a Core. Of course, the Core alone may not show high inhibitory potency toward the target enzyme, but diversity structures installed on the Core are expected to act not only as enhancers to increase the affinity for the target enzyme but also as discriminators to decrease the affinity for the many other related enzymes with which the Core itself can interact. In this Account, we summarize our work to create unique inhibitors of PPases by synthesizing and screening suitable libraries, as well as some biochemical insights obtained by means of biological investigations utilizing the new inhibitors.

2. SEARCH FOR PHOSPHATE-MIMICKING CORE STRUCTURES AMONG NATURAL PRODUCTS: FIRST-GENERATION FOCUSED LIBRARY

PPases regulate phosphorylation states through dephosphorylation of phosphoproteins in various intracellular signal transduction pathways.[11] Protein phosphorylation is one of the most important and fundamental post-translational modifications, and analysis of the functions and regulatory mechanisms of phosphorylation of individual proteins should provide insights into the origin of diseases caused by malfunction in signal transduction. However, few specific inhibitors of PPases are available. We have been developing new inhibitors using focused library approaches, based on the idea that phosphate analogues would be a suitable Core, since PPases interact with and hydrolyze phosphate esters.

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PPases can be roughly categorized into two types, the protein serine/threonine phosphatase (PPs) and protein tyrosine phosphatase (PTPs) superfamilies, based on their substrate specificity.[12] Several potent inhibitors of various PPs (such as PP1, PP2A, and PP2B) have been identified from natural sources.[13] Among them, the clinically used immunosuppressants cyclosporin A and FK506 show selective inhibition of PP2B (calcineurin) via binding to specific proteins designated as immunophilins (cyclophilin and FKBP, respectively). The resulting protein complexes, but not the immunophilins or immunosuppressants alone, interact with the regulatory domain (not the catalytic domain) of PP2B to inhibit the phosphatase activity.[14] However, the immunophilins themselves have important roles in other cellular processes, and we considered that it would be desirable to develop direct inhibitors of PP2B that act independently of binding with immunophilins. To select a suitable Core, we focused on the natural product cantharidin (Figure 2). Cantharidin is a strong inhibitor of PP1

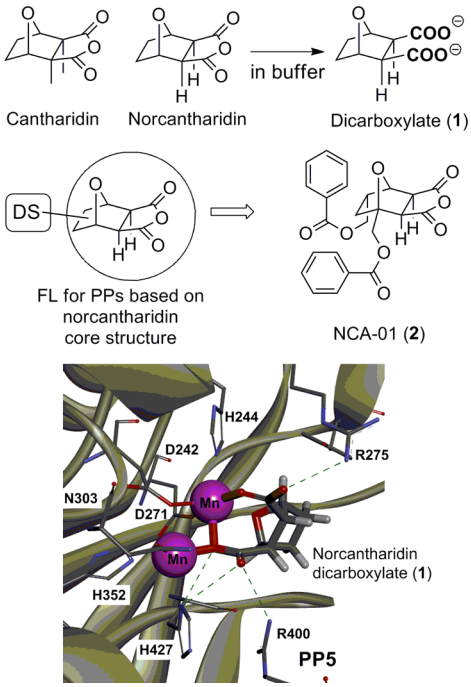

Figure 2. (A) Structures of cantharidin, norcantharidin, and derivatives **1** and **2**, and illustration of a focused library based on norcantharidin core. (B) Crystal structure of PP5 complexed with norcantharidin (PDB 3H61). DS, diversity structure.

and PP2A but a very weak inhibitor of PP2B. On the other hand, preliminary SAR indicated that norcantharidin inhibited PP2B in addition to PP1 and PP2A.[6,15] Furthermore, the dicarboxylate **1** obtained by hydrolysis of norcantharidin showed strong inhibition of all three PPs but not PTP1B, suggesting that the dicarboxylate **1** binds to the conserved active site of PPs as a phosphate mimic but not to that of PTPs. Since norcantharidin is easily converted to **1** under aqueous conditions, we selected norcantharidin as the Core for a library directed at PPs. Construction of the library resulted in identification of a highly selective inhibitor of PP2B, NCA-01 (**2**).[6,16] Its methyl ester was found to be stable in water and cell-permeable, and it inhibited IL2 (interleukin-2) production in Jurkat cells, probably after hydrolysis by an endogenous esterase.[17] The crystal structure of the complex of PP5 phosphatase with **1** was subsequently reported and was consistent with our hypothesis. Norcantharidin was indeed converted to **1**, and critical interactions of the two carboxylic acids with Mn^{2+} ions located at the active site of PP5 were observed.[18]

