SYNTHESIS OF 3-EPISIASTATIN B ANALOGUES† HAVING ANTI-INFLUENZA VIRUS ACTIVITY

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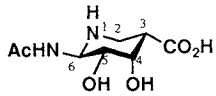
Two epimers of siastatin B, 3-episiastatin B (**3**) and 3,4-diepisiastatin B (**4**), were obtained by the chemical modification of siastatin B. Compound **3** showed marked inhibitory activity against influenza virus neuraminidases and significant inhibition of influenza virus infection *in vitro*.

Two integral membrane glycoproteins, haemagglutinin and neuraminidase, envelope the viral surface of influenza A and B. Infection by influenza virus begins with the binding of haemagglutinin to terminal sialic acid residues of glycoproteins on the surface of the host cell and subsequent fusion of viral and host cell membranes.[1] Neuraminidase [EC 3.2.1.18] is a glycosidase, cleaving the α-2,3- and α-2,6-glycosidic linkages between terminal sialic acid and adjacent sugar residues of glycoproteins and glycolipids.[2] Neuraminidase is thought to facilitate the elution of progeny virus particles from the infected cells[3] and the maintenance of mobility of progeny virus by prevention of self-aggregation.[4] Thus neuraminidase may be an important factor in the spread of the infection.[5] This suggests that a potent inhibitor for influenza virus neuraminidase could prevent or limit influenza infection. Many naturally occuring and synthetic azasugars are potent and specific inhibitors for enzymes associated with carbohydrate metabolism, and they have the potential to produce a number of kinds of beneficial therapeutic effects such as antiviral, antimetastatic, antifungal and antihyperglycemic activity, *etc.*[6] One such, a multifunctional piperidine siastatin B (**1**), which was isolated as an inhibitor of neuraminidase by UMEZAWA *et al.*[7] from a *Streptomyces* culture, structurally resembles sialic acid (*N*-acetylneuraminic acid, **2**).

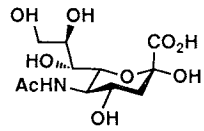
In the course of our studies[8~17] on the relationships between structure and biological activity of siastatin B (**1**), we have demonstrated that several analogues[13~16] having the same equatorial carboxyl group as **1** show potent inhibitory activities for *Streptococcus* sp. and *Clostridium perfringens* neuraminidases,

† The synthesis of 3-episiastatin B analogues (**3** and **4**) were briefly communicated to Natural Products Lett.[13] The anticlockwise numbering was used in Natural Products Lett.[13] on siastatin B analogues according to the first report on isolation of siastatin B in J. Antibiotics.[7] The correct clockwise numbering is employed for siastatin B analogues in this article according to IUPAC rules. Therefore, 3-episiastatin B (**3**) and 3,4-diepisiastatin B (**4**), respectively, in this article are the same compounds as 5-episiastatin B and 4,5-diepisiastatin B in Natural Products Lett.[13]

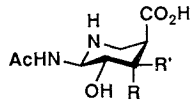
Fig. 1. Structures of siastatin B, *N*-acetylneuraminic acid and their analogues.



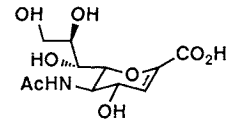
Siastatin B (**1**)



N-Acetylneuraminic acid (**2**)

