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Four New Dicaffeoylspermidine Derivatives From *Lycium barbarum*

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ABSTRACT

Four new dicaffeoylspermidine derivatives, lycibarbarspermidines P–S (**1–4**), were isolated from the fruit of *Lycium barbarum* (wolfberry). The structures were determined by extensive spectroscopic (HRESIMS, 1D NMR, and 2D NMR) analyses and chemical methods. Dicaffeoylspermidine derivatives are a kind of major bioactive and characteristic constituents in wolfberry, and the discovery of **1–4** added new members of this family.

Key words: *Lycium barbarum*, Wolfberry, Dicaffeoylspermidine derivatives, Lycibarbarspermidines
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INTRODUCTION

Lycium barbarum (Solanaceae) is a defoliated shrubbery[1]. As a traditional Chinese medicine (TCM) and functional food worldwide, the fruit of *Lycium barbarum*, called wolfberry and goji berry, has been used to nourish liver and kidney, and brighten eye[2,3]. In our previous research on the constituents of wolfberry, a kind of major bioactive and characteristic constituents, dicaffeoylspermidine derivatives, were firstly reported from wolfberry[4]. Consecutive study resulted in the discovery of four additional dicaffeoylspermidine derivatives, lycibarbarspermidines P–S (**1–4**), from wolfberry. Details of the isolation and structural elucidation of **1–4** are reported herein.

MATERIALS AND METHODS

1. General experimental procedures

UV data were recorded using a JASCO V-550 UV/vis spectrometer (Jasco International Co. Ltd, Tokyo, Japan). IR data were recorded on a JASCO FT/IR-480 plus spectrometer (Jasco International Co. Ltd). Optical rotations were measured on a JASCO P1020 digital polarimeter (Jasco International Co. Ltd). The ESIMS spectra were performed on a Finnigan LCQ Advantage MAX mass spectrometer (Finnigan MAT GmbH, Bremen, Germany). The HRESIMS spectra were obtained on a Micromass Q-TOF mass spectrometer (Waters Corporation, Milford, USA). 1D and 2D NMR spectra were acquired with Bruker AV 300, Bruker AV 400, and Bruker AV 600 spectrometers (Bruker BioSpin Group, Faellanden, Switzerland) using the solvent signals (DMSO-d_6: δ_H 2.50/δ_C 39.5; CD_3OD: δ_H 3.30/δ_C 49.0) as internal standards. The analytical HPLC was performed on a Dionex HPLC system equipped with an Ultimate 3000 pump, an Ultimate 3000 DAD, an Ultimate 3000 column compartment, an Ultimate 3000 autosampler (Thermo Fisher Scientific Inc., Sunnyvale, USA), and an Alltech (Grace) 2000ES ELSD (Alltech Co. Ltd, Portland, USA) using a Cosmosil Packed C_{18} column (4.6 × 250 mm^2, 5 μm) (Nacalai Tesque Inc., Kyoto, Japan) or a Phenomenex Gemini C_{18} column (4.6 × 250 mm^2, 5 μm) (Phenomenex Inc., Los Angeles, USA). The preparative HPLC was performed on a Shimadzu LC-6-AD liquid chromatography (Shimadzu Inc., Kyoto, Japan) with an SPD-20A detector using a Cosmosil Packed C_{18} column (20.0 × 250 mm^2, 5 μm) or a Phenomenex Gemini C_{18} column (21.2 × 250 mm^2, 5 μm). Medium pressure liquid chromatography (MPLC) was equipped with a dual pump gradient system, an UV preparative detector, and a Dr Flash II fraction collector system (Lisui E-Tech Co. Ltd, Shanghai, China). Column chromatography (CC) was performed on HP-20 macroporous resin (Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200–300 mesh, Haiyang Chemical Co. Ltd, Qingdao, China), and ODS (50 μm, YMC Co. Ltd, Tokyo, Japan).

2. Plant materials

Wolfberry was collected from Zhongning County of Ningxia Hui Autonomous Region in China by one of the authors (Kwok-Fai So) in July, 2013. The plant materials were identified by one of the authors (Jia Xiao). A voucher

specimen (LYBA-2013-NX-ZN) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou, China.

