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Anti-influenza virus activity of biflavonoids

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Abstract—Ginkgetin was found to inhibit the influenza virus sialidase. Ginkgetin-sialic acid conjugates showed a significant survival effect in the influenza-virus-infected mice.

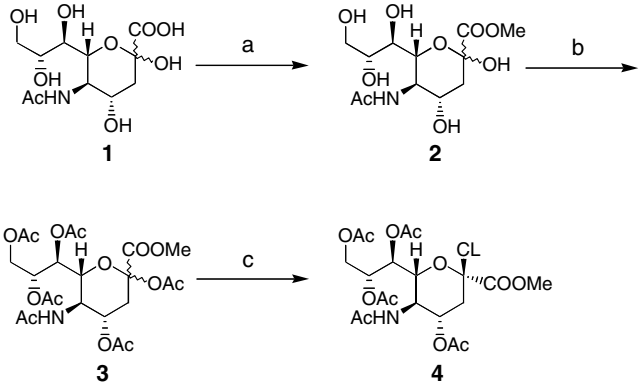
5,7,4′-Trihydroxy-8-methoxyflavone (**F36**) was surveyed as an influenza virus sialidase inhibitory compound among many flavonoids.[1] It also showed an anti-influenza virus activity in Madin–Darby canine kidney (MDCK) cells and in the allantoic sac of embryonated egg.[1–3] In the influenza-virus-infected mice, **F36** revealed a distinct survival effect.[3–5] Compound **F36** was characterized as an 8-methoxy-flavone derivative, which does not frequently occur in nature.[6] On the other hand, biflavonoids including amentoflavone-type compounds were reported to show the inhibitory activity against respiratory syncytial,[7] herpes[8], and measles viruses.[8]

Accordingly, some naturally occurring biflavonoids were screened for the inhibitory activities against influenza virus sialidase.

As the result, it was demonstrated that ginkgetin isolated from *Ginkgo biloba* L. and *Cephalotaxus harringtonia* K. Koch showed a potent inhibitory activity against influenza virus sialidase.

On the other hand, influenza A and B viruses are known to bind themselves on the host cells via sialic acid residue of glycoconjugate receptors at the first stage of infection. It may suggest that synthetic sialic acid-aglycone conjugates are important to inhibit the proliferation of viruses. Therefore, ginkgetin-sialic acid conjugates were synthesized herewith.

N-Acetylneuraminic acid **1** is widely distributed internally in animals playing crucial roles in various biological events such as infection, cell adhesion, immune response, and neural function. The glycosylation of the



Scheme 1. Reagents and conditions: (a) Dowex-50 W-X2, dry MeOH, rt, under Ar, 24 h (92%); (b) Ac_2O, DMAP, dry pyridine, rt, under Ar, 24 h (83%); (c) AcCl, HCl gas, 0 °C to rt, 24 h (98%).

