Bioorganic & Medicinal Chemistry Letters 18 (2008) 6046–6049

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Pterocarpans and flavanones from *Sophora flavescens* displaying potent neuraminidase inhibition

Young Bae Ryu [a,c], Marcus J. Curtis-Long [b], Jin Hyo Kim [a], Seong Hun Jeong [a], Min Suk Yang [a], Keun Woo Lee [a], Woo Song Lee [c,*], Ki Hun Park [a,*]

[a] Division of Applied Life Science (BK21 Program), EB-NCRC, Institute of Agriculture & Life Science, Graduate School of Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] 12 New Road, Nafferton, East Yorkshire YO25 4JP, UK
[c] Bioindustry Research Center, KRIBB, Jeongeup 580-185, Republic of Korea

ARTICLE INFO

Article history:
Received 19 June 2008
Revised 6 September 2008
Accepted 8 October 2008
Available online 11 October 2008

Keywords:
Neuraminidase
Pterocarpan
Sophora flavescens

ABSTRACT

Pterocarpans (**1**–**3**) and flavanones (**4**–**10**) were isolated from *Sophora flavescens* and screened for their ability to inhibit neuraminidase (an enzyme crucial in the proliferation of the influenza virus). The majority of inhibitors were shown to have IC_{50} values of 20 μM or below. Interestingly, pterocarpan **1** emerged as the best inhibitor with an IC_{50} of 1.4 μM. We were thus able to prove that the pterocarpan skeleton is a new class of lead structure for neuraminidase inhibitors. Our studies reveal that the IC_{50} has a marked dependence upon structure in the case of the pterocarpans but much less so for the flavanones. Kinetic analysis disclosed that all inhibitors are noncompetitive. Our molecular docking experiment resulted that the most potent pterocarpan-derived inhibitor **1** may bind to another binding pocket adjacent to the active site.

The influenza virus has historically been and remains today the most likely source of a pandemic affecting humans.[1] The development of vaccines to combat influenza are of restricted usefulness as they are not compatible with the high mutability of the virus.[2] The obvious alternative remains the development of effective drugs targeting highly conserved domains which are essential to ensure viral proliferation. The two targets which have so far yielded fruitful results are M2 and neuraminidase.[3–5] The former is an ion channel important in early stage infection, while the latter, a surface glycoside hydrolase, is essential for maintenance of virus mobility by catalyzing the hydrolysis of the sialic acid-derived linkage between viruses and host cells to release virons from infected cells. Of these two drug classes, neuraminidase inhibitors have been shown to be effective against influenza A and B, but M2 inhibitors are only functional against influenza A.[6] This arguably makes neuraminidase inhibition the more fruitful avenue for further research.

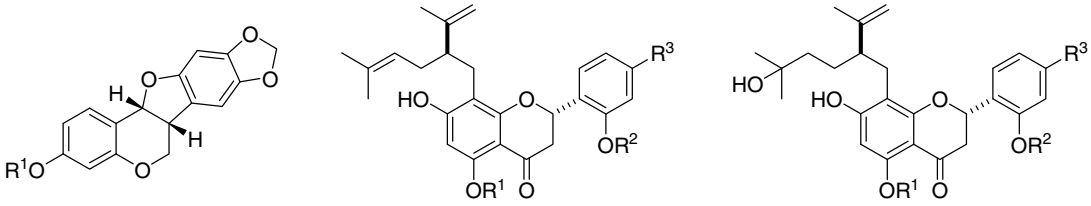
There are currently two commercially available neuraminidase inhibitors, zanamivir and oseltamivir (Tamiflu®)[7] both of which are highly polar cyclic species designed to mimic the native substrate of neuraminidase, sialic acid. Oseltamivir has been used to treat an estimated 50 million people worldwide.[8] Sadly, the viability and cost of these compounds are both affected by prolonged chemical syntheses and variable demand. With the perennial threat of flu pandemics a constant worry to the individual and government alike, and also the incorrect use of drugs potentially leading to accelerated mutation rates, natural product-derived influenza antivirals are perhaps more than ever of paramount importance.

