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# Peptide Inhibitors of CDK2-cyclin A that Target the Cyclin Recruitment-Site: Structural Variants of the C-Terminal Phe

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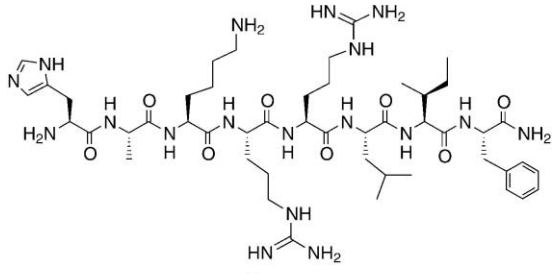
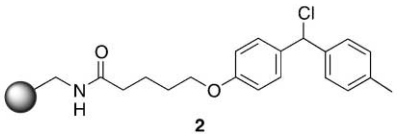
**Abstract**—A focused series of octapeptides based on the lead compound H-His-Ala-Lys-Arg-Arg-Leu-Ile-Phe-$NH_2$ **1**, in which the C-terminal phenylalanine residue was replaced by α and/or β-modified variants, was synthesized using solid-phase chemistry. Both the L-*threo*-β-hydroxy-phenylalanine (β-phenylserine, Pse) and (2*S*)-phenylalaninol derivatives, as competitive binders at the cyclin-recruitment site, displayed potent inhibitory activity towards the CDK2-cyclin A complex. Unexpectedly, the D-*threo*-Pse derivatives also showed inhibitory activity. 

Progression through the eukaryotic cell cycle is driven by the orderly activation of cyclin-dependent kinases (CDK) in association with appropriate cyclin subunits and phosphorylation by CDK-activating kinase at a conserved Thr residue.[1,2] Each phase of the cell cycle is characterized by the presence and activity of different CDK-cyclin complexes, for example, CDK2-cyclin A during the S phase. Crucially, the functions of these complexes are negatively regulated by a host of regulatory proteins, including members of the CIP/KIP family, $p21^{WAF1}$ and $p27^{KIP1}$.[3,4] Loss or mutation of one or more of these negative-regulatory proteins would result in cellular neoplastic phenotypes.[2,4]

The recognition of CDK-cyclin complexes by $p21^{WAF1}$ occurs in part through interaction with a binding groove in the cyclin subunit. This groove is centrally involved in the recruitment or initial binding of macromolecular CDK substrates such as pRb and E2F.[3,4] Thus, selective blockade of the cyclin recruitment-site would prevent recognition and subsequent phosphorylation of CDK substrates, and therefore offers a therapeutic approach towards restoration of $p21^{WAF1}$-like tumour suppression. Our recent efforts led to the discovery of an octapeptide $^{152}$HSKRRLIF$^{159}$ located C-terminal in $p21^{WAF1}$, which displayed potent CDK-cyclin inhibitory activity due to its capacity to bind to the cyclin recruitment-site (C-RS). Comprehensive SAR studies resulted in the identification of the lead compound **1**, in which the native Ser is replaced by an Ala residue.[5] In addition, the C-terminal Phe residue appeared to be a crucial determinant in the binding competence of **1**, since dramatic loss of activity was observed upon truncation or even conservative replacement with Tyr or Trp.



**1**



**2**

We herein report unexpected SAR data derived from a focused series of peptides, in which the Phe residue in **1** is replaced by both α-modified and χ-constrained Phe analogues. These unique peptide derivatives were synthesized using robust solid-phase chemistry and our recently reported polymer-supported linker **2**.[6]

