# Molecular Biosystems

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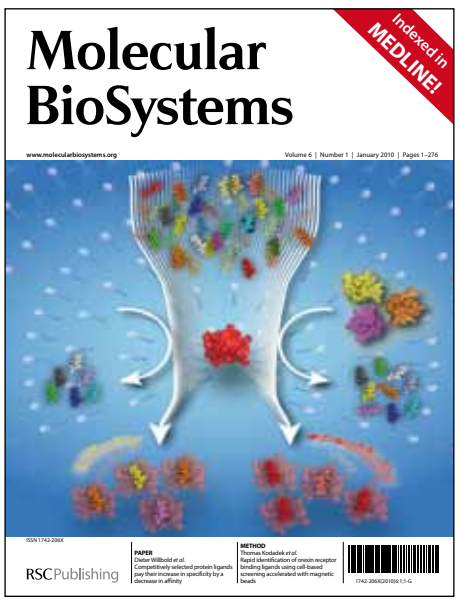


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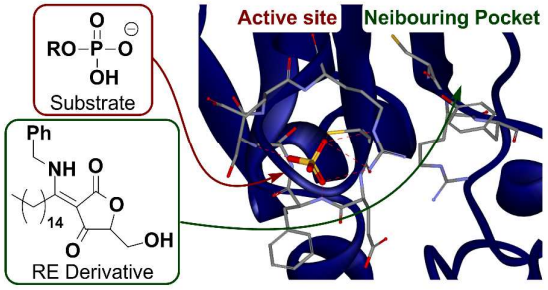
CDC25s inhibitors, RE derivatives, binds to pocket adjacent to catalytic active site, which was revealed by LC-MS analysis using originally developed chemical probes.



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Full Papers

# CDC25A-inhibitory RE Derivatives Bind to Pocket Adjacent to the Catalytic Site

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RE derivatives, which are cell-permeable and non-electrophilic dual-specificity protein phosphatase inhibitors developed in our laboratory, inhibit CDC25A/B non-competitively, as determined by means of kinetic experiments. To identify the binding site of RE derivatives, we designed and synthesized the new probe molecule RE142, having a Michael acceptor functionality for covalent bond formation with the enzyme, a biotin tag to enable enrichment of probe-bound peptide(s), and a chemically cleavable linker to facilitate release of probe-bound peptides from avidin beads. LC-MS analysis indicated that RE142 binds to one of the residues Cys384-Tyr386 of CDC25A, within a pocket adjacent to the catalytic site.

## Introduction

Cell division cycle 25 molecules (CDC25s) are members of the dual-specificity protein phosphatase (DSP) family of enzymes that dephosphorylate both phospho-serine/threonine and phospho-tyrosine residues in the same protein. CDC25s dephosphorylate conserved phospho-tyrosine and phospho-threonine residues on cyclin-dependent kinases (CDKs), thereby activating the kinases and promoting cell cycle progression and transition.[1] In addition to these functions, CDC25s play an important role as checkpoint regulators for handling DNA damage caused by UV light, ionizing irradiation, or chemicals. Therefore, genomic instability is likely to be triggered by misregulation of CDC25s. Mammalian cells express three CDC25 isoforms, CDC25A, CDC25B, and CDC25C. It is believed that all three isoforms contribute to regulation of the activities of CDKs during all phases of the cell cycle, though specific functions of each enzyme at different phases have been reported.[2]

Since CDC25s are associated with oncogenic transformation and are overexpressed in various cancer cells,[3] many medicinal chemists have focused on the development of CDC25 inhibitors since the late 1990s. Many inhibitors have been reported, as reviewed recently,[4] but most of them have a quinone structure and show non-selective inhibitory activity towards all isoforms of CDC25s. Nevertheless, these non-selective inhibitors are considered to be promising lead compounds for cancer therapy, because mice lacking CDC25B and CDC25C survive and exhibit normal cell cycle and checkpoint responses, suggesting that other phosphatases, including CDC25A, are able to functionally compensate for loss of CDC25B and CDC25C.[5] Therefore, subtype non-selective inhibitors may be appropriate for cancer therapy. Indeed, these quinoid-type inhibitors generally show powerful inhibitory activity at the enzyme level and exhibit potent anti-proliferative activity towards various cancer cell lines. However, isoform-selective inhibitors are also required as tools for chemical biology research, e.g., for analysing the characteristics of the individual CDC25 isoforms.[6]

But, it would be difficult to create isoform-selective inhibitors based on quinoid compounds, because of their property of generating reactive oxygen species (ROS) within cells.[7] The active site of the CDC25s has a characteristic P-loop structure with the consensus sequence (H/V)C$X_5$R of all DSPs and protein tyrosine phosphatases (PTPs).[8] The highly conserved cysteine and arginine residues have crucial roles in the catalytic dephosphorylation mechanism. The cysteine residue, which acts as a nucleophile, triggering hydrolysis of the phosphate ester, is especially reactive compared to other cysteine residues due to the low p*K*a value of its thiol moiety. Thus, it may be especially susceptible to ROS. There are thought to be three possible mechanisms through which CDC25s inhibitors inhibit CDC25s and other phosphatases, i.e., reversible interaction of the inhibitor with the active site of phosphatases,[9] covalent-bond formation with a nucleophilic amino acid residue around the active site,[10] and oxidation of the critical cysteine residue by ROS generated by the inhibitor.[7] The former two mechanisms are widely encountered, and indeed, some inhibitors of CDC25s have been shown to exhibit competitive or competitive/non-competitive mixed-mode inhibition.[6c,11] On the other hand, some phosphatases utilize redox reactions as a mechanism of regulation of their enzymatic activity.[12] Therefore, it is plausible that the quinone-type inhibitors of CDC25s may act via ROS-mediated oxidation of the susceptible cysteine residue; this would be consistent with their non-selective inhibition of CDC25 isoforms. Moreover, ROS may oxidize other phosphatases, as well as unrelated cysteine-based enzymes, and therefore quinone-type agents could potentially trigger a range of unrelated events in cells.

