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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3938–3950
Bioorganic & Medicinal Chemistry

The structure–activity relationship of the series of non-peptide small antagonists for p56lck SH2 domain

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Received 18 May 2006; revised 23 June 2006; accepted 24 June 2006
Available online 5 April 2007

Abstract—The antagonists for the SH2 domain are regarded as novel therapeutic candidates for cancer, autoimmune disease, and chronic inflammatory disease. Previously, we identified rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyl-lactic acid; RosA) from *Prunella vulgaris* as an antagonist for the p56lck SH2 domain by screening natural products. RosA not containing phosphotyrosine surrogate had a considerable inhibitory activity for T-cell antigen receptor (TCR)-induced interleukin (IL)-2 expression, and subsequent T-cell proliferation in vitro cell assay. To investigate the structure–activity relationship of RosA and to identify a novel p56lck SH2 antagonist with more potent in vitro T-cell inhibitory activity, we synthesized several analogs of RosA by using rational design. All synthesized compounds were tested in vitro binding activity for the SH2 domain and in vitro T-cell inhibitory activity. All four hydroxyl groups of RosA were essential for binding with the p56lck SH2 domain and T-cell inhibitory activity. Unexpectedly, conformationally less constrained analogs **4** and **9** showed a more potent binding affinity for the SH2 domain than that of RosA, and chirality of the analog did not play an important role in protein binding. We successfully identified several RosA analogs with a more potent T-cell inhibitory activity than that of RosA. Overall results revealed important structural requirements of the p56lck SH2 antagonists for in vitro T-cell inhibitory activity and in vitro protein binding activity.

1. Introduction

The src homology-2 (SH2) domain is a highly conserved non-catalytic module consisting of 100 amino-acid residues that is found in many intracellular signal-transduction proteins.[1] The SH2 domains specifically recognize phosphotyrosine (pY)-containing proteins with high affinity and each SH2 domain appears to have a selectivity toward particular phosphotyrosyl peptide sequences.[2,3] Specific antagonists of the SH2 domains, which inactivate inappropriately hyperstimulated cell signaling, can be developed as novel therapeutic agents to treat a broad range of human diseases such as cancer, autoimmune disease, osteoporosis, and chronic inflammatory disease.[4–7] The p56lck SH2 domain, found in T cells and NK cells, is indispensable for efficient initiation of T-cell antigen receptor (TCR)-signaling as well as activation-induced intracellular Ca^{2+} mobilization, and production of cytokine such as interleukin-2 (IL-2).[8,9] Thus, the antagonists of the p56lck SH2 domain have therapeutic potential for immune disorders such as organ transplantation rejection, graft-versus-host diseases, autoimmune diseases, and chronic inflammatory diseases. A peptide library screen revealed the specificity of phosphopeptides for various SH2 domains and identified a lead peptide, AcpYEEIE, that showed a potent binding affinity for the p56lck SH2 domain (IC_{50} = 0.1–1.8 μM).[3] X-ray structural studies about p56lck/Src SH2 domains in complex with this phosphopeptide revealed that the peptide was anchored by insertion of the pY and pY + 3 side chains into two pockets of SH2 domains (''two-pronged plug two-holed socket'' model).[10,11] Moderately high-affinity peptide-based ligands for the p56lck SH2 domain have been recently and successfully synthesized from AcpYEEIE by using rational design.[12–14] Even though

Keywords: T-cell inhibition; Non-peptide; Antagonist; Inhibitor; SH2; Binding affinity.
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doi:10.1016/j.bmc.2006.06.059

high-affinity peptide-based antagonists for the SH2 domain (IC_{50} = 10 nM to 1 μM) were successfully synthesized, most did not show in vitro cell inhibitory activity for several reasons. First, phosphotyrosine, essential for SH2 domain recognition, is hydrolytically unstable in the presence of protein tyrosine phosphatase.[15] The peptide antagonists could not pass through cell lipid membrane due to high negative charge or peptidic character of the peptide antagonists. Recently, several non-hydrolyzable pY surrogates were applied into SH2 peptide antagonists to develop novel SH2 antagonists with less peptidic character and with a reduced number of negative charges.[14,16,17] However, still most of peptide-based antagonists containing even pY surrogate did not exhibit activity in vitro cell assay.

Even though screening of natural products must be a useful and conceptually straightforward strategy for the development of therapeutic agents, there is little research about chemical antagonist for SH2 domains.[18,19] Recently, we successfully identified a small chemical antagonist for the p56lck SH2 domain by screening natural products from *Prunella vulgaris*.[19] The small chemical antagonist was characterized to be rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyl-lactic acid; RosA) which was frequently found in medicinal plant, herbs, and spices.[20] Interestingly, RosA that does not contain pTyr had considerable inhibitory activity on the interactions between p56lck SH2 and AcpYEEIE in ELISA and had inhibitory activity for T-cell antigen receptor (TCR)-induced interleukin (IL)-2 expression and subsequent T-cell proliferation in vitro.[19,21] Furthermore, the compound marginally improved allograft survival rate in the animal model and the combination of RosA and Rapamycin prolonged synergistically survival in the allograft mouse model.[22]

In this study, we designed and synthesized RosA-based analogs with the aim of investigating the structure–activity relationships and identifying new lead compounds for the p56lck SH2 domain which will exhibit more potent activity in in vitro cell assay. All synthesized compounds were tested for in vitro binding affinity for the p56lck SH2 domain as well as in vitro T-cell inhibitory activity by using IL-2 gene expression assay. We successfully characterized structural requirements of this small chemical antagonist for interacting with p56lck SH2. All four hydroxyl groups of the compounds were essential for the binding with the SH2 domain. Interestingly, **4** and **9** that were conformationally flexible inhibitors, exhibited more potent binding affinity than did RosA, and chirality of the analog did not play an important role in the protein binding. We successfully identified some analogs of RosA which showed more potent inhibitory activity for T-cell antigen receptor (TCR)-induced interleukin (IL)-2 expression than that of RosA. Overall results revealed important structural requirements of the small chemical antagonists for the SH2 domain binding activity and T-cell inhibitory activity.

2. Results and discussion

2.1. Design and chemical synthesis of the RosA analogs

To design the analogs of RosA with a potent binding affinity, information about the binding mode of RosA with p56lck SH2 domain is needed. However, X-ray crystallographic information is not available for the binding mode of RosA with the p56lck SH2 domain. The previous research in which caffeic acid-pYEEIE was identified as the most potent p56lck SH2 antagonist through the screening of unnatural amino acid at the pY-1 position of -pYEEIE may provide a clue for the binding mode of RosA to the p56lck SH2 domain.[23] As shown in Figure 1, RosA (I) shared the similar moiety of the peptide inhibitors (II-IV) for the p56lck SH2

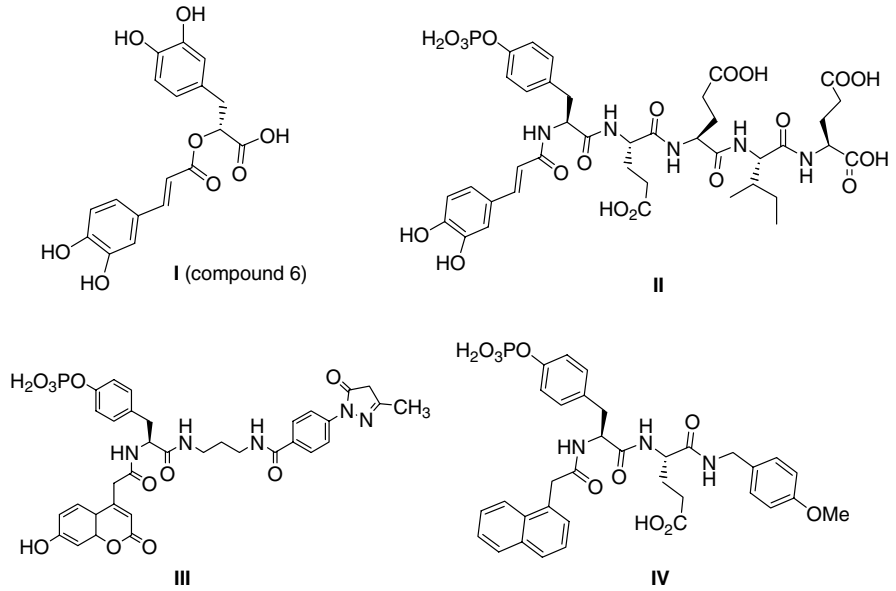
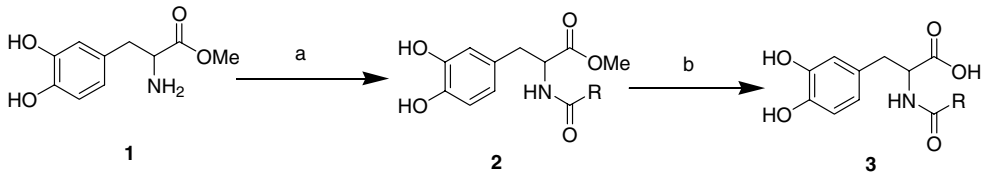


