Betonicosides A—D and Betonicolide, Diterpenoids from the Roots of *Stachys officinalis*

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Four new diterpene glycosides, betonicosides A—D and a new diterpene, betonicolide, were isolated from the roots of *Stachys officinalis*. The structures of these compounds were established on the basis of spectroscopic and chemical evidence.

Key words *Stachys officinalis*; betonicoside; betonicolide; diterpene; Labiatae

Previously we worked on the isolation and structural elucidation of phenylethanoid glycosides from the aerial parts of *Stachys officinalis* TREVISAN (syn. *Betonica officinalis* L.),[1] oleanane-type triterpene saponins and phenylethanoid glycosides from *S. riederi* CHAMISSO,[2,3] and iridoid glycosides and phenylethanoid glycoside from *S. sieboldii* MIQ.[4] As terpenic constituents of *S. officinalis*, iridoid glycosides[5] and a unique diterpene, betolide (**6**) have been reported.[6] We investigated the polar constituents of the roots of *S. officinalis* and isolated four new diterpene glycosides, betonicosides A—D (**1**—**4**) and a new diterpene, betonicolide (**5**).

Betonicoside A (**1**) showed a quasi-molecular ion peak $[M+Na]^+$ at m/z 693 in the FAB-MS, and elemental analysis data was consistent with the formula $C_{32}H_{46}O_{15}$. The ^{1}H-NMR spectrum exhibited three singlet methyl proton signals at δ 0.96, 0.98, 1.38 and a pair of oxymethylene proton signals at δ 4.95 and 5.07 as an AB-type quartet ($J=12.5$ Hz), a low field methine proton signal at δ 7.05 as a singlet, and two anomeric proton signals at δ 4.64 (d, $J=7.5$ Hz) and 4.78 (d, $J=8$ Hz). The ^{13}C-NMR spectrum showed two sets of glucopyranosyl carbon signals at δ 62.6—105.3, an ester carbonyl carbon signal at δ 171.3, six aromatic carbon signals at δ 124.8—155.7 and an acetalic carbon signal at δ 103.1 in addition to twelve upfield sp^3 carbon signals at δ 19.0—55.8. The enzymatic hydrolysis of **1** afforded an aglycone, **1a**, while acid hydrolysis afforded D-glucose as a sugar moiety. These data suggested **1** to be a diterpenic diglucoside. In the nuclear Overhauser effect (NOE) difference spectrum, NOE was observed at a methine proton signal (δ 7.05) on irradiation at an anomeric proton signal at δ 4.78, while no NOE was observed at any proton signals other than its own glucosyl proton signals (H-3, H-5). The low field methine proton signal at δ 7.05 was correlated to an acetalic carbon signal at δ 103.1 in the heteronuclear single quantum coherence (HSQC) spectrum. An anomeric proton signal at δ 4.64 was correlated to an aromatic carbon signal at δ 155.7 in the heteronuclear multiple bond coherence (HMBC) spectrum. On acetylation, **1** gave a nonaacetate, **1b**, whose ^{1}H-NMR spectrum exhibited nine aliphatic acetoxyl signals at δ 2.00, 2.02, 2.03, 2.04, 2.04, 2.08, 2.08, 2.10 and 2.15, as well as downfield shifted oxymethylene proton signals at δ 5.36 and 5.57 as an AB-type quartet. On the basis of the above finding, **1** has one phenolic and one aliphatic glycoside. After assigning all proton signals by the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum, ^{13}C–^{1}H correlation and long-range ^{13}C–^{1}H correlation led to the structure shown in Chart 1. The structure of the aglycone (**1a**) was confirmed to be identical to the sodium borohydride reduction product of betolide (**6**) in ^{1}H-NMR spectrum and $[\alpha]_D$.

