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

YOSHIO NISHIMURA,* YOJI UMEZAWA, SHINICHI KONDO and TOMIO TAKEUCHI
Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

KEIJI MORI, ISAO KIJIMA-SUDA and KENKICHI TOMITA
Tokyo Research Institute, MECT Corporation,
1780 Kitano, Tokorozawa, Saitama 359, Japan

KANETSU SUGAWARA and KIYOTO NAKAMURA
Department of Bacteriology, Yamagata University, School of Medicine,
Iida-Nishi, Yamagata 990-23, Japan

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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.† 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.‡
Neuraminidase is thought to facilitate the elution of progeny virus particles from the infected cells§
and the maintenance of mobility of progeny virus by prevention of self-aggregation.¶ Thus neuraminidase
may be an important factor in the spread of the infection.†† This suggests that a potent inhibitor for
influenza virus neuraminidase could prevent or limit influenza infection. Many naturally occurring 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.‡‡ One such, a multifunctional piperidine
siastatin B (1), which was isolated as an inhibitor of neuraminidase by UMEZAWA et al.‡§ from a Streptomyces
culture, structurally resembles sialic acid (N-acetylneuraminic acid, 2).

In the course of our studies¶¶ † on the relationships between structure and biological activity of
siastatin B (1), we have demonstrated that several analogues‡‡ † having the same equatorial carboxyl
group as I 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.†§ The
anticlockwise numbering was used in Natural Products Lett.†§ on siastatin B analogues according to the first report
on isolation of siastatin B in J. Antibiotics.‡§ 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.†§
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-1-glycosyl bonding to their saccharide chains. We previously communicated the synthesis of 3 and 4. 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 \(\mu\text{M}\) against \(N\)-acetylneuraminidase.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\text{IC}_{50}) (m)</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>53.1 (7.4 \times 10^{-5})</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>93.2 (&lt;1.0 \times 10^{-5})</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plaque forming units (PFU)</th>
<th>Stained area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40 (\mu\text{M})</td>
<td>20 (\mu\text{M})</td>
</tr>
<tr>
<td>3</td>
<td>88.9</td>
<td>55.5</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

a 1,4-conjugated Michael addition of alcohol to the \(\alpha,\beta\)-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 \(^1\text{H}\) NMR spectrum of 7 shows protons at 2.92 (t, \(J=13\text{ Hz}, \text{H-2}\)), 3.10 (ddd, \(J=13, 10 \text{ and } 4.4\text{ Hz}, \text{H-3}\)), 3.91 (dd, \(J=10 \text{ and } 3\text{ Hz}, \text{H-4}\)), 4.11 (broad s, H-5), 4.29 (dd, \(J=13 \text{ and } 4.4\text{ Hz}, \text{H-2}\)), 5.85 (d, \(J=7\text{ Hz}, \text{NH}\)) and 6.14 (dd, \(J=7 \text{ and } 3\text{ Hz}, \text{H-6}\)), indicative of \(\text{C}_2\)-conformer generally observed in the ring-imine protected siastatin B with carbamate fashion.\(^{10,12,16}\)

The \(^1\text{H}\) 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 \text{ and } 8\text{ Hz between H-5 and H-6 in 3 and 4, respectively, are clearly indicative of \(\text{C}_2\)-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)/Fushimi 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 \(\mu\text{M}\) for the influenza virus haemagglutinins. Recently, BRUSEMEISTER 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 neuraminidase18)
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. 1H 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 × g for 15 minutes and
33,000 × 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 × g for 2 hours. The virus-containing band was collected and
centrifuged at 35,000 × 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 CaCl2 (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 Hanks’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% CO2 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-Benzoylcarbonyl)-3,4-didehydro-4-deoxysiastatin B Benzyl Ester (6)

To a solution of N-(tert-benoxycarbonyl)-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 CHCl3. The solution was washed with H2O, dried over MgSO4, and filtered. Evaporation of the filtrate gave an oil which was subjected to column chromatography on silica gel. Elution with a mixture of CHCl3 - CH3OH (10:1) gave a colorless foamy glass of 6 (747 mg, 91%): [α]D +76.4° (c 0.78, CHCl3); 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 (CD3OD, 400 MHz), δ 1.48 (9H, s, COO(CH3)3), 1.91 (3H, s, NCOCH3), 3.80 (1H, broad, J= 19 Hz, H-2), 4.12 (1H, broad, J=5 Hz, H-5), 4.45 (1H, dd, J= 19 and 2 Hz, H-2), 5.23 and 5.26 (2H, ABq, /=14 Hz, -CH2-), 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-benoxycarbonyl)-3-episiastatin B Benzyl Ester (7) and 4-O-Benzyl-N-(tert-benoxycarbonyl)-3,4-diepiastatin B Benzyl Ester (8)

To a solution of 6 (710 mg) in dry benzyl alcohol (8 ml) was added K2CO3 (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 CHCl3 - CH3OH (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 CH3OH: mp 199~200°C; [α]D +4.1° (c 1.5, CH3OH); 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 (CDCl3, 400 MHz) δ 1.45 (9H, s, COO(CH3)3), 1.99 (3H, s, NCOCH3), 2.48 (1H, broad s, OH), 2.92 (1H, broad s, J=6 Hz, H-2ax), 3.10 (1H, t, J=13 and 5.6 Hz, H-2ax), 3.91 (1H, dd, J=10 and 3 Hz, H-3), 4.11 (1H, broad s, J=1/2=6 Hz, H-5), 4.29 (1H, dd, J=10 and 3 Hz, H-4), 4.48 and 4.58 (2H, ABq, /=12 Hz, -OCH2-), 5.13 and 5.20 (2H, ABq, J=12 Hz, -OCH2-), 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, 2xphenyl); MS (FAB) m/z 499 (M+H)+, 443, 340, 232, 154, 136, 91, 57.