Flavonoids of various natural product origins have been shown in a number of communications to exhibit antiviral properties. For instance, catechin derivatives were reported to be strong replication inhibitors of both HIV,[9] and the influenza virus.[10] Isoscutellarin-8-*O*-glucuronide from the leaf of *Scutellaria baicalensis* has also been shown to be a potent mouse liver sialidase inhibitor (IC_{50} 40 μM).[11] Based upon this and subsequent cell-culture results some papers have reported that flavonoids have a great potential to function as neuraminidase inhibitors.[11,12] However, to the best of our knowledge, other than flavonoids, there is currently a paucity of natural molecular scaffolds known to elicit neuraminidase inhibition, and it is with this strongly in mind that we set about this work.

We recently reported[13] that polyphenols from *Sophora flavescens*, a representative polyphenol-rich plant from the legume family known to possess numerous beneficial properties, have glycosidase inhibitory activity. Since glycosidase possesses similar active residues (Asp, Glu)[14] to neuraminidase (Asp, Tyr)[15] we believed that neuraminidase inhibitors may be accessible from this plant. We found that the MeOH (95%) extract from *S. flavescens* at 30 μg/ml inhibited neuraminidase activity by 90%. Subsequent bioactivity-guided fractionation of the MeOH extract led to the isolation of ten polyphenols [seven flavanones (**4**–**10**) and three pterocarpans (**1**–**3**)] as shown in Figure 1.

* Corresponding authors. Tel.: +82 55 751 5472; fax: +82 55 757 0178 (K.H. Park), tel.: +82 63 570 5170 (W.S. Lee).
E-mail addresses: wslee@kribb.re.kr (W.S. Lee), khpark@gsnu.ac.kr (K.H. Park).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.10.033



1 : $R^1 = CH_3$
2 : $R^1 = H$
3 : $R^1 = \beta$-D-glucose

4 : $R^1, R^2 = H, R^3 = OH$
5 : $R^1 = CH_3, R^2 = H, R^3 = OH$
6 : $R^1 = H, R^2 = CH_3, R^3 = OH$
7 : $R^1, R^2, R^3 = H$

8 : $R^1, R^2 = H, R^3 = OH$
9 : $R^1 = CH_3, R^2 = H, R^3 = OH$
10 : $R^1, R^2, R^3 = H$

Figure 1. Chemical structures of isolated compounds from *S. flavescens*.

In this study, we wish to report that the pterocarpan backbone is a novel molecular basis for neuraminidase inhibition. In addition we also report the isolation, structural characterization, and neuraminidase inhibitory activities of flavanones (**4**–**10**). The structures of isolated polyphenols (**1**–**10**) were performed using spectroscopic analysis in conjunction with previously reported data.[13,16–18,24]

The isolated compounds **1**–**10** were identified as pterocarpin (**1**), maackiain (**2**), trifolrhizin (**3**), sophoraflavanone G (**4**), kurarinone (**5**), leachianone A (**6**), kushenol A (**7**), norkurarinol (**8**), kurarinol (**9**), and kushenol T (**10**). The identification of pterocarpans (**1**) was determined on the basis of X-ray crystallographic data (CCDC-690107).[19]

The isolated compounds were tested for their enzymatic inhibitory activities against neuraminidase (E.C. 3.2.1.18) from *Clostridium perfringens* (*C. welchii*). The enzyme assay was based on a literature procedure with some modifications[20] by following the hydrolysis of MU-Neu5Ac (4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid) via fluorescence. The initial velocity (υ_i) was recorded over a range of concentrations and the data were analyzed using a nonlinear regression program [Sigma Plot (SPCC Inc., Chicago, IL)].

All isolated compounds showed a dose-dependent inhibitory effect toward neuraminidase activity (Table 1). As shown in Table 1, both flavanone and pterocarpan derivatives exhibited a significant degree of neuraminidase inhibition. All the lavandulylated flavanones (**4**–**10**) were of almost equal efficiency with IC_{50} values (12–20 μM). However, pterocarpans (**1**–**3**) were significantly affected by subtle structural changes. For instance, the most potent inhibitor was **1** which possessed a methylated phenol moiety. The demethylated analogue **2** was half as potent, whilst the glycosylated species **3** showed almost no activity at all. This possibly hints that this class of inhibitors binds to a relatively hydrophobic area within the enzyme. However, steric interactions between the bulky sugar moiety and the enzyme adversely affecting the IC_{50} in the case of inhibitor **3** cannot be ruled out. Other hints at the importance of hydrophobicity can be found in the differences in IC_{50} values for lavandulyl flavanones (**4**, **5**, **6**, and **7**) and their hydroxylated counterparts (**8** and **9**). Most importantly, however, pterocarpan **1** showed between 10- and 20-fold more activity than flavonoid derived compounds (**4**–**10**) and was 12 times more active than positive control, the glucoside, mangiferin (IC_{50} = 16.2 μM). As shown representatively in Figure 2A as the concentration of com-