Betonicoside B (**2**) showed a quasi-molecular ion peak $[M+Na]^+$ at m/z 531 in the FAB-MS and led to the molecular formula $C_{26}H_{36}O_{10}$ in combination with the ^{13}C-NMR data and elemental analysis data. The ^{1}H-NMR spectrum exhibited three singlet methyl proton signals at δ 0.97, 0.99, 1.37, a pair of oxymethylene proton signals at δ 4.94 and 5.08 as an AB-type quartet ($J=$ 12 Hz), a low field methine proton signal at δ 6.84 as a singlet and an anomeric proton signal at δ 4.63 (d, $J=$ 8 Hz). The ^{13}C-NMR spectrum was similar to that of **1** except for lacking a set of glucosyl carbon signals and its upfield shift (-2.8 ppm) of an oxymethine carbon signal at C-15. Acetylation of **2** afforded a hexaacetate **2b**, whose ^{1}H-NMR spectrum suggested that all hydroxyl groups are alcoholic. On enzymatic hydrolysis, **2** afforded an aglycone **1a**, while acid hydrolysis afforded D-glucose as a sugar moiety. In the HMBC spectrum of **2**, an anomeric proton signal at δ 4.63 was correlated to an aromatic carbon signal at δ 155.4. These data led us to conclude the structure of betonicoside B to be **2**.

The ^{1}H- and ^{13}C-NMR spectra of betonicoside C (**3**) were very similar to those of **2**, suggesting that **3** was composed of **1a** as an aglycone and glucose as a sugar. On acetylation, **3** afforded a hexaacetate, **3b**, which had one aromatic acetoxyl group at δ 2.35. In the ^{1}H-NMR spectrum of **3**, NOE was observed at a methine proton signal at δ 6.83 (s) on irradiation of an anomeric proton signal at δ 4.79 (d, $J=8$ Hz). Thus, the structure of betonicoside C was determined to be **3**.

Betonicoside D (**4**) has the same molecular formula, $C_{26}H_{36}O_{10}$, as **2** and **3** in combination with the ^{13}C-NMR data and elemental analysis data. On enzymatic hydrolysis **4** afforded **1a** as an aglycone, while acid hydrolysis afforded D-glucose as a sugar moiety. On acetylation, **4** afforded a hexaacetate, **4b**, whose ^{1}H-NMR spectrum showed that **4** had a phenolic hydroxyl group (δ 2.35). Comparison of the NMR data of oxymethylene proton [δ 5.42 (2H, s)] and carbon (δ 62.3) signals at C-17 with those of **3** indicated that glucose was attached to C-17. In the NOE difference spectrum, NOE was observed at an oxy-

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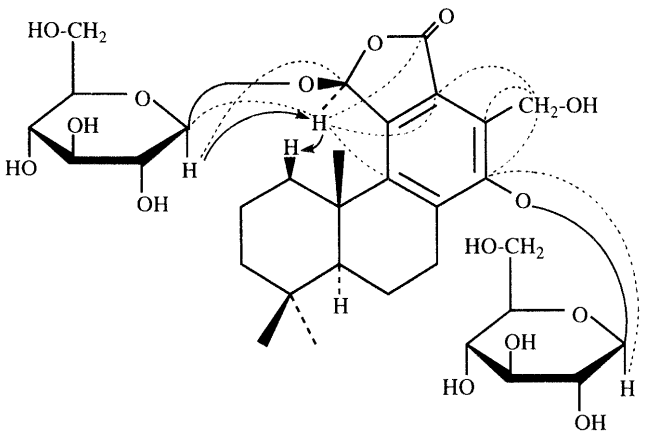

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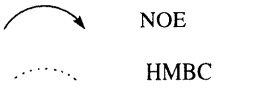
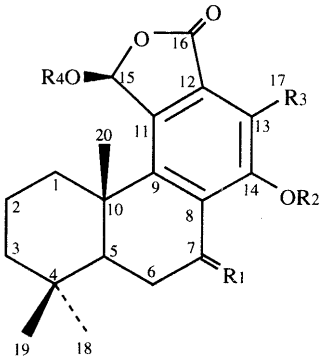