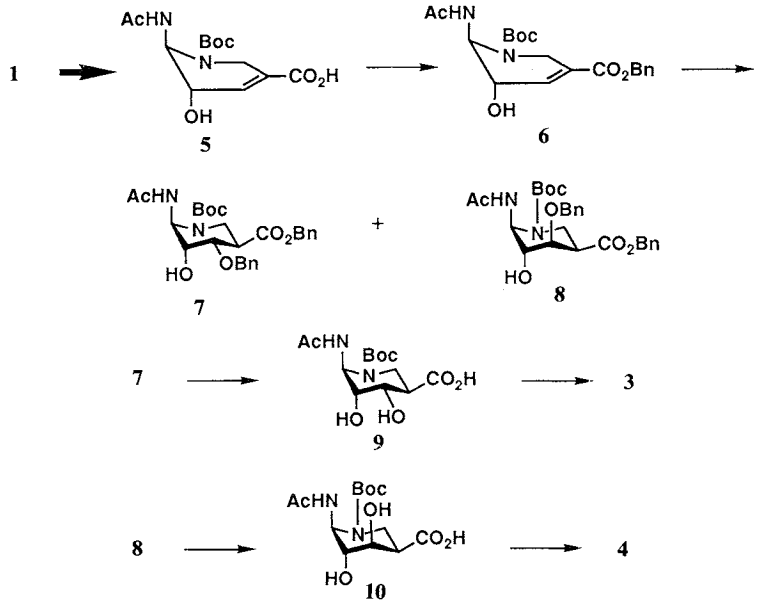


3 R=OH R′=H
4 R=H R′=OH



2,3-Dehydro-2,3-dideoxy-
N-acetylneuraminic acid (**11**)

Scheme 1.



Boc=$COOC(CH_3)_3$, Bn=$CH_2C_6H_5$

whereas none of these compounds are inhibitors of influenza virus neuraminidase. Thus, we became interested in the biological activity of epimeric analogues of siastatin B (**3** and **4**), which more closely resemble a terminal sialic acid residue (**2**) having an axially oriented carboxyl group in glycoprotein or glycolipid as a result of α-L-glycosyl bonding to their saccharide chains. We previously communicated the synthesis of **3** and **4**.[13] Herein, we disclose that the siastatin B analog **3** with an epimeric carboxyl group is a potent inhibitor of influenza virus neuraminidases and influenza virus *in vitro* infectivity.

Synthesis

The synthesis of **3** and **4** is outlined in Scheme 1. Epimerization at the C-3 position was achieved by

Table 1. Inhibition (%) of 3-episiastatin B (**3**), 3,4-diepisiastatin B (**4**) and 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid (**11**) at 100 μM against *N*-acetylneuraminidase.

Compound	*N*-Acetylneuraminidase				
	Influenza virus			Sendai virus (HVJ)/Fushimi	Newcastle disease virus (ND)/Miyadera
	A/FM/1/47 (H1N1)	A/Kayano/57 (H2N2)	B/Lee/40		
3	53.1 (7.4×10^{-5})	25.6 ($>1.0\times10^{-5}$)	67.2 (4.2×10^{-5})	0	0
4	0	2.0	19.8	0	22.4
11	93.2 ($<1.0\times10^{-5}$)	75.3 (2.9×10^{-5})	75.4 (4.9×10^{-5})	47.9	83.0

( ): IC_{50} (M).

Table 2. Inhibition (%) of 3-episiastatin B (**3**) and 2,3-dehydro-2,3-dideoxy-*N*-acetylneuraminic acid (**11**) against influenza virus A/FM/1/47 (H1N1) infection in MDCK cells.

Compound	Plaque forming units (PFU)			Stained area		
	40 μM	20 μM	10 μM	40 μM	20 μM	10 μM
3	88.9	55.5	35.6	97.1	87.2	64.1
11	100	100	89.6	100	100	98.7

a 1,4-conjugated Michael addition of alcohol to the α,β-unsaturated ester **6** with base. Benzyl esterification[16] of **5** derived from siastatin B with benzyl chloride and diisopropylethylamine in *N*,*N*-dimethylformamide gave **6** in 91% yield. Treatment of **6** with potassium carbonate in benzyl alcohol afforded monoepimer **7** and diepimer **8** in a ratio of 1 : 13 in 52% yield. The ^{1}H NMR spectrum of **7** shows protons at 2.92 (t, $J=13$ Hz, H-2_{ax}), 3.10 (ddd, $J=13$, 10 and 4.4 Hz, H-3), 3.91 (dd, $J=10$ and 3 Hz, H-4), 4.11 (broad s, H-5), 4.29 (dd, $J=13$ and 4.4 Hz, H-2_{eq}), 5.85 (d, $J=7$ Hz, NH) and 6.14 (dd, $J=7$ and 3 Hz, H-6), indicative of C_3^6-conformer generally observed in the ring-imine protected siastatin B with carbamate fashion.[10~12,16] The ^{1}H NMR spectrum of **8** is also indicative of the same conformer. Removal of the protecting groups of **7** and **8** by catalytic hydrogenolysis followed by acid hydrolysis gave **3** and **4** in good yields, respectively. The large coupling constants of $J=7.3$ and 8 Hz between H-5 and H-6 in **3** and **4**, respectively, are clearly indicative of C_6^3-conformer in both compounds.

