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Neuraminidase Inhibitors from Mushroom *Microphorus affinis*

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Abstract In the course of screening anti-influenza agents from natural products, four neuraminidase inhibitors were isolated from the methanol extract of mushroom *Microphorus affinis* by purification using solvent partition, silica gel column chromatography, Sephadex LH-20, and semi-preparative HPLC. The chemical structures of these compounds were identified as α-lupeol, methyl linoleate, methyl palmitate, and methyl oleate by means of spectral data including GC-MS, ^{1}H-, and ^{13}C-NMR with IC_{50} values of 5.65, 7.07, 7.12, and 7.52 μM, respectively. They did not inhibit other glycosidases such as glucosidase, mannosidase, and galactosidase, indicating that they were relatively specific inhibitors of neuraminidase. The relationship between the fatty acid structure and inhibitory activity was investigated. The result showed that, in the case of an aliphatic linear hydrocarbon skeleton, at least one carboxyl (presumably any carbonyl) moiety and sixteen carbons were the necessary requirements for potent inhibition, whereas saturated, unsaturated, free, and ester forms did not have any significant effect on the activity.

Key words: Neuraminidase inhibitor, influenza, α-lupeol, methyl linoleate, methyl palmitate, methyl oleate, *Microphorus affinis*

Influenza continues to be a serious health concern with yearly epidemics causing significant morbidity and mortality, even in nations with the most advanced health care systems. Primary infection can lead to a number of complications and secondary infections, particularly in the elderly, those with pre-existing airways disease, and many other high-risk groups [5]. Attempts to control this disease through immunization have been hampered by the rapidity with which the virus mutates [6]. Thus, there has been a long-standing interest in the development of effective and safe antiviral agents with which to treat infected individuals.

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Influenza virus is enveloped, and two glycoproteins are displayed on the viral envelope, hemagglutinin and neuraminidase (sialidase, NA) [4]. The receptor for influenza viruses is a carbohydrate, and sialic acid (*N*-acetyl neuraminic acid, Neu5Ac) is the critical sugar residue which interacts weakly with the viral hemagglutinin and causes the virus to attach to target cells. After infection and replication, progeny virions bud at the plasma membrane of the infected cell [9, 19]. Neuraminidase (EC 3.2.1.18) is a surface glycoprotein that possesses enzymatic activity essential for viral replication in both influenza A and B viruses, and is responsible for catalyzing the cleavage of the α(2-6)- or α(2-3)-ketosidic linkage that exists between a terminal sialic acid and an adjacent sugar residue [7]. The breaking of this bond has several important effects: First, it allows for the release of the virus from the infected cells. Second, it prevents the formation of viral aggregates after being released from the host cells. Third, this enzyme, by cleaving the sialic acid found in respiratory tract mucins, may prevent viral inactivation and promote viral penetration into respiratory epithelial cells [3, 8, 11, 12, 14, 15]. Thus, effective neuraminidase inhibitors can be used for preventing and curing influenza infections.

Materials and Methods

General

Microphorus affinis was collected at Mt. Unmoon, Kyungbuk, Korea and the voucher specimen was deposited in the Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea. Fluorescence was measured with a Shimadzu RF-5301 (Japan) spectrofluorophotometer. The image analyzer was purchased from Bio-profil (France) and ^{1}H- and ^{13}C-NMR spectra were recorded on an Anvance Digital 400, Bruker. Chemical shifts were given in δ (ppm) from TMS. EIMS was measured on a Shimadzu QP-1000A (Japan) at 70 eV. GC-MS (GC-17A equipped with Nu Check standard GLC-87, Nu Check Prep., Inc.,

MN, U.S.A.) was a product of Shimadzu, Japan. GC and GC-MS were performed on a fused silica capillary column (HP-1, 0.25 mm×30 m), and the column was initially heated at 50°C for 2 min, and the temperature was then gradually increased at the rate of 10°C per min up to 300°C. Silica gel (Kieselgel 60, Art. 7734) and pre-coated TLC plates (Kieselgel 60 F254, Art. 5715 and Art. 1.15685) were from Merck. Authentic compounds were purchased from Sigma-Aldrich (U.S.A.).

