Synthesis of Naringenin Amino Acid Esters as Potential CDK2 Inhibitors

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The central role of cyclin-dependent kinases (CDKs) in cell cycle regulation makes them a promising target for studying inhibitory molecules that can modify the degree of cell proliferation.[1-7] The discovery of specific inhibitors of CDKs such as polyhydroxylated flavones has opened the way to investigation and design of antimitotic compounds.[8] Among the polyhydroxylated flavones, flavopiridol (**1**, Figure 1)[8] has completed phase I clinical trial where it showed antitumor effect in patients with refractory neoplasms.[9]

This encouraging result, which is consistent with the ability of **1** to inhibit proliferation of cancer cells *in vitro* makes polyhydroxylated flavone an interesting target for further analogue synthesis. In continuation of our studies aimed toward the development of more potent and more selective anticancer agents, we designed flavone derivatives for further biological evaluation. The flavone naringenin is widely spread in nature and easily extracted from a lot of different plants. Their protective effect against lipid peroxidation of membranes, involved in several physiological and pathological disorders, as aging, inflammation, atherosclerosis, ischemia, toxicity of oxygen and chemical substances has been largely reported.[10-16] Thus, interesting biological activity as well as structural similarity with flavopiridol makes naringenin one of the most interesting candidates for designing novel CDK2 inhibitors. However, derivatives of naringenin have not been studied extensively presumably due to the synthetic difficulties. Particularly, naringenin is awkward to make derivatives due to its low solubility in water as well as in organic solvents.[17] In view of designing naringenin derivatives, the 7-*O* position of naringenin (**2**, Figure 1) is particularly attractive because it is well known that electrophiles react with hydroxyl group preferentially at the 7-position.[18,19] Also, comparison of the biological activity of 7-*O*-substitued naringenin with that of flavopiridol would provide interesting insights into designing novel flavonoid CDK2 inhibitors due to their subtle distinction in three dimensional structures. On the other hand, as a limiting factor of the use of flavonoids is their low water solubility, another aim of this study was to increase the solubility and dissolution rate of naringenin by conjugate formation with polar substituents. Thus, we set out to prepare various naringenin-amino acid conjugates (**2**, Figure 1) because amino acids substituted at the 7-*O* position would serve as an excellent model for our purpose: formation of the amino acid conjugate would benefit naringenin from the various substituent effect as well as increased solubility. Herein we report our efforts to synthesize the title compounds.

Recently, two classes of naringenin derivatives, naringenin 7-*O*-oleic ester and naringenin 7-*O*-cetyl ether, were prepared for biological evaluation of their anti-atherogenic activity by using the mixed anhydride strategy.[18] However, in our case, due to the epimerization problem at the amino acid α-carbon under the reaction conditions, we decided to use rather mild coupling conditions which was successfully exploited for the preparation of the amino acid esters of 5,7-dihydroxy-3-phenoxychromones (**3**, Figure 2).[20]

Thus, a mixture of naringenin (**4**), *N*-Boc-L-alanine, and DMAP in anhydrous THF or DMF was treated with EDCI (Scheme 1) and the reaction was monitored by TLC.

However, the reaction did not proceed at all presumably due to the solubility problem of the naringenin. After extensive survey of the appropriate solvent system for the coupling reaction, methylene chloride (CH_2Cl_2) was found to be the solvent of choice: initial slurry of naringenin and *N*-Boc-L-alanine in anhydrous CH_2Cl_2 became a clear solution as the reaction proceeds, and the reaction was complete in 2

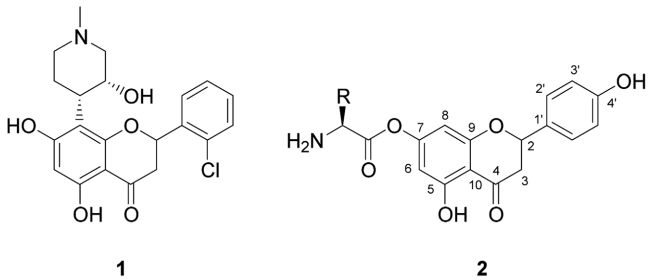

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Figure 1. Structures of flavopiridol (**1**) and naringenin-amino acid ester (**2**).

