New spinosin derivatives from the seeds of Ziziphus mauritiana

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Abstract: Three new acylated flavonoid C-glycosides, 6″-(−)-phaseolospinosin (1), 6″-(3‴,4‴,5‴-trimethoxyl)-(E)-cinnamoylspinosin (2), and 6″-(4‴-O-β-D-gluco-pyranosyl)-benzoylspinosin (3), were isolated from the seeds of Ziziphus mauritiana (Rhamnaceae). A further 19 known compounds including eight spinosin analogues (4–11) were also isolated. Their structures were elucidated by means of spectroscopic analysis and chemical method. Among spinosin derivatives 1, 2, 4, 7, 8, and triterpenoid saponin 14, jujuboside A (14) displayed moderate acetylcholinesterase (AchE) inhibitory activity with an inhibition value of 46.2% at a concentration of 1 μM.

Keywords: Ziziphus mauritiana, Rhamnaceae, seeds, spinosin derivatives, jujuboside A, acetylcholinesterase inhibitory

Introduction

The genus Ziziphus (Rhamnaceae), comprises of approximately 170 species and 12 variants, and is distributed in the warm-temperate and subtropical regions throughout the world. Thirteen of which are distributed in the southern and eastern China. Most Ziziphus species are important sources for their edible fruits and medicinal uses. In the Chinese Pharmacopoeia, the dry seeds of Z. jujuba Mill var. spinosa (Bunge) Hu ex H. F. Chou, commonly named as Semen Ziziphi Spinosae ("Suan-Zao-Ren"), have been used traditionally to tranquilize and relax the mind, soothing nerves (anxiolytic), and reducing sweating (anti-hydronic) effect. Flavonoids, saponins and alkaloids were previously reported as isolated from the seeds of Z. jujuba. 1–6

On the other hand, Z. mauritiana Lam., an evergreen shrub up to 15 m height, is distributed in the southern China. It is also widely growing throughout low-latitudes of Asia, Africa and Australia. 1,2 The dry seeds have been used as a substitute of "Suan-Zao-Ren" (seeds of Z. jujuba var. spinosa) by local people in Yunnan province of China. Cyclopeptide, 1,7–12 triterpenes, 1 flavones, 1 steroids, 1,3,14 and aliphatic compounds 1,14 have previously been identified from the roots, barks and leaves of Z. mauritiana. Interestingly, some of these compounds showed antitumor, anti-HIV, 15 anti-plasmiodial and anti-mycobacterial activities. 11 However, no chemical study was reported on the seeds. As a part of our continuing study of the phytotherapeutic components of traditional Chinese medicines (TCM), 7,10 the phytochemical investigation on the seeds of Z. mauritiana was carried on. This led to the isolation of 11 spinosin derivatives (1–11), along with 11 other known compounds (12–22). Compounds 1–3 are new acylated flavonoid C-glycosides. The acetylcholinesterase (AChE) inhibitory activities of the major isolates were tested, using tacrine as the positive control.

Results and Discussion

The defatted MeOH extract of the air-dried seeds of Z. mauritiana was applied to column chromatography (CC) over Diaion HP20SS, Sephadex LH-20, Chromatorex ODS, Toyopearl HW40C, silica gel, MCI-gel CHP20P, and RP-18, finally to semi-preparative HPLC, to give three new compounds (1–3) and 19 known ones. The known compounds (see Electronic Supplementary Material) were determined to

Figure 1. Chemical structures of flavonoids 1–3
be eight spinosin derivatives [6'-dihydrophaseoyl-(4), 6'-p-coumaryloxy-(5), 6'-feruloyl-(6), 6'-sinapoyl-(7), 6'-4 oxo-\beta-D-glucopyranosyl]-vanillyl-(8), 6'-apigenin-2-\beta-D-glucopyranoside (10), and isospinosin (11)], two flavone C-glycosides [vinilen-2,21 apigenin 6-C-\alpha-L-rhamnopyranosyl-(1→2)-\beta-D-glucopyranoside (13)], one triterpenoid saponin [jujuboside A (14)], six lignans [pinolenosin-4,4'-di-\beta-D-glucoside (15), erythro-dihydroxydihydroconderic alcohol (16), threo-dihydroxydihydroconderic alcohol (17), 2-\beta-(+)-dehydrodi-coniferyl alcohol-4-\beta-D-glucopyranoside (18), 19 larinicesin-4-\beta-D-glucopyranoside (20)], one alkaid [triptophane (21)] and one amino acid [tryptophane (22)].