Compound 8: [α]D +52.7° (c 1.2, CH3OH); IR (KBr) cm⁻¹ 3400, 3030, 2970, 2930, 1735, 1695, 1455, 1390, 1365, 1335, 1310, 1250, 1210, 1165, 1070, 1030, 965, 905; ¹H NMR (CD3OD, 400 MHz) δ 1.44 (9H, s, COO(CH3)3), 1.75 (3H, s, NCOCH3), 3.07 (1H, dd, 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-2eq), 4.37 and 4.49 (2H, ABq, J=11 Hz, -CH2-), 5.13 and 5.57 (2H, ABq, J=12 Hz, -CH2-), 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-Benzoylcarbonyl)-3-episiastatin B (9)

The solution of 7 (22 mg) in CH3OH (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; [α]D +27° (c 1.2, CH3OH); IR (KBr) cm⁻¹ 3430, 2980, 2930, 1700, 1560, 1490, 1440, 1390, 1360, 1310, 1270, 1180, 1130, 1110, 1090, 1005, 955; ¹H NMR (CD3OD, 400 MHz) δ 1.46 (9H, s, COO(CH3)3), 1.97 (3H, s, NCOCH3), 2.83 (1H, ddd, J=12, 11, 4.5 Hz, H-3), 3.05 (1H, t, J=13 Hz, H-2ax), 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-2eq) 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%): \([\alpha_l]^\beta +50.1° (c 1.3, \text{CH}_3\text{OH})\); IR (KBr) cm\(^{-1}\) 3400, 2980, 2930, 1690, 1590, 1530, 1485, 1460, 1425, 1400, 1375, 1350, 1320, 1250, 1170, 1085, 1060, 1010, 975; \(^1\text{H NMR (CD}_3\text{OD, 400 MHz)}\) \(\delta 1.46 (9\text{H, s, COO(CH}_3)_3), 1.95 (3\text{H, s, NCOCH}_3), 2.89 (1\text{H, ddd, }\nu=12, 4\text{ and }2\text{Hz, H-3}), 3.29 (1\text{H, t, }\nu=13\text{Hz, H-2ax}), 3.73 (1\text{H, broad s, H-5}), 4.04 (1\text{H, dd, }J=13\text{ and }4\text{Hz, H-2eq}), 4.26 (1\text{H, broad t, }J=3\text{Hz, H-4})\) 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-Episiasiastatin 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%): \([\alpha_l]^\beta +35° (c0.14, \text{H}_2\text{O})\); IR (KBr) cm\(^{-1}\) 3450, 1745, 1700, 1590, 1430 (sh), 1400, 1310, 1215, 1175, 1105, 1070, 980, 915; \(^1\text{H NMR (D}_2\text{O, 270MHz)}\) \(\delta 1.99 (3\text{H, s, NCOCH}_3), 3.09 (1\text{H, broad q, }\nu=5.6\text{Hz, H-3}), 3.3-3.4 (2\text{H, broad d, H-2}), 3.96 (1\text{H, dd, }J=7.3\text{ and }2.5\text{Hz, H-5}), 4.33 (1\text{H, dd, }J=6\text{ and }2.5\text{Hz, H-4})\) and 5.11 (1H, d, \(J=7.3\text{ Hz, H-6})\); 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 \(\text{CHCl}_3 - \text{CH}_3\text{OH - cone aq ammonia (20 : 10 : 3)}\) to give a colorless amorphous solid of 4 (13 mg, 79%); mp 101 - 103°C; \([\alpha_l]^\beta +25.7° (c 0.34, \text{H}_2\text{O})\); IR (KBr) cm\(^{-1}\) 3450, 1690, 1600, 1430, 1400, 1280, 1240, 1160, 1100, 990, 920; \(^1\text{H NMR (D}_2\text{O, 400MHz)}\) \(\delta 2.91 (1\text{H, q, }\nu=4.5\text{Hz, H-3}), 3.02 (1\text{H, dd, }J=13.8\text{ and }4.5\text{Hz, H-2}), 3.40 (1\text{H, dd, }J=13.8\text{ and }4.5\text{Hz, H-2}), 3.88 (1\text{H, t, }\nu=8\text{Hz, H-5}), 3.94 (1\text{H, dd, }J=8\text{ and }4.5\text{Hz, H-4})\) and 4.73 (1H, d, \(J=8\text{Hz, H-6})\); MS (FAB) \(m/z\) 219 (M+H\(^+\)), 207, 160, 142, 115, 75, 57.

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**References**


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