Biological Activities

The synthetic compounds (**3** and **4**) and the known sialidase inhibitor (**11**) as the reference compound were tested for their inhibitory effects on *N*-acetylneuraminidases of influenza viruses, Sendai virus and Newcastle disease virus. As shown in Table 1, **3** as well as **11** strongly inhibited *N*-acetylneuraminidases from influenza viruses (A/FM/1/47 (H1N1), A/Kayano/57 (H2N2) and B/Lee/40). Compound **11** also showed inhibitory activity against *N*-acetylneuraminidases from Sendai virus (HVJ)/Fusimi and Newcastle disease virus (ND)/Miyadara, whereas **3** did not inhibit these enzymes. On the other hand, **4** showed little or no inhibition for all tested *N*-acetylneuraminidases. All compounds also showed no inhibition at 250 μM for the influenza virus haemagglutinins. Recently, BRUMEISTER *et al.*[18] have presented the crystal structure of the enzymatically active head of the *N*-acetylneuraminidase from influenza virus B/Beijing/1/87 and its complex with *N*-acetylneuraminic acid. The binding mode of *N*-acetylneuraminic acid to *N*-acetylneuraminidase, in which all the large side groups such as the carboxyl group are equatorial, involves

the α-boat rather than the β-chair conformation. It can be assumed that the monoepimeric **3** and the diepimeric **4** could change their conformation from chair to boat when in the enzyme, and the resulting equatorial groups in **3** would be more strongly stabilized by hydrogen bonding with the peptide of the influenza virus neuraminidase than those of **4**. Asp 148 of influenza virus B/Beijing/1/87 neuraminidase[18] could form a hydrogen bond to the 4-OH of **3** similarly to 2-OH of **2** in the boat conformations. As shown in Table 2, **3** exhibited potent antiviral activity against influenza virus A/FM/1/47 infection in MDCK cells *in vitro*. Compound **11** also showed strong antiinfluenza virus A/FM/1/47 activity *in vitro*. Compound **11** inhibits not only influenza virus neuraminidases but also many other neuraminidases, including mammalian ones.[19,20] In contrast, compound **3** specifically affects influenza virus neuraminidases. There results indicate that **3** should prove to be a candidate for synthesis of useful and specific anti-viral agents inhibiting the influenza virus neuraminidase.

Experimental

General

Melting points were determined with a Yanagimoto apparatus and were uncorrected. IR spectra were determined on a Hitachi Model 260-10 spectrophotometer. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. ^{1}H NMR spectra were recorded with a JEOL JNM EX400 spectrometer. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane as an internal standard. Mass spectra were taken by a JEOL JMS-SX102 in the FAB mode.

Viral Strains

The FM/1/47 (H1N1) and Kayano/57 (H2N2) strains of influenza A virus, the Lee/40 strain of influenza B virus, the Fushimi strain of Sendai virus, and the Miyadera strain of Newcastle disease virus (NDV) were used. Stocks of these viruses were all grown in the allantoic cavity of 10-day-old embryonated hen's eggs, and the allantoic fluids were harvested after incubation for 48 hours at 36°C. The viruses were purified partially from the allantoic harvests by differential centrifugations at $4,000 \times g$ for 15 minutes and $33,000 \times g$ for 1 hour, and the resulting virus pellets were further purified by centrifugation through a 5 to 40% potassium tartrate gradient at $25,000 \times g$ for 2 hours. The virus-containing band was collected and centrifuged at $35,000 \times g$ for 1 hour, and the resultant pellet was suspended in phosphate-buffered saline, pH 7.2 (PBS). The MDCK line of canine kidney cells was grown in EAGLE's minimal essential medium (MEM) containing 10% bovine serum.