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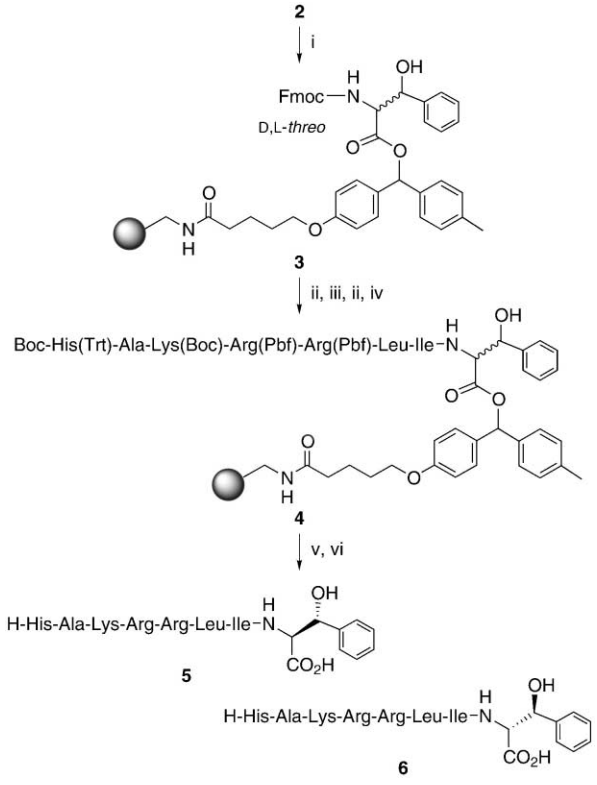
A molecular model of the complex between **1** and cyclin A was constructed,[5,7] which showed that the LIF motif, especially the Phe aromatic group, makes complimentary contacts with the C-RS hydrophobic groove. In order to gain further insights and to refine our understanding of the structure–activity profile of **1**, conformationally χ-constrained derivatives of Phe were installed and evaluated for their binding capacity and kinase-inhibitory activity towards CDK2-cyclin A.

χ-Constrained aromatic amino acids, typically β-methyl or-isopropyl analogues, have been successfully applied to unravel the bioactive conformation of peptides.[8] In this context, our approach is to use the commercially available (2*S*,3*R*;2*R*,3S)-Pse as χ-constrained surrogates of Phe, in which the side-chain has a preferred *trans* conformation. Moreover, the flexibility of this approach allows us to exploit the installed *threo*-Pse residue as synthetic precursor to *Z*-dehydrophenylalanine (Dhp) and *O*-modified peptide derivatives.

The synthetic strategy (Scheme 1) is based on the selective and efficient condensation (>98%) of Fmoc-DL-Pse-OH to the polymer-bound 4-alkoxy-4′-methylbenzhydryl chloride linker **2**[6] to afford the tethered derivative **3**. The peptide chain was then assembled using standard Fmoc solid-phase peptide synthesis conditions,[9] and the N-terminus was capped by treatment with di-*tert*-butyl dicarbonate ($Boc_2O$) to give the polymer-bound peptide **4** in typically >90% yield.



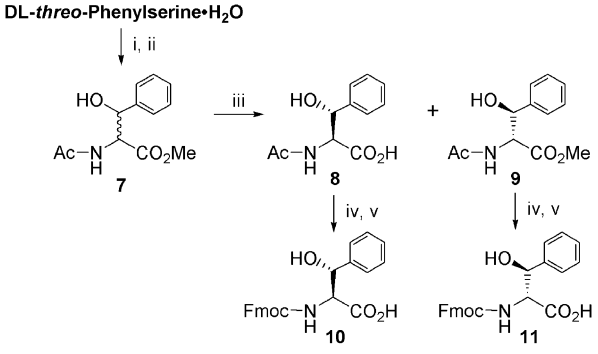

**Scheme 1.** Reagents and conditions: (i) FmocPseOH, DIEA, $CH_2Cl_2$, 24 h; (ii) piperidine-DMF (1:4); (iii) Fmoc-amino acid-TBTU-HOBt-DIEA (1:1:1:2; 4–8 equiv); (iv) $Boc_2O$ (4–8 equiv), DMF; (v) TFA-$H_2O$-*i*$Pr_3SiH$ (90:8:2), 2 h; (vi) separation using RP-HPLC using Kromasil C8 preparative column (10×150 mm).

As anticipated, no acylation of the exposed β-hydroxyl functionality was evident. Acidolytic treatment of **4** using a TFA-based cocktail afforded the peptide diastereoisomers **5** and **6** in high yield and purity. Gratifyingly, the two diastereoisomers were readily separated and purified by RP-HPLC. The identity and purity of peptidic products were established using two different reversed-phase silica HPLC columns[10] and ES–MS.

