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Design of New Inhibitors for CDC2 Kinase Based on a Multiple Pseudosubstrate Structure

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Abstract: New inhibitors have been designed for cdc2 kinase based on a multiple pseudosubstrate structure. The new inhibitors have three different structural components: 3,4-bis(indol-3-yl)maleimide, Ac-Cys-(Ser)-Pro-Lys-Lys-NHMe, and ethyloxy group between the two components. Inhibitory activities toward cdc2 and other protein kinases were investigated, and the compound (**21**) with Ac-Cys-Pro-Lys-Lys-NHMe connected with the triethylene glycol spacer exhibited the most potent inhibition with relatively high selectivity.

Cyclin-dependent kinases (CDKs) play a central role in the cell division cycle. CDKs include several subtypes based on the constituents of a catalytic [cdk1(=cdc2)-cdk8] and a regulatory subunit (cyclin A-cyclin H). The activity of each subtype is regulated at the specific stage of the cell cycle by a number of biochemical events: production, complex formation, chemical modification (phosphorylation or dephoshporylation), and interaction with other regulatory proteins. Recently, CDKs have attracted much attention as a new target for development of therapeutic agents, because CDKs have direct interaction with oncogenes and tumor suppressors; furthermore, deregulation of CDKs and their regulators are found frequently in human cancer. In fact, some chemical inhibitors of CDKs block cell cycle progression and exhibit interesting antitumor activities.[1)]

Although there are a number of inhibitors of kinases, such as staurosporin, UCN-01 and so on, only three types of selective inhibitors have been reported: butyrolactone-I,[2)] flavopiridol,[3)] olomoucine,[4)] and their derivatives. In general, selective inhibitors are searched for randomly from naturally occurring products or synthetic compounds. In this study, we attempted to design new selective inhibitors toward cdc2 kinase based on 3,4-bis(indol-3-yl)maleimide, a PKC inhibitor,[5)] and a partial structure of a peptide substrate (Fig. 1).

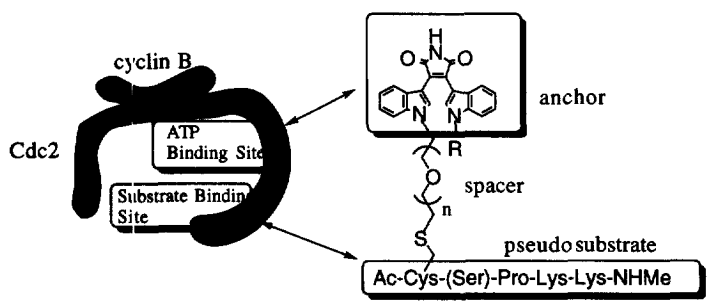

Fig. 1. A Schematic Drawing of Cdc2-Cyclin B Complex and the Design of New Inhibitor.

The cdc2-cyclin B complex is called maturation/M-phase promoting factor (MPF) and regulates the transition from G2- to M-phase.[6)] It phosphrylates a serine or a thereonine residue in a common sequence of Ser/Thr-Pro-X-Lys/Arg (X= amino acid) of substrate proteins.[6)] Cdc2 kinase has an ATP binding site and a

