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BUTANOIC ACID GLUCOSIDE COMPOSITION OF WHOLE BODY AND *IN VITRO* PLANTLETS OF *ANOECTOCHILUS FORMOSANUS*

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Key Word Index—*Anoectochilus formosanus*; Orchidaceae; butanoic acid glucoside; tissue culture.

Abstract—A butanoic acid glucoside, 3-*O*-*β*-D-glucopyranosyl-(3*R*)-4-dihydroxy butanoic acid, with known aliphatic glucosides were isolated from the methanol extract of dried whole plants of *Anoectochilus formosanus* Hay. Butanoic acid glucosides were formed in 3 types of regenerated plantlets *in vitro*. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Anoectochilus formosanus is an orchidaceous phanerophyte, widely used as a folk medicine for hypertension, lung and liver diseases, and underdeveloped children [1]. *Anoectochilus koshunensis* has been studied to isolate some aliphatic and aromatic glucosides [2]. Takatsuki *et al*. also isolated palmitic acid, 1,3-dipalmitin, *p*-coumaric acid, *β*-sitosterol *β*-D-glucopyranoside, from an unidentified *Anoectochilus* species [3]. Since no survey of the components of *A. formosanus* has been reported, we report herein the isolation and structural elucidation of three butanoic acid glucosides from this species. Moreover, tissue culture in liquid media was also investigated in order to set up a maintenance method for the crude drug.

RESULTS AND DISCUSSION

Repeated fractionation of the methanol extract of dried whole plants by chromatographic procedures gave pure samples of 1-*O*-isopropyl-*β*-D-glucopyranoside (**1**), methyl 3-*O*-*β*-D-glucopyranosyl-(3*R*)-4-dihydroxy butanoate (**2**) and 3-*O*-*β*-D-glucopyranosyl-(3*R*)-hydroxybutanolide (**3**) which were identified by comparison with published spectral data [2, 4], and 3-*O*-*β*-D-glucopyranosyl-(3*R*)-4-dihydroxy butanoic acid (**4**) which was a new compound.

Acid hydrolysis of **3** gave D-glucose, which was confirmed by specific rotation measurements, $[\alpha]_D + 49.8°$. The *β*-configuration of the anomeric centre of the D-glucopyranosyl group was suggested by the coupling constant of the anomeric proton (J = 7.9 Hz). In order to confirm the exact structure of **3**, X-ray diffraction analysis of the corresponding peracetate was investigated. The structure of **3**, including the stereochemistry on the hydroxyl group at C-3 was identified unambiguously to be 3-*O*-*β*-D-glycopyranosyl-(3*R*)-hydroxybutanolide, isolated from *A. koshunensis* and named as kinsenoside by Ito *et al*. [2], as indicated in Fig. 1.

The positive ion FAB mass spectrum of **4** showed a $[M + H]^+$ peak at m/z 283 suggesting that it had the molecular formula $C_{10}H_{18}O_9$. The ^{13}C NMR spectrum indicated the existence of a free carboxylic acid group (δ 179.6), an oxygenated carbon at δ 66.0 and a hexose moiety at δ 104.1 (see Experimental). The ^1H NMR spectrum showed some readily assignable signals, such as two methylene groups δ 2.38 (1H, *dd*, J = 14.8, 5.9 Hz), 2.46 (1H, *dd*, J = 14.8, 7.3 Hz) and δ 3.59 (1H, *dd*, J = 12.5, 5.9 Hz), 3.64 (1H, *dd*, J = 12.5, 4.3 Hz), and a methine signal 4.12 (*dddd*, J = 7.3, 5.9, 5.9, 4.3 Hz). These data suggested the presence of 3, 4-dihydroxy butylic acid in **4**. When **4** was exposed to mild acid conditions, compound **3** was obtained. From the above evidence, the structure of **4** was