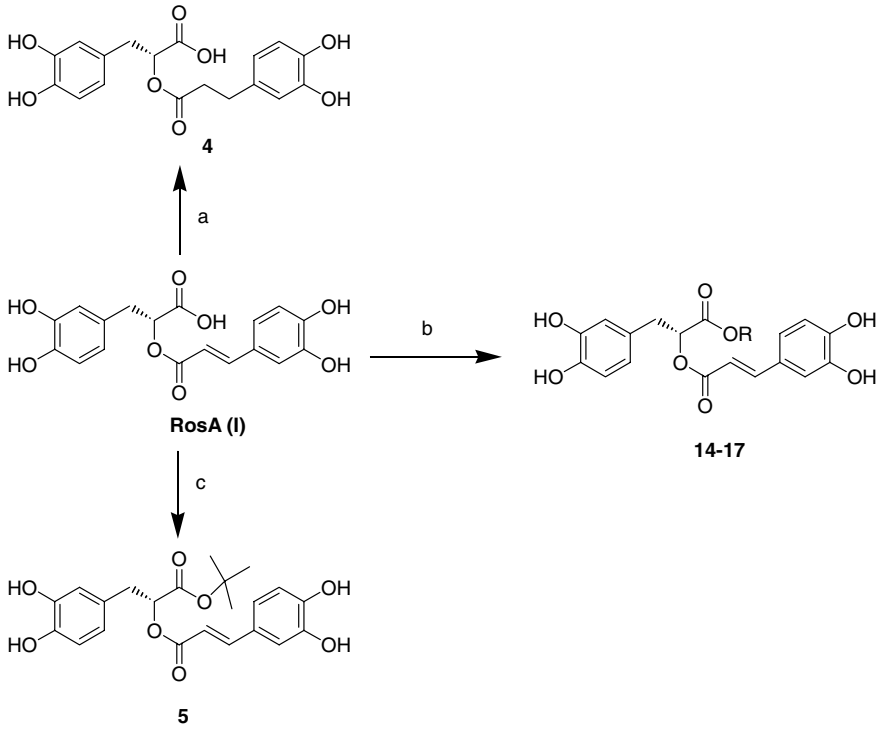
Figure 1. The structures of rosmarnic acid (I) and p56lck SH2 peptide inhibitors (II–IV).

domain previously reported by us as well as the other researchers.[23–25] Comparison of the structures of the RosA and the peptide inhibitor revealed two characteristics. The caffeic acid moiety of RosA may correspond to the amino acid at the pY-1 position of the peptide inhibitors. And the dihydroxyphenylacetic acid (DOPL) moiety may correspond to the pY of the peptide inhibitors. Interestingly, the stereochemistry of the DOPL (*R* form) moiety of RosA is the reverse of that of the pY (*S* form) of the peptide inhibitors. Thus, we synthesized each enantiomer of the RosA analog containing amide bond by coupling caffeic acid with D-DOPA (3,4-dihydroxyphenyl-L-alanine) and L-DOPA, respectively, as shown in Scheme 1. There is some possibility that RosA interacts with the pY+3 binding pocket, but this possibility is likely low because RosA has a considerably different structure compared to small chemical inhibitors screened for the pY+3 binding site of the Lck SH2 domain.[26] According to the molecular model of RosA, there is a considerable conformational constraint in the caffeic acid moiety, and the phenyl and vinyl chains of this moiety are locked into a coplanar orientation. To find out the requirement of the coplanar orientation of this moiety for its binding affinity, the vinyl group of caffeic acid was reduced or the caffeic acid moiety was replaced with 3,4-dihydroxybenzoic acid. Even though RosA containing a carboxylic group exhibited considerable in vitro cell activity, considering the requirement of cell penetration for in vitro cell activity, the acid group of the compound was modified into various aliphatic ester groups, as shown in Scheme 2. As the hydroxyl group of the catechol moiety of RosA was partially negatively charged under physiological condition, the hydroxyl group might play an important role in the interaction with the SH2 domain as well as in cell penetrating ability. To explore the importance of the catechol hydroxyl groups for binding activity and in vitro cell activity, each hydroxyl group of RosA was methylated.

The proposed analogs of RosA were synthesized as outlined in Schemes 1 and 2. As shown in Scheme 1, the series of the analog 2 containing amide bond were synthesized by a coupling reaction of (*R*) or (*S*) 3,4-dihydroxyphenyl-alanine (DOPA) methylester **1** with various dihydroxyphenyl acid compounds including caffeic acid as the similar method previously described.[27] Hydrolysis of the ester of **2** by the treatment of LiOH provided the corresponding acid **3**. RosA analogs containing ester bond were synthesized as outlined in Scheme 2. Reduction of RosA (I) by H_2 in the presence of Pd–C provided compound **4** in a satisfactory yield.



Scheme 1. Reagents and condition: (a) RCOOH, PyBOP, TEA, DMF, DCM; (b) LiOH, 0 °C, DMF/H_2O.



Scheme 2. Reagents and conditions: (a) H_2, Pd/C; (b) $SOCl_2$, ROH, R = CH_3, CH_2CH_3, $CH_2CH_2CH_3$, $CH(CH_3)_2$; (c) isobutylene, dioxane/−10 °C.

Activation of the carboxyl group of RosA, coupled with various alcohols provided ester analogs **14**–**17** in appreciable yields. The reaction of RosA (**I**) with isobutylene in the presence of acid provided compound **5**. All synthesized analogs employed in this study were further purified by preparative HPLC with a Vydac C_{18} column (22 mm × 250 mm) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient. The purity (generally >95% by RP analytical HPLC, UV_{214nm}) and the retention time of the compound were analyzed by using analytical HPLC (5–45% linear gradient of acetonitrile during 40 min). log *P* of each compound was measured according to a literature method and compared with the retention time on a Vydac C_{18} column in HPLC to investigate the hydrophobicity of the compounds.[28]

2.2. In vitro binding activity of RosA and its analogs

The binding affinity of the compounds for the p56lck SH2 domain was measured by using a competitive assay as described.[23] As shown in Figure 2, RosA and the active analogs inhibited the binding of EPQpYEEIPIYL with the p56lck SH2 domain in a concentration-dependent manner and a control phosphopeptide, AcpYEEIE, exhibited approximately 1.8 μM of IC_{50} value, which is highly consistent with the previously reported value.[23,24] The measured IC_{50} values of each compound are summarized in Table 1. Compound **7** bound to the SH2 domain with IC_{50} = 20 μM, that is, just 10-fold less potent than the phosphopeptide, AcpYEEIE. Interestingly, compound **7** containing amide bond showed a more potent binding activity than did RosA. This is an encouraging result because compound **7** has a different scaffold and was easily synthesized by coupling caffeic acid with DOPA. Furthermore, we compared the binding affinity between compound **7** and RosA by using BIAcore. As shown in Figure 3, compound **7** showed almost similar binding affinity to that of RosA in this competition assay. We concluded that replacement of ester bond with amide bond of RosA did not affect the binding affinity for the SH2 domain. Unexpectedly, compound **8** that was an enantiomer of compound **7** showed the similar binding affinity to those of compound **7** and RosA, which strongly suggested that the stereochemistry of the amino α carbon of the antagonist was not a critical factor for the interaction with the SH2 domain. Interestingly, compounds **4** and **9**, which have a less conformationally constrained moiety than RosA, exhibited a more potent binding affinity than did RosA. Compounds **12** and **13** which have dihydroxylphenyl acetic acid and dihydroxylbenzyl acid moiety instead of caffeic acid moiety showed more potent binding affinity than did RosA and compound **7**. The overall results indicated that planarity of caffeic acid moiety of RosA was not essential for binding with the p56lck SH2 domain. Compounds **14**–**17** which have a different size of alkyl ester group showed a less potent binding affinity than did RosA. Particularly, compound **16** containing *n*-propylester showed the lowest binding affinity among the active ester analogs, and compound **18** containing long aliphatic ester did not show binding affinity even at the concentration of 500 μM. The binding affinity of the ester analog series decreased with increasing carbon number of the alkyl chain of the ester. Compounds **19**–**22** which contained at least one methylated hydroxyl group at the phenyl ring moiety did not show any binding affinity, which strongly suggested that all hydroxyl groups of RosA were critically required for the interaction with the p56lck SH2 domain. As catechol compounds were reported to have covalent binding to Src family SH2 domains,[29] we investigated the binding affinity of DOPL (**23**) and (±)-DOPA (**24**). Both compounds did not show binding affinity for the p56lck SH2 domain.

