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Cyanogenetic glycosides and simple glycosides from the linseed meal

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ABSTRACT

Three new cyanogenetic triglycosides linustatins A–C (**1**–**3**), and two new simple glycosides linustatins D and E (**4** and **5**) were isolated from the 70% ethanol extract of flaxseed meal (*Linum usitatissimum* L.). Their structures were elucidated on the basis of spectroscopic analysis and chemical evidence. All of the isolates showed moderate activities against aldose reductase and weak activities against α-glucosidase, DPP-IV, and FBPase at the same concentrations as the positive control drugs.

1. Introduction

Several plants contain compounds capable of liberating hydrogen cyanide during hydrolysis. This ability, which is known as cyanogenesis, has been observed for centuries in plants such as apricots, peaches, and other important food plants. Most cases of cyanide poisoning are caused by consuming plants belonging to the families of Rosaceae, Leguminosae, and Euphorbiaceae or members of the genus *Sorghum*. While some cases of cyanide poisoning are accidental, large numbers of people are exposed daily to low concentrations of cyanogenic compounds that are present in the food they eat. Cyanogenic glycosides, which liberate hydrogen cyanide when sugar is removed, are responsible for this cyanophoric capability [1]. Flaxseed (*Linum usitatissimum* L.), an herbaceous plant belonging to the Linaceae family, is an important industrial crop worldwide; this crop is grown for its fiber and oilseed [2]. Recent pharmacological research shows that flaxseed exhibits strong anti-cancer, immunodepression, antioxidant, hypoglycemic, and hypolipidemic effects [3]. The maximum lignan concentration in flaxseed is 3% (w/w); thus, flax is one of the richest edible sources of lignans [4]. In addition, flaxseed meal is rich in cyanogenic compounds. However, only a few studies on cyanogenic compounds (e.g., linamarin, lotaustralin, linustatin, neolinustatin, and amygdalin) in flaxseed meal have been reported [5,6], largely because of difficulties associated with isolating and purifying these compounds and the inherent instability of aglycones during hydrolysis. Therefore, research on cyanogenetic glucosides must be conducted systematically to develop and utilize flaxseed meal. In previous paper [7], our research group reported four new compounds from the flaxseed meal. On the basis of the above research, continuous research on the flaxseed meal resulted in the isolation of five new compounds linustatins A–E (**1**–**5**) (Fig. 1). Among them, compounds **1**, **2**, and **3** are new cyanogenetic triglycosides, and compounds **4** and **5** belong to new simple glycoside derivatives. Pharmacological tests indicated that all of the isolates present moderate activities against aldose reductase and weak activities against α-glucosidase, DPP-IV, and FBPase at the same concentrations as the positive control drugs.

2. Experimental details

2.1. General experimental procedures

Optical rotations were measured on a P2000 polarimeter (JASCO, Tokyo, Japan). UV spectra were obtained on a JASCO P650 spectrophotometer. IR spectra were obtained on a Nicolet 5700 FT-IR microscope instrument (FT-IR microscope transmission). 1D and 2D NMR spectra were respectively acquired at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR on a Bruker AVANCE III HD 600 MHz (Bruker Corporation, Germany) in MeOH-d_4, and solvent peaks were used as references. ESIMS and HRESIMS data were obtained using an AccuToFCS JMST100CS spectrometer (Agilent Technologies, Ltd., Santa Clara, CA, USA). Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China) and a Pharmadex LH-20 column (Amersham Biosciences, Inc., Shanghai, China). Preparative TLC separation was performed using

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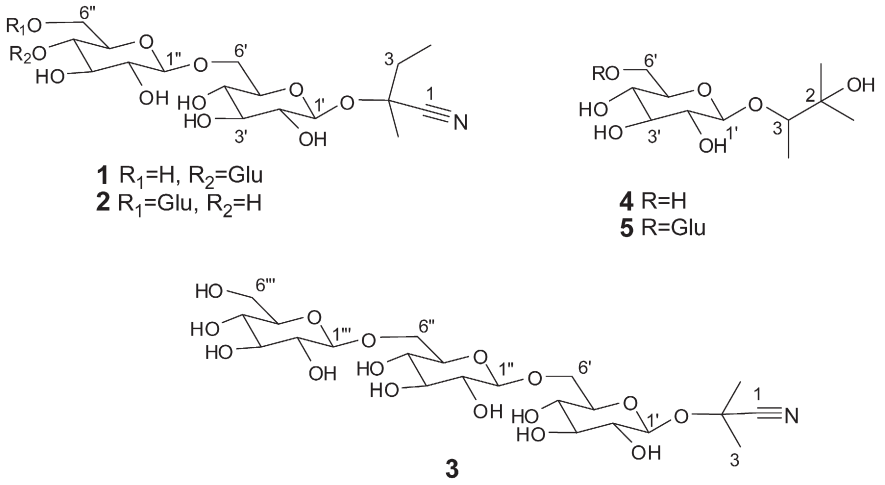

