New dihydrobenzoxanthone derivatives with bacterial neuraminidase inhibitory activity isolated from *Artocarpus elasticus*

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Abstract

Artocarpus elasticus is a popular fruit tree in the tropical regions. Primary screenings of methanol extracts of the root bark confirmed its potent inhibition of bacterial neuraminidase (BNA), which plays an essential role in the pathogenesis of many microbial diseases. Assessments of the responsible phytochemicals were conducted by isolating eight compounds (**1**-**8**) and two of them (**6** and **8**) were identified as new compounds. Among the isolates, the dihydrobenzoxanthones attained the highest BNA inhibition with IC_{50} values of 0.5 ~ 3.9 μM. Further investigation of the inhibitory mechanism by Lineweaver-Burk plots revealed the phytochemicals to function as reversible noncompetitive inhibitors. Fluorescence quenching showed their binding affinities were highly correlated with their inhibitory potential dose-dependently. Molecular docking experiments suggested the dihydrobenzoxanthones (**4** and **6**) as noncompetitive inhibitors of BNA with unique interaction with Tyr435 of BNA in comparison with the mother flavonoid (**7**).

Keywords: *Artocarpus elasticus*, dihydrobenzoxanthone, bacterial neuraminidase.

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44 ## 1. Introduction

45 Bacterial neuraminidase (BNA) is attractive because of its involvement in infections with
46 certain pathogenic bacteria, inflammation during infection, and the production of biofilms
47 [1]. The BNA enzyme (EC 3.2.1.18) hydrolases the glycosidic linkages of terminal sialic acid
48 residues in oligosaccharides, glycoproteins, and glycolipids [2]. This enzyme is strictly
49 specific with respect to the configuration of the glycosidic bond and relatively specific to the
50 position of this bond in the molecule, and usually cleaves α–2→3 and α–2→6 galactose [3].
51 The main function of BNA is to cleave the sialic acid residues of the cellular receptor to
52 which the newly formed particles are attached, after which they can penetrate new cells [4].
53 After infection, the sialic acid linkage plays a key role in the initiation of the inflammatory
54 cascade by producing explosive cytokines leading to sepsis [5]. A number of microorganisms
55 have been found to contain neuraminidase, which critically controls some of them [6]. For
56 example, *Clostridium perfringens* can infect the host cell only in the presence of
57 neuraminidase, because the sialic acid linkage is a target recognition point for the bacteria
58 [7]. Without this, enzyme infection would be limited to one round of replication, rare enough
59 to cause disease. Thus, the BNA is associated with regulating the release of toxins from
60 intestinal infections and the substrate generation needed for bacterial metabolism as a source
61 of energy for growth [7]. The variation of BNA permits the disease to evade human immune
62 responses and therefore necessitates the formulation of new inhibitors.

63 *Artocarpus elasticus* is a member of the Breadfruit (Maraceae) family, which contain
64 around 50 species in the genus [8]. The common name preferred in Malaysia is Terap, which
65 produces edible fruit and seeds that have foul-smelling pulp [9]. *Artocarpus* plants have been
66 used as traditional medicine in Southeast Asian countries; particularly, the leaves of *A.*
67 *elasticus* were used to treat tuberculosis and vomiting, the bark was used to treat
68 inflammation, and the latex was also used in dysentery [10,11]. Earlier reports showed that

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69 the major secondary metabolites in the roots and barks were prenylated flavonoids and
70 dihydrobenzoxanthone derivatives [8,10,13,14]. The main metabolites were found to be α-
71 glucosidase, xanthine oxidase, and human neutrophil elastase inhibitory, antibacterial,
72 anticancer, and antioxidant properties, and inhibition of DNA oxidation activities [10,14–18].
73 The metabolites of *A. elasticus*, particularly dihydrobenzoxanthones, have not been reported
74 to have BNA inhibitory potential.

75 The purpose of our study was the investigation of the potential of the
76 dihydrobezoxanthones isolated from *A. elasticus* to inhibit BNA. The isolation and
77 identification of responsible phytochemicals with spectroscopic methods resulted in the
78 characterization of eight compounds, of which two were new. The kinetic parameters of the
79 inhibitory mode were determined by double reciprocal plots of the Michaelis-Menten
80 equation. The binding affinities between the BNA enzyme and the identified compounds
81 were measured by conducting fluorescence (FS) quenching experiments. The binding site
82 was found by molecular docking experiments, which gave the explanation of BNA inhibition
83 by dihydrobenzoxanthones.