Chart 1



	R1	R2	R3	R4
1	H_2	Glc	CH_2OH	Glc
1a	H_2	H	CH_2OH	H
1b	H_2	Glc $(OAc)_4$	CH_2OAc	Glc $(OAc)_4$
2	H_2	Glc	CH_2OH	H
2b	H_2	Glc $(OAc)_4$	CH_2OAc	Ac
3	H_2	H	CH_2OH	Glc
3b	H_2	Ac	CH_2OAc	Glc $(OAc)_4$
4	H_2	H	CH_2-O-Glc	H
4b	H_2	Ac	CH_2-O-Glc $(OAc)_4$	Ac
5	O	H	CH_2OH	H
6	H_2	H	CHO	H

Glc : β-D-glucopyranosyl

Glc(OAc)4 : tetra-*O*-acetyl-β-D-glucopyranosyl

Chart 2

methylene proton signal at δ 5.42 (2H, s) on irradiation at an anomeric proton signal at δ 4.47 (d, $J=8$ Hz). The HMBC spectrum also supported the structure of **4**. The anomeric configurations of the D-glucosyl moiety of compounds **1**—**4** were determined as β from the J values (7.5—8 Hz) of their proton signals.

Betonicolide (**5**) showed a quasi-molecular ion peak $[M+Na]^+$ at m/z 383 in the FAB-MS, and elemental analysis data was consistent with the formula $C_{20}H_{24}O_6$. The ^{1}H-NMR spectrum exhibited three singlet methyl signals at δ 0.98, 1.04 and 1.43, an oxymethylene proton signal at δ 5.06 (2H, s) and a methine proton signal at δ 6.83 (s). The ^{13}C-NMR spectrum was similar to that of **1a** except for carbon signals around the ketonic carbonyl carbon (δ 208.7). In the HMBC spectrum, the ketonic carbon signal correlated to the proton signal at δ 1.90 (1H, dd, $J=14$, 3 Hz) due to H-5. From these data the structure of betonicolide was determined to be **5**.

In the NOE difference spectra of compounds **1**, **1a** and **2**—**6**, while NOE was observed at the H-1β signal on irradiation of the H-15 signal, no NOE was observed at the H-15 signal on irradiation of the H_3-20 signal. From these data, H-15 was determined to be α in each compound.

Experimental

General Procedure ^{1}H- and ^{13}C-NMR spectra were obtained with a JEOL α-400 FT NMR and the chemical shifts were given in δ ppm with tetramethylsilane as an internal standard. FAB-MS was recorded on a JEOL JMS-SX102 mass spectrometer. Optical rotations were measured with a JASCO DIP-1000 Digital Polarimeter. Gas chromatography (GC) was run on a HITACHI G-3000 gas chromatograph. Preparative, semi-preparative and analytical HPLC were done on a JASCO model 800 instrument.

Extraction and Isolation The dried roots (2 kg) of *S. officinalis* were extracted twice with hot H_2O. The H_2O extract was passed through a Diaion HP-20 (Mitsubishi Kasei Co., Ltd.) column (9×41 cm). After the content of the column was washed with H_2O, the adsorbed materials were eluted with MeOH–H_2O (6:4) and MeOH, successively. The MeOH–H_2O (6:4) eluate (28 g) was chromatographed on a silica gel (600 g) column using $CHCl_3$–EtOAc–MeOH–H_2O [(35:25:35:5)→(30:30:35:5)] to give 16 fractions (frs. 1—16). From fr. 6, compound **1** was isolated by preparative HPLC [Develosil Lop-ODS 5×50 cm×2, CH_3CN–H_2O (25:75)]. The MeOH eluate (7 g) was chromatographed on silica gel (300 g) using $CHCl_3$–MeOH [(90:10)→(70:30)] to give 18 fractions (frs. 17—34). From fr. 18, compound **5** was isolated by preparative HPLC [Develosil Lop-ODS 5×50 cm×2, CH_3CN–H_2O (45:55)]. From frs. 22—24, compounds **2**—**4** were isolated by preparative HPLC [Develosil Lop-ODS 5×50 cm×2, CH_3CN–H_2O (35:65)]. Yield: **1** (0.03%), **2** (0.0025%), **3** (0.0064%), **4** (0.0012%), **5** (0.0010%).