3. Extraction and isolation

The dried wolfberry (19.5 kg) was refluxed three times with 100 L of 60% EtOH–H_2O for 2 h each time. After filtration, the EtOH was removed under reduced pressure to yield a concentrated solution. The solution was passed through a HP-20 macroporous resin column (20 × 100 cm^2), successively eluted with EtOH–H_2O (0:100, 30:70, 50:50, 70:30, 95:5, v/v) to yield 5 fractions (F1–F5). A portion (70.0 g) of F2 (695 g) was separated by open silica gel CC with a successive elution of $CHCl_3$–MeOH–H_2O to yield fractions 2.1–2.10. Fraction 2.8 (10.1 g) was separated by MPLC on ODS CC (3.1 × 19.5 cm^2) with a successive elution of MeOH–H_2O–CF_3COOH to yield fractions 2.8.1–2.8.8. A portion (0.5 g) of fraction 2.8.1 (4.1 g) was separated by preparative HPLC (the Cosmosil C_{18} column), using MeOH–H_2O–CF_3COOH (20:80:0.1, v/v/v) at a flow rate of 8 mL/min to yield fractions 2.8.1.1–2.8.1.5. Fraction 2.8.1.1 (25.1 mg) was purified by preparative HPLC (the Cosmosil C_{18} column), using CH_3CN–H_2O–CF_3COOH (8:92:0.1, v/v/v) at a flow rate of 8 mL/min to yield **2** (t_R: 29.3 min, 17.3 mg). Fraction 2.8.1.2 (147.8 mg) was isolated by preparative HPLC (the Phenomenex C_{18} column), using MeOH–H_2O–CF_3COOH (20:80:0.1, v/v/v) at a flow rate of 8 mL/min to yield **3** (t_R: 17.5 min, 14.8 mg). Fraction 2.8.1.3 (48.3 mg) was isolated by preparative HPLC (the Cosmosil C_{18} column), using CH_3CN–H_2O–CF_3COOH (10:90:0.1, v/v/v) at a flow rate of 8 mL/min to yield **1** (t_R: 19.8 min, 16.5 mg). Similarly, fraction 2.9 (3.8 g) was subjected to MPLC on ODS CC to yield fractions 2.9.1–2.9.9. Fraction 2.9.2 (170.6 mg) was isolated by preparative HPLC (the Cosmosil C_{18} column), using MeOH–H_2O–CF_3COOH (20:80:0.1, v/v/v) at a flow rate of 8 mL/min to yield **4** (t_R: 36.3 min, 13.4 mg).

Lycibarbarspermidine P (**1**, Figure 1): Greenish oil; $[\alpha_D^{27}]$ −21.9 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.14), 224 (3.72), 286 (3.47), 317 (3.30) nm; IR (KBr) ν_{max} 3299, 2934, 1682, 1517, 1436, 1285, 1206, 1140, 1075, 802, 723 cm^{-1}; ESIMS (positive) *m/z* 634.6 $[M + H]^+$; ESIMS (negative) *m/z* 632.4 $[M - H]^-$; HRESIMS (positive) *m/z* 634.2971 $[M + H]^+$ (calcd. for $C_{31}H_{44}N_3O_{11}$, 634.2976); ^1H and ^{13}C NMR data see Table 1.

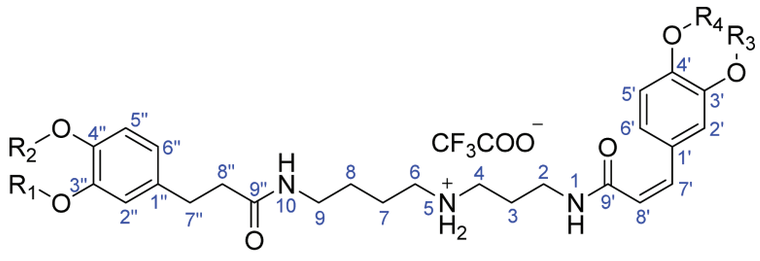
Lycibarbarspermidine Q (**2**, Figure 1): Greenish oil; $[\alpha_D^{27}]$ −24.9 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.41), 283 (3.86) nm; IR (KBr) ν_{max} 3290, 2936, 1681, 1509, 1441, 1280, 1203, 1135, 1072, 801, 723 cm^{-1}; ESIMS (positive) *m/z* 634.4 $[M + H]^+$; ESIMS (negative) *m/z* 632.4 $[M - H]^-$; HRESIMS (positive) *m/z* 634.2974 $[M + H]^+$ (calcd. for $C_{31}H_{44}N_3O_{11}$, 634.2976); ^1H and ^{13}C NMR data see Table 1.