Fig. 1. Structures of compounds **1**–**5**.

high-performance silica gel TLC plates (HSGF254, glass-precoated, Yantai Jiangyou Silica Gel Development Co., Ltd., Yantai, China). HPLC separation was performed on an experimental setup consisting of a Waters 515 pump, Waters 2487 dual λ absorbance detector, and Knauer Smartline RI detector 2300 with a YMC semi-preparative C18-packed column (250 mm × 10 mm i.d., 5 μM). TLC was conducted using glass-precoated silica gel GF-254 plates. Spots were visualized under UV light or by spraying with 7% H_2SO_4 in 95% EtOH followed by heating.

2.2. Plant material

The linseed meal (*L. usitatissimum* L.) was provided by Gansu Puyuan Pharmaceutical Technology Co., Ltd. (Gansu Province, People's Republic of China, May 2009) and identified by Prof. Wang-zhi Song (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100050, China). A voucher specimen (No. ID-S-2540) was deposited at the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China.

2.3. Extraction and isolation

Linseed meal (4.8 kg) was dried, ground and extracted three times with 10 L of 70% EtOH/H_2O under ultrasonication. Each extraction lasted 45 min at room temperature. The combined aqueous EtOH extracts were evaporated to near dryness under vacuum, and the resulting residue (500 g) was suspended in H_2O (5 L). The insoluble solid was removed, and the water-soluble fraction was successively partitioned using petroleum ether (4 × 2 L) and EtOAc (4 × 2 L). The water solution was then absorbed on a macroporous resin and successively eluted with H_2O, 40% EtOH/H_2O, 60% EtOH/H_2O, 70% EtOH/H_2O, and 90% EtOH/H_2O. Exactly 45 g of the 40% EtOH/H_2O fraction was subjected to silica gel CC (80 cm × 10 cm, 200–300 mesh) and eluted using a mixtures of $CHCl_3$/MeOH with increasing polarity (v:v = 4:1, 3:1, 7:3, 2:1, 3:2, 1:1, 2:3, 3:7, 1:4, 1:9, 3 L each) to yield ten fractions, namely, LW-I to LW-X.

Fraction LW-VIII (6.684 g) was loaded on an MPLC with an ODS column (40–63 μm, 4.0 × 50 cm) and eluted using a gradient of increasing MeOH (v:v = 95:5, 50:50, 0:100) in H_2O to produce three subfractions, LW-VIII-A to LW-VIII-C. Subfraction LW-VIII-B (4.0 g) was applied to an ODS column (40–63 μm, 4.0 × 50 cm) and eluted using MeOH/H_2O (v:v = 0:100, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 50 mL each) to afford seven subfractions, LW-VIII-B1 to LW-VIII-B7. Fraction LW-VIII-B2 (1.208 g) was further separated on a silica gel column (mesh 45–75 μm, 1.5 × 20 cm) and eluted using a mixture of $CHCl_3$/MeOH/H_2O (v:v:v = 50:5:0.5–5:5:0.5) to yield six subfractions of LW-VIII-B2-a1 to LW-VIII-B2-a6. Fraction LW-VIII-B2-a4 (32 mg) was loaded on a Sephadex LH-20 column using MeOH/H_2O (v:v = 1:1) as the eluent to produce a subfraction (12 mg) that was subsequently purified by semi-preparative Rp-HPLC using MeOH/H_2O (v:v = 10:90) as the eluent to yield compound **5** (6.88 mg). Fraction LW-VIII-C (1.239 g) was subjected to a Sephadex LH-20 column using a mobile phase of MeOH/H_2O (v:v = 1:1) to produce three subfractions, namely, LW-VIII-C1 to LW-VIII-C3. Subfraction LW-VIII-C1 (906 mg) was further separated on an ODS column (40–63 μm, 4.0 × 20 cm) and eluted using a gradient of increasing CH_3CN (0%–10%) in H_2O to provide three subfractions, namely, LW-VIII-C1-1 to LW-VIII-C1-3. Subfraction LW-VIII-C1-1 (30 mg) was then purified by semi-preparative Rp-HPLC (column, Rp-18, 250 × 10 mm, 5 μm) and eluted using MeCN/1‰TFA/H_2O (v:v = 2:98) to afford compounds **1** (1.6 mg), **2** (5.83 mg), and **3** (5.18 mg).