Recently, we succeeded in developing a series of inhibitors that do not generate ROS in cells. These inhibitors were found in a focused library of enamine derivatives of a natural product, RK-682 (RE derivatives).[13] The RE derivatives have better cell permeability[14] than the original compound, RK-682,[15] and its derivatives.[16] Furthermore, most of the RE derivatives showed strong inhibitory selectivity for VHR, CDC25A, and CDC25B, and did not inhibit CDC25C, MKPs, PTP1B, or CD45. Although DSPs and PTPs share the same catalytic mechanism, their active site structures are different. DSPs such as VHR have a shallower binding pocket for substrate phosphate ester than that of PTPs such as PTP1B.[17] In particular, there is no obvious active site pocket in CDC25s,[18] and hence, development of isoform- and/or enzyme-specific inhibitors is quite challenging. Nevertheless, our RE derivatives, RE1 (**1**) and RE44 (**2**), showed complete selectivity for CDC25A/B over CDC25C as well as MAPKs and PTPs. Furthermore, some of the derivatives showed partial selectivity for VHR, CDC25A, and CDC25B. To create inhibitors with strict isoform selectivity, which would be useful for chemical biology research to clarify the role of individual CDC25 isoforms, it is essential to understand the inhibition mechanism at the molecular level. Here, we report the results of kinetic studies and binding site analysis using our original biotin-conjugated probes. Based on these findings, we propose a unique inhibition mechanism of RE derivatives for CDC25A, via binding at pocket 1 adjacent to the active site.

## Results and Discussion

### Enzyme kinetic studies of RE1 (1) and RE44 (2)

We have reported that benzyl-substituted RE1[14] (**1**) showed inhibitory activity towards CDC25A ($IC_{50}$ = 16.6 μM), CDC25B ($IC_{50}$ = 8.4 μM), and VHR ($IC_{50}$ = 11.4 μM), and that *o*-hydroxybenzyl-substituted RE44[13] (**2**) showed selectivity for CDC25B ($IC_{50}$ = 4.3 μM) over CDC25A ($IC_{50}$ = 13.5 μM) and VHR ($IC_{50}$ = 24.9 μM) (Fig. 1). RE derivatives were designed as neutral phosphate-mimicking molecules, in which the polarized core enamine functionality conjugated with two carbonyl groups was expected to have similar properties to substrate phosphate ester and to bind to the active site P-loop structure of DSPs. Molecular modelling of RE1 (**1**) with VHR suggested that the core structure could bind to the active site, and the results of a preliminary structure-activity relationship study focusing on the long hydrophobic alkyl chain and primary alcohol were consistent with this model. Unfortunately, however, attempts to generate a binding model of RE derivatives with CDC25s using Discovery Studio software failed, probably due to the shallowness of the binding pocket on CDC25s. Therefore, for further refinement of the structure, aimed at improving the inhibitory potency and selectivity of the ligands, we decided that it was necessary to obtain experimental information about the binding mode of RE derivatives with CDC25s. First, to examine whether RE derivatives compete with substrate, we carried out enzyme kinetic experiments to establish the character of the inhibition by RE derivatives. Unexpectedly, as shown in Fig. 2, Lineweaver-Burk plots of RE1 (**1**) for CDC25A and RE44 (**2**) for CDC25B indicated that inhibition was non-competitive, i.e., the inhibitor should bind to both enzyme and substrate-enzyme complex in a similar manner and inhibit the de-phosphorylation. Most CDC25 inhibitors are believed to bind to the active site. In contrast, our kinetic data suggested that RE derivatives showed inhibition without disturbing the binding of a small-molecular substrate, 3-*O*-methylfluorescein phosphate (OMFP).

