Application of a Regioselective Mannich Reaction on Naringenin and its Use in Fluorescent Labeling

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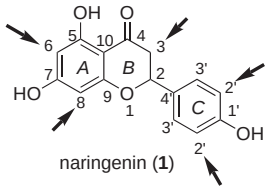
Abstract: A novel strategy for site-specific fluorescent labeling on naringenin (**1**) was established by a new direct Mannich reaction in combination with a Huisgen [3+2]-cycloaddition reaction. High regioselectivity was observed for direct Mannich reactions on naringenin and several other flavonones using a variety of amines and aldehydes.

Key words: naringenin, Mannich reaction, Huisgen cycloaddition, regioselectivity, fluorescent labeling

Naringenin (**1**, Figure 1) is a representative flavonone existing in many plants and fruits (e.g. grapefruit). Recent studies show that naringenin protects against toxins that are found in chemotherapy drugs and some environmental sources. It also enhances lipid and ethanol metabolism, as well as serving as a free-radical scavenger and an antioxidant.[1] More interestingly, such flavonones have also been shown to play an important role in nitrogen fixation in a variety of legume species. Many studies have shown that naringenin is an active small molecule involved in the control of root nodulation, which is a prerequisite for nitrogen fixation. The interaction between naringenin, which is released by the plant, with nod-D protein induces the over-expression of nod genes and leads to the production of NodRm-IV (sulfated lipooligosaccharides), which are Rhizobium nodulation signaling molecules. During nodulation of legumes, the interaction between narigenin and nod-D protein is the first important step.[2] Although genetics show that nod-D and naringenin interact with each other, there is no direct evidence proving this.[3,4] In order to unambiguously demonstrate direct interactions between nod-D and naringenin, suitable naringenin derivatives containing fluorescent labels are required for visualization techniques such as fluorescence resonance energy transfer (FRET).[5] Herein, we report the results of our studies on direct Mannich reactions of unprotected naringenins, as well as their application to the above-mentioned efforts to fluorescently label naringenin derivatives.

Structural analysis indicates that the A ring of naringenin, especially positions C-6 and C-8, might be more reactive towards electrophiles than the C ring (Figure 1). Therefore, relatively mild Mannich reactions[6,7] might provide a potential site-selective derivation protocol for naringenin, which could introduce sites for fluorescent labeling through further elaboration. Other positions in naringenin, such as the α-carbon of the carbonyl on the B ring and the C-2′ of the C ring could also potentially be involved in such Mannich reactions (Figure 1). Previous studies on electrophilic reactions of naringenin under a variety of conditions indicates that mixtures often result through substitution at different positions, including C-6 and C-8 of the A ring and C-2′ of the C ring, as well as C-3 of the B ring.[8–12] In order to acquire pure single Mannich adducts, one frequently adopted approach is to protect one or more phenolic hydroxyls prior to the Mannich reactions. However, such an indirect approach usually requires multi-step transformations. Obviously, it could be of great value to find a regioselective Mannich reaction, which would allow the addition of extended functionalized alkyl groups to naringenin without the need for protecting groups. Such a process would be of great utility in the study of phenolic flavonones. Though the phenolic hydroxyl groups in naringenin are chemically similar, fine differences exist in the chemical and electronic environments of each. After reviewing the literature of other compounds with multiple phenolic hydroxyl groups, we expected the C-6 of naringenin to be the most reactive site for electrophillic reactions.[13] Optimization of appropriate Mannich conditions may provide a highly regioselective facile entrance to naringenin derivatives. Herein, we describe our efforts to achieve such a reaction.



naringenin (**1**)

Figure 1 Structure of naringenin (**1**) and possible sites for Mannich reaction

N-Methyl β-alanine methyl ester (Table 1, **3g**) was chosen as the first amine substrate in combination with paraformaldehyde to find appropriate conditions for a selective Mannich addition. Fortunately, success was achieved after investigating a variety of conditions. Initial studies showed that temperature and duration of the reaction significantly affected the yield of the major product. No