84 2. Materials and Methods

85 *2.1. Instruments and chemicals*

86 NMR spectra were recorded on a Bruker AM500 spectrometer (Karlsruhe, Germany).
87 Mass data detected using a mass spectrometer JEOL JMS-700 (Tokyo, Japan). Forte/R 100
88 (YMC, Kyoto, Japan) recycling HPLC and MPLC with a Triart C18 column (YMC, Japan)
89 were used for the isolation. Analytical grade methanol, acetonitrile, water, and acetic acid for
90 HPLC were purchased from Fisher Scientific (Korea). Enzyme assays were performed on a
91 SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, LLC., San Jose, USA).
92 *C. perfringens* neuraminidase (EC 3.2.1.18), quercetin, and 4-methylumbelliferyl-*α*-D-
93 acetylneuramic acid sodium salt were purchased from Sigma Aldrich (St. Louis, USA).

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94 *2.2. Plant material*

95 *Artocarpus elasticus* root bark was collected in December 2013 in Malaysia by Associate 96 Professor Dr. Mohd Azlan Nafiah. The specimen voucher (TM1016) was issued by the 97 Universiti Pendidikan Sultan Idris, Malaysia.

98 *2.3. Extraction and isolation*

99 The compounds were isolated according to previously published methods, with slight 100 modification, which led to the isolation of new compounds [16]. Extraction of *A. elasticus* 101 bark (300 g) with methanol (10 L) yielded a crude gum with a reddish-brown color (32 g), 102 from which then obtained the chloroform extract by liquid-liquid fractionation (17 g). 103 Chloroform extract was fractionated by MCI GEL CHP20P (30 × 5 cm, 75−150 μm, 500 g) 104 column with methanol in water (20% → 100%) resulting in the fractions A1−10. The 105 fractions A6-10 (around 5 g) were subjected by MPLC (using an ODS column 25 × 3 cm, S-106 10 μm, 12 nm, YMC) by the solvents system methanol in water (0 → 100 %) to produce the 107 subfractions B1-100. The subfractions B20−40, around 2 g, were subjected to recycling 108 HPLC (ODS, 25 × 3 cm, S-5 μm, 12 nm, YMC), to isolate compounds **5** (22 mg), **6** (18 mg), 109 and **8** (25 mg). The same, recycling HPLC was used to process subfractions B40-50, 110 approximately 3 g, to isolate dihydrobenzoxanthone **3** (32 mg), **4** (27 mg), and **7** (19 mg). 111 Subfractions B50-65 (3 g) were similarly recycled on HPLC to give compounds **1** (35 mg) 112 and **2** (26 mg). The detailed spectroscopic data are as follows.

113 *Artoindonesianin W (**1**)*: Brown amorphous powder; Mp 168-170 °C; $[\alpha]_D^{20}$ +77.5 (c 0.1, 114 MeOH). UV (MeOH) λ_{max} (log ε) 205 (4.02), 230 (4.08), 250 (4.08), 260 (4.07), 275 (4.05), 115 375 (4.02); IR (KBr) 3430, 2927, 1655, 1598 cm^{-1}; FABMS, *m*/*z* 383 $[M+H]^+$; HREIMS, *m*/*z* 116 383.1149 $[M+H]^+$ (calcd for $C_{25}H_{26}O_8$ 383.1053); ^1H NMR (500 MHz, acetone-d_6) δ 1.78 117 (3H, s, H-13), 2.43 (1H, dd, *J* = 15.98 Hz, H-9a), 3.39 (1H, dd, *J* = 15.98 Hz, H-9b), 3.89 (3H,

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118 s, 7-OMe), 3.99 (1H, d, J = 6.3 Hz, H-10), 4.29 (1H, s, H-12a), 4.65 (1H, s, H-12b), 6.29 (1,
119 s, H-6), 6.50 (1H, s, H-3′), 6.65 (1H, s, H-8); ^{13}C NMR (125 MHz, acetone-d_6) δ 21.97 (C-13),
120 22.39 (C-9), 38.01 (C-10), 56.39 (7-OMe), 93.17 (C-8), 98.69 (C-6), 103.84 (C-3′), 105.64
121 (C-4a), 106.56 (C-1′), 111.67 (C-3), 111.88 (C-12), 129.42 (C-6′), 136.93 (C-5′), 145.37 (C-
122 11), 151.16 (C-4′), 151.40 (C-2′), 157.67 (C-8a), 161.85 (C-2), 162.92 (C-5), 166.09 (C-7),
123 181.09 (C=O, C-4).