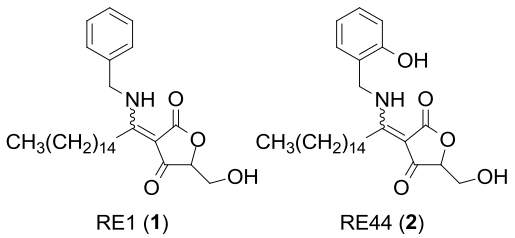



**Fig. 1** Structures of RE derivatives (**1** and **2**).

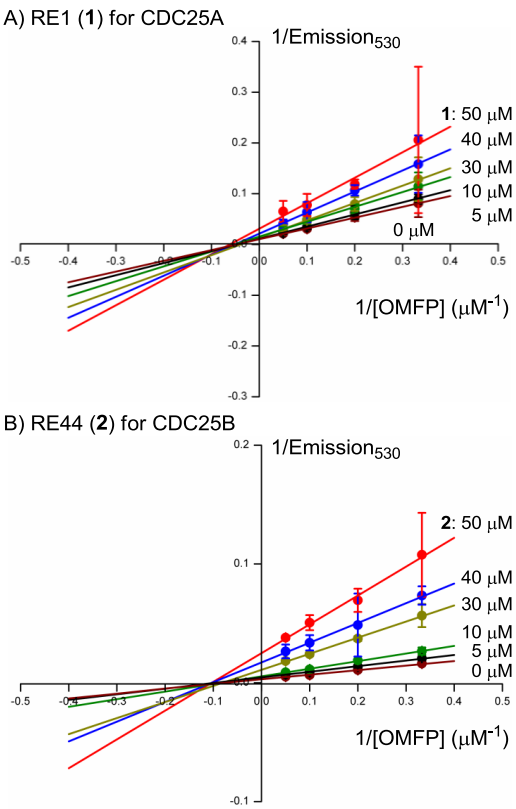



**Fig. 2** Lineweaver-Burk plot of the effect of RE derivatives on CDC25s activities. A) Effects of RE1 (**1**) on the activity of CDC25A, B) Effects of RE44 (**2**) on the activity of CDC25B.

As mentioned above, RE derivatives show high inhibitory selectivity for CDC25A/B, and therefore identification of the binding site of these derivatives would be helpful to understand the origin of the observed selectivity. Thus, we next planned to identify the binding site by installing a covalent bond-forming functionality in the RE derivatives.

### Design and synthesis of modified RE derivatives

Here, we focused on identification of the binding site on CDC25A and carefully examined its crystal structure[18a] in comparison with that of CDC25B[18b] (Figure 3A and 3B). As already reported, the crystal structure of CDC25B complexed with sulfate anions has two possible surfaces available for contact

with small molecules, in addition to the catalytic site. One of them is a pocket adjacent to the catalytic site, the so-called "swimming pool" (pocket 1), and the other is the second sulfate binding site (pocket 2).[19] Pocket 1 is surrounded by positively charged groups and is constituted by residues 442-448, 479, and 531-550 (C-terminus of CDC25B).[18b] In the crystal structure of CDC25A[18a] (Figure 3B) this pocket is not obvious, probably due to the disordered C-terminal structure and the difference in the position of the side chain of R436, which is a part of the catalytic site loop (Fig. 3B). The position of the side chain of R436 on CDC25A was unusual compared to that in the case of CDC25B (R479) and other DSPs/PTPs, and it covers the neighboring binding pocket (pocket 1 in Figure 3B). Therefore, we speculated that pocket 1 might be formed by a change in the orientation of R436 in solution. It is likely that these differences in crystal structure between CDC25A and CDC25B are induced by sulfate ion. In fact, interaction of the R479 side chain in the crystal structures of most other DSPs/PTPs in complexes with oxyanions such as phosphate and sulfate have similar structures to CDC25B, and the homology model of CDC25A incorporating sulfate, constructed based on the crystal structure of CDC25B, indicated the existence of a similar pocket (pocket 1), as shown in Fig. 3C and 3D. Therefore, pocket 1 seems to be a good candidate for the binding site of RE derivatives. The other possible RE-binding site is the second sulfate-binding site, which forms a shallow but wide pocket (pocket 2) in both CDC25A and CDC25B (Fig. 3A~D). If the inhibitor binds to one of these pockets, covalent modification of CDC25s with an RE derivative might be possible by introducing an electrophilic functionality into the RE derivative, because nucleophilic cysteine residues (C384 and C441 in CDC25A, C426 and C484 in CDC25B) exist in, or at least in close proximity to, the binding pockets.

The primary alcohol of RE1 (**1**) was found not to be essential for CDC25-inhibitory activity, since compound **3**[14] (Scheme 1) showed comparable inhibitory activity to RE1 (**1**), and its $IC_{50}$ value for CDC25A was 18.5 μM. Thus, we designed the new RE derivatives **4**~**9** (Scheme 1) possessing leaving groups such as methanesulfonate (mesylate, OMs) and halide instead of the hydroxyl group of **1**, or a Michael acceptor structure (α,β-unsaturated ketone).

