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Prenylated pterocarpans as bacterial neuraminidase inhibitors

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ABSTRACT

During the course of a neuraminidase inhibitor screening program on natural products, four new (**6**, **8**, **11**, and **12**) and eleven known (**1–5**, **7**, **9–10**, and **13–15**) pterocarpan derivatives were isolated as active principles from the EtOAc extract of the stem bark of *Erythrina abyssinica*. Their structures were identified by spectroscopic data analyses. All isolates exhibited significant inhibitory effects on the neuraminidases from *Clostridium perfringens* and *Vibrio cholerae* with IC_{50} values ranging from 1.32 to 77.10 μM and 0.35 to 77.73 μM, respectively. The isolates (**1–3**, **5–8**, **10**, and **13–15**), which possessed noncompetitive inhibition modes in kinetic studies, showed stronger activity against *C. perfringens* neuraminidase (IC_{50} 1.32–19.82 μM) than quercetin (IC_{50} 25.34 μM), which was used as the positive control. In contrast, compounds **4** and **9** behaved as competitive inhibitors and were displayed less effective (IC_{50} 26.39–33.55 μM). Furthermore, calopocarpine, as a neuraminidase inhibitor, produced a decrease of *V. cholerae* adhesion to the host cell. Overall, these results suggest that neuraminidase inhibitors can be used in the development of new treatments to combat infectious diseases.

1. Introduction

Sialidases (neuraminidases) catalyze the removal of sialic acid from a range of sialo-glycoconjugates, and their physiological significance had been linked with the pathogenicity of various bacterial strains.[1] Sialidase activity has been observed in bacteria isolated from a variety of infections, and is often one of many virulence factors in important diseases caused by bacteria.[2] The location of sialic acids at the terminal of various carbohydrate complexes in animal cells has been exploited by a broad spectrum of microbial pathogens. Certain pathogens possess proteins that recognize sialic acid for cell attachment, and many of these pathogens have acquired sialidases to aid in their pathogenesis and/or nutritional requirements.[1]

Many pathogenic and nonpathogenic sialidase-producing bacteria employ sialic acid as a carbon and energy source, and have a permease function to transport the sugar inside the cell. As *Clostridium perfringens* is responsible for the production of life-threatening gas gangrene (myonecrosis) in wound infections and enterotoxemia in humans, its infections are characterized by the release of large amounts of toxins and enzymes that can cause massive tissue destruction in the host, placing the organism into the category of flesh-eating microbes.[3] These bacteria produce large amounts of sialidase which can be detected in the serum and wounds of infected patients.[4]

Cholera is an acute bacterial infection of the intestine caused by the ingestion of food or water contaminated with *Vibrio cholerae*. *V. cholerae* neuraminidase plays a significant role in the pathogenesis of cholera by removing sialic acid from higher order gangliosides to create G_{M1}, which is the binding site for the cholera toxin.[5] There has been little research effort focused on the development of bacterial sialidase inhibitors.[6] Bacterial sialidases are clearly important virulence factors and considerably more study is needed. One obvious requirement is specific and high affinity inhibitors that can block the sialidases in order to allow an assessment of their importance.[1]

During the course of an anti-bacterial screening program on natural products, the extract of the stem bark of *Erythrina abyssinica* showed significant inhibitory activity against both neuraminidases from *C. perfringens* and *V. cholerae*. The genus *Erythrina* (Leguminosae) comprise approximately 110 species of trees and shrubs that are distributed widely in tropical and subtropical regions with representative species being used in indigenous medicine.[7] Alkaloids, benzofurans, flavonoids, chalcones, and pterocarpans have been reported to be constituents of this genus, which have been found to possess a wide range of antioxidant, antimicrobial, and anti-inflammatory activities.[8] To determine the bacterial neuraminidase inhibitors, the EtOAc extract was subjected to a

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succession of chromatographic procedures, including silica gel chromatography, RP-18, and HPLC, to afford a series of pterocarpanoid derivatives as active principles. This paper reports the isolation, structural elucidation, and inhibitory effects of these compounds on the specific neuraminidase enzymes.

2. Result and discussion

A bioassay-guided investigation of the EtOAc extract of the stem bark of *E. abyssinica* resulted in the isolation of four new pterocarpans (**6**, **8**, **11**, and **12**) and 11 known ones (**1**–**5**, **7**, **9**, **10**, and **13**–**15**). The structures of known compounds were determined to be demethylmedicarpin (**1**),[9] neorautenol (**2**),[10] isoneorautenol (**3**),[11] phaseollin (**4**),[12] eryvarin D (**5**),[13] calopocarpin (**7**),[10] erysubin D (**9**),[14] erysubin E (**10**),[14] cristacarpin (**13**),[15] sophorapterocarpan A (**14**),[16] and erystagallin A (**15**),[17] from a comparison of the physical and spectroscopic data (IR, UV, $[\alpha]_D$, NMR, and MS) with those reported in the literature.

Compound **6** was isolated as a yellow amorphous powder with the molecular formula $C_{21}H_{18}O_5$, as determined from the HREI mass spectrum ($[M]^+$, m/z 350.1154). The IR spectrum of compound **6** suggested the presence of a hydroxy group at 3418 cm^{-1}, 2924 (C–C), 1708, 1622 (C=O), 1418 (aromatic absorption), and 1265 (ether function) cm^{-1}. The UV spectrum showed absorption maxima at 210, 214, 242, 299, 336, and 352 nm. The ^1H NMR spectrum showed a characteristic singlet signal at C-6 [δ_H 5.75 (2H, s)], which is reminiscent of a pterocarpene skeleton,[16,19,20] which is supported by carbon resonances at δ_C 67.7, 108.6, and 145.1 in the ^{13}C NMR spectrum. In addition, the ^1H NMR spectrum displayed two singlet signals at δ_H 6.45 and 7.82, a pair of *ortho*-coupled aromatic protons at δ_H 6.89 and 7.18 (each 1H, d, J = 8.4 Hz), and a prenyl group [δ_H 1.67, 1.88 (each 3H, s), 3.64 (2H, d, J = 7.2 Hz), and 5.40 (1H, m)]. These results suggested it to be structurally distinct form of erycristagallin, a pterocarpan isolated from the Bolivian coral tree, *Erythrina crysta-galli*,[18a] and from the bark of *Erythrina variegata*.[18b] A comparison of the ^1H and ^{13}C NMR data of compound **6** strongly suggested the presence of an aldehyde group at δ_H 9.94 (1H, s) and a corresponding carbon resonance at δ_C 196.1 instead of an prenyl group in erycristagallin.[18] The aldehyde group was attached to the C-2 position according to an HMBC experiment from the correlations between the aldehyde proton at δ_H 9.94 to C-2 (δ_C 116.6) and C-3 (δ_C 164.9) (Fig. 1). The presence of a prenyl group at the C-10 position was also confirmed by HMBC showing that the methylene protons at C-1′ (δ_H 3.64) were correlated with the sp^2 quaternary carbons at C-9 (δ_C 156.2), C-10 (δ_C 113.1), and C-10a (δ_C 154.0). Therefore, compound **6** was characterized as 3,9-dihydroxy-10-prenyl-2-formyl-6a,11a-dehydroxypterocarpan named erythribyssin O.

