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Anti-influenza diarylheptanoids from the bark of *Alnus japonica*

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ABSTRACT

This study to investigate anti-influenza components from the bark of *Alnus japonica* resulted in the isolation of two rare acylated diarylheptanoids, named oregonoyl A (**5**) and oregonoyl B (**6**), along with nine known compounds (**1–4** and **7–11**). Their structures were elucidated on the basis of extensive spectroscopic and chemical methods. Antiviral testing of compounds **1–11** against KBNP-0028 (H9N2) avian influenza virus showed that platyphyllone (**10**) was strongly active, and platyphyllonol-5-xylopyranoside (**9**) was moderately active against KBNP-0028 as compared with the positive control, zanamivir, respectively.

Influenza occurs with seasonal variations and reaches peak prevalence in winter, with many people killed worldwide every year.[1,2] To date, only a few organic compounds including amantidine, rimantadine, zanamivir, tamiflu, and ribavirin have been used for influenza therapy.[3] However, drug-resistant influenza viruses are generated quickly.[4] Thereby, it is attractive and urgent to seek new influenza drugs.

Alnus japonica Steud. (Betulaceae) has been used in folk oriental medicine as remedies for fever, hemorrhage, diarrhea, and alcoholism.[5] Previous phytochemical investigation of *A. japonica* has led to the identification of numerous diarylheptanoids, along with several triterpenoids and flavonoids.[6–10] Diarylheptanoids belong to a phenolic class of natural products based on 1,7-diphenylheptane frame, and many diarylheptanoids have been isolated from Zingiberaceae and Betulaceae plants up to date.[11–15] Some of which such as curcumin and oregonin showed various biological properties including anti-inflammatory, anticancer, and antioxidative activities.[6,7,16,17] Our recent study on antioxidative components from *A. japonica* resulted in the isolation of 12 diarylheptanoids.[18] There has more interest in this plant since an ethanol extract of the bark of *A. japonica* exhibited, notably, potent anti-influenza activity against KBNP-0028 (H9N2) in our search for naturally-occurring antiviral agents. The bark of *A. japonica* was collected in Yanzi Province, China in September 2006, and was taxonomically identified by one of us (Y.H. Kim). Voucher specimens (CNU 08102) have been deposited at the College of Pharmacy, Chungnam National University, Korea. Subsequently, the air-dried sample (1.0 kg) was extracted with hot 95% EtOH (3 × 3.0 L). Then, the combined extracts were concentrated in vacuo to give a residue (308 g), which was suspended in 2.0 L of water and successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH (each 2.0 L × 3) to obtain soluble fractions of CH_2Cl_2 (39 g), EtOAc (83 g), and *n*-BuOH (15 g). The EtOAc-soluble fraction, the most active fraction against KBNP-0028 with an EC_{50} value of 31.3 μg/mL, was fractionated over a silica gel column using a gradient of $CHCl_3$–MeOH (15:1–0:1, v/v), followed by silica gel and YMC reversed-phase column chromatography (Supplementary data) to yield two new diarylheptanoids, named oregonoyl A (**5**) and oregonoyl B (**6**), along with nine known ones including platyphyllenone (**1**),[9] hirsutanone (**2**),[19] hirsutanonol (**3**),[19] oregonin (**4**),[19] alnuside A (**7**),[7] alnuside B (**8**),[7] platyphyllonol-5-xylopyranoside (**9**),[20] platyphyllone (**10**),[20] and platyphylloside (**11**)[20] (Fig. 1).

Oregonoyl A (**5**),[21] a yellow syrup, has the molecular formula of $C_{33}H_{36}O_{12}$ established from its HRFABMS (found at *m*/*z* 647.2098 $[M+Na]^+$, calcd for $C_{33}H_{36}O_{12}Na$ 647.2104). The NMR spectra of **5** (Table 1) indicated the presence of two 3,4-dihydrophenyl groups, the same heptane chain as that of oregonin (**4**), and a xylopyranosyl unit to which an acyl group attached. The acyl moiety was indicated as *p*-coumaroyl by the ^1H and ^{13}C NMR spectra.[22,23] The ^1H NMR spectrum of **5** showed four doublets at δ 6.25 (1H, d, J = 16.0 Hz), 6.73 (2H, d, J = 8.0 Hz), 7.34 (2H, d, J = 8.0 Hz), and 7.62 (1H, d, J = 16.0 Hz) attributable to the symmetric pattern of a 1,4-disubstituted aromatic ring and an α,β-unsaturated ketone