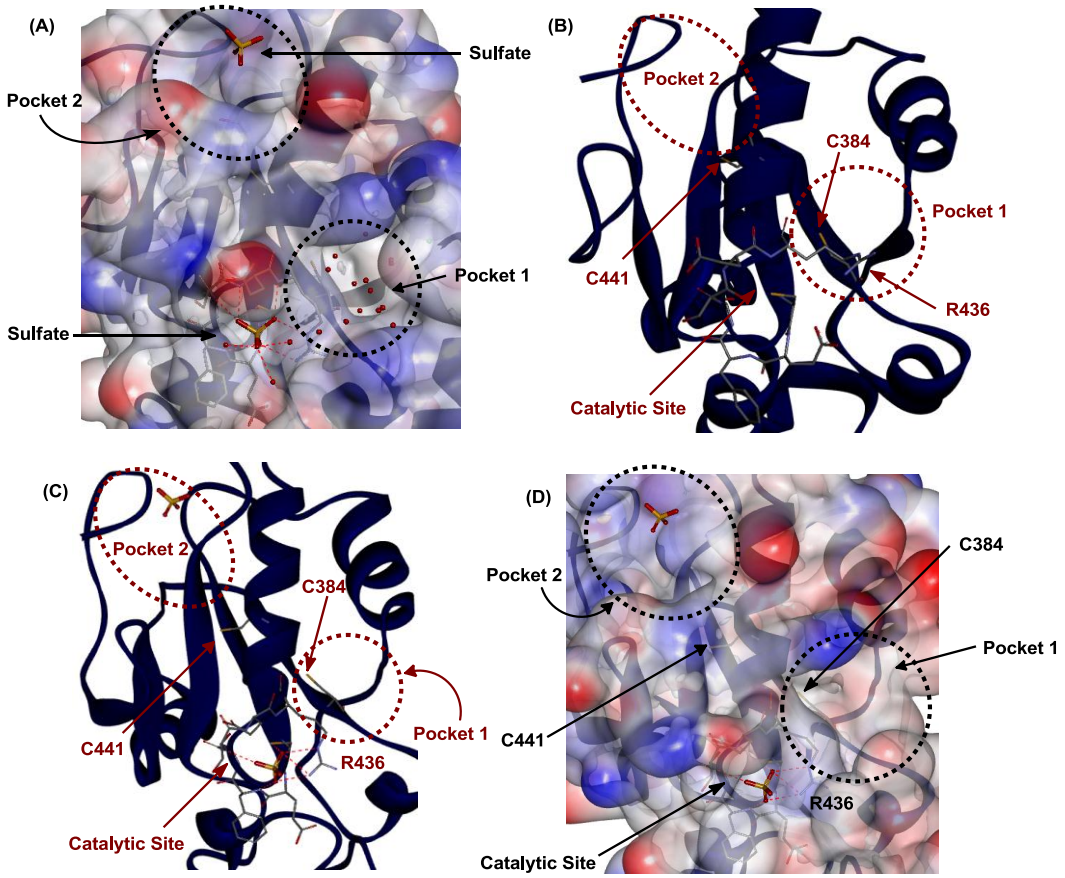


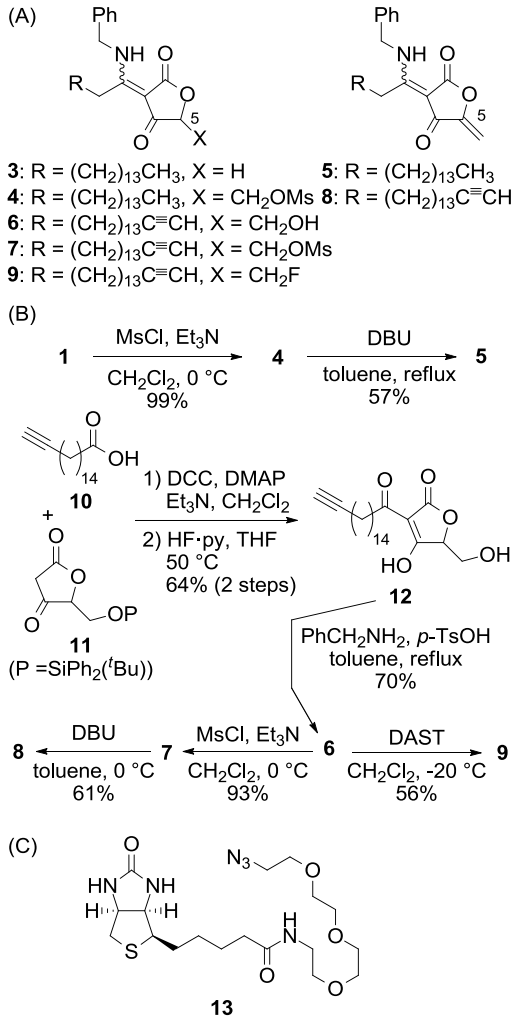

**Fig.3** (A) Crystal structure of CDC25B (PDB ID: 1QB0); (B) Crystal structure of CDC25A (PDB ID: 1C25); (C) Simulated homology model of CDC25A derived from the crystal structure of CDC25B (PDB ID: 1QB0); (D) Surface model of the CDC25A homology model.

### Synthesis of new RE derivatives (4~9)

The new RE derivatives were synthesized as illustrated in Scheme 1. The primary alcohol of **1** was converted to mesylate to afford **4**. Treatment of **4** with DBU in toluene gave a potential Michael acceptor, α,β-unsaturated ketone derivative **5**. Both **4** and **5** was found to be stable under neutral conditions, and could be purified by column chromatography without difficulty. In contrast, the corresponding bromine and chlorine derivatives (X = Br, Cl) were unstable and gradually decomposed in solution.

Alkyne-modified RE derivatives were also prepared in order to analyse covalent bond formation of these molecules with CDC25A utilizing click chemistry" (Huisgen reaction).[20]

Carboxylic acid **10** with a terminal alkyne moiety[21] was connected to the C3 position of tetronic acid derivative **11** by treatment with DCC (1,3-dicyclohexylcarbodimide) and DMAP (4-dimethylaminopyridine) to afford the corresponding 3-acyltetronic acid derivative. Treatment of the crude product with HF-pyridine complex in THF gave **12**. Condensation of **12** with benzylamine in the presence of a small amount of *p*-toluenesulfonic acid (*p*-TsOH) provided enamine **6**. Mesylate **7** and α,β-unsaturated ketone derivative **8** were prepared similarly from **6**. Furthermore, the fluorine-substituted derivative **9** (X = F) was also synthesized by treatment of **6** with DAST (*N*,*N*-diethylaminosulfur trifluoride), and this molecule was found to be stable.