Enzyme Assay

Neuraminidase. Neuraminidase (NA) activity was determined using the method of Myers *et al.* [13] with some modifications. Briefly, a mixture of 10 µl enzyme (2.5×10^{-3} U, from *Clostridium perfringens*, Sigma), 340 µl 0.04 M sodium acetate buffer (pH 5.0), 10 µl sample solution in MeOH, and 40 µl 0.125 mM substrate [2'-(4-methyl-umbeliferyl)-α-D-*N*-acetylneuraminic acid, Sigma] was incubated for 10 min at 37°C. After the reaction was stopped by adding 3.5 ml of 0.1 M glycine-NaOH buffer (pH 10.4), the fluorescence of reactant (A) was measured at Ex. 360 nm/Em. 440 nm. The control (C) was made by adding MeOH instead of the sample solution. The fluorescence of the sample (B) was measured to correct the fluorescence of the sample itself. The percent inhibition was calculated by the following equation: Inhibition (%)=[{C-(A-B)}/C]×100.

Other Glycosidase. Glucosidase (from an almond, Sigma), galactosidase (from bovine liver, Sigma), and mannosidase (from a snail, Sigma) activities were measured according to the method described in the Sigma catalog (2002-2003, Sigma-Aldrich), using *o*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-β-D-mannopyranoside as substrates, respectively.

Extraction and Isolation

Dried *M. affinis* (530 g) was refluxed in 2 l MeOH, and the extract was evaporated to dryness. The MeOH extract (23 g) was suspended in 500 ml of water, and the suspension was partitioned with hexane, $CHCl_3$, and EtOAc, consecutively. The $CHCl_3$ soluble fraction (4.26 g) was chromatographed on a silica gel column (4.5×63 cm, C_6H_6-acetone=40:1→1:0% acetone) to give Fr. I to X. Fr. II (631.1 mg) was rechromatographed on a silica gel (2.6×51 cm, hexane-EtOAc=80:1→100% EtOAc) to give Fr. II-1 to Fr. II-3. Repeated semi-prep HPLC (µBondapak C18, 2.8×300 mm, 1st, 95% MeOH, 2nd, 85% MeOH, RI detector, 1.5 ml/min) afforded MA1 (11.0 mg), MA2 (10.1 mg), and MA3 (10.3 mg). MA4 (7.6 mg) was purified by Sephadex LH-20 (2.4×60 cm, 50%→100% MeOH), followed by HPLC (µBondapak C18, 2.8×300 mm, 95% MeOH, RI detector, 1.5 ml/min).

MA1 [Z-Methyl Linoleate (Z-Linoleic Acid Methyl Ester)]

Pale yellowish oil; $FeCl_3$ negative; $C_{19}H_{34}O_2$ (m.w. 294); EIMS *m/z*: 294 [M^+], 263 [M^+-OMe], ^{1}H-NMR (400 MHz, Chloroform-*d*) δ: 5.36 (4H, m, olefinic), 3.67 (3H, s, -OMe), 2.77 (2H, brt, *J*=6.4 Hz, diallylic), 2.30 (2H, brt, *J*=7.6 Hz, H-2), 2.05 (4H, m, allylic×2), 1.62 (2H, m, H-3), 1.20-1.40 (methylene protons), 0.89 (3H, brt, *J*=7.0 Hz, terminal Me); ^{13}C-NMR (100 MHz, Chloroform-*d*) δ: 174.3 (carbonyl), 130.2, 130.1, 128.1, and 127.9 (olefinic), 51.5 (methoxy), 34.1 (C-2), 31.5 (diallylic), 29.6 and 29.4 (allylic), 22.6-29.2 (eight methylenes), 14.1 (terminal Me); t_R in GLC: 17.50 min.

MA2 [Z-Methyl Oleate (Z-Oleic Acid Methyl Ester)]

Colorless oil; $FeCl_3$ negative; $C_{19}H_{36}O_2$ (m.w. 296); EIMS *m/z*: 296 [M^+], 265 [M^+-OMe], ^{1}H-NMR (400 MHz, Chloroform-*d*) δ: 5.34 (2H, m, olefinic), 3.66 (3H, s, -OMe), 2.30 (2H, brt, *J*=7.6 Hz, H-2), 2.00 (4H, m, allylic×2), 1.62 (2H, m, H-3), 1.20-1.40 (methylene protons), 0.88 (3H, brt, *J*=7.0 Hz, terminal Me); ^{13}C-NMR (100 MHz, Chloroform-*d*) δ: 174.3 (carbonyl), 130.0 and 129.9 (olefinic), 51.4 (methoxy), 34.1 (C-2), 29.7 (allylic×2), 24.9-31.9 (11 methylenes), 14.1 (terminal Me); t_R in GLC: 14.50 min.

