Food and Chemical Toxicology 49 (2011) 1849–1856

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Flavanones and rotenoids from the roots of *Amorpha fruticosa* L. that inhibit bacterial neuraminidase

Young Soo Kim [a], Young Bae Ryu [b], Marcus J. Curtis-Long [c], Heung Joo Yuk [a], Jung Keun Cho [a], Jun Young Kim [a], Kwang Dong Kim [a], Woo Song Lee [b], Ki Hun Park [a,*]

[a] Division of Applied Life Science (BK21 Program), IALS, Gyeongsang National University, Jinju 660-701, Republic of Korea
[b] Korea Research Institute of Bioscience and Biotechnology, Jeongeup 580-185, Republic of Korea
[c] Graduate Programe in Biochemistry and Biophysics, Brandeis University, 415 South Street Woltham, MA 02453, USA

ARTICLE INFO

Article history:
Received 14 March 2011
Accepted 30 April 2011
Available online 6 May 2011

Keywords:
Amorpha fruticosa
Bacterial neuraminidase
Flavanone
Roteoid
Biofilm

ABSTRACT

Neuraminidase is a proven target in anti-viral drug development. It also appears to be important for infection by certain pathogenic bacteria and has been implicated in biofilm formation. Based on activity-guided fractionation, the acetone extract of *Amorpha fruticosa* roots gave four flavanones **1**–**4** and three rotenoids **5**–**7** which were identified as amoradicin (**1**), amorisin (**2**), isoamoritin (**3**), amoricin (**4**), amorphigeni (**5**), dalbinol (**6**), and 6-ketodehydroamorphigenin (**7**), respectively. All isolated inhibitors showed strong neuraminidase inhibition with IC_{50}s between 0.12 and 22.03 μM. In particular, amorisin **2** exhibited 120 nM IC_{50}, which is 30-fold more potent than the positive control, quercetin. In addition, this is the first report detailing rotenoids (IC_{50} = 8.34–16.74 μM) exhibiting neuraminidase inhibition. Kinetic analysis revealed that all inhibitors were noncompetitive. The most active neuraminidase inhibitors (**2**, **3**, **5**, **6**) were proven to be present in the native root in high quantities by HPLC. Finally, at concentrations where no toxicity was observed, **3** and **6** inhibited *Pseudomonas aeruginosa* biofilm production. 29.7% and 21.0% inhibition respectively was observed at 25 μM.

1. Introduction

Sialic acids are nine carbon α-keto aldonic acids, which are characteristically at the terminus of peptidoglycans at the cell surface. Given this exposed position, sialic acid is uniquely able to interact with its microenvironment, and it is known to play pivotal roles in cell–cell recognition (Du et al., 2009). For instance, sialic acid is recognized by selectins and siglecs. However, the strategic position of sialic acid also presents an opportunity for cell recognition by pathogens. Indeed this is a common mechanism of infectivity. One of the classic pathogens which make use of this pathway is the influenza virus. Indeed it is the strong interaction between influenza hemagglutin and sialic acid that leads to agglutination when influenza is added to blood (Varki and Varki, 2007). However, to release virions from the host cell, sialic acid must be liberated from the glycoprotein, and the enzyme neuraminidase is required for this. Neuraminidase is thus a huge anti-influenza therapeutic target. Well-known drugs, such as tamiflu target this enzyme. The neuraminidase family (EC 3.2.1.18) is a group of exo-acting enzymes that hydrolyze terminal sialic acids from a variety of glycoproteins. This enzyme specifically cleaves N-acetylneuraminic acid (Neu5Ac) from cell surface glycoproteins when sialic acid is joined to galactose *via* an α2 → 3 or α2 → 6 linkage (Matrosovich et al., 2004; Shinya et al., 2006; Couceiro et al., 1993). However, neuraminidase is not only mandatory for viral infections: it is also required by certain pathogenic bacteria (Soong et al., 2006; Vimr et al., 2004). In fact, many commensal and pathogenic bacteria use environmental (host) sialic acids as a source of carbon, nitrogen, energy, and amino sugars for cell wall synthesis (Vimr et al., 2004; Chang et al., 2004). Microbial sialic acid metabolism has now been linked with numerous important infectious processes including the formation of biofilms. For such bacterial pathogens, neuraminidase could prove to be an Achilles heel. For instance, in *Pseudomonas aeruginosa*, neuraminidase plays a key role in the initial stages of pulmonary infection by targeting bacterial glycoconjugates. It is this process that facilitates the formation of biofilms (Soong et al., 2006). Thus, in addition to applicability to

Abbreviations: δ, chemical shift; DMSO, dimethyl sulfoxide; HMBC, heteronuclear multiple bond correlation; ^1H–^1H, correlation spectroscopy; HPLC, high performance liquid chromatography; *I*, intensity at ex: 365 nm, em: 450 nm; IC_{50}, the inhibitor concentration leading to 50% activity loss; IR, infrared spectroscopy; *J*, coupling constant; K_m, maximum velocity catalyzed by a fixed [E]; K_i, dissociation constant by inhibition; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; SAR, structure-activity relationship.