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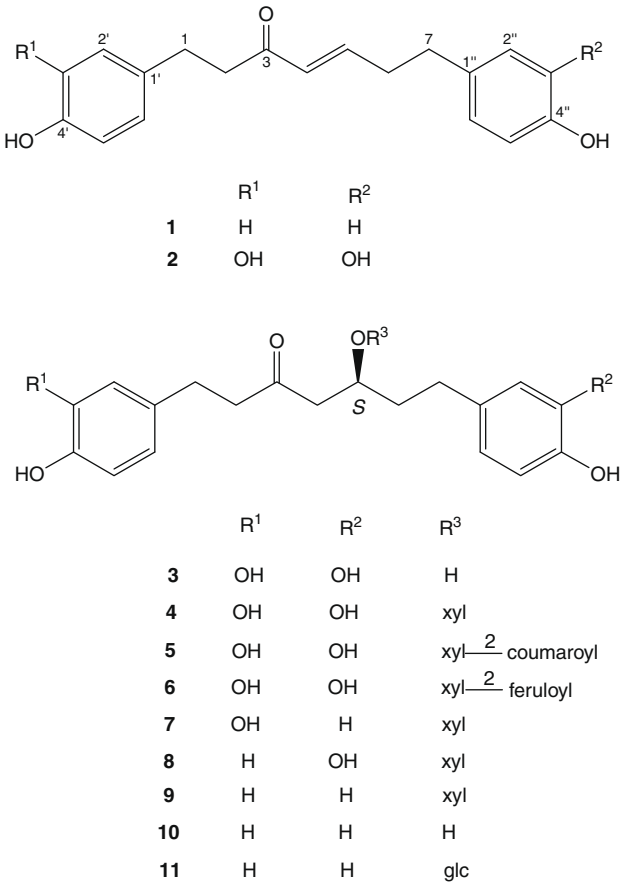

Figure 1. Structures of the diarylheptanoids **1**–**11** (glc: β-D-glucopyranosyl and xyl: β-D-xylopyranosyl).

group with *E*-form of *p*-coumaroyl. The ^{13}C NMR spectrum of **5** showed seven carbon signals, in which four of them (δ 116.9, 127.0, 131.3, and 161.4) resembling for a 1,4-disubstituted phenyl ring and three remaining carbon signals (δ 115.2, 146.9, and 168.4) corresponding for an α,β-unsaturated ketone group. Acylation of the xylopyranosyl moiety was located at C-2‴ due to the downfield-shifted resonance of H-2‴ at δ 4.70 (1H, t, J = 8.0 Hz) and the upfield-shifted resonance of C-3‴ at δ 76.2 as compared with those of oregonin (**4**).[7,23] Comprehensive analyses of the HMQC, HMBC, and H–H COSY spectra of **5** permitted complete assignments of its proton and carbon signals and of the substitution sites. The H–H COSY and selected HMBC correlations of **5** were illustrated in Figure 2. Accordingly, the HMBC correlations of H-5/C-1‴, H-1‴/C-5, and H-2‴/C-9⁗ assured the interlinkage positions at C-5 between the heptane chain and the xylopyranosyl moieties and at C-2‴ between the xylopyranosyl and the coumaroyl moieties, respectively.

Assignment of the (*S*)-configuration to the C-5 position of **5** was accomplished as follows. Acidic hydrolysis of **5** gave aglycone **5a**, which was identical ($[\alpha]_D^{20}$, NMR, MS) to hirsutanonol (**3**), and D-xylose as confirmed by GC (Supplementary data). On the other hand, application of the ^{13}C NMR glycosylation shift rule for secondary alcohols having two surrounding methylene groups could assign the absolute configuration of the C-5 positions in oregonin (**4**) and its derivatives.[14,24,25] Accordingly, it is found that the glycosylation shift at C-4 was −2.4 ppm, while the shift at C-6 was −2.0 ppm between the ^{13}C NMR data of **5** (Table 1) and that of its aglycone, hirsutanonol (Table S4, Supplementary data). This finding suggested the *S*-configuration for the C-5 position of **5** as in oregonin (**4**).[14] Based on the above evidence, oregonoyl A (**5**) was identified as 5*S*-1,7-bis(3,4-dihydroxyphenyl)-5-*O*-β-D-(2-*p*-coumaroyl xylopyranosyl)-heptane-3-one.