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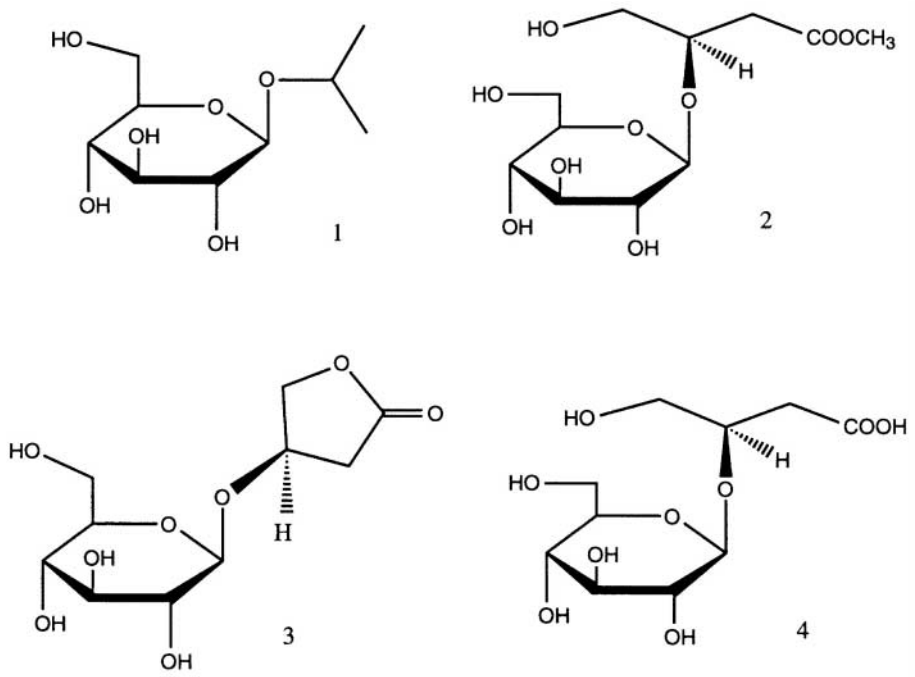

determined to be 3-*O*-*β*-D-glucopyranosyl-4-dihydroxy butanoic acid. From these results the stereochemistry of the C-3 hydroxyl group of **4** was confirmed to be *R*.

Since it is reported that natural sources of *A. formosanus* are becoming exhausted in Taiwan [5], we started to investigate the propagation of this species by tissue culture techniques. As it is known that production of secondary metabolites can be enhanced by the induction of organogenesis, the regenerated plant was assayed for **1–4** at three different stages of culturing: (a) multiple shooting, (b) shoot elongation and (c) rooting. The results suggested that the cultured segments, a, b and c

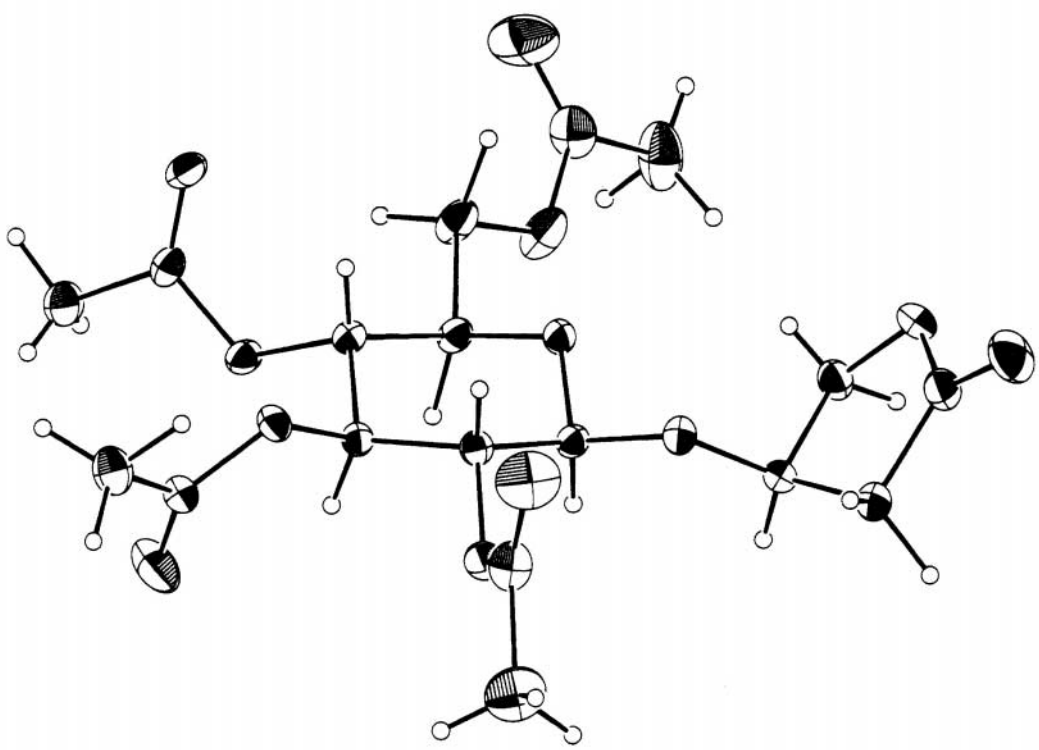

Fig. 1. Computer generated perspective drawing of compound **3a**.

and the original plant had the same pattern of composition, although the concentrations were somewhat different. Therefore, it is evident that micropropagation of this species using plant biotechnological technique will be available to enable maintenance of this important crude drug because Namba *et al*. have indicated that several species of the same genus and a different genus, *Goodyera*, are used by the same name in the Taiwan market [6, 7].