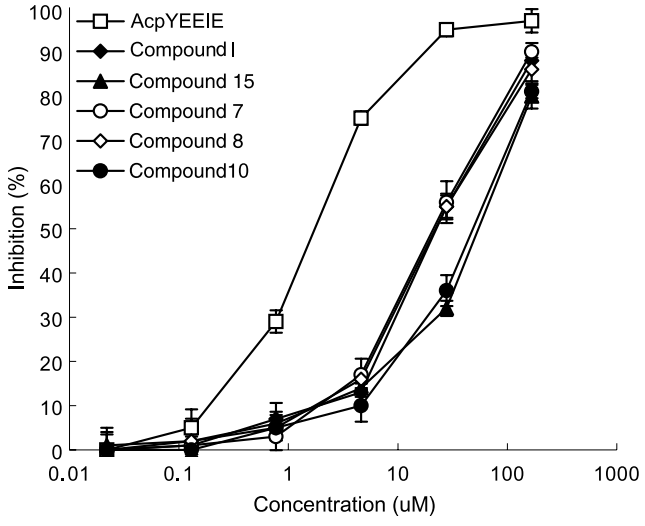


Figure 2. The inhibition of compounds for p56lck SH2-phosphopeptide interaction in ELISA. Each of the compounds is added to a solution containing GST-p56lck-SH2 domain and incubated and then the mixture is added to biotinyl-ε-aminocaproyl-EPQpYEEIPIYL coated wells in duplicate. After washing, the degree of interaction between SH2 domain and biotinyl-ε-aminocaproyl-EPQpYEEIPIYL is detected by incubation with an anti-GST polyclonal antibody, followed by incubation with a peroxidase-conjugated anti-rabbit antibody and peroxidase substrate solution. The color development is monitored at 450 nm. Each value was calculated from three independent experiments performed in duplicate, which provided a standard deviation below 20%.

2.3. In vitro T-cell inhibitory activity of RosA and its analogs

All employed compounds were evaluated for their protein binding affinity as well as for their ability to inhibit T-cell activation. It was demonstrated that point mutation in the p56lck SH2 domain, to disrupt possible interactions with phosphotyrosine-containing molecules, abrogated TCR-induced Ca^{2+} flux and subsequent IL-2 promoter activation.[8,9,30] Thus, in vitro T-cell inhibitory activity was measured by using the blocking of TCR-induced IL-2 gene activation as described in Section 3. The commercially available immune suppressive drug, Cyclosporin A (CsA), was used as a positive control in this assay.[31] As shown in Figure 4, in vitro cell inhibition activity was measured at two different concentrations (10 μM and 30 μM). A positive control, CsA, inhibited T-cell activation almost 100% at 10 μM, whereas, RosA inhibited about 50% under the same condition. AcpYEEIE with a potent binding affinity

Table 1. log *P*, Lck SH2 binding affinity, and T-cell inhibitory activity of the compounds

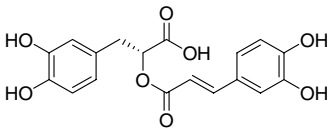
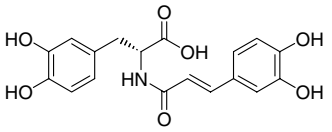
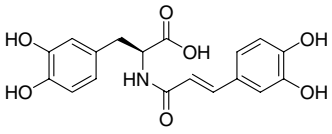
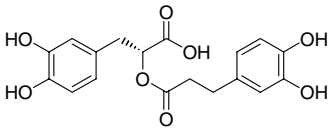
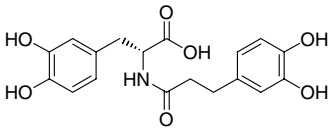
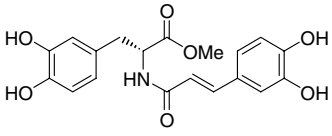
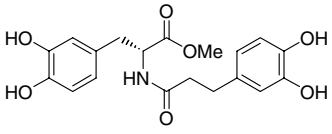
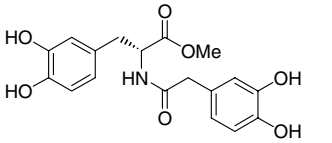
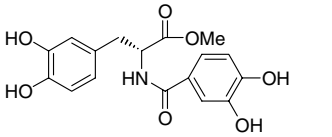
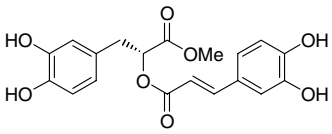
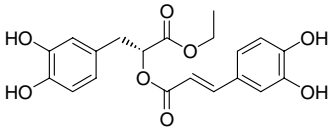
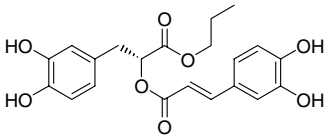
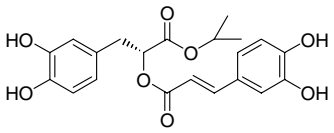
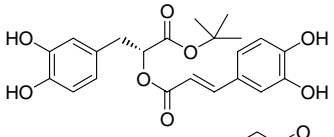
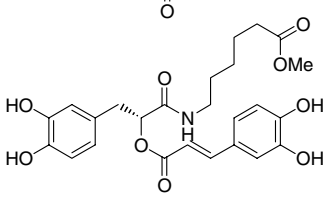
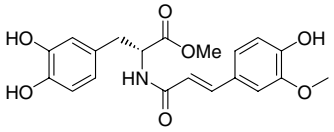
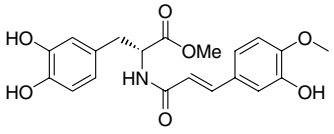
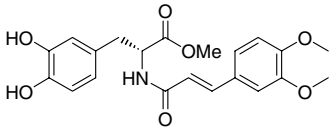
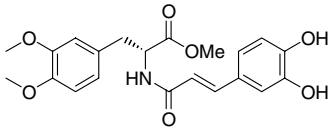
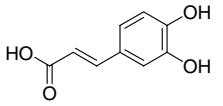
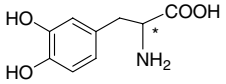
Compound	Structure	IC_{50} value (μM) ELISA[a]	T-cell Inhibition[b] (%)	log *P*[c]	t_R[d] (min)
6(I)		24 ± 2	40.4 ± 1.17	0.834	27
7		20 ± 2	10.3 ± 0.92	0.758	17
8		21 ± 2	11 ± 1.1	0.731	17
9		15 ± 1	7.6 ± 0.81	0.503	13
4		13 ± 1	0 ± 4.52	0.493	13
10		71 ± 2	61.6 ± 3.23	1.386	22
11		32 ± 3	7.5 ± 0.49	0.344	20
12		21 ± 2	23 ± 1.53	0.138	18
13		19 ± 1	0.4 ± 0.06	0.125	17
14		80 ± 4	54.7 ± 5.09	1.479	30
15		91 ± 3	22.7 ± 1	1.499	32
16		145 ± 4	87.5 ± 4.1	1.520	34

Table 1 (*continued*)

Compound	Structure	IC_{50} value (μM) ELISA[a]	T-cell Inhibition[b] (%)	log *P*[c]	t_R[d] (min)
17		110 ± 5	86.1 ± 5.86	1.833	33
5		127 ± 3	80.7 ± 4.5	1.995	32
18		>500	29.4 ± 0.56	1.683	29
19		>500	2.2 ± 0.2	1.313	25
20		>500	5.4 ± 0.4	1.371	26
21		>500	3.5 ± 0.28	1.522	29
22		>500	9.4 ± 0.43	1.535	28
23		>500	2.0 ± 0.2	—	—
24		>500	0.6 ± 0.2	—	—

[a] The average IC_{50} values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 20% and the IC_{50} value of AcpYEEIE was 1.8 μM.