Compound **8** was obtained as a white amorphous powder with the molecular formula, $C_{25}H_{28}O_5$, as determined from the HREI mass spectrum ($[M]^+$, m/z 408.1937). The IR spectrum of compound **8** suggested the presence of a hydroxy group at 3425 cm^{-1}, 2926 (C–C), and 1599–1450 (aromatic ring) cm^{-1}. The ^1H and ^{13}C NMR data (Tables 1 and 2) of compound **8** resembled those of erysubin D (**9**),[14] which was also obtained in this study. Two set of aromatic protons were present for a 1,2,3,4-tetrasubstituted benzene [δ_H 6.95 (1H, d) and 6.39 (1H, d)] and a 1,2,4,5-tetrasubstituted benzene [δ_H 7.20 (1H, s) and 6.22 (1H, s)]. The ^{13}C NMR spectrum showed 25 carbons, 15 were assigned to the pterocarpan skeleton, and five carbon signals were assignable to the prenyl group [δ_C 131.3 (C), 123.7 (CH), 23.5 (CH_2), 25.9 (CH_3), and 18.0 (CH_3)]. The remaining five carbon signals [δ_C 78.2 (C), 70.0 (CH), 31.6 (CH_2), 26.3 (CH_3), and 21.7 (CH_3)] and the corresponding protons [δ_H 3.77 (1H, dd), 3.00 (1H, dd), 2.70 (1H, dd), 1.34 (3H, s), and 1.23 (3H, s)] were typical of a 3-hydroxy-2,2-dimethyldihydropyran moiety.[14,21,22] The HMBC correlations of compound **8** from the aliphatic proton H-4′ (δ_H 3.00, 1H, dd) to C-2 (δ_C 114.2) and C-3 (δ_C 156.0), and from the methylene proton of H-1″ (δ_H 3.26, 2H, d) to C-9 (δ_C 159.8) and C-10 (δ_C 112.0) indicated that the prenyl group was attached to C-10, and the 2,2-(3-hydroxy)-dimethylpyrano ring was fused to C-2 and C-3. Compound **8** contained two chiral centers at C-6a and C-11a, which possessed either *R*,*R* or *S*,*S* configurations according to the stereochemical environment around the C-6a and C-11a.[23] The absolute configuration of a pterocarpan compound may be presumed from the sign of its optical rotation.[23] The absolute config-

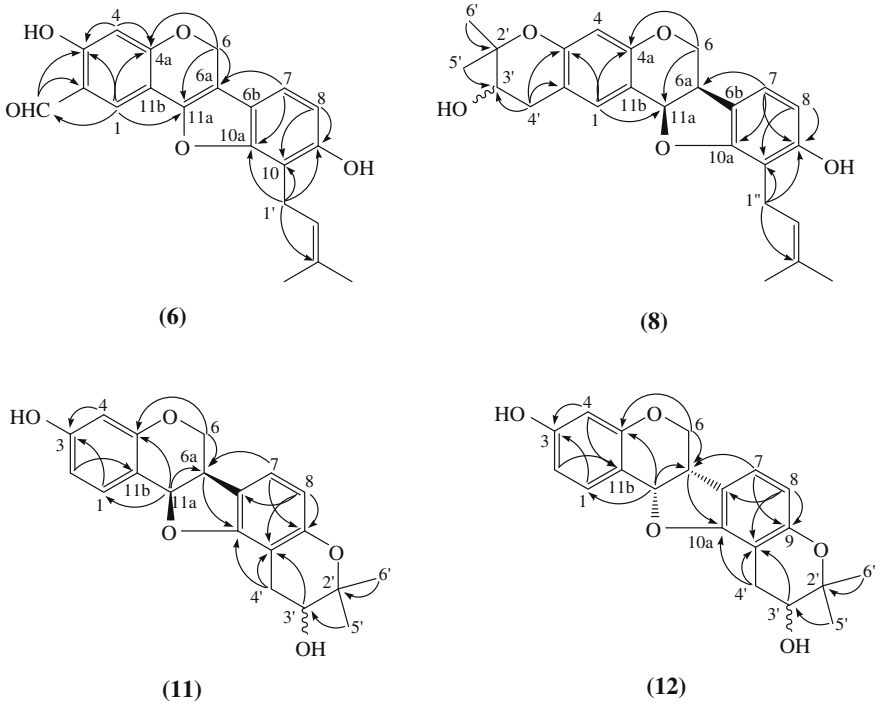


Figure 1. Selected key HMBC (H→C) correlations for the new compounds **6**, **8**, **11**, and **12**.

Table 1
^{1}H NMR spectroscopic data for new compounds **6**, **8**, **11**, and **12** in acetone-d_6

No.	**6**[a] δ_H (J in Hz)	**8**[a] δ_H (J in Hz)	**11**[b] δ_H (J in Hz)	**12**[b] δ_H (J in Hz)
1	7.82 (s)	7.20 (s)	7.34 (d, 8.5)	7.34 (d, 8.0)
2			6.55 (dd, 2.5, 8.5)	6.56 (dd, 2.5, 8.0)
3				
4	6.45 (s)	6.22 (s)	6.35 (d, 2.5)	6.36 (d, 2.5)
4a				
6	5.75 (2H, s)	4.22 (dd, 4.2, 10.2)	4.24 (m)	4.25 (dd, 10.5, 16.5)
		3.57 (t, 10.2)	3.58 (m)	3.56 (dd, 2.0, 16.5)
6a		3.54 (m)	3.58 (m)	3.55 (m)
6b				
7	7.18 (d, 8.4)	6.95 (d, 7.8)	7.03 (d, 8.5)	7.03 (d, 8.0)
8	6.89 (d, 8.4)	6.39 (d, 7.8)	6.28 (d, 8.0)	6.28 (d, 8.0)
9				
10				
10a				
11a		5.45 (d, 7.2)	5.52 (d, 6.5)	5.50 (d, 6.0)
11b				
1′	3.64 (d, 7.2)			
2′	5.40 (m)			
3′		3.77 (dd, 4.8, 7.8)	3.73 (dd, 5.5, 7.5)	3.75 (dd, 6.0, 7.5)
4′	1.67 (s)	3.00 (dd, 5.4, 15.6)	2.80 (dd, 5.5, 16.5)	2.90 (dd, 6.0, 16.5)
		2.70 (dd, 8.4, 15.6)	2.55 (dd, 7.5, 16.5)	2.46 (dd, 7.5, 16.5)
5′	1.88 (s)	1.23 (s)	1.30 (s)	1.33 (s)
6′	9.94 (s)	1.34 (s)	1.23 (s)	1.20 (s)
1″		3.26 (d, 6.6)		
2″		5.25 (m)		
3″				
4″		1.61 (s)		
5″		1.73 (s)		
2-CHO	9.94 (s)			

Compounds were measured in acetone-d_6 at 600 MHz[a] and 500 MHz[b] of ^{1}H NMR).

uration of compound **8** was assigned to the *R* form because the specific optical-rotation of compound **8** was −110.4 (*c* 0.02, MeOH).[21–23] Based on the above data, compound **8** was established as 9-hydroxy-10-prenyl-[2′,2′-(3′-hydroxy)-dimethylpyrano]-(5′, 6′:2,3)-(6a*R*,11a*R*)pterocapan, a new natural prenylated pterocapanoid, and named erythribyssin L.