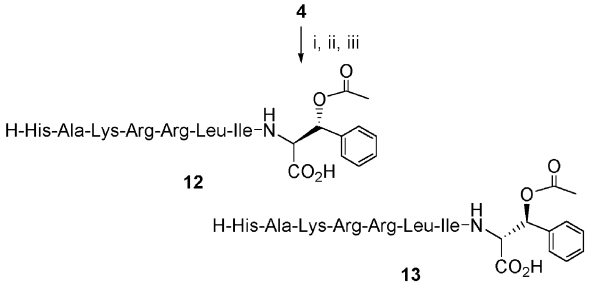
The stereo-identities of the synthesized peptides were established via the chymotrypsin-mediated resolution of Ac-DL-Pse-OMe **7**,[11] outlined in Scheme 2. Thus, a fine suspension of the methyl ester **7** in phosphate buffer was exposed to α-chymotrypsin for 20 h, after which the unreacted Ac-D-Pse-OMe **9** was extracted from the reaction mixture using EtOAc. Following work up of the aqueous phase, the desired product Ac-L-Pse-OH **8** was obtained as a white solid. Next, each derivative **8** and **9** was subjected to acid hydrolysis, lyophilization and, without further purification, was *N*-protected using FmocOSu under mild basic conditions to afford the pure enantiomers **10** (mp 127–129 °C, $[\alpha]_D^{22}$ +27.9° ($c$ = 1.0, MeOH) and **11** (mp 127–129 °C, $[\alpha]_D^{22}$ −29.0° ($c$ = 1.0, MeOH).[12] The stereo-identity of peptide **6** was then established by its co-elution on RP-HPLC with the peptide synthesized using Fmoc-(2*R*,3*S*)-Pse-OH **11** as the initial building block.

The polymer-tethered peptide **4** is an ideal intermediate for selective chemical modification, for example, acylation, of the Pse[8]-residue—this would allow convenient access to peptides that could probe the effect of substituents at the β-position. Following initial evaluation of a series of *O*-acylation conditions, it was established that acetylation was readily accomplished using $Ac_2O$ in the presence of DMAP (Scheme 3). Acidolytic treatment, followed by RP-HPLC purification provided the Pse(Ac)-peptide derivatives **12** and **13**.

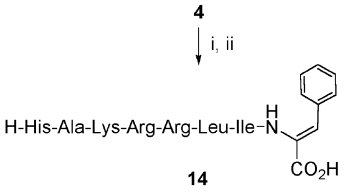
We next focused our attention on the regio-selective dehydration of the polymer-bound peptide **4**. This chemical modification would result in the formation of an α,β-dehydrophenylalanine (Dhp) residue, thus




**Scheme 2.** Reagents and conditions: $HCl_{(g)}$, MeOH, 5 °C to rt, 18 h; (ii) $Ac_2O$ (3 equiv), aq AcONa (4 equiv), 5 °C to rt, 16 h; (iii) α-chymotrypsin (Type II from bovine pancreas), 0.05 M aq phosphate buffer (pH 8), rt, 20 h; (iv) 6 M aq HCl, reflux, 5 h; (v) FmocOSu, aq $Na_2CO_3$, THF, 5 h.




**Scheme 3.** Reagents and conditions: (i) $Ac_2O$ (10 equiv), DIEA (1.3 equiv), DMAP (0.1 equiv), DMF, 24 h; (ii) TFA-$H_2O$-$i$Pr$_3$SiH (90:8:2), 2 h; (iii) RP-HPLC using Kromasil C8 preparative column.




**Scheme 4.** Reagents and conditions: (i) $Et_3N$ (30 equiv), $SOCl_2$ (2×4 equiv), THF-$CH_2Cl_2$, −78° to 5 °C; (ii) TFA-$H_2O$-$i$Pr$_3$SiH-EtSMe (88:8:2:2), 2 h.

introducing elements of conformational rigidity and unique conjugative properties. Recent studies[6] revealed that a polymer-bound *N*-acyl-*threo*-β-hydroxy-aminoacyl entity, on treatment with $SOCl_2$, underwent intramolecular cyclization to a cyclic sulfamidite,[13] which, under mild basic condition, can undergo stereospecific E1-elimination to afford a *Z*-α,β-dehydroaminoacyl residue. We have successfully applied this methodology to effect in situ dehydration of the *threo*-Pse[8]-residue within the polymer-bound peptide **4** to furnish, following acidolysis, the desired *Z*-Dhp-containing peptide derivative **14** (Scheme 4). Peptide **14** was purified by RP-HPLC and obtained in an overall yield of 24%.