**Scheme 1** (A) Structures of RE derivatives (**3**~**9**); (B) Synthesis of new RE derivatives (**4**~**9**); (C) Structure of biotin-azide (**13**).

### Confirmation of covalent bond formation of RE derivatives with CDC25A by MALDI-TOF/MS analysis and western blotting

With these molecules in hand, we first evaluated their inhibitory activity towards CDC25s. As shown in Fig. 4A, all compounds showed potent inhibitory activity for GST-CDC25A, and, as we had hoped, the nature of the C5 substituent on RE compounds did not affect the potency.

To examine whether these derivatives can form a covalent bond with the catalytic domain of CDC25A (catCDC25A), we first tried MALDI-TOF/MS analysis of catCDC25A treated with or without simple derivatives **4** and **5**. In the case of mesylate **4**, a mass peak indicating covalent bond formation with **4** was not observed. In contrast, a small new mass peak corresponding to catCDC25A modified by the Michael acceptor **5** was detected (Figure S1).[22]

However, it proved difficult to identify the binding site using this sample, due to the very low labelling efficiency. Namely, peptide fragments generated by trypsin or endoproteinase lysC digestion of catCDC25A modified with **5** were analysed by LC-MS and compared to those of the control catCDC25A, but no pronounced difference between the control and RE-modified catCDC25A was observed, and no RE-modified peptide fragment was detected.

(A)

| Compounds | $IC_{50}$ Values [μM] CDC25A | CDC25B | CDC25C |
|---|---|---|---|
| **3** | 18.5 | 5.7 | >100 |
| **4** | 7.3 | 5.1 | >100 |
| **5** | 9.2 | 4.8 | 59.2 |
| **6**[a] | 12.7 | 24.1 | 90.2 |
| **7**[a] | 10.1 | 5.3 | >100 |
| **8**[a] | 5.1 | 5.3 | ND |
| **9**[a] | 7.2 | 7.0 | 34.3 |

a) $IC_{50}$ values were determined by two independent experiments.

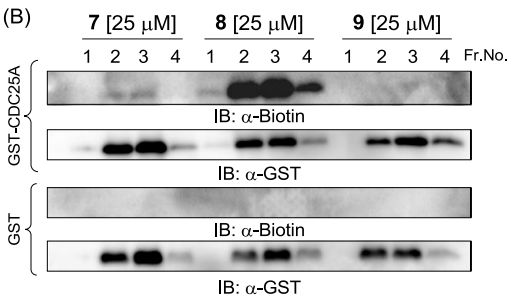



**Fig. 4** Inhibitory activities of RE derivatives towards CDC25s, and covalent bond-forming properties with GST-CDC25A. A) Inhibitory activities of RE derivatives towards GST-CDC25s; B) Western blotting analysis of GST and GST-CDC25A after treatment with alkyne-modified RE derivatives **7**~**9** followed by conjugation with biotin-azide (**13**) using Cu(I)-mediated click chemistry. Alkyne-modified compounds were removed on a Micro Bio-Spin Chromatography 6 column (Bio-Rad) before click chemistry.

Therefore, to further confirm covalent bond formation, we next used the alkyne-modified RE derivatives **7**~**9**. After treatment of GST-CDC25A protein in Tris-based buffer with each compound, a biotinyl group was introduced into the alkynyl tail of the RE derivative by Cu(I)-mediated azide-alkyne Huisgen reaction[20b] with biotin-azide probe **13** (Scheme 1). The GST-CDC25A protein and the free RE derivative were separated by SDS-PAGE, and the protein was blotted onto a membrane. The biotinylated RE derivative-modified protein should be detectable by immunostaining using anti-biotin antibody. However, strong staining was observed not only for GST-CDC25A, but also for the control GST protein treated with the compounds, indicating non-specific labelling of the protein. We speculated that Huisgen reaction and/or sample preparation for SDS-PAGE in the