Betonicoside A (1) An amorphous powder, $[\alpha]_D^{21}$ +88.6° ($c=1.23$, MeOH). *Anal.* Calcd for $C_{32}H_{46}O_{15}\cdot 3/2H_2O$: C, 55.09; H, 7.08. Found: C, 54.86; H, 7.23. FAB-MS m/z: 693 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Betonicoside B (2) An amorphous powder, $[\alpha]_D^{21}$ +78.5° ($c=1.14$, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{10}\cdot H_2O$: C, 59.30; H, 7.27. Found: C, 59.32; H, 7.31. FAB-MS m/z: 531 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Betonicoside C (3) An amorphous powder, $[\alpha]_D^{21}$ +122.7° ($c=1.28$, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{10}\cdot 1/2H_2O$: C, 60.34; H, 7.21. Found: C, 60.29; H, 7.30. FAB-MS m/z: 531 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Betonicoside D (4) An amorphous powder, $[\alpha]_D^{20}$ +73.0° ($c=1.00$, MeOH). *Anal.* Calcd for $C_{26}H_{36}O_{10}\cdot H_2O$: C, 59.30; H, 7.27. Found: C, 59.11; H, 7.41. FAB-MS m/z: 531 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR: Table 1.

Betonicolide (5) An amorphous powder, $[\alpha]_D^{20}$ +159.4° ($c=1.60$, MeOH). *Anal.* Calcd for $C_{20}H_{24}O_6$: C, 66.65; H, 6.71. Found: C, 66.54;

Table 1. ^{1}H-NMR Data of Compounds **1**—**5** in CD_3OD

	1	**2**	**3**	**4**	**5**
Aglycone moiety					
1α	1.43[a]				
1β	2.86 (br d, 12)	2.83 (br d, 11.5)	2.83 (br d, 11.5)	2.79 (br d, 11.5)	2.90 (br d, 14)
2α	1.62 (m)	1.64 (m)	1.59 (m)		1.70 (m)
2β	1.81[a]	1.80[a]	1.72 (m)		1.82 (dt, 13, 2)
3α	1.25[a]				1.32 (dt, 13, 2)
3β	1.47 (m)	1.49 (m)	1.46 (m)	1.46 (m)	1.53[a]
5α	1.21 (dd, 13, 3)	1.26[a]	1.22[a]	1.27[a]	1.90 (dd, 14, 3)
6α	1.96 (m)	1.99 (m)	1.96 (m)		2.81 (dd, 19, 3)
6β	1.80[a]				2.92 (dd, 19, 14)
7α	3.16 (m)	3.13[a]	2.92 (dd, 19, 7)	2.97 (dd, 19, 7)	
7β	3.16 (m)	3.13[a]	2.71 (m)	2.74 (m)	
15α	7.05 (s)	6.84 (s)	6.83 (s)	6.71 (s)	6.83 (s)
17	4.95 (d, 12.5)	4.94 (d, 12)	5.26 (d, 12)	5.42 (s)	5.06 (s)
17	5.07 (d, 12.5)	5.08 (d, 12)	5.32 (d, 12)	5.42 (s)	5.06 (s)
18	0.96 (s)	0.99 (s)	0.97 (s)	0.97 (s)	0.98 (s)
19	0.98 (s)	0.97 (s)	0.96 (s)	0.96 (s)	1.04 (s)
20	1.38 (s)	1.37 (s)	1.35 (s)	1.37 (s)	1.43 (s)
Glucose moiety					
	(at C-14)	(at C-14)	(at C-15)	(at C-17)	
1	4.64 (d, 7.5)	4.63 (d, 8)	4.79 (d, 8)	4.47 (d, 8)	
2	3.55 (dd, 9, 7.5)	3.55 (dd, 8.5, 8)	3.37[a]	3.22 (dd, 8.5, 8)	
3	3.45 (dd, 9, 9)	3.44 (dd, 9.5, 8.5)	3.39[a]	3.35[a]	
4	3.35[a]	3.34 (dd, 9.5, 9.5)	3.42[a]	3.33[a]	
5	3.20[a]	3.16[a]	3.36[a]	3.33[a]	
6	3.63 (dd, 12, 6.5)	3.63 (dd, 12, 6.5)	3.71 (dd, 12.5, 4.5)	3.71 (dd, 11.5, 4)	
6	3.78 (dd, 12, 2.5)	3.77 (dd, 12, 2)	3.86 (dd, 12.5, 1.5)	3.86 (dd, 11.5, 1.5)	
	(at C-15)				
1	4.78 (d, 8)				
2	3.35[a]				
3	3.40[a]				
4	3.33[a]				
5	3.37[a]				
6	3.68 (dd, 11.5, 5)				
6	3.81 (dd, 11.5, 2)				