On the other hand, the catalytic site structures of PTPs are completely different from those of PPs. A unique loop structure (P-loop) containing cysteine and arginine residues is highly conserved in the active site of all PTPs. The PTP superfamily contains two major subclasses, PTPs, which hydrolyze only phosphotyrosine, and dual specificity phosphatases (DSPs), which dephosphorylate both phosphotyrosine and phosphoserine/threonine on the same proteins.[12] Since PTPs and DSPs, such as CDC25s (cell division cycle 25) and VHR (*vaccinia* VH1-related), are considered to be implicated in various diseases, many inhibitors have already been developed, as summarized in review articles.[19−21] In contrast, there are few highly selective and nontoxic inhibitors of DSPs effective *in cellulo*. The major difficulties in the creation of specific DSPs inhibitors are the high structural homology of the P-loop and the shallowness of the active site pocket,[22] which recognizes only the phosphate ester part of large substrates. Hence, development of potent and selective small-molecular inhibitors of DSPs that bind at the active site is challenging.

To address this problem, we planned to utilize a focused library approach with a phosphate-mimicking Core. Among several natural product inhibitors, we chose RK-682 (**3**, Figure 3A), which inhibits VHR.[23] The 3-acyltetronic acid structure is highly acidic (calculated pK_a of **3** = 3.1) and exists in dissociated form under physiological conditions. Since 4-Me-RK-682 (**4**, Figure 3B) showed negligible inhibition of VHR, we expected the anionic form of **3**, which structurally resembles the putative transition state of dephosphorylation (Figure 3C), to be a suitable phosphate-mimicking Core (dotted square, Figure 3A). Figure 3D shows the hypothetical binding mode of the Core with the P-loop, together with two sites at which diversity structures could be installed. In this case, diversity structures were expected to act as discriminators for selective inhibition of individual DSPs via interaction with unique amino acids around the active site. To test this hypothesis, we constructed **FL-1st** (Figure 3E, Scheme 1) and evaluated the inhibitory activities. Indeed, some compounds (**5** and **6**, Figure 3F) with different inhibitory profiles from that of **3** were found in **FL-1st**.[7] But highly selective DSP inhibitors were not obtained. Furthermore, most of the compounds in **FL-1st** were found to be ineffective *in cellulo*, probably due to low cell membrane permeability, which can be attributed to the highly acidic and/or their detergent-like property. In other words, employing a phosphate-mimicking scaffold with a similar acidic nature to the substrate phosphate ester proved to be a reasonable strategy to develop DSP inhibitors that were effective *in vitro* but was unsuccessful in obtaining inhibitors active *in cellulo*. The same problem has been encountered elsewhere; for example, most reported VHR inhibitors contain an acidic functionality such as sulfonic acid,[24] and their effects *in cellulo* either have not been reported or are weaker than expected from the inhibitory potency *in vitro*. Therefore, we concluded that a Core without high acidity should be employed to construct a second-generation library.

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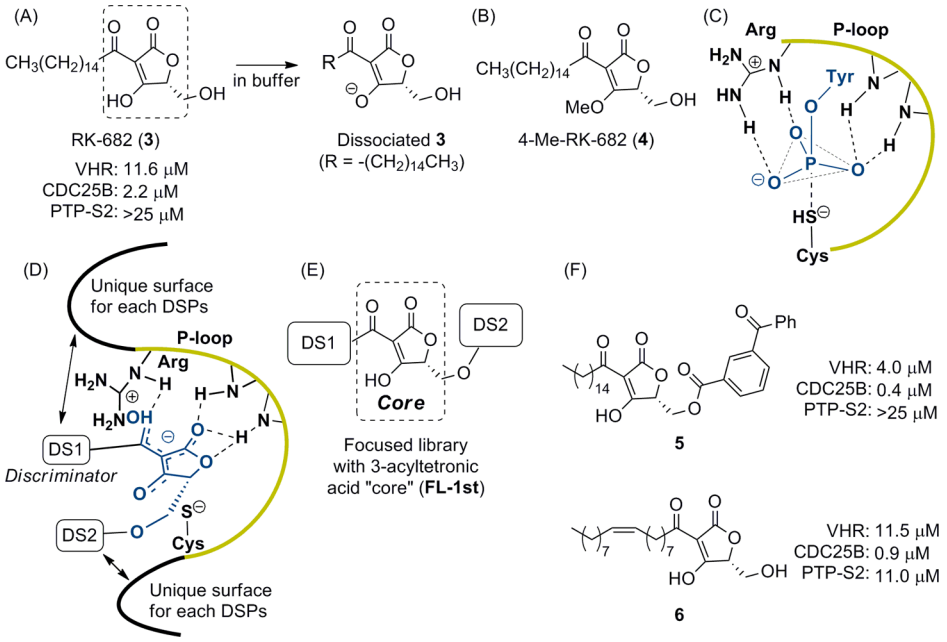
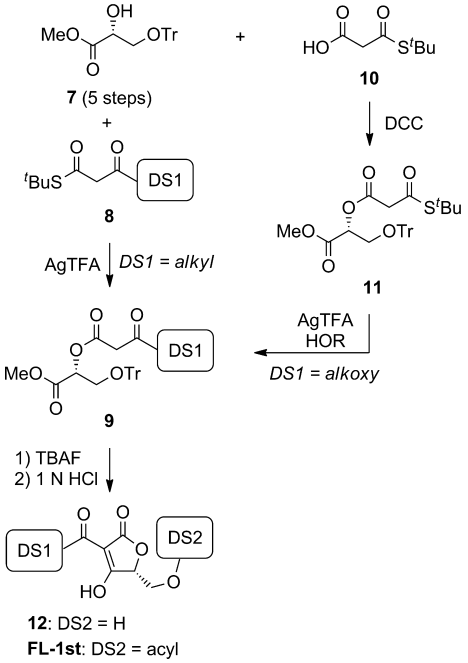