Table 1
NMR data for oregonoyls A (**5**) and B (**6**) in CD_3OD

Position	**5**		**6**	
	δ_C[a]	δ_H[b] (J in Hz)	δ_C[a]	δ_H[b] (J in Hz)
Aglycone				
1	30.1	2.50–2.55 m	30.1	2.50–2.55 m
2	46.3	2.44 m 2.56 m	46.3	2.44 m 2.56 m
3	211.0		210.9	
4	49.0[c]	2.40 m 2.60 m	49.0[c]	2.40 m 2.58 m
5	77.0	4.03 m	77.2	4.03 m
6	38.5	1.68 m 1.72 m	38.5	1.65 m 1.74 m
7	31.6	2.40–2.45 m	31.6	2.40–2.45 m
1′	133.8		133.8	
2′	116.6	6.56 d (2.0)	116.6	6.55 d (2.0)
3′	146.1		146.1	
4′	144.4		144.4	
5′	116.4	6.60 d (8.0)	116.3	6.59 d (8.0)
6′	120.7	6.42 dd (8.0, 2.0)	120.5	6.42 dd (8.0, 2.0)
1″	135.1		135.1	
2″	116.5	6.55 d (2.0)	116.4	6.55 d (2.0)
3″	146.0		146.1	
4″	144.1		144.2	
5″	116.3	6.58 d (8.0)	116.3	6.59 d (8.0)
6″	120.5	6.41 dd (8.0, 2.0)	120.7	6.42 dd (8.0, 2.0)
Xylose				
1‴	103.1	4.43 d (8.0)	103.3	4.44 d (8.0)
2‴	75.3	4.70 t (8.0)	75.3	4.70 t (8.0)
3‴	76.2	3.48 m	76.3	3.47 m
4‴	71.4	3.53 m	71.4	3.53 m
5‴	66.9	3.19 m 3.88 dd (11.6, 5.6)	67.0	3.19 m 3.86 dd (10.8, 5.6)
Acyl moiety				
1⁗	127.0		127.6	
2⁗	131.3	7.34 d (8.0)	111.8	7.08 d (1.6)
3⁗	116.9	6.73 d (8.0)	149.4	
4⁗	161.4		150.8	
5⁗	116.9	6.73 d (8.0)	116.4	6.75 d (8.0)
6⁗	131.3	7.34 d (8.0)	124.2	6.99 dd (8.0, 1.6)
7⁗	146.9	7.62 d (16.0)	147.2	7.58 d (16.0)
8⁗	115.2	6.25 d (16.0)	115.5	6.30 d (16.0)
9⁗	168.4		168.3	
OCH_3			56.4	3.77 s

[a] Recorded at 100 MHz.
[b] Recorded at 400 MHz.
[c] Overlapped with solvent signals. Assignments were confirmed by HMQC, HMBC, and COSY spectra.

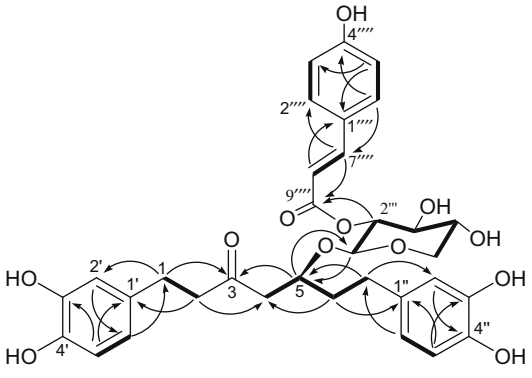

Figure 2. Selected H–H COSY (——) and HMBC correlations (H→C) of **5**.