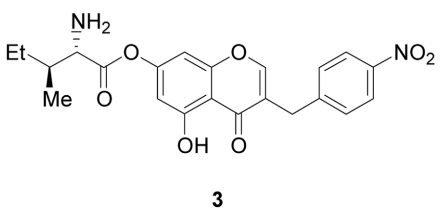

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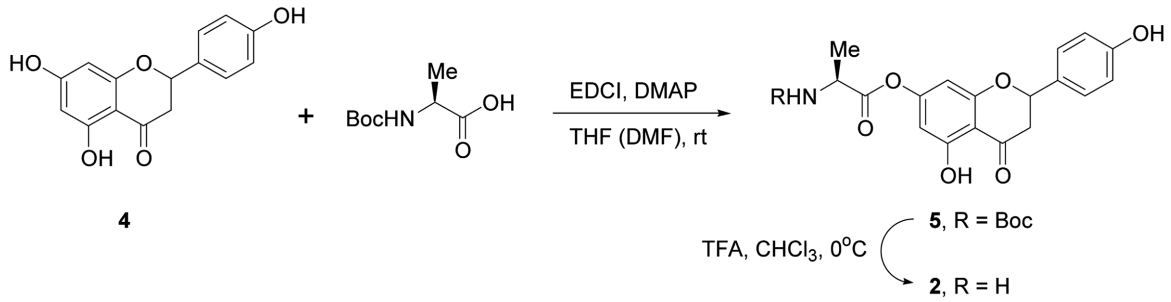
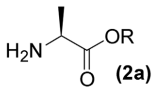
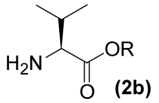
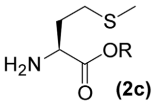
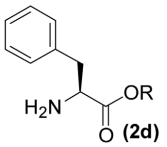
Figure 2. 5,7-Dihydroxy-3-phenoxychromone amino acid ester (**3**).



Scheme 1. Synthesis of naringenin-amino acid ester (**2**) from naringenin (**4**).

hours. However, the naringenen-alanine adduct, thus formed, easily decomposed upon aqueous work-up or purification by column chromatography on silica gel. Usually, the starting materials naringenin and *N*-Boc-L-alanine were recovered after purification presumably due to the hydrolysis of the amino acid ester, and the problem persisted in various EDCI coupling conditions including EDCI-HOAt combinations (data not shown). Considerable experimentation was devoted to investigating the way in which the product of the coupling reaction could survive throughout the purification process. It is general practice to use EDCI with equimolar amount of base because the commercially available EDCI comes as an HCl-salt form. In search of optimum reaction conditions, several bases such as DMAP, TEA and $NaHCO_3$ were tested in combination with EDCI. Interestingly, however, the coupling reaction and purification process smoothly underwent to give the desired compound when the base was not used, which suggests that the base molecule prompts cleavage of the newly formed ester bond presumably by nucleophilic attack of amino acid at the ester carbonyl carbon. Thus, *N*-Boc-L-alanine was condensed with naringenin and the reaction mixture was directly concentrated without aqueous work-up. The residue was filtered through a short silica gel column washing with a 1 : 1 : 1 mixture of hexanes, EtOAc and CH_2Cl_2, and the use of three-component solvent system including CH_2Cl_2 was critical for filtration because the naringenin derivative did not pass through the silica gel column without CH_2Cl_2. The filtrate was concentrated under reduced pressure and immediately treated with TFA in $CHCl_3$ followed by trituration with diethyl ether to give free naringenin-L-alanine ester in good yield (65%, Entry 1, Table 1). No further purification by column chromatography was attempted

Table 1. Preparation of various naringenin-amino acid esters

Entry	Amino Acid	Conditions	Time	Product	Yield
1	Boc-Ala-OH	EDCI, CH_2Cl_2, rt	2 h	 **(2a)**	65%
2	Boc-Val-OH	EDCI, CH_2Cl_2, rt	5 h	 **(2b)**	60%
3	Boc-Met-OH	EDCI, CH_2Cl_2, rt	2 h	 **(2c)**	75%
4	Boc-Phe-OH	EDCI, CH_2Cl_2, rt	6 h	 **(2d)**	56%