Figure 3. (A) Structure and dissociated structure of RK-682 (**3**) and reported inhibitory activities toward PPases. (B) Structure of 4-Me-RK-682 (**4**). (C) Putative transition state in dephosphorylation by PTPs and DSPs. (D) Putative binding mode of compounds in **FL-1st**. (E) Schematic structure of **FL-1st**. (F) Structures of two representative DSP inhibitors (**5** and **6**). DS, diversity structure.

Scheme 1. Synthetic Routes to FL-1st[a]



[a]DS, diversity structure.

3. CORE STRUCTURE MODIFICATION FROM ACIDIC TO NEUTRAL: SECOND-GENERATION FOCUSED LIBRARY

We had initially assumed that the 3-acyltetronate anion Core **13** would be a suitable mimic of phosphate ester in view of its planar structure and anion-delocalized nature (Figure 4A). But we speculated that the anionic structure would not be essential if the critical oxygen atoms have a sufficiently high electron density to act as hydrogen bond acceptors. Therefore, we selected enamine structure **14** (Figure 4A), which is easily obtainable from 3-acyltetronic acid as an equilibrated mixture of *E*- and *Z*-isomers simply by treatment with primary amines. The enamine structure was expected to act as a neutral surrogate (calculated pK_a = 18.3) of an anion-delocalized phosphate ester (Figure 4A). The electrostatic potential map predicted by DFT calculation indicated that electrons were still delocalized into the two oxygen atoms owing to the contribution of the nitrogen lone pair (Figure 4B). Although the total electron density was decreased in enamine **14** compared with 3-acyltetronate **13**, we anticipated that enamine **14** would be a suitable Core for membrane-permeable PPase inhibitors and accordingly designed a second-generation library (**FL-2nd**, RE derivatives, Figure 4C). An additional advantage of this new Core was the acquisition of an additional diversity structure installation site. The availability of three diversity structures would allow preparation of a more diverse library, and the variety of combinations could provide a way to tune the inhibitory potency and selectivity. In other words, electrostatic and hydrogen-bonding interactions between the active site P-loop and enamine Core **14** would be weakened due to the decreased electron density of the Core, but this would be compensated by the potential availability of three diversity structures as enhancers of affinity.

FL-2nd was prepared as shown in Scheme 2A. Its members were racemic compounds, in which Core was initially constructed and then diversity structure (DS-1 and DS-2) were introduced sequentially. The inhibitory potency of these compounds toward several DSPs and PTPs was tested *in vitro*. Surprisingly, most of the RE derivatives inhibited VHR, CDC25A, and CDC25B but not other enzymes, including CDC25C, several mitogen-activated protein (MAP) kinase phosphatases (MKPs), phospholipase A2, and heparanase[25] (Figure 5A). Since the parent natural product **3** showed inhibitory activities toward all of these enzymes, the inhibitory selectivity was dramatically increased by modification of the Core in combination with introduction of an additional substituent on the nitrogen atom. Furthermore, the selectivity