124 *Artobiloxanthone (**2**)*: Brown gum; EIMS *m*/*z* 434 [M]$^+$; HREIMS *m*/*z* 434.1363 [M]$^+$
125 (calcd for $C_{25}H_{22}O_7$ 434.1366); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.51 (3H, s, H-17), 1.54
126 (3H, s, H-18), 1.86 (3H, s, H-13), 2.51 (1H, dd, J = 15.9 Hz, H-9a), 3.47 (1H, dd, J = 15.9 Hz,
127 H-9b), 4.07 (1H, brs, J = 6.2 Hz, H-10), 4.38 (1H, s, H-12a), 4.73 (1H, s, H-12b), 5.73 (1H,
128 d, J = 10.0 Hz, H-15), 6.19 (1H, s, H-6), 6.67 (1H, s, H-3´), 6.99 (1H, d, J = 10.0 Hz, H-14),
129 13.45 (1H, s, 5-OH); ^{13}C NMR (125 MHz, acetone-d_6) δ 21.99 (C-12), 22.27 (C-9), 28.20 (C-
130 18), 28.54 (C-17), 38.14 (C-10), 78.71 (C-16), 99.78 (C-6), 102.03 (C-8), 103.7 (C-3′),
131 105.51 (C-4a), 107.03 (C-1′), 111.43 (C-3), 111.91 (C-13), 116.14 (C-14), 127.88 (C-6′),
132 129.88 (C-15), 136.68 (C-5′), 145.35 (C-4′), 150.92 (C-11), 151.57 (C-2′), 152.31 (C-8a),
133 159.48 (C-7), 161.80 (C-5), 162.57 (C-2), 181.27 (C=O, C-4).

134 *Artoindonesianin P (**3**)*: Brown amorphous powder; EIMS *m*/*z* 368 [M]$^+$; HREIMS *m*/*z*
135 368.0899 [M]$^+$ (calcd for $C_{20}H_{16}O_7$ 368.0896); ^{1}H NMR (500 MHz, acetone-d_6) δ 1.28 (3H, s,
136 H-13), 1.59 (3H, s, H-12), 2.29 (1H, t, J = 15.2 Hz, H-9a), 3.11 (1H, dd, J = 15.2 Hz, H-9b),
137 3.34 (1H, dd, J = 15.2 Hz, H-10), 6.16 (1H, s, H-6), 6.23 (1H, s, H-3′), 6.39 (1H, s, H-8); ^{13}C
138 NMR (125 MHz, acetone-d_6) δ 21.18 (C-9), 23.07 (C-12), 28.54 (C-13), 50.05 (C-10), 94.44
139 (C-11), 95.23 (C-8), 100.11 (C-6), 105.22 (C-1′), 105.27 (C-4a), 103.73(C-3′), 112.96 (C-3),
140 133.92 (C-6′), 138.35 (C-5′), 147.88 (C-4′), 152.16 (C-2′), 158.57 (C-8a), 162.63 (C-5),
141 163.16 (C-2), 165.39 (C-7), 181.98 (C=O, C-4).

142 *Cycloartobiloxanthone (**4**)*: Yellowish brown amorphous powder; EIMS *m*/*z* 434 [M]$^+$;

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143 HREIMS *m*/*z* 434.1364 $[M]^+$ (calcd for $C_{25}H_{22}O_7$ 434.1366). ^{1}H NMR (500 MHz, acetone-d_6)
144 δ 1.41 (3H, s, H-13), 1.51 (3H, s, H-17), 1.53 (3H, s, H-18), 1.74 (3H, s, H-12), 2.41 (1H, t, *J*
145 = 15.5 Hz, H-9a), 3.26 (1H, dd, *J* = 15.2 Hz, H-9b), 3.47 (1H, dd, *J* = 15.2 Hz, H-10), 5.74
146 (1H, d, *J* = 9.95 Hz, H-15), 6.21 (1H, s, H-6), 6.49 (1H, s, H-3′), 6.99 (1H, d, *J* = 9.95 Hz, H-
147 14). ^{13}C NMR (125 MHz, acetone-d_6) δ 20.61 (C-9), 23.10 (C-13), 28.51 (C-18), 28.36 (C-
148 17), 28.71(C-12), 47.78 (C-10), 78.99 (C-16), 93.97 (C-11), 100.19 (C-6), 102.14 (C-8),
149 105.13 (C-4a), 105.64 (C-1′), 105.64 (C-3′), 112.95 (C-3), 116.21 (C-14), 128.16 (C-15),
150 133.96 (C-6′), 138.17 (C-5′), 147.30 (C-4′), 151.84 (C-2′), 152.31 (C-8a), 159.77 (C-5),
151 161.78 (C-2), 162.88 (C-7), 181.69 (C=O, C-4).