Oregonoyl B (**6**),[21] also a yellow syrup, has the molecular formula of $C_{34}H_{38}O_{13}$ based on HRFABMS (found at m/z 677.2241

$[M+Na]^+$, calcd for $C_{34}H_{38}O_{13}Na$ 677.2210). The NMR spectra of **6** (Table 1) were almost similar to those of **5** by means of the presence of two 3,4-dihydroxyphenyl groups, a heptane chain, and a xylopyranosyl unit. Remaining ^1H and ^{13}C NMR signals for an acyl group were in consistent with the feruloyl structure.[22,23] The ^1H NMR showed proton signals at δ 3.77 (3H, s), 6.30 (1H, d, J = 16.0 Hz), 6.75 (1H, d, J = 8.0 Hz), 6.99 (1H, d, J = 8.0, 1.6 Hz), 7.08 (1H, d, J = 1.6 Hz), and 7.58 (1H, d, J = 16.0 Hz) attributable to a methoxy group, a 1,3,4-trisubstituted aromatic ring, and an α,β-unsaturated ketone group. In addition, the ^{13}C NMR spectrum of **6** (Table 1) showed a methoxy carbon signal at δ 56.4; six carbon signals at δ 111.8, 116.4, 124.2, 127.6, 149.4, and 150.8 characteristic for a 1,3,4-trisubstituted aromatic ring; and three carbon signals at δ 115.5, 147.2, and 168.3 revealing an α,β-unsaturated ketone group, respectively. Like oregonoyl A (**5**), acylation of the xylopyranosyl unit at C-2‴ was suggested due to the downfield-shifted resonance of H-2‴ at δ 4.70 (t, J = 8.0 Hz) and the upfield-shifted resonance of C-3‴ at δ 76.3 in comparison with those of oregonin (**4**).[7,23] Furthermore, ^1H, ^{13}C NMR assignments, and partial structures of **6** were completely assured by the HMQC, HMBC, and H–H COSY spectra (Fig. 3).

Assignment of the (*S*)-configuration to the C-5 position of **6** was also achieved by the same manner as **5**. Acidic hydrolysis of **6** gave aglycone **6a**, which was identical ($[\alpha]_D^{20}$, NMR, MS) to hirsutanonol (**3**), and D-xylose as confirmed by GC (Supplementary data). Similarly, application of the ^{13}C NMR glycosylation shift rule showed that the shift was larger for C-4 (−2.4 ppm) than for C-6 (−2.0 ppm) when the ^{13}C NMR spectrum of **6** (Table 1) was compared to that of its aglycone, hirsutanonol (**3**) (Table S4, Supplementary data). Therefore, the C-5 position is assigned the *S*-configuration as in oregonoyl A (**5**). Thus, oregonoyl B (**6**) was identified as 5*S*-1,7-bis(3,4-dihydroxyphenyl)-5-*O*-β-D-(2-feruloyl xylopyranosyl)-heptane-3-one.

Compounds **1–11** were screened for in vitro anti-influenza activity against KBNP-0028 (H9N2)[26] avian influenza virus, using the egg-bit assay[27–29] as described.[30] Zanamivir (LGM Pharma Corp., FL, USA), an approved antiviral drug, was referred as the positive control with an EC_{50} of 16.9 μM. Of the isolates, notably, platyphyllone (**10**) showed potent activity with an EC_{50} value of 29.9 μM; platyphyllonol-5-xylopyranoside (**9**) displayed moderate activity with an EC_{50} value of 56.1 μM, respectively; platyphylloside (**11**) exhibited weak activity with an EC_{50} value of 105.0 μM; whereas platyphyllenone (**1**) was negligibly active (EC_{50} = 282.8 μM). Besides, the others were considerably inactive due to their inhibition less than 50% at concentrations up to 100 mg/mL or 350 μM, equivalently (Table 2). Accordingly, the above results suggested that the presence of two 4-hydroxyphenyl moieties in the diarylheptanoids might be important with their anti KBNP-0028 activity. The cytotoxic concentration which inhibited the viability of chicken embryo fibroblast (CEF) cells by 50% (CC_{50}) was calculated from MTT assay results.[31] All of the tested diarylheptanoids were nontoxic at concentrations larger than 500 μM or 250 μg/mL, equivalently. The selective index (SI), the ratio of CC_{50} to EC_{50}, ranged from at least 3 to more than 26.6 (Table 2). Thereby, platyphyllone(**10**) was a promising anti-influenza agent on the basis of the SI value. This is the first report about antiviral components of *A. japonica*.

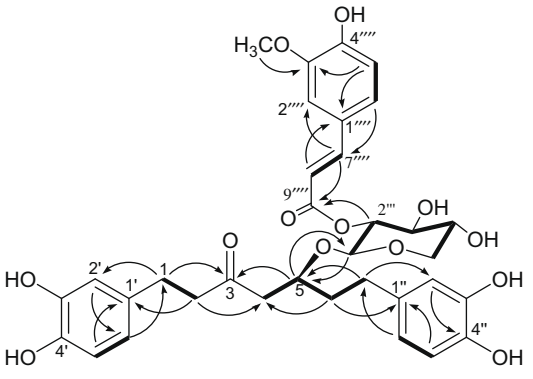

Figure 3. Selected H–H COSY (——) and HMBC correlations (H→C) of **6**.