Lycibarbarspermidine R (**3**, Figure 1): Greenish oil; $[\alpha_D^{27}]$ −27.2 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.54), 283 (3.92), 319 (3.87) nm; IR (KBr) ν_{max} 3300, 2941, 1679, 1510, 1438, 1284, 1206, 1135, 1073, 802, 723 cm^{-1}; ESIMS (positive) *m/z* 634.4 $[M + H]^+$; HRESIMS (positive) *m/z* 634.2966 $[M + H]^+$ (calcd. for $C_{31}H_{44}N_3O_{11}$, 634.2976); ^1H and ^{13}C NMR data see Table 1.

Lycibarbarspermidine S (**4**, Figure 1): Greenish oil; $[\alpha_D^{27}]$ −32.0 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.52), 280 (3.93), 314 (3.74) nm; IR (KBr) ν_{max} 3326, 2930, 1678, 1508, 1434, 1281, 1207, 1131, 1075, 802, 723 cm^{-1}; ESIMS (positive) *m/z* 796.5 $[M + H]^+$; HRESIMS (positive) *m/z* 796.3511 $[M + H]^+$ (calcd. for $C_{37}H_{54}N_3O_{16}$, 796.3504); ^1H and ^{13}C NMR data see Table 1.

4. Acid hydrolysis

Acid hydrolysis was performed according to the method described by Tanaka et al.[5] with minor modifications. The sample (1.0 mg) was hydrolyzed with 2 M of HCl for 1 h at 90 °C. After extracted with EtOAc twice, the H_2O layer was evaporated in vacuo to furnish a monosaccharide residue using an Eyela N-1001 rotary evaporator (Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The residue was dissolved in pyridine (1.0 mL) containing L-cysteine methyl ester hydrochloride (1.0 mg) and heated at 60 °C. After 1 h, 10 μL of *o*-tolyl isothiocyanate was added to the reaction mixture and further reacted at 60 °C for 1 h. Then, the reaction mixture was directly analyzed by the Dionex HPLC system and detected by an UV detector (at 254 nm). Analytical HPLC was performed on the Cosmosil C_{18} column with isocratic elution of CH_3CN–H_2O–HCOOH (25:75:0.1, v/v/v) for 40 min at a flow rate of 0.8 mL/min. The standard monosaccharides of D-Glc and L-Glc were subjected to the same method.



1: R_1 = H, R_2 = H, R_3 = β-D-Glc, R_4 = H
2: R_1 = H, R_2 = H, R_3 = H, R_4 = β-D-Glc
3: R_1 = H, R_2 = β-D-Glc, R_3 = H, R_4 = H
4: R_1 = H, R_2 = β-D-Glc, R_3 = H, R_4 = β-D-Glc

Figure 1. Chemical structures of **1**–**4**.

Table 1. NMR Data of **1**–**4** (δ_H in ppm, *J* in Hz).