152 *Artorigidinone B (**5**)*: Brown amorphous solid; EIMS *m*/*z* 502 $[M]^+$; HREIMS *m*/*z*
153 502.1994 $[M]^+$ (calcd for $C_{30}H_{30}O_7$ 502.1992). ^{1}H NMR (500 MHz, acetone-d_6) δ 1.32 (3H, s,
154 H-13), 1.42 (3H, s, H-17), 1.54 (3H, s, H-23), 1.62 (3H, s, H-22), 1.65 (3H, s, H-12), 1.72
155 (2H, m, H-18), 2.08 (2H, m, H-19), 2.35 (1H, t, *J* = 15.5, 15.2 Hz, H-9a), 3.18 (1H, dd, *J* =
156 7.15, 15.2 Hz, H-9b), 3.39 (1H, dd, *J* = 7.15, 15.2 Hz, H-10), 5.11 (1H, d, *J* = 6.85 Hz, H-10),
157 5.63 (1H, dd, *J* = 2.25, 10.0 Hz, H-15), 6.13 (1H, s, H-6), 6.40 (1H, s, H-3′), 6.95 (1H, dd, *J* =
158 1.75, 10.0 Hz, H-14). ^{13}C NMR (125 MHz, acetone-d_6) δ 16.77 (C-23), 19.46 (C-9), 21.97
159 (C-13), 22.53 (C-19), 24.90 (C-22), 25.25 (C-17), 28.51 (C-12), 41.29 (C-18), 46.53 (C-10),
160 80.37 (C-16), 92.87 (C-11), 95.85 (C-6), 100.8 (C-8), 104.05 (C-3′), 104.40 (C-4a), 104.50
161 (C-1′), 111.83 (C-3), 115.50 (C-14), 123.93 (C-15), 131.28 (C-21), 132.95 (C-6′), 137.03 (C-
162 5′), 145.04 (C-2′), 150.58 (C-4′), 151.15 (C-8a), 159.01 (C-7), 160.55 (C-2), 161.79 (C-5),
163 180.52 (C=O, C-4).

164 *Artonin W (**6**)*: Dark brown solid; UV (MeOH) λ_{max} (log ε) 230 (4.65), 250 (4.65), 280
165 (4.59), 340 (3.95), 400 (3.93); IR (KBr) 3300, 2920, 2850,1650, 1740, 1600, 1570, 1460,
166 1350, 1270, 1160, 1130, 1110, 1020 cm^{-1};

167 *Norartocarpetin (7)*: Yellowish white amorphous powder; EIMS *m*/*z* 286 $[M]^+$; HREIMS

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168 m/z 286.0472 $[M]^+$ (calcd for $C_{15}H_{10}O_6$ 286.0477). ^1H NMR (500 MHz, acetone-d_6) δ 6.23
169 (1H, d, J = 2.1 Hz, H-6), 6.49 (1H, d, J = 2.1 Hz, H-5′), 6.55 (1H, dd, J = 2.3, 8.75 Hz, H-3′),
170 6.61 (1H, d, J = 2.3 Hz, H-8), 7.07 (1H, s, H-3), 7.83 (1H, d, J = 8.75 Hz, H-6′). ^{13}C NMR
171 (125 MHz, acetone-d_6) δ 93.63 (C-8), 98.53 (C-6), 103.46 (C-3′), 104.33 (C-10), 107.71 (C-
172 5′), 108.24 (C-3), 109.88 (C-1), 130.06 (C-6′), 157.95 (C-2′), 158.40 (C-5), 161.65 (C-9),
173 161.92 (C-2), 162.45 (C-4′), 163.86 (C-7), 182.54 (C=O, C-4).

174 *Atrocarmin N (**8**):* White amorphous powder; UV (MeOH) λ_{max} (log ε) 230 (4.41), 250
175 (4.41), 280 (4.34), 360 (3.30); IR (KBr) 3390, 2920, 2850, 1730, 1650, 1460, 1375, 1250,
176 1100 cm^{-1}

177 *2.4. Bacterial neuraminidase inhibitory activity assay*

178 Neuraminidase activity was evaluated using a slight modification of a previously
179 described methods [19,20], using 4-methylumbelliferyl-N-acetyl-α-$_D$-neuraminic acid sodium
180 salt hydrate as the substrate and 50 mM sodium acetate (pH 5.0) as buffer. The FS was
181 measured using a UV-Vis spectrophotometer at 37 ºC, where at an emission wavelength was
182 450 nm and an excitation wavelength was 365 nm. Briefly, in a 96-well black immuno-
183 microplate (SPL Life Science, Korea), 20 μL of an aqueous solution of the substrate (1 mM)
184 was mixed in 160 μL of buffer. After this, 10 μL of the inhibitors and 10 μL of enzyme (0.2
185 units/mL) were immediately added to the mixture. The inhibitor concentration leading to 50%
186 activity loss (IC_{50}) was calculated using the following Eq. (1):