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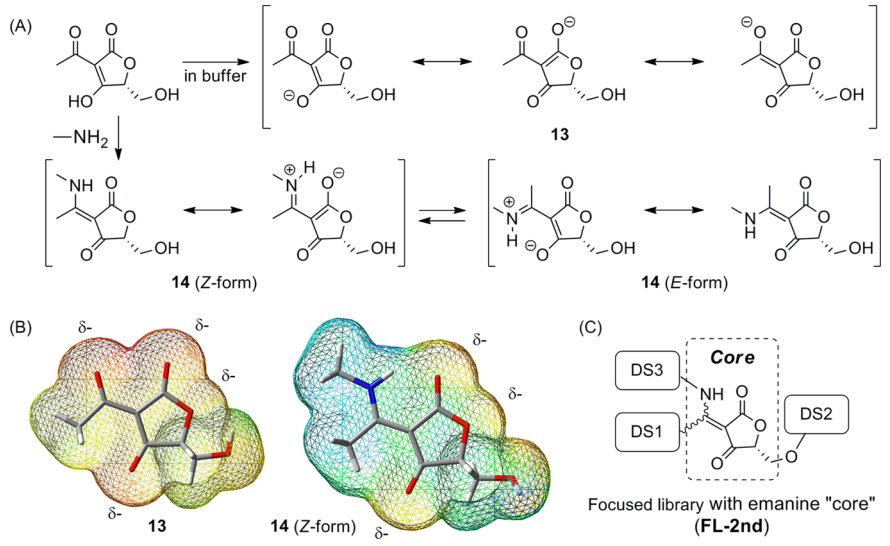
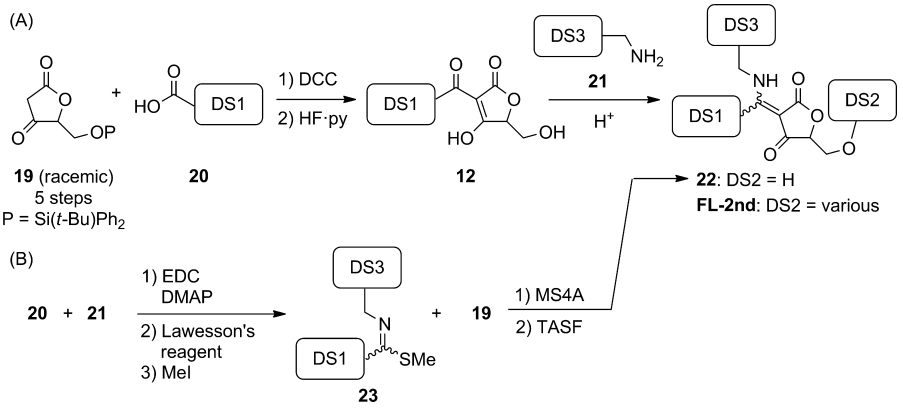
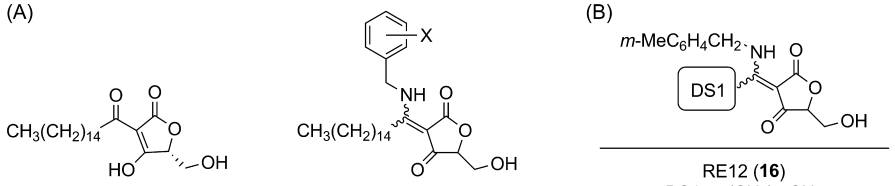


Figure 4. (A) Resonance structures of 3-acyltetronate **13** and equilibrium between *E*- and *Z*-isomers of enamine **14**. (B) Electrostatic potential map of **13** and **14** (Gaussian09, B3LYP/6-311G+(d,p); images were made by Gaussview 5.0). (C) Schematic illustration of **FL-2nd** with the enamine core. DS, diversity structure.

Scheme 2. Synthesis of FL-2nd (A) through 3-Acyltetronic Acid 12 or (B) by Direct Coupling between 20 and 21[a]



[a]DS, diversity structure.