Table 1
Inhibitory effects of isolated compounds **1**–**10** on neuraminidase activities

Compound	IC_{50}[a] (μM)	Inhibition type (K_i, μM)
1	1.4 ± 0.36	Noncompetitive (1.7 ± 1.1)
2	3.2 ± 1.1	Noncompetitive (2.1 ± 0.9)
3	237 ± 25.1	Noncompetitive (190 ± 20)
4	13.5 ± 1.08	Noncompetitive (20.0 ± 3.7)
5	15.1 ± 2.0	Noncompetitive (18.8 ± 1.7)
6	20.1 ± 0.8	Noncompetitive (30.4 ± 3.0)
7	14.8 ± 1.5	Noncompetitive (17.1 ± 0.8)
8	18.3 ± 1.2	Noncompetitive (22.7 ± 2.1)
9	17.0 ± 1.6	Noncompetitive (19.7 ± 2.4)
10	12.1 ± 2.4	Noncompetitive (10.8 ± 0.8)
Mangiferin[b]	16.2 ± 2.2	ND

[a] All compounds were examined in a set of experiments repeated three times (ND, not detected).
[b] Mangiferin was used as a positive control.[21]

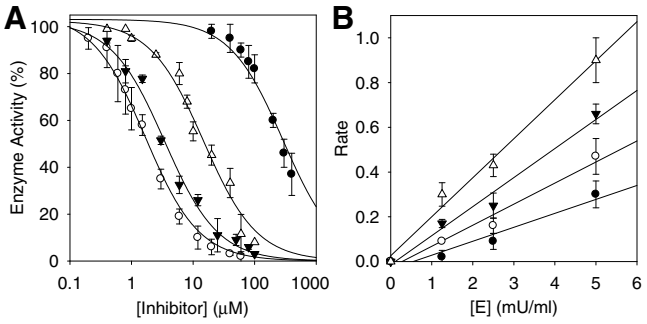


Figure 2. Effect of compounds on the activity of neuraminidase for the hydrolysis of MU-Neu5Ac at 37 °C. (A) Dose-dependent manner of compounds [**1** (○), **2** (▼), **3** (●), and **4** (Δ), respectively]. (B) Relationship of the hydrolytic activity of neuraminidase with enzyme concentrations at different concentrations of compound **1**. Concentration of compound **1** for curve from top to bottom: 0, 0.6, 1, and 10 μM.

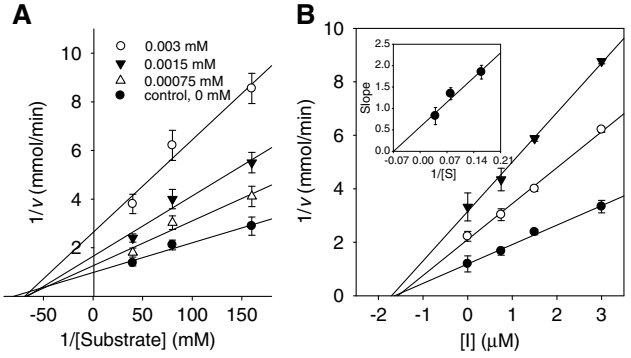


Figure 3. Graphical determination of the type of inhibition for compound **1**. (A) Lineweaver–Burk plot for inhibition of compound **1** on neuraminidase for the hydrolysis of substrate. In the presence of different concentrations of compounds were 0 (●), 0.00075 (Δ), 0.0015 (▼), and 0.003 mM (○), respectively. (B) Dixon plot; inset is replot of *slope* versus the corresponding 1/[S] of compound **1**.

pounds **1**–**4** was increased, the enzyme activity rapidly decreased. Increasing the concentration of inhibitor **1** resulted in lowering of the slope of the line, indicating that these compounds were reversible inhibitors (Fig. 2B).