[b] T-cell Inhibition (%) values were obtained at 10 μM and from three independent experiments performed in duplicate, which provided a standard deviation below 10%. Cyclosporin A as a control inhibited T-cell activation completely at 10 μM, whereas RosA inhibited T-cell activation by about 40% at 10 μM.

[c] Log P values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 5%.

[d] Retention times were obtained with analytical HPLC system (5–45% linear gradient of acetonitrile during 40 min).

(IC_{50} = 1.8 μM) did not show T-cell inhibition activity even at 100 μM, which can be attributed to its low cell penetrating activity as well as low stability against phosphatase. As in vitro cell inhibition activity of RosA analogs was more clearly differentiated at the concentration of 10 μM, the percent inhibition of T-cell activation at this concentration is summarized in Table 1. Both compounds **7** and **8** (IC_{50} = 20 μM) were not as effective as RosA (IC_{50} = 24 μM), for inhibiting IL-2 expression in vitro cell assay; both compounds are four times less active than RosA in in vitro cell assay. Considering their low log *P* value and the short retention time on the C_{18} reverse phase HPLC column, the low in vitro cell activity of both compounds must be due to their low cell penetration. This suggestion was supported by the fact that compound **10** (methyl ester form of compound **7**) showed increased inhibition activity than did RosA in in vitro cell assay even though the binding affinity of

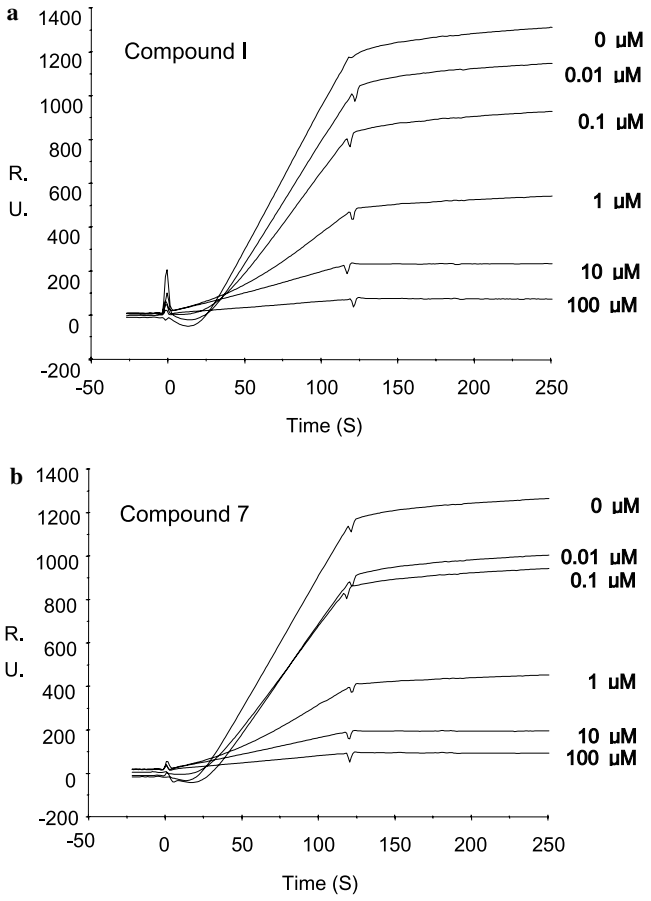


Figure 3. The inhibition of the RosA (a) and compound **7** (b) for GST-p56lck-SH2 domain by using BIAcore. The BIAcore instrument used in this study was manufactured by BIAcore AB (BIAcore 3000, Uppsala, Sweden). First, biotinyl-ε-aminocaproyl-EPQpYEEIPIYL at 10 μg/ml in the running buffer (50 mM Tris, 150 mM NaCl, pH 7.5) was injected at a flow rate 5 μl/min for 5 min onto streptavidin coated chip surface. For the competitive binding assay, the GST-p56lck-SH2 domain was diluted to 100 nM in the running buffer. Samples were titrated into GST-p56lck-SH2 domain from 100 to 0 μM and allowed to come to equilibrium in the running buffer; 10 μl of each reaction mixture containing samples and SH2 protein was passed over the surface with looped injections at a flow rate of 5 μl/min, with 5 μl of 100 mM NaCl and the resonance unit was measured in the running period.

compound **10** (IC_{50} = 70 μM) was lower than that of compound **7**. Compounds **4** and **9**, with a potent binding affinity (IC_{50} = 13–15 μM) showed almost no inhibition activity in vitro cell activity. Both compounds showed a relatively low log *P* value compared to those of the other analogs with in vitro cell activity and no in vitro cell activity of compounds **4** and **9** must be due to their low cell penetration ability. Compounds **11**–**13** with a high protein binding affinity exhibited a low T-cell inhibitory activity. Considering the low log *P*, we assumed that low cell inhibitory activity of the analogs might be due to low cell penetration. However, we observed the discrepancy between log *P* and HPLC retention time of compounds **11**–**13**.

We assumed that an inconsistency between log *P* value and the retention time must be due to the low stability of these compounds in buffer solution. Thus, we investigated the stability of the compounds in buffer solution at pH 7.4 by using LC–mass spectrometer; the ester compounds were rapidly converted into the corresponding acid or unidentified small molecule maybe due to the proximity effect of hydroxyl group of catechol moiety and the half lives of the compounds were around 3–4 h (data not shown). Unexpected low log *P* values of these compounds can be explained by the rapid hydrolysis of the ester group of the compounds. We assumed that low in vitro T-cell inhibitory activities of the compounds might be due to the low cell penetrating ability and low stability in the buffer solution.

The acid group of RosA was modified into various alkyl esters and in vitro cell activity was compared. Methylation of RosA (compound **14**) resulted in the decrease of protein binding affinity three times (IC_{50} = 80 μM) but an increase of the inhibition activity in in vitro cell assay. Interestingly, IL-2 gene expression was totally inhibited at 30 μM concentration of compound **14**, whereas only 50% inhibition was observed at 30 μM concentration of RosA as shown in Figure 4. The comparison between protein binding affinity and in vitro cell activity of compounds **6** and **14**–**18** showed the following trend; as the chain length of C-terminal ester group was increased, their binding activity was steadily reduced, whereas in vitro cell inhibition activity was improved by an increase of the hydrophobicity if the antagonist had still considerable binding affinity. This result indicates that there are two factors, the protein binding activity and cell penetrating activity of the antagonist, which contribute to determining in vitro cell activity. As the chain length of the C-terminal ester group of the antagonist increased for the cell penetrating activity, the binding activity for the protein was reduced. Thus, the analog with potent in vitro cell inhibition activity must have optimal balance between binding affinity and hydrophobicity.

Compound **15** having a considerable protein binding affinity (IC_{50} = 90 μM), exhibited the lowest T-cell inhibitory activity among the ester analog series. As the ethylester analog had an advantage over the methylester analog because the ethylester analog was hydrolyzed to release less toxic ethanol, we measured in vitro T-cell inhibitory activity of compound **15** several times. Compound **15** exhibited low T-cell inhibitory activity in several independent experiments, but we could not explain this low in vitro cell inhibitory activity of compound **15** by its hydrophobicity. Compound **18** with no protein binding affinity showed marginal cell inhibition activity, which suggested the possibility that hydrophobic compounds may show in vitro cell activity through non-specific effects in in vitro cell assay. Methylation of any hydroxyl group of catechol moiety of the inhibitors (**19**–**22**) resulted in the loss of binding affinity as well as in vitro cell inhibition activity.