Fraction LW-V (193.0 mg) was subjected to silica gel CC (mesh 45–75 μm, 1.5 × 20 cm) and eluted using $CHCl_3$/MeOH/H_2O (v:v:v = 85:5:0.5–5:5:0.5) to afford nine subfractions, namely, LW-V-A to LW-V-I. Compound **4** (4.92 mg) was obtained by separating LW-V-H (30.6 mg) on a semi-preparative Rp-HPLC and elution using MeOH/H_2O (v:v = 10:90).

Linustatin A (2-[(*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2-methyl-(2*R*)-butanenitrile, **1**). White amorphous powder; $[\alpha]_D^{20} = -26.9$ (c 0.13, MeOH); IR ν_{max}: 3406, 2924, 2597, 2515, 1720, 1670, 1622, 1546, 1436, 1131, 801, 669, 602, 473 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; (+)-ESIMS *m/z* 608 $[M + Na]^+$, (−)-ESIMS *m/z* 584 $[M-H]^-$, 620 $[M + Cl]^-$; HR-ESI-MS $[M + Na]^+$ *m/z* 608.2169 (calcd for $C_{23}H_{39}NO_{16}Na$, 608.2161).

Linustatin B (2-[(*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2-methyl-(2*R*)-butanenitrile, **2**). White amorphous powder; $[\alpha]_D^{20} = -26.8$ (c 0.49, MeOH); IR ν_{max}: 3406, 2890, 2519, 1675, 1433, 1380, 1291, 1203, 1167, 1068, 899, 837, 800, 721, 666, 604 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; (+)-ESIMS *m/z* 608 $[M + Na]^+$, (−)-ESIMS *m/z* 584 $[M-H]^-$, 620 $[M + Cl]^-$; HR-ESI-MS $[M + Na]^+$ *m/z* 608.2175 (calcd for $C_{23}H_{39}NO_{16}Na$, 608.2161).

Linustatin C (2-[(*O*-β-D–glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2-methyl-propionitrile, **3**). White amorphous powder; $[\alpha]_D^{20} = -27.1$ (c 0.43, MeOH); IR ν_{max}: 3390, 2975, 2894, 1677, 1434, 1368, 1203, 1138, 1076, 1049, 880, 841, 801, 722, 625 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; (+)-ESIMS

Table 1
^{1}H NMR spectroscopic data of compounds **1**–**5**.

No.	1	2	3	4	5
1				1.11 (s)	1.11 (s)
3a	1.82 (dt, 7.4,14.1)	1.83 (dt, 7.3,14.6)	1.68 (s)	3.53 (q, 6.6)	3.57 (q, 6.6)
3b	1.90 (dt, 7.4,14.1)	1.92 (dt, 7.3,14.6)			
4	1.03 (t, 7.4)	1.03 (t, 7.3)	1.66 (s)	1.18 (d, 6.0)	1.18 (d, 6.6)
5	1.56 (s)	1.57 (s)		1.11 (s)	1.11 (s)
1′	4.56 (d, 7.8)	4.57 (d, 7.8)	4.64 (d, 7.8)	4.35 (d, 8.4)	4.36 (d, 7.8)
2′	3.15 (m)	3.15 (m)	3.22 (m)	3.15 (m)	3.16 (m)
3′	3.32 (m)	3.32 (m)	3.34 (m)	3.28 (m)	3.28 (m)
4′	3.32 (m)	3.26 (m)	3.31 (m)	3.24 (m)	3.30 (m)
5′	3. 43 (m)	3. 42 (m)	3.47 (m)	3.20 (m)	3.40 (m)
6′a	3.71 (dd, 11.4, 6.0)	3.71 (dd, 11.4, 4.8)	3.50 (m)	3.80 (dd, 12.0, 2.4)	4.07 (dd, 12.0, 2.4)
6′b	4.09 (dd, 11.4, 1.8)	4.10 (dd, 11.4, 1.8)	4.5 (m)	3.61 (dd, 12.0, 5.4)	3.73 (dd, 12.0, 6.0)
1″	4.39 (d, 7.8)	4.34 (d, 7.8)	4.37 (d, 7.8)		4.35 (d, 7.8)
2″	3.21 (m)	3.15 (m)	3.22 (m)		3.16 (m)
3″	3.50 (m)	3.32 (m)	3.34 (m)		3.28 (m)
4″	3.50 (m)	3.26 (m)	3.31 (m)		3.20 (m)
5″	3.38 (m)	3.47 (m)	3.57 (m)		3.20 (m)
6″a	3.80 (m)	3.70 (dd, 11.4, 4.8)	3.50 (m)		3.81 (dd, 12.0, 2.4)
6″b	3.80 (m)	4.08 (dd, 11.4, 1.8)	4.15 (m)		3.61 (dd, 12.0, 6.0)
1‴	4.35 (d, 7.8)	4.31 (d, 7.8)	4.37 (d, 7.8)		
2‴	3.18 (m)	3.15 (m)	3.22 (m)		
3‴	3.32 (m)	3.32 (m)	3.41 (m)		
4‴	3.24 (m)	3.26 (m)	3.31 (m)		
5‴	3.30 (m)	3.22 (m)	3.28 (m)		
6‴a	3.60 (dd, 12.0, 5.4)	3.62 (dd, 12.0, 5.4)	3.68 (dd, 12.0, 4.8)		
6‴b	3.81 (dd, 12.0, 1.8)	3.81 (dd, 12.0, 2.4)	3.30 (br d, 10.2)		