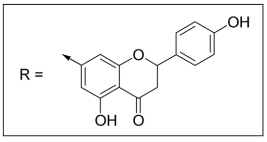


Table 2. Comparison of ^1H and ^{13}C NMR spectra

Position	δ of ^1H NMR / ppm (*J*, Hz)			δ of ^{13}C NMR / ppm		
	2b	**2c**	**4**	**2b**	**2c**	**4**
2	5.43 (dd 3.2, 12.8)	5.43 (dd 2.9, 12.8)	5.43 (dd 2.8, 12.7)	78.42	78.42	78.4
3_{ax}	3.26 (dd 12.8, 17.2)	3.26 (dd 12.9, 17.2)	3.26 (dd 12.8, 17.1)	41.98	41.97	42.0
3_{eq}	2.67 (dd 3.2, 17.2)	2.67 (dd 3.2, 17.2)	2.69 (dd 2.8, 17.1)	41.98	41.97	42.0
6	5.87 (s)	5.88 (s)	5.90 (s)	95.82	95.80	95.9
8	5.87 (s)	5.88 (s)	5.90 (s)	94.99	94.98	95.0
2	7.31 (d 8.8)	7.31 (d 8.6)	7.32 (d 8.5)	128.33	128.33	128.2
3'	6.79 (d 8.8)	6.79 (d 8.6)	6.81 (d 8.5)	115.15	115.16	115.2
5-OH	12.14 (br s)	12.14 (br s)	12.15 (br s)	–	–	–
7-OH	–	–	8.13 (br s)	–	–	–
4'-OH	9.59 (br s)	9.59 (br s)	9.67 (br s)	–	–	–

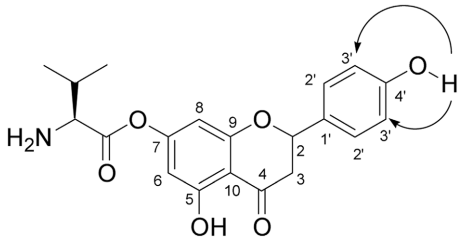


Figure 3. HMBC correlation between $H_{O4'}$ and $C_{3'}$.

solvents resulted in alcoholysis of the ester to give naringenin and free amino acid. By using the same protocol, the coupling reaction of naringenin with *N*-Boc-valine, *N*-Boc-phenylalanine and *N*-Boc-methionine smoothly underwent to give the desired products in 60%, 75% and 56% yields, respectively (Entry 2-4, Table 1).

In order to confirm the regioselectivity of the coupling reaction, various NMR techniques such as ^{1}H NMR, ^{13}C NMR, DEPT135, COSY, NOESY, HMBC and HMQC were employed. The ^{1}H and ^{13}C spectra, however, did not show any difference compared with those of naringenin[10,21,22] (Table 2), and the NOE contacts between H_6/H_8 (5.87 ppm, **2b**) and H_α (3.68 ppm, **2b**) were too weak to observe (Figure 3).

Thus, the only way to make sure the site of substitution was to assign two deuterium-exchangeable hydroxyl protons (12.14 and 9.59 ppms, **2b**). Naringenin 5-OH is known to be observed at around 12.15 ppm in DMSO-d*6* due to the formation of intramolecular hydrogen bond with C_4-carbonyl group. Thus, the connectivity around the hydroxyl proton at 9.59 ppm (4'-OH) was investigated by HMBC experiment, which showed strong correlation with $C_{3'}$ (115.16 ppm, Figure 3).

The biological activity of the synthesized naringenin-amino acid esters (**2a–2d**) as potential CDK2 inhibitors is being evaluated. Also, a complete structure-activity relationship study of naringenin conjugates with various natural amino acids is on the way and the result will be published in due course.

In conclusion, naringenin amino acid ester was prepared by EDCI coupling method under neutral conditions. Due to the poor solubility of naringenin, solvent played a key role in this transformation. Regioselectivity of the coupling reaction was confirmed by HMBC NMR spectroscopy.

Experimental Section

Nuclear magnetic resonance spectra were recorded on a Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR with tetramethylsilane as the internal standard. Chemical shifts (d) are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). TLC was performed on silica gel 60 F_{254} purchased from Merck. Column chromatography was performed using either silica gel-60 (220-440 mesh) for flash chromatography.

General procedure for preparation of naringenin-amino acid ester. Preparation of naringenin-alanine ester (2a) is representative: A stirred mixture of naringenin (**4**, 300 mg, 1.10 mmol) and *N*-Boc-L-alanine (261 mg, 1.38 mmol) in anhydrous CH_2Cl_2 (10 mL) was treated with EDCI (528 mg, 2.75 mmol) at room temperature, and the resulting turbid mixture was stirred until a clear solution was obtained (2-3 h). After evaporation of the volatiles, the residue was briefly filtered though a short silica gel column washing with a 1 : 1 : 1 mixture of hexanes, EtOAc and CH_2Cl_2. The filtrate was concentrated under the reduced pressure and then used for the next step without further purification. The naringenin-alanine adduct (**5**) obtained above was dissolved in $CHCl_3$ (5 mL) and treated with TFA (5 mL) at 0 °C and the reaction mixture was slowly warmed to rt. After stirring for 12 h, the volatiles were evaporated and the residue was triturated with ether three times to give a TFA salt of the desired compound **2a** (327 mg, 0.72 mmol, 65% yield) as a dark yellow syrup.