	1[a]		**2**[b]		**3**[b]		**4**[a]	
No.	δ_C	δ_H[c]	δ_C	δ_H[c]	δ_C	δ_H[c]	δ_C	δ_H[c]
1		8.23, t (5.6)						8.21, t (4.9)
2	35.8	3.17, q (7.0)	36.9	3.32	36.9	3.31	35.7	3.18, q (6.1)
3	25.9	1.75, quint (6.7)	27.4	1.86, quint (6.8)	27.4	1.86, quint (7.0)	25.9	1.75, quint (6.8)
4	44.9	2.87	46.4	2.90	46.4	2.91	44.8	2.88
5		8.43, br s						8.40, br s
6	46.6	2.86	48.6	2.91	48.6	2.90	46.6	2.87
7	23.1	1.52, quint (7.1)	24.4	1.50	24.4	1.49	23.1	1.52, quint (6.4)
8	26.3	1.41, quint (6.9)	27.4	1.48	27.4	1.46	26.2	1.41, quint (6.9)
9	37.8	3.03, q (6.6)	39.3	3.13, t (6.6)	39.3	3.13, t (6.5)	37.8	3.04, q (5.9)
10		7.84, t (5.6)						7.85, t (5.4)
1′	126.8		132.2		128.6		129.9	
2′	119.0	7.80, d (1.9)	117.8	7.12, d (2.5)	117.5	7.07, br s	117.2	7.30, s
3′	144.7		147.8		145.9		146.0	
4′	147.6		147.1		147.4		145.7	
5′	115.3	6.76, d (8.3)	117.8	7.13, d (8.8)	116.0	6.72, d (8.6)	115.6	7.04, s
6′	126.0	7.24, dd (8.3, 1.9)	122.9	6.90, dd (8.4, 1.6)	123.4	6.84, dd (8.0, 1.1)	122.0	7.04, s
7′	137.3	6.52, d (13.1)	138.3	6.69, d (12.7)	139.1	6.63, d (12.4)	136.2	6.53, d (13.0)
8′	120.9	5.80, d (12.9)	123.2	5.94, d (12.5)	121.3	5.83, d (12.6)	122.4	5.86, d (12.8)
9′	166.6		171.1		171.4		166.5	
1″	132.1		133.7		137.8		136.2	
2″	115.7	6.56, d (2.0)	116.8	6.62, d (1.4)	117.3	6.70, br s	115.8	6.64, d (1.8)
3″	145.0		146.0		148.1		146.6	
4″	143.3		144.5		145.1		143.6	
5″	115.4	6.61, d (8.0)	116.5	6.66, d (8.0)	118.8	7.06, d (8.3)	116.9	6.99, d (8.2)
6″	118.7	6.41, dd (8.0, 1.9)	120.8	6.51, dd (8.2, 0.8)	121.0	6.62, br d (7.6)	118.9	6.54, dd (8.5, 1.7)
7″	30.7	2.61, t (7.4)	32.3	2.74, t (7.5)	32.3	2.79, t (7.4)	30.6	2.67, t (7.4)
8″	37.6	2.26, t (7.5)	39.3	2.39, t (7.5)	38.9	2.41, t (7.0)	37.2	2.30, t (7.5)
9″	171.6		175.7		175.4		171.4	
1‴	102.4	4.65, d (7.3)	103.8	4.80, d (7.1)	104.4	4.72, d (7.0)	101.9	4.70, d (7.3)
2‴	73.3	3.29	74.8	3.49	74.8	3.46	73.3	3.26*2
3‴	76.0	3.28	77.6	3.46	77.6	3.45	75.9*1	3.29*3
4‴	69.6	3.21	71.4	3.40	71.2	3.39	69.8	3.16
5‴	77.3	3.31	78.4	3.42	78.3	3.39	77.2	3.32*4
6‴	60.7	3.75, br d (11.9), Ha 3.54, dd (11.9, 5.4), Hb	62.5	3.90, dd (11.8, 0.6), Ha 3.70, dd (12.0, 5.2), Hb	62.3	3.87, d (11.7), Ha 3.71, br d (11.4), Hb	60.8	3.72*5, Ha 3.47, dt (11.8, 5.4), Hb
1⁗							102.7	4.58, d (7.4)
2⁗							73.3	3.29*2
3⁗							75.8*1	3.27*3
4⁗							69.8	3.16
5⁗							77.2	3.29*4
6⁗							60.8	3.70*5, Ha 3.47, dt (11.8, 5.4), Hb
3′-OH								8.58*6, br s
4′-OH		8.97*, s						
3″-OH		8.73*, br s						8.45*6, br s
4″-OH		8.67*, br s						
2‴-OH								5.54*7, br s
3‴-OH								5.10*8, d (3.3)
4‴-OH								5.07*9, d (5.1)
6‴-OH								4.61*10, t (5.4)
2⁗-OH								5.46*7, br s
3⁗-OH								5.09*8, d (3.3)
4⁗-OH								5.04*9, d (5.1)
6⁗-OH								4.60*10, t (4.4)

*Assignment may be interchanged.
[a]Measured in DMSO-d_6 (^{1}H NMR for 600 MHz, ^{13}C NMR for 150 MHz).
[b]Measured in CD_3OD (^{1}H NMR for 400 MHz, ^{13}C NMR for 100 MHz).
[c]The indiscernible signals due to overlap or having the complex multiplicity are reported without designating multiplicity.

RESULTS AND DISCUSSION

Compound **1** was obtained as a greenish oil. Its molecular formula was established as $C_{31}H_{43}N_3O_{11}$ by HRESIMS (*m/z* 634.2971 $[M + H]^+$, calcd. for $C_{31}H_{44}N_3O_{11}$, 634.2976), indicating 12 degrees of unsaturation. The ^{13}C NMR spectrum along with the DEPT-135 experimental data showed that **1** contained 31 carbons, including two