Neuraminidase Inhibition Assay

To 50 μl of the virus suspension containing 1 to 10 μg of each virus was added an equal volume of the test sample at the final concentration of 100 μM, and the mixture was incubated for 15 minutes at 37°C. The reaction mixture then received 100 μl of fetuin solution (10 mg/ml) as the substrate either in 0.2 M phosphate buffer pH 6.0 containing 2.5 mM $CaCl_2$ (for influenza A and B viruses) or in 0.2 M acetate buffer pH 5.0 (for Sendai virus and NDV), and the mixture was further incubated at 37°C for 5 hours (influenza A and B viruses) or 3 hours (Sendai virus and NDV). Neuraminic acid liberated from the substrate was determined by the thiobarbituric acid method of AMINOFF.[21]

Haemagglutination Inhibition Assay

The test sample (50 μl) was serially diluted in two-fold steps with PBS and then mixed with an equal volume of the virus suspension containing 16HA units of each virus. After incubation for 15 minutes at 4°C, 100 μl of chicken erythrocyte suspension (0.5% in PBS) was added to each mixture, and the HA patterns were read after a further incubation at 4°C for 1 hour.

Plaque Assay

The assay was carried out by the modified method of SCHULMAN and PALESE.[22] Monolayers of MDCK

cells were washed with HANK's BSS, and then incubated with 0.2 ml of the mixture consisting of equal volumes of the virus suspension (50 PFU) and the test sample each prepared in EAGLE's MEM. After adsorption for 1 hour at 34°C, the cell culture received 1.5 ml of agar overlay medium consisting of EAGLE's MEM, 10 μg/ml of Trypsin (DIFCO) and 0.8% Ager Noble. After incubation for 2 days at 34°C under 5% CO_2 atmosphere, 1.5 ml of the second agar overlay medium containing 0.001% Neutral Red was added, and the plaque numbers were counted on the next day. The test samples were included in both the first and second agar overlay media at the concentrations indicated in the text.

N-(*tert*-Butoxycarbonyl)-3,4-didehydro-4-deoxysiastatin B Benzyl Ester (**6**)

To a solution of *N*-(*tert*-butoxycarbonyl)-3,4-didehydro-4-deoxysiastatin B (**5**) (630 mg) in DMF (6.3 ml) were added diisopropylethylamine (3.5 ml) and benzyl chloride (1.15 ml), and the mixture was stirred at room temperature for 18 hours. Evaporation of the solvent gave an oil, which was dissolved in $CHCl_3$. The solution was washed with H_2O, dried over $MgSO_4$, and filtered. Evaporation of the filtrate gave an oil which was subjected to column chromatography on silica gel. Elution with a mixture of $CHCl_3$-CH_3OH (10:1) gave a colorless foamy glass of **6** (747 mg, 91%): $[\alpha]_D^{21}$ +76.4° (*c* 0.78, $CHCl_3$); IR (KBr) cm^{-1} 3425, 2980, 1710, 1660, 1540, 1480, 1460, 1410, 1395, 1375, 1330, 1240, 1170, 1130, 1080, 1050, 990, 960, 920; ^1H NMR (CD_3OD, 400 MHz), δ 1.48 (9H, s, $COO(CH_3)_3$), 1.91 (3H, s, $NCOCH_3$), 3.80 (1H, broad d, J=19 Hz, H-2), 4.12 (1H, broad d, J=5 Hz, H-5), 4.45 (1H, dd, J=19 and 2 Hz, H-2), 5.23 and 5.26 (2H, ABq, J=14 Hz, $-CH_2-$), 6.07 (1H, broad s, H-6), 6.99 (1H, m, H-4) and 7.25~7.45 (5H, m, phenyl); MS (FAB) *m/z* 391 $(M+H)^+$, 335, 307, 289, 232, 154, 136, 91, 57.

4-*O*-Benzyl-*N*-(*tert*-butoxycarbonyl)-3-episiastatin B Benzyl Ester (**7**) and 4-*O*-Benzyl-*N*-(*tert*-butoxycarbonyl)-3,4-diepisiastatin B Benzyl Ester (**8**)

To a solution of **6** (710 mg) in dry benzyl alcohol (8 ml) was added K_2CO_3 (800 mg), and the mixture was stirred at room temperature overnight. The mixture was directly subjected to column chromatography on silica gel. Elution with a mixture of $CHCl_3$-CH_3OH (30:1) gave **7** (34 mg, 3.8%) and **8** (437 mg, 48.3%) as a colorless solid and a colorless foamy glass, respectively.