Using the procedures outlined in Schemes 1–4, a corresponding series of Ser[2]-peptide derivatives **17**–**22** (see Table 1) was also synthesized. Though the Ser[2]-derivative of **1** is known to be ~100-fold less active as an inhibitor of CDK2-cyclin A,[5] these derivatives **17**–**22** are necessary to establish that the observed structure–activity profile is due to localized and not long-range structural effects.

To date, we are not aware of any studies that appraise the effect of C-terminal homochiral β-amino alcohols on the bioactivity of peptides that target C-RS. Hence, the (2*S*)-phenylalaninol[8]-derivatives **16** and **22** were synthesized by solid-phase procedure (Scheme 5), which relies on the highly efficient condensation of *N*-Fmoc-phenylalaninol (FmocPhe[ψ$CH_2$OH]) to the linker-resin **2** (96%) and the chemical robustness of the benzhydryl methyl ether bond in **15**.

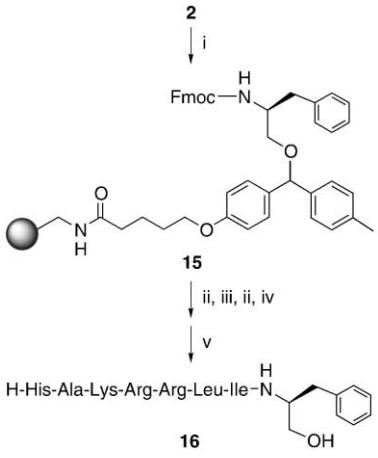
All the synthetic peptide derivatives were screened in vitro for their ability to inhibit the kinase activity of CDK2-cyclin A using retinoblastoma protein (pRb) as substrate,[5] and in competitive binding assay[5] using the CDK2-cyclin A-p21$^{WAF1}$ peptide system (Table 1). Both sets of screening results revealed similar structure–activity profiles and consistency.

The (2*S*,3*R*)-Pse[8] peptide **5** showed similar competitive binding potency as **1** but was a better inhibitor of kinase function. Analysis of the molecular model generated for the cyclin A-peptide **1** complex (Fig. 1) suggests that the (2*S*,3*R*)-Pse residue can be accommodated since the Phe *pro-S* β-hydrogen projects into an unoccupied cyclin groove volume that is large enough for a hydroxyl group. Moreover, the steric constraint in the $\chi_1$-space imposed by the β-OH group may impose more favorable interaction of the phenyl ring with the cyclin groove. We propose that the Pse side-chain makes excellent complimentary contact with the cyclin groove resulting in an enhancement of activity. This is profoundly obvious with the (Ser[2],L-Pse[8])-derivative **17**, which showed a considerable enhancement in activity compared to the corresponding Ser[2]-peptide.

**Table 1.** Inhibition ($IC_{50}$ μM)[a] of CDK2-cyclin A complex determined using competitive binding assays and a functional kinase assay (pRb as substrate)

| Peptide derivatives | Competitive binding assays | | Kinase assay |
|---|---|---|---|
| | Cyclin A | CDK2/cyclin A | |
| **1** | 0.043±0.010 | 0.041±0.011 | 0.039±0.012 |
| L-Pse[8]-OH, **5** | 0.074±0.014 | 0.028±0.007 | 0.002 |
| D-Pse[8]-OH, **6** | 0.12±0.04 | 0.11±0.08 | 0.30 |
| L-Pse(Ac)[8]-OH, **12** | 0.23±0.06 | 0.24±0.11 | 0.32±0.09 |
| D-Pse(Ac)[8]-OH, **13** | 0.19±0.05 | 0.16±0.07 | 0.20±0.25 |
| *Z*-Dhp[8]-OH, **14** | 0.060±0.008 | 0.054±0.019 | 0.070±0.011 |
| L-Phe[8][ψ$CH_2$OH], **16** | 0.022±0.001 | 0.018±0.005 | 0.024±0.011 |
| Ser[2] | 4.3 | n.d. | 3.4 |
| Ser[2],L-Pse[8]-OH, **17** | 0.25±0.06 | 0.19±0.13 | 0.56±0.45 |
| Ser[2],D-Pse[8]-OH, **18** | 0.76±0.06 | 0.71±0.87 | 1.20 |
| Ser[2],L-Pse(Ac)[8]-OH, **19** | 1.32±0.16 | 0.80±0.14 | n.d. |
| Ser[2],D-Pse(Ac)[8]-OH, **20** | 1.82±0.59 | 0.60±0.28 | n.d. |
| Ser[2],*Z*-Dhp[8]-OH, **21** | 0.20±0.09 | 0.22±0.17 | 1.3±0.6 |
| Ser[2],L-Phe[8][ψ$CH_2$OH], **22** | n.d. | 0.31±0.16 | 0.43±0.12 |

[a]Values are means of three experiments; n.d. = not determined.