187 $$\text{Activity (\%)} = [1 + ([I] / IC_{50})] \times 100 \quad (1)$$

188 *2.5 Bacterial neuraminidase inhibitory kinetic assay*

189 Using the same approach as in the activity experiments, the kinetic behavior of the
190 enzyme was determined using the same substrate with different concentrations of 0.5, 1, and
191 2 mM with different concentrations of inhibitors. The data were analyzed using a Sigma Plot
192 (Chicago, USA) to determine the variables of curves. Lineweaver-Burk plots were employed

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193 to determine kinetic values such as the Michaelis-Menten (K_m) constant and maximum
194 velocity (V_{max}). The dissociation constants of the enzyme and inhibitors (K_i) were calculated
195 from the Dixon plots.

196 *2.6 Measurement of binding affinity to the enzyme*

197 To 180 μL of buffer with 10 μL of 0.5 unit/mL neuraminidase in the 96-well black
198 immuno-plates, 10 μL of the inhibitors with different concentrations (15.6 ~ 250 μM) were
199 added. The spectra of the fluorescent emissions were recorded from 300 to 400 nm with the
200 emission slits of the spectrophotometer adjusted to 2.0 nm at an excitation wavelength of 260
201 nm.

202 *2.8. Molecular docking study*

203 The crystal structure of *C. perfringens* neuraminidase (PDB ID: 5TSP), complexed with
204 the inhibitor CHES, was obtained from the RCSB Protein Data Bank (www.rcsb.org) [21].
205 The resolution of the crystal structure was 1.24 Å. Subsequently, the protein was prepared by
206 using the *Clean Protein* tool of Discovery Studio v2018 (DS). The water molecules and
207 heteroatoms were removed and the protein was minimized employing the *Minimize and*
208 *Refine Protein* module available with DS. The molecular docking study was carried out with
209 the three-dimensional (3D) structures of three inhibitors, compounds **7**, **4**, and **6**, prepared
210 using the *Sketching* tool (PubChem Sketcher v2.4) after which their geometry was optimized
211 employing the Avogadro program. The binding modes of the three inhibitors at the *C.*
212 *perfringens* neuraminidase docking site were explored using the GOLD Suite 5.2.2
213 (Cambridge Crystallographic Data Center, UK) using genetic algorithms [22].
214 Correspondingly, the docking site was defined within 10 Å around the allosteric site with
215 reference to the quercetin docking study as reported previously [23]. In molecular docking
216 simulations, each ligand generated 50 conformations keeping all other default parameters
217 invariable. The optimal binding modes of the inhibitor with the enzyme were designated from

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218 by the highest GOLD fitness score.

219 3. Results and Discussion

220 *3.1. Isolation of BNA inhibitors from A. elasticus root bark*

221 The methanolic extract of the dried root bark of *A. elasticus* was separated by solvent 222 partition to obtain a chloroform portion (IC_{50} = 32 μg/mL) with strong BNA inhibition 223 potential. Activity guided fractionation resulted in the isolation of eight compounds by 224 repeated column chromatography, MPLC, and recycling HPLC. Compounds **1-5** and **7** were 225 identified as artoindonesianin W, artobiloxanthone, artoindonesianin P, 226 cycloartobiloxanthone, artorigidinone B, and norartocarpetin (Fig. 1)by spectroscopic 227 analysis (Supplementary material Fig. S1-19, S27-29) and comparison with previous reports 228 [16,24,25]. The other two compounds (**6** and **8**) emerged as new phytochemicals and were 229 named artonin W and artocarmin N, respectively.