Compound **11** was obtained as a yellowish amorphous powder with absorption bands at 3410 (OH), 2928 (C–C), 1566, 1468 (aromatic ring) and 1193 cm^{-1} in the IR spectrum. The HREIMS of compound **11** showed a molecular ion peak at *m/z* 340.1313 $[M]^+$ (calcd $C_{20}H_{20}O_5$ 340.1311). The ^{1}H and ^{13}C NMR spectra of compound **11** showed the characteristic signals of a pterocarpan skeleton as shown in Tables 1 and 2.[21–23] In addition, the ^{1}H NMR of compound **11** revealed an ABX-type aromatic spin system at [δ_H 7.34 (1H, d, J = 8.5 Hz), 6.55 (1H, dd, J = 2.0, 8.5 Hz), and 6.35 (1H, d, J = 2.0 Hz)], two *ortho*-coupled aromatic protons [δ_H 7.03 (1H, d, J = 8.5 Hz) and 6.28 (1H, d, J = 8.0 Hz)]. The ^{13}C NMR spectrum showed 20 carbons, of which 15 were assigned to the pterocapan skeleton. The remaining five carbon signals [δ_C 76.8 (C), 68.4 (CH), 26.1 (CH_2), 25.2 (CH_3), and 19.7 (CH_3)] and corresponding protons [δ_H 3.73 (1H, dd, J = 5.5, 7.5 Hz), 2.80 (1H, dd, J = 5.5, 16.5 Hz), 2.55 (1H, dd, J = 7.5, 16.5 Hz), 1.30 (3H, s), and 1.23 (3H, s)] were a typical of a 3-hydroxy-2,2-dimethyldihydropyran moiety.[14,21,22] The presence of the dihydropyran moiety was also evident from the EI mass spectrum, which showed the characteristic fragment at *m/z* 269 $[M-71]^+$, all of these observations resembled those of rautandiol A.[24] However, the HMBC experiments with the aliphatic protons at C-1′ (δ_H 2.80 and 2.55) and sp^2 quaternary carbons at C-9 (δ_C 154.1), C-10 (δ_C 104.2), and C-10a (δ_C 158.3) showed that the dihydropyran moiety in compound **11** was fused to the C-9 and C-10 positions. The absolute stereochemistry at C-6a and C-11a was determined to be the *R* form from the negative optical rotation of −74.9 (*c* 0.02, MeOH).[9,14–17] The negative [MeOH, λ_{max} = 250 nm ($\Delta\varepsilon$ = −2.68] and a positive [MeOH, λ_{max} = 305 nm ($\Delta\varepsilon$ = +8.98)] Cotton effect observed in the circular dichroic (CD) spectrum of compound **11** confirmed this assignment.[9,17,21,25] Based on the above mentioned evidence, the structure of compound **11** was determined to be 3-hydroxy-[2′,2′-(3′-hydroxy)-dimethylpyrano]-(5′,6′:10,9)-(6a*R*,11a*R*)pterocapan, named erythribyssin D.

Table 2
13C NMR spectroscopic data for new compounds **6**, **8**, **11**, and **12** in acetone-d_6

No.	**6**[a] δ_C (ppm)	**8**[a] δ_C (ppm)	**11**[b] δ_C (ppm)	**12**[b] δ_C (ppm)
1	123.7	133.1	132.3	133.2
2	110.2	114.2	109.5	110.5
3	162.9	156.0	158.7	159.7
4	104.3	104.8	103.0	104.0
4a	155.5	155.3	156.9	157.8
6	158.6	67.2	66.4	67.4
6a	103.5	41.0	40.0	40.8
6b	117.8	119.0	117.8	118.7
7	119.0	122.8	122.5	123.5
8	114.7	108.1	108.7	109.6
9	157.3	159.8	154.1	155.1
10	115.1	112.0	104.2	105.2
10a	156.5	156.8	158.3	159.2
11a	161.4	79.0	78.5	79.5
11b	105.7	115.3	112.2	113.1
1′	23.4			
2′	122.4	78.2	76.8	77.8
3′	132.9	70.0	68.4	69.5
4′	18.0	31.6	26.1	27.1
5′	25.9	20.7	19.7	20.6
6′		26.3	25.2	26.1
1″		23.5		
2″		123.7		
3″		131.3		
4″		25.9		
5″		18.0		
2-CHO	196.1			

Compounds were measured in acetone-d_6 at 150 MHz[a] and 125 MHz[b] of ^{13}C NMR.

Table 3
Inhibitory effects of isolated compounds **1**–**15** on neuraminidase activities

Compounds	*Clostridium perfringens*		*Vibrio cholerae*
	IC_{50}[a] (μM)	Inhibition type (K_i, μM)	IC_{50}[a] (μM)
Demethylmedicarpin (**1**)	6.39 ± 0.40	Noncompetitive (3.83 ± 0.30)	29.54 ± 1.94
Neorautenol (**2**)	19.82 ± 0.88	Noncompetitive (21.74 ± 2.14)	53.11 ± 3.98
Isoneorautenol C (**3**)	14.12 ± 0.19	Noncompetitive (10.96 ± 0.62)	64.75 ± 6.19
Phaseolin (**4**)	33.55 ± 2.07	Competitive (4.84 ± 0.68)	31.40 ± 1.55
Eryvarin D (**5**)	2.09 ± 0.08	Noncompetitive (2.55 ± 0.11)	3.30 ± 0.53
Erythribyssin O (**6**)	1.32 ± 0.16	Noncompetitive (1.21 ± 0.22)	0.35 ± 0.02
Calopocarpin (**7**)	1.57 ± 0.06	Noncompetitive (2.09 ± 0.15)	7.55 ± 2.23
Erythribyssin L (**8**)	2.79 ± 0.33	Noncompetitive (4.13 ± 0.86)	27.14 ± 2.29
Erysubin D (**9**)	26.39 ± 1.94	Competitive (15.20 ± 1.27)	26.39 ± 1.78
Erysubin E (**10**)	1.30 ± 0.12	Noncompetitive (0.24 ± 0.01)	19.48 ± 1.94
Erythribyssin D (**11**)	77.10 ± 2.17	NT[c]	46.20 ± 5.89
Erythribyssin M (**12**)	205.40 ± 4.03	NT[c]	77.73 ± 11.01
Crystacarpin (**13**)	2.28 ± 0.31	Noncompetitive (0.31 ± 0.05)	22.03 ± 2.24
Sophorapterocarpan A (**14**)	2.01 ± 0.16	Noncompetitive (1.78 ± 0.28)	11.59 ± 3.17
Erystagallin A (**15**)	2.04 ± 0.08	Noncompetitive (1.80 ± 0.07)	27.74 ± 0.40
Quercetin[b]	25.34 ± 1.58	NT[c]	NT[c]

[a] Results are expressed as IC_{50} values (μM), determined by regression analyses and expressed as the mean ± SD of three replicates.
[b] The compound was used as positive control.
[c] NT = not determined.