Measured in MeOH-d_4 at 600 MHz, with assignments confirmed by ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC.

m/z 594 $[M + Na]^+$, (−)-ESIMS m/z 570 $[M-H]^-$, 606 $[M + Cl]^-$; HR-ESI-MS $[M + Na]^+$ m/z 594.2005 (calcd for $C_{22}H_{37}NO_{16}Na$, 594.2005).

Table 2
^{13}C NMR spectroscopic data of compounds **1**–**5**.

No.	1	2	3	4	5
1	121.6	121.7	122.2	26.0	26.1
2	76.1	76. 1	72.5	74.3	74.3
3	35.1	34.9	28.6	85.9	85.5
4	8.82	8.86	27.4	17.6	17.6
5	24.2	24.3		24.3	24.4
1′	100.5	100.5	100.9	106.4	106.1
2′	74.8	74.8	74.8	75.7	75.1
3′	77.9	78.0	78.0	78.1	77.9
4′	71.3	71.6	71.5	71.6	71.5
5′	77.3	76.9	77.0	78.0	77.2
6′	69.9	70.0	69.9	62.7	69.8
1″	104.6	104.9	104.9		104.9
2″	75.0	75.1	75.1		75.6
3″	75.9	77.8	77.8		77.9
4″	80.6	71.6	71.6		71.6
5″	76.2	77.2	77.0		78.0
6″	61.8	70.5	70.1		62.7
1‴	104.4	104.9	105.0		
2‴	74.9	75.2	75.1		
3‴	77.9	77.7	77.7		
4‴	71.4	71.6	71.6		
5‴	78.1	77.9	77.9		
6‴	62.4	62.7	62.7		

Measured in MeOH-d_4 at 150 MHz, with assignments confirmed by DEPT, HMQC and HMBC.

Linustatin D (2-methyl-2,3-dihydroxybutyl-3-*O*-β-D-glucopyranoside, **4**). White amorphous powder; $[\alpha]_D^{20} = -8.4$ (c 0.41, MeOH); IR ν_{max}: 3359, 2980, 2923, 1725, 1675, 1452, 1382, 1316, 1255, 1175, 1078, 1040, 949, 892, 866, 755, 652, 612, 583, 524 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; (+)-ESIMS m/z 289 $[M + Na]^+$, (−)-ESIMS m/z 265 $[M-H]^-$; HR-ESI-MS $[M + Na]^+$ m/z 289.1267 (calcd for $C_{11}H_{22}O_7Na$, 289.1258).

Linustatin E (2-methyl-2,3-dihydroxybutyl-3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-gluco pyranoside, **5**). White amorphous powder; $[\alpha]_D^{20} = -24.2$ (c 0.57, MeOH); IR ν_{max}: 3388, 2979, 2913, 1649, 1589, 1446, 1379, 1288, 1171, 1077, 1041, 949, 899, 621 cm^{-1}; ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; (+)-ESIMS m/z 451 $[M + Na]^+$, (−)-ESIMS m/z 427 $[M-H]^-$, 463 $[M + Cl]^-$; HR-ESI-MS $[M + Na]^+$ m/z 451.1800 (calcd for $C_{17}H_{32}O_{12}Na$, 451.1786).