IC_{50}	RK-682 (**3**)	RE1 (**15**) X = H	RE12 (**16**) X = *m*-Me	RE44 (**17**) X = *o*-OH
VHR	4.9 ± 0.8 μM	11.4 ± 0.5 μM	1.6 ± 0.2 μM	24.9 ± 2.2μM
CDC25A	6.2 ± 0.2 μM	16.6 ± 1.3 μM	>100 μM	13.5 ± 3.4 μM
CDC25B	5.3 ± 0.4 μM	8.4 ± 0.5 μM	>100 μM	4.3 ± 0.2 μM
CDC25C	13.0 ± 0.8 μM	>100 μM	>100 μM	>100 μM
MKP-1	9.9 ± 0.2 μM	>100 μM	>100 μM	>100 μM
MKP-3	4.3 ± 0.1 μM	>100 μM	>100 μM	>100 μM
PTP1B	3.7 ± 0.6 μM	>100 μM	>100 μM	>100 μM
HL60	>100 μM	9.5 ± 2.0 μM	9.3 ± 0.1 μM	2.4 ± 0.2 μM

IC_{50}	for VHR[a]: >100 μM for HeLa cells: >30 μM
	for VHR[a]: 7.7 ± 2.0 μM for HeLa cells: 7.8 ± 0.5 μM

a) in the presence of 0.001% NP-40

Figure 5. (A) Structures of four representative DSP inhibitors (**3**, **15**−**17**) and their inhibitory activities toward several PPases and proliferation of HL60 cells. (B) Inhibitory activities of **16** and **18** toward VHR in the presence of NP-40 and proliferation of HeLa cells. DS, diversity structure.

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was found to be variable depending on the substituent (discriminator) on the nitrogen. Among the compounds obtained, RE12 (**16**) was a potent and selective VHR inhibitor,[26] and RE44 (**17**)[27] showed preferential inhibition of CDC25A/B (especially CDC25B) over VHR (Figure 5A).

As we expected, the RE derivatives showed improved cell membrane permeability. As shown in Figure 5A, RE derivatives showed antiproliferative activity toward HL-60 cells, though the parent compound **3** was inactive even at 100 μM. It was difficult to investigate whether RE derivatives inhibit DSP activity in cells, both because their inhibitory activity was only moderate and because of the variability of phosphorylation states of DSP substrates in cells. Eventually, we confirmed the inhibitory effects of RE derivatives on VHR and CDC25s by using NIH3T3 or tsFT210 cells, in which the cell cycle was synchronized at G0 or G2/M phase by serum starvation or culture at 39 °C, respectively. Serum-stimulation of G0-arrested NIH3T3 cells triggered a synchronized and transient increase of the phosphorylation level of extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs) through activation of the MAP kinase cascade. VHR-inhibiting RE derivatives **15** and **16** retarded dephosphorylation of these MAP kinases. Hyperphosphorylation of these MAP kinases is known to block cell cycle progression, and indeed **15** and **16** caused G0/G1 arrest. VHR, MKPs, and atypical DSPs are considered to be responsible for dephosphorylation of ERKs and JNKs. Since **15** and **16** do not inhibit MKPs, suppression of dephosphorylation would be caused by the inhibition of VHR by RE derivatives in cells.[26] On the other hand, interesting results were obtained in tsFT210 cells having two point mutations on the cdc2 gene. Dephosphorylation of the cdc2 gene product, cyclin-dependent kinase 1 (CDK1), is essential for G2-M phase transition. Dephosphorylation of CDK1 did not occur in tsFT210 cells at 39 °C, and the cells were arrested at G2 phase. However, when the cells were cultured at 32 °C, dephosphorylation of CDK1 proceeded, triggering cell cycle progression from G2/M phase to G1 phase. CDC25A/B inhibitors **15** and **17** inhibited both dephosphorylation of CDK1 and cell cycle progression. It is noteworthy that the selective VHR inhibitor RE12 (**16**) is much less potent than **15** and **17**.[27] Subtype-selective inhibitors would be key tools to explore the biological functions of CDC25s. Indeed, our results indicate that CDC25C has little role in cell cycle progression of tsFT210 cells, since RE derivatives lacking CDC25C-inhibitory activity arrested cell cycle.

Overexpression of CDC25A/B has been reported in several cancer cell lines, and thus, many CDC25 inhibitors have been developed in both academic institutes and pharmaceutical companies. As summarized in several excellent reviews,[28,29] most of the potent inhibitors, including the natural product adociaquinone B,[30] are quinone derivatives, which equally inhibit all three subtypes of CDC25s with submicromolar IC_{50} values; most of them also strongly inhibit proliferation of cancer cells. Thus, constructing a library with a quinone moiety as the Core is superficially attractive. But it was reported that oxidation of the catalytic cysteine residue of CDC25s by reactive oxygen species (ROS) generated by electrophilic quinone derivatives is involved in their mode of action.[31] ROS can have undesirable effects in cells. In contrast, the RE derivatives did not generate ROS either *in vitro* or in cells, and this may be one of the reasons for the low toxicity and high selectivity of RE derivatives among the subtypes of CDC25.