Compound **12** was also obtained as a yellowish amorphous powder with IR absorption bands at 3406 (OH), 2927(C–C), 1606, 1468, 1374, 1279, 1142, and 1062 cm^{-1}. The HRESIMS of compound **12** showed a quasimolecular ion peak at *m/z* 363.1216 $[M+Na]^+$ (calcd $C_{20}H_{20}O_5Na$ 363.1208). A comparison of the IR, UV, MS, ^1H, and ^{13}C NMR spectral data of compound **12** with those of compound **11** (Tables 1 and 2, see also Supplementary data) indicated compound **12** to be an optical isomer of compound **11**. The HMBC spectrum of compound **12** showed similar long-range correlations to those of compound **11** (see Tables S.3 and S.4 in Supplementary data). A positive optical rotation value +53.7 (*c* 0.02, MeOH) was produced from the $[\alpha]_D$ experiment. This suggests that the absolute stereochemistry at C-6a and C-11a of compound **12** is (6a*S*:11a*S*),[15–17,26] whereas compound **11** has an *R,R* configuration at C-6a, C-11a according to its negative optical rotation value. The CD spectrum of compound **12** was examined to provide further evidence of this identification. The spectrum revealed a positive [MeOH, λ_{max} = 250 nm ($\Delta\varepsilon$ = +7.49] and negative [MeOH, λ_{max} = 310 nm ($\Delta\varepsilon$ = −2.25)] Cotton effect. Therefore, compound **12** was characterized as 3-hydroxy-[2′,2′-(3′-hydroxy)-dimethylpyrano]-(5′,6′:10,9)-(6a*S*,11a*S*)pterocapan, named erythribyssin M.

In vitro enzyme assays were tested with some modification to determine the enzymatic inhibitory activities of the isolated metabolites on both *C. perfringens* (Sigma, N2876) and *V. cholerae* bacterial neuraminidases.[27,28] All the data were analyzed using a nonlinear regression program [Sigma Plot 11.0 (SPCC Inc., Chicago, IL)].

All isolates revealed dose-dependent inhibitory effects against both bacterial neuraminidase activities (Table 3 and Fig. 2). Compounds **1**–**3**, **5**–**8**, **10**, and **13**–**15** possessed noncompetitive inhibition modes from kinetic studies and showed greater potency against *C. perfringens* neuraminidase (IC_{50} 1.30–19.82 μM) than quercetin (IC_{50} 25.34 μM), which was used as the positive control. On the other hand, compounds **4** and **9** behaved as competitive inhibitors and were less effectives (IC_{50} 26.39–33.55 μM). Among the isolates, compounds **5**–**8**, **10**, and **13**–**15**, which possessed at least a prenyl moiety, hydroxy and/or methoxy functional group in the structures, showed the most potency with IC_{50} values ranging from 1.30 ± 0.12 to 2.79 ± 0.33 μM (against *C. perfringens*) and from 0.35 ± 0.02 to 29.54 ± 1.94 μM (against *V. cholerae*). While compound **1**, which is the basic skeleton of pterocarpan (3,9-hydroxy-6a*R*:11a*R*-pterocarpan), exhibited inhibitory activity with IC_{50} of 6.39 ± 0.40 μM (*C. perfringens*) and 29.54 ± 1.94 μM (*V. cholerae*). These results suggest that the addition of a prenyl group, hydroxy and/or methoxy substituents into the A- and/or B-ring might be responsible for the increase of activity on both the neuraminidase. Compounds **2**–**4**, which were fused as the 2,2-dimethylpyran moiety on the A- or B-ring, showed less activity than compound **1** on both enzyme assays (Table 3).

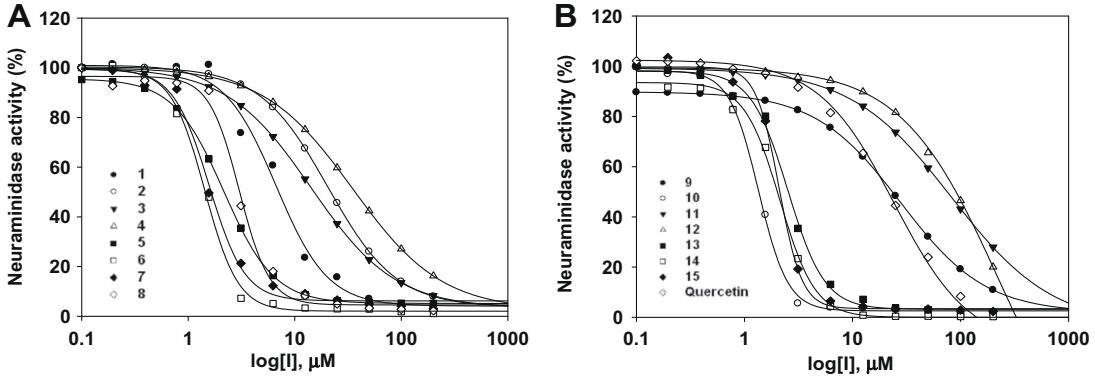
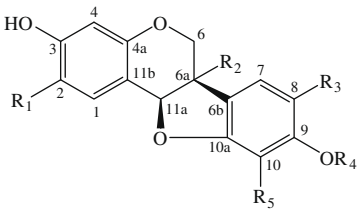


Figure 2. Effects of the isolated compounds (**1**–**15**) on the activity of neuraminidase from *C. perfringens* for the hydrolysis of 4-methylumbelliferyl-α-D-*N*-acetyl neuraminic acid at room temperature. (A) Concentration-dependent inhibition of neuraminidase by the isolated compounds **1**–**8**. (B) Concentration-dependent inhibition of neuraminidase by the isolated compounds **9**–**15**, and the positive control, quercetin. The inhibitor concentrations are displayed on logarithmic scales. The IC_{50} was identified from the midpoint (neuraminidase activity = 50%) of the semilog plot.



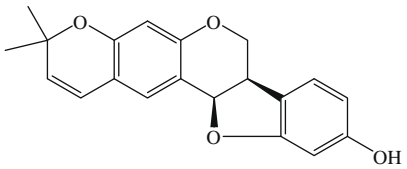
1 $R_1 = R_2 = R_3 = R_4 = R_5 = H$

7 R_1 = prenyl, $R_2 = R_3 = R_4 = R_5 = H$

13 $R_1 = R_3 = H$, $R_2 = OH$, $R_4 = CH_3$, R_5 = prenyl

14 $R_1 = R_2 = R_4 = R_5 = H$, R_3 = prenyl

15 $R_1 = R_5$ = prenyl, $R_2 = OH$, $R_3 = H$, $R_4 = CH_3$



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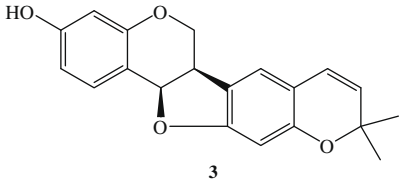
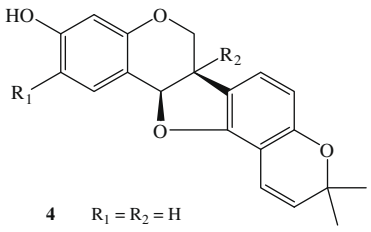
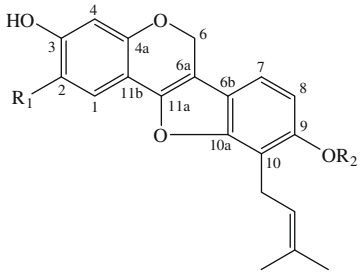