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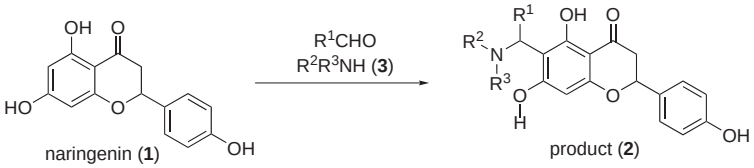
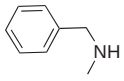
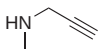
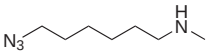
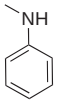
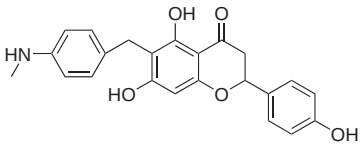
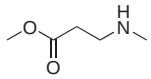
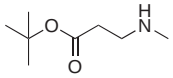
reaction was observed at room temperature in ethanol. Only a highly polar product (difficult to purify) was afforded when the reaction was heated to reflux for one hour. However, when the reaction was stirred at 50 °C for 16 hours, a single Mannich product **2g** was generated in 61% isolated yield. To better understand this reaction, *N*-methylbenzylamine (**3a**) was selected as the secondary amine substrate to replace *N*-methyl β-alanine methyl ester (**3g**). Similarly, temperature was found to be an important factor affecting the regioselectivity. A mixture of Mannich adducts (C-6 vs C-8, 4:1) was obtained when the reaction was carried out at 65 °C (Table 3, entry 1); a single product (**2a**, C-6 adduct) was afforded in 77% isolated yield (Table 3, entry 2) when the reaction was performed at 55 °C. To our knowledge, this is the first demonstration that a direct Mannich reaction on naringenin without any protecting groups can be well controlled to afford a single product regioselectively.

A variety of secondary amines and aldehydes in various combinations were examined under similar conditions. As

Table 1 Mannich Reactions of Naringenin with Secondary Amines and Aldehydes



Entry	Aldehyde	Amine	Conditions	Products	Yields[a]
1	$(HCHO)_n$	 **3a**	2 h, 55 °C	**2a**: R^1 = H; R^2 = Me; R^3 = Bn	77%
2	$(HCHO)_n$	 **3b**	2 h, 55 °C	**2b**: R^1 = H; R^2,R^3 = -($CH_2CH_2OCH_2CH_2$)-	80%[b]
3	$(HCHO)_n$	 **3c**	24 h, 60 °C	**2c**: R^1 = H; R^2 = R^3 = Et	66%
4	$(HCHO)_n$	 **3d**	8 h, 45 °C	**2d**: R^1 = H; R^2 = Me; R^3 = $CH_2C{\equiv}CH$	68%
5	$(HCHO)_n$	 **3e**	3 h, 60 °C	**2e**: R^1 = H; R^2 = Me; R^3 = $(CH_2)_6N_3$	90%[c]
6	$(HCHO)_n$	 **3f**	24 h, 60 °C	 **2f**	72%[c,d]
7	$(HCHO)_n$	 **3g**	16 h, 50 °C	**2g**: R^1 = H; R^2 = Me; R^3 = $CH_2CH_2CO_2Me$	61%
8	$(HCHO)_n$	 **3h**	8 h, 20 °C	**2h**: R^1 = H; R^2 = Me; R^3 = $CH_2CH_2CO_2$-*t*-Bu	66%[e]
9	CH_3CHO	 **3b**	7 h, 35 °C	**2i**: R^1 = CH_3; R^2,R^3 = -($CH_2CH_2OCH_2CH_2$)-	75%

[a] Isolated yields.
[b] Structure of **2b** was determined by single-crystal X-ray analysis.
[c] Reagents: $ZnCl_2$ (0.1 equiv), EtOH, 60 °C.
[d] Two by-products, **4a** and **4b**, were obtained.
[e] Structure of **2h** was confirmed by 2D-NMR studies (HMQC and HMBC).

the results show (Table 1), all reactions, irrespective of electrophiles, performed well under mild conditions, to afford predominantly the C-6 substituted derivatives. These products are very polar and easily absorbed onto silica gel with moderate isolated yields obtained in most cases following chromatographic purification. The regioselectivities and chemical yields of the naringenin Mannich reactions can be optimized by changing the reaction temperatures and reaction times (Table 1).