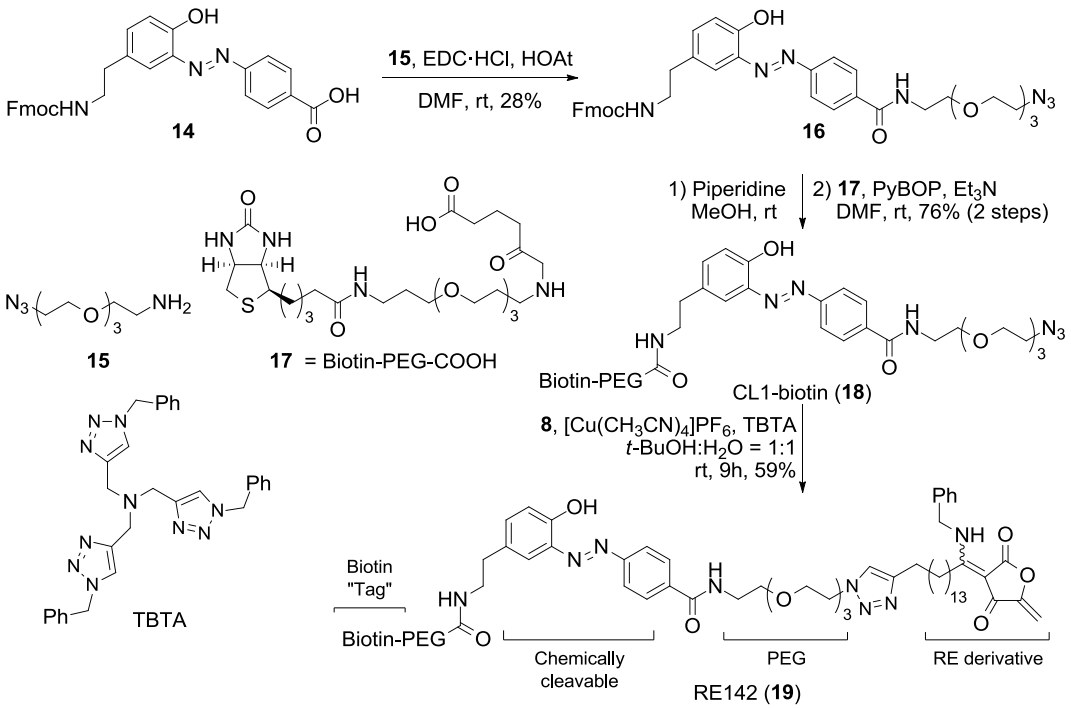


presence of residual free RE derivative may have caused the non-specific binding to GST (Figure S2),[22] and therefore, remaining small molecules were removed by gel filtration before the Huisgen reaction. The flow-through fractions from the gel filtration column (Fr. No. 1~4) were subjected to the Huisgen reaction and analysed by western blotting. As shown in Fig. 4B, selective and significant covalent bond formation of GST-CDC25A with Michael acceptor **8** was confirmed, and no labelling of GST protein was observed. It is noteworthy that a faint band of GST-CDC25A labelled with mesylate **7** was also observed, suggesting that mesylate **7** could also form a covalent bond with CDC25A, although the efficiency was very low. In contrast, no band was detected for the fluorine derivative **9**. These results indicated that a Michael acceptor-type molecule could form a covalent bond more efficiently than electrophiles in the $S_N2$-type reaction mode. Based on these experimental results, we designed a new Michael acceptor-type RE derivative for identification of the binding site on CDC25A.

### Design and synthesis of multi-functional probe RE142 (19) with biotin moiety and chemically cleavable diazobenzene linker

As mentioned above, it was difficult to detect a very small quantity of modified peptide in the presence of a large amount of unmodified peptides by mass analysis. Affinity purification utilizing the specific interaction of a tag with a tag-binding protein, such as biotin-avidin, is well established as a powerful tool to enrich target molecules. But, to identify the binding site of a small molecule on a target protein, especially if the small molecule-modified protein is a minor component in a mixture of unmodified or unrelated proteins, enrichment of the modified protein/peptide is essential. In the case of the biotin-avidin system, the strength of the interaction sometimes makes elution of the desired proteins/peptides difficult and results in low yield. To overcome these issues, a chemically cleavable linker, which anchors the small molecule and tag and facilitates isolation of the objective protein/peptide, can be used.[23] Therefore, we designed a new probe, CL1-biotin (**18**), equipped with biotin tag, a chemically cleavable linker, and an azide group for coupling to the alkynylated molecule **8**, for identification of the binding site. As a chemically cleavable linker, we employed diazobenzene, which is cleaved by reducing salt $Na_2S_2O_4$.[24] Since we assumed that low solubility of protein/peptide modified by **5** (having a long hydrophobic alkyl chain) might also be an issue, the above parts were connected with a poly-ethylene glycol (PEG) spacer. We expected that introduction of hydrophilic functionality into the new RE probe would improve the solubility of the peptide fragment linked to RE derivative in aqueous buffer solution.




**Scheme 2** Structures and synthesis of CL1-biotin (**18**) and RE142 (**19**).

Synthesis of CL1-biotin (**18**) was started from Bogyo's intermediate (**14**, Scheme 2).[24c] Condensation of **14** with commercially available $N_3$-PEG-$NH_2$ (**15**) provided **16**, although the chemical yield was not satisfactory. After removal of the Fmoc group, introduction of commercially available biotin-PEG-COOH (**17**) afforded **18**. However, when we performed Western blotting experiments similar to Figure 4B using the new CL1-biotin probe **18** instead of the simple biotin-azide probe **13**, an unexpectedly strong band was observed for the control GST. It was found that a significant level of biotin labelling of GST occurred under Huisgen reaction conditions even in the absence of RE derivatives. We speculate that some reactive species may be generated by Cu(I)-mediated oxidation of the diazo-phenol moiety of **18** and this species may react with surface amino acid residue(s) on GST. Therefore, we decided to connect **8** and **18** before the reaction with CDC25A. The Huisgen reaction of **18**

with **8** in *t*-BuOH-water proceeded without difficulty to give the new probe molecule for binding site analysis, RE142 (**19**), in 59% yield.