We subsequently progressed to analyze the mode of inhibition using both Lineweaver–Burk (Fig. 3A) and Dixon plots (Fig. 3B). Both of these experiments revealed that all isolated compounds exhibited noncompetitive inhibition (for instance all lines in the Lineweaver–Burk plot met at a nonzero point on the *x*-axis). A replot of slope versus the corresponding 1/[S] (inset Fig. 3B) is a straight line with a gradient of $K_m/V_{max}K_i$ and an intercept of $1/V_{max}K_i$ on the *y*-axis, proving the concordance of both graphs and affirming that all compounds are noncompetitive inhibitors.

In view of these results, a tentative in silico molecular docking simulation was undertaken to investigate potential protein–ligand interactions responsible for the enhanced selectivity and potency of **1**. The X-ray structure of neuraminidase determined by Smith[22] was obtained from the protein data bank (PDB Code 1l7f). Molecular docking studies were carried out using LigandFit[23] (Discovery Studio 2.0, Accelrys, San Diego, USA), with prior removal of binding water molecules and cocrystallized inhibitor. For the initial exploration, various sites were searched and analyzed by docking of compound **1** to the enzyme.

Figure 4A shows compound **1** docked with neuraminidase. Our modeling calculations unveiled two potential binding sites at which the enzyme could most favorably dock with neuraminidase (sites I and II). In both instances the methoxy group on the A ring was found to interact wit arginine residues. H-bond interactions were observed in the both sites: in site II to Arg371 and Arg118 and in site I to Arg292 (Fig. 4B and C). Although the site II seems to be more favorable in H-bond interaction than the site I, Interestingly the overall docking scores showed in different way. The site I was found to be the best scored binding site (LigScore2 = 4.56) and the site II, which is the enzyme's active site, was turned out to be the next favorable binding site (LigScore2 = 3.75). And the mesh diagram also shows better alignment with the pocket of site I (Fig. 4D and inset). Theses results mean that the binding pocket of the site I matches compound **1** more closely than the site II or the compound **1** interact more favorably with the site I than the site II. If it is true that the new inhibitor binds to another active site like an allosteric site rather than the original active site, this result can be well consistent with our kinetic analysis data which showed **1** to be a noncompetitive inhibitor.