Relatively high concentrations of these prototype RE derivatives were required for growth inhibition of some cell lines, such as VHR-overexpressing HeLa cells. Therefore, RE derivatives could still be improved. We speculated that the long alkyl chain might be associated with low solubility in aqueous media, as well as nonspecific hydrophobic binding with various biomolecules that would limit access of the RE derivatives to the target phosphatases. But we found that shortening the alkyl chain or addition of a small amount of neutral detergent NP-40 to the assay buffer diminished their inhibitory potency *in vitro* (Figure 5B).[32] Thus, we planned to replace the straight alkyl chain with other hydrophobic groups containing aromatic rings. For SAR study of diversity structure DS1, we established a new direct coupling method between the tetronic acid derivative **19** and thioimidates **23**, which were readily obtainable from **20** and **21** (Scheme 2B).[33] This method avoids the use of inconvenient amphiphilic 3-acyltetronic acid derivatives such as **21** as synthetic intermediates and facilitates the synthesis of new derivatives with various DS1 units. Based on the side-chain structure of SA compounds[24] that are sulfonate-based VHR inhibitors, the benzyloxyphenyl group was introduced as DS1 with various connection modes. Among the synthesized compounds, RE176 (**18**) was identified as a VHR inhibitor effective even in the presence of NP-40 (Figure 5B).[33] Interestingly, RE176 (**18**) was a potent antiproliferative agent for HeLa cells, whereas VHR-selective RE12 (**12**) was less potent.

4. BINDING SITE OF RE DERIVATIVES: ROLE OF THE NEW CORE STRUCTURE

We identified several DSPs inhibitors, but lack of structural information for enzyme−inhibitor complexes as to their binding mode hampered rational design of superior inhibitors. Although the structure of VHR in complex with an SA compound was recently reported,[24] no CDC25−inhibitor complex structure is available. For **FL-1st**, we had chosen an anionic phosphate mimic as the Core, which was expected to interact with the active-site P-loop. However, kinetic analysis of RE derivatives **15** and **17** with CDC25A and CDC25B, respectively, revealed noncompetitive inhibition with respect to small-molecular substrate 3-*O*-methylfluorescein phosphate (OMFP).[34] Therefore, we set out to identify the binding site of RE derivatives. For this purpose, alkyne-modified RE derivatives **24**−**26** were designed based on **15**; they were expected to bind covalently with CDC25s (Figure 6). Copper(I)-mediated azide−alkyne cycloaddition technology was adopted to evaluate the efficiency of covalent bond formation, and Michael acceptor-type **24** was found to possess the best reactivity with CDC25A among the compounds examined. Nevertheless, binding site identification with **24** was unsuccessful, presumably because of low covalent bond-forming efficiency or low solubility of the resulting peptide−**24** conjugate. Thus, we decided to transform **24** into RE142 (**27**, Figure 6), which incorporates the biotin moiety as a tool for enrichment of the target peptide conjugate, PEG linkers to increase the solubility, and the chemically cleavable diazobenzene moiety[35,36] to enable facile elution from avidin beads. The procedure finally adopted for binding site identification is illustrated in Figure 7. Analysis of the peptides covalently modified with cRE142 (**28**, Figure 6) by means of LC-MS/MS revealed that cRE142 (**28**) bound to one of residues C384, R385, and Y386. Consideration of their chemical reactivities suggested that C384 is most plausible candidate for the

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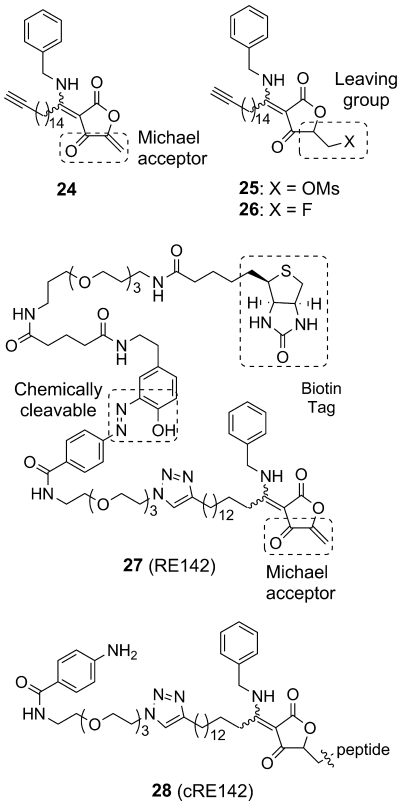

Figure 6. Structures of **24**–**26**, RE142 (**27**), and cRE142 (**28**).

covalently modified amino acid residue.[34] Modification of more reactive catalytic C430 by RE142 (**27**) was not detected, demonstrating that **27** has specificity for its binding site.