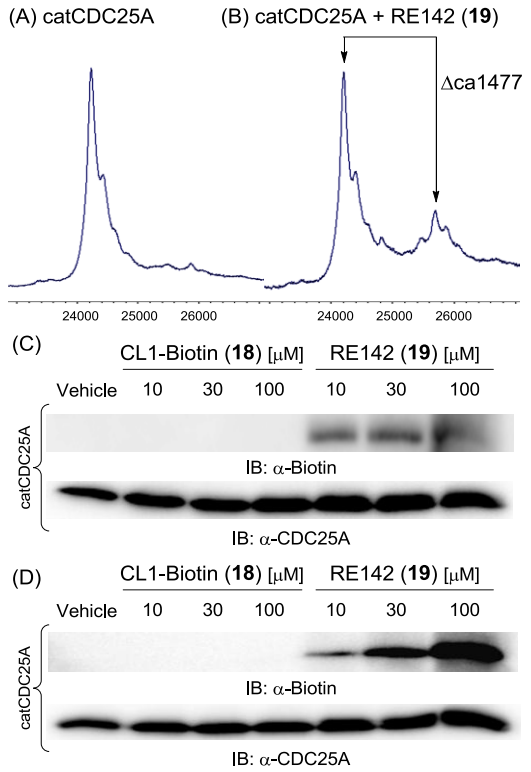



**Fig. 5** Confirmation of covalent-bond formation of RE142 (**19**) with catCDC25A. A) MALDI-TOF/MS analysis of catCDC25A (calculated average M.W. = 24,204); B) MALDI-TOF/MS analysis of catCDC25A after treatment with compound **19** (6.6 mM, calculated M.W. = 1476.81); C) Western blotting analysis of catCDC25A after treatment with CL1-biotin (**18**) or RE142 (**19**) in Tris-based buffer; D) Western blotting analysis of catCDC25A after treatment with CL1-biotin (**17**) or RE142 (**19**) in phosphate-based buffer.

### Covalent bond formation of RE142 (19) with CDC25A

The newly synthesized RE142 (**19**) retained moderate inhibitory activity towards GST-CDC25A ($IC_{50}$ = 59.2 μM) and GST-CDC25B ($IC_{50}$ = 56.3 μM) but GST-CDC25C ($IC_{50}$ = >100 μM), although its potency was about 10 times less than that of **5** or **8**. MALDI-TOF/MS analysis of catCDC25A after treatment with **19** (6.6 mM, molar ratio 1:200) resulted in the appearance of a new small peak corresponding to catCDC25A protein with one molecule of **19** (Fig. 5A and 5B). Since **19** has a biotinyl group, covalent bond formation was directly detectable by Western blotting analysis without further transformation. As shown in Figure 5C, mixing of CL1-biotin (**18**) with catCDC25A in the absence of Cu(I) salt did not give any biotin-labelled protein. In contrast, the protein band corresponding to biotin-labelled catCDC25A was observed when catCDC25Awas treated with RE142 (**19**) in Tris-based buffer (pH = 8.2, Fig. 5C), but its formation showed poor dose-dependency. Since the use of a high concentration of **19** in Tris-buffer resulted in formation of a suspension, we suspected that the solubility of **19** was insufficient, and so we examined several other conditions. We found that phosphate-based buffer (pH = 8.0) gave a clear solution even with 100 μM **19**, and in this case, dose-dependent labelling of catCDC25A was observed (Fig. 5D). (3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate) (CHAPS) was also found to be useful as a detergent for improving the solubility of **19** and did not disturb the binding of **19** with catCDC25A (data not shown). Overall, these results indicated that RE142 (**19**) is suitable for analysis of the binding site of RE derivatives on CDC25A.

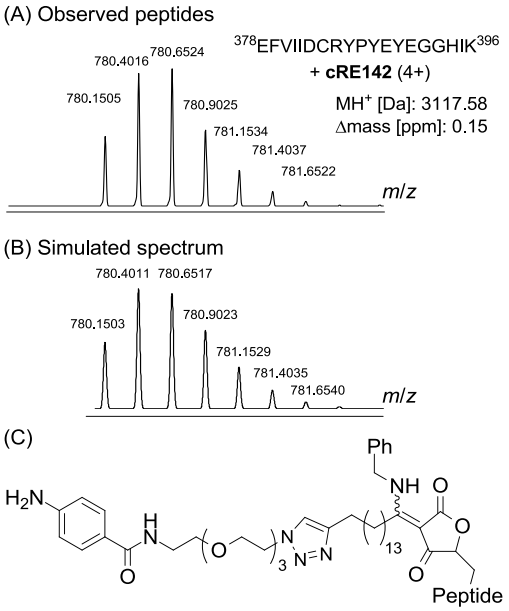



**Fig. 6** LC-MS analysis of digested catCDC25A after treatment with RE142 followed by cleavage of the diazobenzene linker with $Na_2S_2O_4$. A) Observed mass spectrum of cleaved RE142 (cRE142)-modified peptide; B) Simulated mass spectrum of cRE142-modified peptide; C) Structure of cRE142-modified peptide.