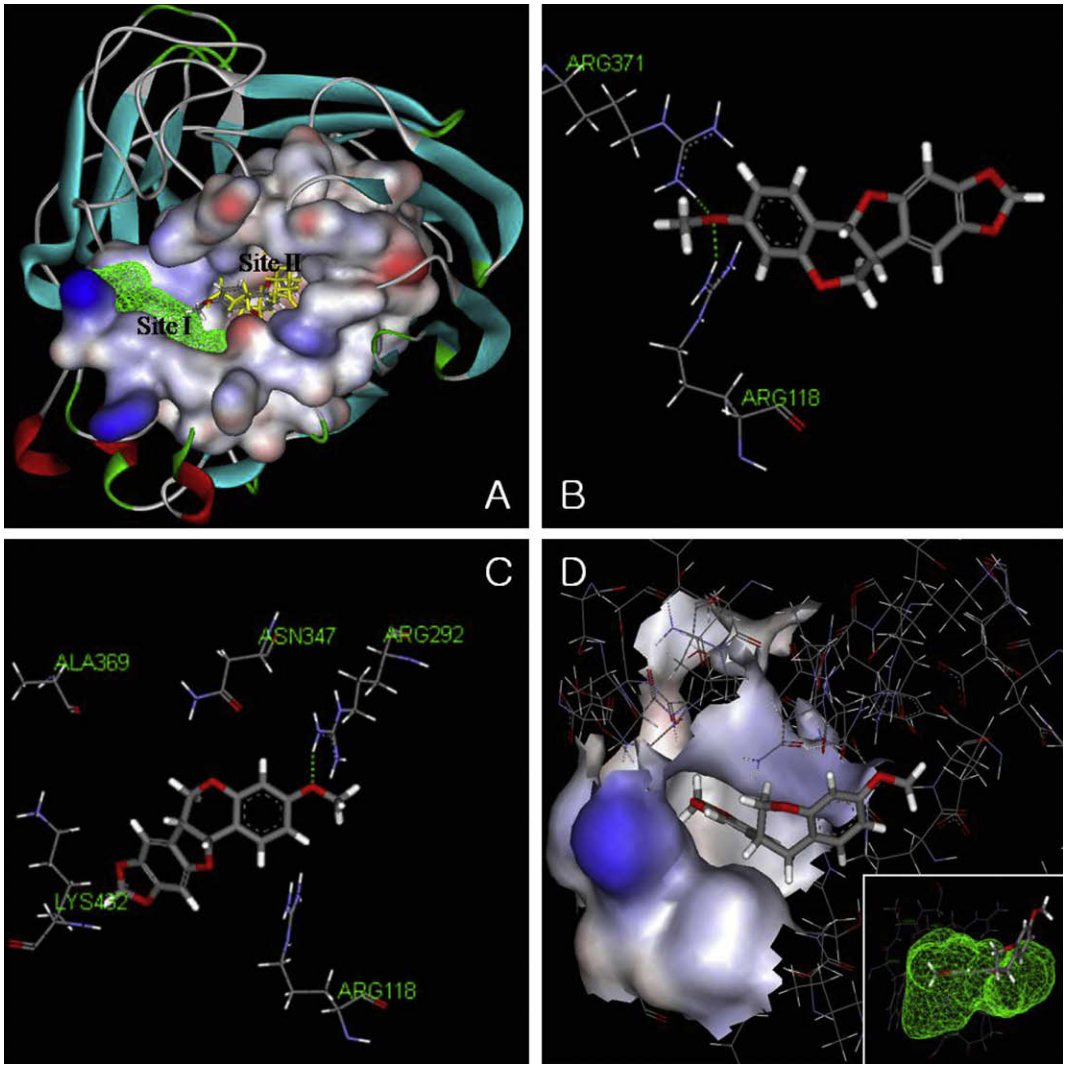


Figure 4. Comparison of *in silico* molecular docking modes for compound **1**. Representative binding mode of the most stable docking sites. (A) Two low energy binding sites found using computer simulations (yellow color: cocrystallized inhibitor[22] in original X-ray structure). Hydrogen bond interactions between compound **1** and neuraminidase in site II (B) and site I (C). (D) Compound **1** docked in site I [inset: **1** (shown as mesh surface) in site I].

Although this preliminary molecular modeling study cannot be final proof for this critical argument, it can be a positive sign for the possibility of the mechanism.

In summary, this work has shown that pterocarpans can serve as a new class of lead structure for neuraminidase inhibitors. Although all isolated compounds apart from **3** showed a significant inhibitory activities (12–20 μM), pterocarpan **1** exhibited the most potent neuraminidase inhibitory activity with IC_{50} values of 1.4 μM. The molecular docking experiment suggests that pterocarpan may bind to another binding pocket near the active site and we think that this can be a good clue supporting for the result of the kinetic analysis of **1**. We believe that this lead structure may be highly beneficial for the development of new drugs to combat this serious disease.

Acknowledgments

This research was supported by a grant from KRIBB Research Instiative Program and the MOST/KOSEF to the Environmental Biotechnology National Core Research Center (R15-2003-012-02001-0). Y.B. Ryu was supported by a scholarship from the BK21 Program.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.10.033.