Based on the crystal structure of CDC25B complex with sulfate salt, the shallow active-site pocket surrounded by the P-loop and the neighboring deeper pocket designated as the "swimming pool"[37] are proposed to be potential binding sites for inhibitors (Figure 8A, right).[38] Some inhibitors, such as NSC663284[31,39] and vitamin K analogues,[40] covalently bind to an amino acid residue in the active site. Others are proposed to interact with the neighboring binding site on the basis of molecular modeling,[41,42] though no experimental evidence is available to support this idea. The neighboring pocket is not obvious in the crystal structure of CDC25A in the absence of any ligand (Figure 8A, left).[43] But a homology model of CDC25A constructed from the crystal structure of CDC25B with sulfate suggested that the neighboring pocket would be available in CDC25A (Figure 8A, center).[34] In this model, C384 as well as R385 and Y386 are located at the bottom of the swimming pool. The experimental finding that RE142 (**27**) modified one of these residues is therefore clear evidence that RE derivatives bind to this neighboring pocket.[34]

However, the mechanism through which RE derivatives noncompetitively inhibit enzymatic activity of CDC25A/B remains to be established. We speculate that RE derivatives influence critical conformational changes that lead to the transition state of dephosphorylation by interacting with the swimming pool site. It has already been proposed that the swimming pool in CDC25B is deformed by binding of the native substrate CDK2(pT14pY15)–cyclin A complex.[44] A plausible hypothesis is that RE derivatives alter the orientation of R436 residue in CDC25A (corresponding to R479 in CDC25B); this amino acid is essential for the enzymatic activity.[34] The fact that the side-chain orientations of this arginine residue in the crystal structures of CDC25s with and without the sulfate are different indicates that it is conformationally flexible (Figure 8A), and this lends support to our hypothesis.

The C-terminal region in both CDC25A/B is highly flexible,[45] and this region (after A551 in CDC25B) is invisible in crystal/solution structures. However, the C-terminus of CDC25B was shown to be involved in recognition of the native substrate CDK2(pT14pY15)–cyclin A complex and in the enzymatic activity, suggesting that interaction between the C-terminus of CDC25A/B and protein substrates plays an important role. The two arginine residues of CDC25B (R556 and R562) are likely to be especially important, because double mutation of these residues resulted in a dramatic decrease of the dephosphorylation activity of CDC25B toward the native substrate, though not toward artificial small-molecule substrates.[46] We speculate that RE derivatives interact with arginine residue(s) in the C-terminus as a mimic of Y15 phosphate in CDK2 or of acidic amino acid residues (D38/E42 in CDK2 are candidates to interact with the two arginines[46]) (Figure 8B). In this connection, it is interesting that these basic amino acid residues are conserved in CDC25A (K513 and R519) but not in CDC25C (L460 and L466) (Figure 8C). This is consistent with the idea that interaction of RE derivatives with the C-terminal region of CDC25A/B is the origin of the observed high selectivity of RE derivatives for CDC25A/B over CDC25C.

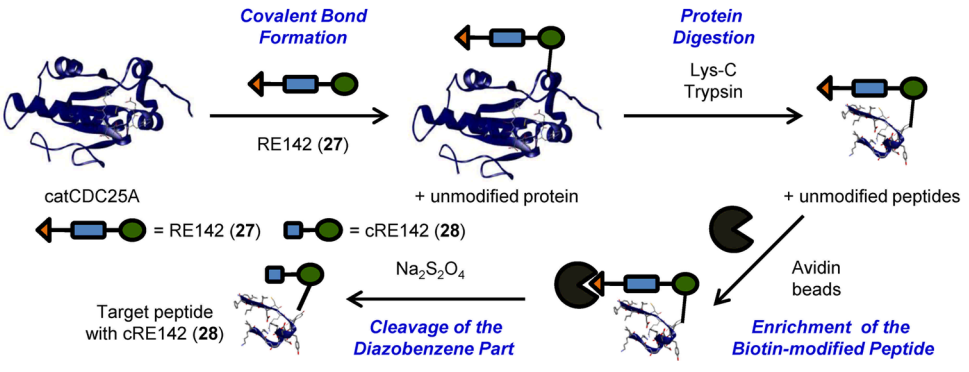

Figure 7. Schematic representation of the procedure for binding site identification using RE142 (**27**).