### Identification of RE-modified peptide by LC-MS analysis

As shown in Figure 5, we confirmed the generation of RE142-modified catCDC25A protein by MALDI-TOF/MS and western blotting analysis, but the efficiency of covalent bond formation was still low, and enrichment of the modified peptide was required. After treatment of catCDC25A with RE142 (**19**, 1 mM) in the presence of 2% CHAPS, proteins were precipitated with TCA/acetone to remove excess RE142 (**19**) and CHAPS. The resulting protein precipitate was dissolved in 7 M guanidine-HCl, and treated with DTT and iodoacetamide (IAA) to reduce and cap the thiol groups of cysteine residues. Then it was digested sequentially with endoproteinase lysine-C (Lys-C) and trypsin to afford a peptide mixture including the RE142 (**19**)-modified peptide.[22] Enrichment of the modified peptide was conducted with NeutrAvidin beads at 37 °C. The beads were washed several times, then treated with cleavage buffer containing 0.5 M $Na_2S_2O_4$ and 0.005% SDS to elute the peptide. These treatments reduced the diazobenzene group of the RE-142-labeled peptide with release of cleaved RE142 (cRE142) having the structure shown in Figure 6C. NanoLC-MS analysis of the eluted peptides confirmed the formation of the cRE142-modified peptide corresponding to $^{378}$EFVIIDCRYPYEYEGGHIK$^{396}$ + cRE142. The observed mass numbers were well matched with the calculated values, as shown in Fig. 6A and 6B (the error was just 0.15 ppm). These results strongly indicate that the observed ion at

m/z 780 ([M+4H]$^{4+}$) is that of the cRE142-modified peptide. Further MS/MS analysis indicated that RE142 was bound to one of residues C384, R385, or Y386 (Figure S5 and Table S2).[22] Interestingly, these amino acid residues are located at binding pocket 1, shown in Figure 3. Although we cannot exclude the possibility that RE142 also binds to the other amino acid residues of cat CDC25A, this was the only modified peptide fragment that we found. These results strongly suggest that RE142 binds to pocket 1.

### Plausible mechanism of inhibition of CDC25s by RE derivatives

Although we could not determine which amino acid residue was modified by RE142 among the above three residues, R385 is an unlikely candidate, because it is buried in the folded protein (Fig. 7) and the nucleophilicity of the guanidine residue is generally low. Y386 is located at the surface near pocket 1, and a phenol group can react with the Michael acceptor. However, it is well known that a thiol group is a much better nucleophile than a phenolic hydroxyl group as a Michael donor, and C384 seems to be the most plausible candidate, based on the chemical reactivity. Although the conformation of the side chain residue of C384 in the reported crystal structure is poorly placed to react directly with RE142, a molecular modelling study[22] suggested that covalent bond formation of the thiol group with the exo-methylene group of **4** is possible. Based on these considerations we think that C384 is the most plausible candidate.

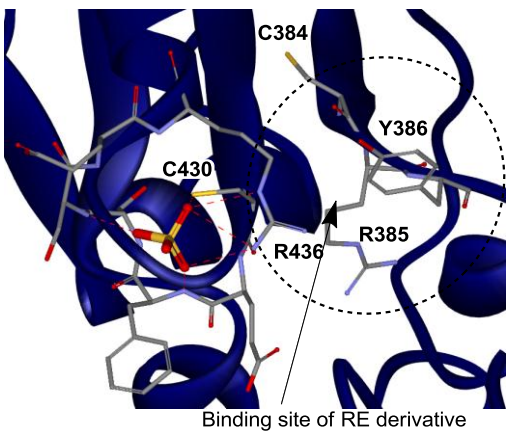



**Fig. 7** Plausible binding site of RE derivatives on CDC25A.

As discussed previously, pocket 1 in the crystal structure of CDC25A without sulfate anion is covered by R436 (Fig. 3A). In contrast, in the model of the sulfate complex (Fig. 3B), R436 flips and faces the P-loop, forming multiple hydrogen bonds to the sulfate oxygen atoms, and pocket 1 is opened. It is likely that the side chain of R436 is flexible in the absence of substrate. If this is the case, RE derivatives could bind to pocket 1 either in the absence or presence of substrate phosphate ester (Fig. 7). The observed non-competitive inhibition mode is reasonable, if RE derivatives bind at pocket 1. For the hydrolysis of the phosphate ester, nucleophilic attack of the thiolate residue of C430 and stabilization of the resulting meta-phosphate-like transition state by hydrogen bonding from the R436 are proposed to be critical.[2e,25] Therefore it is possible that the binding of RE derivatives to pocket 1 disturbs the stabilization of the transition state by affecting the side chain conformation of the critical R436, thereby inhibiting the hydrolysis.

## Conclusions

We designed a series of RE derivatives from natural product RK-682 as phosphate-mimicking molecules. Initially, we considered that, like other phosphatase inhibitors, RE derivatives would bind to the active site of CDC25A and CDC25B in a competitive manner to substrate phosphate ester. However, kinetic experiments showed that the inhibition was non-competitive. Binding site analysis using a covalent bond-forming RE derivative revealed that the RE derivative binds to a pocket adjacent to the catalytic site. Although this binding pocket has been already identified in the literature as a promising target for drug development, to our knowledge, the present RE derivatives are the first class of compounds that have been shown to bind to this pocket. Although we have not yet analysed the binding site of RE derivatives on CDC25B, a similar binding mechanism might be anticipated, based on the observation of non-competitive inhibition in the kinetic study of RE44 (**2**, Fig. 2B). Although the molecular basis on the observed selectivity of the RE derivatives is still unclear partially due to the lack of the structural information of CDC25C with high resolution, our finding that RE derivatives bind at pocket 1 adjacent to the active site represents the first experimental evidence supporting the idea that a strategy of targeting this pocket would be effective for developing inhibitors of the biologically and pharmaceutically important CDC25s.

## Notes and references

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