Compound 1 was isolated as a yellow amorphous powder. Its molecular formula was deduced to be C_{20}H_{22}O_{10} by the basis of HREIMS (m/z 870.2990 [M]+). The IR spectrum showed absorption bands at 3430 and 1708 cm\(^{-1}\) due to hydroxyl and carbonyl groups, respectively. The UV spectrum exhibited maximum absorptions at 210, 272, and 332 nm. All the protons and carbons of 1 appeared as pair signals in the \(^1\)H and \(^13\)C NMR spectra, which is characteristic of signals arising from a spinosin skeleton. In addition, the remaining 15 carbon resonances were further classified by DEPT spectrum as six quaternary carbons, including a ketone (\(\delta_c\), 208.37/208.35, C-4\(^\text{m}\)) and a carbonyl (\(\delta_c\), 164.95/164.78, C-15\(^\text{m}\)), three olefinic methines, three methylene including one oxygen-bearing (\(\delta_c\), 76.66, C-11\(^\text{m}\)) and three quaternary methyl (\(\delta_c\), 20.72/20.70, 15.35/15.28, 19.15/19.11, C-10\(^\text{m}\), C-12\(^\text{m}\), C-13\(^\text{m}\)). The \(^1\)H NMR spectrum displayed the presence of two trans-coupled olefinic protons (\(\delta_h\), 6.40/6.39, 7.89/7.81, each 1H, d, J = 15.7 Hz, H-7\(^\text{m}\), H-8\(^\text{m}\)), one singlet olefinic proton at \(\delta_h\) 5.50/5.50 (1H, s, H-16\(^\text{m}\)) and three methyl singlets (\(\delta_h\), 1.91/1.87, 0.88/0.87, 1.06/1.03 (each 3H, s)). The aforementioned data of 1 were closely related to those of 6'-dihydrophaseoylspinossin (4). The difference between 1 and 4 was the sesquiterpene moiety, featuring with an additional ketone in 1, relative to the oxygen-bearing methine in 4. In the HMBC spectrum of 1 (Figure 2), correlations of both H-3\(^\text{m}\) (\(\delta_h\), 2.61/2.27) and H-5\(^\text{m}\) (\(\delta_h\), 2.76/2.30) with C-4\(^\text{m}\) (\(\delta_c\), 208.37/208.35), could assign the additional ketone (\(\delta_c\), 208.37/208.35) to be C-4\(^\text{m}\). The above data of the sesquiterpene moiety of 1 was essentially identical to those of phaseic acid. The position of the phaseic acid moiety in 1 was revealed to be at C-6\(^\text{m}\) by the HMBC correlation of the H-6\(^\text{m}\) (\(\delta_h\), 3.87/3.83) with the carbonyl carbon at \(\delta_c\) 164.95/164.78 (C-15\(^\text{m}\)). Other HMBC and \(^1\)H-\(^1\)H COSY correlations (Figure 2), with electronic supplementary material further confirmed the structure of 1 as shown in Figure 1.

