Non-Phosphopeptide Inhibitor for *Lck* SH2 Domain: Solid-Phase Synthesis and Structure Activity Relationship of Rosmarinic Acid Analogs

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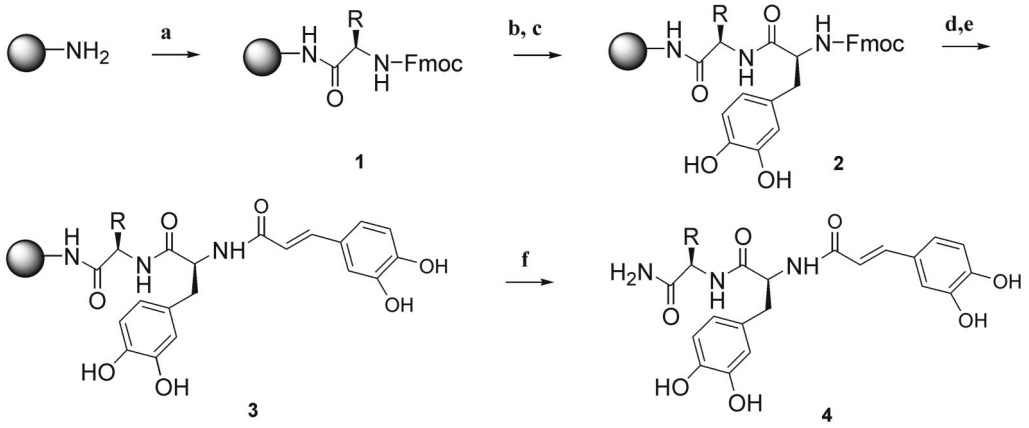
SH2 domains play a critical role in organizing coherent signal transducing complexes that are essential for the appropriate cellular response to extracellular stimuli.[1] Ligands that are able to disrupt these inappropriately hyperstimulated pathways, by blocking SH2 domain-dependent interactions, may ultimately be developed to therapeutic agents.[2-4] For example, ligands directed against the *lck* SH2 domain could serve in various capacities, such as for the treatment of autoimmune disease and T cell-based leuckemias and lymphomas. Recent studies about peptide inhibitors for SH2 domain revealed that SH2 domains including *src* and *lck* exhibited a marked preference for the sequence -pYEEIE-, and that short peptides bearing this sequence exhibited a reasonably high affinity for *src* family SH2 domains.[3-5]

Although moderately high-affinity phosphopeptide-based ligands for SH2 domains have been reported, their utility as therapeutic agents is obscure because of low resistance against phosphatase and protease and cell penetration ability. As an alternative way to develop non-phosphopeptide inhibitor for SH2 domains, natural chemical compounds were screened. Previously, we reported that (R) or (S) rosmarinic acid (RosA) had considerable inhibitory activities on *lck* SH2-pYEEI interaction in Enzyme linked immunosorbent assay (ELISA).[6] Furthermore, (R) RosA was reported to inhibit T-cell antigen receptor (TCR)-induced interleukin (IL)-2 expression and subsequent T-cell proliferation *in vitro*.[7]

Considering the recent rational design of non-phosphopeptide inhibitors for SH2 domain, the size of the inhibitors was commonly similar to that of tripeptide or tetrapeptide because this size seemed to be optimum for the interactions of inhibitors with the surface of SH2 domains including pY pocket and for the cell penetration. Although RosA had an inhibition activity for T-cell proliferation *in vitro* cell assay, the binding affinity of the compound for *lck* SH2 domain was not potent. As RosA was smaller than tripeptide, it is possible to design RosA analogs with high binding affinity to SH2 domain by appending amino acids at the C-terminus of RosA.

In the present study, novel non-phosphopeptide inhibitors on the basis of the structure of RosA were synthesized by appending natural amino acids at the C-terminus of RosA in solid phase synthesis and their binding affinities to *lck* SH2 domain were investigated.



Scheme 1. (a) 3 equiv Fmoc-AA-OH, DCC, HOBt, DMF; (b) Piperidine, DMF; (c) 3 equiv Fmoc-DOPA, DCC, HOBt, DMF; (d) Piperidine, DMF; (e) 3 equiv caffeic acid, DCC, HOBt, DMF; (f) TFA/Thioanisole/H_2O (9/0.5/0.5, v/v/v). Rink amide resin was used as solid support.