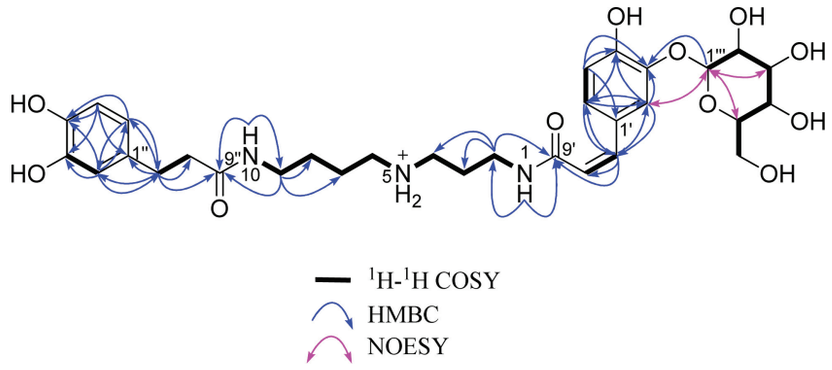


Figure 2. Key 2D NMR correlations of **1**.

carbonyls, fourteen aromatic or olefinic carbons (including eight sp^2 methine carbons), five oxygenated sp^3 methine carbons, and ten sp^3 methylene carbons (including an oxygenated carbon). The nonexchangeable proton resonances were assigned to the relevant carbon atoms by HSQC data. Based on detailed analysis of the ^{1}H–^{1}H COSY and HMBC data (Figure 2), the planar structure of **1** was established, and the assignments of all proton and carbon resonances were provided in Table 1. In the planar structure, there was a hexose unit. A precise comparison of ^{13}C NMR data of the sugar unit with those of glycosides recorded in the literatures[6–8] suggested that the sugar chain of **1** was a glucopyranosyl. The relative configuration of the glucopyranosyl was established as β from the coupling constant of the anomeric proton (H-1‴) located at δ_H 4.65 (d, 1H, J = 7.3 Hz). The absolute configuration of the glucopyranosyl was determined by HPLC analysis of products obtained from acid hydrolysis and derivatization reactions by L-cysteine methyl ester hydrochloride and *o*-tolyl isothiocyanate[5]. Analytical HPLC was performed on the Cosmosil C_{18} column with isocratic elution of CH_3CN–H_2O–HCOOH (25:75:0.1, v/v/v) for 40 min at a flow rate of 0.8 mL/min, and the peaks of the derivatives from standard monosaccharides and sample were recorded at t_R 18.9 (D-Glc), 17.4 (L-Glc), and 19.0 (**1**) min, respectively. These evidences revealed that **1** contained the D-glucose moiety. The key NOESY correlation between H-1‴ and H-2′ confirmed that the glycosylation occurred at C-3′ position (Figure 2). The aglycone of **1** consisted of a spermidine moiety, a *cis*-caffeoyl unit, and a dihydrocaffeoyl unit. N-5 existed as amine salt based on the fact that N-5 bore two protons. When measured in DMSO-d_6, the chemical shifts of H-5 and H/C-3/4/6/7 of the amine salt of lycibarbarspermidines distributed in 8.34–8.47 (H-5), 1.67–1.78/25.9–26.2 (H/C-3), 2.76–2.90/44.6–44.9 (H/C-4), 2.82–2.89/46.5–46.6 (H/C-6), and 1.50–1.53/23.1 (H/C-7), respectively. While, the corresponding chemical shifts of the free base form were assigned in 1.48–1.58/29.5 (H/C-3), 2.42–2.51/46.8–46.9 (H/C-4), 2.41–2.46/48.9–49.0 (H/C-6), and 1.30–1.35/26.8–27.0 (H/C-7), respectively[4]. Thus, the chemical shifts of H-5 and H/C-3/4/6/7 of **1** confirmed the deduction of the amine salt existence. Combined with the preparative method involved with 0.1% CF_3COOH, **1** was inferred as trifluoroacetate. Since the formation of amine salt, the proton signal from CF_3COO^- was not observed in the ^{1}H NMR spectrum. In the ^{13}C NMR spectrum, only two set of very weak carbon signals from CF_3COO^- (about 116–120 and 160 ppm) were detected, and this phenomenon could be explained by the following two reasons: (1) the two carbon signals of CF_3COO^- are quaternary carbons; (2) both of them are splitted by three fluorine atoms[9]. Therefore, **1** was identified as (2*Z*)-*N*-{3-[(4-{[3-(3,4-dihydroxyphenyl) propanoyl]amino}butyl)amino]propyl}-3-[3-(*β*-D-glucopyranosyloxy)-4-hydroxyphenyl]prop-2-enamide, and named lycibarbarspermidine P.