Figure 2. Key HMBC and ROESY correlations of compound 1

The relative configuration of compound 1 was determined on the basis of the coupling constants and ROESY experiment (Figure 2). The trans double bond between C-2\(^\text{m}\) and C-8\(^\text{m}\) was deduced due to the large coupling constant (15.7 Hz) of J-\(^\text{J}_{\text{H}-6\text{-H}-7}\), while the cis double bond between C-9\(^\text{m}\) and C-14\(^\text{m}\) was indicated by the ROESY correlations of the olefinic H-14\(^\text{m}\) (\(\delta_h\), 5.50/5.50) with Me-10\(^\text{m}\) (\(\delta_h\), 1.91/1.87). In the ROESY spectrum of 1, correlations of H-7\(^\text{m}\) and H-8\(^\text{m}\) with both Me-12\(^\text{m}\) and Me-13\(^\text{m}\) were observed. In addition, the H-3\(^\text{m}\) and H-3\(^\text{m}\) were correlated with H-11\(^\text{m}\) and Me-12\(^\text{m}\), respectively. These data reveal that the unsaturated side chain -CH(7)-CH(8)-C(9)-CH(14)-COO(15) of phaseic acid moiety in 1 oriented to the same side of both Me-13\(^\text{m}\) and Me-13\(^\text{m}\), while the CH-11\(^\text{m}\) and O-(C(6)) were oriented to the opposite side of Me-12\(^\text{m}\), Me-13\(^\text{m}\) and C-7\(^\text{m}\) side chain. Alkaline hydrolysis of 1 with 0.5% NaOH yielded spinosin (10) and phaseic acid (1a). Compound 1a showed the similar NMR spectroscopic data and optical rotation value ([\(\alpha\)]\text{D}\) of...
Table 2. $^1$H (600 MHz) and $^{13}$C (150 MHz) NMR spectroscopic data of compounds 2 and 3 (DMSO-$d_6$; 313 K; $\delta$ in ppm)

| position | $\delta_H$ (Hz) | $\delta_C$ ( ppm) |
|----------|-----------------|------------------|
| 2        | 164.10/163.89, C | 6.70/6.46 (IH, s) |
| 3        | 103.17/102.92, CH | 102.89/102.74, CH |
| 4        | 182.22/181.78, CH | 182.23/181.69, CH |
| 5        | 160.81/159.46, CH | 160.70/159.48, CH |
| 6        | 108.76/108.74, CH | 108.81/108.68, CH |
| 7        | 165.23/163.60, CH | 165.09/163.56, CH |
| 8        | 6.69/6.69 (s) | 6.64/6.53 (IH, s) |
| 9        | 90.55/89.88, CH | 90.47/89.85, CH |
| 10       | 157.01/156.85, C | 156.91/156.73, C |
| 11       | 104.45/103.96, C | 104.41/103.90, C |
| 2',6'    | 7.80/7.79 (d, 8.8) | 7.82/7.75 (d, 8.7) |
| 3',5'    | 6.88/6.83 (d, 8.8) | 6.93/6.92 (d, 8.7) |
| 1''      | 115.76/115.70, C | 115.77/115.66, C |
| 4''      | 161.23/161.15, C | 161.29/161.34, C |
| 7-OCH$_3$| 3.90/3.87 (s) | 3.87/3.80 (s) |
| 1''      | 70.98/70.64, CH | 71.02/70.67, CH |
| 2''      | 81.65/80.13, CH | 81.59/80.26, CH |
| 3'       | 7.81/7.68, CH | 7.84/7.99, CH |
| 4'       | 70.33/70.27, CH | 70.33/70.27, CH |
| 5'       | 81.93, CH | 81.91/81.90, CH |
| 6''      | 6.51/6.49 (m) | 6.52/6.48 (m) |
| 1''      | 105.70/105.70, CH | 105.61/105.16, CH |
| 2''      | 74.71/74.43, CH | 74.54/74.51, CH |
| 3''      | 76.31, CH | 76.15/76.11, CH |
| 4''      | 68.66/68.51, CH | 68.96/68.84, CH |
| 5'       | 7.25/72.94, CH | 7.15, CH |
| 6''      | 62.41/62.29, CH | 62.74/62.21, CH |
| 1''      | 129.46/129.21, C | 122.93/122.80, CH |
| 2''      | 105.33/105.18, CH | 130.83/130.64, CH |
| 3''      | 152.98/152.87, C | 115.88/115.86, CH |
| 4''      | 139.43/139.38, C | 161.00/160.95, C |
| 5''      | 152.88/152.87, C | 115.88/115.86, CH |
| 6''      | 105.53/105.18, CH | 130.83/130.64, CH |
| 7''      | 144.32/144.15, CH | 167.44/164.65, C |
| 8''      | 116.80/116.29, CH | 167.44/164.65, C |
| 9''      | 168.06/165.96, CH |  |
| 3''-OCH$_3$| 3.82/3.79 (s$^b$) | 55.98/55.90, CH$_3$ |
| 4''-OCH$_3$| 3.71/3.70 (s) | 60.80/60.60, CH$_3$ |
| 5''-OCH$_3$| 3.82/3.79 (s$^b$) | 55.98/55.90, CH$_3$ |
| 1''''    | 5.00/4.96 (d, 7.5) | 100.03/99.91, CH$_3$ |
| 2''''    | 3.28$^a$ | 73.18, CH |
| 3''''    | 3.34$^a$ | 77.15/76.59, CH |
| 4''''    | 3.18$^a$ | 69.71/69.68, CH |
| 5''''    | 3.44$^a$ | 78.77/78.79, CH |
| 6''''    | 3.70 (IH, m)/3.50$^b$ | 60.69/60.67, CH$_3$ |
| 5-OH     | 13.59 (s) | 13.64 (s) |