*2.4. Absolute configurations of the sugars for compounds **1**, **2**, **3**, **4**, and **5***

The absolute configurations of glucose were determined according to a reported procedure [8]. Compound **1** (1 mg) was stirred in 1 M HCl (1 mL) at 100 °C for 1 h. The reaction mixture was dried under vacuum. Then water was added to the mixture, and the acidic solution was evaporated to remove HCl. After drying under vacuum, the residue was dissolved in pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (2 mg) and heated at 60 °C for 1.5 h. o-Toylisothiocyanate (2 μL) was subsequently added, and the mixture was heated at 60 °C for 2 h. Each reaction mixture was directly analyzed by HPLC [consisting of a Waters 515 pump, Waters 2487 dual λ absorbance detector, and Knauer Smartline RI detector 2300 with a COSMOSIL packed column C18-AR II (250 mm × 4.6 mm i.d., 5 μM); temp, 35 °C; flow, 0.8 mL/min; eluate, CH_3CN-H_2O (25:75) containing 50 mM H_3PO_4] at 250 nm. The reaction conditions for D- and L-glucose were the same as described above. Retention times of authentic sugar derivatives, D-glucose (11.72 min), and L-glucose (10.58 min) were used for comparison with those from reaction mixtures. A peak at 11.63 min of the sugar derivative from **1** coincided with the derivative of D-glucose. The absolute configurations of the sugars for **2**–**5** were identified using the same method as **1**.

2.5. Assessment of inhibitory activities of α-glucosidase, lipase, DPP-IV, aldose reductase, and FBPase

Detailed procedures for conducting assays for α-glucosidase, lipase, DPP-IV, aldose reductase, and FBPase inhibition are provided in a previous study [9–13]. Acarbose, orlistat, INDP-2, epalrestat, and FBPI377-6 were used as positive controls.

3. Results and discussion

Compound **1** was isolated as a white amorphous powder. HR-ESI-MS results showed a pseudomolecular ion $[M + Na]^+$ at m/z 608.2169 (calcd., 608.2161), which agrees with the molecular formula $C_{23}H_{39}NO_{16}$. The IR spectrum of compound **1** shows the presence of a hydroxyl group at 3525 and 3406 cm^{-1}, and a cyano group at 2515 cm^{-1}. The ^{1}H NMR spectrum of compound **1** (Table 1) shows one methyl signal at δ_H 1.56 (3H, s, H-5) and one ethyl signals at δ_H 1.03 (3H, t, J = 7.4 Hz, H-4), 1.82 (1H, dt, J = 7.4, 14.1 Hz, H-3a), and 1.90 (1H, dt, J = 7.4, 14.1 Hz, H-3b). Two quaternary C signals at δ_C 76.1 and 121.6 ppm were observed in the ^{13}C NMR and DEPT spectra (Table 2); Combination of these results with the signal at 2515 cm^{-1} in IR spectrum, suggests the presence of 2-methyl-2-hydroxyl-butanenitrile moieties. The presence of three anomeric proton signals at δ_H 4.56 (1H, d, J = 7.8 Hz, H-1′), 4.39 (1H, d, J = 7.8 Hz, H-1″), and 4.35 (1H, d, J = 7.8 Hz, H-1‴) in the ^{1}H NMR spectrum as well as three anomeric C signals at δ_C 100.5, 104.6, and 104.4 ppm and 15 characteristic C signals at δ_C 62.0–90.0 ppm in ^{13}C

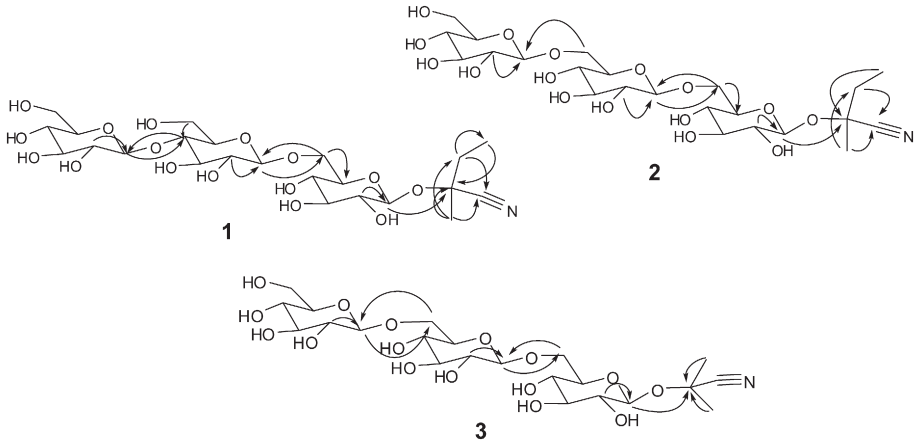

Fig. 2. Significant HMBC correlations of compounds **1**, **2**, and **3**.

