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NEW RESVERATROL DIMER GLUCOSIDES AND TRIMERS IN STEM AND ROOT OF *WELWITSCHIA MIRABILIS*

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Abstract- Two new resveratrol dimer glucosides, mirabilosides A (**1**) and B (**2**), and two new resveratrol trimers, mirabilols A (**3**) and B (**4**) were isolated from stem and root of *Welwitschia mirabilis* (Welwitschiaceae) together with four known stilbene oligomers (gnetins C, E, F and I). The structures of isolated compounds were determined by spectroscopic analysis.

Welwitschia mirabilis is an absolutely unique gymnosperm and commonly called “Wonderplant”. The plant is endemic in Namib Desert of South West Africa and belongs to the family of Welwitschiaceae, which comprises one genus and only one species. It is classified in Gnatale along with Ephadraceae and Gnetaceae, but each family has diverse morphological characteristics, which has been controversy on whether the members are common origin.[1-3] In view of the controversy of Gnatale, the phytochemical study of *W. mirabilis* has been projected to find biological markers that can be used for the relationship among the members in the order. The existence of stilbenoids in Welwitschiaceae[4] and Gnetaceae[5-9] has been revealed in the previous study. In this paper we report the isolation and structure elucidation of two new stilbene glucosides (mirabilosides A and B) and two stilbene trimer derivatives (mirabilols A and B) isolated from methanol extract of root and stem of *W. mirabilis* in addition to four known stilbenoids; gnetins C, E, F and I. The structures of isolated compounds were determined by spectroscopic analysis.

RESULT AND DISCUSSION

MeOH extract (18 g) of the dried powdered stem and root of *Welwitschia mirabilis* was subjected to chromatography on reversed phase ODS. Further repeated fractionation of the fractions on reverse phase