Compound **7** was recrystallized from CH_3OH: mp 199~200°C; $[\alpha]_D^{21}$ +4.1° (*c* 1.5, CH_3OH); IR (KBr) cm^{-1} 3440, 3330, 3050, 2990, 2950, 2905, 1740, 1675, 1540, 1505, 1465, 1430, 1400, 1380, 1330, 1315, 1265, 1220, 1200, 1165, 1145, 1105, 1085, 1075, 1040, 1025, 1005, 975, 955, 920; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.45 (9H, s, $COO(CH_3)_3$), 1.99 (3H, s, $NCOCH_3$), 2.48 (1H, broad s, OH), 2.92 (1H, t, J=13 and 5.6 Hz, H-2_{ax}), 3.10 (1H, ddd, J=13, 10 and 4.4 Hz, H-3), 3.91 (1H, dd, J=10 and 3 Hz, H-4), 4.11 (1H, broad s, $J_{1/2}$=6 Hz, H-5), 4.29 (1H, dd, J=13 and 4.4 Hz, H-2_{eq}), 4.48 and 4.58 (2H, ABq, J=12 Hz, $-OCH_2-$), 5.11 and 5.20 (2H, ABq, J=12 Hz, $-OCH_2-$), 5.85 (1H, d, J=7 Hz, $-NHCO-$), 6.14 (1H, dd, J=7 and 3 Hz, H-6) and 7.15~7.35 (10H, m, 2×phenyl); MS (FAB) *m/z* 499 $(M+H)^+$, 443, 340, 232, 154, 136, 91, 57.

Compound **8**: $[\alpha]_D^{26}$ +52.7° (*c* 1.2, CH_3OH); IR (KBr) cm^{-1} 3400, 3030, 2970, 2930, 1735, 1695, 1455, 1390, 1365, 1335, 1310, 1250, 1210, 1165, 1070, 1030, 965, 905; ^1H NMR (CD_3OD, 400 MHz) δ 1.44 (9H, s, $COO(CH_3)_3$), 1.75 (3H, s, $NCOCH_3$), 3.07 (1H, ddd, J=13, 5 and 3 Hz, H-3), 3.92 (1H, t, J=3 Hz, H-5), 4.10 (1H, t, J=3 Hz, H-4), 4.14 (1H, dd, J=14 and 5 Hz, H-2_{eq}), 4.37 and 4.49 (2H, ABq, J=11 Hz, $-CH_2-$), 5.13 and 5.17 (2H, ABq, J=12 Hz, $-CH_2-$), 5.95 (1H, s, with a small coupling, J=~3 Hz, H-6) and 7.1~7.4 (10H, m, phenyl); MS (FAB) *m/z* 499 $(M+H)^+$, 443, 340, 232, 154, 136, 91, 57.

N-(*tert*-Butoxycarbonyl)-3-episiastatin B (**9**)

The solution of **7**(22 mg) in CH_3OH (5 ml) was stirred with 10% Pd/C (7 mg) under atmospheric pressure of hydrogen at room temperature for 5 hours. After filtration, evaporation of the filtrate gave colorless crystals of **9** (13.8 mg, 98%): mp 144~146°C; $[\alpha]_D^{21}$ +27° (*c* 1.2, CH_3OH); IR (KBr) cm^{-1} 3430, 2980, 2930, 1700, 1560, 1490, 1440, 1390, 1360, 1310, 1270, 1180, 1130, 1110, 1090, 1005, 955; ^1H NMR (CD_3OD, 400 MHz) δ 1.46 (9H, s, $COO(CH_3)_3$), 1.97 (3H, s, $NCOCH_3$), 2.83 (1H, ddd, J=12, 11, 4.5 Hz, H-3), 3.05 (1H, t, J=13 Hz, H-2_{ax}), 3.81 (1H, broad s, H-5), 4.00 (1H, dd, J=11 and 3 Hz, H-4), 4.17 (1H, dd, J=12 and 4.5 Hz, H-2_{eq}) and 6.03 (1H, d, J=2 Hz, H-6); MS (FAB) *m/z* 319 $(M+H)^+$, 307, 289, 263, 219, 154, 136, 107, 89, 57.