MA3 [Methyl Palmitate (Palmitic Acid Methyl Ester)]

Colorless oil; $FeCl_3$ negative; $C_{17}H_{34}O_2$ (m.w. 296); EIMS *m/z*: 270 [M^+], 239 [M^+-OMe], ^{1}H-NMR (400 MHz, Chloroform-*d*) δ: 3.68 (3H, s, -OMe), 2.33 (2H, brt, *J*=7.0 Hz, H-2), 1.60 (2H, m, H-3), 1.20-1.40 (methylene protons), 0.88 (3H, brt, *J*=7.0 Hz, terminal Me); ^{13}C-NMR (100 MHz, Chloroform-*d*) δ: 176.5 (carbonyl), 52.4 (methoxy), 35.2 (C-2), 24.1-33.5 (13 methylenes), 14.8 (terminal Me); t_R in GLC: 8.15 min.

MA4 (α-Lupeol)

Colorless powder; negative to $FeCl_3$; $C_{30}H_{50}O$ (m.w. 228); EIMS *m/z*: 426 [M^+]; ^{1}H-NMR (400 MHz, chloroform-*d*) δ: 4.69 (1H, brs, H-29a), 4.56 (1H, brs, H-29b), 3.18 (1H, dd, *J*=5.0 and 11.0 Hz, H-3), 2.39 (1H, m, H-19), 1.94 (3H, s, H-27), 1.93 (1H, m, H-21a), 1.71 (1H, m, H-15b), 1.68 (3H, s, H-30), 1.68 (1H, m, H-12b), 1.68 (1H, m, H-1a), 1.67 (1H, m, H-13), 1.61 (1H, m, H-2a), 1.54 (1H, m, H-2b), 1.54 (1H, m, H-6a), 1.49 (1H, m, H-16a), 1.42 (1H, m, H-11a), 1.42 (1H, m, H-22a), 1.41 (2H, m, H-7), 1.39 (1H, m, H-6b), 1.38 (1H, m, H-16b), 1.36 (1H, m, H-18), 1.33 (1H, m, H-21b), 1.29 (1H, d, *J*=1.5 Hz, H-9), 1.25 (1H, m, H-11b), 1.20 (1H, m, H-22b), 1.07 (1H, m, H-12a), 1.03 (3H, s, H-26), 1.01 (1H, m, H-15a), 0.97 (3H, s, H-23), 0.90 (1H, m, H-1b), 0.83 (3H, s, H-25), 0.79 (3H, s, H-28), 0.77 (3H, s, H-24), 0.69 (1H, d, *J*=9.0 Hz, H-5); ^{13}C-NMR (100 MHz, Chloroform-*d*) δ: 151.0 (s, C-20), 109.3 (t, C-29), 79.0 (d, C-3), 55.2 (d, C-5), 50.4 (d, C-9), 48.2 (d, C-18), 48.0 (d, C-19), 43.0 (s, C-17), 42.8 (s, C-14), 40.8 (s, C-8), 40.0 (t, C-22), 38.9 (s, C-4), 38.7 (t, C-1), 38.0 (d, C-13), 37.2 (s, C-10), 35.6 (t, C-16), 34.2 (t, C-7), 29.8 (t, C-21), 28.0 (q, C-23), 27.4 (t, C-15), 27.4 (t, C-2), 25.1 (t, C-12),

20.9 (t, C-11), 19.3 (q, C-30), 18.3 (t, C-6), 18.0 (q, C-28), 16.1 (q, C-25), 15.9 (q, C-26), 15.3 (q, C-24), 14.5 (q, C-27).