NMR spectrum indicates the presence of three β-D-glucopyranose groups in compound **1**. Comparison of the NMR data of **1** and neolinustatin reported in the literature [6] demonstrated that the two compounds exhibited similar structures, and the major difference between them was the replacement of a C-4″ hydroxyl in neolinustatin by a D-glucopyranose unit in compound **1**. These evidences suggested that compound **1** was a cyanogenetic glucoside with three D-glucopyranoses. In the HMBC spectrum (Fig. 2), correlations of H-1′/C-2; H-3, H-4, and H-5/C-2; H-1″/C-6′; H_2-6′/C-1″; H-1‴/C-4″; and H-4″/C-1‴, together with their corresponding chemical shifts, verified the connections of C-2 and C-1′, C-1″ and C-6′, and C-1‴ and C-4″. The coupling constants (J = 7.8, 7.8, 7.8 Hz) of the anomeric protons and chemical shifts (δ_C 100.5, 104.6, 104.4) of the anomeric C demonstrated that three sugar moieties should be β-anomeric configuration [14]. Hydrolysis of compound **1** in the presence of acid produced D-glucose; This result, together with its NMR data, demonstrated that all three glycosyl groups were D-glucopyranoses. Thus, compound **1** was determined as 2-[(*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2–methyl-butanenitrile, and subsequently named linustatin A.

Compound **2** exhibited the molecular formula $C_{23}H_{39}NO_{16}$, as indicated by the HR-ESI-MS results (m/z 608.2175 $[M + Na]^+$; calcd. for $C_{23}H_{39}NO_{16}Na$, 608.2161) and NMR data. Its IR spectrum showed a hydroxyl group at 3406 cm^{-1} and a cyano group at 2515 cm^{-1}. The NMR data of **2** (Tables 1 and 2) were similar to those of compound **1**, which showed the presence of a 2-methyl-2-hydroxyl-butanenitrile moiety and three β-D-glucopyranose moieties. This suggested that compound **2** could be an isomer of **1**. The ^{13}C NMR data of the sugar moiety of compounds **1** and **2** were compared, and the downfield values at C-6′ and C-6″ (i.e., δ_C 69.9 and 70.0 for C-6′, δ_C 61.8 and 70.5 for C-6″) were observed; which suggested that three β-D-glucopyranose moieties were linked through C-1‴/C-6″ and C-1″/C-6′. This association was also confirmed by the HMBC correlations (Fig. 2) of H-1″ with C-6′, H_2-6′ with C-1″, H-1‴ with C-6″, and H_2-6″ with C-1‴. The HMBC correlations of C-2 with H-1′, H-3, H-4, and H-5 indicated that C-2 was connected to C-1′. In addition, hydrolysis of compound **2** in the presence of acid produced D-glucose. This result, together with the coupling constants (J = 7.8, 7.8, 7.8 Hz) of the anomeric protons and chemical shifts (δ_C 100.5, 104.9, 104.9) of the anomeric carbons, confirmed the presence of β-D-glucopyranose in compound **2**. Thus, compound **2** was determined as 2-[(*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2-methyl-butanenitrile, and named linustatin B.