References and notes

1. Webster, R. G.; Walker, E. J. *Am. Sci.* **2003**, *91*, 122.
2. Ghedin, E.; Sengamalay, N. A.; Shumway, M.; Zaborsky, J.; Feldbylum, T.; Subbu, V.; Spiro, D. J.; Sitz, J.; Koo, H.; Bolotov, P.; Dernovoy, D.; Tatusova, T.; Bao, Y.; St. George, K.; Taloy, J.; Lipman, D. J.; Fraser, C. M.; Taubenberger, J. K.; Salzberg, S. L. *Nature* **2005**, *437*, 1162.
3. De Clercq, E.; Neyts, J. *Trends Pharm. Sci.* **2007**, *28*, 280.
4. Hay, A. J.; Wolstenholme, A. J.; Skehel, J. J. *EMBO J.* **1985**, *4*, 3021.
5. Colman, P. M. In *The Influenza Viruses: Influenza Virus Neuraminidase, Enzyme and Antigen*; Kruh, R. M., Ed.; Plenum: New York, 1999; pp 175–218.
6. Heins, J. R.; Planp, J. *S. D. J. Med.* **2004**, *74*, 3.
7. Moscona, A. *N. Engl. J. Med.* **2005**, *335*, 1363.
8. 'Roche update on Tamiflu for pandemic influenza preparedness', Roche Media News, 2007-04-26.
9. (a) Fassina, G.; Buffa, A.; Benelli, R.; Varnier, O. E.; Noonan, S. K.; Li, C.; Liu, S. T. *Biochem. Biophys. Res. Commun.* **2002**, *16*, 939; (b) Nakane, H.; Ono, K. *Biochemistry* **1990**, *29*, 2841.
10. (a) Song, J.-M.; Lee, K.-H.; Seong, B.-L. *Antiviral Res.* **2005**, *68*, 66; (b) Song, J. M.; Park, K. D.; Lee, K. H.; Byun, Y. H.; Park, J. H.; Kim, S. H.; Kim, J. H.; Seong, B. L. *Antiviral Res.* **2007**, *76*, 178.
11. Nagai, T.; Miyaichi, Y.; Tomimori, T.; Yamada, H. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 25.
12. Nagai, T.; Miyaichi, Y.; Tomimori, T.; Yamada, H. *Chem. Pharm. Bull.* **1990**, *38*, 1329.
13. Kim, J. H.; Ryu, Y. B.; Kang, N. S.; Lee, B. W.; Heo, J. S.; Jeong, I.-Y.; Park, K. H. *Biol. Pharm. Bull.* **2006**, *29*, 302.
14. Rye, C. S.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573.
15. Luo, M.; Air, G. M.; Brouillette, W. J. *J. Infect. Dis.* **1997**, *176*, 62.
16. (a) Kang, T. H.; Jeong, S. J.; Ko, W. G.; Kim, N. Y.; Lee, S. H.; Inagaki, M.; Miyamoto, T.; Higuchi, R.; Kim, Y. C. *J. Nat. Prod.* **2000**, *63*, 680; (b) Kuroyanagi, M.; Arakawa, T.; Hirayama, Y.; Hayashi, T. *J. Nat. Prod.* **1999**, *62*, 1595; (c) Sato, S.; Takeo, J.; Aoyama, C.; Kawahara, H. *Bioorg. Med. Chem. Lett.* **2007**, *15*, 3445; (d) Aratanechemuge, Y.; Hibasami, H.; Katsuzaki, H.; Imai, K.; Komiya, T. *Oncol. Rep.* **2004**, *12*, 1183.
17. (a) Hwang, E. M.; Ryu, Y. B.; Kim, H. Y.; Kim, D.-G.; Hong, S.-G.; Lee, J. H.; Curtis-Long, M. J.; Jeong, S. H.; Park, J.-Y.; Park, K. H. *Bioorg. Med. Chem.* **2008**, *16*, 6669; (b) Ryu, Y. B.; Westwood, I. M.; Kang, N. S.; Kim, H. Y.; Kim, J. H.; Moon, Y. H.; Park, K. H. *Phytomedicine* **2008**, *15*, 612.
18. *Spectroscopic data of pterocarpin (**1**):* Mp 112–115 °C; [α] −56.7 (*c* 1.0, CH_3OH); ^1H NMR (500 MHz, $CDCl_3$) δ 4.15 (1H, dd, *J* = 11.1, 5.0 Hz, H-2a), 3.58 (1H, dd, *J* = 11.1, 5.0 Hz, H-2b), 3.4 (1H, m, H-3), 5.40 (1H, d, *J* = 6.9 Hz, H-4), 7.31 (1H, d, *J* = 8.5 Hz, H-5), 6.55 (1H, dd, *J* = 8.5, 2.5 Hz, H-6), 6.4 (1H, s, H-8), 6.64 (1H, s, H-2′), 6.39 (1H, d, *J* = 2.5 Hz, H-5′), 5.73 (2H, dd, *J* = 13.8, 1.4 Hz, H-1″), 3.71 (OCH_3).