RESULTS AND DISCUSSION

In the course of screening for neuraminidase inhibitors from Basidiomycetes, *M. affinis* showed strong activity. The methanolic extract of the above samples were successively partitioned with *n*-hexane, chloroform, and EtOAc. The chloroform soluble fraction exhibited the highest activity. The activity-guided purification of the chloroform soluble fraction of *M. affinis* afforded four inhibitors, MA1, MA2, MA3, and MA4.

MA1 was obtained as a pale yellowish oil, and was negative to $FeCl_3$. The molecular weight was determined as 294 from the EIMS spectrum. A fragment ion at *m/z* 263 indicated the presence of methoxyl group. In the ^{1}H-NMR spectrum, the typical methylene protons of fatty acid appeared at δ 1.30 to 1.32 with olefinic protons at δ 5.36 (4H), which could be assigned as the unsaturated parts of a fatty acid backbone. A methoxyl signal was detected at δ 3.67 (3H, s). From these spectral data, MA1 was postulated as linoleic acid methyl ester. The structure was confirmed by the comparison of NMR data and retention time in GC with that in the reported reference [16, 20] and of an authentic sample, respectively.

MA2 showed [M$^+$] at *m/z* 296 in the EIMS spectrum. [M$^+$-OMe] fragment ion was observed at *m/z* 265. The ^{1}H-NMR data were very similar to those of MA1 except for the number of olefinic protons, suggesting that MA2 was an analogue of MA1. In ^{13}C-NMR spectrum, two olefinic signals (δ 129.9 and 130.0), thirteen methylene carbons, one methoxyl (δ 51.4) group, a carbonyl carbon (δ 174.3), and a methyl signal (δ 14.1) were detected. MA2 was identified as oleic acid methyl ester by comparing its t_R and NMR data with that of an authentic sample and of the reference [16], respectively.

The molecular ion peak of MA3 was detected at *m/z* 270 in the EIMS spectrum. Fragment ion at *m/z* 239 indicated the presence of a methoxyl group. No olefinic resonances were detected in ^{1}H- and ^{13}C-NMR. The structure was established by comparing its t_R in GC with an authentic sample and referring to the reported NMR data [15].

MA4 was purified as white powder. The molecular ion peak was found at *m/z* 426. In ^{1}H-NMR, terminal methylene proton signals were detected at d 4.69 and 4.56 with an α-carbinol proton at δ 3.18. Six singlet methyl signals appeared at δ 0.77- 1.68. Thirty carbon signals including an oxygenated carbon (δ 79.0), two olefinic carbons (δ 151.0 and 109.3), seven methyl carbons (δ 14.5- 19.3), six quaternary carbons, and eleven methylene carbons were observed in the ^{13}C-NMR and DEPT spectra, indicating that MA4 had a triterpene skeleton. In combination with

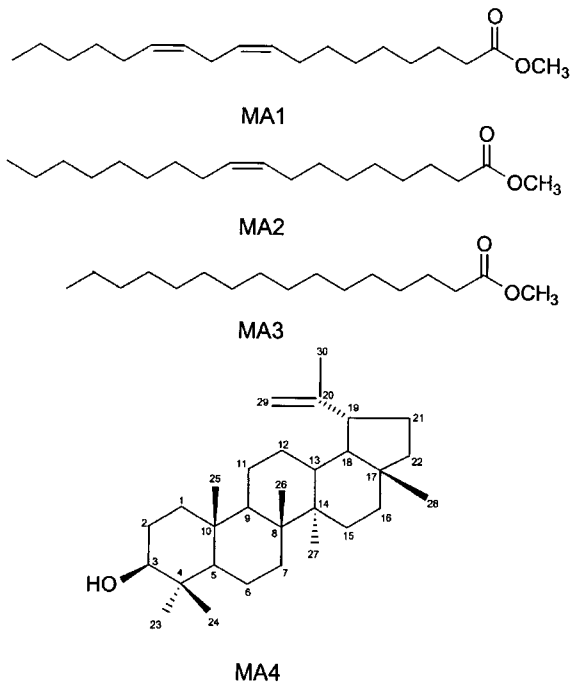

Fig. 1. Structures of MA1, MA2, MA3, and MA4.
MA1, methyl linoleate; MA2, methyl oleate; MA3, methyl palmitate; MA4, α-lupeol.