* Corresponding author. Tel.: +82 55 772 1965; fax: +82 55 772 1969.
E-mail address: khpark@gnu.ac.kr (K.H. Park).

0278-6915/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2011.04.038

viral infections, development of inhibitors of neuraminidase provides a new weapon for the treatment of bacterial pathogens that require hydrolysis of sialic acid.

In our continuing search for neuraminidase inhibitors of natural origin (Ryu et al., 2010), the acetone extract of the roots of *Amorpha fruticosa* was found to strongly inhibit bacterial neuraminidase. *A. fruticosa* L. belongs to the Leguminosae family and is a representative polyphenol rich plant. Its main bioactive constituents are prenylated flavanones (Rozsa and Hohmann, 1988; Ohyama et al., 1998), isoflavones (Li and Wang, 1993), rotenoids (Dat et al., 2008), and stilbenes (Dat et al., 2008). Many of these exhibit antitumor (Li and Wang, 1993), antimicrobial (Mitscher et al., 2001) and TNF-α inhibition activities (Cho et al., 2000). However, to the best of our knowledge, there is no report of the roots of *A. fruticosa* eliciting neuraminidase inhibition and it is with this strongly in mind that we set about this work. In this study, we isolated eight phenolic compounds from the roots of *A. fruticosa* and their structures were identified using spectroscopic methods. The isolated compounds were evaluated separately for their inhibitory activities against neuraminidase. Their inhibition mechanisms were ascertained using Lineweaver–Burk and Dixon plots. We also assessed the relative amount of these extracts within the native root using an HPLC chromatogram profile and proved the most active compounds to be present in abundance. Finally, we were able to show that compounds **3** and **6** were able to inhibit biofilm formation, even though they were used at a concentration at which exhibited no toxicity to the cells.

2. Materials and methods

2.1. General

Preparative column chromatography (CC) was carried out using either silica gel (230–400 mesh, Merck), RP-18 (ODS-A, 12 nm, S-150 mM, YMC), or Sephadex LH-20 (Amersham Bioscience). Test chromatographic separations were carried out by Thin-Layer Chromatography (TLC) (E, Merck Co., Darmastdt, Germany), using commercially available glass plates pre-coated with silicagel and visualized using UV illumination at 254 and 366 nm or by spraying with H_2SO_4 staining reagent. Melting points were measured on a Thomas Scientific capillary apparatus. Fluorometric determination was measured on a SpectraMax M2^e Multimode Reader (Molecular Devices, USA). NMR spectra were recorded on a Bruker AM 500 spectrometer with TMS as an internal standard. Chemical shifts are expressed in δ values. Mass spectrometry, EIMS and HREIMS, were obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan).

2.2. Chemicals and reagents

Organic solvents were of first grade and stock solutions and buffers were prepared with milliQ water. For biochemical assays, neuraminidase (E.C. 3.2.1.18. from *Clostridium perfringens*) and 4-methylumbellifery-α-D-*N*-acetylneuraminic acid sodium salt hydrate were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Plant materials

The roots of *A. fruticosa* were collected at Munsan in Chinju, South Korea, in July 2009 and identified by Prof. Myong, Gi Chung. A voucher specimen (KHPark 240211) of this raw material is deposited at the Herbarium of Gyeongsang National University (GNUC).

2.4. Extraction and isolation

The acetone extract was determined to be the principle source of neuraminidase inhibitors as it exhibited the highest inhibition. The root bark (3.5 kg) was air-dried, pulverized, and extracted with acetone for a week, at room temperature. The filtered extract was evaporated to dryness under reduced pressure at a temperature below 35 °C to afford acetone-soluble extracts (87 g, 2.4%). The acetone extract was subjected to column chromatography on silica gel (10 × 40 cm, 230–400 mesh, 850 g) using hexane–acetone mixtures [100:1 (1L), 60:1 (2L), 40:1 (2L), 20:1 (2L), 10:1 (2L), 5:1 (2L), 1:1 (2L), 1:2 (4L)] to give 10 fractions (A–J). Fraction B (3.3 g) was fractionated by silica gel CC employing a gradient of hexane to acetone, resulting in 6 subfractions (FB.1–FB.6). Subfraction FB.2, enriched with compound **4** (210 mg) was further purified by CC on Sephadex LH-20 eluting with 70% acetone to yield compound **4** (8 mg). Subfraction FB.4 (425 mg), enriched with compounds **3** and **2**, was further purified by reversed-phase CC (ODS-A, 12 nm, S-150 μM) eluting with acetonitrile (ACN):H_2O (4:1) to afford compounds **2** (30 mg) and **3** (130 mg). Fraction C (4.1 g) was fractionated by silica gel CC and further purified by reversed-phase CC eluting with MeOH:H_2O (3:2) to afford compounds **1** (15 mg) and **7** (10 mg). Fraction E (5.2 g) was fractionated by Sephadex LH-20 employing 80% MeOH, to give ten subfractions (FE.1–FE.10). Subfraction FE.8 (1.5 g) was rechromatographed on Sephadex LH-20, to yield compounds **5** (150 mg) and **6** (250 mg). Subfraction FE.5 (400 mg) was purified by reverse-phase CC eluting with ACN:H_2O (5:1) to afford compound **8** (50 mg). All isolated compounds **1–8** were characterized and identified by spectroscopic methods, as well as by comparison with published data.