19. *X-ray crystal structure analysis of **1**:* $C_{34}H_{28}O_{10}$, *M* = 596.56, monoclinic, space group, $P2_1$, *a* = 7.0459(11) Å, *b* = 8.0714(12) Å, *c* = 12.0720(19) Å, α = 90, β = 97.952(3)°, γ = 90, *V* = 679.94(18) Å^3, *Z* = 1, *T* = 173(2) K, *D* = 1.457 Mg/m^3, μ = 0.108 mm^{-1}, *F*(000) = 312, R_1 = 0.0404, R_w = 0.1077 for 2699 [*R*(int) = 0.0547] independent reflections.
20. (a) Potier, M.; Mameli, L.; Belisle, M.; Dallaire, L.; Melancon, S. B. *Anal. Biochem.* **1979**, *94*, 287; (b) Engstler, M.; Talhouk, J. W.; Smith, R. E.; Schauer, R. *Anal. Biochem.* **1997**, *250*, 176.
21. Li, X.; Ohtsuki, T.; Shindo, S.; Sato, M.; Koyano, T.; Preeprame, S.; Kowithayakorn, T.; Ishibashi, M. *Planta Med.* **2007**, *73*, 1995.
22. Smith, B. J.; McKimm-Breshkin, J. L.; McDonald, M.; Fernley, R. T.; Varrghese, J. N.; Colman, P. M. *J. Med. Chem.* **2002**, *45*, 2207.
23. Venkatachalam, C. M.; Jiang, X.; Oldfield, T.; Waldman, M. *J. Mol. Graph. Model.* **2003**, *21*, 289.
24. *Procedure for extraction and isolation:* The dried root of *S. flavescens* (2 kg) was air-dried, pulverized, and extracted with methanol for a week at room temperature. The combined extract was concentrated in vacuo to afford a dark orange gum (53 g). After concentration, the resultant extract was separated by VLC chromatography, performed using $CHCl_3$ (23 g) and MeOH (28 g). The $CHCl_3$ extract was subjected to column chromatographed on silica gel (4 × 60 cm, 230–400 mesh, 400 g) using hexane/EtOAc (80:1 → 1:1) mixtures to furnish fractions A–G. Fraction C (2.9 g) was subjected to silica gel chromatography (2 × 50 cm) eluting with hexane/EtOAc (40:1 → 1:2) to give five fractions (fr. C.1–C.5); fr. C.2 was resubjected to silica gel chromatography with hexane/acetone (30:1 → 1:1) to yield compound **4** (19 mg) and **7** (28 mg). Fr. D (2.7 g) was applied to a silica gel column [eluent: hexane/EtOAc (20:1 → 1:1)] to afford three subfractions fr. D.1–D.3. Fr. D.2 was resubjected to silica gel column {(1.5 × 30 cm, 230–400 mesh, 90 g) [eluent: hexane/Et_2O (40:1 → 1:2)]} to yields compound **6** (11 mg) and **2** (18 mg). Fr. E (3.3 g) was subjected to silica gel column chromatography with hexane/EtOAc (10:1 → 1:1) to yield compound **5** (32 mg) and this column was evaporated and recrystallized from hexane to give compound **1** (19 mg). MeOH phase (28 g) was chromatographed on silica gel {(6 × 60 cm, 230–400 mesh, 800 g, eluent: $CHCl_3$/acetone (40:1 → 1:1) to give fraction A–I. Fr. G (3.1 g) was applied to a silica gel column [eluent: $CHCl_3$/MeOH (90:1 → 4:1)] to afford seven subfractions fr. G.1–G.7. Fr. G.3–G.5 were subjected to silica gel column [eluent: $CHCl_3$/MeOH (60:1 → 2:1)] to yields compound **9** (12 mg). Fr. H (2.2 g) was chromatographed on silica gel [eluent: $CHCl_3$/MeOH (20:1 → 1:1)] to yield compound **10** (34 mg) and **3** (55 mg). Fr. I was repeatedly chromatographed over silica gel using [eluent: $CHCl_3$/MeOH (20:1 → 1:1)] and then on Sephadex LH-20 [eluent: MeOH (2 × 90 cm)] to yield compound **8** (26 mg). All of flavonoids (**4**–**10**) were described as previously reported.[13,17]