As SH2 domains recognize the proteins containing pY, which is dephosphorylated by protein tyrosine phosphatase (PTPase), the SH2 inhibitors may interact with PTPase. Compounds **6**–**8** that showed potent binding affinity for p56lck SH2 domain were selected and

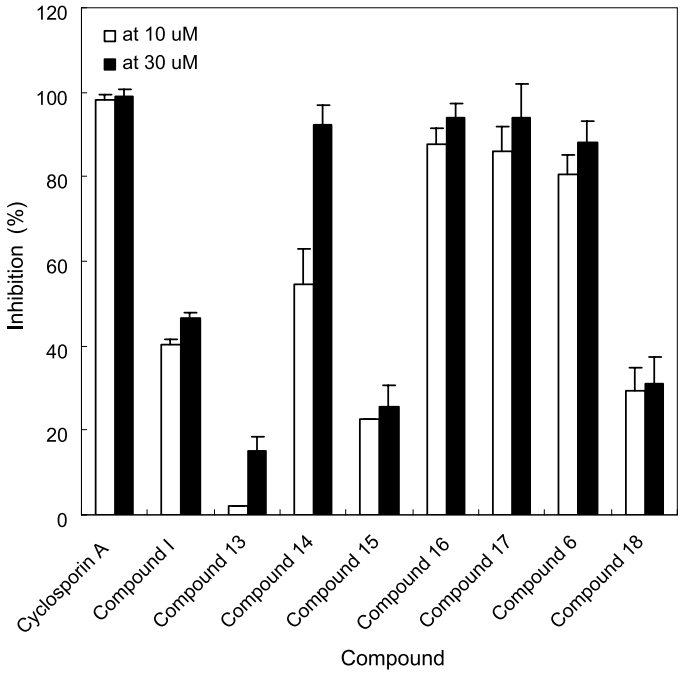


Figure 4. The intracellular inhibition of the rosmarinic acid and its derivatives (IL-2 Luc assay). Jurkat cells were transfected with the IL-2 Luc reporter, treated with 10 μM and 30 μM of Cyclosporin A, rosmarinic acid, and it derivatives for 2 h and activated with immobilized anti-CD3 antibody or PMA plus ionomycin for 16 h. After stimulation, the cells were washed, lysed, and assayed for luciferase activity with microplate luminometer. Each value was calculated from three independent experiments performed in duplicate, which provided a standard deviation below 10%.

examined against human PTPase 1B. Enzyme assay proved that all tested compounds did not show inhibition activity even at 100 μM (data not shown). RosA, a lead compound, was proven to have no inhibition activity for Lck, a protein tyrosin kinase.[19] These results indicate that the small chemical antagonists employed in this study might have a selectivity for the p56lck SH2 domain over other proteins of T-cells.

In conclusion, we synthesized several analogs of RosA and evaluated their binding affinity for the p56lck SH2 domain and inhibition activity for T-cell. Four hydroxyl groups in catechol moieties of the antagonists were essential for the binding with the p56lck SH2 domain. Replacement of the ester bond of RosA with an amide bond resulted in retention of binding affinity but a decrease of in vitro cell inhibition activity. Interestingly, a conformationally less constrained analog showed more improved binding affinity for the SH2 domain, while *R* and *S* form analogs showed similar binding affinity for the protein and in vitro cell inhibition activity. The chemical antagonists with a potent cell activity required an optimal balance between the protein binding affinity and cell penetrating ability. Overall results revealed a number of important features regarding the design of novel p56lck SH2 antagonists with more improved in vitro binding affinity as well in vitro cell activity.

3. Materials

PyBOP and DCC for the solution phase amide coupling were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Piperidine, acetic anhydride, methyl alcohol, dicyclohexylcarbodiimide (DCC), *N*-methylpyrrolidone (NMP), and *N*-hydroxybenzotriazole (HOBt) were purchased from Applied Biosystems, Inc. (Foster city, CA, USA). Trifluoroacetic acid (TFA), (+)-biotin, ε-aminocaproic acid, 3,4-dihydroxycinnamic acid, 3,4-dihydroxy-L-phenylalanine, 3,4-dihydroxy-D-phenylalanine, and *N*,*N*-dimethylformamide (DMF) were purchased from Aldrich (Milwaukee, WI, USA). SH2-GST fusion Lck (120–226) and polyclonal rabbit anti-GST antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated mouse anti-rabbit antibody, peroxidase substrate (1-step Turbo TMB-ELISA, trimethylbenzidine), and streptavidin coated 96-well plates were purchased from Pierce (Rockford, IL, USA). RosA was obtained from Indofine Chemical Company (Somerville, NJ, USA). CyclosporinA was kindly provided by Dr. G. Lee (Hanmi Pharm. Co., Korea). All chemicals were of reagent grade and used without further purification.

3.1. General chemistry

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 MHz spectrometer with DMSO-d_6 as solvent, δ values in ppm (TMS as internal standard). Coupling constants (*J*) were recorded in hertz. Electron spray ionization (ESI)-mass spectra were recorded on VG platform II mass spectrometer. Flash chromatography was performed on silica gel 60H (Merck) using the different solvent systems as indicated below. Analytical HPLC was performed on Gilson HPLC system equipped with UV detector and reversed phase C_{18} column, Delta PAK at a flow rate of 1 ml/min. Retention times were

obtained by using 5–45% linear gradient of acetonitrile during 40 min.

3.1.1. 3-(3.4-Dihydroxy phenyl)-2-[3-(3,4-dihydroxy phenyl)-acryloylamino]-propionic acid methyl ester (10). DOPA (D form 2.0 g, 10.14 mmol, 1 equiv) was suspended in methanol (40 ml) and to the solution was added thionyl chloride (7.4 ml, 101.4 mmol, 10 equiv) at 0 °C ice bath. The reaction mixture was stirred for 18 h under N_2 gas and then excess methanol and thionyl chloride were evaporated. The crude product was purified by crystallization (methanol/ethyl acetate) and the yield was 93%. DOPA methyl ester (2.0 g, 8.07 mmol, 1 equiv) was dissolved in DMF, (10 ml) and to the reaction mixture was added caffeic acid (1.45 g, 8.07 mmol, 1 equiv) and it was diluted with dichloromethane (DCM, 20 ml). To the reaction mixture were added PyBOP (4.2 g, 8.07 mmol, 1 equiv) and tri-ethylamine (TEA, 3.4 ml, 24.21 mmol, 3 equiv) at 0 °C ice bath. The reaction mixture was stirred for 18 h under N_2 gas and excess dichloromethane was evaporated in vacuo. Then the reaction mixture was diluted with ethyl acetate (10 ml) and sequentially washed with 1 N aqueous HCl, 10% aqueous $NaHCO_3$, distilled water, and brine. The organic layer was dried over $MgSO_4$ and the crude product was purified by silica gel column chromatography (normal phase, eluting sequentially with 5:4:1, *n*-hexane/ethyl acetate/methanol) to afford the 85% yield of the title compound.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.21–2.37 (m, 2H), 3.08 (s, 3H), 3.93–3.95 (m, 1H), 5.85–5.94 (m, 2H), 6.07–6.10 (m, 2H), 6.21–6.23 (m, 1H), 6.30–6.32 (m, 1H), 6.42 (s, 1H), 6.70 (d, J = 16 Hz, 1H), 7.85 (d, J = 8 Hz, 1H), 8.28 (br, 2H), 8.72 (br, 1H), 8.92 (br, 1H).

^{13}C NMR (DMSO-d_6, 400 MHz): 25.9, 51.8, 59.7, 112.9, 113.9, 116.4, 119.9, 122.9, 126.2, 127.8, 133.9, 140.0, 143.9, 144.9, 145.5, 147.4, 148.8, 165.3, 172.3.

ESI MS: $(M+H)^+$ calcd *m/e* 374.12, obsd *m/e* 373.90.

3.1.2. 3-(3,4-Dihydroxy-phenyl)-2-[3-(3,4-dihydroxy-phenyl)-acryloylamino]-propionic acid (7,8). Compound **10** (1.0 g, 2.67 mmol, 1 equiv) was dissolved in acetone (30 ml) and to the reaction mixture was added dropwise distilled water (20 ml) and concentrated HCl solution (10 ml) in 0 °C ice bath so as to reduce the precipitation of the dissolved compound and then it was refluxed under drying tube ($CaCl_2$) for 20 h at 80 °C. After the excess acetone was evaporated, the reaction mixture was washed with *n*-hexane (20 ml, ×3) and extracted with ethyl acetate (20 ml, ×3). The collected organic layer was washed sequentially with distilled water, brine and dried over anhydrous $MgSO_4$ and evaporated in vacuo. The crude product was purified by flash silica gel column chromatography (normal phase, elution with 4:5:1 *n*-hexane/ethyl acetate/methanol) to afford the 83% yield of the title compound **7**. Compound **8** was synthesized from the enantiomer of **10** by the same procedure and the yield was 81%.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.21–2.37 (m, 2H), 3.93–3.95 (m, 1H), 5.85–5.94 (m, 2H), 6.07–6.10(m, 2H), 6.21–6.23(m, 1H), 6.30–6.32 (m, 1H), 6.42 (s, 1H), 6.70 (d, J = 16Hz, 1H), 7.85 (d, J = 8 Hz, 1H), 8.28 (br, 2H), 8.72 (br, 1H), 8.92 (br, 1H).

