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A LIGNAN AND FOUR TERPENOIDS FROM *BRUCEA JAVANICA* THAT INDUCE DIFFERENTIATION WITH CULTURED HL-60 PROMYELOCYTIC LEUKEMIA CELLS

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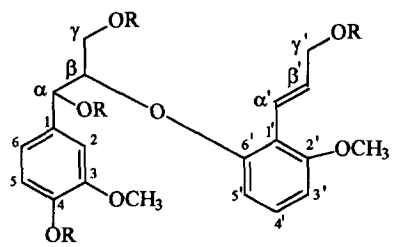
Key Word Index—*Brucea javanica*; Simaroubaceae; seeds; guaiacylglycerol-β-*O*-6′-(2-methoxy)cinnamyl alcohol ether; lignan; simaroubolides; human promyelocytic leukemia (HL-60) cells; cell differentiation.

Abstract—A novel lignan, guaiacylglycerol-β-*O*-6′-(2-methoxy)cinnamyl alcohol ether, three known simaroubolides, brusatol, dehydrobrusatol, yadanziolide C, and the known terpenoid, blumenol A, were obtained as active compounds from an ethyl acetate-soluble extract of *Brucea javanica*, using a bioassay based on the induction of cell differentiation with human promyelocytic leukemia (HL-60) cells. Also obtained were the known coumarinolignan, cleomiscosin A, and the known quassinoid glycoside, bruceoside B, which were inactive in the HL-60 cell test system. The structure of the new lignan was determined by a combination of 1D and 2D NMR techniques. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Brucea javanica (L.) Merr. (Simaroubaceae) is distributed from southeast Asia to northern Australia. Several quassinoids, inclusive of 15-*O*-benzoylbrucein D, bruceantarin, bruceantin, bruceantinol, bruceantinoside A, bruceins A-G and Q, brucein E 2-*O*-β-D-glucoside, bruceolide, bruceosides A-C, brusatol, dehydrobruceantinol, dehydrobruceins A and B, dehydrobrusatol, dihydrobrucein A, yadanzigan, yadanziolides A–D, and yadanziosides A–P, with a broad range of bioactivity (e.g., antitumour, antimalarial, antiprotozoal), have been isolated previously from this plant [1–14]. As part of our search for cancer chemopreventive natural products, seeds of *B. javanica* were selected for fractionation since an EtOAc extract significantly induced cell differentiation with human promyelocytic leukemia (HL-60) cells. We have previously demonstrated that HL-60 cell differentiation is a valid novel system to assist in the discovery of potential cancer chemopreventive agents of natural origin [15]. Bioassay-guided fractionation of the EtOAc extract of *B. javanica* using the HL-60 test system led to the isolation and identification of five active compounds, inclusive of a new lignan, guaiacylglycerol-β-*O*-6′-(2-methoxy)cinnamyl alcohol ether (**1**), three known simaroubolides, brusatol, dehydrobrusatol, and yadanziolide C, and the known terpenoid, blumenol A. Two further known compounds, cleomiscosin A and bruceoside B, were also isolated in this investigation, and found to be inactive in the HL-60 test system. We report herein the structure elucidation of compound **1**.



1: R = H

2: R = Ac

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RESULTS AND DISCUSSION

A molecular formula of $C_{20}H_{24}O_7$ was assigned to **1** from its HRFAB-mass spectral data ($[M+H]^+$, *m/z* 377.1519). The UV spectrum (λ_{max} 215, 265, 287, 300 nm) was similar to those of other lignans [16] and the IR spectrum revealed characteristic absorption bands at 3380 (OH), 2993 (aliphatic CH) and 1601,