3



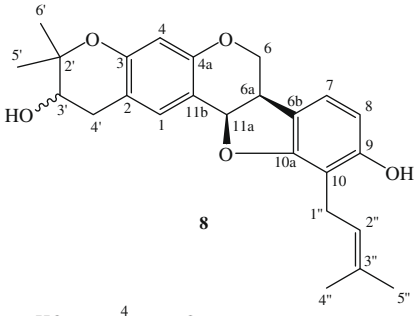
4 $R_1 = R_2 = H$

10 R_1 = prenyl, $R_2 = OH$

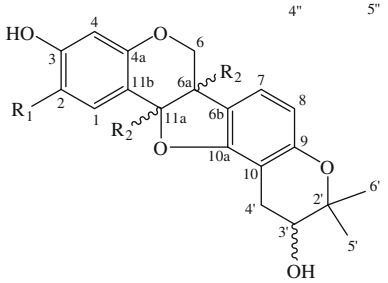


5 $R_1 = H$, $R_2 = CH_3$

6 $R_1 = CHO$, $R_2 = H$



8

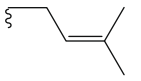


9 R_1 = prenyl, R_2 = α-H

11 $R_1 = H$, R_2 = α-H

12 $R_1 = H$, R_2 = β-H

Prenyl group =



Compounds **11** and **12**, showed weak activity against both neuraminidases with respective IC_{50} values of 77.10 ± 2.17 and 205.40 ± 4.03 μM against *C. perfringens*, and 46.20 ± 5.89 and 77.73 ± 11.01 μM against *V. cholerae*. The decrease in activity of these compounds might be explained by the presence of a 2′,2′-(3′-hydroxy)-dimethylpyran ring fused to the C-9 and C-10 position, and the absence of prenyl and methoxy groups. Furthermore, compound **11** possessed the *R,R* configuration at C-6a and C-11a, while compound **12** had the *S,S* configuration. This different stereochemistry might also be responsible for the change in their activities, in which the *R,R* form exhibited a stronger inhibitory effect than the *S,S* form. In addition, compound **9** had the *R,R* configuration and 2′,2′-(3′-hydroxy)-dimethylpyran moiety fused to the B-ring at the C-9 and C-10 position. However, its activity was 2- to 7-times higher than that of compounds **11** and **12**. This significant change might be due to an additional prenyl group attached to the C-2 position in compound **9**. Compound **10** (1.30 ± 0.12) which possessed a hydroxy group at C-6a showed significant activity than **9** (26.39 ± 1.94) against *C. perfringens*. The same manners were also found for compounds **13** (2.28 ± 0.31) and **15** (2.04 ± 0.08). However, compounds **9** (26.39 ± 1.78), **10** (19.48 ± 1.94), **13** (22.03 ± 2.24), and **15** (27.74 ± 0.40) showed similar activity against *V. Cholerae*. This result suggests that hydroxy group which attached to C-6a (compounds **10**, **13**, and **15**) was not increased the inhibitory activity against *V. Cholerae*. However, the aldehyde group showed the significant enhancement of inhibitory activity on both neuraminidases. Finally, compounds **5** and **6** which are 6a,11a-dehydropterocarpans with a prenyl group and methoxy moiety showed the highest activities against both enzymes with IC_{50} of 2.09 ± 0.08 and 1.32 ± 0.16 (*C. perfringens*), and 3.30 ± 0.53 and 0.35 ± 0.02 μM (*V. cholerae*), respectively. This led to the conclusion that 6a,11a-dehydropterocarpan derivatives possess stronger activity toward bacterial neuraminidases than 6a,11a-dihydropterocarpans. Overall, the 6a,11a-dehydropterocarpan-type compounds can be considered the lead compounds for the treatment of bacterial infections.

As shown in Figure 2, all the isolates (**1**–**15**) showed a dose-dependent inhibitory effect against the neuraminidase activity, and the inhibition modes were all reversible (Fig. 3). The mechanism of enzyme inhibition by each compound was examined in order to determine the relative affinity of each compound for neuraminidase. For the confirming whether the inhibition caused by the compounds is rapidly reversible, slowly reversible, or irreversible, the resulting progress curves after dilution of compounds

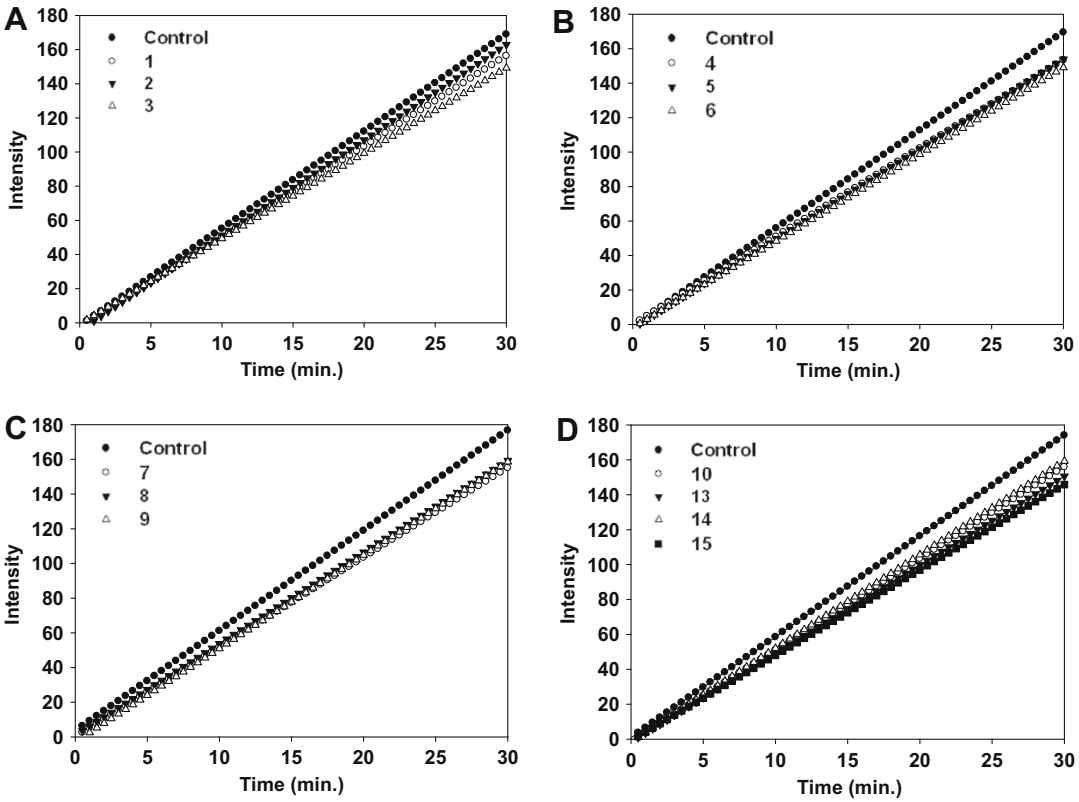


Figure 3. Recovery of the neuraminidase activity after the rapid dilution of a pre-incubated mixture of the enzyme–compound. The control (closed circles) was pre-incubated and diluted in the absence of a compound. The progress curves were linear with a slope equal to approximately 91% of the slope of the control, indicating that all compounds were rapidly reversible.

1–**15** was examined as Figure 3A–D. The progress curves are linear with a slope equal to approximately 91% of the control slope, which suggests that all compounds are reversible inhibitors.