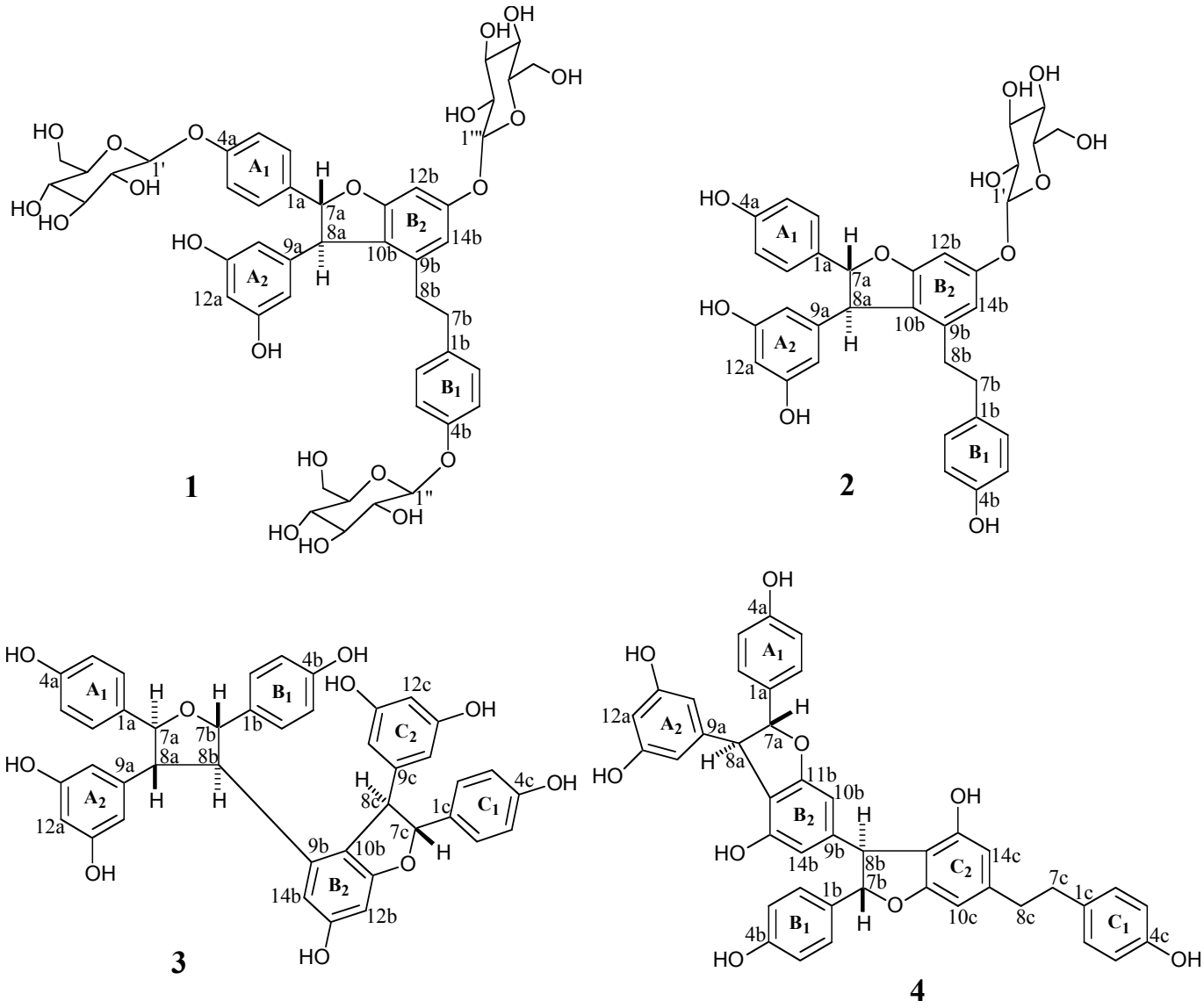

Figure 1

ODS and Sephadex L-H 20 resulted in the isolation of **1**-**4**.

Mirabiloside A (**1**), $[\alpha]_D$ + 3°, a white amorphous powder, showed a positive reaction to Gibbs reagent. It exhibited an $[M–H]^-$ ion peak at *m/z* 941 in the negative FAB-MS, indicating the molecular weight to be 942. The molecular formula of $C_{46}H_{54}O_{21}$ was established by the HR-FAB-MS $[M-H]^-$ at *m/z* 941.3101. Analysis of the ^1H NMR spectrum (Table 1) revealed the presence of two sets of *p*–substituted phenyl groups [ring A_1 and B_1], a set of *meta*–coupled aromatic protons on a 1,2,3,5-tetrasubstituted benzene ring [B_2] and a set of 3,5-dihydroxyphenyl group [A_2]. The spectrum also exhibited the presence of a set of methine protons coupled in *J*= 5.2 Hz [H-7a/H-8a], a set of protons in CH_2CH_2 [H-7b/H-8b] and three anomeric protons [δ 4.78 (1H, d, *J*= 7.6 Hz, Glc(a)-H-1"), 4.84 (1H, d, *J*= 7.2 Hz, Glc(b)-H-1'"), 4.87 (1H, d, *J*= 7.2 Hz, H-Glc(c)-H-1')] in addition to two phenolic hydroxyl groups [δ 9.28 (2H, br s, OH-11a, 13a)]. The ^1H and ^{13}C NMR spectral data (Table 1) were similar to those of gnetin F.[4]

Table 1 ^{1}H and ^{13}C NMR Spectral Data of Compounds (**1** - **4**)