EXPERIMENTAL

General

^{1}H and ^{13}C NMR: JEOL EX270 (TMS as an int. std.): 270 MHz and 67.5 MHz, respectively.

Plant material

Dried whole plants and the cultured plantlets were purchased from Qiling Anoectochilus Cultural Station in Nantou district of Taiwan, where the seedlings were produced by *in vitro* culture of mature seeds collected in Taiwan. This plant was continuously subcultured *in vitro* in our laboratory as described below and identified as *A. formosanus* Hay. A voucher specimen is deposited in the Herbarium of Faculty of Pharmaceutical Sciences, Kyushu University.

Extraction and isolation

Dried whole plants (200 g) were percolated with MeOH at room temp. The MeOH extract was concd to dryness by evapn *in vacuo* and the residue (64 g) suspended in H_2O. After removal of $CHCl_3$ and *n*-BuOH sol. materials by partition, the *aq*. portion was concd to dryness (32 g) by evapn *in vacuo*. The *aq*. extract was applied to a column of Diaion HP-20 and eluted with H_2O to afford frs 1–3. Fr. 2 (25 g) was applied to a silica gel column eluting with $CHCl_3$–MeOH–H_2O (8:2:0.2–6:4:1) successively to afford frs 4–7. Further purification of frs 4, 5 and 7 by silica gel CC ($CHCl_3$–MeOH–H_2O) yielded **1** (75 mg), **2** (1.96 g) and **4** (980 mg), respectively. Purification of fr. 6 by silica gel CC ($CHCl_3$–EtOH, 7:5) yielded **3** (9.83 g). The known compounds, **1** [4], **2** [2] and **3** [2] were identified by comparison with published data, respectively.

1-O-*Isopropyl-β-*D*-glucopyranoside* (**1**)

Colourless needles, mp 123–124°. Positive FAB-MS: *m/z* 245 $[M + Na]^+$, 223 $[M + H]^+$. ^{1}H NMR (pyridine-d_5): δ 4.89 (1H, *d*, *J* = 7.6 Hz, G-1), 4.54 (1H, *dd*, *J* = 11.8, 2.3 Hz, G-6), 4.38 (1H, *dd*, *J* = 11.8, 5.3 Hz, G-6), 4.24 (1H, *m*, G-3), 4.23 (1H, *dq*, *J* = 6.3, 6.3 Hz), 4.21 (1H, *m*, G-4), 3.99 (1 H, *m*, G-2), 3.96 (1 H, *m*, G-5), 1.26 (3H, *d*, *J* = 6.3 Hz), 1.21 (3H, *d*, *J* = 6.3 Hz), 1.21 (3H, *d*, *J* = 6.3 Hz). ^{13}C NMR (pyridine-d_5): δ 22.1 (C-1), 23.9 (C-3), 62.9 (G-6), 70.8 (G-4), 71.8 (C-2), 75.3 (G-2), 78.4 (G-5), 78.6 (G-3), 102.7 (G-1).

Methyl 3-O-*β-*D*-glucopyranosyl-*(3R)-4-*dihydroxy butanoate* (**2**)

Amorphous. Positive FAB-MS: *m/z* 319 $[M + Na]^+$, 297 $[M + H]^+$. ^{1}H NMR (pyridine-d_5): δ 5.05 (1H, *d*, *J* = 7.3 Hz, G-1), 4.72 (1H, *dddd*, *J* = 7.3, 5.6, 5.4, 5.2 Hz), 4.55 (1H, *dd*, *J* = 11.9, 2.1 Hz, G-6′, 4.35 (1H, *dd*, *J* = 11.9, 5.6 Hz, G-6), 4.23 (1H, *m*, G-3), 4.17 (1H, *dd*, *J* = 11.2, 5.4 Hz, H-4′), 4.16 (1H, *m*, G-4), 4.02 (1H, *dd*, *J* = 11.2, 5.2 Hz, H-4), 3.98 (1H, *m*, G-2), 3.95 (1H, *m*, G-5), 3.61 (3H, *s*, OCH_3), 3.02 (1H, *dd*, *J* = 15.8, 5.6 Hz, H-2′), 2.94 (1H, *dd*, *J* = 15.8, 7.3 Hz, H-2). ^{13}C NMR (pyridine-d_5): δ 37.6 (C-2), 51.5 (OCH_3), 62.6 (G-6), 65.1 (C-4), 71.6 (G-4), 75.0 (G-2), 78.3 (G-5), 78.4 (G-3), 79.1 (C-3), 104.8 (G-1), 172.2 (C-1).