**Scheme 5.** Reagents and conditions: (i) FmocPhe[$\psi$$CH_2OH$], DIEA, $ClCH_2CH_2Cl$-THF (3:1), 24 h; (ii) piperidine-DMF (1:4); (iii) Fmoc-amino acid-TBTU-HOBt-DIEA (1:1:1:2; 4–8 equiv); (iv) $Boc_2O$ (4–8 equiv), DMF; (v) TFA-$H_2O$-$i$$Pr_3SiH$ (90:8:2), 2 h.

The most striking and unexpected observation is that the (2*R*,3*S*)-Pse[8]-, i.e., D-*threo*-Pse-containing peptides **6** and **18** retained good potency, although they are somewhat less potent compared to the (2*S*,3*R*)-Pse[8] peptides **5** and **17**, respectively. We are currently exploring the implications of these results.

Furthermore, the above hypothesized complimentary interactions between the peptide ligand and C-RS is highly fastidious, since the presence of a bulky β-acetoxy group within Pse[8]-residue in peptides **12**, **13**, **19** and **20** resulted in decreased binding competency and kinase inhibitory activity. Interestingly, the potency order appears to be reversed for the (2*S*,3*R*)- and (2*R*,3*S*)-forms in both pairs of peptide derivatives, although the differences may not be significant. Nevertheless these results suggest that the β-acetoxy group precludes the complementary interactions more in the context of the L-derivatives.

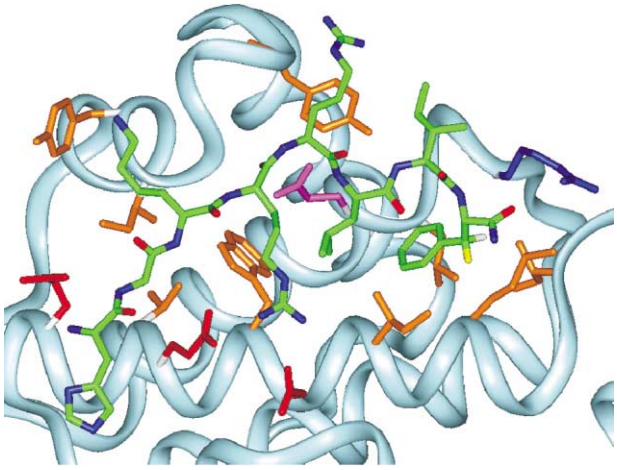


**Figure 1.** Molecular model of cyclin A-peptide **1** complex.[5,7] To aid viewing of the model, only a portion of the cyclin groove is shown. The *pro-S* β-H is highlighted in yellow, the cyclin groove $Arg^{250}$ residue's side-chain in blue.

The introduction of Dhp residue (peptides **14** and **21**) led to a potency enhancement in the context of the $Ser^2$ peptide. This appeared to indicate that a reduction in the degree of rotational freedom of the phenyl ring is tolerated. The other significant observation was that the introduction of C-terminal 2*S*-phenylalaninol produced a small improvement in potency. Here, it is likely that the α-hydroxyl group makes a unique H-bond contact with the guanidino function of $Arg^{250}$ in cyclin A (see Fig. 1).

These encouraging results not only highlight a generic methodology for probing structure–activity relationships using β-hydroxyamino acid building blocks, but also provide a framework for the design of peptidomimetics and small molecules that target the cyclin recruitment-site.

## Acknowledgements

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## References and Notes

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60% **B** in 20 min at 1.3 mL $min^{-1}$. Columns dimension: 4.6×150 mm; effluent was monitored at 220 nm; **A**: 0.06% aq TFA; **B**: 0.06% TFA in MeCN–$H_2O$ (9:1).
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