No	**1**[a]		**2**[a]		**3**[b]		**4**[b]	
	δ H (*J*= Hz)	δ C	δ H (*J*= Hz)	d C	δ H (*J*= Hz)	δ C	δ H (*J*= Hz)	δ C
1a		134.84		131.5		133.2		133.6
2a(6a)	7.22 *d* (8.4)	127.1	7.05 *d* (8.0)	127.3	6.78 *d* (8.8)	129.5	7.22 *d* (8.8)	127.95
3a(5a)	7.04 *d* (8.4)	116.5	6.75 *d* (8.0)	115.5	6.58 *d* (8.8)	115.1	6.86 *d* (8.8)	116.1
4a		157.4		157.5		156.9		158.0
7a	5.45 *d* (5.2)	92.4	5.30 *d* (6.0)	95.7	5.30 *d* (8.8)	84.9	5.40 *d* (6.0)	93.7
8a	4.27 *d* (5.2)	55.5	4.15 *d* (6.0)	55.2	4.27 *dd* (8.8, 12.4)	55.8	4.43 *d* (6.0)	55.9
9a		145.5		145.5		140.6		145.9
10a(14a)	6.08 *d* (2.0)	105.9	6.02 *d* (2.0)	107.1	5.75 *d* (1.6)	108.8	6.21 *d* (1.6)	106.8
11a(13a)		158.9		158.1		158.1		159.4
12a	6.11 *t* (2.0)	101.5	6.09 *t* (2.0)	102.2	5.88 *t* (1.6)	101.3	6.27 *t* (1.6)	101.0
1b		134.95		131.40		131.3		134.1
2b(6b)	6.88 *d* (8.4)	129.3	6.75 *d* (8.4)	129.2	7.07 *d* (8.4)	128.9	7.24 *d* (8.8)	127.64
3b(5b)	6.85 *dd* (8.4)	116.3	6.59 *d* (8.4)	115.0	6.83 *d* (8.4)	115.8	6.88 *d* (8.8)	116.1
4b		155.8		155.7		156.9		157.9
7b	2.45 *m*	34.9	2.468 *m*	36.25	4.62 *d* (9.6)	89.0	5.46 *d* (4.2)	93.4
8b	2.46 *m*	34.6	2.569 *m*	35.98	3.45 *dd* (9.6, 12.4)	50.7	4.48 *d* (4.2)	55.8
9b		139.7		139.6		136.6		146.6
10b		121.6		121.7		122.3	6.32 *br s*	101.0
11b		160.3		160.1		162.0		162.7
12b	6.52 *d* (2.0)	95.7	6.45 *d* (2.0)	96.8	6.17 *d* (2.0)	96.4		114.1
13b		158.8		158.6		159.4		155.2
14b	6.46 *d* (2.0)	109.8	6.43 *d* (2.0)	110.0	6.47 *d* (2.0)	106.0	6.27 d *br s*	108.2
1c						131.7		133.4
2c(6c)					6.95 *d* (8.8)	129.3	7.07 *d* (8.2)	130.1
3c(5c)					6.81 *d* (8.8)	116.0	6.78 *d* (8.2)	115.8
4c						158.2		156.2
7c					5.07 *d* (9.4)	94.7	2.835 *m*	39.1
8c					3.04 *d* (9.4)	56.5	2.820 *m*	37.5
9c						145.9		145.5
10c					6.03 *d* (1.6)	109.2	6.37 *br s*	102.1
11c						158.5		162.6
12c					6.34 *t* (1.6)	103.0		113.1
13c						158.5		155.0
14c					6.03 *d* (1.6)	109.2	6.34 *br s*	109.5

Measured on 400 MHz (^{1}H) and 100MHz (^{13}C). a: $(CD_3)_2SO$ and b: $(CD_3)_2CO$

The presence of three anomeric protons and the molecular weight suggested that **1** is a triglucoside of gnetin F. The correlations in COLOC spectrum (Figure 2) between C-2a(6a)/H-7a, C-10a(14a)/H-8a, C-7b/H-2b(6b) and C-8b/H-14b revealed the linkages of C-7a/C-1a, C-8a/C-9a, C-7b/C-1b and C-8b/C-9b,

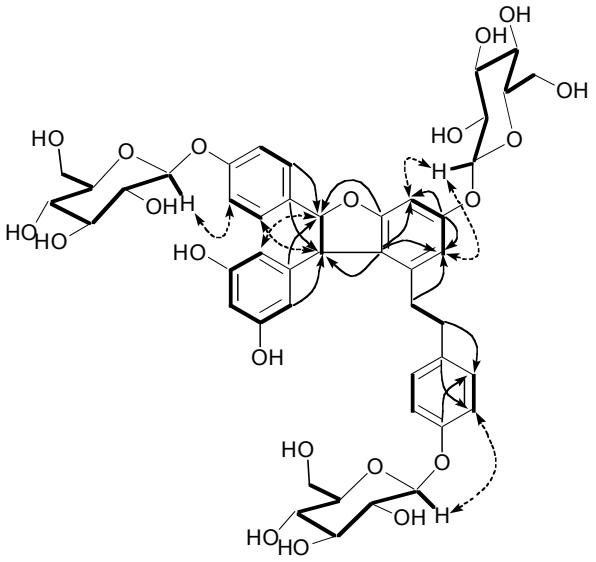

Figure 2 — Correlations in ^{1}H-^{1}H COSY, ↷ COLOC and ↶ in NOESY spectrum of **1**