DEPT data, the terminal methylene (δ 109.3), seven singlet methyl signals, and an α-carbinol carbon (δ 79.0) strongly suggested that the triterpene skeleton was a lupeol. The stereochemistry of H-3 was determined to be an α rather than a β from its chemical shift [1]. The structure of MA4 was finally identified as α-lupeol.

The structures are presented in Fig. 1.

All compounds inhibited neuraminidase in a dose-dependent manner (Fig. 2). The IC_{50} values of MA1, 2, 3,

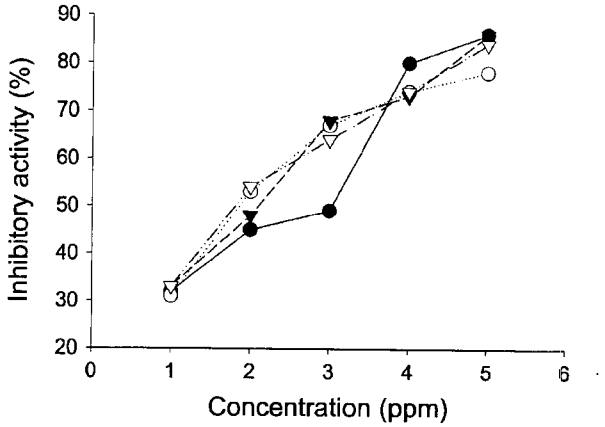

Fig. 2. Concentration-dependant inhibition of neuraminidase by isolated compounds.
-○-, MA1 (methyl linoleate); -▼-, MA2 (methyl oleate); -▽-, MA3 (methyl palmitate); -●-, MA4 (α-lupeol).

Table 1. Inhibitory activity against other glycosidases.

Enzyme	MA1		MA2		MA3		MA4	
	5[a]	20	5	20	5	20	5	20
Glucosidase	1.1[b]	3.2	3.2	3.5	8.1	10.3	3.0	5.1
Galactosidase	0.8	2.1	2.0	3.0	1.0	4.1	0.4	8.3
Mannosidase	0.6	2.2	8.1	5.3	5.0	5.4	4.4	1.5
Neuraminidase	76.5	ND[c]	87.1	ND	85.6	ND	87.3	ND

[a]ppm.
[b]Presented in %.
[c]Not determined.

and 4 were 7.07, 7.12, 7.52, and 5.65 μM, respectively. To test the enzyme specificity, the inhibitory activities on other glycosidases such as glucosidase, galactosidase, and mannosidase were compared with that of neuraminidase. Up to 5 ppm of the isolated compounds inhibited only less than 10% of the above enzyme activities (Table 1). Thus, they were thought to be relatively specific inhibitors of neuraminidase.

To establish a structure-activity relationship, inhibitory activities of 36 compounds, which were structurally related to the isolated fatty acids, were compared. In the case of linear fatty acids, the number of carbon was very critical for the stronger activity. C9 and C10 fatty acids did not have any activity, however, inhibition increased dramatically up to C16. Generally, the larger the fatty acid, the stronger the activity. The changes became insignificant from C16 to C23. The degree of unsaturation was not as significant as carbon number, although unsaturated fatty acids seemed to be twice as strong as saturated ones. Esterification of fatty acid appeared not to have an effect on the activity. The alcohols even larger than C14 did not show any inhibitory activity, indicating that the carbonyl moiety was essential

Table 2. Neuraminidase-inhibiting activity of related compounds.