2.4.1. Amoradicin (*1*)

Pale yellow oil; EIMS *m/z* 438; HREIMS [M]$^+$ 438.2044 (calcd for $C_{26}H_{30}O_6$ 438.2042); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (1H, brm, H-6′), 6.89 (1H, brm, H-5′), 6.84 (1H, s, H-2′), 3.78 (3H, s, OCH_3), 3.31 (2H, d, *J* = 6.4 Hz, H-1″), 3.26 (2H, d, *J* = 6.4 Hz, H-1‴), 2.80–3.00 (2H, m, H-3), 1.63 (3H, s, H-5‴), 1.79 (3H, s, H-4″), 1.69 (3H, s, H-5″), 1.65 (3H, s, H-4‴); ^{13}C NMR (125 MHz, $CDCl_3$) δ 198.4 (C-4), 165.6 (C-7), 159.9 (C-8a), 158.7 (C-5), 144.5 (C-4′), 144.3 (C-3′), 132.2 (C-3‴), 131.9 (C-3″), 131.7 (C-1′), 123.3 (C-2‴), 123.0 (C-2″), 119.3 (C-6′), 116.0 (C-5′), 115.7 (C-8), 114.6 (C-2′), 113.7 (C-6), 108.8 (C-4a), 78.8 (C-2), 62.0 (OCH_3), 43.7 (C-3), 26.1 (C-5‴), 26.1 (C-5″), 23.1 (C-1″), 22.6 (C-1″), 18.2 (C-4″), 18.2 (C-4‴).

2.4.2. Amorisin (*2*)

Pale yellow oil; EIMS *m/z* 492; HREIMS [M]$^+$ 492.2496 (calcd for $C_{30}H_{36}O_6$ 492.2512); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.70 (1H, s, H-2′), 6.62 (1H, s, H-6′), 3.25 (2H, d, *J* = 6.83 Hz, H-1⁗), 3.21 (2H, d, *J* = 6.41 Hz, H-1″), 3.17 (2H, d, *J* = 6.62 Hz, H-1‴), 2.63–2.86 (2H, t, H-3), 1.70 (3H, s, H-4″), 1.65 (3H, s, H-5″), 1.64 (3H, s, H-4‴), 1.63 (3H, s, H-5‴) 1.59 (3H, s, H-5⁗), 1.59 (3H, s, H-4⁗); ^{13}C NMR (125 MHz, $CDCl_3$) δ 197.4 (C-4), 163.0 (C-7), 158.3 (C-8a), 144.3 (C-3′), 142.9 (C-4′), 135.0 (C-3⁗), 134.8 (C-3‴), 134.8 (C-3″), 131.0 (C-8), 128.3 (C-6), 122.3 (C-2⁗), 122.1 (C-2″), 122.0 (C-2‴), 119.7 (C-6′), 111.3 (C-2′), 107.7 (C-5′), 107.0 (C-4a), 103.2 (C-1′), 79.0 (C-2), 43.5 (C-3), 29.5 (C-1⁗), 26.1 (C-4⁗), 26.1 (C-4‴), 26.1 (C-4″), 22.3 (C-1″), 21.6 (C-1‴), 18.2 (C-5⁗), 18.2 (C-5″) 18.2 (C-5‴).