^{13}C NMR (DMSO-d_6, 400 MHz): 25.9, 51.8, 59.7, 112.9, 113.9, 116.4, 119.9, 122.9, 126.2, 127.8, 133.9, 140.0, 143.9, 144.9, 145.5, 147.4, 148.8, 165.3, 172.3.

ESI MS: $(M+H)^+$ calcd *m/e* 360.10, obsd *m/e* 359.70.

3.1.3. 3-(3,4-Dihydroxy-phenyl)-2-[3-(3,4-dihydroxy-phenyl)-propionylamino]-propionic acid (4). This was synthesized by the catalytic hydrogenation of compound **7** over Pd/C catalyst and the yield was 95%.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.21–2.25 (m, 2H), 2.45–2.47 (m, 2H), 2.64–2.75 (m, 2H), 4.27–4.30 (m, 1H), 6.33–6.37 (m, 2H), 6.50–6.57 (m, 4H), 8.23 (d, J = 8 Hz, 1H), 8.64 (br, 1H), 8.73 (br, 1H), 8.80 (br, 1H).

^{13}C NMR (DMSO-d_6, 400 MHz): 30.5, 36.4, 37.2, 53.9, 115.3, 115.6, 116.3, 118.6, 119.8, 127.8, 132.0, 143.3, 143.9, 145.0, 171.6, 172.3.

ESI MS: $(M+H)^+$ calcd *m/e* 362.12, obsd *m/e* 361.75.

3.1.4. 3-(3,4-Dihydroxy-phenyl)-2-[3-(3,4-dihydroxy-phenyl)-propionyloxy]-propionic acid (9). This was synthesized by the catalytic hydrogenation of D form of RosA over the Pd/C catalyst and the yield was 94%.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.39–2.41 (m, 2H), 2.68–2.72 (m, 2H), 2.86–2.94 (m, 2H), 4.76–4.78 (m, 1H), 6.28–6.30 (m, 1H), 6.38–6.40 (m,1H), 6.51–6.57 (m, 4H), 8.23 (d, J = 8 Hz, 1H), 8.64 (br, 1H), 8.73 (br, 1H), 8.80 (br, 1H).

^{13}C NMR (DMSO-d_6, 400 MHz): 29.6, 35.7, 74.4, 115.3, 115.5, 115.6, 116.6, 118.6, 119.8, 128.4, 131.2, 143.4, 143.7, 144.8, 145.0, 171.2, 171.7.

ESI MS: $(M+H)^+$ calcd *m/e* 363.10, obsd *m/e* 361.68.

3.1.5. 3-(3,4-Dihydroxy-phenyl)-2-[3-(3,4-dihydroxy-phenyl)-propionylamino]-propionic acid methyl ester (11). This was also synthesized by the catalytic hydrogenation of compound **10** over palladium/carbon catalyst and the yield was quantitative.

^{1}H NMR (DMSO-d_6, 400 MHz): 2.29–2.36 (m, 2H), 2.71–2.77 (m, 2H), 3.01–3.02 (m, 2H), 3.57 (s, 3H), 4.34–4.32(m, 1H), 6.40–6.41 (m, 2H), 6.56–6.60 (m, 4H), 8.25(d, J = 8 Hz, 1H), 8.64 (br s, 1H), 8.73 (br s, 1H), 8.8 (br s, 1H), 8.84 (br s,1H).

^{13}C NMR (DMSO-d_6, 400 MHz): 30.5, 36.4, 37.1, 51.7, 53.9, 115.4, 115.6, 116.3, 118.6, 119.8, 127.8, 132.03, 143.3, 143.9, 145.03, 171.7, 172.3.

ESI MS: $(M+H)^+$ calcd *m/e* 376.13, obsd *m/e* 375.29.

3.1.6. 3-(3,4-Dihydroxy-phenyl)-2-[2-(3,4-dihydroxy-phenyl)-acetylamino]-propionic acid methyl ester (12). (3,4-Dihydroxy-phenyl)-acetic acid (0.2 g, 1.18 mmol, 1 equiv) was dissolved in DMF (10 ml) and to the reaction mixture was added DOPA methyl ester (0.2 g, 1.01 mmol, 1 equiv) and PyBop (4.2 g, 8.07 mmol, 1 equiv) and tri-ethylamine (TEA, 3.4 ml, 24.21 mmol, 3 equiv) at 0 °C ice bath. The reaction mixture was stirred for 18 h under N_2 gas and excess dichloromethane was evaporated. And then the reaction mixture was further proceeded as in compound **10**. The crude product was purified by preparative HPLC using the solvent system (0.1% TFA H_2O/acetonitrile) to afford the title compound and the yield was 45%.

^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.65–2.84 (m, 2H), 3.22 (s, 2H), 3.56 (s, 3H), 4.25–4.34 (m, 1H), 6.38–6.42 (m, 2H), 6.58–6.60 (m, 4H), 8.75 (br, 4H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 36.4, 41.2, 51.7, 54.0, 115.3, 116.4, 116.7, 119.7, 126.7, 126.8, 127.7, 128.1, 143.8, 143.9, 144.8, 150.0, 170.7, 173.2.

ESI MS: $(M+H)^+$ calcd *m*/*e* 362.12, obsd *m*/*e* 361.75.

3.1.7. 2-(3,4-Dihydroxy-benzoyl amino)-3-(3,4-dihydroxy-phenyl)-propionic acid methyl ester (13). (3,4-Dihydroxy)-benzoic acid (0.2 g, 0.78 mmol, 1.2 equiv) was dissolved in DMF (10 ml) and to the reaction mixture were added DCC (0.4 g, 1.94 mmol, 3 equiv) and HOBt (0.26 g, 1.94 mmol, 3 equiv). After activating 3,4-dihydroxy benzoic acid for 30 min, to the reaction mixture were added DOPA methyl ester (0.2 g, 0.78 mmol, 1.2 equiv) dissolved in DMF (5 ml) and DMAP (0.24 g, 1.94 mmol, 3 equiv) and then it was stirred for 18 h. The crude product was further proceeded as mentioned earlier in compound **10** and purified by preparative HPLC by using the same solvent system as in compound **12** and the yield was 48%.

^{1}H NMR (DMSO-d_6, 400 MHz): 3.30–3.33 (m, 2H), 4.02 (s, 3H), 4.87–4.90 (m, 1H), 6.91–6.93 (m, 1H), 7.01–7.03 (m, 1H), 7.05–7.06 (m, 1H), 7.15–7.19 (m, 1H), 7.59–7.61 (m, 1H), 7.66–7.67 (m, 1H), 8.81 (d, J = 7.4 Hz).

^{13}C NMR (DMSO-d_6, 400 MHz): 36.4, 52.4, 55.3, 115.4, 115.8, 115.9, 116.9, 120.0, 120.4, 125.5, 129.1, 144.4, 145.4, 145.5, 149.2, 172.7, 173.3.

ESI MS: $(M+H)^+$ calcd *m*/*e* 348.10 obsd *m*/*e* 347.32.

3.1.8. 3-(3,4-Dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-1-methoxy carbonyl-ethyl ester, 3-(3,4 dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-1-ethoxy carbonyl-ethyl ester, 3-(3,4 dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-1-propoxy carbonyl-ethyl ester, and 3-(3,4 dihydroxy-phenyl)-acrylicacid 2-(3,4-dihydroxy-phenyl)-1-isopropoxycarbonyl-ethyl ester, (14, 15, 16, and 17). Rosmarinic acid (0.1 g, 0.277 mmol, 1 equiv) was, respectively, suspended in methanol for compound **14**, ethanol for compound **15**, 1-propanol for compound **16**, and 2-propanol for compound **17**, and to the solution was added dropwisely thionyl chloride (0.2 ml, 2.77 mmol, 10 equiv) at 0 °C ice bath. Each reaction mixture was equally stirred for 18 h under N_2 gas and further proceeded as previously discussed in compound **10**. The crude product was purified by preparative HPLC by using same solvent system as in compound **12**. The yields for **14**, **15**, **16**, and **17** were 93%, 84%, 52%, and 47%, respectively.