Table 2
Antiviral activity of compounds **1–11** against the influenza virus KBNP-0028 (H9N2) on the basis of the egg-bit assay (data is the means ± SD of three different experiments)

Compound	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
1	282.8 ± 8.1	>845	>3
2	>350	>500	NC[d]
3	>350	>500	NC
4	>350	>500	NC
5	>350	>500	NC
6	>350	>500	NC
7	>350	>500	NC
8	>350	>500	NC
9	56.1 ± 3.8	>560	>10
10	29.9 ± 2.5	>796	>26.6
11	105.0 ± 3.8	>525	>5
Zanamivir[e]	16.9 ± 1.2	>753	>44

[a] EC_{50}: 50% effective concentration.
[b] CC_{50}: 50% cytotoxic concentration.
[c] SI: selective index.
[d] NC: not calculated.
[e] Positive control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.12.057.

References and notes

1. Yen, H. L.; Webster, R. G. *Curr. Top. Microbiol. Immunol.* **2009**, *333*, 3.
2. Scalera, N. M.; Mossad, S. B. *Postgrad. Med.* **2009**, *121*, 43.
3. Wiwanitkit, V. *Biologics* **2009**, *3*, 377.
4. Choi, W. Y.; Kim, S. J.; Lee, N. J.; Kwon, M. H.; Yang, I. S.; Kim, M. J.; Cheong, S. G.; Kwon, D. H.; Lee, J. Y.; Oh, H. B.; Kang, C. *Antiviral Res.* **2009**, *84*, 199.
5. Lee, S. J. *Korea folk Medicine*; Seoul National University Publishing Center: Seoul, Korea, 1996.
6. Kim, H. J.; Yeom, S. H.; Kim, M. K.; Shim, J. G.; Paek, I. N.; Lee, M. W. *Arch. Pharm. Res.* **2005**, *28*, 177.
7. Kuroyanagi, M.; Shimomae, M.; Nagashima, Y.; Muto, N.; Okuda, T.; Kawahara, N.; Nakane, T.; Sano, T. *Chem. Pharm. Bull.* **2005**, *53*, 1519.
8. Lee, M. W.; Tanaka, T.; Nonaka, G. I.; Nishioka, I. *Phytochemistry* **1992**, *31*, 2835.
9. Nomura, M.; Tokoroyama, T.; Kubota, T. *Phytochemistry* **1981**, *20*, 1097.
10. Wada, H.; Tachibana, H.; Fuchino, H.; Tanaka, N. *Chem. Pharm. Bull.* **1998**, *46*, 1054.
11. Kikuzaki, H.; Usuguchi, J.; Nakatani, N. *Chem. Pharm. Bull.* **1991**, *39*, 120.
12. Kuroyanagi, M.; Noro, T.; Fukushima, S.; Aiyama, R.; Ikuta, A.; Itokawa, H.; Morita, M. *Chem. Pharm. Bull.* **1983**, *31*, 1544.
13. Ma, J.; Jin, X.; Yang, L.; Liu, Z. L. *Phytochemistry* **2004**, *65*, 1137.
14. Gonzalez-Laredo, R. F.; Helm, R. F.; Chen, J.; Karchesy, J. J. *J. Nat. Prod.* **1998**, *61*, 1292.
15. Giang, P. M.; Son, P. T.; Matsunami, K.; Otsuka, H. *Chem. Pharm. Bull.* **2006**, *54*, 139.
16. Lee, M. W.; Kim, N. Y.; Park, M. S.; Ahn, K. H.; Toh, S. H.; Hahn, D. R.; Kim, Y. C.; Chung, H. T. *Planta Med.* **2000**, *66*, 551.
17. Lee, W. S.; Kim, J. R.; Im, K. R.; Cho, K. H.; Sok, D. E.; Jeong, T. S. *Planta Med.* **2005**, *71*, 295.