230 Compound **6** was obtained as a dark brown solid with the molecular formula $C_{35}H_{38}O_7$ 231 and 17 degrees of unsaturation, as confirmed by HREIMS of *m*/*z* 570.2612 (calcd. 570.2618). 232 ^{1}H and ^{13}C NMR data in combination with the molecular formula indicated a hexacyclic 233 skeleton with two aromatic rings, corresponding dihydrobenzoxanthone **6** (Table 1, Fig. 2, 234 and Supplementary material Fig. S20-26). The presence of 6 methines (sp^2), 4 methylenes 235 (sp^3), 7 methyls, and 18 quaternary carbons in **6** was elucidated by DEPT analysis. The allylic 236 endomethylene H9a/9b (δ_H 2.30/δ_H 3.15), hydrogen bonding hydroxyl group C5-OH (δ_H 13.7) 237 and *α*, *β*-unsaturated carbonyl C4 (δ_C 180.5) were consistent with the features of the 238 dihydrobenzoxanthone skeleton and ring D. The 2,2-dimethyldihydropyran motif (ring F) 239 was deduced by HMBC correlations between oxygenated quaternary carbon C11 (δ_C 92.8) 240 and two methyl carbons H12/13 (δ_H 1.58/δ_H 1.28). The conjunction of rings D and F were 241 reduced by HMBC correlations of H10 (δ_H 3.35) and methyl carbons C12/13 (δ_C 27.4/δ_C 242 22.6). The furan motif (ring E) was deduced from the AB coupling of H15 (δ_H 5.58) and H14

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243 (δ_H 6.93) and the oxygenated quaternary carbon C16 (δ_C 80.3). The 2-methyl-2-pentenyl
244 group was easily confirmed by successive COSY connectivities from H18 (δ_H 1.68) to
245 H22/23. The position of the pentenyl group was confirmed by the strong HMBC correlation
246 of H18 and C16. The other 3,3-dimethylallyl group on C6 was confirmed by successive
247 COSY connectivities (H24→ H27/28) and HMBC correlation H24 (δ_H 3.27) to C5/C7 (δ_C
248 158.7/δ_C 156.5). The isolated methine H3′ (δ_H 6.40, s) was confirmed by HMBC correlation
249 between H3′ and the oxygenated aromatic carbons C2′/C5′ (δ_C 146.0/δ_C 137.0). Thus,
250 compound **6** was determined as 1,3,8-trihydroxy-5,5,11-trimethyl-9-(3-methylbut-2-en-1-yl)-
251 11-(4-methylpent-3-en-1-yl)-5a,6-dihydro-5*H*,7*H*,11*H*-benzofuro[3,4-*bc*]pyrano[3,2-
252 *h*]xanthen-7-one and named artonin W.

253 On the HREIMS spectrum of compound **8**, the molecular ion [M^+] 340.0945 (calcd
254 340.0947) corresponded to the molecular formula $C_{19}H_{16}O_6$. A tricyclic xanthone skeleton
255 was deduced by counting 12 degrees of unsaturation between 9 olefin functionalities, and the
256 hydrogen bonding hydroxyl group C5-OH (δ_H 12.8). The ^{1}H NMR spectrum had two isolated
257 aromatic signals (δ_H 6.34, 6.44), an isopentyl motif (5.25, 5.30, and 2.17, each *s*), and an
258 acetyl group (δ_H 2.99, 3H, *s*) [25]. The location of the acetyl group on C4 was confirmed with
259 the HMBC correlation of H17 and C16, and the hydrogen bonding hydroxyl group C15-OH
260 (δ_H 14.6). The isopenthyl motif was placed on C10 because of the HMBC correlation of H13
261 (δ_H 2.17) and C9 (δ_C 131.7). The CH_3O motif on C7 was easily confirmed by the HMBC
262 correlation of CH_3O (δ_H 3.91) and C6 (δ_C 93.2). Therefore, the structure of compound **8** was
263 identified by spectroscopic data (Table 1 and Supplementary material Fig. S30-36) as 5-
264 acetyl-1,6-dihydroxy-3-methoxy-7-(prop-1-en-2-yl)-9*H*-xanthen-9-one and the compound
265 was named atrocarmin N.