1510, 1464 and 1429 (benzenoid) cm^{-1}. Comparison of the ^1H and ^{13}C NMR spectral data of **1** with those of guaiacylglycerol-β-*O*-4′-sinapyl ether [17] indicated that these two compounds differ only in ring B. Thus, in the ^1H–^1H COSY spectrum of **1**, coupling between an OH multiplet at δ 5.30 (OH-α) and a 1 H multiplet at δ 4.68 (H-α) was apparent, as was a correlation with another 1H multiplet located at δ 4.29 (*H*-β) and a broad 2H singlet at δ 3.58 (HOC$\underline{H}_2$-γ). These proton resonances corresponded to carbon peaks at δ 71.56 (C-α), 83.64 (C-β) and 60.07 (C-γ), respectively, in the ^1H–^{13}C HETCOR NMR spectrum of **1**, revealing the glyceroyl portion of a guaiacylglycerol moiety. Moreover, a doublet of doublets (J = 8.0, 2.5 Hz) at δ 6.67 (H-5) was coupled to a 1H broad doublet (J = 8.0 Hz) at δ 6.76 (H-6). Irradiation of this proton in a homonuclear decoupling ^1H NMR experiment resulted in an obvious collapse of the doublet located at δ 6.67 into a singlet, and also in a dramatic change of a 2H multiplet at δ 6.98 that included H-2, to which this proton was *meta*-coupled. This confirmed the existence of an ABX proton system due to a guaiacyl moiety. The ABX proton system was further evident from the APT and ^1H–^{13}C HETCOR NMR spectra of **1** which displayed three protonated carbon signals at δ 114.53 (C-5), 119.16 (C-6) and δ 111.35 (C-2), respectively.

It was apparent from the ^1H–^1H COSY and ^1H–^{13}C HETCOR NMR spectra of compound **1**, that ring B was formed from an *O*-disubstituted cinnamyl alcohol moiety exhibiting an ABC proton system composed of a 1H triplet at δ 6.85 (J = 8.0 Hz, H-4′), a 1H doublet at δ 6.92 (J = 8.0 Hz, H-5′) and a second doublet within the multiplet resonating at δ 6.98 (J = 8.0 Hz, H-3′). ^{13}C NMR resonance peaks at δ 115.32 (C-5′), 119.03 (C-4′), 109.76 (C-3′), respectively, were correlated to the H-5′, H-4′ and H-3′ proton signals in the HETCOR NMR spectrum. Irradiation of the triplet at δ 6.85 in a homonuclear decoupling ^1H NMR experiment, resulted in the collapse of the doublet due to H-5′ into a singlet at δ 6.92 and also in a change of the shape of the multiplet at δ 6.98 suggesting that the H-3′ doublet was part of the multiplet. Furthermore, two equivalent olefinic carbon signals at δ 128.52 were assigned to C-α′ and C-β′, respectively. The corresponding proton signals at δ 6.44 and 6.23, respectively, and their mutual coupling constant (J = 16.0 Hz) suggested a *trans* configuration of the two olefinic protons. H-β′ exhibited additional coupling with a broad 2H singlet at δ 4.08 (HOC$\underline{H}_2$-γ′).

On the basis of the 1D NOE NMR experiment of **1**, it was established unequivocally that the methoxy groups in rings A and B were in the *meta* and *ortho* positions, respectively, since there was a 6.52% NOE effect on the multiplet located at δ 6.98 from the two equivalent methoxy signals at δ 3.72. SINEPT [18] and FLOCK [19] NMR experiments supported this evidence and suggested that the junction between the two units of **1** linked C-β and C-6′ *via* an ether bridge. Thus, this was supported by peak enhancement at δ 71.56 (C-α) when the H-2, H-6 signals were irradiated ($^3J_{CH}$ = 8.0 Hz) in the SINEPT NMR spectrum, as well as by the presence of 3-bond correlations between H-4′ and carbon peaks at δ 147.78 (C-6′) and 149.59 (C-2′) in ring B, and between H-2 and carbon signals at δ 119.16 (C-6) and 145.60 (C-4) in ring A, respectively, in the ^1H–^{13}C FLOCK NMR spectrum. Irradiation of H-β ($^3J_{CH}$ = 6 Hz) in a SINEPT NMR experiment with a long delay time (2 sec) enhanced the peak at δ 147.72 (C-6′).