Compound **3**

Colourless oil. Positive FAB-MS: *m/z* 287 $[M + Na]^+$ 265 $[M + H]^+$. ^{1}H NMR (pyridine-d_5): δ 4.90 (1H, *d*, *J* = 7.9 Hz, G-1), 4.87 (1H, *m*, H-3), 4.71 (1H, *dd*, *J* = 10.2, 1.6 Hz, H-4′), 4.55 (1H, *dd*, *J* = 11.8, 2.3 Hz, G-6′), 4.43 (1H, *dd*, *J* = 10.2, 4.6 Hz, H-4), 4.35 (1H, *dd*, *J* = 11.8, 5.6 Hz, G-6), 4.24 (1H, *m*, G-3), 4.21 (1H, *m*, G-4), 3.95 (1H, *m*, G-5), 2.85 (2H, *m*, H-2). ^{13}C NMR (pyridine-d_5): δ 35.7 (C-2), 62.7 (G-6), 71.4 (G-4), 74.7 (G-2), 74.8 (C-4), 75.2 (C-3), 78.3 (G-5), 78.7 (G-3), 104.1 (G-1), 175.9 (C-1). Compound **3** was identified as 3-*O-β-*D-glucopyranosyl-(3*R*)-hydroxybutanolide by comparison with lit. ^{1}H and ^{13}C NMR data [2].

Acidic hydrolysis of **3**

Compound **3** (27 mg) was dissolved in 1 M H_2SO_4 (2 ml) and heated at 95° for 1 hr. The reaction mixt. was dild with H_2O (2 ml) and extracted with Et_2O. The aq. layer was neutralized (Ba $(OH)_2$) and the ppts filtered off. The filtrate was desalted by chromatography over LH-20 (MeOH) and then purified by silica gel CC ($CHCl_3$–MeOH–H_2O, 6:4:1) to give D-glucose (15 mg). Syrup $[\alpha]_D^{23}$ + 49.8° (*c* = 1.5, H_2O).

Acetylation of **3**

A soln of **3** (14 mg) in Ac_2O (0.5 ml) and dry pyridine (0.5 ml) was left standing at room temp. overnight. The crude acetate was purified by silica gel CC (hexane–Me_2CO, 3:1) to give **3a** (16 mg) as colourless prisms (from EtOH), mp 157–159°. Positive FAB-MS: *m/z* 433 $[M + H]^+$. ^{1}H NMR (pyridine-d_5): δ 5.13 (1H, *d*, *J* = 7.9 Hz, G-1), 4.88 (1H, *dddd*, *J* = 5.9, 4.6, 2.8, 1.2 Hz, H-3), 4.51 (1H, *dd*, *J* = 12.5, 4.6 Hz, H-4), 4.43 (1H, *dd*, *J* = 12.5, 2.8 Hz, H-4′), 2.97 (1H, *dd*, *J* = 17.8, 5.9 Hz, H-2), 2.78 (1H, *dd*, *J* = 17.8, 1.2 Hz), 2.07, 2.05, 2.04, 2.03 (each 3H, *s*, CH_3CO). ^{13}C NMR (pyridine-d_5):

δ 20.4 ($CH_3\times3$), 20.6 (CH_3), 99.9 (G-1), 169.6, 169.8, 170.3, 170.5 ($CH_3\underline{C}O\times4$), 175.3 (C-1).

X-ray analysis of **3***-peracetate*

Crystal data: $C_{18}H_{24}O_{12}$, $M_r=432$, monoclinic, space group $P21$; a = 11.291 (3) Å, b = 17.361 (4) Å and c = 5.676 (2) Å, $\alpha=\beta=\gamma=102.23$ (2) degrees, $V=1087.4$ (5) Å^3, z = 2, $D_\chi=1.356$ Mg/m^3, crystal size, $0.5\times0.4\times0.4$ mm. A crystal was mounted on a Mac Science MXC18 diffractometer. $R=0.048$ ($\omega R=0.048$).

Compound **4**.