18. Tung, N. H.; Kim, S. K.; Ra, J. C.; Zhao, Y. H.; Sohn, D. H.; Kim, Y. H. *Planta Med.* **2009**, *75*. doi:10.1055/s-0029-1240595.
19. Lee, M. W.; Park, M. S.; Jeong, D. W.; Kim, K. H.; Kim, H. H.; Toh, S. H. *Arch. Pharm. Res.* **2000**, *23*, 50.
20. Smite, E.; Lundgren, L. N.; Andersson, R. *Phytochemistry* **1993**, *32*, 365.
21. *Oregonoyl A* (**5**): yellow syrup; $[\alpha]_D^{20}$ −9.6 (*c* 0.32, MeOH); UV λ_{max}^{MeOH} (nm) (log ε) 280 (2.94), 220 (3.22); IR (film) ν_{max} 3416. 1648, 1430, 1044 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; FABMS *m*/*z* 647.3 $[M+Na]^+$; HRFABMS *m*/*z* 647.2098 $[M+Na]^+$ (calcd for $C_{33}H_{36}O_{12}Na$, 647.2104). *Oregonoyl B* (**6**): yellow syrup; $[\alpha]_D^{20}$ −12.4 (*c* 0.34, MeOH); UV λ_{max}^{MeOH} (nm) (log ε) 280 (3.14), 218 (3.40); IR (film) ν_{max} 3412. 1654, 1426, 1040 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; FABMS *m*/*z* 677.2 $[M+Na]^+$; HRFABMS *m*/*z* 677.2241 $[M+Na]^+$ (calcd for $C_{34}H_{38}O_{13}Na$, 677.2210).
22. Chakravarty, A. K.; Sarkar, T.; Takahisa, N.; Nobuo, K.; Kazuo, M. *Chem. Pharm. Bull.* **2002**, *50*, 1616.
23. Cioffi, G.; Escobar, L. M.; Braca, A.; Tommasi, N. D. *J. Nat. Prod.* **2003**, *66*, 1061.
24. Seo, S.; Tomita, Y.; Tori, K.; Yoshimura, Y. *J. Am. Chem. Soc.* **1978**, *100*, 3331.
25. Ohta, S.; Aoki, T.; Suga, T. *J. Chem. Soc., Perkin Trans. 1* **1984**, *1*, 1635.
26. Kwon, H. J.; Cho, S. H.; Ahn, Y. J.; Kim, J. H.; Yoo, H. S.; Kim, S. J. *Open Vet. Sci. J.* **2009**, *3*, 9.
27. Fulton, F.; Armitage, P. *J. Hyg.* **1951**, *49*, 247.
28. Meyer, W. J.; Wood, J. M.; Major, D.; Robertson, J. S.; Webster, R. G.; Katz, J. M. *Virology* **1993**, *196*, 130.
29. Hardy, C. T.; Young, S. A.; Webster, R. G.; Naeve, C. W.; Owens, R. J. *Virology* **1995**, *211*, 302.
30. Anti-influenza testing: A H9N2 subtype avian influenza virus, A/chicken/Korea/KBNP-0028/2000(H9N2) (KBNP-0028) was propagated in the allantoic cavity of 10-day-old SPF embryonated chicken eggs (ECE; Hy-Vac, Adel, Iowa). To test the anti-influenza activity of the isolated compounds, an egg-bit assay was used as reported previously with slight modification. Briefly, egg-bits were prepared from 10- to 11-day-old SPF ECEs. Each egg-bit was placed into a well of a 24-well culture plate. The allantois was infected with 100 μL of KBNP-0028 solution corresponding to 100 times the 50% egg-bit infection dose ($EBID_{50}$) and allowed to incubate for 30 min. One milliliter of 199+F10 (1:1; Gibco-BRL, Grand Island, NY, USA) medium containing 0.075% sodium bicarbonate and gentamicin (100 μg/mL) was added to each well. *A. japonica* isolates were evaluated for antiviral activity at concentrations of 125, 100, 50, 25, 12.5, 6.3, and 3.1 μg/mL. Each sample dose was performed in duplicate and the egg-bits were incubated for five days. Plate hemagglutination tests were performed by mixing 25 μL of the culture fluid with the same volume of washed chicken RBC (0.1%). The isolate concentration required to reduce the degree of hemagglutination of KBNP-0028 by 50% relative to control wells without isolate (EC_{50}) was calculated by plotting the percent hemagglutination inhibition versus isolate concentration.
31. Mosmann, T. J. *Immunol. Methods* **1983**, *65*, 55.