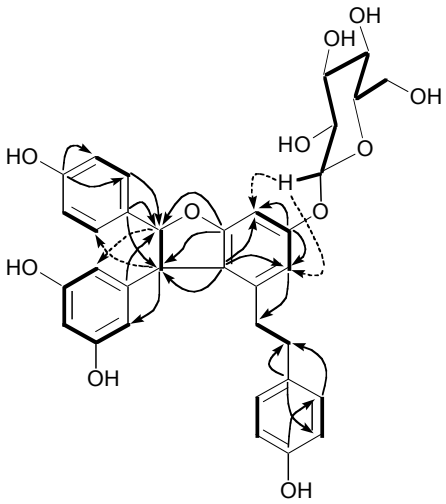

Figure 3 — Correlations in ^{1}H-^{1}H COSY, ↷ COLOC and ↶ in NOESY spectrum of **2**

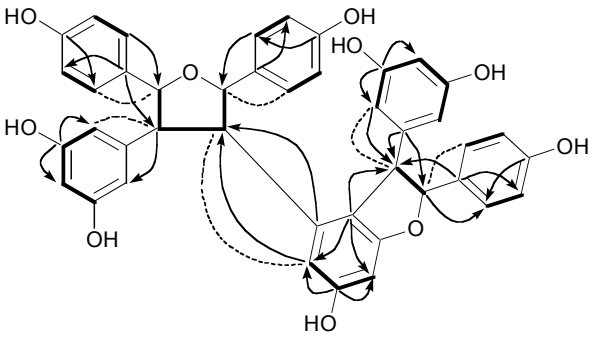

Figure 4 ⌒ Correlations in ^{1}H-^{1}H long range COSY, — ^{1}H-^{1}H COSY and ↷ COLOC spectrum of **3**

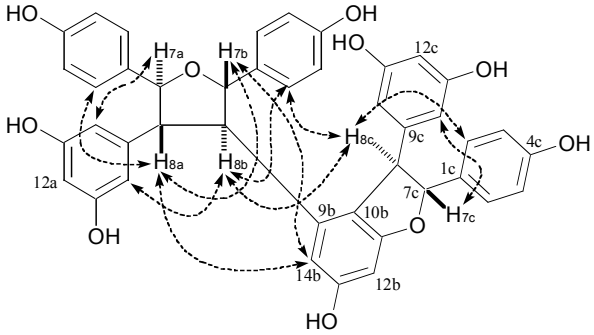

Figure 5 Correlations in NOESY spectrum of **3**

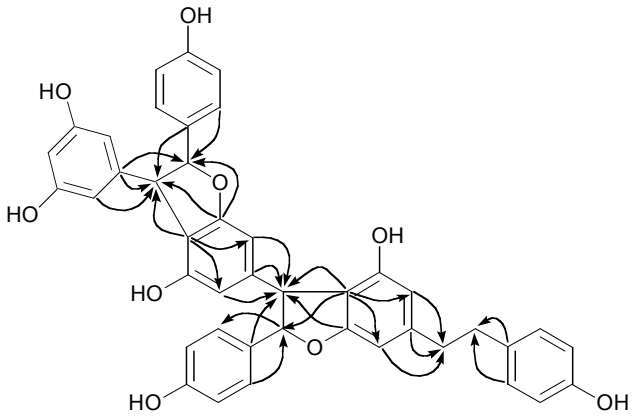

Figure 6 Correlations in COLOC spectrum of **4**

respectively. The correlations between C-11b/H-7a, C-10b/H-8a in COLOC also showed the connection

of a resveratrol unit, ring A_1-7a-8a-ring A_2 with another resveratrol unit, ring B_1-7b-8b-ring B_2, at C-10b and C-11b, respectively. Thus the planar structure of the aglycone was characterized as gnetin F. As compared to those of gnetin F, the chemical shifts of at H-3a(5a), H-3b(5b) and H-12b(14b) were observed in down field by about 0.28 ppm , which suggested that the three glucose molecules attached at C-4a, C-4b and C-13b. The results of NOESY experiment (Figure 2) substantiated not only the positions of glucoses, but also the configuration of the dihydrofuran ring to be *trans*. Subsequently the relative structure of mirabiloside A was determined to be gnetin F-4a, 4b, 13b-tri-*O*-glucoside in Figure 1.