266 *3.2. Bacterial neuraminidase inhibitory activity*

267 The bacterial neuraminidase (BNA) inhibitory activity was screened by following the

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268 hydrolysis of the fluorogenic substrate in the presence or absence of the test compounds [19].
269 The Michaelis-Menten constant (K_m = 100 μM) was determined for the enzyme concentration
270 (0.2 unit/mL) *vs.* the substrate concentrations (50 ~ 200 μM). All isolated
271 dihydrobenzoxanthones (**1**-**6**) exhibited extremely significant inhibition of BNA with IC_{50}s
272 ranging from 0.5 to 3.9 μM (Table 2 and Fig. 3A). This is the first report to confirm that the
273 dihydrobenzoxanthone structure is effective to inhibit BNA. Biogenetically, the
274 dihydrobenzoxanthone derivatives could arise from 3-prenylflavone by oxidative C-C′ bond
275 formation [26]. In comparison to norartocarpetin (flavone), the tested dihydrobenzoxanthones
276 were 10 ~ 60 fold active to BNA inhibition: **6** (IC_{50} = 0.5 μM) *vs.* **7** (IC_{50} = 33.4 μM).
277 Additionally, these compounds were much more effective inhibitors (6 ~ 40 fold) than
278 quercetin, which is considered to be the flavonoid with the highest number of hydroxyl
279 groups. The potency of these inhibitors (**1**-**6**) was affected by subtle changes in the structure,
280 with more effective inhibition observed when the pyran motif (ring E) was present. This is
281 apparent from a comparison with compound **4** (IC_{50} = 1.3 μM) and compound **3** (IC_{50} = 3.9
282 μM). The prenyl group in compound **5** (IC_{50} = 1.1 μM) and compound **6** (IC_{50} = 0.5 μM)
283 improved the inhibitory potency significantly. However, the newly isolated xanthone (**8**) has
284 moderate inhibitory potential with IC_{50} of 119.5 μM.

285 The kinetic study showed that all the tested compounds inhibited BNA in a dose-
286 dependent manner as shown in Fig. 3A. The dihydrobenzoxanthones (**1**-**6**) showed a similar
287 relationship between enzyme activity and concentration, and were shown to be reversible
288 inhibitors. An increase in the concentrations of inhibitor **6** resulted in a decrease of the slope
289 of the lines, forming a family of straight lines by the enzyme concentration. The fact that all
290 lines passed through the origin (Fig. 3B) indicated that **6** is a reversible inhibitor. We then
291 further investigated the inhibitory mechanism of inhibitors (**1**-**6**) using Lineweaver-Burk and
292 Dixon plots. The kinetic plots showed that dihydrobenzoxanthones (**1**-**6**) were

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293 noncompetitive inhibitors. Increasing the concentration of the inhibitors resulted in a family
294 of lines with a common intercept on the x-axis (Fig. 3C-3F, Supplementary materials Fig.
295 S37-S38). This observation was in agreement with noncompetitive inhibitory behavior,
296 typified by K_m remaining constant and V_{max} decreasing [27]. The K_i values of compounds
297 were determined on the basis of the Dixon plots (Table 2 and Supplementary materials Fig.
298 S37-38). As shown in Fig. 3D and 3F the K_i values of compounds **6** and **7** were determined to
299 be 0.5 μM and 30.8 μM, respectively.

300 *3.3. Binding affinity to enzyme*

301 Strong correlation of the inhibitory potencies with the binding affinities to the enzyme
302 would be reasonable evidence for enzyme inhibition. The binding affinity of an inhibitor to
303 the BNA enzyme was assessed by measuring the FS quenching of the intrinsic protein
304 because the BNA enzyme has strong FS intensity resulting from nine Trp, twenty-two Tyr,
305 and eight Phe residues [19]. None of the other components of the reaction mixture are able to
306 affect the FS intensity under the measurement conditions (i.e., emission from 300 to 400 nm).
307 FS quenching is described by the Stern-Volmer Eq. (2):

308 $$F_0 - F = 1 + K_{SV}[\mathrm{Q}] \quad (2)$$

309 where F_0 and F are the FS intensities in the presence of different concentrations of the
310 quencher $[Q]$. K_{SV} is the Stern-Volmer quenching constant that can reflect the quenching
311 duration. Quenching-induced extinction of the FS intensity was shown by all isolated
312 compounds according with the concentration and inhibitory potential. For the determination of
313 the correlation between the change of the FS intensities and the quencher amount can be used
314 following Eq. (3):

315 $$\log[(F_0 - F)/F] = \log K_A + n\log[Q]_f \quad (3)$$

316 where $[Q]_f$ is the concentration of the quenchers; K_A is the binding constant for the
317 acceptable fluorophores; n is the number of binding sites per BNA molecule. The values of K_A

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318 and n can be calculated by the linear plots of Eq. (3), obtained by log $[(F_0 - F)/F]$ *vs.* log $[Q]_f$.
319 The values of n approximately equals to 1, which is indicate that binding site existing in BNA
320 for inhibitors is only one. The FS spectra in Fig. 4A-C and Supplementary Materials Fig. S39
321 show that the FS intensities decreased in proportion to the inhibitor concentrations. A
322 comparison of the insets in Fig. 4A and 4B shows that the FS reduction ratios correspond with
323 the inhibitory activities in the case of **6** (IC_{50} = 0.5 μM) *vs.* **3** (IC_{50} = 3.9 μM). The binding
324 constants (K_{SV}) of dihydrobenzoxanthones (**1**-**6**) were measured in the range of 1.26 ~ 5.07×10^5
325 $L\cdot mol^{-1}$ by using Eq. (2). The values of K_{SV} are in agreement with the order of their inhibitory
326 potencies (IC_{50}) as shown in Table 3 and Fig. 4D. The most active compound **6** (IC_{50} = 0.5 μM)
327 had the highest K_{SV} value of 5.07×10^5 $L\cdot mol^{-1}$.