The inhibition mode of the isolated compounds was tested using both the double reciprocal Lineweaver–Burk plot, which is the most straightforward means of diagnosing an inhibitor model (Figs. 4A, I and S.5 in Supplementary data), and the Dixon plot by plotting $1/v$ as a function of [I] for each substrate concentration (Figs. 5A, I and S.6 in Supplementary data). The $-K_i$ values were determined from the x-axis value at which the lines intersect. Table 3 lists the K_i values for the isolated compounds **1**–**15**. Most of the isolates, except for compounds **4** and **9**, exhibited noncompetitive inhibition because the V_{max} values decreased with concentration without changing K_m for the substrate, and the lines intersected at a value of $1/[S]$ under zero on the x-axis (at 1/(intensity/min) = 0) (Fig. 4A). In contrast, the pattern of straight lines with intersecting y intercepts (Figs. 5I and S.5I in Supplementary data) is characteristic of competitive inhibitors, indicating that compounds **4** and **9** are competitive. The lines intersect at their y intercepts indicated that the two compounds do not affect the apparent V_{max}. The slopes of the lines, which are given by K_m/V_{max}, vary among the lines because of the effect imposed on K_m by the inhibitors. These results further support the competitive inhibition of compounds **4** and **9**.

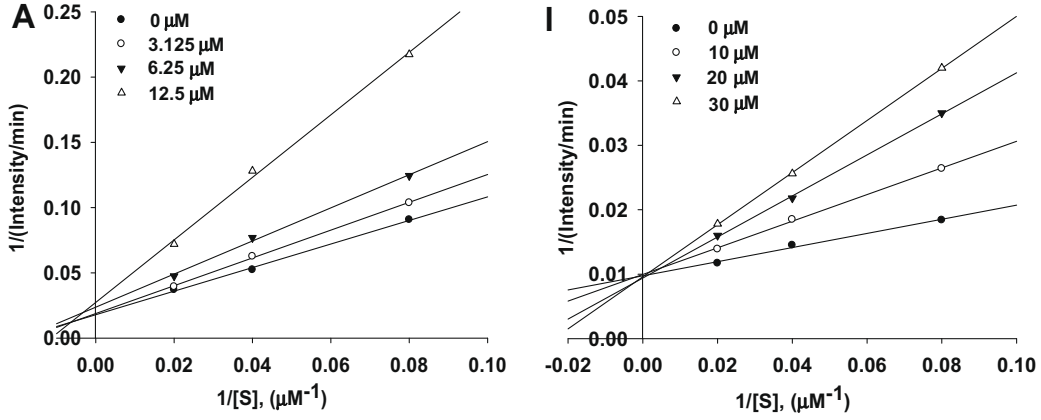


Figure 4. Graphical determination of the inhibition type of the isolated compounds. Lineweaver–Burk plots for the inhibition of compounds **1** (A) and **9** (I) on the neuraminidase-catalyzed hydrolysis of the substrate was determined. The data is expressed as the mean reciprocal of intensity/min for $n = 3$ replicates at each substrate concentration.

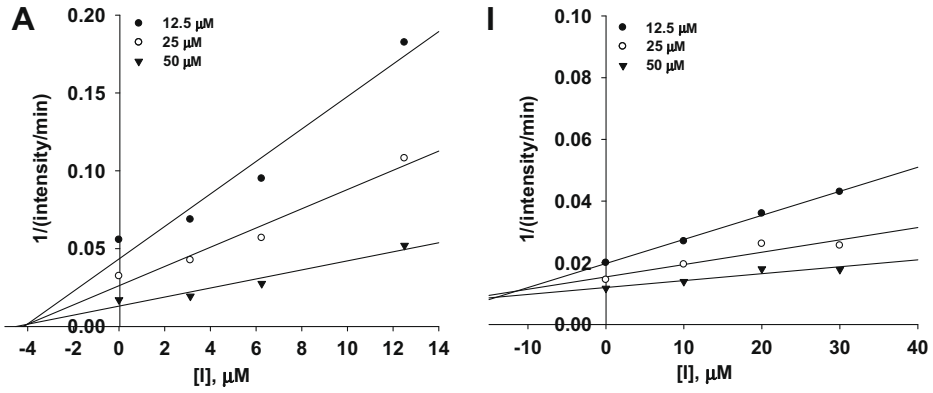


Figure 5. Dixon plots for the inhibition of compounds **1** (A) and **9** (I) on the neuraminidase-catalyzed hydrolysis of the substrate was determined. The K_i value was determined from the negative *x*-axis value at the point of intersection of the three lines.

To determine if calopocarpin (**7**) affects *V. cholerae* growth, the number of live bacteria in a LB broth culture were counted as described in Section 3.11.[29] Calopocarpin (3 and 10 μg/mL) did not inhibit the growth of *V. cholerae* in the LB broth (Fig. 6A). Adhesion is a critical virulence trait for many bacteria,[30] and *V. cholerae* neuraminidase may play a key role in the pathogenesis of cholera.[31] Neuraminidase was reported to promote the adhesion of *Streptococcus pneumoniae*, *Neisseria meningitides*, and *Staphylococcus aureus* to host cells.[32] In addition, it was reported that vaccines containing mucinases composed of neuraminidase and protease prevented the adhesion of *V. cholerae* and reduced the delivery of cholera toxin (CT) to receptors.[33] Therefore, the effect of calopocarpin, a neuraminidase inhibitor, on *V. cholerae* adhesion was also examined. Bacterial adhesion to the host cells was assayed by Giemsa staining, as described in Section 3.12. Calopocarpin (3 and 10 μg/mL) inhibited the adhesion of *V. cholerae* to the host cells (Fig. 6B). This suggests that neuraminidase inhibitors have the potential to develop as a new treatment to combat infectious diseases.

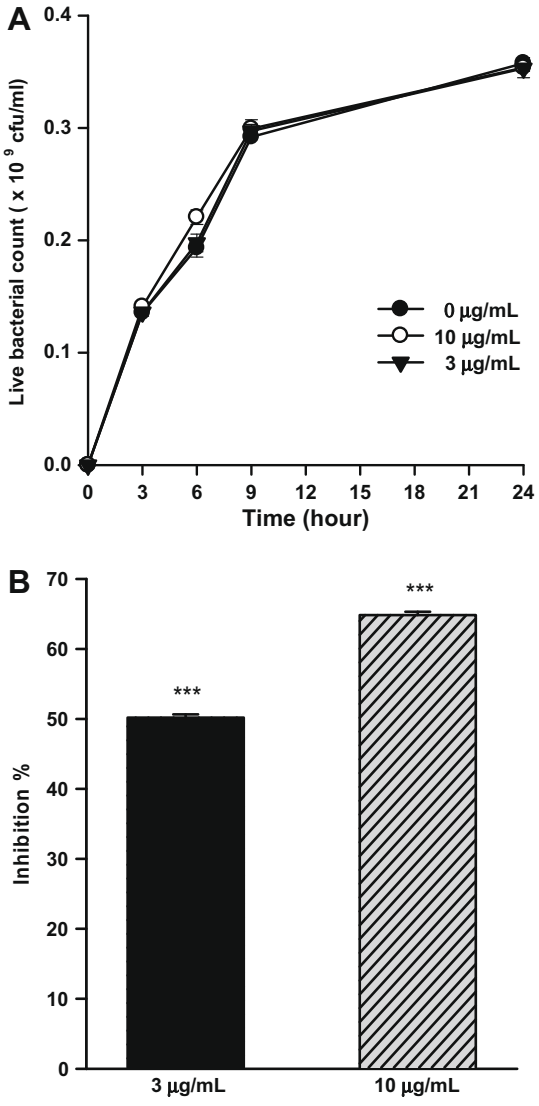


Figure 6. The effects of calopocarpin (**7**) on *Vibrio cholerae* growth (A) and the adhesion of *Vibrio cholerae* to HeLa cells (B).