3.1.8.1. Compound 14. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.96 (br, 2H), 3.64 (s, 3H), 5.11–5.12 (m, 1H), 6.28 (d, J = 16 Hz, 1H), 6.51 (d, J = 8 Hz, 1H), 6.63–6.65 (m, 2H), 6.78 (d, J = 8 Hz, 1H), 7.02–7.07 (m, 2H), 7.50 (d, J = 16 Hz, 1H), 8.76 (s, 1H), 8.8 (s, 1H), 9.17 (s, 1H), 9.68 (s, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 30.7, 52.0, 72.8, 112.8, 114.9, 115.4, 115.7, 116.7, 121.7, 125.3, 126.6, 134.9, 144.1, 145.0, 145.6, 146.4, 148.7, 165.9, 169.9.

ESI MS: $(M + H)^+$ calcd *m*/*e* 375.10, obsd *m*/*e* 374.79.

3.1.8.2. Compound 15. ^{1}H NMR (DMSO-d_6, 400 MHz): 1.66(t, J = 6 Hz, 3H), 3.40 (br, 2H), 4.50–4.52 (m, 2H), 5.56 (m, 1H), 6.72 (d, J = 15.6 Hz), 6.93–6.94 (m, 1H), 7.07–7.09 (m, 2H), 7.20–7.21 (m, 1H), 7.44–7.50 (m, 2H), 7.94 (d, J = 15.6 Hz, 1H), 9.19 (br, 2H), 9.58 (br, 1H), 10.08 (br, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 13.8, 36.1, 60.6, 72.7, 112.8, 114.8, 115.3, 115.6, 116.6, 120.0, 121.5, 125.2, 126.5, 144.0, 144.8, 145.5, 146.1, 148.6, 165.8, 169.3.

ESI MS: $(M+H)^+$ calcd *m*/*e* 389.12, obsd *m*/*e* 388.77.

3.1.8.3. Compound 16. ^{1}H NMR (DMSO-d_6, 400 MHz): 0.83(t, J = 7.2 Hz, J = 7.6 Hz, 3H), 1.51–1.56 (m, 2H), 2.97 (d, J = 6.8 Hz, 2H), 4.0 (t, d = 6.4 Hz, 6.4 Hz, 2H), 5.09 (t, J = 7.2 Hz, 6.0 Hz, 1H), 6.29 (d, J = 16 Hz, 1H), 6.50–6.52 (m, 1H), 6.78 (d, J = 8 Hz, 1H), 7.01–7.07 (m, 2H), 7.51 (d, J = 16 Hz, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 10.1, 21.4, 36.2,66.1, 72.9, 112.9, 114.9, 115.4, 115.7, 116.7, 120.1, 121.7, 125.3, 126.6, 144.1, 145.0, 145.6, 146.3, 148.7, 165.9, 169.5.

ESI MS: $(M+H)^+$ calcd *m*/*e* 403.13, obsd *m*/*e* 402.56.

3.1.8.4. Compound 17. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 1.08–1.17 (m, 6H), 2.95 (d, J = 6.4Hz, 2H), 4.84–4.90 (m, 1H), 5.02 (t, J = 6.4 Hz, 1H), 6.30 (d, J = 16Hz, 1H), 6.50–6.53 (m, 1H), 6.63–6.66 (m, 2H), 6.76–6.78 (m, 1H), 7.02–7.07 (m, 2H), 7.51 (d, J = 16 Hz, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 21.4, 21.5, 25.5, 36.2, 62.0, 68.4, 73.0, 113.5, 115.0, 115.4, 115.8, 116.8, 120.2, 121.7, 125.3, 126.5, 144.1, 145.0, 145.6, 146.3, 148.7, 165.9, 168.9.

ESI MS: $(M+H)^+$ calcd *m*/*e* 403.13, obsd *m*/*e* 402.56.

3.1.9. 3-(3,4 Dihydroxy-phenyl)-acrylicacid 1-*tert*-butoxy-carbonyl-2-(3,4-dihydroxyphenyl) ethyl ester (6). RosA was dissolved in dioxane containing 1% sulfuric acid and then the resulting solution was cooled to −10 °C. Liquid isobutylene was added into the solution and stirred at 0 °C for 48 h. The excess solvent was removed by reduced pressure and the crude product was further purified by preparative HPLC and the yield was 38%.

^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 1.33 (s, 9H), 2.93 (d, J = 6.4 Hz, 2H), 4.95 (t, J = 6.0 Hz, J = 6.8Hz, 1H), 6.29 (d, J = 16 Hz, 1H), 6.51–6.53 (m, 1H), 6.63–6.67 (m, 2H), 6.78 (d, J = 8 Hz, 1H), 7.01–7.07 (m, 2H), 7.51 (d, J = 16Hz, 1H), 8.79 (br, 2H), 9.18 (br, 1H), 9.68 (br, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 27.5, 36.1, 73.1, 81.2, 113.0, 114.9, 115.3, 115.7, 116.8, 120.2, 121.5, 125.3, 126.7, 144.0, 144.9, 145.5, 146.0, 148.6, 165.8, 168.4.

ESI MS: $(M+H)^+$ calcd *m/e* 417.15, obsd *m/e* 417.60.

3.1.10. 3-(3,4 Dihydroxy-phenyl)-acrylic acid 2-(3,4-dihydroxy-phenyl)-1-(5-methoxy carbonyl-pentylcarbamoyl)-ethyl ester (18). 6-Aminocaproic acid methyl ester (1 g, 6.8 mmol, 1.2 equiv) was dissolved in DMF (5 ml) and to the reaction mixture was added RosA (2.45 g, 6.8 mmol, 1 equiv) and diluted in dichloromethane (20 ml). To the reaction mixture were added PyBop (1.2 equiv) and tri-ethylamine (3 equiv) at 0 °C ice bath. The reaction mixture was stirred for 18 h under N_2 gas. The reaction was further proceeded as mentioned earlier in compound **10** and the crude product was purified by preparative HPLC using the same solvent system as previously mentioned in compound **12** and the yield was 52%.

^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 1.19–1.23 (m, 2H), 1.34–1.37 (m, 2H), 1.45–1.51 (m, 2H), 2.18–2.29 (m, 2H), 2.78–2.89 (m, 2H), 3.02–3.03 (m, 2H), 3.57 (s, 3H), 5.01–5.04 (m, 1H), 6.25 (d, J = 16Hz, 1H), 6.48 (d, J = 8Hz, 1H), 6.59–6.63 (m, 2H), 6.77 (d, J = 8 Hz, 1H), 7.00 (d, J = 8 Hz, 1H), 7.03 (s, 1H), 7.46 (d, J = 16 Hz, 1H), 7.99 (br, 1H), 8.69 (s, 1H), 8.75 (s, 1H), 9.16 (br, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 24.1, 25.7, 28.6, 33.2, 37.1, 38.2, 51.2, 113.6, 114.7, 115.3, 115.7, 116.7, 120.0, 121.5, 125.4, 127.5, 143.8, 144.9, 145.6, 148.5, 165.8, 168.8, 173.4.

ESI MS: $(M+H)^+$ calcd *m/e* 488.18, obsd *m/e* 487.30.

3.1.11. 3-(3,4-Dihydroxy-phenyl)-2-[3-(4-hydroxy-3-methoxy-phenyl)-acryloylamino]-propionic acid methyl ester, 3-(3,4 dihydroxy-phenyl)-2-[3-(3-hydroxy-4-methoxy-phenyl)-acryloylamino]-propionic acid methyl ester, and 3-(3,4 dihydroxy-phenyl)-2-[3-(3,4-dimethoxy-phenyl)-acryloylamino]-propionic acid methyl ester (19, 20, and 21). D-DOPA methyl ester (0.2 g, 0.94 mmol, 1 equiv) was dissolved in DMF/DCM and to the mixture was added the same equivalent of 3-methoxycaffeic acid for compound **19**, 4-methoxy caffeic acid for compound **20**, and 3,4 dimethoxy caffeic acid for compound **21**, respectively, and added 1.2 equiv of PyBop and 3 equiv of triethyl amine. Then each reaction mixture was equally stirred for 18 h under nitrogen environment and further proceeded as in compound **10**, and the crude product was purified by HPLC by using the same solvent system as mentioned in compound **12**. The yields for **19**, **20**, and **21** were 65%, 67%, 72%, respectively.