Group	Condensed structure	Compound name	IC_{50} (μM)
Saturated	$CH_3(CH_2)_6CH_2COOH$ (9:0)	Pelargonic acid	>160
	$CH_3(CH_2)_7CH_2COOH$ (10:0)	Capric acid	>160
	$CH_3(CH_2)_9CH_2COOH$ (12:0)	Lauric acid	110.0
	$CH_3(CH_2)_{11}CH_2COOH$ (14:0)	Myristic acid	90.0
	$CH_3(CH_2)_{13}CH_2COOH$ (16:0)	Palmitic acid	16.5
	$CH_3(CH_2)_{15}CH_2COOH$ (18:0)	Stearic acid	13.4
	$CH_3(CH_2)_{17}CH_2COOH$ (20:0)	Arachidic acid	11.2
	$CH_3(CH_2)_{19}CH_2COOH$ (22:0)	Tricosanic acid	6.6
Unsaturated	$CH_3(CH_2)_3CH{=}CH(CH_2)_7COOH$ (14:1)	Myristoleic acid	88.2
	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$ (16:1)	Palmitoleic acid	9.8
	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$ (18:1)	Oleic acid	5.1
	$CH_3(CH_2)_4[CH{=}CHCH_2]_2(CH_2)_6COOH$ (18:2)	Linoleic acid	8.9
	$CH_3(CH_2)_4[CH{=}CHCH_2]_4(CH_2)_2COOH$ (20:2)	Arachidonic acid	5.6
Me ester	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOMe$ (16:1)	Methyl palmitoleate	11.3
	$CH_3(CH_2)_4[CH{=}CHCH_2]_2(CH_2)_6COOH$ (18:2)	Methyl linoleate	7.1
	$CH_3(CH_2)_4[CH{=}CHCH_2]_4(CH_2)_2COOH$ (20:4)	Methyl arachidonate	6.2
	$CH_3CH_2[CH{=}CHCH_2]_3(CH_2)_6COOH$ (18:3)	Methyl linolenate	11.4
Alcohol	$CH_3(CH_2)_{12}CH_2OH$ (14:0)	Myristyl alcohol	>100
	$CH_3(CH_2)_{14}CH_2OH$ (16:0)	Palmityl alcohol	>100
	$CH_3(CH_2)_{16}CH_2OH$ (18:0)	Stearyl alcohol	>100
Dioic acid	$(COOH)_2$ (1)	Oxalic acid	>280
	$(CH_2COOH)_2$ (4)	Succinic acid	>210
	$HOOC(CH_2)_9COOH$ (11)	Undecanedioic acid	>100
	$HOOC(CH_2)_{20}COOH$ (22)	Dodecanedioic acid	3.6
Ketone	$CH_3(CH_2)_5CO(CH_2)_5CH_3$ (13:0)	Dihexyl ketone	70.7
	Geranyl-$COCH_3$ (12:2)	Geranyl acetone	90.5
Aromatic	C_6H_5-COOH (7)	Benzoic acid	>210
	C_6H_5-CH_2COOH (8)	Phenyl acetic acid	>180
	C_6H_4-$(COOH)_2$ (7)	*m*-Phthalic acid	>150
	C_6H_5-CH=CHCOOH (9)	Cinnamic acid	>160
	C_6H_5-$(CH_2)_5COOH$ (12)	6-Phenylhexanoic acid	>130
	$C_{10}H_6(COOH)_2$ (12)	1,4-Naphthalenedicarboxylic acid	>120
	$C_{10}H_6(COOH)_2$ (12)	1,6-Naphthalenedicarboxylic acid	>120
	$C_{10}H_7COOH$ (11)	2-Naphthylacetic acid	>130
	$(C_6H_5)_2CHCOOH$ (14)	Diphenylacetic acid	>120

for the stronger activity. The activity of higher ketones (C12 and C13) exhibited almost the same activity of C12 and C14 fatty acids. Since C16 aldehydes and ketones were not obtainable, a definite conclusion could not be drawn about what type of carbonyl group was necessary for the inhibitory activity. Likewise, it was not clear whether the number of carboxyl groups was responsible for the stronger activity or not, since only three dioic acids were tested, even though C22 dioic acid was the strongest in the activity. Aromatic acid did not show any activity at all, regardless of the carbon number. Consequently, in the case of an aliphatic linear hydrocarbon skeleton, at least one carboxyl (presumably any carbonyl) moiety and sixteen carbons were the necessary requirements for the potent inhibition, whereas saturated, unsaturated, free, and ester forms did not have a significant effect on the activity.

Many neuraminidase inhibitors, having a Neu5Ac2en (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid) skeleton, have been developed [2, 18]. Only one report on natural inhibitors, however, has been published by our research group [10]. The fatty acids could be rapidly metabolized in the human body, therefore, it might be difficult to use them directly as an antiviral agent. The fatty acid derivatives, which are not easily degraded by metabolic pathways such as β-oxidation, would be a good candidate in the development of anti-influenza agents.

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