2.4.3. Isoamoritin (*3*)

Pale yellow oil; EIMS *m/z* 506; HREIMS [M]$^+$ 506.2670 (calcd for $C_{31}H_{38}O_6$ 506.2668); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.82 (1H, s, H-2′), 6.67 (1H, s, H-6′), 3.73 (3H, s, OCH_3), 3.29 (2H, m, H-1″), 3.26 (2H, m, H-1‴), 3.23 (2H, m, H-1⁗), 2.71–2.96 (2H, m, H-3), 1.67 (3H, s, H-5‴), 1.67 (3H, s, H-4‴), 1.67 (3H, s, H-5″), 1.67 (3H, s, H-4″), 1.65 (3H, s, H-4⁗), (1.65 (3H, s, H-5⁗); ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.7 (C-4), 162.7 (C-7), 159.7 (C-5), 158.0 (C-8a), 149.4 (C-3′), 145.4 (C-4′), 135.9 (C-1′), 135.4 (C-3″), 135.0 (C-3‴), 134.4 (C-3⁗), 133.7 (C-5′), 122.5 (C-2″), 122.4 (C-2‴), 122.2 (C-2⁗), 119.2 (C-6′), 111.4 (C-2′), 107.7 (C-6), 106.8 (C-8), 103.2 (C-4a), 78.8 (C-2), 61.6 (OCH_3), 43.8 (C-3), 28.6 (C-1″), 26.2 (C-5″), 26.15 (C-4″), 22.3 (C-1‴), 21.6 (C-1⁗), 18.3 (C-5⁗), 18.3 (C-4‴), 18.2 (C-4⁗), 18.2 (C-5⁗).

2.4.4. Amoricin (*4*)

Pale yellow oil; EIMS *m/z* 504 [M]$^+$; HREIMS *m/z* 504.2513 (calcd for $C_{31}H_{36}O_6$ 504.2512); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.94 (1H, s, H-2′), 6.79 (1H, s, H-6′), 6.65 (1H, d, *J* = 10.0 Hz, H-1″), 5.52 (1H, d, *J* = 10.0 Hz, H-2″), 3.73 (3H, s, OCH_3), 3.39 (2H, m, H-1⁗), 3.24 (2H, m, H-1‴), 2.83–3.01 (2H, m, H-3), 1.81 (3H, s, H-4⁗), 1.78 (3H, s, H-5‴), 1.70 (3H, s, H-4⁗), 1.68 (3H, s, H-5⁗), 1.50 (3H, s, H-4″), 1.47 (3H, s, H-5″); ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.7 (C-4), 160.2 (C-7), 159.6 (C-8a), 149.4 (C-4′), 145.4 (C-3″), 135.8 (C-3⁗), 135.4 (C-3‴), 133.6 (C-1′), 131.4 (C-5′), 126.5 (C-2″), 122.9 (C-2‴), 122.4 (C-2⁗), 119.3 (C-6′), 116.0 (C-1″), 111.5 (C-2′), 109 (C-8), 103.2 (C-6), 103.0 (C-4a), 78.9 (C-3″), 78.9 (C-2), 61.6 (OCH_3), 43.7 (C-3), 43.7 (C-3), 29.7 (C-1‴), 28.8 (C-4″), 28.7 (C-5″), 26.2 (C-4‴), 26.1 (C-5⁗), 21.3 (C-1⁗), 18.2 (C-5‴), 18.2 (C-4⁗).

2.4.5. Amorphigeni (*5*)

Needles; EIMS *m/z* 410 [M]$^+$; HREIMS *m/z* 410.1368 (calcd for $C_{23}H_{22}O_7$ 410.1336); ^{1}H NMR (500 MHz, CDCl3) δ 7.77 (1H, d, *J* = 8.55 Hz, H-11), 6.84 (1H, s, H-1), 6.44 (1H, d, *J* = 8.55 Hz, H-10), 6.38 (1H, s, H-4), 5.32 (1H, t, *J* = 9.05, H-5′), 5.20 (2H, d, *J* = 12.9 Hz, H-8′), 4.86 (1H, m, H-6a), 4.54 (1H, m, H-6b), 4.19 (2H, d, *J* = 4.05 Hz, H-7′), 4.11 (1H, m, H-6a), 3.78 (1H, d, *J* = 4.05 Hz, H-12a), 3.69 (3H, s, OMe), 3.74 (3H, s, OMe), 3.32 (1H, m, H-4′a), 3.02 (1H, m, H-4′b); ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.3 (C-12), 167.3 (C-9), 158.3 (C-9), 149.9 (C-2), 147.1 (C-6′), 144.3 (C-3), 130.40 (C-11), 113.9 (C-11a), 113.3 (C-8), 112.9 (C-8′), 110.8 (C-1), 105.32 (C-10), 105.3 (C-12b), 101.3 (C-2), 85.9 (C-5′), 72.6 (C-6a), 66.6 (C-6), 63.2 (C-7′), 45.12 (C-12a), 29.7 (C-4′).

2.4.6. Dalbinol (*6*)

Amorphous solid; EIMS *m/z* 426 [M]$^+$; HREIMS *m/z* 426.1318 (calcd for $C_{23}H_{22}O_8$ 426.1315); ^{1}H NMR (500 MHz, CDCl3) δ 7.74 (1H, d, *J* = 7.74 Hz, H-11), 6.47 (1H, s, H-1), 6.44 (1H, d, *J* = 8.55 Hz, H-10), 6.40 (1H, s, H-4), 5.29 (1H, m,