The tetraacetate derivative of **1** exhibited a $[M]^+$ ion peak at m/z 544 in the EI-mass spectrum. Also, two fragments at m/z 323 ($[M]^+ - 221$) and 222 ($[M]^+ - 322$) were observed which represented the acetylated guaiacylglycerol and *O*-6′-(2-methoxy)cinnamyl alcohol units of **1**, respectively. Thus, the structure of **1** was established as guaiacylglycerol-β-*O*-6′-(2-methoxy)cinnamyl alcohol ether. Compound **1** was found to be a mixture of *erythro* and *threo* isomers since in its ^{13}C NMR spectrum duplicate resonances were apparent for C-α at δ 70.88 and 71.56 and for C-β at δ 83.64 and 84.23 (approximate ratio 3:1). A similar phenomenon has been reported for a structurally related compound, guaiacylglycerol-β-coniferyl aldehyde ether [20].

Six constituents of *B. javanica* of known structure were identified as blumenol A, bruceoside B, brusatol, cleomiscosin A, dehydrobrusatol and yadanziolide C, by comparison of their physical and spectroscopic data with literature values [21–27]. The terpenoid, blumenol A, and the coumarinolignann, cleomiscosin A, have not been reported from this species before.

The novel lignan **1** and blumenol A were weakly active in the HL-60 test system (ED_{50} values 3.6 and 20 μg ml^{-1}, respectively). However, the most potent constituent of *B. javanica* seeds in inducing the differentiation of HL-60 cells obtained in this investigation was the quassinoid brusatol (ED_{50} 0.006 μg ml^{-1}). The potent activity of this compound was abrogated by glycosylation at the C-3 position, as in bruceoside B ($ED_{50} > 20$ μg ml^{-1}). Modification of the ring-A functionality of brusatol as evident in dehydrobrusatol led to a nearly hundred-fold diminution of activity (ED_{50} 0.8 μg ml^{-1}). The non-esterified quassinoid yadanziolide C (ED_{50} 0.6 μg ml^{-1}) exhibited similar potential for HL-60 cell differentiation. Finally, cleomiscosin A was inactive in this test system ($ED_{50} > 20$ μg ml^{-1}).

EXPERIMENTAL

General. Mps: uncorr., ^1H and ^{13}C NMR: TMS int. standard. EI-MS: direct inlet system, 70 eV. TLC: silica gel 60 F_{254} plates, sprayed with 10% v/v H_2SO_4; 110°, 10 min.

Plant material. Seeds of *B. javanica* were purchased in a Hong Kong herbal market in April 1994. A voucher specimen has been deposited at the Program for Collaborative Research in the Pharmaceutical Sci-

ences, Department of Medicinal Chemistry, College of Pharmacy, University of Illinois at Chicago.

Extraction and isolation. Ground seeds of *B. javanica* (4.5 kg) were exhaustively defatted with petroleum ether (bp 60–90°; 25 l), and extracted with MeOH (40 l). The extract was suspended in 250 ml of H_2O and partitioned with EtOAc (5 × 400 ml) to give, on drying, 120 g of EtOAc-soluble residue. This residue induced cell differentiation in a human promyelocytic leukemia (HL-60) cells (ED_{50} 0.17 $\mu g\ ml^{-1}$). CC fractionation of the residue, using silica gel as stationary phase and eluting with $CHCl_3$ and MeOH mixtures of increasing polarity (0–50%), afforded 13 frs. Frs 6–8 were active in the HL-60 test system. Thus, CC of fr. 6 over silica gel, developed in hexane–$CHCl_3$ (50:50) then with $CHCl_3$ and increasing quantities of MeOH (0–25%), combined with repeated prep. TLC purification in $CHCl_3$–MeOH (97:3), afforded blumenol A (25 mg, 0.0005% w/w), brusatol (452 mg, 0.010% w/w) and dehydrobrusatol (26 mg, 0.0005% w/w). CC of fr. 7 in $CHCl_3$ with increasing amounts of MeOH (0–10%) and prep. TLC developed in EtOAc–MeOH (99:1) produced cleomiscosin A (14 mg, 0.0003% w/w). CC of fr. 8 over silica gel, in EtOAc–MeOH mixtures of increasing polarity (5–50%), combined with prep. TLC purification performed in EtOAc–MeOH (95:5) furnished **1** (18 mg, 0.0004% w/w), yadanziolide C (23 mg, 0.0005% w/w) and bruceoside B (137 mg, 0.0032% w/w).