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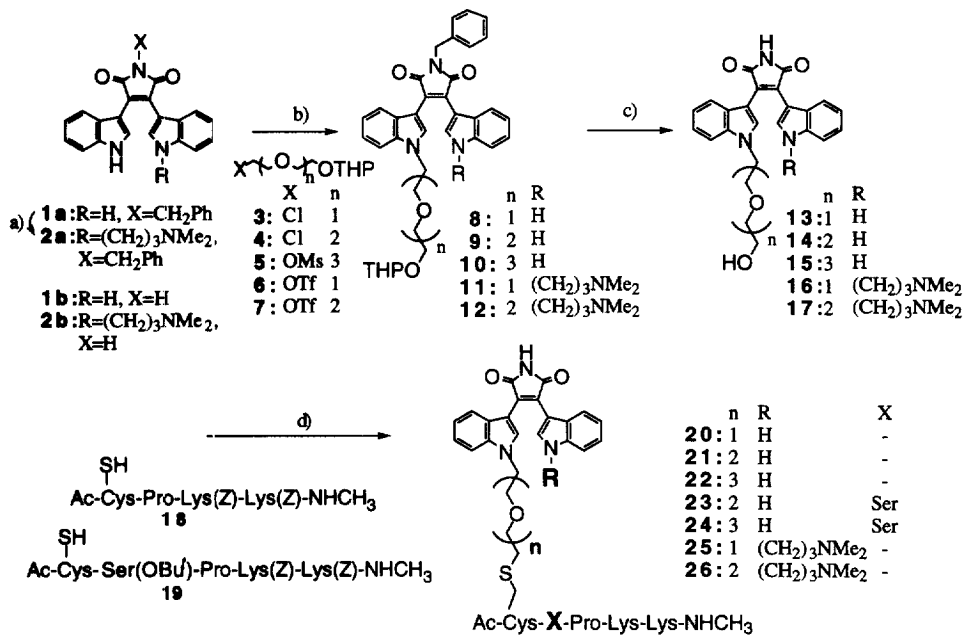
substrate binding site, and catalyzes transfer of a phosphoryl group from ATP to peptide substrates. Pseudosubstrate peptide inhibitors which mimic a partial sequence of the substrate or inhibitory factors have been used as selective inhibitors in biological studies.[7] However, the potency of the peptide inhibitors is usually low and may not be useful in a practical sense. New compounds were originally designed to have three different structural components: 3,4-bis(indol-3-yl)maleimide as an anchor which has high affinity to an ATP binding site of PKC, Ac-Cys-Pro-Lys-Lys-NHMe as a pseudo-peptide substrate, and an ethyloxy group as a spacer between the two components (Fig. 1). The structure of 3,4-bis(indol-3-yl)maleimide was chosen, because it showed moderate inhibitory activity to cdc2 kinase in a preliminary study.[8] Thus, the multiple pseudosubstrate type constituents of 3,4-bis(indol-3-yl)maleimide and Ac-Cys-Pro-Lys-Lys-NHMe were expected to produce high affinity and selectivity, respectively.

Scheme 1[a]



[a] a) NaH, $Cl(CH_2)_3NMe_2$, DMF (66 %), b) NaH in DMF, rt, **3** for **8** (62 %), **4** for **9** (62 %), **5** for **10** (88 %); NaH in DME, rt, **6** for **11** (70 %), **7** for **12** (47 %), c) i) *p*-TsOH, CH_2Cl_2, MeOH, ii) 5N KOH, EtOH, then $AcONH_4$, 140 °C, **13** (73 %), **14** (84 %), **15** (82 %), **16** (63 %), **17** (50 %), d) i) TsCl, DMAP, THF, ii) **18** or **19** (5eq) , NaH (3eq), DMSO, iii) CF_3COOH, thioanisole, *m*-cresol, **20** (7 %), **21** (19 %), **22** (27 %), **23** (45 %), **24** (21 %), **25** (18 %), **26** (10 %) for three steps.

The synthesis started with 1-benzyl-3,4-bis(indol-3-yl)maleimide (**1a**)[5] (Scheme 1). The spacer was introduced with a THP protected ether (**3-7**), then the *O*-THP protecting group was removed under acidic condition. The *N*-benzyl protecting group was removed by alkaline hydrolysis followed by the treatment with $AcONH_4$ to give the non-protected skeleton (**13-17**). The pseudosubstrate peptides (**18** and **19**) were synthesized by the conventional solution chemistry using *N*-Boc derivatives of L-amino acids and DCC-HOBT in DMF as a coupling reagent. A cystein residue, *N*-acetyl-*S*-benzoylcystein, was condensed using DEPC in DMF as a coupling reagent, followed by removal of the benzoyl group with 0.2 N aqueous NaOH under argon atmosphere. The terminal hydroxyl group of the spacer (**13-17**) was tosylated, and reacted with **18** or **19** in

the presence of NaH in DMSO. The *N*-carbobenzyloxy and *O*-*t*-butyl protecting groups were removed in TFA in the presence of thioanisole and *m*-cresol, and the crude products were purified by reverse-phase HPLC.[9]