Mirabiloside B (**2**), $[\alpha]_D$ + 113°, a white amorphous powder, showed a positive reaction to Gibbs reagent. It exhibited an $[M–H]^-$ ion peak at *m/z* 617 in the negative FAB-MS and $[M–H]^-$ at *m/z* 617.2028 in the HR-FAB-MS, which was in consistence with the molecular formula of $C_{34}H_{33}O_{11}$. The ^1H and ^{13}C NMR spectra (Table 1) showed the presence of one glucose moiety [δ 4.82 (Glc-H-1)]. The signals due to the aglycone were closely similar to those of **1**, which suggested that **2** is a mono glucoside of gnetin F. The H-H COSY, C-H COSY and COLOC spectra (Figure 3) allowed complete assignment of the protonated and quaternary carbon atoms in **2**. The position of the glucose molecule was deduced at C-13b by DIFNOE experiment in acetone solution. Irradiation of the anomeric proton at δ 4.82 (Glc-H-1) resulted in a significant NOE effect at H-12b and H-14b, thus the position of the glucose moiety was established to be at C-13b. The structure of mirabiloside B was characterized as gnetin F-13b-*O*-glucoside.

Mirabilol A (**3**), $[\alpha]_D$ + 130°, a white amorphous powder, showed a positive reaction to Gibbs reagent. It exhibited an $[M–H]^-$ ion peak at *m/z* 697 in the negative FAB-MS and $[M–H]^-$ ion peak at *m/z* 697.2062 in the HR-FAB-MS that was corresponding to the molecular formula of $C_{42}H_{34}O_{10}$. In the ^1H NMR spectrum (Table 1) the presence of three sets of *ortho*–coupled aromatic protons in an A_2X_2 spin system [δ 6.58 (2H, *d*, *J*= 8.8 Hz, H-3a, 5a), 6.78 (2H, *d*, *J*= 8.8 Hz, H-2a, 6a); 6.81 (2H, *d*, *J*= 8.4 Hz, H-3c, 5c), 6.95 (2H, *d*, *J*= 8.8 Hz, H-2c, 6c); 6.83 (2H, *d*, *J*= 8.4 Hz, H-3b, 5b), 7.07 (2H, *d*, *J*= 8.4 Hz, H-2b, 6b)], a set of *meta*–coupled aromatic proton on a 1,2,3,5-trtrsubstituted benzene ring [δ 6.17 (1H, *br s*, H-12b), 6.47 (1H, *br s*, H-14b)] and two sets of 3,5-dihydroxyphenyl groups [δ 5.75 (2H, *d*, *J*= 1.6 Hz, H-10a(14a), 5.88 (2H, *t*, *J*= 1.6 Hz, H-12a); 6.03 (2H, *d*, *J*= 1.6 Hz, H-10c, 14c), 6.34 (1H, *t*, *J*= 1.6 Hz, H-12c)) were observed. A pair of mutually coupled methine protons on a dihydrofuran ring [δ 3.04 (1H, *d*, *J*= 9.4 Hz, H-8c), 5.07 (1H, *d*, *J*= 9.4 Hz, H-7c)] and a set on a tetrahydrofuran ring [δ 4.62 (1H, *d*, *J*= 9.6 Hz, H-7b), 3.45 (1H, *dd*, *J*= 9.4, 12.4 Hz, H-8b), 4.27 (1H, *dd*, *J*= 8.8, 12.4 Hz, H-8a), 5.30 (1H, *d*, *J*= 8.8 Hz, H-7a)] were also observed in the spectrum in addition to eight hydroxyl groups. Analysis of ^1H-^1H long range COSY, C-H COSY and COLOC (Figure 4) spectra allowed the assignment of all protons and carbons in **3**. In the COLOC spectrum the correlations observed between C-2a(6a)/H-7a, C-8a/H-10a(14a), C-2b(6b)/H-7b, C-9b(14b)/H-8b, C-7c/H-2c(6c) and C-10c(14c)/H-8c revealed the

linkages of all methine protons with the respective aromatic rings. Furthermore, the correlations between C-8b/H-7a(8a), C-10b/H-8c(7c) in the COLOC spectrum showed the connection of a resveratrol unit, ring A_1-7a-8a-ring A_2 to a second resveratrol unit, ring B_1-7b-8b-ring B_2 which in turn is connected to a third resveratrol unit, ring C_1-7c-8c-ring C_2. The relative configuration of H-7a(α), H-8a(β), H-7b(β), H-8b(α), H-7c(β) and H-8c(α) was deduced by the correlations observed in the NOESY spectrum (Figure 5). The structure of **3** showed a closed resemblance to neparensinol C isolated from *Kobresia neparensis*[10], but the differences found in the chemical shifts, coupling constant values and the configurations of methine protons on the tetrahydrofuran ring revealed that **3** is an isomer of the resveratrol trimer.