328 *3.4. Molecular docking study*

329 Molecular docking is an excellent method to predict the binding mode of a given small
330 molecule at the active site of a protein [28]. Prior to the docking study, we collected three-
331 dimensional information about the protein structure and prepared the structure for use in
332 docking studies. Molecular docking simulation was employed to investigate the proper
333 binding modes of compounds **7**, **4**, and **6** at the binding pocket of *C. perfringens*
334 neuraminidase. Interestingly, the *C. perfringens* neuraminidase target has two defined
335 binding sites, the catalytic site and the potential allosteric site [19]. The second binding site
336 was used for the current study because these new compounds are noncompetitive inhibitors.

337 The molecular docking results showed that the docking scores of compounds **7**, **4**, and **6**
338 were 44.3, 47.9, and 57.5, respectively. The highest docking score of compound **6** indicated
339 that its binding affinity for the pocket is the highest. Subsequently, the binding structures
340 were refined by minimizing the energy of the three best-docked complexes using DS and the
341 resulting structures were thoroughly examined for the binding modes/intermolecular
342 interaction analyses. The results revealed that compound **7** has six conventional hydrogen

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343 bonds with Glu369, Tyr320, Asn386, Asn401, Ala402, and Asn448, one carbon hydrogen
344 bond with Gly404, two π-alkyl interactions with Leu371, and one π-sigma interaction with
345 Phe362. Several other residues were found to interact with compound **7** by van der Waals
346 interactions to hold the compound in the binding pocket (Fig. 5A and 6A). Compound **4** has
347 three conventional hydrogen bonds with Tyr435, Pro446, and Asn448, and two π-alkyl
348 interactions with Leu371 and Ile416. Several other residues interact with compound **4** by van
349 der Waals interactions to accommodate the compound in the binding pocket (Fig. 5C and
350 6B). Compound **6** has three conventional hydrogen bonds with Asn386, Tyr435, and Glu445,
351 one carbon hydrogen bond with Ser444, many π-alkyl interactions, one π-anion interaction
352 with Glu445, and several van der Waals interactions (Fig. 5E and 6C).

353 In the electrostatic surface model, the binding patterns of the more potent compounds **4**
354 and **6** are significantly different from those of compound **7** (Fig. 5B, 5D, 5F). The former two
355 compounds undergo additional interactions between the extra moiety of the compound and
356 the pocket entrance region that includes Tyr435. Subsequently, their overall main plane axis
357 angles are shifted or different from those of compound **7**. Based on this observation, it can be
358 inferred that the interaction of the inhibitor with the tyrosine may play an important role in
359 enhancing the inhibitory potency. The intermolecular interaction diagram (Fig. 6B and 6C)
360 clearly shows that compound **6** underwent additional interactions with the bottom of the
361 pocket gorge between the extra ethylene group and Tyr430, Ala425, His346, and Tyr320 etc.
362 We consider this additional interaction to contribute to the superior affinity of this compound
363 compared with compound **6**.

364 4. Conclusion

365 This study led to the discovery that the dihydrobenzoxanthones of *A. elasticus* are mainly
366 responsible for neuraminidase inhibition, which is associated with pathogenesis from

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367 microbial diseases. The structures of these dihydrobenzoxanthones, including the new
368 compounds **6** and **8** were fully characterized by spectroscopic data. Their ability to inhibit
369 BNA were confirmed by different techniques involving enzyme kinetics, FS quenching, and
370 molecular docking experiments. The new compounds were all found to be reversible
371 noncompetitive inhibitors. The inhibitory potencies (IC_{50}) were doubly confirmed by
372 determining the binding affinities (K_{SV}) using a FS quenching assay. The unique binding sites
373 and binding residues of the dihydrobenzoxanthones were suggested by molecular docking
374 experiments. This is the first report that dihydrobenzoxanthones are an appropriate skeleton
375 for BNA inhibition.

376

377 **Declaration of Competing Interest**

378 The authors declare that they have no conflict of interests.

379

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386

387 **Appendix A. Supplementary Material**

388 Spectroscopic data (^{1}H NMR, ^{13}C NMR, 2D NMR and MS data), BNA enzyme kinetics
389 and FS effect on enzyme of isolated compounds.

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