3.1.11.1. Compound 19. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.73–2.90 (m, 2H), 3.61 (s, 1H), 3.80 (s, 1H), 4.45–4.50 (m, 1H), 6.44–6.47 (m, 1H), 6.97–6.99 (m, 1H), 7.12 (d, J = 2Hz, 1H), 7.31 (d, J = 15.6 Hz, 1H), 8.31 (d, J = 7.6 Hz, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 36.8, 51.8, 54.1, 55.5, 110.6, 115.4, 115.6, 116.3, 118.2, 119.8, 121.8, 126.2, 127.8, 139.8, 143.9, 145.0, 147.8, 148.4, 165.4, 172.4.

ESI MS: $(M+H)^+$ calcd *m/e* 388.13, obsd *m/e* 387.50.

3.1.11.2. Compound 20. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.73–2.89 (m, 2H), 3.61 (s, 3H), 3.79 (s, 3H), 4.46–4.48 (m, 1H), 6.44–6.49 (m, 2H), 6.59–6.63 (m, 2H), 6.92–6.97 (m, 3H), 7.26 (d, J = 15.6 Hz, 1H), 8.4 (d, J = 7.2 Hz, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 36.4, 51,8, 55.6, 112.1, 113.4, 115.4, 116.3, 118.7, 119.8, 120.4, 127.6, 127.8, 139.7, 144.9, 146.7, 149.3, 165.2, 172.4.

ESI MS: $(M+H)^+$ calcd *m/e* 388.13, obsd *m/e* 387.45.

3.1.11.3. Compound 21. ^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.74–2.90 (m, 2H), 3.61 (s, 3H), 3.78 (s, 3H), 3.79 (s, 3H), 4.51 (q, J = 2.8 Hz, J = 5.4Hz, J = 2.8Hz, 1H), 6.45–6.47 (m, 1H), 6.56–6.63 (m, 3H), 6.99 (d, J = 8.4 Hz, 1H), 7.09–7.15 (m, 2H), 7.35 (d, J = 15.6 Hz, 1H), 8.34 (d, J = 7.6Hz, 1H), 8.73 (br, 1H), 8.78 (br, 1H).

^{13}C NMR (DMSO-d_6/TMS, 400 MHz): 31.4, 52.5, 56.0, 56.2, 110.5, 112.3, 116.0, 116.9, 119.8, 120.4, 122.2, 128.1, 128.4, 140.1, 144.6, 145.6, 149.5, 150.8, 165.9, 172.9.

ESI MS: $(M+H)^+$ calcd *m/e* 402.15, obsd *m/e* 401.80.

3.1.12. 2-[3-(3,4-dihydroxy-phenyl)-acryloylamino]-3-(3,4-dimethoxy-phenyl)-propionic acid methyl ester (22). 3,4 Dimethoxy phenylalanine (0.2 g, 0.887 mmol, 1 equiv) was suspended in methanol (5 ml) and add 4 equiv of thionyl chloride at 0 °C ice bath. The reaction mixture was stirred under nitrogen atmosphere for 18 h. The excess methanol was evaporated in vacuo and further proceeded as in compound **10**.

Then, add 1.1 equiv of caffeic acid, 1.2 equivalent of PyBop, and 4 equiv of tri-ethylamine were added to the above product and stirred under nitrogen atmosphere for 18 h. The reaction was further proceeded as in

compound **10** and purified by flash column chromatography by using 5:4:1 (Hx/EtOAc/MeOH) to afford the yield of 83% of the title compound.

^{1}H NMR (DMSO-d_6/TMS, 400 MHz): 2.96 (d, 2H), 3.57 (s, 3H) 3.65 (s, 6H), 4.48–4.49 (m, 1H), 6.35 (d, J = 15.6 Hz, 1H), 6.45–6.47 (m, 1H), 6.67–6.69 (d, J = 8 Hz, 2H), 6.78 (m, 3H), 6.88 (s, 1H), 7.17 (d, J = 15.6 Hz, 1H), 8.35 (d, J = 7.6 Hz, 1H), 9.2 (br, 1H), 9.4 (br, 1H).

^{13}CNMR(DMSO-d_6/TMS,400 MHz): 36.4, 51.8, 54.9, 55.4, 111.6, 112.9, 113.8, 115.7, 117.5, 120.5, 121.0, 126.1, 129.5, 140.0, 145.6, 147.5, 147.6, 148.4, 165.4, 172.3.

ESI MS: $(M+H)^+$ calcd *m/e* 402.15, obsd *m/e* 401.33.

3.2. ELISA binding assay

The assays were performed as we described previously.[19,23] Briefly, an enzyme-linked immunosorbant assay was employed to measure the binding affinity of synthesized derivatives for each SH2 domains. 100 μl of biotinyl-ε-aminocaproyl-EPQpYEEIPIYL (10 ng/ml in 50 mM Tris, 150 mM NaCl, pH 7.5) was added to each well of streptavidin-coated 96-well micro titer plates. The plates were shaken overnight at 4 °C and rinsed with TBS (50 mM Tris, 150 mM NaCl, pH 7.5, 3× 200 μl). Each well was then blocked with 100 μl of a solution containing 2% BSA and 0.2% Tween 20 in TBS (1 h at 37 °C). The wells were then rinsed with 4× 200 μl of a standard BSA-T-TBS solution (0.2% BSA, 0.1% Tween 20, TBS). A 50-μl solution of samples (1 mM, in BSA-T-TBS) and a 50-μl solution of the SH2-GST fusion proteins (100 ng/ml, in BSA-T-TBS) were added in each well and the plate was shaken for 1 h at room temperature. The solutions were removed and each well rinsed with 4× 200 μl BSA-T-TBS. 100 μl of polyclonal rabbit anti-GST antibody (1 μg/ml in BSA-T-TBS) was then added to each well and incubated for 1 h at room temperature. Following subsequent washing steps with BSA-T-TBS (4× 200 μl), 100 μl of horseradish peroxidase-conjugated mouse anti-rabbit antibody (2 μg/ml in BSA-T-TBS) was added to each well and subsequently incubated for 1 h at room temperature. After a series of final wash steps (4× 200 μl BSA-T-TBS; 2× 300 μl and TBS), 100 μl of peroxidase substrate (1-Step Turbo TMB-ELISA, trimethylbenzidine) was added to each well and incubated for 5–15 min. 100 μl of 1 M sulfuric acid solution was introduced to stop the peroxidase reaction and absorbance was measured at 450 nm with a plate reader.

3.3. Luciferase assay

The assays were performed as we described previously.[19] Briefly, a total of 2×10^6 Jurkat T cells were transfected with 2 μg of IL-2-Luciferase (gifts of Gerald Crabtree, Stanford University, Stanford, CA) using Lipofectamine according to manufacturer's protocol (GibcoBRL). After incubation with DNA-Lipofectamine mixtures for 24 h, cells were pre-incubated in the presence or absence of rosmarinic acid or its derivative (10 μg/ml) for 2 h before stimulation. Cells were activated either with anti-CD3 mAb (5 μg/ml, UCHT1, IgG_{2a} isotype) coated on the plate or with PMA (5 μg/ml) and ionomycin (0.5 μg/ml) for 16 h. In the rosmarinic acid or its derivative treated group, they were present through the whole 16 h incubation process. After stimulation, the cells were washed, lysed, and assayed for luciferase activity according to the manufacturer's instructions (Luciferase Assay System kit, Promega, Madison, WI) with Microplate luminometer LB96V (Perkin-Elmer, Foster City, CA).

3.4. Estimation of log P

The relative hydrophobicity was assessed for each of the rosmarinic acid and its derivatives using a typical method involving measuring the partitioning of the compound between 1-octanol and distilled water. HPLC-grade 1-octanol was pre-saturated with distilled water, and aqueous phase distilled water was saturated with 1-octanol before use. The derivatives were each dissolved in 1-octanol/water phase at final concentration of 1 mM, and an equal volume of 1-octanol/water was added. The tubes were then vortexed continuously for 30 min. The final concentration of compound in both 1-octanol and aqueous phases was measured by HPLC with C_{18} column. The partition coefficient, P, was determined by dividing the concentration of the derivative in 1-octanol by the concentration in the aqueous phase.

Acknowledgment

This work was supported by the grant (R01-2006-000-10956-0) from the Basic Research Program of the Korea Science & Engineering Foundation. B.P. Joshi was recipients of BK21(II) fellowship.

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