Amorphous. $[\alpha]_D$ −5.8° (*c* = 1.2, MeOH). Positive FAB-MS: *m/z* 283 $[M+H]^+$. ^{1}H NMR (CD_3OD) δ: 4.44 (1H, *d*, *J* = 7.9 Hz, G-1), 4.12 (1H, *dddd*, *J* = 7.3, 5.9, 5.9, 4.3 Hz, H-3), 3.85 (1H, *dd*, *J* = 11.9, 1.7 Hz, G-6′), 3.65 (1H, *dd*, *J* = 11.9, 6.1 Hz, G-6), 3.64 (1H, *dd*, *J* = 12.5, 4.3 Hz, H-4′), 3.59 (1H, *dd*, *J* = 12.5, 5.9 Hz, H-4), 3.37 (1H, *dd*, *J* = 8.9, 8.9 Hz, G-3), 3.30 (1H, *dd*, *J* = 8.9, 8.9 Hz, G-4), 3.36 (1H, *m*, G-5), 3.19 (1H, *dd*, *J* = 8.9, 7.9 Hz, G-2), 2.47 (1H, *dd*, *J* = 14.9, 7.3 Hz, H-2), 2.38 (1H, *dd*, *J* = 14.9, 5.9 Hz, H-2′). ^{13}C NMR (CD_3OD) δ: 41.3 (C-2), 62.7 (G-6), 66.0 (C-4), 71.5 (G-4), 75.4 (G-2), 77.9 (G-5), 78.0 (G-3), 80.8 (C-3), 104.1 (G-1), 179.6 (C-1).

Conversion of **4** *to* **3** *by acid treatment*

A soln of **4** (20 mg) in H_2O (1 ml) was treated with 0.05 M H_2SO_4 (0.2 ml), then left to stand at room temp. for 2 hr. The mixt. was neutralized ($Ba(OH)_2$) and the ppts filtered off. The filtrate was evapd under a stream of N_2 and the residue chromatographed over silica gel ($CHCl_3$–EtOH, 7:3) to give a colourless oil (11 mg). The ^{1}H and ^{13}C NMR were superimposable on those of **3**.

Tissue culture

Shoot tops (1 cm length) were dissected from shoot buds of *in vitro* plantlets and cultured on Murashige and Skoog (MS) medium [8] at 25° under a 16 hr photoperiod for 2 months. The shoot tops propagated were subcultured on MS liquid medium without hormone on a reciprocating shaker (60 rpm) at 25° under a 16 hr photoperiod for 2 months to form adventitious shoots, which were propagated by repeated subculturing in the same medium under the same conditions.

Isolation of **1**–**4** *from plantlets of cultured tissues.*

Fresh tissues of stage of a, b and c (each 100 g) were homogenized in MeOH, respectively. After filtration, the solvent was evapd and the residue suspended in H_2O and extracted with $CHCl_3$ and *n*-BuOH, successively. The aq. soln extract was purified by Diaion HP-20 and silica gel CC as described above, successively, to afford **1** (2 mg from plantlets of stage a and c, 3 mg from b), **2** (27 mg from plantlets of stage a, 48 mg from b and 37 mg from c), **3** (508 mg from plantlets of stage a, 473 mg from b and 493 mg from c) and **4** (13 mg from plantlets of stage a, 11 mg from b and 9 mg from c). They were identified by the direct comparisons of ^{1}H and ^{13}C NMR.

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REFERENCES

1. Kan, W.-S., in *Pharmaceutical Botany*, National Research Institute of Chinese Medicine, Taiwan, 1986, p. 647.
2. Ito, A., Kasai, R., Yamazaki, K. and Sugimoto, H., *Phytochemistry*, 1993, **33,** 1133.
3. Takatsuki, S., Wang, J.-D., Narui, T. and Okuyama, T., *J. Jap. Botany*, 1992, **67,** 121.
4. (a) Seo, S., Tomita, Y., Tori, K. and Yoshimura, Y., *J. Am. Chem. Soc.*, 1978, **100**(11), 3311; (b) Kasai, R., Suzuo, M., Asakawa, J. and Tanaka, O., *Tetrahedron Lett.*, 1977, 175; (c) Tori, K., Seo, S., Yoshimura, Y., Arita, H. and Tomita, Y., *Tetrahedron Lett.*, 1977, 179.
5. Liu, S. Y., Tsay, H. S., Huang, H. C., Hu, M. F. and Yeh, C. C., *Jour. Agric. Res. China*, 1987, **36**(4), 357.
6. Lin, C.-C. and Namba, T., *Shoyakugaku Zasshi*, 1981, **35,** 262.
7. Lin, C.-C. and Namba, T., *Shoyakugaku Zasshi*, 1981, **35,** 272.
8. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15,** 473.