Compound **3** was isolated as an amorphous powder. Its molecular formula was determined as $C_{22}H_{37}NO_{16}$ from the positive HR-ESI-MS data at m/z 594.2005 $[M + Na]^+$ (calcd. for $C_{22}H_{37}NO_{16}Na$, 594.2005). The IR spectrum of **3** showed the presence of hydroxyl group at 3390 cm^{-1} and cyano group at 2502 cm^{-1}. Comparison of the NMR data (Tables 1 and 2) of compounds **3** and **2** indicated that the two compounds had an identical sugar chain; the major difference between them was the replacement of aglycone of 2-methyl-2-hydroxyl-butanenitrile in compound **2** by 2-methyl-propionitrile [δ_H 1.66 (3H, s, H-4) and 1.68 (3H, s, H-3) in ^1H NMR, and δ_C C-1 (122.2), C-2 (72.5), C-3 (28.6), and C-4 (27.4) in ^{13}C NMR] in compound **3**. The correlations of H-1′/C-2, H-1″/C-6′, H_2-6′/C-1″, H-1‴/C-6″, and H_2-6″/C-1‴ in the HMBC spectrum (Fig. 2) substantiated the connections between C-1′ and C-2, C-1″ and C-6′, and C-1‴ and C-6″. In addition, hydrolysis of compound **3** in the presence of acid produced the same D-glucose obtained from compound **2**. These above evidences confirm the presence of β-D-glucopyranose in **3**. Therefore, compound **3** was determined as 2-[(*O*-β-D–glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2-methyl-propionitrile, and named linustatin C.

Compound **4** was obtained as an amorphous powder, and its molecular formula was $C_{11}H_{22}O_7$ based on the positive HRESIMS data at m/z 289.1267 $[M + Na]^+$ (calcd., 289.1258). The IR spectrum of compound **4** exhibits the presence of a hydroxyl group at 3359 cm^{-1} and saturated aliphatic protons at 2980, 2924, and 1382 cm^{-1}. Signals at δ_H 1.18 (3H, d, J = 6.0 Hz, H-4), 1.11 (3H, s, H-1), 1.11 (3H, s, H-5), and 3.53 (1H, q, J = 6.0 Hz, H-3) and δ_C 26.0, 24.3, 17.6, 85.9, and 74.3 in the NMR spectrum of compound **4** (Tables 1 and 2) indicate the presence of a 2-methyl-2,3-dihydroxybutyl moiety. Anomeric proton and C signals at δ_H 4.35 (1H, d, J = 8.4 Hz, H-1′) and δ_C 106.4 ppm, together with five characteristic C

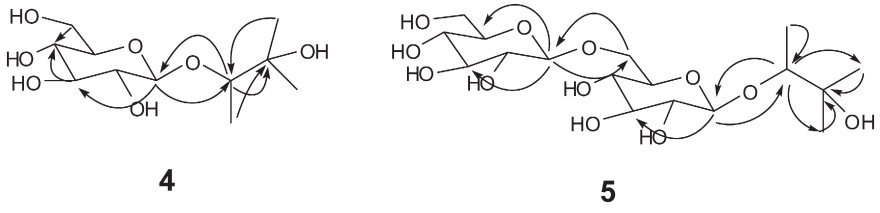

Fig. 3. Significant HMBC correlations of compounds **4** and **5**.

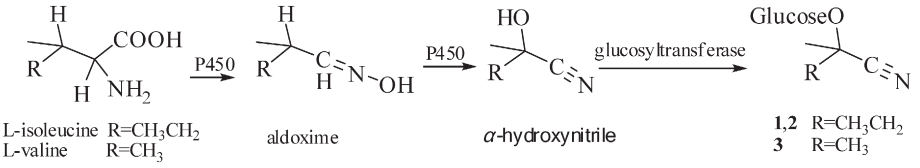

Fig. 4. The proposed biosynthetic pathway for the cyanogenic glucosides **1**–**3**.

signals from δ_C 62.7 ppm to 78.1 ppm, confirm the presence of a glycosyl group. In the HMBC spectrum (Fig. 3) correlations of H-1′ with C-3 and H-3 with C-1′ indicate that C-1′ is connected to C-3. Hydrolysis of compound **4** in the presence of an acid produced D-glucose. This result, together with the corresponding NMR data, demonstrates the presence of β-D-glucopyranose. Therefore, compound **4** was identified as 2-methyl-2,3-dihydroxybutyl-3-*O*-β-D-glucopyranoside according to the results of DEPT, ^{1}H–^{1}H COSY, HMBC, and HSQC experiments; this compound was subsequently named linustatin D.