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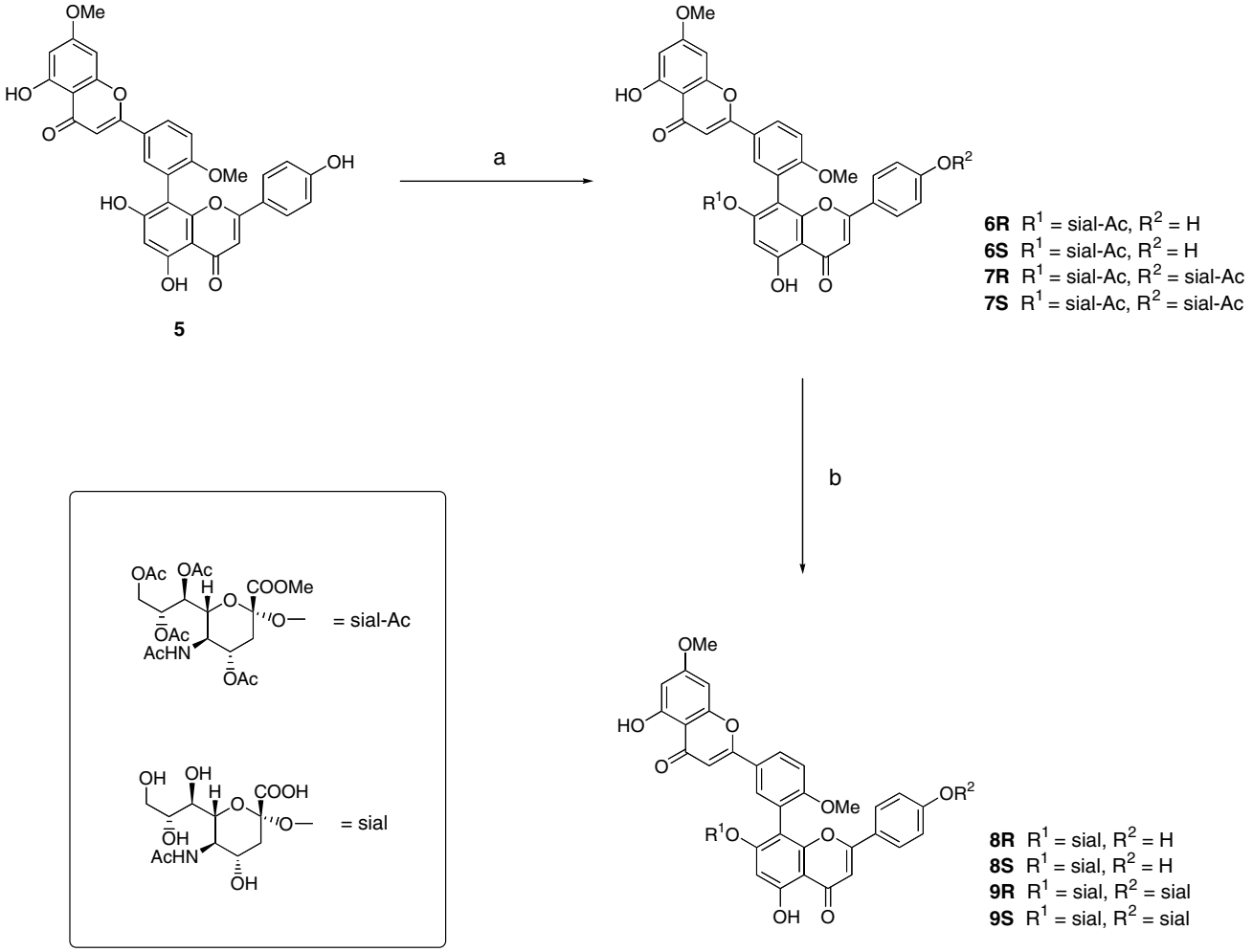
arylic hydroxyl group to sialic acid is performed by Williamson's method.[9,10] The synthetic route for ginkgetin-neuraminoside is outlined in Schemes 1 and 2. In the treatment of **1** with strong cation-exchange resin (Dowex-50W-X2), MeOH afforded **2**.[11] Followed by acetylation of **2** with Ac_2O, DMAP, and pyridine,[9,10] compound **4** was prepared from **3** by AcCl and HCl gas.[9] The glycosides of **6**–**7** were prepared from **4** and ginkgetin **5** with NaH, DMF.[9,10,12] When *N*-acetylneuraminic acid was combined with C-7″ position, it caused a stereochemical obstruction to yield atropic isomers (*R* and *S* formation). These atropisomeric compounds, **6R** and **6S**, **7R** and **7S**, were separated by HPLC, respectively. Compounds **8R**, **8S**, **9R**, and **9S** were prepared from **6R**, **6S**, **7R**, and **7S** by hydrolysis of the acetyl group with NaOMe, MeOH, and H_2O, respectively.

The influenza A and B virus sialidase inhibitory activities[13] of ginkgetin, ginkgetin-sialic acid conjugates **6R**–**9S**, are shown in Table 1, and **F36** was used as a positive standard. The IC_{50} values of ginkgetin against A/PR/8/34 (H1N1) and A/Guizhou/54/89 (H3N2) sialidases were 55.00 and 9.78 μg/mL, respectively. **6R**, **6S**, **7R**, and **7S**, which are ginkgetin acetylated neuraminosides, showed lower inhibitory activities against A/PR/8/34 (H1N1) and B/Ibaraki/2/85 sialidases comparing with **F36**. However, **6S** and **7R** showed the increase in the activity against A/Guizhou/54/89 (H3N2) (Table 1).

The atropic isomers of ginkgetin mononeuraminoside (**8R**, **8S**) and bisneuraminoside (**9R**, **9S**), respectively, inhibited both A/PR/8/34 (H1N1) and A/Guizhou/54/89 (H3N2) sialidases more potently than **F36**.

Especially, **8R** showed the most potent inhibitory activity whose IC_{50} value against A/PR/8/34 (H1N1) sialidase was 5.50 μg/mL, and that against A/Guizhou/54/89 (H3N2) sialidase was 0.82 μg/mL. On the other hand, **8R**, **8S**, **9R**, and **9S** showed lower activities than **F36** against B/Ibaraki/2/85 sialidase (Fig. 1).