With the exception of *N*-methylaniline (**3f**), all secondary amines gave single products. In the case of *N*-methylaniline (Table 1, entry 6), the reaction occurred at the *para*-position of the phenyl ring under the general reaction conditions, affording **2f** in 12% yield along with two by-products **4a** and **4b** (Figure 2).[14] This might be due to the higher electron density at the *para*-position of the benzene ring. To improve the yield of this reaction, we also tried the addition of Lewis acids and other solvents (e.g. CH_3COOH–H_2O, 1:1 or 1:3; DMF; EtOH, cat. HCl; $AlCl_3$, CH_2Cl_2). The most efficient conditions were found to be 0.1 equivalent of $ZnCl_2$ in ethanol at 60 °C, which increased the chemical yield to 72%. Such conditions also can be applied to the Mannich reaction of secondary amine **3e**, which gave no reaction either at room temperature or by heating; addition of 0.1 equivalent of $ZnCl_2$ greatly improved the reaction (Table 1, entry 5) and afforded the corresponding adduct **2e** in 90% yield.

Two additional flavonones, **5** and **7**, with structural features similar to naringenin (**1**), were also examined under these reaction conditions (Table 2). Both gave similar regioselectivities favoring the C-6 position (**6** and **8**, respectively).

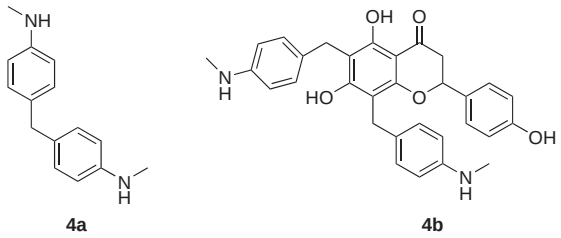
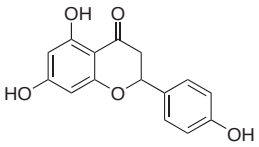
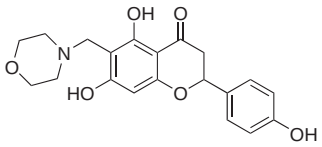
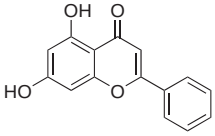
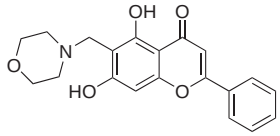
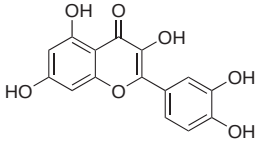
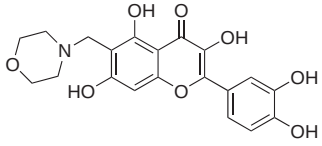


Figure 2 By-products of the Mannich reaction of naringenin with *N*-methylaniline and paraformaldehyde

Significant temperature effects on the regioselectivity (C-6 vs. C-8) were observed in Mannich reactions of naringenin with three secondary amines (Table 3). Reaction with *N*-methyl-*N*-propargylamine (**3d**) and paraformaldehyde gave C-6 and C-8 adducts in almost equal amounts at 65 °C, while a 50:1 ratio (C-6 vs C-8) resulted when the reaction was performed at 45 °C (Table 3, entry 3 and 4). Results with the other two amines were also in good agreement with the above observations. These results demonstrate that the A ring of naringenin is the most electron-rich with the C-6 position being the predominant site for Mannich reactions.[13]

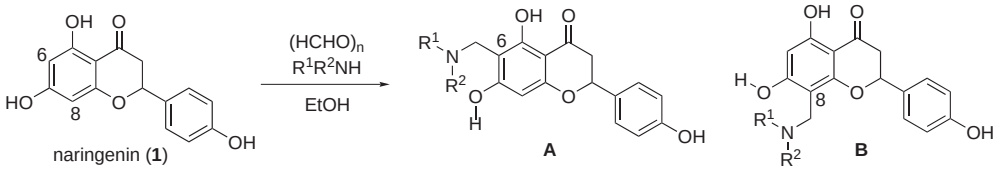
The direct and highly regioselective Mannich reactions described above afford a facile entry to bio-labeling naringenin derivatives with fluorescent groups. With azido derivative **2e** in hand, a Huisgen [3+2] cycloaddition was devised as the key step for coupling with the fluorescein derivative **9** (Scheme 1) to generate the corresponding fluorescent compound **10**.