2-Amino-propionic acid 5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-chroman-7-yl ester (2a): ^{1}H NMR (DMSO-d*6*, 400 MHz) δ 11.99 (s, 1H), 9.65 (br s, 1H), 9.47 (br s, 2H), 7.33 (d, J = 8.8 Hz, 2H), 6.81 (d, J = 8.4 Hz, 2H), 6.41 (s, 2H), 5.61 (dd, J = 12.8, 2.4 Hz, 1H), 4.39 (q, J = 6.8 Hz, 1H), 3.46 (dd, J = 17.2, 12.8 Hz, 1H), 2.83 (dd, J = 17.2, 2.8 Hz, 1H), 1.53 (d, J = 7.2 Hz, 3H); ^{13}C NMR (DMSO-d*6*, 100 MHz) δ 198.27, 168.07, 162.42, 162.27, 157.94, 156.78, 128.47, 128.34, 115.24, 106.30, 102.21, 101.28, 78.91, 64.92, 48.17, 15.56.

2-Amino-3-methyl-butyric acid 5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-chroman-7-yl ester (2b): The title compound was obtained in 60% yield as a dark yellow syrup: ^{1}H NMR (DMSO-d*6*, 400 MHz) δ 12.14 (br s, 1H), 9.59 (br s, 1H), 7.93 (br s, 2H), 7.31 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 8.8 Hz, 2H), 5.87 (s, 2H), 5.43 (dd, J = 12.8, 3.2 Hz, 1H), 3.68 (d, J = 3.5 Hz, 1H), 3.26 (dd, J = 17.2, 12.8 Hz, 1H), 2.67 (dd, J = 17.2, 3.2 Hz, 1H), 2.18 (dq, J = 3.2, 7.2 Hz, 1H), 0.96 (d, J = 7.2 Hz, 3H), 0.84 (d, J = 7.0 Hz, 3H); ^{13}C NMR (DMSO-d*6*, 100 MHz) δ 196.35, 167.40, 163.48, 162.93, 157.92, 157.72, 128.86, 128.33, 115.15, 101.73, 95.82, 94.99, 78.42, 59.13, 41.98, 31.05, 18.67, 17.30.

2-Amino-3-phenyl-propionic acid 5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-chroman-7-yl ester (2c): The title compound was obtained in 56% yield as a dark yellow syrup: ^{1}H NMR (CD3COCD3, 400 MHz) δ 7.43-7.30 (m, 5H), 7.42 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 6.36 (s, 1H), 6.34 (s, 1H), 5.60 (dd, J = 13.0, 2.9 Hz, 1H), 5.32-5.21 (m, 1H), 3.68-3.59 (m, 1H), 3.51-3.45 (m, 1H), 3.35 (dd, J = 17.1, 13.0 Hz, 1H), 2.88 (dd, J = 17.1, 3.0 Hz, 1H); ^{13}C NMR (CD3COCD3, 100 MHz) δ 198.96, 167.76, 163.68, 163.60, 158.90, 158.01, 135.29, 130.61, 130.01, 129.74, 129.04, 128.54, 116.22, 107.21, 103.11, 102.14, 80.26, 55.27, 43.61, 37.11.

2-Amino-4-methylsulfanyl-butyric acid 5-hydroxy-2-(4-hydroxy-phenyl)-4-oxo-chroman-7-yl ester (2d): The title compound was obtained in 75% yield as a dark yellow syrup: ^{1}H NMR (DMSO-d*6*, 400 MHz) δ 12.14 (br s, 1H), 9.62 (br s, 1H), 7.31 (d, J = 8.6 Hz, 2H), 6.79 (d, J = 8.6 Hz,

2H), 5.88 (s, 2H), 5.43 (dd, J = 12.8, 2.9 Hz, 1H), 3.67 (t, J = 6.5 Hz, 1H), 3.26 (dd, J = 17.2, 12.9 Hz, 1H), 2.67 (dd, J = 17.2, 3.2 Hz, 1H), 2.03 (s, 3H), 1.96-1.86 (m, 2H), 1.83-1.74 (m, 2H); ^{13}C NMR (DMSO-d*6*, 100 MHz) δ196.36, 171.50, 163.47, 162.93, 157.72, 157.68, 128.85, 128.33, 115.16, 101.74, 95.80, 94.98, 78.42, 52.03, 41.97, 40.12, 29.16, 14.45.

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