a) Overlapped with other signals. Assignments are based on ^{1}H–^{1}H COSY and/or spin decoupling, HSQC and HMBC spectra, and *J* values in parentheses are expressed in Hz.

Table 2. ^{13}C-NMR Data of Compounds **1**—**5** in CD_3OD

C No.	**1**	**2**	**3**	**4**	**5**
Aglycone moiety					
1	38.0	38.0	37.5	37.3	37.2
2	20.0	20.0	20.0	20.0	19.7
3	42.5	42.4	42.5	42.4	41.7
4	34.7	34.7	34.6	34.6	34.5
5	50.6	50.6	51.3	51.2	49.9
6	19.0	19.1	18.9	19.0	37.2
7	26.8	27.1	26.7	27.1	208.7
8	140.9	142.8	134.5	134.1	120.0
9	150.8	150.1	149.4	149.7	155.2
10	41.7	41.6	41.1	41.3	42.5
11	140.8	140.7	133.8	137.3	133.6
12	124.8	125.2	122.5	123.4	132.1
13	133.0	132.9	121.2	119.1	128.3
14	155.7	155.4	158.3	157.6	164.5
15	103.1	100.3	103.5	99.4	99.7
16	171.3	171.8	171.1	171.3	169.8
17	55.8	56.1	59.0	62.3	52.9
18	33.9	33.9	34.0	34.0	33.1
19	22.2	22.2	22.2	22.2	21.8
20	22.2	22.7	22.7	22.7	19.7

C No.	**1**	**2**	**3**	**4**	**5**
Glucose moiety					
	(at C-14)	(at C-14)	(at C-15)	(at C-17)	
1	105.3	105.3	104.1	103.9	
2	75.7	75.7	75.2	75.0	
3	77.9	77.9	78.6	78.1	
4	71.9	71.9	71.0	71.5	
5	78.0	78.0	78.6	78.2	
6	62.9	63.0	62.6	63.0	
	(at C-15)				
1	103.6				
2	75.1				
3	78.5				
4	71.1				
5	78.5				
6	62.6				

Assignments are based on HSQC and HMBC spectra.

H, 6.81. FAB-MS *m/z*: 383 $[M+Na]^+$. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Enzymatic Hydrolysis of 1—4 **1** (10 mg) was hydrolyzed with cellulase (Sigma, C-0901, from *Penicillium funiculosum*) (20 mg) in an acetate buffer (pH 4.5, 2 ml) at 40 °C for 2 d. The reaction mixture was diluted with H_2O and extracted with EtOAc twice. The concentrated EtOAc extract was subjected to semi-preparative HPLC [YMC ODS A-323 1 × 25 cm, CH_3CN–H_2O (52.5 : 47.5), UV 205 nm] to give an aglycone **1a** (1 mg) as an colorless powder, $[\alpha]_D^{23}$ +70.0° (c=0.10, MeOH). ^{1}H-NMR ($CDCl_3$): δ 0.96, 0.98, 1.34 (each 3H, s, Me), 5.43 (1H, d, J=14 Hz, H-17), 5.56 (1H, d, J=14 Hz, H-17), 6.75 (1H, s, H-15). This aglycone was identical to the $NaBH_4$ reductant of betolide (**6**). **2—4** (each 1 mg) were hydrolyzed in the same manner as **1** to give an aglycone **1a**, which was confirmed by HPLC [YMC R-ODS-7, 4.6 mm × 25 cm, CH_3CN–H_2O (55 : 45), 1.0 ml/min, UV 220 nm, t_R 13.5 min].