N-(*tert*-Butoxycarbonyl)-3,4-diepisiastatin B (**10**)

Compound **10** was obtained as a foamy glass from **8** by a similar procedure to that used for the preparation of **9** (99%): $[\alpha]_D^{26}$ +50.1° (*c* 1.3, CH_3OH); IR (KBr) cm^{-1} 3400, 2980, 2930, 1690, 1590, 1530, 1485, 1460, 1425, 1400, 1375, 1350, 1320, 1250, 1170, 1085, 1060, 1010, 975; ^1H NMR (CD_3OD, 400 MHz) δ 1.46 (9H, s, $COO(CH_3)_3$), 1.95 (3H, s, $NCOCH_3$), 2.89 (1H, ddd, J=12, 4 and 2 Hz, H-3), 3.29 (1H, t, J=13 Hz, H-2_{ax}), 3.73 (1H, broad s, H-5), 4.04 (1H, dd, J=13 and 4 Hz, H-2_{eq}), 4.26 (1H, broad t, J=3 Hz, H-4) and 5.99 (1H, broad s, H-6); MS (FAB) *m/z* 319 $(M+H)^+$, 307, 289, 263, 219, 176, 154, 136, 107, 89, 57.

3-Episiastatin B (**3**)

Compound **9** (12.8 mg) was dissolved in 4 M hydrogen chloride in dioxane (0.25 ml), and the mixture was allowed to stand at room temperature for 4 hours. The resulting crystals were taken by centrifugation. The crystals were washed with dioxane to give a hydrochloride of **3** as hygroscopic crystals (9.9 mg, 97%): $[\alpha]_D^{24}$ +35° (*c* 0.14, H_2O); IR (KBr) cm^{-1} 3450, 1745, 1700, 1590, 1430 (sh), 1400, 1310, 1215, 1175, 1105, 1070, 1025, 980, 915; ^1H NMR (D_2O, 270 MHz) δ 1.99 (3H, s, $NCOCH_3$), 3.09 (1H, broad q, J=5.6 Hz, H-3), 3.3~3.4 (2H, broad d, H-2), 3.96 (1H, dd, J=7.3 and 2.5 Hz, H-5), 4.33 (1H, dd, J=6 and 2.5 Hz, H-4) and 5.11 (1H, d, J=7.3 Hz, H-6); MS (FAB) *m/z* 219 $(M+H)^+$, 207, 160, 142, 115, 75, 57.

3,4-Diepisiastatin B (**4**)

Compound **10** (24 mg) was dissolved in 1 M hydrochloric acid (0.5 ml), and the mixture was allowed to stand at room temperature overnight. Evaporation of the solvent gave a solid. The solid was subjected to preparative TLC on silica gel developed with a mixture of $CHCl_3$ - CH_3OH - conc aq ammonia (20 : 10 : 3) to give a colorless amorphous solid of **4** (13 mg, 79%); mp 101~103°C; $[\alpha]_D^{24}$ +25.7°C (*c* 0.34, H_2O); IR (KBr) cm^{-1} 3450, 1690, 1600, 1430, 1400, 1280, 1240, 1160, 1100, 990, 920; ^1H NMR (D_2O, 400 MHz) δ 2.07 (3H, s, $NCOCH_3$), 2.91 (1H, q, J=4.5 Hz, H-3), 3.02 (1H, dd, J=13.8 and 4.5 Hz, H-2), 3.40 (1H, dd, J=13.8 and 4.5 Hz, H-2), 3.88 (1H, t, J=8 Hz, H-5), 3.94 (1H, dd, J=8 and 4.5 Hz, H-4) and 4.73 (1H, d, J=8 Hz, H-6); MS (FAB) *m/z* 219 $(M+H)^+$, 207, 160, 142, 115, 75, 57.

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