Mirabilol B (**4**), $[\alpha]_D$ – 43°, a white amorphous powder, showed a positive reaction to Gibbs reagent. The negative FAB-MS exhibited an $[M–H]^-$ ion peak at *m/z* 681, the molecular formula of $C_{42}H_{34}O_9$ was deduced by the HR-FAB-MS $[M–H]^-$ ion at *m/z* 681.2112. The presence of three 4-hydroxyphenyl groups [ring A_1, B_1, C_1], two sets of *meta*-coupled aromatic protons on a 1,2,3,5-tetrasubstituted benzene ring [B_2, C_2] and a set of 3,5-dihydroxyphenyl group [A_2] were observed in the ^{1}H NMR spectrum (Table 1). Two sets of mutually coupled methine protons [H-7a/8a, H-7b/8b] and a set of methylene protons [δ 2.82 (2H, m, H-8c), 2.84 (2H, m, H-7c)] were also observed in the spectrum. Analysis of the ^{1}H and ^{13}C NMR spectral data including 2D technique (Figure 6) revealed a closed resemblance to those of gnetin E[5] except for the appearance of a CH_2CH_2 proton signal in place of CH=CH. Then **4** is a gnetin E derivative dihydrogenated at C-7c and C-8c. The structure was further confirmed by the results of catalytic hydrogenation of gnetin E. The relative configuration of the methines was same as that of gnetin E, thus the structure of mirabilol B was characterized as 7c,8c-dihydrognetin E.

Olefinic moiety, which is usually found in stilbene oligomers was hydrogenated in **1**, **2** and **4**. These stilbene oligomers are rare in nature, but they are commonly found in *Gnetum africanum* (Gnetaceae) and *Welwitschia mirabilis*. This finding is of significant importance clue to the relationship of the families. Gnetins C, E, F and I[4] were also isolated from the methanol extract of stem and root of *W. mirabilis*. Further phytochemical investigation of the methanol extract of *W. mirabilis* is still in progress.

EXPERIMENTAL

General Method

^{1}H and ^{13}C NMR spectra were recorded on EX-400 and AL-300 (JEOL). Chemical shift were shown as δ values with trimethylsilane (TMS) as an internal reference. Peak multiplicities were quoted in Hz. The negative FAB-MS and HR-FAB-MS were recorded on JMS-DX-300 spectrometer equipped with JMA

3500 data analysis system (JEOL). UV spectrum was recorded on 2200 UV spectrophotometer (Shimadzu) and optical rotation was measured on P-1020 polarimeter. Silica gel 60 (70–230, mesh), Sephadex LH-20, Fuji Silysia Chemical Chromatorex ODS (100-200, mesh) and Sep-Pak C_{18} Cartridges ODS were used for column chromatography. Kiesel-gel 60 F_{256} (Merck) 0.25 mm was used for analytical and 0.5 mm for preparative TLC.

Plant Material

Stem and root of *Welwitschia mirabilis* cultivated was purchased in Japan in April 2002. A voucher specimen is deposited in Gifu Prefectural Institute of Health and Environmental Sciences, Gifu, Japan.

Extraction and Isolation

The dried stem and root (250 g) of *W. mirabilis* was powdered and extracted with MeOH (2 L x weekly x 3) and 70% MeOH (2L x weekly x 3) at rt. After concentration of the solvent, MeOH extract (18 g) and 70% MeOH extract (40 g) were obtained. The MeOH extract was subjected to chromatography on reversed phase ODS eluted with H_2O followed by increasing concentration of MeOH to give five fractions (Fr. 1–5). Further chromatography of Fr. 3 on reserved phase ODS chromatography eluted with a mixture of H_2O–MeOH (95:5) and increasing the concentration of MeOH gave 17 fractions (Fr. 3a–3r). Compound **1** (80mg) was obtained from Fr. 3i by repeated chromatography of the fraction on Sep-Pak eluted with H_2O–MeOH (85:15). Compounds (**3**) (75 mg) and (**4**) (200 mg) were obtained from fractions Fr. 4 and 5 by repeated chromatography on Sephadex LH-20 eluted with MeOH. Chromatography of Fr. 3q on Sephadex LH-20 and further purification by PTLC developed with a mixture of EtOAc–$CHCl_3$–MeOH–H_2O (15:8:4:1) gave **2** (18.5mg).