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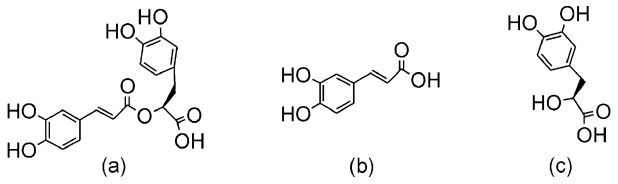


Figure 1. Structures of rosmarinic acid (a), caffeic acid (b), and 3,4-dihydroxylphenylacetic acid (c).

Parallel Solid-phase Synthesis of Rosmarinic Acid Analogs

To develop more potent SH2 inhibitor analogs, natural amino acid was appending at the C-terminus of RosA in solid phase synthesis described in Scheme 1. As RosA, a natural plant metabolite, was naturally isolated, it is difficult to get RosA as g scale. In addition, the coupling efficiency of RosA to amino acids attached on the resin in solid phase was low [data not shown]. RosA was structurally divided into two compounds, caffeic acid and 3,4-dihydroxylphenylacetic acid as shown in Figure 1. However, 3,4-dihydroxylphenylacetic acid is not commercially available, whereas structurally similar compound, L-3-(3,4-dihydroxylphenyl)alanine (L-DOPA) is commercially available and can be more efficiently coupled with the acid compound.

Thus, using caffeic acid, L-DOPA, and natural amino acids as a monomer, we synthesized RosA analogs containing RosA moiety and amino acid moiety in solid phase synthesis, shown in Scheme 1. Fmoc protected natural amino acids except cysteine were coupled with amino group on Rink amide resin by using DCC/HOBt coupling reagent. After removal of the Fmoc-group of amino acid attached resin by treatment of 50% piperidine in DMF, Fmoc-DOPA[8] was coupled with the amino acid on resin by using DCC/HOBt coupling reagent. After deprotection of Fmoc group, caffeic acid was introduced to the resin by using the same coupling reagent. Each coupling reaction in solid phase was repeated until no color change of the resin was monitored in ninhydrin test.[9] Cleavage and deprotection were achieved by treatment with a mixture of trifluoroacetic acid (TFA)/H_2O/thioanisole (9/0.5/0.5, v/v/v) at room temperature for 8-9 h. Each compound was purified by preparative reverse phase high performance liquid chromatography. The yields of each product were over 70% and the products were obtained in high purity (generally >95% by RP analytical HPLC, UV_{214nm}). Electrospray mass spectrometry on a Platform II from VG (Manchester, UK) was used to characterize the mass and purity of the product generated from solid phase synthesis.

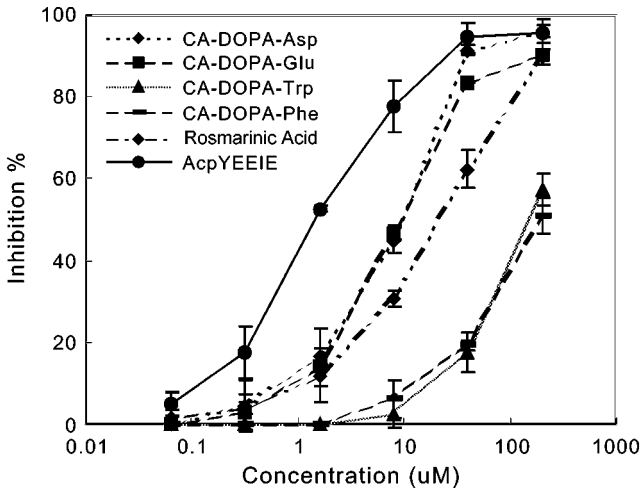
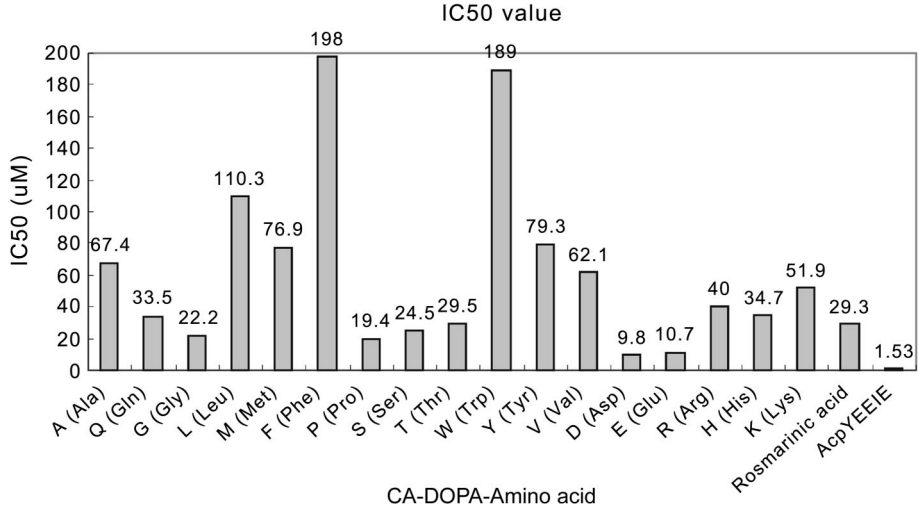