Next, proliferation of influenza virus and the cytotoxicities were tested for compounds **6R**–**9S** by using MDCK cells infected with A/PR/8/34 (H1N1) (Fig. 2). The assay was carried out by the described method.[14] It means that the prevention of virus proliferation reflects the decrease of the sialidase activity, and the damage of MDCK cells treated by compounds causes to decrease formasan



Scheme 2. Reagents and conditions: (a) **4**, NaH, dry DMF, rt, under Ar, 24 h (5% for **6R**, 4% for **6S**, 12% for **7R**, 8% for **7S**); (b) NaOMe, dry MeOH, H_2O, rt, 24 h (**6R** → **8R**, 96%; **6S** → **8S**, 76%; **7R** → **9R**, 80%; **7S** → **9S**, 69%).

Table 1. Inhibition of influenza A and B virus sialidases by ginkgetin-sialic acid conjugates

Compound	Sialidase inhibitory activity [IC_{50} (μg/mL)]		
	A/PR/8/34 (H1N1)	A/Guizhou/54/89 (H3N2)	B/Ibaraki/2/85
F36	9.78	8.95	6.58
Ginkgetin	55.00	9.78	>100.00
6R	79.20	>10.00	>100.00
6S	>100.00	1.10	>100.00
7R	>100.00	1.35	>100.00
7S	>100.00	>10.00	>100.00
8R	5.50	0.82	9.11
8S	6.35	0.99	8.64
9R	6.61	6.57	9.05
9S	6.99	0.94	9.34

formation. Ginkgetin, which was found as a sialidase inhibitor in the former experiment, showed a cytotoxicity, whereas its neuraminoside derivatives have no cytotoxic effects to MDCK cells. Among these compounds **6R**–**9S**, O-acetylation of sialic acid moiety did not affect the inhibitory activity of virus proliferation. Mononeuraminoside, **8R** and **8S**, significantly showed the inhibitory activity of virus proliferation in comparison to bisneuraminosides, **9R** and **9S**, at least 3- and 8-fold, respectively. These results revealed that neuraminosylation of ginkgetin lowered the cytotoxicity and enhanced inhibitory activity against influenza virus sialidase of aglycone.

The efficacies of intranasally administered compounds, **8R**–**9S** and **F36**, were tested in the influenza virus A/PR/8/34 (H1N1)-infected mice on the basis of the survival days (Table 2).[15]

The control mice began to die from day 8 after the virus infection, and the survival rate was 22% at day 21. **F36** as a positive control gave the similar result in comparison to the control. On the other hand, compounds **8R**, **8S**, **9R**, and **9S** prolonged the survival days. Especially, compound **8R** exhibited the highest survival rates, 75% at day 10 and 62.5% at day 21 after the A/PR/8/34 (H1N1) virus infection ($p = 0.0385$, Logrank test, Kaplan–Meier method), while **8S** showed almost the same results, 78% at day 10 and 56% at day 21 ($p = 0.0714$),

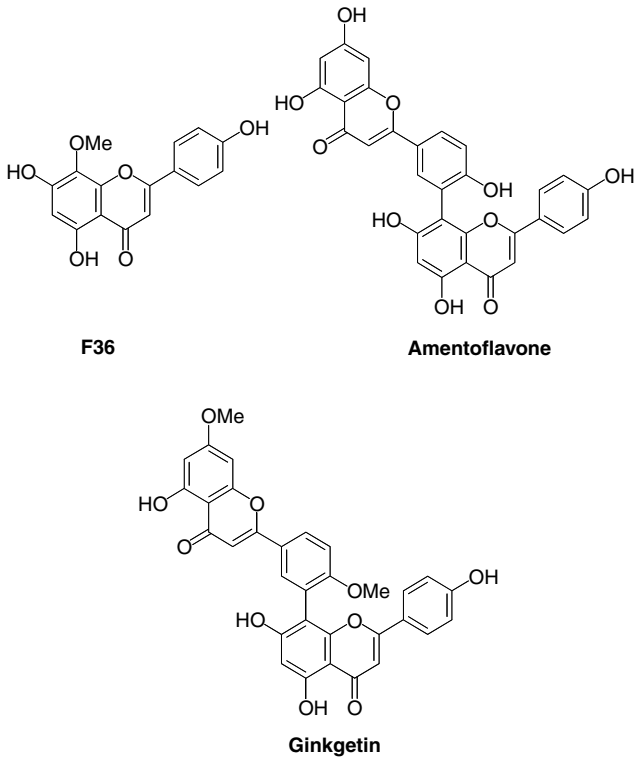


Figure 1.