Guaiacylglycerol-β-O-6′-(2-methoxy)cinnamyl alcohol ether (**1**). Oil: $[\alpha]_D^{20}$ +5.4° (MeOH; *c* 0.1); UV λ_{max}^{MeOH} nm (log ε): 215 (4.0), 265 (3.8), 287 (3.5), 300 (3.3); IR ν_{max}^{film} cm^{-1}: 3380, 2937, 1601, 1510, 1464, 1429, 1275, 1132, 1037l; ^1H NMR (300 MHz, DMSO-d_6): δ 3.00 (1H, *bm*, OH-γ), 3.58 (2H, *bs*, H-γ), 3.72 (6H, *s*, 2 OMe), 4.08 (2H, *bs*, H-γ′), 4.29 (1H, *m*, H-β), 4.68 (1H, *m*, H-α), 4.85 (1H, *bs*, OH), 5.30 (1H, *m*, OH), 6.23 (1H, *m*, H-β′), 6.44 (1H, *d*, *J* = 16.0 Hz, H-α′), 6.67 (1H, *dd*, *J* = 8.0, 2.5 Hz, H-5), 6.76 (1H, *bd*, *J* = 8.0 Hz, H-6), 6.85 (1H, *t*, *J* = 8.0 Hz, H-4′), 6.92 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.98 (2H, *m*, H-2, H-3′); ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 55.41 (*q*, O<u>C</u>H$_3$), 55.57 (*q*, O<u>C</u>H$_3$), 60.07 (*t*, C-γ), 61.62 (*t*, C-γ′), 70.88 (*d*, C-α), 71.56 (*d*, C-α), 83.64 (*d*, C-β), 84.23 (*d*, C-β), 109.76 (*d*, C-3′), 111.35 (*d*, C-2), 114.53 (*d*, C-5), 115.01 (*d*, C-5′), 115.35 (*d*, C-5′), 119.03 (*d*, C-4′), 119.46 (*d*, C-6), 128.52 (*d*, C-α′, C-β′), 130.05 (*s*, C-1′), 132.89 (*s*, C-1), 145.60 (*s*, C-4), 146.91 (*s*, C-3), 147.78 (*s*, C-6′), 149.65 (*s*, C-2′); FAB-MS *m/z* (rel. int., %): 399 $[M + Na]^+$ 377 $[M + H]^+$; HRFAB-MS *m/z* found: 377.1519 $[M + H]^+$; $C_{20}H_{24}O_7$ requires: 377.1522.

Acetylation of **1**. Compound **1** (10 mg) was acetylated with pyridine–Ac_2O (1:1) at room temp. for 24 hr and the reaction mixture was worked up in the usual manner. Guaiacylglycerol-β-*O*-6′-(2-methoxy)cinamyl alcohol ether tetraacetate (**2**) was further purified by prep. TLC with $CHCl_3$–MeOH (98:2) as mobile phase.