Table 2 Mannich Reactions of Naringenin-Like Flavonoids with Paraformaldehyde and Morpholine[a]

Entry	Flavonoid	Conditions	Products	Yields
1	 **1**	55 °C, 2 h	 **2b**	80%[b]
2	 **5**	40 °C, 3 h	 **6**	79%[b]
3	 **7**	r.t., 4 h	 **8**	90%[c]

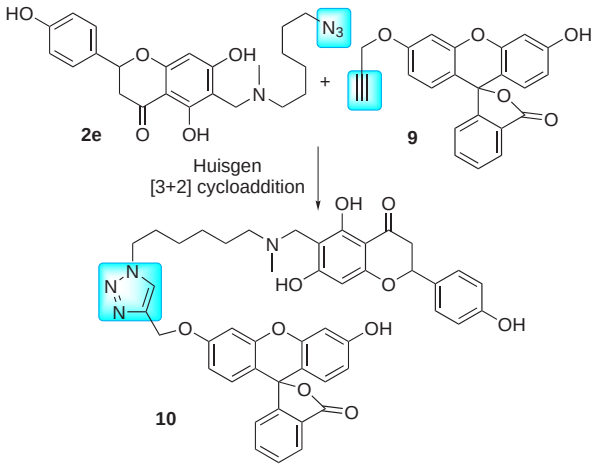
[a] Reaction conditions: flavonoid (1 mmol), paraformaldehyde (1.5 mmol), morpholine (1.5 mmol), EtOH (4 mL).
[b] Isolated yields following purification by silica gel column chromatography.
[c] Crystallization from H_2O as the hydrochloride salt.

Table 3 Effects of Temperature on the Regioselelctivity of Mannich Reactions

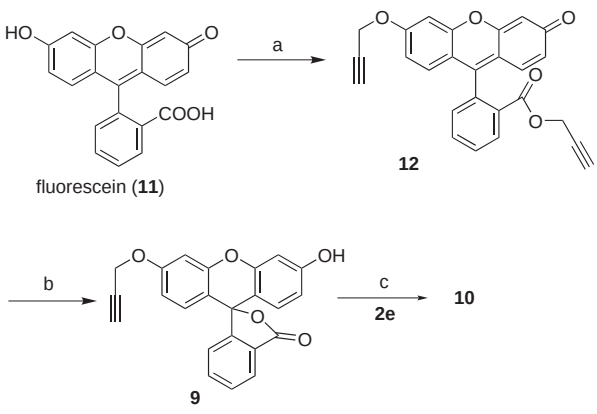


Entry	R^1R^2NH	Temperature	Yield[a]	Ratio (A/B)[b]
1	**3a**	65 °C	68%	4:1
2	**3a**	55 °C	77%	A only (**2a**)
3	**3d**	65 °C	30%	1:1
4	**3d**	45 °C	68%	50:1 (**2d**, major product)
5	**3c**	75 °C	20%	ca. 50:1
6	**3c**	60 °C	66%	A only (**2c**)

[a] Isolated yields.
[b] Measured by ^{1}H NMR spectroscopy.



Scheme 1 Naringenin fluorescent-labeling strategy utilizing Mannich adduct **2e**.



Scheme 2 *Reagents and conditions*: a) 3-bromopropyne, K_2CO_3, DMF, 100%; b) 1 M LiOH, THF–H_2O (1:1), 95%; c) **2e**, $CuSO_4·5H_2O$ (cat.), ascorbic acid, *t*-BuOH, H_2O, 67%.