Acid Hydrolysis of 1—4[7] A solution of each glycoside (1 mg) in 5% H_2SO_4 aq. (3 drops) and dioxane (3 drops) was heated in a boiling water bath for 2 h. The reaction mixture was diluted with H_2O and extracted with EtOAc three times. The H_2O layer was passed through an Amberlite IRA-60E column. The eluate was concentrated. The residue was dissolved in H_2O (0.03 ml). After the addition of D-cysteine (0.05 mg) and pyridine (0.015 ml), the mixture was warmed at 60 °C for 1 h. The solvent was blown off under an air stream. After dryness, the residue was trimethylsilylated and checked by GC to be identical to authentic D-glucose. The GC conditions: column, Supelco capillary column SPB™-1, 0.25 mm × 27 m; column temperature, 230 °C; carrier gas, N_2; t_R, L-glucose (16.1 min), D-glucose (16.5 min). From **1—4**, D-glucose was detected.

Acetylation of 1—4 **1** (50 mg) was acetylated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) at room temperature overnight. The usual workup afforded nonaacetate **1b** (50 mg) as a colorless powder. From **2** (2 mg), **3** (7 mg) and **4** (2 mg), **2b** (2 mg), **3b** (7 mg) and **4b** (2 mg) were obtained in the same manner as **1**, respectively. **1b**: ^{1}H-NMR ($CDCl_3$): δ 0.94, 0.96, 1.27 (each 3H, s, Me), 2.00, 2.02, 2.03, 2.04, 2.04, 2.08, 2.08, 2.10, 2.15 (each 3H, s, OAc), 4.87 (1H, d, J=8 Hz, anomeric H), 5.36 (1H, d, J=12 Hz, H-17), 5.57 (1H, d, J=12 Hz, H-17), 6.80 (1H, s, H-15). **2b**: ^{1}H-NMR ($CDCl_3$): δ 0.94, 0.96, 1.20 (each 3H, s, Me), 2.03, 2.04, 2.06, 2.08, 2.14, 2.17 (each 3H, s, OAc), 4.90 (1H, d, J=8 Hz, anomeric H), 5.24 (1H, d, J=12 Hz, H-17), 5.61 (1H, d, J=12 Hz, H-17), 7.57 (1H, s, H-15). **3b**: ^{1}H-NMR ($CDCl_3$): δ 0.93, 0.93, 1.21 (each 3H, s, Me), 2.00, 2.01, 2.01, 2.07, 2.09, 2.35 (each 3H, s, OAc), 5.19 (1H, d, J=8 Hz, anomeric H), 5.49 (1H, d, J=12 Hz, H-17), 5.51 (1H, d, J=12 Hz, H-17), 6.81 (1H, s, H-15). **4b**: ^{1}H-NMR ($CDCl_3$): δ 0.93, 0.95, 1.29 (each 3H, s, Me), 1.97, 1.98, 2.02, 2.08, 2.20, 2.35 (each 3H, s, OAc), 4.64 (1H, d, J=8 Hz, anomeric H), 4.88 (1H, br d, J=12 Hz, H-17), 5.32 (1H, d, J=12 Hz, H-17), 7.58 (1H, s, H-15).

$NaBH_4$ Reduction of Betolide (6) Betolide (**6**) (20 mg), isolated from the MeOH extract of the roots of *S. officinalis*, was reduced with $NaBH_4$ (40 mg) in MeOH (2 ml) at room temperature overnight. The reaction mixture was diluted with H_2O and extracted with ether three times. The ether extract was subjected to preparative TLC [Silicagel PF_{254}, $CHCl_3$–MeOH (97 : 3)] to give **1a** (12 mg) as a colorless powder. The ^{1}H-NMR data and $[\alpha]_D$ were identical to those of the aglycone **1a**.

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