The genus *Erythrina* includes approximately 120 species that occur in tropical and subtropical regions. *E. abyssinica* is used widely as an important folk medicine.[34] The bark and leaves of *E. abyssinica* are used primarily to cure ailments, such as toothache, earache, tuberculosis, and the roots are used to treat syphilis, asthma, venereal diseases, and leprosy,[35] and are particularly effective against malaria and microbial infections.[34] These traditional uses indicate the importance of *E. abyssinica* as an medicinal plant in African countries.[36] The shortage of Western doctors in these areas has forced the inhabitants to consult traditional healers if not by choice then certainly by necessity. Overall, these results suggest that *E. abyssinica* can be used as beneficial source for the development of safer and cheaper drugs for the treatment of bacterial infections. Further biological and phytochemical investigations should be focused on the 6a,11a-dehydropterocarpan derivatives from the genus *Erythrina*.

3. Experimental section

3.1. General experimental procedures

The optical rotations were determined on a Rudolph Autopol AP 589 polarimeter using a 100 mm glass microcell. The IR spectra were recorded on a Nicolet 6700 FT-IR (Thermo electron Corp.). The NMR spectra were obtained on a Varian Inova 500 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The EIMS and HREIMS data were obtained on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63–200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out on Silica Gel 60 F_{254} and RP-18 F_{254} plates from Merck. HPLC was carried out using a Gilson system with a UV detector and an OptimaPak C18 column (10 × 250 mm, 10 μm particle size, RS Tech Corp., Korea).

3.2. Plant material

The stem bark of *E. abyssinica* was collected in June 2005 in Mukono, Uganda. The sample was authenticated by Professor John Silike-Muruumu, and its voucher specimen (No. 0001) was deposited at the Department of Botany, Makerere University, Uganda.

3.3. Extraction and isolation

The dried stem bark of *E. abyssinica* (3 kg) was extracted with EtOAc at room temperature for one week. The EtOAc-soluble extract was concentrated to yield a dry residue (156 g). The crude extract was tested in vitro to determine the inhibitory effect on the neuraminidase activity. The result showed that this EtOAc-soluble extract was active with >70% inhibition at a concentration of 20 μg/mL. A partial fraction (55 g) was subjected to silica gel column chromatography (10 × 60 cm; 63–200 μm particle size) using a gradient of *n*-hexane/acetone (from 20:1 to 0:1) to yield five combined fractions (F.1–F.5) according to their TLC profiles. The five fractions were tested in vitro using both enzyme assays. Fractions F.2–F.4 exhibited strong inhibition against *C. perfringens* and *V. cholerae* (see Table S.5 in Supplementary data). Fraction F.2 (10 g) was subjected to reversed phase C18 (RP-18) column chromatography (6.0 × 60 cm; 40–63 μm particle size), and eluted with MeOH/H_2O (from 5:5 to 5:0, 2 L for each step) to afford five subfractions (F.2-1–F.2-5). Subfraction F.2-1 with inhibitory activity was further purified using a semi-preparative Gilson HPLC system [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeCN/H_2O containing 0.1% formic acid (0–38 min: 51% MeCN, 38–40 min: 51–100% MeCN, 40–45 min: 100% MeCN, 45–47 min: 100–51% MeCN, 47–50 min: 51% MeCN); UV detection at 205 and 254 nm] to give compounds **1** (t_R 25.5 min, 5.4 mg), **2** (t_R 34.5 min, 8.6 mg), and **3** (t_R 42.8 min, 6.7 mg). Further purification of subfraction F.2-4 by HPLC using an isocratic solvent system of 78% MeCN in H_2O containing 0.1% formic acid, over 25 min with UV detection at 205 and 254 nm led to the isolation of compounds **4** (66.3 mg, t_R = 17.5 min), **5** (227.8 mg, t_R = 19.0 min), compound **6** (5.8 mg, t_R = 22.5 min). In the same manner, fraction F.3 (7 g) was also subjected to reversed phase C18 (RP-18) column chromatography (6.0 × 60 cm; 75 μm particle size) and eluted with MeOH/H_2O (from 4:6 to 4:0, 2 L for each step) to afford five subfractions (F.3-1 to F.3-5). Subfraction F.3-2 was further purified using a semi-preparative Gilson HPLC system [Shodex Packed C18 column (10 × 250 mm, 10 μm particle size); solvent MeCN in H_2O containing 0.1% formic acid (0–30 min: 55% MeCN, 30–35 min: 55–100% MeCN, 45–55 min: 100% MeCN, 55–57 min: 100–55% MeCN, 57–60 min: 51% MeCN), UV detection at 205 and 254 nm] to afford compounds **7** (t_R 25.2 min, 16.4 mg) and **8** (t_R 32.0 min, 4.3 mg). Compounds **9** (7.5 mg, t_R = 30.1 min) and **10** (17.2 mg, t_R = 34.9 min) were isolated from F.3-4 by semi-preparative Gilson HPLC with 60% MeCN containing 0.1% formic acid as the mobile phase over 38 min with UV detection at 205 and 254 nm. Fraction F.4 [eluted with *n*-hexane/acetone (from 2:1 to 1:1), 4.2 g] was subjected to reversed phase C18 (RP-18) column chromatography (6.0 × 60 cm; 75 μm particle size) using a stepwise gradient of MeOH/H_2O (from 3:7 to 3:0; each 2 L) to yield five subfractions (F.4-1–F.4-5). Subfraction F.4-3 with inhibitory activity against neuraminidases was purified by semi-preparative HPLC [Gilson System 321 pump equipped with a model UV/vis-155 detector, RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeCN/H_2O containing 0.1% formic acid (0–80 min: 50% MeCN, 80–85 min: 50–100% MeCN, 85–95 min: 100% MeCN, 95–97 min: 100–50% MeCN, 97–100 min: 50% MeCN); flow rate 2 mL/min; UV detection at 205 and 254 nm] to yield compounds **11** (10.6 mg; t_R = 36.7 min), **12** (8.9 mg; t_R = 42.5 min), **13** (16.0 mg; t_R = 60.2 min), **14** (6.6 mg, t_R = 65.7 min), and **15** (15.5 mg, t_R = 74.9 min).

3.4. Erythribyssin O (6)

Yellow amorphous powder; IR (KBr): ν_{max} 3418, 2924, 1708, 1622, 1418, 1265, 1160–1032 cm^{-1}; UV (*c* 0.025, MeOH) λ_{max} nm: 210, 214, 242, 299, 336, and 352 nm; ^1H (600 MHz) and ^{13}C (150 MHz, acetone-d_6) NMR data, see Tables 1 and 2; HREIMS *m*/*z* 350.1154 $[M]^+$, (calcd $C_{21}H_{18}O_5$ 350.1146).