Chemically stable acetylene-terminated fluorescein derivative **9** was prepared from commercially available fluorescein in two steps (Scheme 2). Treatment of fluorescein (**11**) with excess propargyl bromide in the presence of K_2CO_3 gave **12**[15] in quantitative yield. All initial efforts at selective and/or direct preparation of **9** resulted in mixtures which could not be purified efficiently. Hydrolysis of propargyl ester **12** was carried out smoothly using 1 M LiOH in THF–H_2O (1:1), affording **9**[16] in excellent yield. The 'click' reaction[17,18] between azido-naringenin derivative **2e** and the acetylene-fluorescein derivative **9** was accomplished in *t*-BuOH and H_2O using $CuSO_4·5H_2O$ as a catalyst and ascorbic acid to afford the fluorescein-labeled naringenin **10** in 67% yield.[19] UV analysis of **10** (in MeOH) indicated typical fluorescein absorptions at 453 nm (ε = 15967 $M^{-1}cm^{-1}$) and 478 nm (ε = 13271 $M^{-1}cm^{-1}$) in addition to the naringenin absorptions at 279 nm and 324 nm. Fluorescence characterization of **10** shows λ_{em} at 523 nm (Φ = 0.20, in MeOH, using λ_{exc} 478 nm and quinine sulfate as a standard).

In summary, direct Mannich reactions onto naringenin were investigated without the use of phenolic protection.[20] High regioselectivity for the C-6 position achieved under optimized conditions indicated that electronic density in the naringenin A ring could be exploited. Further studies on related substrates showed such regioselectivity could also be achieved under similar conditions. Based on Mannich adduct **2e**, a quick and convenient fluorescent labeling strategy for naringenin was established, affording the first fluorescein-labeled naringenin derivative **10**. Further biologically relevant studies with nod-D protein, utilizing this fluorescently labeled compound are currently underway and will be reported in due course. In addition, the Mannich adduct **2d** having terminal acetylene functionality offers alternate fluorescent labeling options using 'click' chemistry. This work is also under investigation in our laboratory.

Acknowledgment

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(19) Data for **10**: IR (KBr): 2937, 1759, 1637, 1616, 1505, 1459, 1364, 1250, 1174, 1110, 1085, 834 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz): δ = 8.24 (1 H, s), 8.00 (1 H, d, *J* = 7.5 Hz), 7.79–7.69 (2 H, m), 7.32 (1 H, s), 7.28 (2 H, d, *J* = 8.4 Hz), 7.09 (1 H, d, *J* = 2.7 Hz), 6.81–6.74 (4 H, m), 6.65 (1 H, d, *J* = 9.3 Hz), 6.59 (2 H, d, *J* = 1.2 Hz), 5.66 (1 H, s), 5.35 (1 H, dd, *J* = 12.6, 2.4 Hz), 5.22 (2 H, s), 4.36 (2 H, t, *J* = 6.6 Hz), 3.83 (2 H, s), 3.15 (1 H, dd, *J* = 17.1, 12.6 Hz), 2.69 (2 H, t, *J* = 7.2 Hz), 2.61 (1 H, dd, *J* = 17.1, 2.4 Hz), 2.40 (3 H, s), 1.82 (2 H, t, *J* = 6.6 Hz), 1.56 (2 H, br m), 1.27 (4 H, br m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 194.8, 173.4, 169.1, 162.6, 161.7, 160.2, 158.1, 152.8, 152.3, 142.6, 136.1, 130.6, 129.7, 129.5, 129.4, 128.7, 126.6, 125.1, 125.0, 124,5, 115.6, 113.4, 112.9, 111.9, 109.8, 102.7, 102.2, 100.0, 99.8, 96.6, 83.4, 78.5, 62.1, 55.7, 52.1, 49.8, 42.3, 40.4, 29.9, 26.1, 25.9, 25.3. HRMS: *m*/*z* calcd for $C_{46}H_{43}N_4O_{10}$ ($M + H^+$): 811.2974; found: 811.2993.
(20) **General Procedure**: To a solution of naringenin (1 mmol) and paraformaldehyde or acetaldehyde (1.5 mmol) in EtOH (4 mL) was added the secondary amine (1.5 mmol) at r.t. The mixture was then heated to 40–65 °C, depending on the secondary amine (see Table 1), and the reaction was monitored by TLC. After the reactants were consumed, EtOAc (30 mL) and diluted HCl (30 mL, pH 3) were added to the mixture. Then the pH of the aqueous phase was adjusted to 7. The aqueous phase was extracted with EtOAc (3 × 15 mL). The combined extracts were dried over anhyd Na_2SO_4. The crude product was purified by silica gel chromatography.