Guaiacylglycerol-β-O-6′-(2-methoxy)cinnamyl alcohol ether tetraacetate (**2**). Oil: $[\alpha]_D^{20}$ −3.8° ($CHCl_3$; *c* 0.2); UV λ_{max}^{MeOH} nm (log ε): 205 (1.2), 225 (1.3), 323 (2.3); IR ν_{max}^{film} cm^{-1}: 2928, 1741, 1628, 1604, 1510, 1495, 1480, 1371, 1228, 1136, 1032; ^1H NMR (300 MHz, $CDCl_3$): δ 2.00 (3H, *s*, OAc), 2.09 (6H, *s*, 2 OAc), 2.30 (3H, *s*, OAc), 3.79 (3H, *s*, OMe), 3.81 (3H, *s*, H-OMe), 4.71 (2H, *d*, *J* = 6.5 Hz, H-γ′), 6.15–7.10 (8H, *m*, H-α, H-β, arom. H); ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 21.00 (*q*, <u>C</u>H$_3$COO-), 55.80 (*q*, O<u>C</u>H$_3$), 55.83 (*q*, O<u>C</u>H$_3$), 55.83 (*q*, O<u>C</u>H$_3$), 62.43 (*t*, C-γ), 62.94 (*t*, C-γ′), 73.53 (*d*, C-α), 80.11 (*d*, C-β), 110.17 (*d*, C-3′), 111.78 (*d*, C-2), 118.17 (*d*, C-5), 119.20 (*d*, C-5′), 119.71 (*d*, C-4′), 120.00 (*d*, C-6), 122.52 (*d*, C-α′), 124.32 (C-β′), 131.05 (*s*, C-1′), 131.75 (*s*, C-1), 139.60 (*s*, C-4), 147.07 (*s*, C-3), 147.78 (*s*, C-6′), 150.89 (*s*, C-2′), 168.02 (*s*, CH$_3$<u>C</u>OO-), 169.44 (*s*, CH$_3$<u>C</u>OO-), 170.79 (2*s*, CH$_3$<u>C</u>OO-); EI-MS *m/z* (rel. int., %): 544 $[M]^+$ (1), 323 (1), 281 (1), 222 (11), 174 (14), 147 (5), 84 (25), 43 (100).

Brusatol. Crystals from MeOH: mp 268°, lit, 274–277° [21]; $[\alpha]_D^{20}$ +45.1° (acetone; *c* 0.1), lit. 43.6°, acetone [22]; UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with lit. values [21].

Dehydrobrusatol. Oil: $[\alpha]_D^{20}$ +60° (MeOH: *c* 0.1), lit. +57°, MeOH [23]; UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with lit. values [23].

Yadanziolide C. Crystals in MeOH: mp 289°, lit. 292–297° [24]; $[\alpha]_D^{20}$ +25° (MeOH; *c* 0.5), lit. +29°, MeOH [24]; UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with lit. values [24].

Blumenol A. Yellowish oil: $[\alpha]_D^{20}$ +244° ($CHCl_3$; *c* 0.3), lit. +256°, $CHCl_3$ [25]; UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with literature values [25].

Cleomiscosin A. Crystals in MeOH: mp 261°, lit. 250–252° [26]; $[\alpha]_D^{20}$ +2.5 (MeOH; *c* 0.07), lit. 0°, MeOH [26]); UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with lit. values [26].

Bruceoside B. Amorphous powder: $[\alpha]_D^{20}$ +7.2° (MeOH; *c* 2.04), lit. +3.73°, MeOH [27]; UV, IR, ^1H NMR, ^{13}C NMR and EI-MS, consistent with lit. values [27].

Biological testing. Compound **1** was active in the HL-60 cell differentiation test [15, 28] (ED_{50}: 3.6 $\mu g\ ml^{-1}$), and the simaroubolides brusatol, dehydrobrusatol, yadanziolide C and the terpenoid blumenol A exhibited ED_{50} values of 0.006, 0.8, 0.6 and 20 $\mu g\ ml^{-1}$, respectively, in the same assay. Cleomiscosin A and bruceoside B were not significantly active (ED_{50} > 20 $\mu g\ ml^{-1}$) in the HL-60 assay. In all cases, ED_{50} values are given that were derived from NBT reduction assays conducted with cultured HL-60 cells, as described previously [15].