$^a$overlapped by solvent; $^b$overlapped with each other.

21.7, c 0.098, MeOH) with those of the reported (-)-phaseic acid ([$\delta_H$]$_{21}$ = 18.0, c 0.10, MeOH). Therefore, the structure of compound 1 was deduced as shown in Figure 1 and named 6''-(3''',4''',5'''-trimethoxy)spinosin derivative acylated with a 3,4,5-trimethoxy-(E)-cinnamoyl moiety. In the HMBC spectrum of compound 1, correlations between the H-6'' ([$\delta_H$] 4.05/3.65) and C-3'''' ([$\delta_C$] 166.08/165.96) indicated the linkage of the cinnamoyl moiety with C-6'' ([$\delta_C$] 62.41/62.29) of spinosin unit. Other HMBC correlations (Figure 3) were used to further confirm the structure of 2 as shown in Figure 1. Thus, the structure of compound 2 was determined as 6''-(3''',4'''',5'''-trimethoxy) (E)-cinnamoyl-spinosin.

Compound 3 was isolated as a yellow amorphous powder. On the basis of the HREIMS (m/z 890.2523, calc. for C$_34$H$_{46}$O$_{24}$: 890.2523, m/z 890.2523), the EI mass spectrum of 3 exhibited the characteristic features of a spinosin skeleton. In addition, a set of signals arising from one carbonyl ([$\delta_C$] 166.08/165.96, C-9''''$^b$), three methoxyl ([$\delta_C$] 55.98/55.90 (OCH$_3$ × 2), 60.80/60.60), four olefinic methines ([[$\delta_C$] 105.53/105.18 (CH × 2), 144.32/144.15 (CH), 116.80/116.29 (CH)], and four aromatic quaternary carbons ([[$\delta_C$] 129.46/129.21 (C), 152.98/152.87 (C × 2), 139.43/139.38 (C)]) were observed in the $^{13}$C NMR (DEPT) spectra. The H NMR spectrum displayed the existence of a symmetric 1,3,4,5-tetra-substituted aromatic ring [$\delta_H$] 6.86/6.69 (2H, s), one trans double bond [$\delta_H$] 6.72/6.70, 6.40/6.30 (each 1H, d, J = 13.8 Hz, H-7'', H-8'') and three methoxyl groups [$\delta_H$] 3.82/3.79 (6H, s, 3''', 5'''-OMe); [$\delta_H$] 3.71/3.70 (3H, s, 4'''-OMe). The aforementioned data suggested that 2 was a

Figure 3. Key HMBC (H $\rightarrow$ C) correlations of compound 2.