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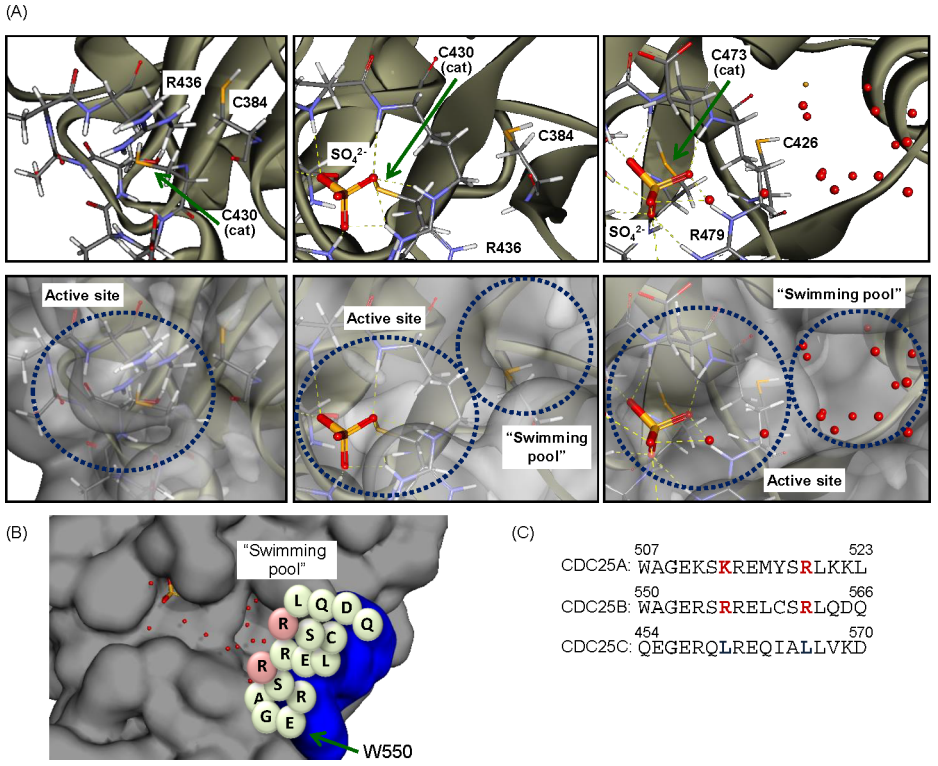

Figure 8. (A) Representations of the active site in the crystal structure of CDC25A (PDB 1C25, left) and in a homology model of CDC25A complexed with sulfate (center) made from the crystal structure of CDC25B in the presence of sulfate salt (PDB 1QB0, right), together with surface models. Water molecules are shown as red dots; the active site pocket surrounded by the P-loop and the neighboring pocket (so-called swimming pool) are shown as blue dotted circles. (B) Schematic model of CDC25B. In the crystal structure, the "swimming pool" is partially constituted by C-terminal amino acids (shown in blue); other C-terminal amino acids after A551 are invisible in the crystal structure. The C-terminal region including the two arginine residues (R556 and R562) can be located near the swimming pool. (C) C-Terminal amino acid sequences of CDC25s.

5. CONCLUSIONS

We have presented an Account of our work to develop PPases inhibitors by employing a focused library approach and some results of biological studies using the developed inhibitors. In the focused library approach, appropriate selection of the Core is a central issue, and we found that modification from 3-acyltetronic acid in our first-generation library to its enamine derivative as a Core dramatically increased selectivity for DSPs, as well as improving cell membrane permeability. We identified RE derivatives with unique enzyme and subtype selectivities, and these are expected to be useful chemical probes to investigate biological functions of PPases. Interestingly, Core modification changed the mechanism of inhibition, which accounts for the high selectivity toward VHR and CDC25A/B. These RE derivatives are the first inhibitors proven experimentally to bind the neighboring pocket of CDC25A, not the active site pocket. The tetronic acid derivative **3** was a competitive inhibitor of VHR, but interaction with a second phosphate-binding site near the active site was also suggested on the basis of kinetics and molecular modeling studies.[47] Thus, our work has identified the neighboring pocket, which likely plays a role in recognition of the specific native protein substrate, as a promising target for novel DSPs inhibitors.[48] Further expansion of the library size of **FL-2nd** may lead to identification of selective inhibitors of other DSPs, such as MKPs.[49]

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The manuscript was jointly written by both authors. Both authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This Account is dedicated to Prof. Iwao Ojima for his 70th birthday.

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