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REFERENCES

1. Polonsky, J. (1985) *Fortsch. Chem. Org. Naturst.* **47**, 221.
2. Bhatnagar, S., Polonsky, J., Sevenet, T. and Prange, T. (1985) *Tetrahedron Letters* **26**, 1225.
3. Fukamiya, N., Okano, M., Miyamoto, M., Tagahara, K. and Lee, K. H. (1992) *J. Nat. Prod.* **55**, 468.
4. Lin, L.-Z., Cordell, G. A., Ni, C-Z. and Clardy, J. (1990) *Phytochemistry* **23**, 2121.
5. Sakaki, T., Yoshimura, S., Ishibashi, M., Tsuyuki, T., Takahashi, T., Honda, T. and Nakanishi, T. (1985) *Bull. Chem. Soc. Jpn.* **58**, 2680.
6. Sakaki, T., Yoshimura, S., Tsuyuki, T., Takahashi, T., Honda, T. and Nakanishi, T. (1986) *Bull. Chem. Soc. Jpn.* **59**, 3541.
7. Sakaki, T., Yoshimura, S., Tsuyuki, T., Takahashi, T. and Honda, T. (1986) *Chem. Pharm. Bull.* **34**, 4447.
8. Sakaki, T., Yoshimura, S., Sakaki, T., Ishibashi, M., Tsuyuki, T., Takahashi, T. and Honda, T. (1985) *Bull. Chem. Soc. Jpn.* **58**, 2673.
9. Yoshimura, S., Ogawa, K., Tsuyuki, T., Takahashi, T. and Honda, T. (1988) *Chem. Pharm. Bull.* **36**, 841.
10. Phillipson, J. D. and Darwish, F. A. (1981) *Planta Med.* **41**, 209.
11. Polonsky, J., Baskevitch, Z. and Muller, J. (1969) *C. R. Acad. Sci. C* **268**, 1392.
12. Stöklin, W. and Geissman, T. A. (1968) *Tetrahedron Letters* 6007.
13. Duncan, G. R. and Henderson, D. B. (1968) *Experientia* **24**, 768.
14. Lee, K. H., Imakura, Y. and Huang, H. C. (1977) *Chem. Commun.* 69.
15. Suh, N., Luyengi, L., Fong, H. H. S., Kinghorn, A. D. and Pezzuto, J. M. (1995) *Anticancer Res.* **15**, 233.
16. Ayres, D. C. and Loike, J. D. (1990) *Lignans, Chemical, Biological and Clinical Properties*, p. 69. Cambridge University Press, Cambridge, UK.
17. Deyama, T., Ikawa, T., Kitagawa, S. and Nishibe, S. (1987) *Chem. Pharm. Bull.* **35**, 1803.
18. Bax, A. (1984) *J. Magn. Reson.* **57**, 314.
19. Reynolds, W. F., McLean, S., Perpick-Dumont, M. and Enriquez, R. G. (1989) *Magn. Res. Chem.* **27**, 162.
20. Deyama, T., Ikawa, T., Kitagawa, S. and Nishibe, S. (1987) *Chem. Pharm. Bull.* **35**, 1785.
21. Lee, K. H., Hayashi, N., Okano, M., Nozaki, H. and Ju-Chi, M. (1984) *J. Nat. Prod.* **47**, 550.
22. Sim, K. S., Sims, J. J., and Geissman, T. A. (1968) *J. Org. Chem.* **33**, 429.
23. Sakaki, T., Yoshimura, S., Ishibashi, M., Tsuyuki, T., Takahashi, T., Honda, T. and Nakanishi, T. (1985) *Bull. Chem. Soc. Jpn.* **58**, 2680.
24. Yoshimura, S., Sakaki, T., Ishibashi, M., Tsuyuki, T., Takahashi, T. and Honda, T. (1985) *Bull. Chem. Soc. Jpn.* **58**, 2673.
25. Gonzalez, A. G., Gulluno, J. A., Ravelo, A. G. and Jimenez, I. A. (1994) *J. Nat. Prod.* **57**, 400.
26. Arisawa, M., Handa, S. S., McPherson, D. D., Lankin, D. C., Cordell, G. A., Fong, H. H. S. and Farnsworth, N. R. (1984) *J. Nat. Prod.* **47**, 300.
27. Lee, K. H., Imakura, Y., Wu, R. Y., Hall, I. H. and Huang, H. L. (1979) *J. Org. Chem.* **44**, 2180.
28. Tanaka, H., Abe, E., Miyaura, C., Kuribayashi, T., Konno, K., Nishi, Y. and Suda, T. (1982) *Biochem. J.* **204**, 713.