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The air-dried seeds (20 kg) of *Z. mauritiana* were powdered and extracted three times per 2 hours with MeOH at 60 °C. The extracts was suspended in H$_2$O and degreased by petroleum ether. The H$_2$O-soluble layer (1.2 kg) was applied to a Diaion HP20SS column, eluting with H$_2$O/MeOH (1:0→0:1), to give fractions I-IV. Compound 10 (13.6 g) was obtained by crystallization from fraction III, and the remaining part was further subjected to Sephadex LH-20 chromatography column, eluting with MeOH–H$_2$O (10:90, 30:70, 50:50, 70:30 and 100:0) to derive the subfractions (III-1 and III-2). Subfraction III-1 (20.5 g) was subjected to Chromatodex ODS column, and eluted with H$_2$O/MeOH (1.0 → 0.1), to derive seven fractions (III-1→7). Fraction III-1-3 (2.0 g) was subjected to Sephadex LH-20, MCI-gel, RP-18, silica gel and semi-preparative HPLC, and obtained compounds 12 (7 mg), 13 (8 mg), 16 (4 mg), 17 (5 mg), 18 (31 mg) and 22 (26 mg). Fraction III-1-4 (5.0 g) was successively separated by Toyopearl HW40C, Sephadex LH-20, silica gel and Sephadex LH-20, then compound 3 (40 mg), 11 (40 mg), 19 (69 mg) and 20 (63 mg) were obtained. Compound 8 (10 mg) was obtained from Fr. III-1-6 (1.1 g) by repeated CC over Toyopearl HW40C, MCI, RP-18 and finally purified by HPLC using MeCN–H$_2$O (18:82) as elution. The sub-fraction III-1-7 (3.2 g) was successively subjected to Toyopearl HW40C, MCI-gel, Sephadex LH-20, and silica gel to afford compounds 1 (139 mg), 4 (170 mg), 7 (113 mg) and 15 (47 mg). Furthermore, fraction III-2 (12.6 g) was successively separated by Rp-18, Toyopearl HW40C and preparative HPLC using MeCN–H$_2$O$_2$ and purified by semi-preparative HPLC using MeCN–H$_2$O$_2$, and isolate compound 9 (4 mg). Similarly, Fraction IV was subjected to Sephadex LH-20 chromatography column, and eluted with MeOH–H$_2$O (30:70, 50:50, 70:30 and 90:10) and was successively separated by Toyopearl HW40C, Rp-18, silica gel, Sephadex LH-20, MCI-gel and silica gel were used to isolate compounds 2 (280 mg), 5 (11 mg), 6 (200 mg), 14 (137 mg) and 21 (109 mg).

**Experimental Section**

**General Experimental Procedures.** Optical rotations were determined with a Jasco P-1020 polarimeter. UV (in MeOH) spectra were obtained with the Shimadzu UV-2401 PC spectrophotometer. The Bruker Tensor-27 infrared spectrophotometer was developed by Ellman et al. with slight modification. Interstingly, AChE inhibition of compound 8 was only 7.8% at 1 μM concentration.
6""(3‴,4‴,5‴)-Trimethoxy-cinnamoylspinosin (2): yellow amorphous powder; \( \alpha \ell^\circ = 63.4 \) (c 0.2, MeOH); UV (MeOH) \( \lambda_{\text{max}} (\log e) 309 (4.52) \) and 206 (4.65) nm; IR (KBr) \( \nu_{\text{max}} 3411, 2938, 1706, 1654, 1508, 1491, 1450, 836 \) and 778 cm\(^{-1}\). \( \delta \) (MeOH) 6.58 (1H, d, \( J = 17.6 \) Hz, H-14), 7.35 (1H, d, \( J = 7.4 \) Hz, H-11a), 7.39 (1H, d, \( J = 7.4 \) Hz, H-11b), 5.70 (1H, s, H-15), 6.35 (1H, d, \( J = 15.6 \) Hz, H-7), 8.02 (1H, s, J = 15.6 Hz, H-8). \( ^{13} \)C NMR (150 MHz, DMSO-d\(_6\)): \( \delta \) (C) 150.8 (C-9), 168.3 (C-15), 208.7 (C-10). HREIMS m/z 828.2475 (calcd. for C\(_{41}\)H\(_{40}\)O\(_{3}\) [M]\(^\circ\), 828.2477).