Compounds **2** and **3** were obtained as greenish oils. Their molecular formulas were the same as that of **1**, indicating that they were isomers of **1**. The detailed analyses of 1D and 2D NMR data of **2** and **3** (Tables S2–S3, Supporting Information) deduced their planar structures, respectively. The planar structures of **2** and **3** had the same aglycone as that of **1**, along with a hexose unit. Unlike those of **1**, the glycosylations of **2** and **3** occurred at C-4′ and C-4″ positions, respectively. The key ROESY correlations of **2** and **3** confirmed these results. A precise comparison of ^{13}C NMR data of the sugar unit with those of glycosides recorded in the literatures[6,10-11] suggested that the sugar chains of **2** and **3** were glucopyranosyls. The relative configurations of the glucopyranosyls were established as β from the coupling constants of the anomeric protons (H-1‴). The glucopyranosyls of **2** and **3** were the D-Glc according to HPLC analysis of products obtained from acid hydrolysis and derivatization reactions by L-cysteine methyl ester hydrochloride and *o*-tolyl isothiocyanate[5]. Combined with the preparative method and the chemical shifts of H/C-3/4/6/7 in CD_3OD compared with lycibarbarspermidine J[4], **2** and **3** were inferred as trifluoroacetates. Just like the situation in **1**, the carbon signals of CF_3COO^- were hardly observed in the ^{13}C NMR spectra of **2** and **3**. Therefore, **2** and **3** were identified as (2*Z*)-*N*-{3-[(4-{[3-(3,4-dihydroxyphenyl)propanoyl]amino}butyl)amino] propyl}-3-[4-(*β*-D-glucopyranosyloxy)-3-hydroxyphenyl] prop-2-enamide (named lycibarbarspermidine Q) and (2*Z*)-3-(3,4-dihydroxyphenyl)-*N*-(3-{[4-({3-[4-(*β*-D-glucopyranosyloxy)-3-hydroxyphenyl]propanoyl}amino)butyl]amino} propyl)prop-2-enamide (named lycibarbarspermidine R), respectively.

Compound **4** was obtained as a greenish oil. Its molecular formula was established as $C_{37}H_{53}N_3O_{16}$ by HRESIMS (*m/z* 796.3511 $[M + H]^+$, calcd. for $C_{37}H_{54}N_3O_{16}$, 796.3504), indicating 13 degrees of unsaturation. The detailed analysis of 1D and 2D NMR data of **4** (Table S4, Supporting Information) built up its planar structure. The planar structure of **4** consisted of the same aglycone as that of **1** and two hexose units. A precise comparison of ^{13}C NMR data of the sugar unit with those of glycosides recorded in the literatures[6–8] suggested that both of the two hexose units of **4** were glucopyranosyls. The relative configurations of the two glucopyranosyls were established as β from the coupling constants of the anomeric protons (H-1‴ and H-1⁗). Both of the two glucopyranosyls were D-Glc according to HPLC analysis of products obtained from acid hydrolysis and derivatization reactions by L-cysteine methyl ester hydrochloride and *o*-tolyl isothiocyanate[5]. The key NOESY correlations between H-1‴ and H-5′ and between H-1⁗ and H-5″ confirmed that the two glycosylations occurred at C-4′ and C-4″ positions, respectively. N-5 existed as amine salt based on the fact that N-5 bore two protons and its chemical shifts of H-5 and H/C-3/4/6/7 in DMSO-d_6[4]. Combined with the preparative method, **4** was inferred as trifluoroacetate. Just like the situation in **1**–**3**, the carbon signals of CF_3COO^- were hardly observed in the ^{13}C NMR spectra of **4**. Therefore, **4** was identified as (2*Z*)-3-[4-(β-D-glucopyranosyloxy)-3-hydroxyphenyl]-*N*-(3-{[4-({3-[4-(β-D-glucopyranosyloxy)-3-hydroxyphenyl]propanoyl}amino) butyl]amino}propyl)prop-2-enamide, and named lycibarbarspermidine S.

In conclusion, four new constituents, lycibarbarspermidines P–S (**1**–**4**), were identified from wolfberry. They belong to the family of dicaffeoylspermidine derivatives, a rare kind of plant secondary metabolites. Dicaffeoylspermidine derivatives are a kind of major bioactive and characteristic constituents in wolfberry, and the discovery of **1**–**4** added new members of this family and gave insight into the chemical components of wolfberry.

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