3.5. Erythribyssin L (8)

White amorphous powder; $[\alpha]_D^{25}$ −110.4 (*c* 0.02, MeOH); IR (KBr): ν_{max} 3425, 2973, 2926, 1599, 1450 cm^{-1}; UV (*c* 0.03, MeOH) λ_{max} nm: 210, 214, 232, 286, 320 nm; ^1H (600 MHz) and ^{13}C (150 MHz, acetone-d_6) NMR data, see Tables 1 and 2; HREIMS *m*/*z* 408.1937 $[M]^+$, (calcd $C_{25}H_{28}O_5$ 408.1948).

3.6. Erythribyssin D (11)

Yellowish amorphous powder; $[\alpha]_D^{25}$ −74.9 (*c* 0.02, MeOH); IR (KBr) ν_{max} cm^{-1}: 3410, 2928, 1566, 1468, 1193, 1119, 1061, 1041; UV (*c* 0.025, MeOH) λ_{max} nm (log ε): 209 (4.79), 233 (4.23), 282 (3.81); CD (*c* 0.55 MeOH): $[\theta]_{250}$ −2.68; $[\theta]_{270}$ −1.75, $[\theta]_{292}$ 0, $[\theta]_{3.05}$ +8.98; $[\theta]_{329}$ +5.01; ^1H (500 MHz) and ^{13}C (125 MHz, acetone-d_6) NMR data, see Tables 1 and 2; HREIMS *m*/*z* 340.1313 $[M]^+$ (calcd for $C_{20}H_{20}O_5$ 340.1311).

3.7. Erythribyssin M (12)

Yellowish amorphous powder; $[\alpha]_D^{25}$ +53.7 (*c* 0.02, MeOH); IR (KBr) ν_{max} cm^{-1}: 3406, 2927, 1606, 1468, 1374, 1279, 1142, 1062; UV (*c* 0.025, MeOH) λ_{max} nm (log ε): 209 (4.77), 2.30 (4.14), 282 (3.83); CD (*c* 0.55 MeOH): $[\theta]_{250}$ +7.49, $[\theta]_{270}$ +8.58, $[\theta]_{304}$ 0, $[\theta]_{310}$ −2.25, $[\theta]_{336}$ +11.68; ^1H (500 MHz) and ^{13}C (125 MHz, acetone-d_6) NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 363.1216 $[M+Na]^+$ (calcd for $C_{20}H_{20}O_5Na$ 363.1208).

3.8. Neuraminidase (*C. perfringens*) inhibition assay

The neuraminidase activity was evaluated using a slight modification of the method reported by Myers et al.[27] Neuraminidase from *C. perfringens* (Sigma, N2876) 0.05 U/mL in 0.04 M sodium acetate buffer (pH 5.0) was used as the enzyme and 1 mM 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid sodium salt hydrate (4-MU-NANA, Sigma, M8639) in acetate buffer was used as the substrate. A mixture of 10 μL enzyme, 340 μL acetate buffer, 10 μL isolated compounds (dissolved in DMSO and diluted to the corresponding concentrations in acetate buffer), and 40 μL substrate in a cuvette was incubated for 10 min at 37 °C. The reaction was quenched by adding of 3.5 mL of a 0.1 M glycine–NaOH buffer at pH 10.4. Free 4-methylumbelliferone was determined in a Spectramax M2 spectrofluorometer (Molecular Devices, USA) using excitation light at 360 nm and fluorescence emission at 440 nm. The unhydrolyzed substrate had an excitation maximum at 315 nm and a fluorescence maximum at 374 nm, and only slightly interfered with the 4-methylumbelliferone determination.[28] For the enzyme kinetic study, 4-methylumbelliferone was quantified immediately without adding of stop solution (glycine–NaOH buffer). The experimental data was analyzed using Sigmaplot 11.0 (SPCC Inc., Chicago, IL) according to the following equation:

$$\% \text{ Inhibition} = \frac{100}{1 + (IC_{50}/[I])}$$

3.9. Reversibility of the isolated compounds

The reversibility of inhibition was examined by measuring the recovery of enzymatic activity after a rapid and large dilution of the enzyme inhibitor complex.[28] The enzyme and compounds were pre-incubated for 15 min at an enzyme of concentration equal to 100 times that needed for the activity assay (0.125 U/mL), and at a concentration of compounds equal to 10 times the IC_{50} value. The mixture was then diluted 1/100 in an acetate buffer containing the enzyme substrates to initiate the reaction. The progress curve for this sample was then measured and compared with that of a similar sample of enzyme incubated and diluted in the absence of an inhibitor. After dilution, the enzyme concentration would be equal to that used in a typical concentration–response experiment but upon dilution, the inhibitory concentration would change from $10 \times IC_{50}$ to $0.1 \times IC_{50}$. These inhibitory concentrations corresponded to approximately 91% and 9% inhibition (fractional activity = 0.09 and 0.91), respectively.

3.10. Neuraminidase (*V. cholerae*) inhibition assay

The *V. cholera* neuraminidase (Sigma, N7885) activity was assessed using a fluormetric assay based on the method developed by Potier et al., which measures the hydrolysis of 4-MU-NANA (Sigma, M8639).[28] All compounds were dissolved in DMSO and diluted to the corresponding concentrations in MES buffer [50 mM 2-(*N*-morpholino)-ethanesulfonic acid, containing 6 mM $CaCl_2$, pH 5.6]. The enzyme inhibitory assay was carried out in 96-well plates containing the enzyme (8.3 U, 10 μL) and 10 μL of the inhibitor in MES buffer. The samples were made up to 95 μL using an enzyme buffer and incubated at 37 °C for 30 min with constant shaking, prior to the addition of the 4-MU-NANA substrate (25 μM final concentration, 5 μL). The reactions were quenched after a further incubation at 37 °C for a 30 min in a glycine solution (0.25 M, pH 10.0, 100 μL). The fluorescence was measured using a Spectramax M2 spectrofluorometer with excitation and emission wavelengths of 360 and 440 nm, respectively. The assay was performed at least in triplicate. The level of inhibition was measured as a percentage of the control (incubations performed in the absence of inhibitor). The sample measurements were corrected for background fluorescence not produced by the enzyme-catalyzed hydrolysis of the substrate, by subtracting a blank sample containing 4-MU-NANA in MES buffer.

3.11. Effect on *V. cholerae* growth

The effect of calopocarpin on *V. cholerae* 16961 growth was examined. A bacterial suspension was diluted 1/1000 in fresh LB broth with different concentrations of calopocarpin (3 and 10 μg/mL), and cultured in a 37 °C shaking incubator at 200 rpm. The effect of calopocarpin on *V. cholerae* growth was determined by counting live bacterial numbers after 10 μL of the diluted suspension was plated on HI agar.

3.12. Assay of bacterial adherence to HeLa cells

Bacterial adhesion to HeLa cells, a cervical carcinoma cell line, was assayed as described previously.[30] The HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO® Invitrogen, USA). The HeLa cells seeded into 4-well LabTec chamber slides (Nunc, Inc., Naperville, IL) were preincubated in serum-free DMEM with or without calopocarpin (3 or 10 μg/mL) for 1 h, and then infected with the *V. cholerae* cells at a multiplicity of infection (MOI) of 250. After 30 min incubation at 37 °C, the HeLa cells were washed thoroughly three times with pre-warmed DMEM media, fixed with methanol at rt, stained with a Giemsa solution (Merck, Darmstadt, Germany), and visualized by optical microscopy (Nikon). The bacterial cells adhering to 90 HeLa cells were counted and the adhesion number was calculated as the mean number of bacteria adhered per HeLa cell.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.005.

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