Inhibitory activities of the compounds were determined by measuring phoshporylation of H1 histone using active human cdc2-cyclin B complexes in the presence and the absence of the inhibitors.[2a, 10] The IC_{50} values (concentration necessary for 50 % inhibition) are listed in Table 1. The compounds **1b** and **2b**[11] which were reported to be potent inhibitors of PKC[5] showed moderate inhibition to cdc2 kinase. The introduction of the diethyleneglycol spacer (**13**) enhanced inhibitory activity, but the triethyleneglycol (**14**) or the tetraethyleneglycol (**15**) spacer diminished the activity. These inhibitory activities of **14** and **15** reached similar levels with the addition of propylamine side chain (**16** and **17**). Introduction of peptide pseudosubstrate into the compound **14** significantly enhanced the activity, and the potency of IC_{50}=285 μM of **14** was improved to IC_{50}=4.5 μM by **21**. On the other hand, **13** bacame less potent (IC_{50}=4 μM by **13** to 98 μM by **20**), and almost no change was caused by the modification of **15** to **22**. Because either **14** or peptide pseudosubstrate**18** itself, the components of **21**, did not show high potency (IC_{50}>1 mM), it was apparent that the high activity was enabled by the suitable length of the spacer connecting the two pseudosubstrates parts. The propylamine side chain disturbed the enhancement effect of the peptide pseudosubstrate (**23** and **24**). The peptide sequence with a serine residue did not display any inhibition (**25** and **26**), again suggesting the major contribution of the peptide pseudosupstrate to the inhibitory potency of **21**. Although there was no direct evidence, the serine-containing compounds might be phosphorylated by the enzyme.

Table 1. Inhibitory Activities of the Compounds Toward CDC2 Kinases.

Compounds	n	R	Pseudopeptide[b]	IC_{50} (μM) for CDC2
1b	-	H	-	18
2b	-	$(CH_2)_3NMe_2$	-	19
13	1	H	-	4
14	2	H	-	285
15	3	H	-	55
16	2	$(CH_2)_3NMe_2$	-	22
17	3	$(CH_2)_3NMe_2$	-	35
20	1	H	CPKK	98
21	2	H	CPKK	4.5
22	3	H	CPKK	31
23	1	$(CH_2)_3NMe_2$	CPKK	220
24	2	$(CH_2)_3NMe_2$	CPKK	570
25	2	H	CSPKK	>1000
26	3	H	CSPKK	>1000
18[a]	Ac-Cys-Pro-Lys-Lys-$NHCH_3$			>1000

a) All protective groups were removed, b) Only peptide sequences of the structure in Scheme 1 are shown.

Inhibition specificity of the compounds was investigated semi-quantitatively using PKA, PKC, PTK, CAMK, and EGFR (Table 2).[12] As already reported, the compounds **1b** and **2b** displayed selectivity to PKC.[5] The compound **13** also showed higher inhibitory activity to PKC than to cdc2 kinase. On the other hand, inhibition activity of **21** to PKC was smaller than that of **13**, showing relative selectivity of **21** to cdc2.

In conclusion, we have developed new cdc2 selective inhibitors which were designed based on the multiple-substrate structure. Although further investigation of the new inhibitors on the selectivity and on the effect on the cell cycle division is needed, this multiple-substrate concept will be applicable for the design of selective inhibitors to other types of protein kinases.[13]

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Table 2. Inhibition of Several Protein Kinases.

Compounds	Conc.(μM)	CDC2 IC_{50}(μM)	PKA	PKC	PTK	CAMK	EGFR
1b	240	18	++	++	++	+	-
1b	24		+	++	+	-	-
1b	2.4		-	++	-	-	-
2b	240	19	++	++	++	-	-
2b	24		+	++	+	-	-
2b	2.4		-	++	-	-	-
13	50	4	+	++	+	-	-
13	16		-	++	-	-	-
13	5		-	++	-	-	-
21	90	4.5	-	++	+	-	-
21	28		-	++	-	-	-
21	9		-	+	-	-	-

++: inhibition more than 75%, +: partial inhibition, - no inhibition

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8) We are grateful to Professor H. Kobayashi, Faculty of Medicine, Kyushu University, Japan, for inhibition assay with use of cdc2 kinase from Xenopus egg extracts.

9) Column: Nacalai tesque 5C18-AR300, 0.1 % TFA-CH_3CN linear gradient. All compounds used in this study showed satisfactory IR, ^{1}H-NMR, and High Resolution FABMS spectra.

10) Phosphorylation was done using cdc2-cyclin B, H1 histone (1 mg), and ATP (10 mM-1 mCi) in the buffer containing Tris•HCl (20 mM), b-mercaptoethanol (10 mM), EDTA (1 mM), and $MgCl_2$ (10 mM) at pH 7.4. The reaction mixture was passed through a nitrocellulose filter, and the remaining radioactivity of phoshprylated histone on the filter was measured with a liquid scintillation counter.

11) Debenzylated compounds **1b** and **2b** were obtained from **1a** and **2a**, respectively, by hydrolysis in 5 M KOH in EtOH, followed by the treatment with $AcONH_4$.

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