Figure 2. The inhibition activity of RosA analogs on *lck* SH2-AcpYEEIE interaction by using ELISA.

Biological Results and Discussion

The inhibition activity of RosA analogs on *lck* SH2-AcpYEEIE- interaction was investigated by using the previously reported ELISA method.[10] As shown in Figure 2,

Table 1. The binding affinity of RosA analogs, RosA, and Ac-pYEEIE for *lck* SH2 domain[a]



[a]Average IC_{50} values were measured from three independent experiments performed in duplicate, which provided a standard deviation below 20%.

synthesized rosmarinic analogs, RosA, and Ac-pYEEIE-inhibited the binding of EPQpYEEIPIYL with *lck* SH2 domain in a concentration-dependent manner. Table 1 summarized the results obtained in the ELISA assay. Appending natural amino acids at the C-terminus of RosA have a considerable effect on the binding affinity. The most potent RosA analogs in this series had negatively charged amino acid at the C-terminus. Appending Asp and Glu amino acids at the C-terminus improved binding affinity three times on the basis of IC_{50} value. Positively charged amino acids were well distributed on the surface of *lck* SH2 domain including pY, pY-1, and pY-2 binding sites.[11] Thus charge interactions between the compounds and *lck* SH2 domain must play a dominant role in the binding affinity. The result is consistent with the previous reported screening result for phosphopeptide inhibitors for *src* SH2 and *lck* SH2 domains by using combinatorial peptide libraries in which the phosphopeptide with more negatively charged amino acids exhibited more potent binding affinity to the proteins.[5,10] Appending hydrophilic amino acid (Ser, Thr, Gly, and Gln) retained or improved inhibition activity slightly, whereas the introduction of small aliphatic amino acid such as Ala, Val, Leu, and Met at the C-terminus resulted in the decrease of inhibition activity. Interestingly, the introduction of Pro at the C-terminus improved inhibition activity, which suggesting the possibility of developing novel RosA analog inhibitors for SH2 domain by introducing various secondary amines at the C-terminus of RosA. The introduction of aromatic amino acids (Trp and Phe) except Tyr resulted in a considerable loss of binding affinity to *lck* SH2. However, introduction of charged amino acids (Arg, Lys, and His) had a little effect on the binding affinity, which indicated that charged side chains of the amino acids were away from the surface of *lck* SH2.

Accordingly, overall result indicated that the increase of negative charge at the C-terminus of RosA had a positive effect on the binding affinity to *lck* SH2 domain while the increase of hydrophobicity by appending aromatic ring and aliphatic chain at the C-terminus induced a considerable negative effect on the binding affinity to the protein. Considering the binding affinity only, RosA-Glu and RosA-Asp could be valuable lead compounds. However, considering binding affinity as well as molecular weight and charge reduction for cell penetration, RosA-Pro could be a novel candidate to be developed for effective SH2 inhibitors.

We did not have direct information about RosA binding site for *lck* SH2 domain, however, the append of amino acids at the C-terminus of RosA provided important information for the binding mode of RosA with *lck* SH2 domain and for the design of novel non-phosphopeptide SH2 inhibitors.

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