Mirabiloside A (1): A white amorphous powder; $[\alpha]_D$ + 3° (*c*= 0.215, MeOH); Negative FAB-MS: $[M–H]^-$ *m/z* 941; HR-FAB-MS: $[M–H]^-$ *m/z* 941.3101 (Calcd 941.3079 for $C_{46}H_{53}O_{21}$); UV λmax (MeOH) nm: 227, 280; ^{1}H NMR [400 MHz, $(CD_3)_2SO$] sugar moiety: δ 3.19 [3H, *m*, Glc-H-(2',2",2'")], 3.27 [3H, *m*, Glc-H-(4',4",4'")], 3.30 [3H, *m*, Glc-H-(3',3",3'")], 3.35 [3H, *m*, Glc-H-(5',5",5'")], 3.49, 3.70 [3H each, *m*, Glc-H-(6',6",6'")], 4.78 (1H, *d*, *J*= 7.6 Hz, Glc-H-1"), 4.84, 4.87 (1H each, *d*, *J*= 7.2 Hz, Glc-H-1'",1'); ^{13}C NMR [100 MHz, $(CD_3)_2SO$] sugar moiety: δ 60.9, 61.0 [3 x C-(6',6",6'")], 69.1, 70.6 [3 x C-(2',2",2'")], 73.38, 73.44, 73.48 [3 x C-(4',4",4'")], 76.75, 76.83, 76.90 [3 x C-(3',3",3'")], 77.0, 77.1, 77.3 [3 x C-(5',5",5'")], 100.5 [3 x C-(1',1",1'")]; ^{1}H and ^{13}C NMR spectra of the aglycone unit are shown in Table 1.

Mirabiloside B (2): A white amorphous powder; $[\alpha]_D$ + 113° (*c*= 0.104, MeOH); Negative FAB-MS: $[M–H]^-$ *m/z* 617; HR-FAB-MS: $[M–H]^-$ *m/z* 617.2028 (Calcd 617.2023 for $C_{34}H_{33}O_{11}$); UV λmax

(MeOH) nm: 218, 283; ^{1}H NMR [400 MHz, $(CD_3)_2SO$] sugar moiety: δ 3.19 (1H, *m*, Glc-H-4'), 3.23 (1H, *m*, Glc-H-2'), 3.28 (1H, *m*, Glc-H-3'), 3.33 (1H, *m*, Glc-H-5'), 3.48, 3.73 (1H each, *m*, Glc-H-6'), 4.82 (1H, *d*, *J*= 7.6 Hz, Glc-H-1'); ^{13}C NMR [100 MHz, $(CD_3)_2SO$] sugar moiety: δ 62.7 (C-6'), 71.4 (C-4'), 74.7 (C-2'), 77.7 (C-3'), 77.99 (C-5'), 102.1 (C-1'); ^{1}H and ^{13}C NMR spectra of the aglycone unit are shown in Table 1.

Mirabilol A (3): A white amorphous powder; $[\alpha]_D$ + 130° (*c*= 0.10, MeOH); Negative FAB-MS: $[M–H]^-$ *m/z* 697; HR-FAB-MS: $[M–H]^-$ *m/z* 697.2062 (Calcd 697.2073 for $C_{42}H_{33}O_{10}$); UV λmax (MeOH) nm: 237, 284; ^{1}H and ^{13}C NMR spectra of the aglycone moiety are listed in Table 1.

Mirabilol B (4): A white amorphous powder; $[\alpha]_D$ – 43° (*c*= 0.10, MeOH); Negative FAB-MS: $[M–H]^-$ *m/z* 681; HR-FAB-MS: $[M–H]^-$ *m/z* 681.2112 (Calcd 681.2125 for $C_{42}H_{33}O_9$); UV λmax (MeOH) nm: 232, 278, 283, 327; ^{1}H and ^{13}C NMR spectra of the aglycone unit are shown in Table 1.

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