6""(4‴-O-β-D-Glucopyranosyl)-benzoylspinosin (3): yellow amorphous powder; \( \alpha \ell^\circ = 83.2 \) (c 0.2, MeOH); UV (MeOH) \( \lambda_{\text{max}} (\log e) 333 (4.31), 307 (4.25), 251 (4.42) \) and 204 (4.68) nm; IR (KBr) \( \nu_{\text{max}} 3437, 2919, 1704, 1653, 1608, 1510, 1491, 1449, 839 \) and 770 cm\(^{-1}\). \( \delta \) (MeOH) 6.13 (1H, d, \( J = 17.4 \) Hz, H-5a), 2.50 (1H, overlapped with H-5b), 3.51 (1H, d, \( J = 15.6 \) Hz, H-3b), 2.78 (1H, d, \( J = 7.4 \) Hz, H-3a), 2.50 (1H, overlapped with solvent, H-5a), 2.67 (1H, d, \( J = 17.6 \) Hz, H-3b), 2.78 (1H, d, \( J = 17.4 \) Hz, H-5b), 3.51 (1H, d, \( J = 7.4 \) Hz, H-11a), 3.75 (1H, d, \( J = 7.4 \) Hz, H-11b), 5.70 (1H, s, H-14), 6.35 (1H, d, \( J = 15.6 \) Hz, H-7), 8.02 (1H, s, J = 15.6 Hz, H-8). \( ^{13} \)C NMR (150 MHz, DMSO-d\(_6\)): \( \delta \) (C) 15.49 (C-12), 19.30 (C-13), 20.91 (C-10), 48.31 (C-2), 52.04 (C-3), 53.13 (C-5), 76.71 (C-11), 81.35 (C-1), 86.14 (C-6), 121.34 (C-14), 131.14 (C-8), 132.26 (C-7), 150.8 (C-9), 168.3 (C-15), 208.7 (C-4). TLC analysis also indicated the presence of spinosin in the aqueous layer (CHCl\(_3\)-MeOH-H\(_2\)O 8:2:0.2, R\(_c\) 0.23). Similarly to 1, alkaline hydrolysis of 2 and 3 (each 10 mg) was carried out, separately. The existence of spinosin was confirmed by TLC analysis (CHCl\(_3\)-MeOH-H\(_2\)O 8:2:0.2, R\(_c\) 0.23).

Acid Hydrolysis of 1–3. Compounds 1–3 (each 10 mg) was dissolved in 2 N HCl (2 mL) and refluxed at 80 °C for 10 h. The reaction mixture was neutralized with Amberlit IRA-401 and a sugar residue was obtained. Identification of D-glucose was performed by comparison of OD (+) with authentic samples.

Acetylcholinesterase (AChE) Inhibitory Activity. The AChE inhibitory activity was assayed using the spectrophotometric method developed by Ellman et al.\(^{[4]}\) with slightly modification. In brief, the reaction mixture (200 \( \mu \)L in total) containing phosphate buffer (pH 8.0), testing compound (50 \( \mu \)M in DMSO), and AChE (0.02 U/mL) was incubated for 20 min (30 °C). Then, the reaction was initiated by the addition of 40 \( \mu \)L of solution containing DTNB (0.625 mM) and acetylthiocholine iodide (0.625 mM). The hydrolysis of acetylthiocholine was monitored at 405 nm every 30 seconds for one hour. Tacrine was used as positive control with final concentration of 0.333 \( \mu \)M. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

\[ \% \text{ inhibition} = \left( \frac{E - S}{E} \right) \times 100 \]

(E is the activity of the enzyme without test compound and S is the activity of enzyme with the test compound).

Electronic Supplementary Material
Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s13659-013-0028-5 and is accessible for authorized users.

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