Compound **5** was obtained as a white amorphous powder, and its molecular formula was determined as $C_{17}H_{32}O_{12}$ based on the positive HRESIMS data at *m/z* 451.1800 $[M + Na]^+$ (calcd. for $C_{17}H_{32}O_{12}Na$, 451.1786). The IR spectrum of compound **5** demonstrates the presence of a hydroxyl group at 3388 cm^{-1} and saturated aliphatic protons at 2979, 2913, and 1379 cm^{-1}. The NMR data (Tables 1 and 2) of compound **5** are similar to those of compound **4**, in which the presence of a 2-methyl-2,3-dihydroxybutyl moiety as well as a β-D-glucopyranose moiety was observed. The remaining characteristic NMR data of compound **5** [δ_H 4.35 (d, J = 7.8 Hz, H-1″) and δ_C 104.9, 75.6, 77.9, 71.6, 78.0, and 62.7] suggest the presence of another β-D-glucopyranose moiety. The ^{13}C NMR data of the sugar moiety in compounds **4** and **5** were compared, and the C-6′ in the β-D-glucopyranose group of compound **5** was observed at a lower field (δ_C 69.8). This result suggests that C-1″ of the second β-D-glucopyranose group is linked to C-6′ of the first β-D-glucopyranose. HMBC correlations (Fig. 3) of H-1″ with C-6′ and H_2-6′ with C-1″ confirm this assumption, while HMBC correlations of H-3 with C-1′ and H-1′ with C-3 indicate that the sugar chain is linked to C-3. Hydrolysis of compound **5** in the presence of an acid produced D-glucose; this result, together with the NMR data, demonstrates that both glycosyl groups of compound **5** are β-D-glucopyranose moieties. Finally, compound **5** was determined as 2-methyl-2,3-dihydroxybutyl-3-*O*-β-D-gluco pyranosyl-(1 → 6)-β-D-glucopyanoside and named linustatin E.

From biogenesis point of view, cyanogenic glucosides are amino acid-derived secondary plant metabolites. Five protein amino acids, valine, leucine, isoleucine, phenylalanine, and tyrosine, are considered to be the primary precursors of their aglycones. The biosynthetic pathway is catalyzed by two membrane-bound multifunctional cytochrome P450s and an apparently soluble glucosyltransferase [15,16]. Therefore, the possible pathways for cyanogenic glucosides **1**, **2**, and **3** are proposed and outlined in Fig. 4. The first step catalyzed by P450 proceeds via two successive N-hydroxylations of the amino group of the parent amino acids (L-isoleucine for **1** and **2**, L-valine for **3**), followed by decarboxylation and dehydration. The formed aldoxime is subsequently converted to an α-hydroxynitrile through the action of a second cytochrome p450. This reaction involves an initial dehydration reaction that forms a nitrile and is followed by hydroxylation of the alpha carbon to generate a cyanohydrin. Glycosilation of the cyanohydrin catalyzed by a UDPG-glycosyltranferase is the final step for the synthesis of cyanogenetic glucosides. However, it seems difficult to rationalize the biogenetic origin of simple glycosides **4** and **5** by the amino pathway since the absence of cyano group. As far as them, biosynthesis through glycosilation of aliphatic alcohol is probably a reasonable interpretation, although more investigation needs to be done.

The inhibitory activities of compounds **1**–**5** against α-glucosidase (40 μM), lipase (10 μM), DPP-IV (10 μM), aldose reductase (10 μM), and FBPase (10 μM) were evaluated. Isolates of compounds **1**, **2**, **3**, **4**, and **5** showed moderate activities against aldose reductase with inhibition rates of 30.0%, 35.5%, 30.0%, 30.0%, and 28.4%, respectively (epalrestat was used as the positive control, Table 3). In addition, all compounds showed weak activities against α-glucosidase, DPP-IV, and FBPase at the same concentration as the positive control drugs (i.e., acarbose, INDP-2, epalrestat, and FBPI377-6, Table 3). All of the compounds were no inhibitory effects on lipase at the same concentration as the positive drug orlistat.

Table 3
Assessment of α-glucosidase, lipase, DPP-IV, aldose reductase, and FBPase inhibitory activity (%) of compounds **1**–**5**.

Compounds	α-glucosidase[b]	Lipase[a]	DPP-IV[a]	Aldose reductase[a]	FBPase[a]
1	6.6	3.5	18.6	30.0	13.1
2	9.8	0.0	8.1	35.5	23.3
3	29.5	3.2	14.0	30.0	18.2
4	11.5	0.0	22.1	30.0	23.3
5	6.6	0.0	14.0	28.4	19.9
Acarbose[c]	99.6				
Orlistat[c]		99.8			
INDP-2[c]			99.0		
Epalrestat[c]				99.4	
FBPI377-6[c]					96.6

[a] Concentration: 1×10^{-5} M.
[b] Concentration: 4×10^{-5} M.
[c] Positive controls.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.08.008.

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