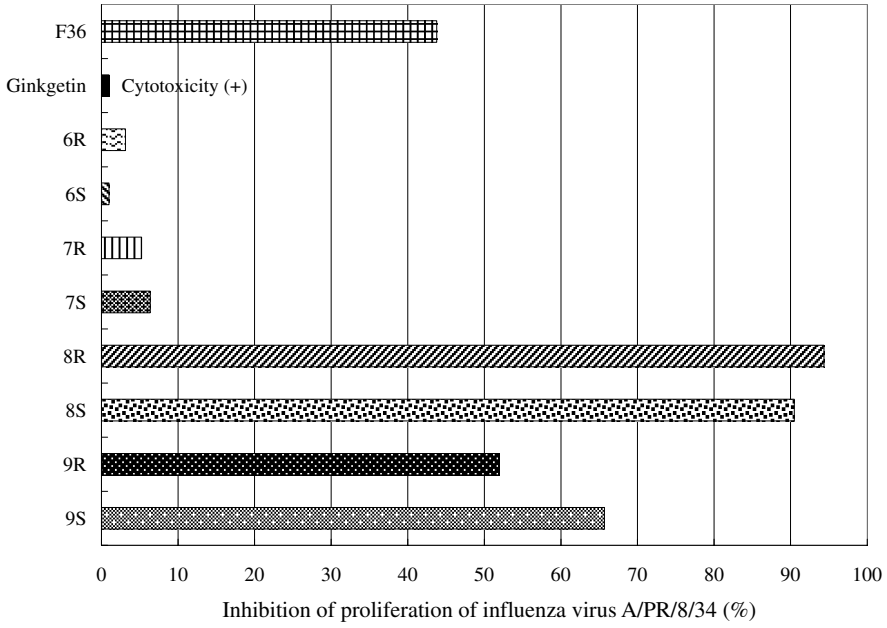


Figure 2. Anti-influenza virus activity of ginkgetin-sialic acid conjugates on MDCK cells (concentration of samples was 12.5 μg/mL).

Table 2. Effects of ginkgetin-sialic acid conjugates on survival rates of influenza virus-infected mice[a]

Compound[b]	No. of survivors/total no. of mice	
	10 days after infection	21 days after infection
Control (saline)	3/9	2/9
F36	5/9	2/9
8R	6/8	5/8
8S	7/9	5/9
9R	5/9	4/9
9S	8/9	4/9

[a] Mice were dosed with compound and then infected with influenza virus A/PR/8/34 (H1N1).

[b] A single dose of ginkgetin-sialic acid conjugates and **F36** were administered intranasally at 0.5 mg/kg.

compared with those of **8R**. Bisneuraminoside **9R** and **9S** exhibited slightly weak results in comparison to mononeuraminosides, **8R** and **8S**, respectively.

In summary, we presented new influenza virus sialidase inhibitors, which were prepared by the conjugation of biflavonoid ginkgetin from the plant source and sialic acid from the animal origin. We also revealed that the ginkgetin-sialic acid conjugates remarkably presented potent anti-influenza virus activities in vivo.

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13. Influenza virus sialidase inhibition activity (A/PR/8/34, H1N1; A/Guizhou/54/89, H3N2; B/Ibaraki/2/85) was determined in a fluorometric assay. The reaction mixture containing 0.1 mM 4-MU-NeuAc (50 μl), sample solution (10 μl), influenza vaccine (10 μl), and buffer was incubated at 37 °C for 10 min in 96-well microtiter plate.
14. In vitro anti-influenza virus activity was assayed by using MDCK cells. Influenza virus A/PR/8/34 was inoculated into confluent monolayers of MDCK cells in 96-well culture plate, and then sample solutions were put into the well. The plate was cultured at 37 °C for 3 days under a 5% CO_2 atmosphere. Inhibition of proliferation of influenza virus was determined in a fluorometric assay by measuring sialidase activity and the viable cells were determined by MTT assay.
15. In vivo anti-influenza virus activity was assayed in the influenza virus A/PR/8/34-infected mice. BALB/c mice (female, 6-week-old) were anesthetized by an intraperitoneal injection of amobarbital sodium. Test samples were dissolved in saline and 10 μl of the solution was administered to the mouse intranasally. Then 20 μl mouse-adapted influenza virus A/PR/8/34 suspension in 0.1% BSA in PBS was infected by intranasal inoculation. The survival rates were observed for 21 days.